2
Review of Literature

2.1 NITRATE CONTAMINATION IN WATER

Nitrate indisputably is one of the most common contaminants in the groundwater and wastewater worldwide. Sources of nitrate pollution include industrial operations, agricultural runoff, inappropriate animal, and human waste disposal etc. [Mohseni-Bandpi *et al*., 2013; Della Rocca *et al*., 2007]. Certain wastes such as those arising from nuclear fuel cycle operations mainly from reprocessing plants are characterized by high nitrate content with initial nitrate concentrations ranging from 10 g/l to 50g/l [Francis and Callahan 1975; Fredrickson *et al*. 2004; Genders *et al*., 1996]. Three types of wastes are released from nuclear reprocessing plants which are high level, intermediate level, and low-level waste. The high-level waste is acidic and contains high levels of nitric acid. Intermediate waste is also acidic and contains nitrate in the order of 50000mg/l. Low-level wastes are neutral and contain relatively low nitrate [Sandhya *et al*., 2012].

To regulate the nitrate contamination in the environment WHO (World Health Organization) has set the limit of nitrate contamination in drinking water as 50mg/l NO_3^- or 10mg/l NO_3^--N [WHO, 1996]. In addition, Central Pollution Control Board, India also has set the standard limit of discharge of nitrate concentration in water as 50mg/l [CPCB, 1998]. Nitrate is one of the nutrients causing eutrophication in lakes killing aquatic life forms. It causes the blue baby syndrome in infants (methemoglobinemia) and is carcinogenic [Mirvish, 1975]. Nitrate has also been identified as a major groundwater contaminant in many countries such as India, UK, North America, Australia, and China [Shrimali and Singh, 2001].

A number of physicochemical methods are adopted to remove nitrate from wastewater. These include methods such as ion exchange, reverse osmosis, nanofiltration, and electro-dialysis etc. These methods carry out nonspecific nitrate ion removal [Hell *et al*., 1998; Islam and Suidan, 1998; Ergas and Reuss, 2001]. In addition, the application of these methods is limited by the cost associated with the process and generation of concentrated nitrate waste which further needs disposal. Biological removal of nitrate is a promising method as it can selectively remove nitrate and with a carefully developed process, complete reduction of nitrate to harmless molecular nitrogen is achievable [Mohseni-Bandpi *et al*., 2013; Ghafari *et al*., 2008].

Biological denitrification/Dissimilatory nitrate reduction/Anaerobic respiration is a process which operates in the absence of oxygen. The process is carried out by denitrifying bacteria which use nitrate as a terminal electron acceptor in their electron transport chain. In the denitrification process, nitrate/nitrite is converted into harmless nitrogen (N_2) gas. Denitrifying bacteria are ubiquitous and play a very important role in the nitrogen cycle in the natural environment [Gamble *et al*., 1977]. The process of biological denitrification is also exploited in industrial wastewater treatment for nitrate removal. Different types of wastewater treatment systems have been used for denitrification such as soil-aquifer, packed beds, use of granular activated carbon (GAC), and rotating biological contactors [Ghafari *et al*., 2008].

Conventional wastewater treatment processes such as activated sludge are very energy intensive and generate a huge amount of sludge which further needs disposal [Sun *et al*., 2016;

Wei *et al.,* 2003]. Considering the high energy demand of the conventional wastewater treatment, the processes that couple waste treatment with energy generation are sought after and a large number of researchers are working to develop an effective method of harnessing the energy from waste.
Microbial fuel cell (MFC) technology is one such waste to energy process which is widely researched. MFCs have been demonstrated as effective wastewater treatment devices [Franks *et al.,* 2010]. The electrical energy produced by these systems is low but can certainly offset the total energy requirements of the process. MFCs convert chemical energy hidden in the organic substrates to electrical energy using bacteria. A number of studies have proven that MFCs are sustainable waste treatment systems [Rabaey and Verstraete, 2005; Lovley, 2006b].

The idea of MFC dates back to 1911. At this time, M.C. potter first demonstrated that the electrical energy can be generated using *Escherichia coli* and *Saccharomyces cerevisiae*. M. C. Potter stated in his article that "The disintegration of organic compounds by microorganisms is accompanied by the liberation of electrical energy" [Potter, 1910].

In 1931, Barnett Cohen obtained 0.2 mA of current by using potentiostat poised half-cell [Cohen, 1931]. Despite the merits associated with the findings of MC Potter, the idea of MFC was not researched for a long time. In 1980, A British researcher H. Peter Bennetto relived the idea of MFC and used the pure culture of bacteria and artificial electron mediators to harness the electrical power from organic substrates [Bennetto, 1990]. In 1999, Kim et al. discovered that artificial mediators are not necessary for electron transfer to take place [Kim *et al.*, 1999]. This was an important breakthrough. The research on MFCs speeded since then and attracted the attention of many environmental biotechnologists and electrochemical engineers around the world. Significant progress in the field of MFC technology has been achieved and the technology is getting better every single day. In 2009, the discovery of exo-electrogenic bacteria *Geobacter sulfurreducens* was included in the 50 best types of research by Time magazine [Time, 2009].

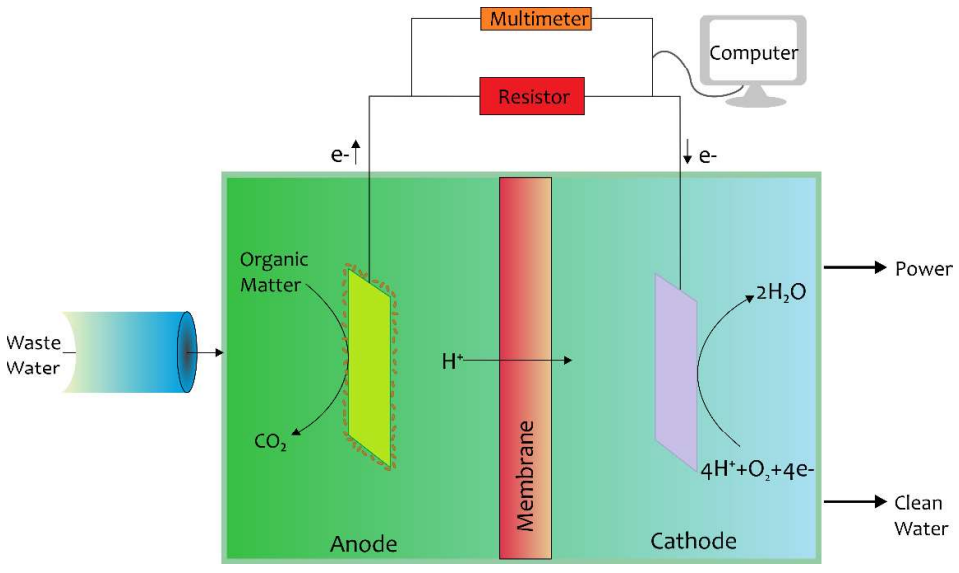


Figure 2.1: Schematic of a basic MFC design

2.2 MFC PRINCIPLE AND ITS COMPONENTS

A typical MFC consists of two chambers namely anode and cathode separated by a proton exchange membrane (PEM). In the anode chamber, anaerobic bacteria carry out the degradation of organic matter resulting in the production of carbon dioxide (CO_2), electrons, and protons. The electrode acts as a terminal electron acceptor. This phenomenon is called exoelectrogenesis and the bacteria which can carry out this process are called as exoelectrogens. Exoelectrogens are the microorganisms which are capable of this kind of extracellular electron transfer. The electrons travel from the anode through the external circuit constituting the flow of current. The external circuit comprises of an electrically conducting wire and a load which connects the anode to the cathode. Proportional to the magnitude of the current flowing through the external circuit, protons from the anode chamber pass through the PEM to the cathode chamber maintaining charge balance. The electron acceptor present in the cathode chamber accepts the electrons flowing through the cathode and gets reduced. A number of electron acceptors have been tested at MFC cathode. It includes chemicals such as ferricyanide, hydrogen peroxide, iron, oxygen, manganese dioxide, permanganate, nitrate, nitrite, etc. Oxygen is the most commonly used electron acceptor in the cathode due to its availability, high redox potential (+0.82 V), and ease of operation. In the anode organic substrate such as glucose, acetate, fumarate, citrate, butyrate, glycerol, cellulose etc. are used widely [Choudhury *et al.*, 2017]. The basic MFC schematic is showing in Figure 2.1.

An example reaction on the electrode surface is shown below:

Anode- $CH_3COO^- + 2H_2O \longrightarrow 2CO_2 + 7H^+ + 8e^-$ (E_{anode} = -0.28V) (2.1)

Cathode- $O_2 + 4e^- + 4H^+ \longrightarrow 2H_2O$ ($E_{cathode}$ = +0.82V) (2.2)

The overall cell voltage will be:

$$E_{cell} = E_{cathode} - E_{anode}\ (+1.1V) \tag{2.3}$$

Equation 2.3 shows the ideal cell voltage which the system can generate. The observed voltage values differ from the ideal value due to different losses in the MFC, the details of which are mentioned in section 2.4.

The MFC performance is commonly measured in terms of power density and current density which is absolute power normalized with electrode surface area or anode/cathode chamber volume [Holzman, 2005]. The power output from MFC is affected by a number of factors. Some of these factors include MFC configuration, electrode material, anode/cathode catalysts, type of membrane material, electrode spacing, microbial characteristic/biofilm properties, and operational conditions in both the chambers (Inoculum source, substrate type, pH etc.) [Du *et al*., 2007; Do *et al.,* 2018].

The three main components of MFC is anode, cathode, and membrane. The description of these three main components is summarized in Table 1.1.

2.2.1 Anode

The anode is placed in the anodic chamber which is completely anaerobic. The oxidation reactions happen on the surface of the anode and anode acts as the terminal electron acceptor. The required characteristics of anode material are (i) high electrical conductivity, (ii) corrosion resistance (iii) high surface area (iv) biocompatibility (v) mechanical strength and chemical stability [Rinaldi *et al*., 2008; Guo *et al*., 2015]. Carbon-based materials are most widely used in the anode chamber. The carbon-based electrode is used in the form of carbon brush, carbon cloth, carbon paper, carbon felt, reticulated vitreous carbon, graphite plate, graphite fiber brush, graphite rod, granular graphite etc. [Zhou *et al*., 2011; Hindatu *et al.,* 2017]

2.2.2 Cathode

The cathode is placed in the cathode chamber of MFC which can be biotic and abiotic. The reduction reactions happen on the surface of the electrode. In abiotic cathode, electrons are transferred to oxygen which acts as a final electron acceptor in the cathode chamber. Carbon-based electrodes are the most preferred materials for the cathode. In addition, a suitable electrode catalyst and electron mediators are needed in order to overcome the electrode overpotential and enhance the rate of the reduction reaction. Platinum is commonly used as a catalyst in the MFC due to its excellent catalytic ability for oxygen reduction [Liu *et al*., 2004]. But due to its high cost and toxicity, its application is limited as the catalyst [Bard and Faulkner, 2001]. Moreover, the ionic species such as phosphate, sulfate, chloride etc. prevailing in MFC can lower/diminish the catalytic activity of platinum [Zhao *et al*., 2006]. To overcome this limitation researchers around the world have utilized non-pt based catalyst such as transition metal (iron and cobalt), metal oxides (manganese (IV) dioxide, lead oxide- PbO_2), carbon nanostructure such as carbon nanotubes, conductive polymers such as polyaniline etc. [He *et al*., 2017]. Microorganisms can also serve as electrode catalysts in the biotic cathodes [Shukla *et al*., 2004; Huang *et al*., 2011]. The biotic cathodes have a number of advantages over the abiotic ones. These include the lower cost of operation, simultaneous wastewater treatment, and bioremediation, lower chemical use etc. [He and Angenent, 2006; Song *et al*., 2015]. Also, using algae in the cathode chamber can produce the oxygen by the photosynthetic reaction which can replace the cost of external oxygen supply [Strik *et al*., 2008]. The success of the process depends on the acquisition and acclimatization of the microbial consortia based on the desired application. For example, the success of denitrifying cathode depends on the composition and activity of denitrifying bacterial consortia present in the system.

2.2.3 Membrane/Separator

The membrane is not the indispensable component of MFC as the studies on the use of membrane-less or sediment MFCs are continuously increasing [Reimers *et al*., 2001; Liu *et al*., 2004]. The major purpose of the membrane in the conventional MFC design is the separation of anodic and cathodic chambers and maintaining the redox differences. The separator should be impermeable to oxygen, organic acids, resistant to biofouling, and most importantly should have high ionic conductivity [Logan *et al*., 2006]. The most commonly used proton exchange membrane in MFC is Nafion 117. However, its application is limited by its high cost. In order to address this, researchers have tested several other materials as potential separators. These include ultrafiltration membranes, J-cloth, nylon fibers, glass fibers, ceramics, earthenware, and biodegradable shopping bags etc. [Santoro *et al*., 2017].

Table 2.1: Description of the different components of the MFC system

MFC components	Description
MFC Configuration	Dual chamber, Single chamber, Upflow, Stacked, Tubular, Multichamber
Anode	Carbon brush, Carbon cloth, Carbon paper, Carbon felt, Graphite rod, Graphite fiber brush, Graphite plate, Granular graphite, Reticulated vitreous carbon
Cathode	Pt coated electrode, Graphite felt, Catalyst (Metal oxides, Carbon nanotubes, Polyaniline, Transition Metal), Granular Graphite, Carbon Cloth, Carbon Paper
Separator	J-cloth, Nylon fibers, Glass fibers, Ceramics, Earthenware, Nafion 117, Salt Bridge, Ulterax, Porcelain septum

2.3 ELECTROCHEMICALLY ACTIVE MICROORGANISM

A number of fundamental studies have focused on the mechanism of extracellular electron transfer by exoelectrogenic bacteria or exoelectrogens. It has been found that electrons are transferred by three different mechanisms namely using electron mediators or shuttles, by direct electron transfer or by bacterial nanowires [Logan and Regan, 2006a; Logan, 2009]. Some examples of exoelectrogenic bacteria include *Shewanella putrefaciens, Geobacter sulferreducens, Geobacter metallireducens* and *Rhodoferax ferrireducens.* [Lovley, 2006a].

Electron transfer by mediators involves small molecular weight electron carriers or shuttles which transfer the electrons from microbe to the electrode [Costa *et al.,* 2018]. These mediators undergo cyclic oxidation-reduction during the process. Bacteria such as *Shewanella oneidensis*, *Pseudomonas acaliphila, and Pseudomonas aerugionsa* have been identified to release these kinds of mediators. Rabaey et al. identified that a redox mediator pyocyanin secreted by *P. aeruginosa* and found it responsible for its electrochemical activity [Rabaey *et al*., 2004].

Direct electron transfer involves a direct transfer of electron by an outer membrane to the electrode [Bond *et al.,* 2010]. The discovery of direct electron transfer was done in the culture of *S. putrefaciens* and it was proposed that electrons get directly transferred from bacterial cell to electrode through outer membrane c-type cytochrome while metabolizing lactate [Kim *et al*., 1999].

Some bacteria have the specialized structure called as nanowires by which electron transfer takes place. In 2005, Lovley and coworkers have discovered the nanowires in the *Geobacter*, this important discovery helped unravel the electron transfer mechanism and also fuelled the research on the other possible applications of these nanowires in electrochemical systems [Reguera *et al*., 2005].

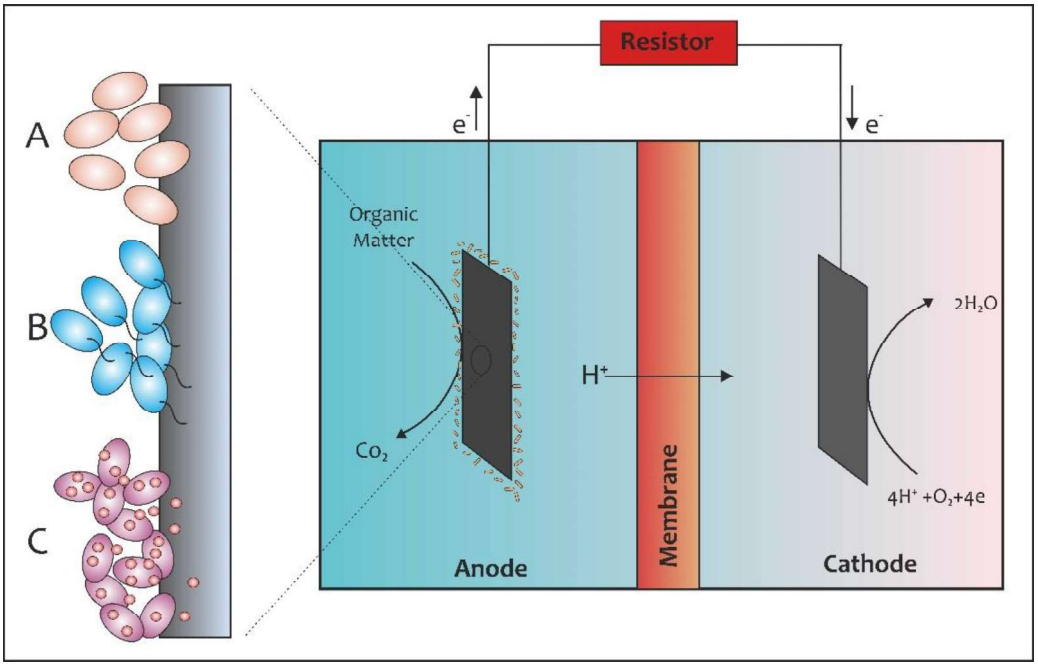


Figure 2.2: Electron transfer mechanism at the anode electrode surface, A- Direct electron transfer, B- Electron transfer via conductive nanowires and C- Electron transfer through endogenous mediators. Modified from- [Logan, 2009]

2.4 DIFFERENT TYPES OF LOSSES IN MFC

The losses which happen in MFC can be quantified using current-voltage polarization curve (Figure 2.3). The losses lower the operating voltage which is calculated using equation 2.4 [Appleby and Foulkes, 1988]:-

$$V_{cell} = E_{cathode} - |\eta_{actc} + \eta_{concc}| - E_{anode} - |\eta_{acta} + \eta_{conca}| - iR_i \quad (2.4)$$

$\eta_{act,a}$ and $\eta_{act,c,}$ are activation polarization losses on anode and cathode, respectively. On the other hand, $\eta_{conc,a}$ and $\eta_{conc,c}$ are concentration polarization in anodic and cathodic chamber respectively. The third component is ohmic losses (η_{ohm}) which occur due to the resistance in the flow of ions present in the electrolyte and resistance in the flow of electrons from the electrode. It is expressed as iR_i in which i is current flowing in the MFC system and R_i is the total internal resistance of the system. Ohmic losses can be minimized by reducing the electrode spacing, or by increasing the solution conductivity [Hoogers G, 2002].

Concentration losses occur due to the slow mass transfer when the substrate concentration becomes the limiting factor. This process limits the current production in the MFC system. Concentration losses can be minimized by increasing the mass transfer rate to/from the reactive species. This can be attained by the stirring and bubbling the solution which in turn reduce the concentration gradient in the bulk solution. Activation energy is needed for any electrochemical reaction (oxidation/reduction) to takes place for reacting species and these losses happen during the electron transfer process from/ to a compound at the electrode surface. These compounds can be redox mediators present in the bacterial surface or in the solution. The activation losses can be minimized by adding the external mediators for the efficient electron transfer process, using efficient catalysts, increasing the electrode surface area & the operating temperature, or by promoting the enriched biofilm on the electrode surface [Logan *et al*., 2006].

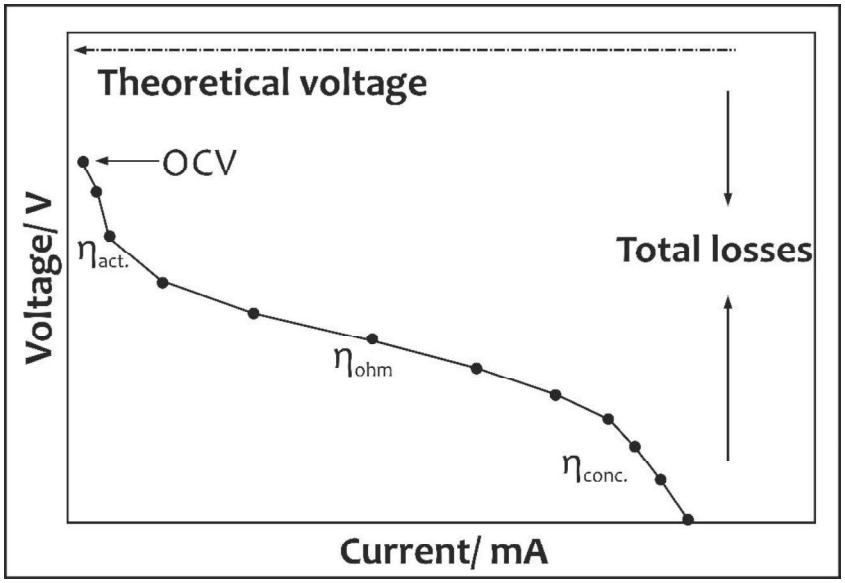


Figure 2.3: The ideal current-voltage polarization curve showing the three different losses. Source- [Zhao *et al.*, 2009]

2.5 ELECTROCHEMICAL TECHNIQUES AND PARAMETERS TO STUDY MFC PERFORMANCE

There are various techniques and parameters to study the MFC performance. These techniques involve the electrochemical assessment of the MFC and are important for the basic characterization of MFC. The techniques are briefly described below:

2.5.1 Voltage and Power Density Measurement

The performance of MFC can be evaluated by measuring voltage and power generation. The cell voltage is defined as a potential difference between the anode and cathode. The overall cell voltage is calculated using equation 3. The open circuit voltage (OCV) is the term used for voltage value in the open circuit (Anode and cathode not connected by any load). The OCV is a measurement of cell voltage at infinite resistance.

The potential of anode/cathode also called Open Circuit Potential (OCP) can be obtained by measuring the voltage against the reference electrode. The examples of reference electrodes are standard hydrogen electrode (SHE) (0V), silver-silver chloride (Ag/AgCl) electrode (+0.197 V v/s the SHE), saturated calomel electrode (0.242V v/s the SHE).
Power of the MFC system can be calculated by connecting anode and cathode via appropriate load/resister using the formula:

$$P = IE_{cell} \quad (2.5)$$

Where E_{cell} is the voltage of the system and current is calculated using Ohm's law-

$$I = E_{cell}R \quad (2.6)$$

Where R is the external resistance applied to the MFC.

The power density is calculated by normalizing power with either electrode projected surface area or reactor volume. The unit of power is Watt (W) and power density is represented by either W m^{-3} or W m^{-2}. Maximum power density can be calculated by analyzing the polarization curve. The polarization curve analysis is the important tool for characterization and analysis of MFC system [Hoogers G, 2002]. This curve shows the relationship between voltage and current density. This curve can be made for the anode, cathode, or full cell using a potentiostat or a multimeter. The voltage and current response at variable resistances are measured and plotted. The curve helps to elucidate the optimum load a system can take up based on its kinetics. The three losses (mentioned in section-2.4) can be estimated by analyzing the polarization curve.

2.5.2 Columbic Efficiency

The electron transfer process which happens between anode and microbes occur through a complex respiratory chain process and this process differs from microbe to microbe. The electron transfer efficiency is measured in terms of columbic efficiency. This is the ratio of the total coulombs transfer to the anode from the substrate oxidation to maximum possible coulombs if all the substrate removal produces current [Logan *et al.*, 2006]. The columbic efficiency can be calculated by the given equation:-

$$\varepsilon = \frac{M\int_0^t Idt}{FnV_{An}\Delta COD} \quad (2.7)$$

Where, where M= 32 (molecular weight of oxygen), n= 4 (the number of electrons exchanged per mole of oxygen), F is Faraday's constant, V_{An} is the volume of anodic chamber, and ΔCOD is the difference in COD over a time period.

2.5.3 Cyclic Voltammetry

Cyclic voltammetry (CV) is a potentiodynamic electrochemical measurement to study the redox reaction occurring at the electrode surface. In this technique, the voltage is ramped

with time within a particular potential range and current response is recorded. The CV is recorded generally in a three-electrode system with a working electrode (anode or cathode), a reference electrode and counter electrode (anode or cathode). The graph of current at the working electrode is plotted as a function of applied potential which gives the cyclic voltammogram. The characterization of electron transfer interaction between microorganism and electrode can be done using CV. Researchers have elucidated various electron transfer processes using CV [Rabaey *et al.,* 2004; Fricke *et al.,* 2008]. A study where CV confirmed the oxidation of acetate (Figure 2.4) was done in the presence of 1mM acetate [Chae *et al.,* 2009]. The CVs with the biofilm and the bulk solution were obtained and the peaks were absent for the bulk solution which indicated the absence of soluble electron mediators. Also, peaks were absent in the absence of acetate confirming acetate as a source of electrons.

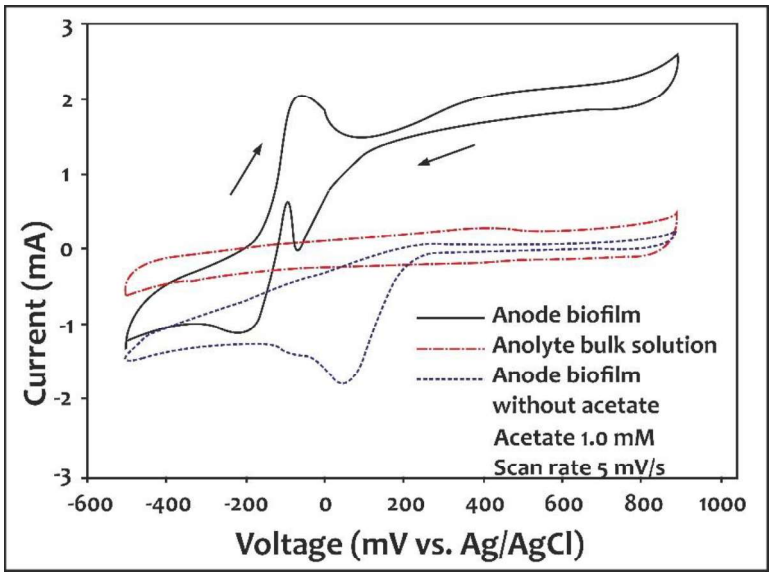


Figure 2.4: Cyclic voltammogram showing the mechanism of electron transfer in MFC anode-The CV was done in anolyte bulk solution v/s anode biofilm to see the presence of mediators; CV with or without a substrate to see the electron origin. The arrows indicate the scan direction forward scan (→) and reverse scan (←). Source- [Chae *et al.*, 2009]

2.5.4 Chronoamperometry

Chronoamperometry (CA) is a technique which can measure the diffusion coefficients while also elucidating the kinetics and mechanisms of electrode reactions. CA is also done in the three electrode system. To obtain a CA curve, the voltage of the working electrode is ramped and the current response is recorded as a function of time [Scott and Yu, 2015]. The biofilm formation is responsible for the current generation at the electrode surface. The biofilm is responsible for the electron transfer process. The technique is primarily used in the MFC to study the biofilm activity and its growth on electrode surface [Rosenbaum *et al.,* 2007].

2.5.5 Electrochemical Impedance Spectroscopy

Electrochemical Impedance Spectroscopy (EIS) is a powerful technique used to measure the charge transfer, mass transfer, and ohmic resistance of the MFC [Dominguez-Benetton *et al.,* 2012]. In this technique, the small amplitude of AC voltage is applied on the system (either electrode or complete cell) and current response is measured through the cell in terms of its magnitude and phase shift [Scott and Yu, 2015]. The data obtained from EIS can be represented by the Nyquist plot (Figure 2.5) and Bode plot. In the Nyquist plot the x-axis and y-axis are the real part (Z_{Re}) and the imaginary part (Z_{lm}) of the impedance ($Z(\omega) = Z_{Re} + jZ_{lm}$), respectively. EIS is analyzed by using the most appropriate equivalent circuit which may comprise of the resistor, capacitor, and Warburg elements in series/parallel.

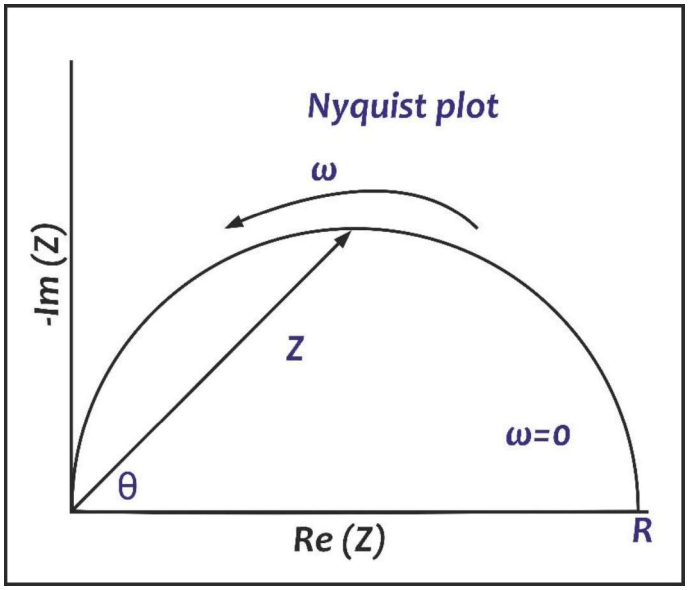


Figure 2.5: Representation of EIS data in the Nyquist plot. Source- [Scott and Yu, 2015]

2.5.6 Butler Volmer and Tafel Equation

Butler-Volmer equation is a governing equation in electrochemical kinetics. This equation establishes a relationship between electrical current occurring on an electrode and electrode potential, by considering that both anodic and cathodic reactions occur on the same electrode. In other words, this equation links the current density at an electrode to the overpotential. This equation is expressed as follows [Bard and Faulkner, 2001]:-

$$i = i_o[e^{(1-\alpha)\eta F/RT} - e^{-\alpha\eta F/RT}] \quad (2.8)$$

Where α = measure of symmetry

Tafel equation is a distinctive case of Butler-Volmer equation which relates the overpotential of an electrode to the current density (Figure 2.6). In a low and medium temperature fuel cell, the main reason for overpotential is activation losses. This voltage drop is represented as follows [Bard and Faulkner, 2001]: -

$$\eta = a + b \log I \quad (2.9)$$

where, η= Overpotential due to activation losses.

a=electron transfer coefficient.

$$a = -\frac{2.303\,RT}{\alpha nF}\log i_o \quad (2.10)$$

b= Tafel slope.

$$b = \frac{2.303\,RT}{\alpha nF} \quad (2.11)$$

i_o = exchange current density.

Exchange current density is the current density at equilibrium, where the anodic and cathodic current is equal. Activation polarization is affected by the rate of electro-reactant's transportation to the electrode surface and the sluggish kinetics of the electrode. Hence, it is essential to increase the exchange current density for optimal performance. When exchange current density is higher, the reaction is more favorable in one direction, which in turn accelerates the electrode kinetics & leads to the reduction in activation losses. Exchange current density can be enhanced by increasing pressure, temperature and concentration of electro-reactants. It can also be enhanced by using appropriate electrode catalyst and by increasing the surface area of electrode.

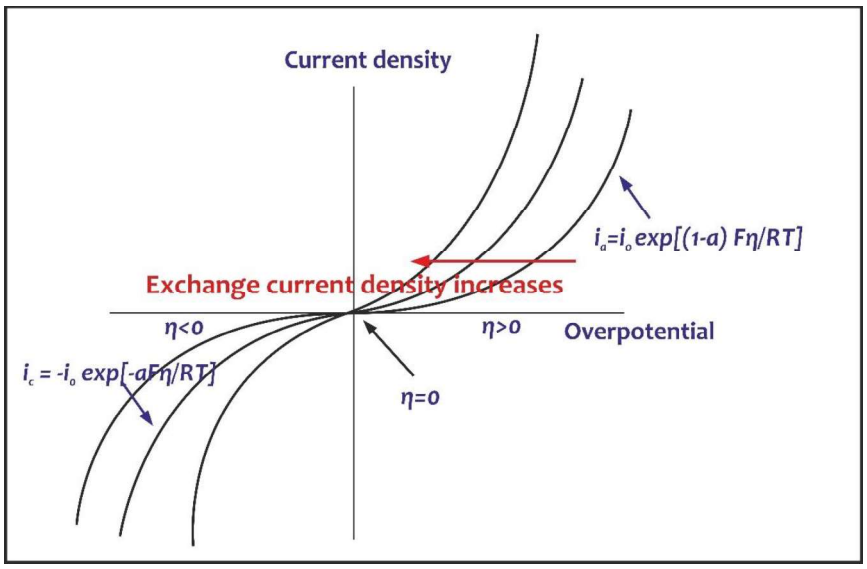


Figure 2.6: Tafel plot showing the relationship between current density and overpotential. Source- [Scott and Yu, 2015]

2.6 MFC CONFIGURATION

The configuration of the MFC has a direct impact on the internal resistance of the device which in turn affects the power output from the system. Researchers across the world have developed designs which contribute towards lowering either the internal resistance or lower the cost associated with the design. Some of the design considerations involve reducing the distance between anode and cathode, mixing requirements, membrane placement, number of chambers, effective volume, ion transfer efficiency etc. Some of the MFC configurations are discussed below:

2.6.1 Two/Dual Chamber MFC

This is most commonly used MFC design as it can cater to wide range of MFC applications. This design is generally built in "H" shape and consist of two chambers namely anode and cathode which are physically separated by proton exchange membrane like Nafion or salt bridge [Du *et al.*, 2007; Logan and Regan 2006b; Logan *et al.*, 2006]. This type of system is generally used at the lab scale as increasing their scale is difficult and the power outputs are generally low [Logan *et al.*, 2006].

2.6.2 Single Chamber MFC

Single chamber MFCs are the system of choice when the aim is to remove COD and produce power. The systems have relatively lower cost and simple designs as only one chamber is involved. The cathode is air exposed in most of the cases with atmospheric oxygen acting as the electron acceptor. Liu and Logan studied the electricity generation using single-chamber MFC where anode was carbon paper without any catalyst and cathode were made either of rigid carbon paper without PEM or Pt catalyst loaded carbon electrode bonded with PEM (Figure 2.7 A). They achieved the power output of 262 ±10 mWm^{-2} (with PEM) and 494 ±21 mWm^{-2} (without PEM) [Liu and Logan, 2004]. Further, Liu et al. demonstrated production of electricity (26 mWm^{-2}) using single-chamber MFC which consisted of single cylindrical plexiglass chamber containing eight graphite rods placed in a concentric arrangement around a

single cathode consisted of carbon/ platinum catalyst /PEM layer fused to a plastic tube [Liu *et al*., 2004].

2.6.3 Tubular MFC

These kinds of MFCs contain a single cylindrical/tubular chamber and are generally membrane less. The height of the chamber ensures lower redox potential at the bottom and high redox potential at the top of the chamber allowing it to serve as both as the anodic and cathodic chamber. In most of the cases, the system is up flow wherein the feed containing high COD enters the bottom of the reactor and flow upwards while passing through the anodic and cathodic biofilms. This type of configuration was first studied by Rabaey et al. (Figure 2.8B). They obtained the power output of 90 Wm^{-3} (with acetate feed) and 66 W m^{-3} (with glucose feed) net anodic volume [Rabaey *et al*., 2005]. A similar configuration (Figure 2.8A) with sucrose as a feed generated power output of 170 mWm^{-2} [He *et al*., 2005]. Further, You *et al* constructed the graphite-granule, membrane-less tubular air cathode MFC using the cylindrical plexiglass tube and achieved a volumetric power density of 50.2 Wm^{-3} [You *et al*., 2007]. This configuration, in general, is easily scalable and gives high power output as demonstrated by the studies cited above.

2.6.4 Stacking MFC

Stacking two or more MFCs in series or parallel generates what is called a bio-battery with enhanced voltage/current. The power output from a single unit is low but stacked MFC offer high output albeit at an enhanced cost. Aelterman et al. constructed stacked MFC wherein six individual MFC units were connected in series and anode/cathode was made up of graphite granules (Figure 2.7 B). High power densities of 258 Wm^{-3} were obtained in the study [Aelterman *et al*., 2006]. Another design involved a series of cassette electrodes. In this system, air cathode was placed on two ends of the cathode box to prevent air or water leakage and cathode box was sandwiched between two proton exchange membranes offering a scalable and high power producing (129 W m^{-3}) device [Shimoyama *et al*., 2008].

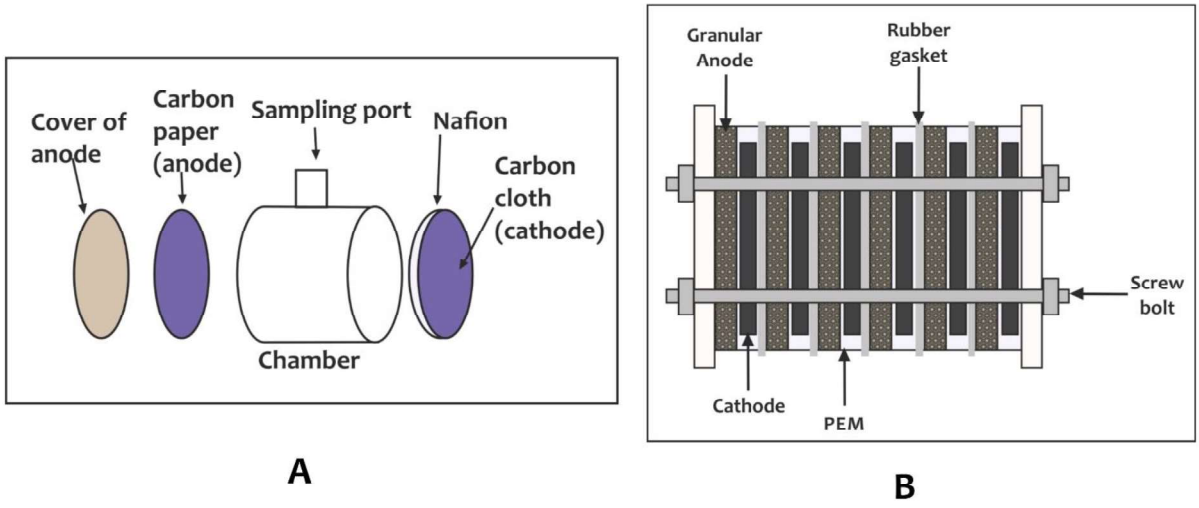


Figure 2.7: MFC configurations A- Single chamber MFC. Source- [Liu and Logan, 2004], B- Stacking MFC Source- [Aelterman *et al*., 2006]

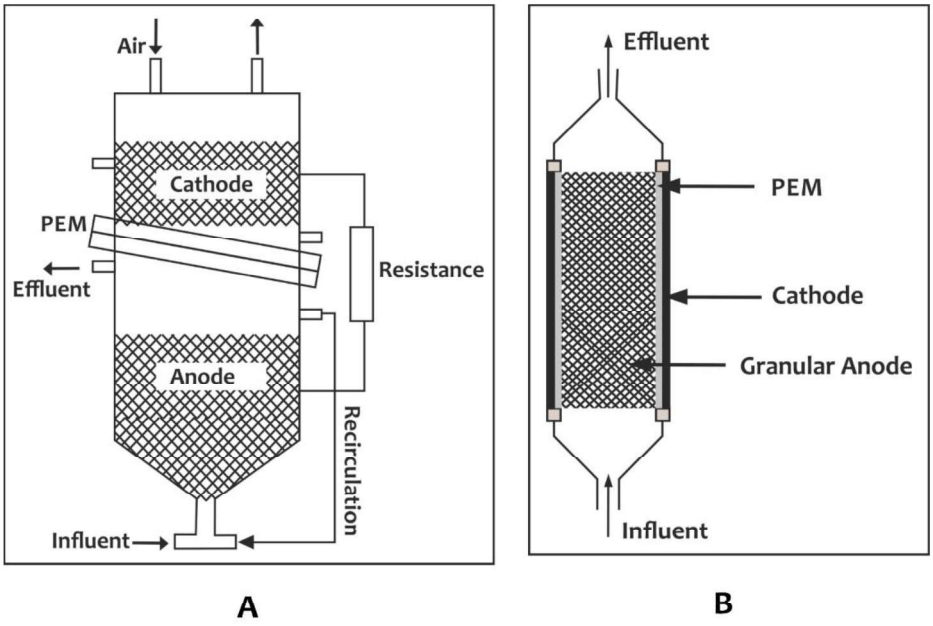


Figure 2.8: MFC configurations A- Upflow MFC. Source- [He *et al.*, 2005], B- Tubular Single chambered MFC. Source- [Rabaey *et al.*, 2005]

2.7 APPLICATION OF MFC

Microbial fuel cells at first catch attention due to their ability to produce electrical energy. However, the power outputs from MFCs are significantly low when compared to chemical fuel cells. A chemical fuel cell can generate power density of 140kW m^{-3} [Arends and Verstraete, 2012]. On the other hand, MFCs have been reported to give power output <6Wm^{-2}; ≤500Wm^{-3} which is significantly less when compared to chemical fuel cell [Zhao *et al.*, 2009]. Therefore, MFCs cannot serve as standalone energy devices. The sustainability of the MFC system can be increased if it is coupled with another high-value application. This includes wastewater treatment, biomass/algae generation, biosensors, powering remote sensors, bio-remediation, Carbon dioxide capture etc. (Figure 2.9). Large numbers of researches are underway worldwide to make MFC technology commercially feasible. Some of the applications of MFC are discussed briefly below:

2.7.1 Wastewater Treatment

As discussed earlier, conventional wastewater treatment is very energy intensive and integrating MFC technology with wastewater treatment can offset the total energy requirements of the treatment process. It is estimated that 4-5% of the country's electrical energy is invested for water infrastructure which includes water treatment, water distribution, wastewater collection, and wastewater treatment. About 1.5% of the total electricity is invested in the wastewater treatment process alone [Logan, 2008]. It is estimated that wastewater contains 9.3 times more energy than what is needed to treat water [Shizas and Bagley, 2004]. About 0.5 $kWhm^{-3}$ of energy is required for the aeration process. It is also estimated that domestic, animal, and food process wastewater contain a total of 17GW of energy [Logan, 2008]. Wastewater generated from domestic, agricultural, and industrial settings has been tested in the MFC as a potential electron donor [Pandey *et al.*, 2016].

MFCs have been used to produce power from different types of wastewater such as industrial wastewater, chemical wastewater, food processing wastewater, domestic wastewater, textile dyes, brewery wastewater, starch wastewater, landfill leachate, and even cellulose-containing wastes [Scott and Yu, 2015; Franks *et al.*, 2010].

Another advantage associated with the use of MFC is that the bacterial sludge formed at the end of the treatment process is considerably less when compared to activated sludge process [Hernández-Fernández *et al*., 2015]. It is estimated that the amount of excess sludge which needs to be disposed off can be 50 to 90% higher when using conventional technology rather than MFC [Holzman, 2005]. Sludge needs dewatering and disposal which adds to the treatment cost and lesser sludge means lesser hassle in managing it.

2.7.2 Electricity Generation

MFC can convert chemical energy present in the organic matter into the electrical energy with the aid of microorganism. The efforts are underway to enhance the power output and columbic efficiency of the device. Columbic efficiency as high as 97% is obtainable in MFC as opposed to other thermodynamic systems. The reports of CE values in the order of 80%, 89%, and 97% exist in the literature [Chaudhuri and Lovley, 2003; Rabaey *et al*., 2003; Rosenbaum *et al*., 2006]. The overall power output from MFCs to date is very low for large scale application. The electricity produced from MFC can be stored in the rechargeable devices such as a capacitor. The charged devices can power devices which require low power such as wireless sensors etc. [Shantaram *et al*., 2005]. A study worth mentioning here is of a MFC which could power a robot named as EcoBot I [Ieropoulos *et al*., 2003]. EcoBot-II had similar energy system and it could perform sensing, information processing, communication, and actuation [Ieropoulos *et al*., 2005]. MFCs can also power the implantable devices in the human body by utilizing nutrients supplied by the body [Rapoport *et al*., 2012].

2.7.3 Algae Cultivation and CO_2 Sequestration

Photosynthetic MFCs is the term used for MFCs which integrate with them algae degradation or production. Algae biomass serves as low cost readily available electron donor substrate at the anode. On the other hand, algal cultivation at cathode results in the generation of oxygen which acts as an electron acceptor. In addition, the algal biomass is harvested for bioenergy generation. The Carbon dioxide produced at the anode is fed to the cathode for capture by algal photosynthesis [Xiao and He, 2014]. Algae also serve to treat water contaminated with nitrates and phosphates at the cathode [Zhang *et al*., 2011]. A large number of studies have been done on photosynthetic MFCs [Rosenbaum *et al*., 2010; Khandelwal *et al*., 2018]. This version of MFC is certainly beneficial when considering a value addition to MFC technology.

2.7.4 Bioremediation

Numerous studies prove the applicability of MFCs for bioremediation. The number of pollutants can either be oxidized or reduced at the MFC anode or cathode. The success of the process depends on the effectiveness of the microbial consortia which can be acclimatized for the given pollutant. In this context, electron donors such as textile dyes, petroleum hydrocarbons, phenolic compounds, ammonium compounds have been remediated at the anode [Pandey *et al*., 2016]. The metals such as mercury, arsenic, chromium, and ions such as nitrate, and sulfate can be reduced and precipitated at the cathode. Among all these compounds nitrate (NO_3/N_2 +0.74V), iron (Fe^{3+}/Fe^{2+} +0.77V) and manganese (MnO_2/Mn^{2+} +0.60V) have a high redox potential but sulfate (SO_4^{2-}/H_2S -0.22V) has relatively lower redox potential [Thauer *et al*., 1977; Rhoads *et al*., 2005]. Use of electron acceptors other than oxygen at the cathode also eliminates the risk of oxygen diffusion at the anode thereby imparting high columbic efficiency to the process [Al-Mamun *et al*., 2016].

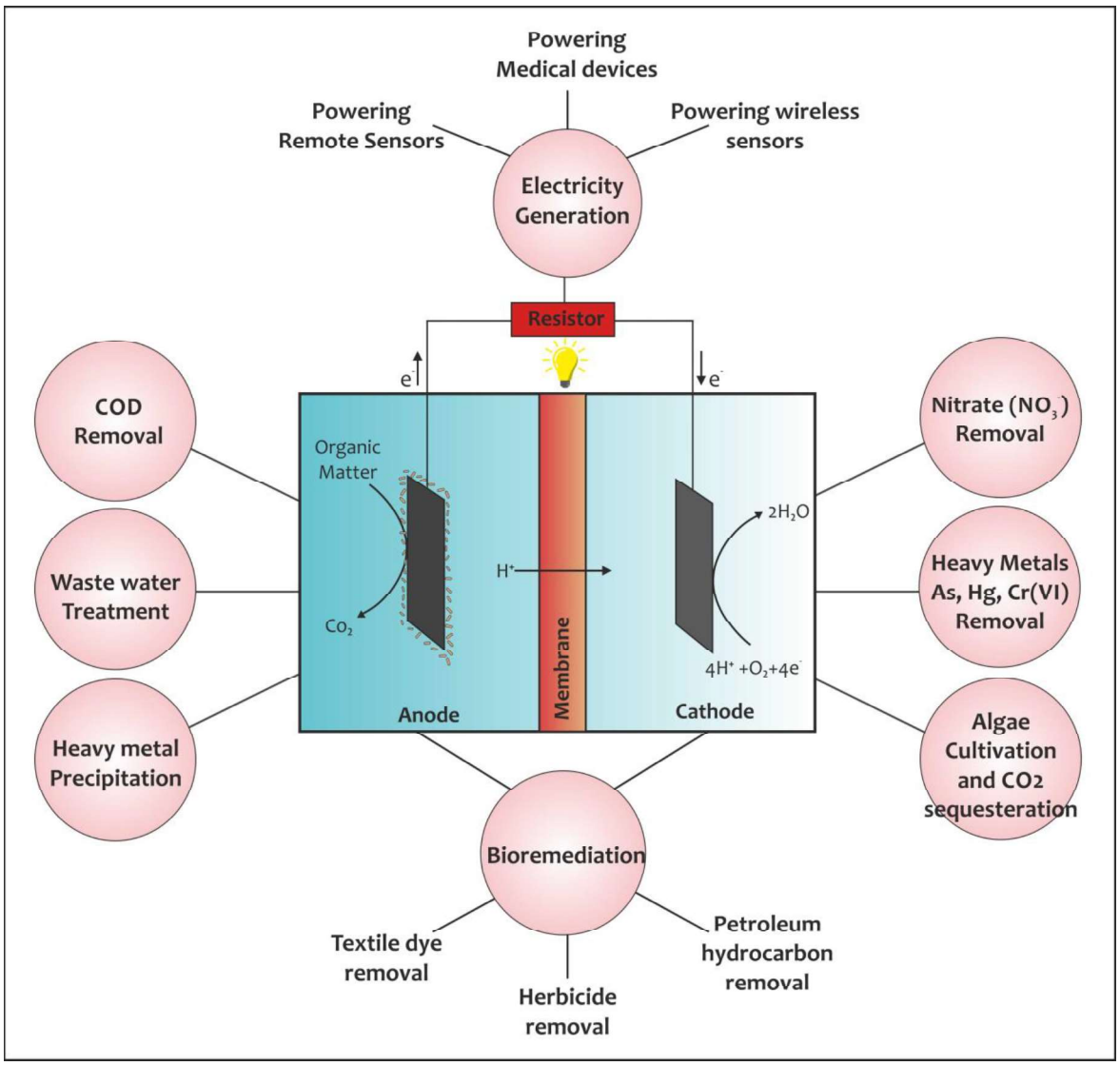


Figure 2.9: Figure depicting the various applications of MFC technology

2.8 DENITRIFICATION IN MICROBIAL FUEL CELL

Biological denitrification can take place in two different conditions namely autotrophic denitrification (AD) and heterotrophic denitrification (HD) [Park and Yoo, 2009]. Both these processes differ in the nature of electron donor for nitrate reduction with heterotrophic denitrifiers utilizing organic electron donors (ethanol, glucose, glycerol, acetic acid, and lactic acid, methanol, and starch) and autotrophic denitrifiers utilizing inorganic electron donors [Sevda *et al.*, 2018].

Denitrification process can be presented using below mentioned equation:-

$$2NO_3^- + 10e^- + 12H^+ \longrightarrow N_2 + 6H_2O \quad (E_0 = +0.74V) \qquad (2.12)$$

The process of the AD is preferred when denitrification is intended in a MFC. This is because the exclusion of organic matter at the cathode is necessitated for maintaining the redox potential difference between anode and cathode. The AD is based on the activity of chemolithotrophic autotrophs which can accept electron flowing through the cathode to reduce nitrate while fixing carbon dioxide [Ghafari *et al.*, 2009]. A number of studies report cathodic AD with different nitrate removal rates and power outputs [Clauwaert *et al.*, 2007; Virdis *et al.*, 2008; Vilar-Sanz *et al.*, 2013; Oon *et al.*, 2016; Al-Mamun *et al.*, 2016; Ding *et al.*, 2018]. While the power output from these systems is considerably higher, the nitrate removal rates are low

because the autotrophs are slow growers and stoichiometrically reduce lesser nitrates during energy metabolism. Also, the source of electrons is only the current flowing through the cathode, a fraction of which is utilized for cell growth and the remaining for nitrate reduction. Highest nitrate removal rate reported so far by the cathodic AD is 0.498±0.043 kg NO_3^--N m^{-3} Net Cathodic Compartment (NCC) d^{-1} at an initial nitrate loading rate of 0.684 kg NO_3^--N m^{-3} NCC d^{-1} while using a 1.1Ω external resistor [Clauwaert *et al*., 2009]. The most widely used inorganic carbon source in the autotrophic denitrification is sodium bicarbonate ($NaHCO_3$).

Nitrate removal rates realized for heterotrophic denitrification are generally higher than autotrophic denitrification owing to high growth rates supported by heterotrophic mode of metabolism [Ghafari *et al*., 2008]. This is desirable when the nitrate content in wastewater is high. Zhang et al. reported heterotrophic denitrification at dual chamber MFC anode and the most obvious problem encountered by them was lower columbic efficiency as the nitrate competed with the electrode for electrons. Yet, the nitrate removal rates were high and in the order of 1.26 ± 0.01 kg NO_3^--N m^{-3} d^{-1} [Zhang *et al*., 2013c]. Heterotrophic denitrification at the cathode is rarely attempted and only a few reports exist in the literature. The presence of organic matter at cathode has been found to lower the power output [Vilar-Sanz *et al*., 2013; Ding *et al*., 2018]. The summary of prominent denitrification studies is given in Table 2.1. A brief overview of nitrate removal methods using MFC are described below:

2.8.1 Bio-Electrochemical Denitrification Combined with MFC

The bioelectrochemical system (BES) applies external voltages with a help of power supply [Virdis *et al*., 2009; Kondaveeti and Min, 2013; Van Doan *et al*., 2013; Tong *et al*., 2013; Kondaveeti *et al*., 2014; Chen *et al*., 2016; Liu *et al*., 2016]. This is done by poising the electrodes at a fixed potential and testing the removal of contaminants. The catalysts are microbes which can interact with the electrode surface. Gregory et al. did the first study on bioelectrochemical denitrification wherein the electrode enriched with G. *metallireducens* biofilm and poised at -500mV reduced nitrate [Gregory *et al*., 2004]. Park et al. used a biofilm electrode reactor treating an aqueous solution containing a high concentration of nitrate and observed the evolution of nitrogen gas. The total nitrate removal was 98% at an applied current of 200mA [Park *et al*., 2005].

Kondaveeti and Min in 2013 ascertained the role of microorganisms by comparing the biotic and abiotic cathodes at an applied voltage of 0.7 V for nitrate reduction. The nitrate removal rate was high with biotic cathode with a value of 0.204 mg NO_3^--N cm^{-2} d^{-1} [Kondaveeti and Min, 2013].

2.8.2 Autotrophic Cathodic Denitrification

A number of studies report this kind of denitrification considering that it supports high power output. The denitrification is either done alone or involves the total nitrogen removal. In the latter case, cathodic denitrification is preceded by a nitrification process wherein the reduced form of nitrogen like ammonia, amine, and nitrite undergoes oxidation in the presence of oxygen. Some of the prominent studies include the following; Clauwaert et al. used a tubular reactor [Clauwaert *et al*., 2007] to perform denitrification using MFC. The system supported nitrate removal rate of 0.146 kg NO_3^--N m^{-3} NCC d^{-1} with the power density of 8 Wm^{-3} NCC. Lefebvre et al. designed a novel type of two-chambered MFC involving an auto-heterotrophic denitrifying biofilm on the cathode. The system generated a power density of 9.4 mWm^{-2} for a period of more than 1.5 months [Lefebvre *et al*., 2008].

Nitrate is a common contaminant in groundwater; therefore, it can serve as a catholyte. Groundwater possesses little or no organic matter thus favoring autotrophic denitrification [Puig *et al*., 2012; Pous *et al*., 2012; Zhang and Angelidaki, 2013]. Puig et al. demonstrated the autotrophic denitrification with the power density of 4.4Wm^{-3} [Puig *et al*., 2012]. The major limitation of the groundwater is its low conductivity which limits the current flow. This also

leads to the incomplete denitrification which is undesirable.

Autotrophic denitrification studies were subsequently done by several groups using different MFC configuration, electrode materials, and reactor volume etc. With an aim to simplify the MFC configuration, a research group developed a membrane-less MFC. This membrane less MFC achieved removal rates of 0.64 kg COD m^{-3} of liquid cathodic volume (LCV) d^{-1} and 0.186 g NO_3-N m^{-3} of LCV d^{-1} with a power output of 0.0712 W m^{-3} of LCV [Zhu *et al*., 2013].

AI-mamun et al. developed a denitrifying MFC with a biocathodic chamber lying between two anodes. In this system, simultaneous removal of 229.5 ± 18 mg L^{-1} COD at the anode and 88.9 g m^{-3} NCC d^{-1} nitrogen at cathode was achieved with a volumetric power density of 7±0.4 Wm^{-3} NCC [Al-Mamun *et al*., 2016]. Oon et al. studied the performance of denitrifying MFC in continuous mode for 180days and concluded that the removal efficiency of COD and nitrate were higher in the closed-circuit mode as compared to open circuit mode. This system achieved a total nitrate removal of 88±4% and a power density of 669 mWm^{-3}[Oon *et al*., 2016].

Vilar-Sanz et al. have done studies on nitrate-nitrogen removal in both autotrophic and heterotrophic conditions in the microbial fuel cell at different time phases and they have concluded that current density and coulombic efficiency were higher in the autotrophic mode [Vilar-Sanz *et al*., 2013]. In this study 15±2.3 and 11±7 Am^{-3} NCC of current density was achieved in autotrophic and heterotrophic conditions respectively.

As mentioned earlier, the reduced forms are nitrogen is first oxidized by a process of nitrification before denitrification. This involves either a separate nitrifying reactor equipped with oxygen or a stratified aerobic/anaerobic cathode biofilm for simultaneous nitrification and denitrification (SND) [Virdis *et al*., 2008; Virdis *et al*., 2010; Ryu *et al*., 2013; Sayess *et al*., 2013; Xie *et al*., 2011; Zhang *et al*., 2013b]. The process of SND is described in Figure 2.10.

Virdis et al. developed a MFC system where COD removal was done at the anode and the anode effluent primarily contaminated with ammonium was directed into the aerobic bioreactor for the nitrification process. The effluent from the nitrification reactor served as the catholyte wherein denitrification eliminated nitrate as nitrogen gas. This configuration achieved the nitrogen removal rate of 0.41kg m^{-3} d^{-1} NCV (Net cathode volume) and power density of 34.6Wm^{-3} [Virdis *et al*., 2008]. The C and N mass balance studies by these authors showed that ammonium ion diffused through the cation exchange membrane into the cathode chamber resulting in high nitrogen concentration in the effluent. The problem of diffusion was circumvented by the authors by integrating nitrification and denitrification in the same chamber which led to 94.1±0.9% nitrogen removal [Virdis *et al*., 2010]. The authors discussed the optimal dissolved oxygen concentration for simultaneous nitrification and denitrification (SND). Also, microstructural studies on the biofilm revealed stratification wherein the nitrifying bacteria were present in the outer layers and the putative denitrifying bacteria were present in the inner biofilm (close to electrode surface) [Virdis *et al*., 2011].

The concept of SND was successfully attempted in the membrane less MFC using a rotating cathode electrode [Yu *et al*., 2011; Sayess *et al*., 2013]. The rotating electrode is an effective method with simple design and operation and very low maintenance and energy consumption [Cortez *et al*., 2008].

Zhang et al. used single chamber MFC with a rotating biocathode on top of the fixed anode and achieved power density of 585mWm^{-3} [Zhang *et al*., 2013b]. A more precise dissolved oxygen (DO) control in cathode chamber was achieved by Xie et al. They used oxic/anoxic coupled MFC and achieved a power density of 14Wm^{-3} (NCC) and 7.2Wm^{-3} in oxic and anoxic

MFC respectively [Xie *et al*., 2011]. The system involved one MFC consisting of dual oxic biocathode and the other MFC consisting of dual anoxic biocathode. The MFCs with oxic/anoxic two stage biocathode were also scaled up to 50L with a power density of 43.1 W m^{-3} [Liang *et al*., 2013]. Zhang and He developed a configuration involving a dual cathode MFC with one cathode aerobic and the other anaerobic. The CEM was used to separate anode and aerobic cathode and AEM was used to separate anaerobic cathode and anode. This strategy helped in preventing ammonium ion leaching into the cathode chamber. The system achieved 96.5% removal at the nitrogen loading rate of 0.01kg N m^{-3} d^{-1} in batch mode [Zhang and He, 2012b]. Further, to optimize reactor structure and improve nitrogen removal rate the batch operated dual chamber MFC was modified into the continuously operated dual cathode tubular type of MFC [Zhang and He, 2012a].

A cooperative MFC system was designed wherein two MFCs were combined together to remove carbon and total nitrogen from the wastewater [Zhang and He, 2013]. First MFC removed the total carbon present in the wastewater and the resulting effluent was fed into the second MFC which removed nitrogen. The system removed 99% COD, 98% ammonium and 96% total nitrogen. Zhang et al. attempted the field application of MFC system. This involved the two 4 L tubular MFCs installed in a municipal water treatment facility. The system operated for more than 400 days on primary effluents for sequential C and N removal. The denitrifying MFC was able to remove 77% of nitrogen with an effluent nitrate concentration of 4.9±3.8 mg/L [Zhang *et al*., 2013a]. The process of SND was further employed in the single chamber MFC with air cathode enriched with nitrifying biofilm [Yan *et al*., 2012]. In this configuration, diethylamine-functionalized polymer was employed as a platinum catalyst binder to enhance the nitrifying biofilm establishment and achieved 96.8% ammonia removal and power density of 900±25 mWm^{-2}. Equipping the system with an additional air cathode or diffusion cloth significantly increased ammonia and COD removal rates by up to 119% and 35% [Yan and Regan, 2013].

Nitrogen removal can also be achieved in MFC by using algae which are known to assimilate nutrients such as nitrogen and phosphorous [Leite *et al*., 2013]. In the context, a sediment type of photo MFC was developed which can remove carbon and nitrogen by a synergistic interaction between microalgae and electrochemically active bacteria. The total organic carbon and nitrogen removal was 99.6% and 87.6% respectively with a stable power density of 68±5 mWm^{-2} [Zhang *et al*., 2011]. An integrated photo-bioelectrochemical system was developed by Xiao et al. for ammonia and COD removal. [Xiao *et al*., 2012].

2.8.3 Anodic Heterotrophic Denitrification

Anodic denitrification being mostly heterotrophic offers high nitrate removal rates and can carry out denitrification in high strength nitrate wastes [Zhang *et al*. 2013c]. Anodic denitrification, however, leads to a potential loss in the device's columbic efficiency as nitrate competes with the anode as an electron acceptor [Sukkasem *et al*. 2008; Lee *et al*. 2012]. Effect of different nitrate concentrations on the performance of single chambered air cathode MFC was tested using sodium acetate as a carbon source and 85% of nitrate removal was achieved in less than 8h [Sukkasem *et al*., 2008].

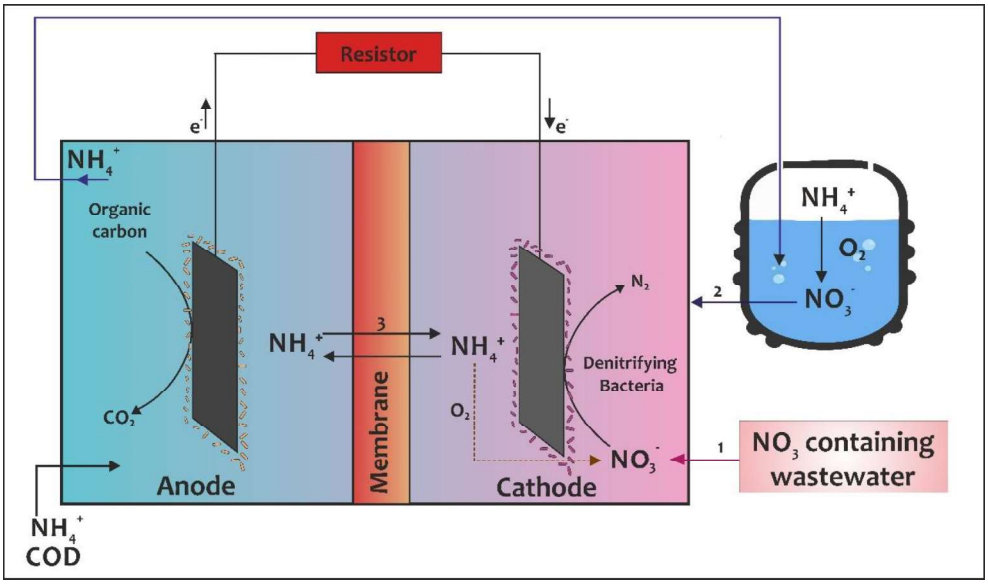


Figure 2.10: Nitrate removal using MFC (1)-Waste water contaminated with only nitrate undergoes denitrification (2) Waste water contaminated with NH_4^+ (first treated in aerobic nitrifying reactor) fed to the cathode for denitrification (3) Waste water contaminated with NH_4^+ treated with SND in cathode chamber Source-[Virdis *et al.*, 2008; Virdis *et al.*, 2010]

2.8.4 Cathodic Heterotrophic Denitrification

Heterotrophic cathodic denitrification is rarely attempted as the presence of organic matter at the cathode lowers the redox potential difference resulting in lower power output. In this context, the performance of double-chambered MFC fed with decomposed cyanobacteria solution was investigated for simultaneous nitrification and denitrification. This system achieved a removal efficiency of 0.064±0.0005 kg TN m^{-3} d^{-1} and 0.063 ± 0.005 kg NH_4^+-N m^{-3} d^{-1}. Authors attributed the enhanced nitrogen removal efficiency to the enriched heterotrophic denitrifying bacteria at cathode chamber [Zhu *et al.*, 2016]. Vilar-Sanz et al. also observed enhanced nitrogen removal rate (with low nitrate in the effluent as compared to autotrophic effluent) in the heterotrophic mode [Vilar-Sanz *et al.*, 2013].

It can be concluded from the literature that high rate heterotrophic denitrification can be done in a MFC albeit at a lower columbic efficiency or power output. Wastewater is heterogeneous and characterized by the presence of both organic carbon and nitrate. It also has different strengths based on their concentrations. A robust MFC system should be able to take up these wastes and carry out high rate nitrate removal without compromising heavily on the power output.

Table 2.2: Summary of prominent denitrification studies in MFC

Denitrification types	MFC Configuration	Substrate used	Rate of Nitrate removal / % N removal	Current Density/Power density/Voltage	Reference
Cathodic denitrification (Autotrophic)	Tubular Reactor	Sodium bicarbonate	0.146 Kg NO_3^--N m^{-3} (NCC) d^{-1}	35 A m^{-3} NCC	[Clauwaert *et al.*, 2007]
	Dual Chamber	Sodium bicarbonate	0.483 Kg NO_3^--N m^{-3} (TCV) d^{-1}	3.4A m^{-2}	[Zhang and Angelidaki, 2013]
	Dual Chamber	Sodium bicarbonate	0.41 kg NO_3^--N m^{-3} (NCC) d^{-1}	133.3±1.0 A m^{-3} NCC	[Virdis *et al.*, 2008]
	Sediment type-Photo microbial fuel cell	CO_2 from anode	87.6% Nitrogen removal	68.5 mW m^{-2}	[Zhang *et al.*, 2011]
	Dual chamber Membrane less MFC	Sodium bicarbonate	0.186 g NO_3^--N m^{-3} (LCV) d^{-1}	0.844 A m^{-3}	[Zhu *et al.*, 2013]
	Up flow tubular dual anode system	Sodium bicarbonate	88.9 g m^{-3} NCC d^{-1} N	7 ± 0.4 W m^{-3} (NCC)	[Al-Mamun *et al.*, 2016]
	Tubular dual Cathode MFC	Sodium bicarbonate	0.20kg NO_3^--N m^{-3} d^{-1}	43 A m^{-3}	[Zhang and He, 2012]
	Baffled reactor MFC	Sodium bicarbonate	148.3±1.4g $Nm^{-3}NCVd^{-1}$	15±0.4Wm^{-3} NCV	[Al-Mamun *et al.*, 2017]
Anodic denitrification (Heterotrophic)	Single chamber	Acetate	85%	410mV	[Sukkasem *et al.*, 2008]
	Dual Chamber	Sulfide/Acetate	-	2.58 mW m^{-2}	[Lee *et al.*, 2012]
	Single Chamber	Organic substrate	48%	631 mA m^{-2}	[Velvizhi *et al.*, 2014]
	Dual Chamber	Methanol	1.26 ± 0.01 kg NO_3^--N m^{-3} d^{-1}	1314.41 ± 24.60 mW m^{-3}	[Zhang *et al.*, 2013c]
Cathodic Denitrification (Heterotrophic)	Dual Chamber (Rectangular)	Effluent from air-cathode	Influent NO_3^--N-29.6±5.3mg/l Effluent NO_3^--N 20.7±4.8 mg/l	11±7.0 A m^{-3}	[Vilar-sanz *et al.*, 2013]
	Dual Chamber	Decomposed Cyanobacteria solution	0.064±0.005 Kg TN m^{-3} d^{-1}	1.28 mW m^{-2}	[Zhu *et al.*, 2016]
	Dual Chamber	Organic matter (COD/N ratio -5)	742.95 mg/L d^{-1}	262 mV	[Ding *et al.*, 2018]

2.9 INOCULUM SOURCES IN DENITRIFYING MFC

The composition and activity of the microbial consortia in the wastewater treatment system determine the process efficiency to a very large extent. The variations in microbial consortia are also inevitable with time yet the redundancy in the microbial metabolisms allows the process to continue even after these variations. For example, the depletion of one acetate utilizing microbial population can be taken over by another population not really affecting the substrate removal kinetics. Yet, the source of microbial consortia is very important as bacterial populations in a consortium should have enough diversity to allow redundancy to operate even under highly variable conditions. For example, a denitrifying bacterial community should have effective denitrifiers which can carry out the process at continuously variable environmental conditions like batch MFC cathode wherein the pH gradually rises from acidic to alkaline. Also, to be functional in a MFC the microbial population has to be electroactive. While the majority of studies utilize activated sludge as a source of microbial consortia, and the reports on microbial community analysis ascertain the existence of high diversity indices and fairly decent denitrification rates, the scope for improvement always exist especially in nitrate removal rates.

In this regard, it is worthwhile to test different sources of microbial consortia. Some of the studies have utilized activated sludge, municipal wastewater, and sewage water as inoculum sources [Sevda *et al*., 2018]. In one of the study, microbial diversity of biocathode was increased by inoculating denitrifying cathode with different aerobic and anaerobic sludge and sediment [Clauwaert *et al*., 2007]. Denitrifying sludge was also utilized for inoculating the cathode chamber [Ding *et al*., 2018; AI mamun *et al*., 2016]. The scope of research in this regard is high as eventually, the microbes are the catalysts driving the desired reaction.

2.10 MICROBIAL COMMUNITY ANALYSIS OF DENITRIFYING BIOCATHODE

Microbial community analysis is an important aspect of the MFC studies. It also helps elucidate the most probable pathway of bioremediation. The denitrification rate depends on the activity of denitrifying bacteria which are phylogenetically and metabolically diverse and belong to more than 60 genera including representatives from the *Bacteroidetes, Proteobacteria*, and *Firmicutes* [Demaneche *et al*., 2009]. These bacteria are generally found in soil, sediment, wastewater treatment systems, and many aquatic habitats. Study of the denitrifying bacterial community in MFC has been attempted by numerous researchers. Study of bacterial community structure is very important as these are the key players in the MFC system and identifying those help in the better understanding of the system. The power generation and denitrification rate in a MFC system are largely dependent on the bacterial community structure.

Microbial community analysis is generally done using 16S rRNA sequencing method. In this context, an analysis of the microbial community in dual chamber MFC cathode revealed *Betaproteobacteria* as the dominant phylum [Chen *et al*., 2008]. Also, *Nitrosomonas* sp and *Azovibrio* were enriched in the presence of nitrate. These bacteria can oxidize ammonia to nitrite or reduce nitrite to nitric oxide. The same group analyzed the microbial community in a MFC operating for 400 days and the community dynamics was evident. The *Betaproteobacteria* were replaced by *Gammaproteobacteria* as the most abundant division in the final stage [Chen *et al*., 2010].

In another study, Microbiology of denitrifying bacterial community was characterized using 16S rRNA (cDNA) PhyloChip and fluorescent in situ hybridization (FISH). This involved isolation of the biofilm in two different conditions: 1) a loop format where the effluent from the anode is subjected to a cathodic nitrifying reactor, 2) a non-loop format where anodic and cathodic feed streams were separate [Wrighton *et al*., 2010]. The loop MFC performed better in terms of power and current output and the microbial community analysis revealed that this was

due to the high bacterial richness and diversity. The *Proteobacteria*, *Firmicutes,* and *Chloroflexi* were dominant in denitrifying biocathode. In another study, the bacterial community structure in AD biocathode was studied by 454 pyrosequencing of the 16S rRNA gene. It was found that *Proteobacteria* was the predominant phylum, and *Bacteroidet*es, *Chloroflexi*, and *Planctomycetes* were the subdominant groups [Xiao *et al*., 2015]. In a study involving autotrophic denitrification with nitrite as an electron acceptor, *Oligotropha carboxidovorans* was observed as a dominant species [Puig *et al*., 2011]. The microbial community analysis in different operating processes namely, denitrifying, denitratating, and anammox also revealed interesting observations. The bacterium *Pseudomonas Glycocaulis,* and SM1A02 were found to dominate the microbial community which indicated their versatility to carry out different processes based on the prevailing substrates and environmental conditions. Also, *Pseudomonas stutzeri* was found to be key functional microorganism [Ding *et al*., 2018]. The studies primarily suggest that while there are commonalities and differences too. These differences affect the diversity index and also the performance of the system. Upon careful analysis, it also reflects the microbial synergism wherein the survival of one population is dependent on the other even if the latter is not in significant numbers. The scope of improving the microbial community exists and this involves bringing together the best of the denitrifying bacteria to increase the efficiency of the system.

2.11 GENES INVOLVED IN DENITRIFICATION PATHWAY

The enzymatic pathway (Figure 2.11) used by denitrifying bacteria in order to carry out denitrification is comprised of four steps catalyzed by four enzymes [Zumft, 1997; Philippot, 2002]:

2.11.1 Nitrate Reductase

Nitrate reductase is the first enzyme in the denitrification pathway which converts nitrate (NO_3^-) into the nitrite (NO_2^-). Bacteria have two types of dissimilatory nitrate reductase which occupy different cellular locations. One is membrane bound (*Nar*) and another is periplasmic bound (*Nap*) nitrate reductase. The membrane-bound nitrate reductase is comprised of three subunits: (i) a catalytic subunit, encoded by *narG*, which contains a molybdopterin cofactor, (ii) a soluble β subunit, encoded by *narH*, containing four (4Fe–4S) clusters and (iii) the γ subunit, encoded by *narI*, which contains two *b*-type haems. On the other hand, *napA* and *napB* genes encode the periplasmic bound nitrate reductase which is a heterodimer. NapA is the large subunit which contains a molybdopterin cofactor catalytic subunit and a (4Fe–4S) cluster. NapB is a c cytochrome.

2.11.2 Nitrite Reductase

The reduction of nitrite (NO_2^-) to nitric oxide (NO) is a very important step in this pathway. It is catalyzed by two enzymes a copper (*nirK*) and a cytochrome cd1-nitrite reductase (*nirS*).

2.11.3 Nitric Oxide Reductase

Nitric oxide reductase is responsible for converting nitric oxide (NO) to dinitrous oxide (N_2O). This enzyme is found to be a complex of two subunits encoded by the *norC* and *norB* genes. The *norC* gene is responsible for encoding of the membrane-anchored c-type cytochrome and *norB* gene encodes the cytochrome b subunit which is consist of 12 transmembrane regions.

2.11.4 Nitrous Oxide Reductase

This is the last enzyme in the denitrification pathway which converts nitric oxide (N_2O) to nitrogen gas (N_2). The nitrous oxide reductase is comprised of two similar subunits and contains eight copper ions. The *nos* genes are organized in three transcriptional units encoding *nosZ*, the catalytic subunit, the *nosR* gene, and the *nosDFYL* genes.

These four genes are the key molecular markers for denitrification pathway. Among all these genes, nitrous oxide reductase plays a very important role because this is the enzyme which is responsible for converting dinitrous oxide into N_2 gas. Nitrous oxide is a potent greenhouse gas and its accumulation is harmful. The highest numbers of denitrifying genes were found in the Gammaproteobacteria class. This includes *P. aeruginosa*, *P. stutzeri*, and *P. fluorescens* with 32, 33 and 20 denitrifying genes sequenced, respectively [Philippot, 2002].

A successful denitrification process, therefore, ensures complete reduction of nitrate to molecular nitrogen. Identification of molecular markers is, therefore, an important step towards ensuring the successful denitrification process. In this context, the bacterial community structure was assessed in MFC two different conditions namely heterotrophic and autotrophic denitrification [Vilar-sanz *et al*., 2013]. In this study, the functional genes of denitrification pathway (*narG, nirS, nirK, nosZ*) were used to identify the key microorganisms and found that cathodic biofilm of the MFC had the highest number of *nirS*-type denitrifiers at all the conditions and its high abundance is associated with N_2O emissions.

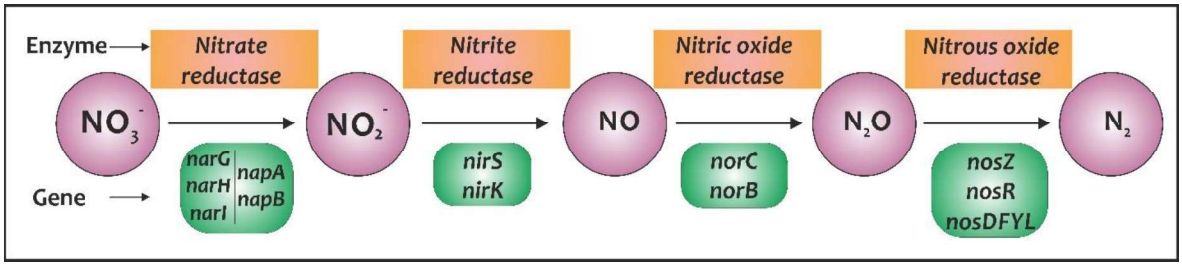


Figure 2.11: Different genes and enzymes involved in the denitrification pathway

2.12 URANIUM REMOVAL MECHANISM AND STUDIES

Nuclear wastes which are emerging from nuclear power plants are generally rich in heavy metals particularly radionuclides like uranium [Lloyd and Macaskie, 2000]. Uranium is an element of great concern due to its radioactivity and WHO has set the permissible limit of uranium disposal at 30µg/l. The contamination of uranium in water and soil can cause many chronic radiation diseases such as leukemia due to its acute toxicity [Priest, 2001]. Uranium is generally found in two states, one is soluble hexavalent state U (VI) and another one is insoluble tetravalent state U (IV). Uranium predominates in industrial effluents as a salt of U (VI) and can leach into groundwater. The conventional methods for the uranium remediation from wastewater are highly expensive and not effective [Gavrilescu *et al*., 2009]. Bioremediation of uranium contaminated groundwater is a propitious strategy for reduction of uranium in its insoluble form [Williams *et al*., 2013]. Microbial interaction with uranium has been studied by many researchers worldwide [Gorby and Lovley, 1992; Lloyd and Macaskie, 2000; Merroun and Selenska-Pobell, 2008; Lovely *et al*., 1991]. Uranium removal can involve any of the four processes which include; 1. Bio-reduction 2. Biosorption 3. Bioaccumulation and 4. Biomineralization [Newsome *et al*., 2014]. All the four mechanisms are described in Figure 2.12.

2.12.1 Bioreduction

In the absence of oxygen, bacteria use different types of electron acceptors. In the bioreduction process, bacteria utilize U (VI) as a final electron acceptor when organic matter is in abundance. Microorganism has the capability to convert U (VI) into U (IV), which could potentially immobilize uranium [Anderson and Lovely, 2002]. The bioreduction process has been achievable using both Fe (III) and sulfate-reducing bacteria because of similarities in the reduction potential of Fe (III) and U (VI) [Petrie *et al*., 2003; Ganesh *et al*., 1997]. Nitrate is generally a co-contaminant in nuclear wastewater because of the use of the nitric acid in the

processing of the uranium [Finneran *et al*., 2002; Spain and Krumholz, 2011]. Nitrate is a more favorable electron acceptor as compare to U (VI) at neutral pH [Edwards *et al*., 2007]. Therefore, nitrate is removed before U (VI). Also, high nitrate concentration promotes the re-oxidation of the U (IV) by anaerobic nitrate-dependent oxidizers [Beller, 2005]. The instability of U (IV) under oxidizing conditions renders this process unfeasible.

2.12.2 Biosorption

In the biosorption process, uranium is immobilized on the microbial cell surface resulting from ionic interactions with the ligands present on the cell surface. These include groups such as carboxyl, amine, hydroxyl, and phosphate [Beveridge and Murray, 1980; Lloyd and Macaskie, 2000]. The process of biosorption has not been utilized for the industrial application due to the fact that desorption can happen very rapidly from the cell surface and cell surface saturates quickly leaving no further scope of biosorption.

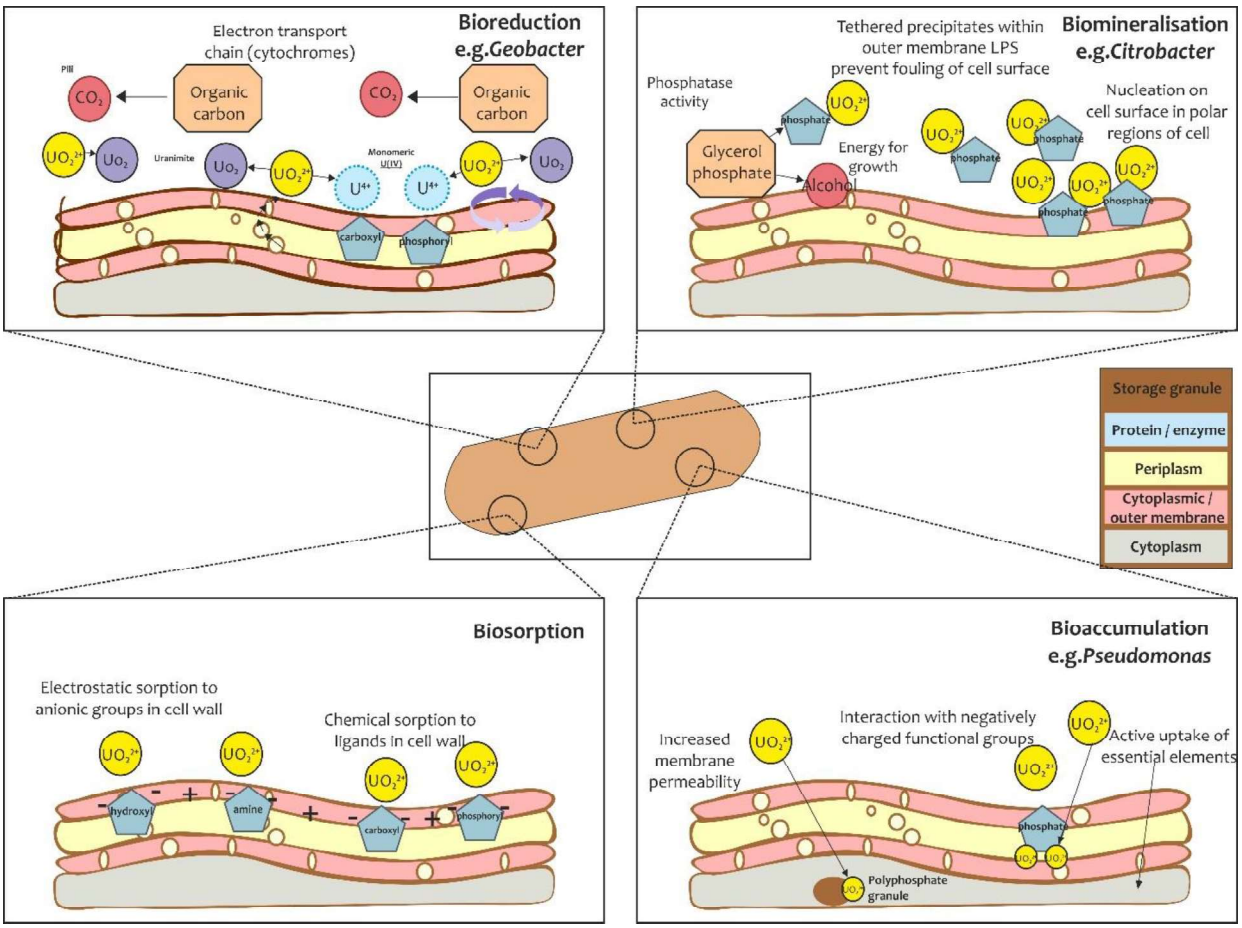


Figure 2.12: Different strategies of U (VI) bioremediation by bacteria. Source- [Newsome *et al*., 2014]

2.12.3 Bioaccumulation

Another process for uranium bioremediation is bioaccumulation. The microbial cell has the capability to accumulate many metals which are necessary for cell functioning such as Fe, Cu, Zn, Co but uranium has no significant role in the biological function of the cell. Uranium may be accumulated in the cell due to the increased cell permeability resulting from uranium toxicity [Suzuki and Banfield, 1999].

2.12.4 Biomineralization

Another process of uranium removal is biomineralization. This is a promising process when nitrate/oxygen content is high and there is a risk of reoxidation of U (IV) [Moon *et al.*, 2007]. In this process, uranium is precipitated by the interaction of phosphate (PO_4^{3-}) which is released by bacterial phosphatase activity and converted into the stable uranyl phosphate. Uranyl phosphate forms a stable complex. The process of biomineralization, however, competes with the chemical process involving phosphate addition for uranyl precipitation. The phosphate ions if added in excess can leach into the groundwater creating secondary pollution. Biological phosphate release by a phosphatase enzyme, however, is a controlled process and matches the uranyl precipitation kinetics thereby eliminating the risk of secondary phosphate pollution.

The phosphatase activity is generally present in the bacteria as phosphorous is one of the essential nutrients of the bacteria. Some bacteria also store P as polyphosphate granules. Most of the bacteria have the ability to hydrolyze organophosphates (via hydrolytic cleavage catalyzed by phosphatase), inorganic phosphate (via enzyme oxidation) and phosphonates (via cleavage catalyzed by C–P lyases). It was found that 80% of the soil microbial population including *Bacillus*, *Serratia*, *Proteus*, *Arthrobacter* and *Streptomyces* species and various fungi can hydrolytically break organophosphates using phosphatase activity [Newsome *et al.,* 2014].

The initial studies of uranium biomineralization were done using *citrobacter* sp. harboring phosphatase activity. The cells released phosphate as a polycrystalline HUO_2PO_4 (Hydrogen uranyl phosphate) at the cell surface [Macaskie *et al.,* 1992]. In this context, a flow-through bioreactor was designed in which cells were immobilized on a polyacrylamide gel. The system could achieve removal yield as high as 9 g U (VI) per gram of bacterial dry weight after a three week period. The accumulated metal, however, blocked the reactor in three weeks' time [Macaskie, 1990]. It was also found later, that uranium uptake is catalyzed by the activity of perplasmic-acid type phosphatase and amino acid sequencing of peptide fragments revealed that it was class phoN enzyme which is non-specific acid phosphatase (NSAP) [Macaskie *et al.,* 1994].

In recent years, many studies have been done on uranium biomineralization using different phosphate sources and harboring different microorganisms showing different uranium removal efficiencies. Different phosphorous sources were used such as glycerol phosphate, phytic acid, tributyl phosphate, phosphate rock etc. [Newsome *et al*., 2014].

Bacterial species such as *Serratia, Proteus*, *Bacillus Citrobacter, Rahnella*, *Pseudomonas,* and *Arthrobacter* have been identified by researchers as effective the uranium biomineralizers [Macaskie *et al.,* 2000]. Uranium biomineralization process has been demonstrated with *P. aeruginosa*-J007 strain isolated from a uranium mining site (India) [Choudhary and Sar, 2011]. Beazley et al. isolated two bacterial strains a *Bacillus* sp. and a *Rahnella* sp. from ORFRC (Oak Ridge Field Research Center, Department of Energy, US) site contaminated subsurface soil. The isolates exhibited phosphatase activity and resulted in 73-95% uranium after 120h of incubation [Beazley *et al*., 2007]. In yet another study, an anaerobic biomineralizer named *Rahanella* strain was isolated which could remove uranium in the presence of high nitrate at pH5.5 under anaerobic conditions [Beazley *et al*., 2009]. These two strains were able to grow under aerobic condition showing positive phosphatase activity and biomineralize uranium at pH 5.5 [Martinez *et al*., 2007]. Yung et al. isolated *Caulobacter crescentus* isolated from uranium contaminated sites showing the alkaline phosphatase (Pho Y) activity which can precipitate U (VI) into the meta-autunite form on the cell surface [Yung and Jiao, 2014].

Further, the phosphatase gene was cloned and expressed in *E.coli* making these engineered strains capable of uranium precipitation [Kulkarni *et al*., 2013; Basnakova *et al*.,

1998]. In another study, acid phosphatase gene encoding the phoN was expressed in *Deinococcus radiodurans* strain R1, a natural radioresistant bacterium [Appukuttan *et al*., 2006]. Uranium biomineralization is not unique to bacteria as many fungi species such as *Aspergillus niger* and *Paecilomyces javanicus* **[**Liang *et al*., 2015] utilize organophosphate**.** The model substrate used for uranium biomineralization is glycerol 3 phosphate (G-3-P) and glycerol 2 phosphate (G-2-P). These substrates act both as carbon and phosphorous source. Recently, phosphate rock was used a phosphorous source for biomineralization process mediated by a fungi *Penicillium funiculosum* [Hu *et al.,* 2018]. Bioreactors containing *E. coli* were able to liberate phosphate from phytic acid (a plant product), which could precipitate uranium as hydrogen uranyl phosphate (HUP) [Paterson-Beedle *et al.,* 2010]. Tributylphosphate (TBP), a solvent used during nuclear fuel reprocessing for the extraction of actinides was found as an another source of carbon and phosphorus in mixed cultures [Thomas and Macaskie, 1996].

2.13 Simultaneous Removal of Nitrate and U (VI)

Nitrate is a common co-contaminant in the nuclear wastes. It was found that the concentration of nitrate was 160mM in the one of the uranium contaminated sites at ORFRC, US. As discussed earlier, nitrate is a more favorable electron acceptor as compared to U (VI). Also, high nitrate concentrations promote the reoxidation of U (IV). In addition, other contaminants like iron, manganese, and calcium also compete with U (VI) as electron acceptors further lowering the rate of U (VI) reduction [Wu *et al.,* 2006]. The biomineralization process eliminates these limitations by rendering U (VI) reduction not necessary. Soluble U (VI) undergoes precipitation as insoluble phosphate salt. Also, both nitrate and U (VI) can be simultaneously removed wherein nitrate undergoes reduction supporting growth and metabolism of denitrifying bacteria which also produce phosphatase activity. The phosphatase enzyme liberates phosphate for precipitating U (VI) as uranyl phosphate. In this context, Beazley et al. found that *Rahnella* sp. Y9602 (gram-negative, facultative anaerobe) can remove nitrate and simultaneously precipitate U (VI) (uranyl acetate- 200μM) in the form of chernikovite [H_2 $(UO_2)_2(PO_4)_2$] anaerobically. The species could not carry out complete denitrification as the process was accompanied with nitrite accumulation [Beazley *et al*., 2009]. Remediation of low-level alkaline waste was also attempted by an electrochemical method in a fuel cell operating at current densities of 300-600 mA cm^{-2} and 80°C [Genders *et al*., 1996]. Technetium and uranium underwent reduction. However, in the presence of nitrate (120mM), U (IV) re-oxidized to U (VI). Re-oxidation did not happen at low nitrate concentrations [Istok *et al.,* 2004]. Further, isolation of a facultative anaerobic bacteria was carried out from ORFRC site contaminated with U (VI) and nitrate (55mM). The isolate precipitated U (VI) as uranyl phosphate while reducing nitrate [Shelobolina *et al.,* 2003; Shelobolina *et al.,* 2004].

Keeping in view the literature studies, an attempt was made to carry out the process of U (VI) biomineralization in the presence of nitrate at the MFC biocathode. The process was found to produce power, eliminate nitrate, and bioprecipitate U (VI).

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