



AFRICA CENTER OF EXCELLENCE FOR WATER MANAGEMENT

ADDIS ABABA UNIVERSITY



**Clay-based Microbial Fuel Cell System for Petroleum Refinery Wastewater Treatment and  
Green Energy generation simultaneously**

**By:**

**Kabo Benedict Jongman**

A Master's thesis submitted to the Africa Center of Excellence for Water Management, Addis Ababa University in partial fulfillment of the requirements for The Degree of Master of Science in Water Management (Water and Wastewater Technology)

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Addis Ababa, Ethiopia

Africa Center of Excellence for Water Management  
Addis Ababa University School of  
Graduate Studies

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Application of Clay-based Microbial Fuel Cell System for Petroleum Refinery Wastewater Treatment through Energy Recovery

By:

Kabo Benedict Jongman

A MASTER’S THESIS SUBMITTED TO

AFRICA CENTER OF EXCELLENCE FOR WATER MANAGEMENT

ADDIS ABABA UNIVERSITY

APPROVED BY BOARD OF EXAMINERS

This is to certify that we have read this MSc research and that in our opinion; it is fully adequate, in scope and quality, as a Master’s thesis proposal for The Degree of Master of Science in Water Management (Water and Wastewater Technology)

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## DECLARATION

I, Kabo Benedict Jongman, declare that this MSc research titled “*Clay-based Microbial Fuel Cell System for Petroleum Refinery Wastewater Treatment and Green Energy generation simultaneously*” has been developed by me and has not been submitted to any other institution for award of any academic qualification. The content of the proposal has not been plagiarized and where works of other researchers have been used, they have been appropriately cited.

**Candidate's Name**

**Signature**

**Date**

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## ABSTRACT

The increasing demand for energy and the growing concern for environment pollution have led to the exploration of sustainable and efficient methods for wastewater treatment coupled with energy recovery. Microbial Fuel Cell have emerged as a promising technology to convert organic matter in wastewater into electricity. This study was focusing on the application of clay based (MFC) microbial fuel cell as a novel approach to treat petroleum wastewater while simultaneously generating electrical energy by using microorganisms extracted from diesel oil-contaminated soil collected from old garage. In this study, microbial fuel cells (MFCs) were constructed using low cost ceramic cylindrical pot (900ml) as an ion exchange membrane. An inoculum derived from oil-contaminated soil, 10% of the volume of the anode inoculum was fed to the anode chamber for MFC1, MFC2 and no inoculation was added to the MFC control. The cathode chamber was filled with 1M potassium ferricyanide in a 1M phosphate buffer solution at a ratio of 1:1. All MFCs were operated in batch mode with synthetic petroleum wastewater containing (810 ml) for 30 days for open circuit voltage and at an external resistance of 10 $\Omega$ , 100 $\Omega$ , and 1000  $\Omega$  for 14 days for closed circuit voltage. Carbon cloth (projected surface area of 546cm<sup>2</sup> and 455.22 cm<sup>2</sup>) was used as both the cathode and anode respectively. The treatment efficiency of clay-based MFCs were evaluated by the removal efficiencies of COD and BOD. For MFC1, BOD removal efficiency of 89%, COD removal efficiency was 96%, and maximum daily open circuit voltage of 188.92 mV. MFC2 demonstrated BOD removal efficiency of 91%, COD removal efficiency of 98%, and a maximum average daily open circuit voltage of 210.35 mV. In contrast, MFC control displayed lower removal efficiencies with BOD removal efficiency of 49%, COD removal efficiency of 30%, and a maximum average daily open circuit voltage of 58.28 mV. The maximum power density achieved was 9.99 $\mu$ W/cm<sup>2</sup> for the highest performing MFC2. These results highlights the effectiveness of MFC1 and MFC2 in terms of organic matter removal and power generation compared to MFC control, displaying the potential of clay-based MFCs for wastewater treatment and energy production. This research just laid a foundation for the use of clay based MFC in treatment of petroleum wastewater and energy recovery, more studies have to be carried out to optimize and maximise its efficiency.

**Keywords:** Biological Oxygen Demand, Chemical Oxygen Demand, Clay-based membrane, Energy recovery, Green energy, Microbial Fuel Cell

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## **LIST OF ABBREVIATIONS**

BES-Bio electrochemical Systems

BOD-Biological Oxygen Demand

CCV-Closed Circuit Voltage

COD-Chemical Oxygen Demand

DCMFC-Dual Chamber Microbial Fuel Cell

IEM-Ion Exchange Membrane

MEC-Microbial Electrochemical Cell

MFC-Microbial Fuel Cell

MLSS-Mixed-liquor Suspended Solids

OCV-Open Circuit Voltage

PEM-Proton Exchange Membrane

PWWT-Petroleum Wastewater Treatment

SCMFC-Single Chamber Microbial Fuel Cell

## CHAPTER ONE

### 1. INTRODUCTION

#### 1.1. Background

According to the United Nation's world, population prospect in the year 2022 the global population is growing at 0.88% per year in 2023, which means the estimated population growth is about 70 million people per year. Such population growth means there will be increase in the demand of goods and services to cater for the needs of humanity. Due to this, there is an increase in industrialization and worldwide demand for the hydrocarbons have escalated to soaring levels, leading to the mushrooming of petroleum industries and demand for petrochemical products. For decade's crude oil and petroleum products, have the largest share in the gross inland energy consumption amongst the 27 European Union member states (Energy Data Edition, 2020). Despite the decrease in the production of oil as it is a non-renewable resource, crude oil and its derived products still play a major role in the energy statics globally (Basu & Sun, 2020). In spite of their importance to everyday life, the petroleum industries have devastating impacts on the environment, human life and aquatic life through contamination due to discharges, spillages, waste generation and even transportation of this petroleum product (Eldos et al., 2022). Due to the mentioned risk exposure to contamination, the soil and water systems get affected. The spillages are subsequently carried as runoff to different aquatic systems including surface water and underground sources (Palaniandy & Feroz, 2018). This contamination deposits different pollutants which consists of different hydrocarbons such as phenols, cyanide, benzene and metal contaminants such as Chromium, Lead, Zinc, Copper and Vanadium to aquatic environments (Aljuboury et al., 2017). The treatment of wastewater from petroleum refineries presents significant environmental challenges due to the complex mixture of pollutants it contains, including hydrocarbons, heavy metals, and phenolic compounds. These contaminants can adversely affect aquatic ecosystems and pose risks to human health (Shah et al., 2016). Traditional treatment methods, such as physical and chemical processes, often fall short in terms of efficiency and sustainability due to the high cost and complex technology involved. Consequently, there is a growing interest in innovative biological approaches for wastewater treatment, particularly those that manipulate microbial metabolism processes and the generation of green energy.

Based on the shortcomings and gaps that were exposed in this section, MFC was found to be a more attractive alternative to treating petroleum wastewater. This is because through using the MFC technology there is a simultaneous treatment and energy generation. MFC also have enhanced removal of organic compounds. The conventional methods such as physical, biological and chemical methods currently used produce a large volume of sludge, which create environmental problems(Varjani, 2020). On top of that, the microbial fuel cell has the potential to recover valuable resources such as metals and nutrients (Fang & Achal, 2019). Therefore, the bone of contention for this thesis is using the clay based microbial fuel cell as a novel approach to treatment and generation of energy from refinery petroleum wastewater. Clay based because clay was used as an ion exchange membrane for the microbial fuel cell.

Clay materials, are known for their abundant availability and unique physicochemical properties, have been explored as components of MFCs. They can enhance microbial adhesion, provide structural stability, and improve ion exchange, which is crucial for the efficiency of MFC systems (Baker et al., 2020). The incorporation of clay into MFC designs not only supports the electrochemical processes but also may enhance the treatment efficiency of complex wastewaters such as those generated by petroleum refining (Ahn et al., 2019). The attributes that are needed to make a good membrane separator are effective ion transportation, ability to operate in a wide range of pH ranges, operational durability and efficient microbial interactions. Traditional proton exchange membranes are usually expensive and sometimes fall short to serve their functions, thus in this paper clay was used as a cheaper and prominent membrane separator that can be used in the MFC to achieve maximum power recovery and water treatment. By far from the studies of the different literatures, shows that clay has all the desired traits because of its double nanosheet structure which has a negative charge which aids in attracting and transporting hydrogen ions to the cathode chamber, high resistance to complex environment and enhanced conducive microbial environment. The incorporation of clay may facilitate the immobilization of microbial communities that adapt at degrading petroleum hydrocarbons.

Research indicates that certain microbial species can utilize hydrocarbons as carbon sources, thereby reducing the concentration of harmful pollutants in the wastewater (Vinas et al., 2005). The synergy between the electroactive microbial communities and the clay matrix may further enhance the degradation rates and overall treatment efficiency of the MFC. .As result this thesis was focused in using the clay based MFC to treat petroleum wastewater and to generate green energy.The use of clay-based MFCs for petroleum refinery wastewater treatment is relatively

novel. Therefore in this research paper, the MFC technology was used to treat petroleum wastewater and simultaneously recover green energy from the process as an attempt to attaining both ecological sustainability and reducing dependency from the use of fossil fuels like petroleum products as energy sources.

## **1.2. Statement of the problem**

The petroleum industry is one of the largest and most important industries in the world, providing fuel and energy to various sectors. However, the production and refining processes of petroleum result in the generation of large volumes of wastewater that contain high concentrations of organic compounds, heavy metals and other pollutants. The discharge of untreated petroleum wastewater into the environment can have severe ecological and human health consequences. Conventional wastewater treatment methods, such as biological treatment and chemical precipitation, have limitations in effectively treating petroleum wastewater due to its complexity and toxicity. The reliance in fossil fuels for energy even by some conventional treatment methods leads to secondary pollution. Therefore, there is a need for innovative and sustainable technologies that can efficiently treat petroleum wastewater and minimize its environmental impacts while recovering green energy. The microbial fuel cell have emerged as a promising technology for wastewater treatment, as they can simultaneously treat wastewater and generate electricity through metabolic activity of microorganisms. MFCs utilize the electron transfer capabilities of bacteria to convert organic matter into electrical energy. However, the low conductivity of the wastewater and high internal resistance of the system often limit the performance of MFCs. To overcome these limitations clay based membranes was used as a potential solution. Clay minerals have unique properties, such as high surface area, ion exchange capacity and electrical conductivity, which can enhance the performance of MFCs. By incorporating clay-based membranes into MFCs, it is possible to improve the efficiency of organic matter degradation, increase power generation, and enhance the removal of pollutants from petroleum wastewater. Despite the advantages of using clay-based membranes in MFCs for petroleum wastewater treatment, there is a lack of comprehensive research and understanding of MFC constructed from a locally available clay as MFC construction materials inoculated with microorganisms extracted from diesel contaminated soil from Addis Ababa. Therefore, it is a perfect timing for the evaluation of the locally available clay-based microbial fuel cells inoculated with microorganisms extracted from diesel contaminated soil for an economical and sustainable way of treating petroleum wastewater.

### **1.3. Research objectives**

#### **1.3.1. General objective**

The general objective of this research was to:

-To construct a locally available clay-based microbial fuel cell technology and inoculated with microorganisms extracted from diesel contaminated soil to treat refinery petroleum wastewater and generate green energy simultaneously

#### **1.3.2. Specific objectives**

The specific objectives are to:

1. Prepare and characterize synthetic petroleum wastewater for enrichment of petroleum degrading microbial consortia from oil-contaminated soil.
2. To extract, enrich and characterize petroleum degrading microbial consortia from an diesel oil polluted soils for MFCs establishment.
3. Evaluate the efficiency of the clay-based MFC inoculated with extracted microorganisms for the removal of degradable organics from petroleum wastewater
4. Evaluate the capability of the clay-based MFC in producing green electricity from petroleum wastewater

### **1.4. Research questions**

The following are the research question that will help in addressing the objectives:

1. Can petroleum wastewater be degraded using homemade clay-based MFC constructed from locally available clay?
2. Are microbes contained in the contaminated soil exoelectrogenic?
3. How effective is the clay-based MFC in the pollutant removal and power generation of the clay-based MFC for treating petroleum wastewater?

### **1.5. Significance of the study**

The outcome of this study will help to solve the problem of petroleum wastewater pollution using a cheaper alternative of a clay-based MFC, cheaper because unlike traditional Microbial Fuel Cells clay will be used to make a separation membrane. Since this is the first time, a clay based MFC is used to treat petroleum WWT, the research findings will contribute as a foundation and source of information to the scientific community for further improvements.

Policy makers, practitioners, can also use the output of this study and private sector to consider the use of clay based Microbial Fuel Cell technology as a sustainable and economically feasible way of treatment of wastewater and energy recovery.

### **1.6 Scope of the study**

This study is limited to the preparation of a clay based Microbial Fuel Cell with a ceramic membrane as an alternative to the traditional ion exchange membranes for removal efficiency of COD and BOD from synthetic petroleum wastewater and generation of electricity. It also focuses on extracting exoelectrogens from oil-contaminated soils as the source of inoculum for the MFC.

## CHAPTER TWO

### 2. LITERATURE REVIEW

#### 2.1. Background on petroleum industry and petroleum wastewater

Petroleum industry is a very significant industry throughout the entire human civilization, without it the world's economy and welfare would tremble. Despite the advances in finding the alternative source of energy petroleum industry is the bone of contention when it comes to fuelling the world and her industries (Corporation & Road, 2012). Petroleum industry is so significant in the modern society that it still drives economies, geopolitics and even wars because of it being the pillar for other industries such as the transportation, construction and food industries. During the pre-modern era, crude oil was used to caulk boats, lubricate cartwheels and impregnate wood for torches. The main source of energy during this era was natural gas which was used for heating and cooking. In the 19<sup>th</sup> century engineers and entrepreneurs started to drill with a deliberate intention of harvesting petroleum giving birth to the modern industry was born. The main use of crude oil by the time was for harvesting kerosene, which was used as a fuel for lanterns in-order to replace the whale fat, which was traditionally used, but very scarce to find (*Oil and Gas Production Handbook An Introduction to Oil and Gas Production, Transport, Refining and Petrochemical Industry*, n.d.). As the hands of time continued to moving the invention of motor engines lead to the creation of automobiles, which will change the whole of human civilization. This invention lead to a shift even in the petroleum industry as now the crude oil was mainly extracted for gasoline and other transportation fuels and lubricants, which were at the 19<sup>th</sup> century treated and disposed as waste. Gasoline production dominated the petroleum industry in 1915. During the 20<sup>th</sup> century United States of America lead the world and adopted the automobile technology which lead to a vast mushrooming of the petroleum industry (Harayama et al., 1999). As time proceeded and advances in technology were made, the use of automobile spread all over the world and the discovery of other useful products from crude oil made the petroleum industry to grow in to a prominent and large industry that exist today. Even though the petroleum industry is very crucial in developments and other industries it comes with severe implications on the environment and ecology at large (Harayama et al., 1999). Petroleum industries are one of the main pollution contributors in the world. The pollution from the petroleum industry ranges from air pollution, water pollution and soil contamination. This industry is responsible for the disposal of various kinds of hazardous substances which are carcinogenic, phytotoxic and

genotoxic .About 65.7% of the discharge is from municipal and industrial water, urban and river runoff ,oceanic dumping 26.% are contribution of transportation, accidents ,blasting and 8.5% is from the coastal refineries, offshore production. Relying on these fossil fuels especially petroleum products have some several disadvantages which continue to threaten the humankind existence and the ecology at large. According to Corporation & Road in 2012 the petroleum industry is a water consuming industry both in the extraction process and the refining process. It is estimated that refining process generates about 1.5 times the volume of waste water for each barrel of crude oil processed (Eldos et al., 2022). Petroleum hydrocarbon released in to the sea, normally during transportation, leading to the pollution of several sites, and can eventually reach the coasts. Oil spills ranging from low level discharges to catastrophic accidents threatened coastal environments; large spills commonly are followed by clean-up efforts, but complete containment is rare(Corporation & Road, 2012) . As solubility of petroleum hydrocarbon in water is generally low, certain fractions of it float in water and form thin surface films, which will facilitate agglomeration of particles and natural organic matter, and impact on oxygen transfer (Hazaimeh & Ahmed, 2021). Other heavier fractions will accumulate in the sediment at the bottom of the water, which may affect bottom-feeding fish and organisms (Freije, 2015).Therefore it is evident that the petroleum industry leads to contamination of aquatic environments. Petroleum as a raw material consist of different contaminants, which are mainly hydrocarbons (Basu & Sun, 2020).This hydrocarbon constituents includes crude oil, diesel, kerosene and heavy oil.

**Table 1:Some parameter of petroleum wastewater according to different studies**

Parameters	The references				
	(Nikolopoulo u et al., 2010)	(Hazaimah & Ahmed, 2021)	(Chouksey, 2004)	(Uhegbu et al., 2011)	(Freije, 2015)
pH(mg/L)	7	6.7	8.3	6.5-6.8	7.19-9.22
COD(mg/L)	1343	450	1250	500-1000	220
BOD(mg/L)	846	174			
TSS(mg/L)	74	150	150	90-300	
Ammonia(mg/L)					
Phenols(mg/L)				20.0	
Sulphides(mg/L)				15-30	
Turbidity(mg/L)	83			150-350	20
Oil & Grease(mg/L)	240	870		400-1000	
TDS(mg/L)				3000-5000	
TOC(mg/L)	398	119			

## 2.2. Environmental and ecological impacts of Petroleum wastewater.

Crude oil produces different significant products which are very crucial for other industries which make the backbone of many economies ,some of the significant amounts end up one way or the other in the wastewater leading to contamination of both the aquatic environment and the soils(Hazaimah & Ahmed, 2021).This contaminations leads to some consequences which are life threatening both the human beings ,plants and marine life and the ecological balance as all organisms are linked by the food web(Chouksey, 2004).Improper handling of such waste water leads to bioaccumulation which means the chemicals end up being in animal cells and spreading in the ecosystems. According to Bryan & Ejumudo, in 2019,

petroleum pollutants has the following effects on the land, plants and human beings. Mallesh & Krishna in 2022 explained that crude oil as a contaminant leads to an increase in total carbohydrates, proteins, amino acids and proline in shoots which reduces the amount of chlorophyll contents in the leaves (Nikolopoulou et al., 2010). On top of that it reduces the total carbohydrates in the roots of plants called Jojoba (Simmondsiachinesis). Crude oil pollution has an adverse effect on growth, yield and leaf chlorophyll content. According to Ahmed (2018) study the grain yield of grain yield was significantly reduced at 95% level in areas which are in close proximity to the petroleum industries and on top of that Diesel, significantly affected the germination, GSI and seedling growth of *Schinusterebinthifolius*. Poses threats to the survival and development of plants *Vigna unguiculata* (Njoku et al., 2018). Plant growth parameters were affected adversely in Eggplant.

A number of the more soluble aromatic components, particularly the alkyl benzenes, have extremely low taste and odour thresholds in water and can render drinking-water unacceptable to consumers at relatively low levels of contamination (Freije, 2015). Several such components have been considered separately in the Guidelines: benzene, toluene, ethyl benzene, and xylene. Other components of petroleum products will also be detectable by odour and/or taste in drinking-water at concentrations of a few micrograms per litre (Harayama et al., 1999). The differing chemical and physical properties of petroleum hydrocarbons mean that they will behave differently in the environment. Persistence of petroleum hydrocarbon compounds in the environment is reflected by physical properties such as volatility, so that generally the persistence increases as the boiling point increases (Faustine, 2008). The main processes affecting environmental concentrations are volatilization, biodegradation and dissolution in water. Only a small proportion of the hydrocarbon constituents of petroleum products will be significantly soluble in water. The hydrocarbons present in contaminated drinking water will not, therefore, reflect the hydrocarbon composition of the petroleum oil. Most petroleum hydrocarbon mixtures contain very low concentrations of PAHs (Lami et al., 2021). A number of these are considered above under the appropriate carbon fractions. The major concern regarding PAHs is the potential carcinogenicity of some molecules (IPCS, 1998a). Benzo (a) pyrene and benz (a) anthracene are classified as probable human carcinogens, and a small number have been shown to induce skin tumours in skin painting studies in laboratory mice. Benzo (a) pyrene is normally considered to be the most potent carcinogenic PAH, but the carcinogenic potency of most PAHs is not well characterized. In conclusion the findings from other studies show that the contamination from the petroleum industry is really needs to be

given thorough attention as it has fatal effects on the environment and her organisms including human beings.

The effects of petroleum waste in human beings can be through ways in which they are assimilated to the body, this classes include the following inhalation, ingestion ,ocular exposure, dermal exposure ,neurotoxicity and nephrotoxicity .Inhalation refer to the process of breathing oxygen and breathing out of carbon dioxide by vertebrae organisms .During the inhalation process air which is a mixture of different gases is inhaled in to the lungs to oxygenate the blood for metabolism process(Faustine, 2008).Therefore, in situations where there is severe pollution of petroleum waste, some constituents of crude oils including the volatile hydrocarbons like PAH and benzene can be inhaled in to the lungs of humans with other gases. Inhalations of such gases has both long-term and short effects on the body, which include headaches, dizziness, drowsiness and euphoria (Aspects et al., n.d.). Aspiration of the petrochemical pollutants into the lungs causes pneumonitis with choking, coughing, breathlessness and cyanosis .The other way in which petroleum waste can end up in to the human boy is through ingestion. Ingestion is the biological process of getting food in to the body through the mouth. As mentioned in the early chapters of this paper petroleum waste pollution ends up contaminating the soil and water resources. In the soil some of the toxic chemicals are absorbed in the crop cells when from the soils. Water contamination leads to bioaccumulation and bio magnification of the substances in the aquatic life like fish which is eaten by human being (Lami et al., 2021).So by such routes petroleum waste end up finding their ways in to the human body. Oral intoxication may result in diarrhoea, Irritation of the aero digestion and gastrointestinal tracts. Petroleum waste can end up intoxicating the human body through ocular exposure, which is exposure through the eyes. Petroleum waste pollutions is a major issue when it comes to water pollution, water being a necessity of life most human activities are centred around water. For example swimming, fishing and drinking etc. Some communities especially those located near water bodies use this water even for bathing and drinking. In such cases there are high chances that the water comes in to contact with their eyes.

If the water is contaminated with petroleum waste water use face the threats of optical irritation and even blindness in situation where the contamination is severe. Eye exposure may also lead to hyperaemia. Human can also be exposed to petroleum waste intoxication through dermal contact. Which refers to the direct skin contact with the pollutants .Hazaimah & Ahmed in their research in 2021 showed that petroleum waste is in high concentrations in areas which are

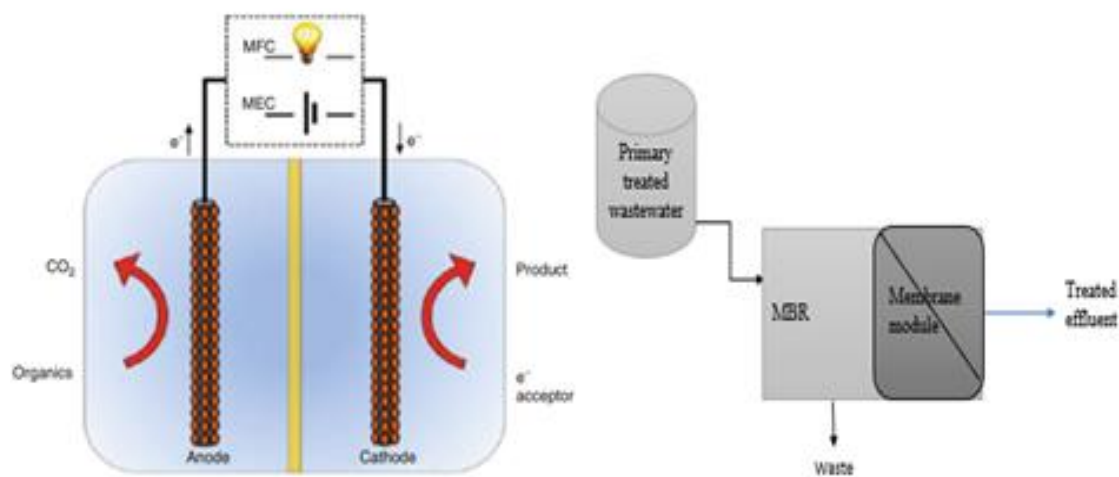
located in close vicinity with the oil drilling plant, storage tanks or refinery factories .Due to spillages, expelling of waste water from the petroleum industry with no treatment the aquatic environment ends up being polluted. Communities living around water bodies have developed a dependency in the water bodies as a way of life and centre of their socio economic activities. Through activities like swimming ,fishing ,bathing and washing .In situation where the water is polluted those people are at risk of getting in direct contact with the polluted water and this may lead to transient pain with erythema, blistering and superficial burns on the skin. Some individuals may especially susceptible to those effects .Excessive exposure under poor personal hygiene may also worsen the situation and lead to oil ache and folliculitis. Lastly intoxication of the petroleum waste to human may lead to neural disorder which includes drowsiness, neurasthenia and altering the sensory motor speed. In a study to develop risk caused by exposure of carcinogenic PAHs, MAHs and heavy metals existing in the soil due to petroleum waste pollution with simulation risk analysis software (RECOLANv12), results showed that the estimated cancer risk is higher than the US EPA acceptable standards. Which clearly indicates that petroleum pollution is a devastating healthconcern (Mariana et al., 2017).

### **2.3. Fundamental principles of bio electrochemical systems**

Fundamentally, BESs are electrochemical systems that involve anodic and cathodic reactions combined with the physiological process by microorganisms. Though MFC is the basic technology, together with more other derived technologies such as the microbial electrolysis cells (MECs), microbial desalination cells and the microbial electrosynthesis cells, such systems are called the bioelectrochemical systems (BES)(Foorginezhad et al., 2023). In BES sytems bio mediation occurs which refers to the use of microbes to degrade toxic waste in to less harmful or untoxic substances. Bioelectrochemical remediation includes a combination of bioremediation with electrochemical remediation, in which bioremediation is achieved through the use of electron active bacteria (EAB) to facilitate the exchange of electrons with the electrodes and subsequently leading to the generation of bioelectric field. BES can sometimes be coupled with Bioelectrokinetics through reverse polarization. This generally refers to after harvesting the bioelectric energy it is reversed back in to the wastewater or polluted soil to enhance the degradation of pollutants.

Bioelectrokinetics is a remediation technology through which direct current from a DC power source is applied either to the wastewater or contaminated through electrodes in order to enhance the degradation of toxins. This is because the weak current that will be passed through

the soil or the wastewater through the electrodes increases the temperature and leads to the production of bio surfactants by micro-organisms, thus facilitating a more feasible surfactants and substrate interaction. Bio electro kinetics degrade dissolved organic and inorganic substances through three main processes which are electro migration, electroosmosis and electrophoresis. Electromigration is one of the main processes that are utilized in electrokinetics and it involves degradation of toxins through the attraction of ions to oppositely charged electrodes. Electro osmosis on the other hand is the refers to be removal of toxins through the migration of pore fluids through the soil and lastly electrophoresis refers to the remediation through the transportation of biological colloids through the electrodes(Lan et al., 2023).The microbial fuel cells (MFCs) and MECs are the two most advancing technologies within BESs that can produce electricity, hydrogen or other useful chemicals while removing pollutants from wastewater (Fig.1). In fact, MFCs do have greater applications beyond wastewater treatment that include their use as biosensors (Taylor et al., 2014).



**Figure 1: Schematic overview of Microbial Fuel Cell or Microbial Electrical Cell configurations in Bio electrochemical System (Niju, 2023)(Left) and a simple membrane bioreactor as applied for wastewater treatment (Right)**

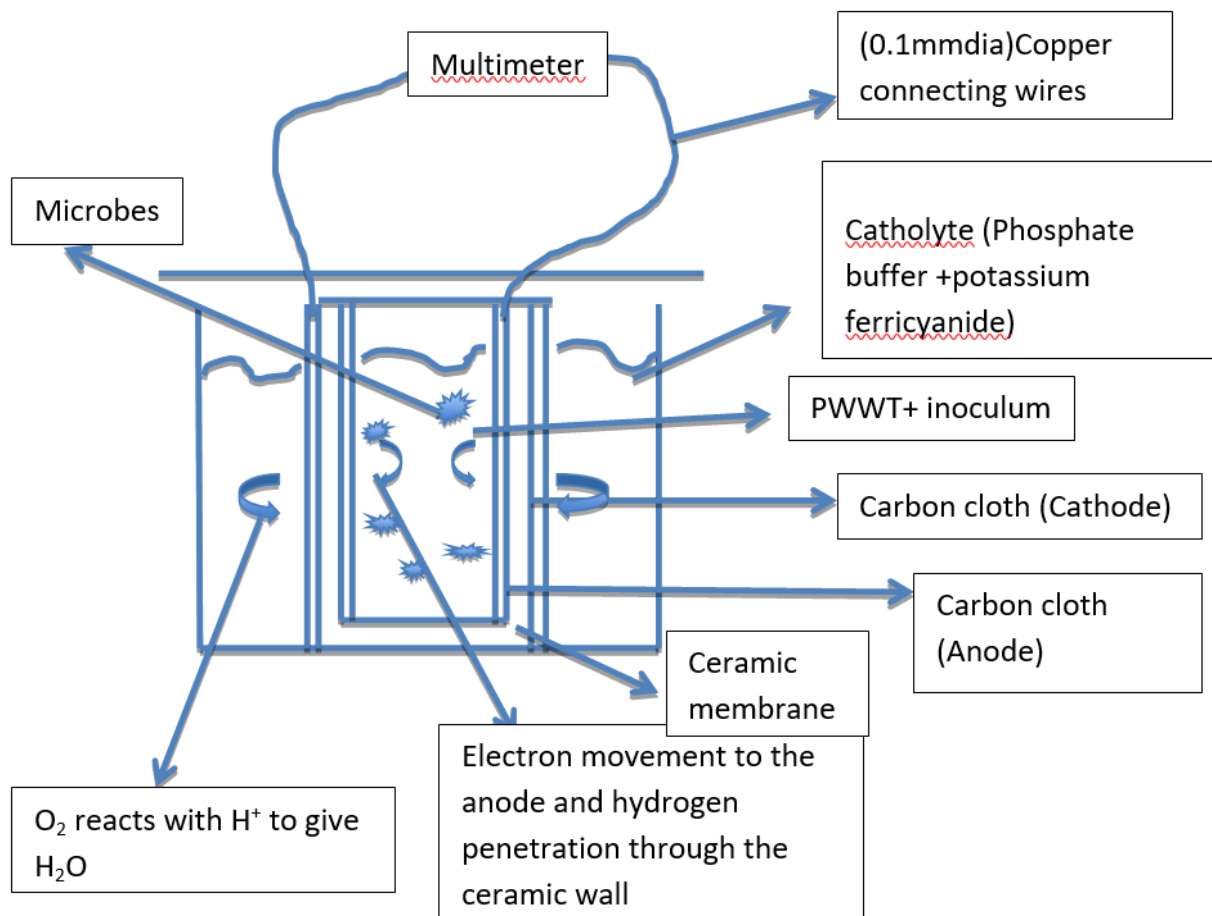
### 2.3.1. Overview of the BES and functioning of the MFC

The use of microorganisms to generate energy gained its recognition from the 19th century after being introduced by a scientist called Michal C. Potter in 1911(Maqsood et al., 2022).In 1962 Davis and Yabough took the concept and made a microbial fuel cell using a hydrocarbon as feedstock for the microorganism. They used an MFC with glucose as a substrate, and

microorganisms *Escherichia coli* or yeast in the anode, and investigated the catalytic effects of thionine and resorufin as a redox mediator. As Kim, et al. did in their work in which they evaluated an MFC type biosensor for lactate using a metal-reducing bacterium without an electrochemical mediator. Then it was recognized that the mediators were not required and consequently increased the interest in MFCs. In the following years, the interest in MFCs continues to rise. It is observed that between 2000 and 2005, published MFCs research did not exceed 100 articles per year, and the trend remained upward until 2007 with 177 publications. This slightly increase could be attributed to the disclosure of the feasibility of working MFCs without the use of chemical mediators. After that, a tremendous increase is observed in the following 17 years, in 2021 with 1,383 and in the first two months of 2022 with 392 published articles. This confirms that the research and technology have captured the attention of the scientific community, due to the capability of the MFCs to transform organic waste into electricity. The growing participation of the scientific community in the development of MFCs is also reflected in the increase of MFCs review articles. It is evident from the findings of (Maqsood et al., 2022; *Microbial Fuel Cell for Wastewater Treatment: Heavy Metal Removal, Sewage Sludge Treatment, and Its Potential Application in Wastewater Reuse in Irrigation*, n.d.) that MFC have always been an interesting topic from past years. Gajda and co-worker in 2017 also concur with the study conducted by Oh and his colleagues in 2010 by explaining that Microbial fuel cell has always been an interesting topic since the 20th century through using the bacteria to get energy from feedstock which was then used as glucose, lactose or wastewater. (Alzate-gaviria, n.d.) stated that the use of the MFC technology to treat wastewater started in 2014. The main reason that encouraged this step was the potential for energy saving from the wastewater treatment aeration and sludge treatment. In addition some of the attractive factors which lead to MFC being adopted in wastewater technology was that it produces less sludge as compared to activated sludge and aerobic systems. (Tsekouras et al., 2022). Throughout the years, MFC technology have been used to generate energy from feedstock, waste water, metal recovery, nutrients and salt reduction. With a wide variety of different wastewater it can degrade such as domestic, industrial (brewery, distillery and dairy), municipal and agricultural wastewater. The Microbial Fuel Cell technology have been used to even fuel some devices in real life application this devices include wireless sensors, oceanographic instruments and self-powered biosensors.

### **2.3.2. Key components and the working principles of the Microbial Fuel Cell(MFC)**

Microbial Fuel Cell(MFC) is a an electrochemical device that uses the microbial metabolism activities to generate energy from the substrate which can be different substances including glucose solutions, wastewater, swine waste and urine(Gajda et al., 2017). Microbial fuel cells (MFCs) are chemical reactor systems that generate electricity from the biodegradation of organic materials with the help of suitable microbial substrate (Tsekouras et al., 2022).Alzategaviria in 2021 explained MFC as electrochemical systems in agreement with Gajda's study in 2017.All the definitions of the MFC from different articles correspond with the topic but the preferred definition from the combination of all definitions would be ,MFC refers to a process of creating a conducive environment for a particular inoculum of microorganism through feeding it with petroleum wastewater and maintaining other conditions so that the microorganism degrade the pollutants and release protons and electrons which are then harvested to make electrical energy .Generally the microbial fuel cell is made up of electrodes which in a single chamber MFC are not separated but separated by a proton exchange membrane in a dual chamber MFC set up. The electrodes of the MFC can be made of different material for example metals, clay, graphite, coal, activated carbon or carbon paper .The function of the electrodes is to interact with the electrolyte and carry electron with the aid of an external connecting wire. The flow of electrons leads to the flow of current. MFCs can be dual chambered or single chambered .In a dual chamber MFC the cathode and anode are separated by a membrane separator which is the only distinction from the single chambered MFC. Traditionally, the membrane separator is usually made of Nafion, salt bridge paper, clay or some polymers(Tan et al., 2020). The main aim of this membrane is to firstly separate the cathodic chamber and the anodic chamber the reason being in a dual chamber MFC the anodic side is kept in anaerobic conditions therefore oxygen interaction with the microorganisms can lead to microbial shock which can greatly reduce the efficiency of the MFC both in energy generation and treatment as shown in figure 2. Secondly the membrane separator acts as a bridge for protons to cross to the cathodic side from the anodic side(Gajda et al., 2017b). The anode chamber is kept under anaerobic condition and this is the chamber through which wastewater or the substrate is feed to the MFC with a certain inoculum of relevant microorganisms, which will be acting on the wastewater to release protons and electrons (Roy et al., 2023). The cathode chamber will be filled with distilled water and kept inn aerobic conditions to encourage the reaction of hydrogen and oxygen to for a water molecule hence completing the circuit for the electrons to continuously flow and current.

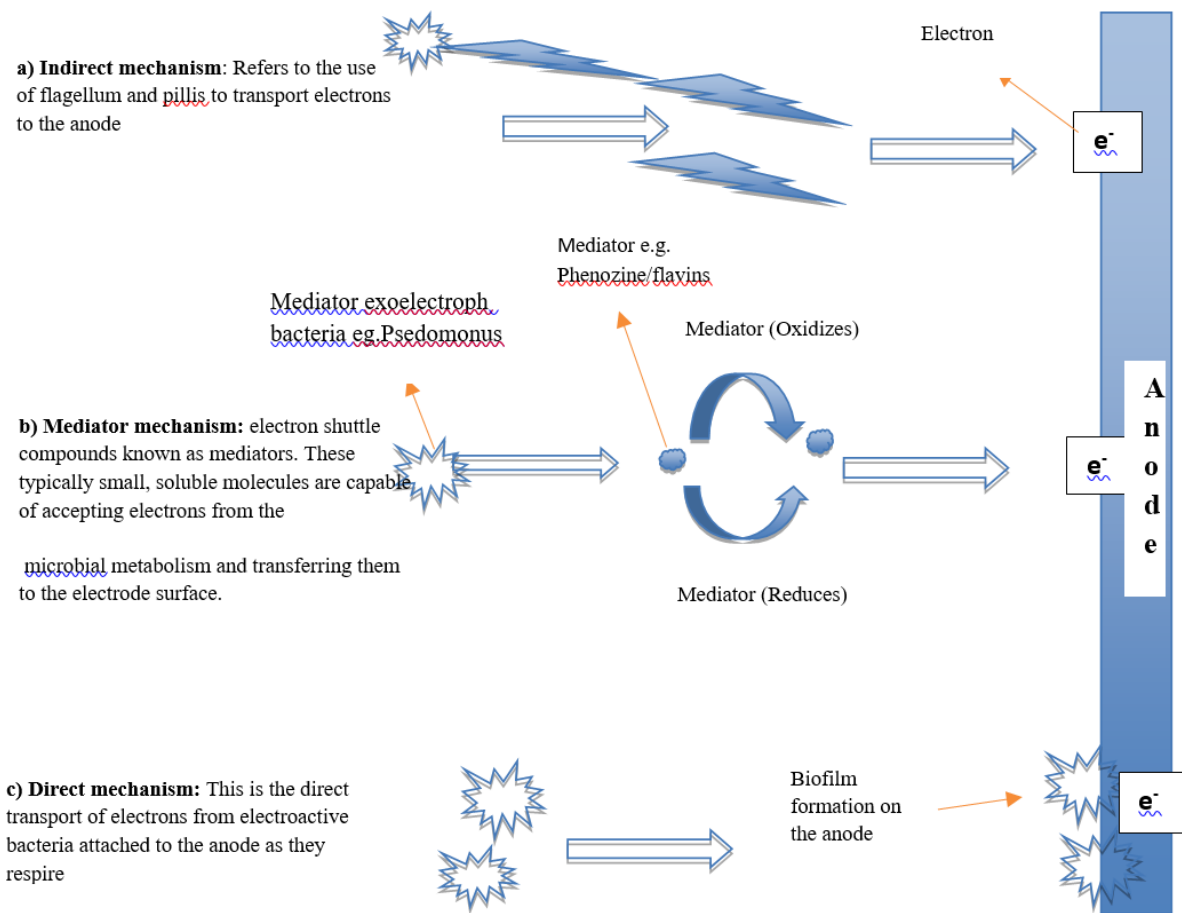


**Figure 2: Clay based Microbial Fuel Cell design**

(Bahan et al., 2011) stated that there are mainly 5 groups of microorganism which fall within the umbrella of exoelectrogens group. These microorganisms include firmicutes, proteobacteria, Acidobacteria, fungi and algae. There are also some organisms which are known to aid as electron exchange with the electrodes such as Clostridium, Geobacter sp. and Aeromonas Hydrophilia. This organisms also show electricity producing properties. In the MFC microbes degrade different pollutants such as organics, inorganics and heavy metals complexes to produce electron and protons as they respire. However, in some instances, microbes can transfer the electrons in an insoluble form of electron acceptors, For example Geobacter has pili which act as an electron conductor. It is preferable that microbes grow on the anode and provide electrons directly to the electrode rather than using the microbe that rely on electron acceptors to transfer electrons. This microorganisms which can release electron in to soluble form include Shewanella, Rhodospseudomonas palustris, Thermicola poten, Escherichia Coli

and *Geobacter*. This unique property of the electro-trophs make it possible for the impurities like heavy metals to be removed by the process of reduction and some through oxidation process (Malik et al., 2023). Some microbes can act as electron shuttles and generate electrons from the electrodes to enhance reduction of fermentation and inorganic compounds. These shuttle bacteria include *Acinetobacter calcoaceticus*.

There are many types of microbes that have the ability to degrade organic, inorganic, heavy metal complexes, release proton, and electrons in the process (Alzate-gaviria, n.d.). The electrons produce in the anode chamber travel from the anode to the cathode through the aid of an external circuit. While protons move directly to the cathode in a single chamber or diffuse through a proton exchange membrane in a dual chamber MFC. The protons will then react with oxygen in the cathode chamber to make water molecules. MFC depends on the electro active microbes to remove pollutants sometimes this microbes are called exoelectrogens (Chandra & Sharma, 2015). This are micro-organism, mostly bacteria which generate electrical energy by oxidation of organic matter and transferring the electrons to an electron acceptor outside their shell. In a nutshell MFC generate electrical energy and treat wastewater through using microbial activity to catalyse the degradation and oxidation of the substrate.



**Figure 3 Different mechanisms of electron transfer in the anode of the Microbial Fuel Cell a) Indirect b) Mediator mechanism and c) direct**

As shown in figure 3 to generate energy in the MFC exoelectrogens have to generate electrons to the electrode using four possible mechanisms. These mechanisms are namely short-range electron transfer, which mainly depends on a redox active protein release electrons, soluble electro shuttling molecules, long-range electron transport by conductive pili, and lastly direct inter species electron transfer as shown in figure 3. Through these mechanisms, the MFC is said to be effective in remediating multiple toxic substances including heavy metals, organic and complex inorganic toxins from wastewater, at low carbon emission and simultaneously provide green energy.

### 2.3.3. Factors affecting Microbial Fuel Cell performance

#### 2.3.3.1. Clay as a suitable ion exchange membrane for the MFC

Clay is an abundant resource that is available all over the world and its occurrence is due to the continuous gradual weathering of stones either chemically or physically. Zaeni and colleagues in 2023 stated that hand-crafted pottery products made from clay serve as an alternative as membrane separators in the MFC due to the corrosion-resistant, strength and ion exchange properties of the montmorillonite which is one of the earthenware crystals. (Das et al., 2020) also concur that clay is not only a good material for a membrane separator but also as an electrode due to its high ionic conductivity and hydrophilicity, which is beneficial for solid state electrolytes. The uniqueness of clay due to its properties makes clay to be a superior membrane separator to replace the traditional MFC membrane separators. Some of the advantages that come with using the clay-based separators are enhanced ion selectivity, high mechanical strength, chemical and biological compatibility and cost effectiveness. Clay-based membrane separators have high or enhance ion selectivity. This is because clay-based minerals have a double-layered structure that allows selective ion transport. Some clay minerals such as talc, sepiolite and halloysites are collectively made of porous structures with fibrous nanorods disorderly arranged. Montmorillonite (MMT) and vermiculite. MMT has the chemical formula of  $(\text{NaCa})_{0.33}(\text{Al,Mg})_2(\text{Si}_{90}\text{Al}_{10})(\text{OH})_2 \cdot n\text{H}_2\text{O}$ . Consists of alumina octahedral nanosheet sandwiched by two sheets of tetrahedral sheet. The weak bonding between the MMT layers allows for free ion movement through the inter layer, which leads to high rate of ion exchange which is a desirable trait for being used in the MFC membrane separator. These structural arrangements lead to clay having the following properties: High adsorption capacity, chemical stability and high temperature endurance. One of the major properties that makes clay to have a high cationic exchange capacity is that clay carries an overall negative charge. This is because the lattices of the clay minerals undergo isomorphous substitution within the layers, as a result there is a substitution as a result of substitution of ions such as  $\text{Al}^{+3}$  for  $\text{Si}^{+4}$  or presence of molecules containing the hydroxyl groups create a negative charge of the clay mineral. As negatively charged due to electrostatic forces of attraction clay particles will attract cations while repelling anions resulting in the enhance ion selectivity property (Samhari et al., 2020). Hydrogen ions being small particles the clay has an affinity to allowing them through. This property is particularly beneficial in the MFCs, as it promotes efficient proton transport from the anode to the cathode thus facilitating ion electron transfer and enhancing the performance of the MFC. Clay-based membrane

separator also have a benefit of having high mechanical stability. This phenomena is due to the strong interlayer bonding with the clay particles. This stability enable the separator to withstand different pressures variations and prevents the membrane from deformation or rupture. The robustness of clay based membrane separators makes the suitable for long-term operation with less replacement hence lowering the operational cost of the ceramic MFC. Clay based membrane separators are a prowess feature to be added to the MFC technology due to their chemical and biological compatibility. What this means is that clay minerals are naturally occurring minerals with excellent chemical and biological compatibility (You et al., 2019). They are non-toxic and do not release harmful substance into the MFC system, ensuring viability of microorganisms involved in the MFC. Moreover, the clay-based membranes have been found to promote microbial growth and attachment of electroactive bacterial on the anode, leading to high efficiency in both energy generation and wastewater treatment in the MFC. Lastly the clay based membrane separators are cost effective (Ray S. Pamintuan et al., 2019). Clay minerals are found abundantly all over the world thus this reduces the cost associated with membrane fabrication. In addition to that, the clay based membrane separators have a longer operational life span as compare to the traditional proton exchange membranes (Raychaudhuri et al., 2021). Clay also offer the potential for modification as it react differently to different materials thus it can be manipulated to enhance its properties, which will make the MFC more prominent and efficient. Len and colleagues in 2021 conclude that all types of clays have desirable features, which are needed for a membrane separator. Concur with this finding by stating that He believes that ceramics should replace the ion exchange membrane because they provide electroactive microorganism with a more favourable environment for their metabolism to run at an optimal, especially in situation where there is less external resistance and therefore higher currents generated from the MFC (Raychaudhuri et al., 2021).

In conclusion, the following summarises properties of clay that enables it to be a lucrative ion exchange membrane for the MFC

1. Enhanced Ion Selectivity: Clay minerals have a double-layered structure that allows for selective ion transport, attracting cations while repelling anions. This property promotes efficient proton transport from the anode to the cathode, facilitating ion-electron transfer and enhancing MFC performance.
2. High Mechanical Stability: Clay-based membrane separators exhibit strong interlayer bonding with clay particles, enabling them to withstand pressure differentials and prevent

membrane deformation or rupture. This robustness makes them suitable for long-term operation with less frequent replacements, thereby lowering operational costs.

3. Chemical and Biological Compatibility: Clay minerals are naturally occurring and exhibit excellent chemical and biological compatibility. They are non-toxic and do not release harmful substances into the MFC system, ensuring the viability of microorganisms involved in the MFC. Additionally, clay-based membranes promote microbial growth and attachment of electroactive bacteria on the anode, leading to high efficiency in both energy generation and wastewater treatment in the MFC.

4. Cost-Effectiveness: Clay minerals are abundantly available worldwide, reducing the costs associated with membrane fabrication. Moreover, clay-based membrane separators have a longer operational lifespan compared to traditional Proton Exchange Membranes (PEMs) and Ion Exchange Membranes (IEMs). Clay also offers the potential for modification to enhance its properties, making MFCs more efficient and prominent.

### **2.3.3.2. Inoculum used in Microbial Fuel Cell**

Studies show that different inoculum in the anode chamber of the MFC have different outcomes both in the pollutants removal efficiency and energy generation. This is because different microbes have different optimal conditions for them to perform at their level best. Therefore, it is very important for a suitable inoculum to be used based on the operating condition of the MFC (eg temperature, oxygen, pH,) and even the type of substrate digested. Some microorganisms are anoxic, facultative, aerobes and anaerobes. Facultative anaerobic bacterium, *Aeromonas hydrophilic*, was reported to be electrochemically active and useful in the anode chamber of MFC (Oh et al., 2010). The facultative anaerobe *Enterobacter aerogenes* grows rapidly under aerobic conditions, consuming oxygen to oxidize organic substrates, and also grow under anaerobic conditions, degrading substrates into hydrogen and a residue. Due to the property of H<sup>2</sup> production and O<sub>2</sub> consumption, they are very suitable for a MFC (Fuel et al., n.d.). A relative large current density (ca. 600 mA/m<sup>2</sup>) was reported by using glucose as a substrate and a stainless-steel net electrode plated with platinum black. *Ochrobactrum anthropi* YZ-1 showed that it can produce high energy in the MFC while acting a various substrates, including acetate, lactate, propionate, butyrate, glucose, sucrose, cellobiose, glycerol and ethanol ((Ma et al., 2021)). A *Klebsiella pneumonia* strain L17 biofilm developed on an anode also showed the ability to degrade starch and glucose to generate electricity (Zhang

et al., 2008b). *Klebsiella pneumonia* biofilm cells showed direct electron transfer from fuels to electrode. Bacteria of *Shewanella* type are known for diversity of terminal electron acceptors they can reduce. This allows them to couple the oxidation of organic matter to the reduction of the various terminal electron acceptors that they encounter in their stratified environments, and are one of the primary families of bacteria used in MFCs (Gajda et al., 2017)). *Shewanella oneidensis* (originally known as *Shewanella putrefaciens*) is a nonfermenting, motile, facultative anaerobic bacterium found in suboxic sediments (Biffinger et al., 2008 b). *Shewanella oneidensis* MR-1 is the wild type strain while *S. oneidensis* DSP10 is a spontaneous rifampin-resistance mutant more recently used in MFCs (Ringeisen et al., 2006). By increasing the acidity, the *shewanella oneidensis* MR-1 culture increases the current output (Biffinger et al., 2008b). The ability of *shewanella oneidensis* to grow under anaerobic and aerobic conditions, to use a wide variety of electron acceptors and to secrete mediators to aid in electron transfer, makes *Shewanella oneidensis* a provocative choice for a significantly wider variety of power applications in aerobic or microaerophilic environments (Biffinger et al., 2008a). However, to date, only a limited number of carbon containing electron donors (lactate, formate, pyruvate, amino acids) have supported metal reduction by *Shewanella oneidensis* under anaerobic conditions. *Shewanella oneidensis* grown with glucose in the presence of oxygen can generate more power than under strictly anaerobic conditions, where the elimination of oxygen should typically increase the fuel cell efficiency and increase power output (Biffinger et al., 2008a). A power increase with oxygen exposure is an indication that aerobic *S.oneidensis* can effectively utilize complex carbon sources as electron donors in MFCs. Using *Geobacter sulfurreducens* with acetate as fuel in a continuous flow 'ministack' system, higher current (4.56 A/m<sup>2</sup>) and power densities (1.88 W/m<sup>2</sup>, 2.15 kW/m<sup>3</sup>) are reported as compared to the power output when mixed anaerobic sludge is used as inoculum (Nevin et al., 2008). Phototrophic purple non-sulfur bacterium (*Rhodospseudomonas palustris* DX-1) can efficiently generate electricity by direct electron transfer in MFCs using wide range of substrate (volatile acids, yeast extract, and thiosulfate) making it another highly useful culture for very high power generation (2720 mW/m<sup>2</sup>) compared to mixed culture.

The use of mixed culture can develop higher current in the MFC due to wide acceptance of different forms of organic matter present in the real wastewaters as a substrate. Studies have shown that the MFC inoculated with mixed anaerobic sludge can also generate current density comparable with the selected pure cultures (Jadhav and Ghangrekar, 2008). Domestic wastewater can also be used as a inoculum (Zhan et al., 2008). In order to enhance power

production in MFC and to reduce the start-up period of the MFC it is beneficial to use a mixed culture as inoculum with some pretreatment. For biohydrogen production heat pretreatment is widely used (Indexed & Author, 2018)). Such heat treatment is also reported to be effective for pretreatment of the inoculum to enhance power production in MFC ((Emmanuel et al., 2016).

### **2.3.3.3. Temperature**

Temperature plays a crucial role in the performance of Microbial Fuel Cells (MFCs) due to its impact on the metabolic activity of microorganisms involved in waste degradation. Different types of microbes have varying temperature requirements for optimal performance. These microorganisms can be categorized into three main types based on their preferred temperature ranges: psychrophilic (cold-loving), mesophilic (moderate-temperature loving), and thermophilic (heat-loving) organisms (Roy et al., 2023).

For MFCs, the operating temperature affects the performance of the anode and overall system efficiency. The optimal operating temperature can vary depending on the specific microbial strains and substrates used. For instance, an operating temperature of 35°C has been reported as optimal when using *Geobacter sp.* and domestic wastewater as substrates in MFCs (Zhan et al., 2008). At this temperature, the system demonstrated improved performance compared to lower temperatures. In contrast, at a temperature of 30°C, the system showed a lag phase of 30 hours, which was half the duration observed at 22°C (Min et al., 2008). This indicates that lower temperatures can significantly delay the startup of microbial processes. Additionally, at 0°C, the maximum power density achieved was 70 mW/m<sup>2</sup>, whereas at 22°C, it was 43 mW/m<sup>2</sup>. This suggests that higher temperatures generally support higher power densities (Min et al., 2008).

Furthermore, a decrease in operating temperature from 30°C to 20°C has been associated with reduced Coulombic Efficiency (CE) and Chemical Oxygen Demand (COD) removal efficiency (Feng et al., 2008). Conversely, thermophilic operation at 55°C has been reported to yield a power density of 37 mW/m<sup>2</sup> with a CE of 89% over 100 days (Wrighton et al., 2008). In MFCs utilizing marine sediments, operation at 60°C resulted in ten times more power compared to 22°C (Mathis et al., 2008). Similarly, successful current production has been noted in MFCs operating at 50°C (Duteanu & Ghangrekar, 2010). Overall, temperature affects the rate of metabolic reactions in microorganisms, and different microbial strains have distinct optimal temperature ranges. Therefore, the choice of operating temperature in MFCs should be aligned with the characteristics of the microorganisms used to achieve optimal performance.

Increasing operating temperature also reduces the internal resistance of the cell. A reduction in internal resistance from 1000  $\Omega$  (at 22°C) to 430  $\Omega$  at 30°C is reported (Kuleshova et al., 2022). Also, CE can be increased at higher temperature (43% at 30°C) as compared to only 8% at 22°C (Y. Zhou et al., n.d.). With increase in temperature biochemical reaction rate increases, and hence results in an increase of biomass growth rate due to increase in the substrate utilization rate. Higher growth rate would also result in faster microbial attachment on the electrode. Conductivity of anolyte and catholyte will increase with increasing temperature, reducing internal resistance of the cell and enhance power output (Al-asheh & Aidan, 2022). Overall, it can be said that the MFC performs well in the mesophilic temperature range and the optimum performance can be obtained at around 35°C.

#### **2.3.3.4. Operating pH**

The enrichment of electrochemically active bacterial culture in the anode chamber of the MFC depends on the operating pH range. MFCs are typically operated at pH values between 6 and 8 in the anode chamber and neutral or little higher pH in the cathode chamber (Merino Jimenez et al., 2017). The anodic microbial process prefers around neutral pH and microbial activity decreases at higher or lower pH. An anode operating pH between 7 and 8 is reported to produce maximum CE and current (Pandit et al., 2021). Reduction in current and CE is reported at pH 6 and 9. It is reported that, the current increases significantly with increase in pH from 6 to 9 (especially from 7.5 to 8.0) and current decrease sharply at pH values above 9.0 (Al-asheh & Aidan, 2022). The acidity of electrolyte has a pronounced influence on both bacterial metabolism as well as Cathodic oxygen reduction reaction (ORR) rates. Increasing the acidity of anode will increase the driving force for cathodic oxygen reduction by 59 mV/pH unit as long as catalytic activity remains high (Liu et al., 2018). The higher current and voltage with higher electrochemical activity results in high pH difference across the membrane; hence, higher pH difference between anodic and cathodic electrolyte was reported to favour higher current and voltage (Beni et al., 2021). Also, it is reported that at an anodic pH of 6.5 the MFC gave maximum current. Acidophilic pH (6.0) in anodic chamber was reported to be effective with respect to power output as compared to the corresponding neutral (7.0) and alkaline pH (8.0) (Oh et al., 2010). However, substrate degradation was observed to be higher at neutral pH, followed by alkaline and acidophilic operation. It is reported that the MFC could tolerate an initial (feed solution) pH as high as 10 (Ma et al., 2021) with optimal initial pH between 8

and 10 to produce higher current. The bacterial metabolism exhibited a buffer effect and changed the electrolyte pH. The results presented so far suggest that, the operational pH between 6 and 7 may give optimum power production from the MFC. However, operation of MFC at feed pH up to 10 is possible. Higher pH in the anode chamber favors higher COD removal but reduces power. Higher pH difference between anode and cathode appears to improve power output of the MFC. The favourable pH and difference of pH between anode and cathode depends on the type of species used in anode, the electron donor used as substrate, catholyte used and proton flux through the membrane.

#### **2.3.3.5. Hydraulic retention time and wastewater concentration**

The applied Organic Loading Rate (OLRs) will have a marked influence on both power yield and substrate degradation rate in the MFC. OLRs in the range of 0.05 to 2.0 kg COD/m<sup>3</sup>.d are typically used by researchers to achieve maximum power from the MFC. The OLRs used in the anode chamber of the MFC by researchers are comparable with those used in activated sludge process. However, these are only comparable with the OLRs adopted for sewage treatment in high rate anaerobic processes, such as UASB reactor and anaerobic filters, and far less than the OLRs used in case of industrial wastewater treatment in UASB reactors. While treating wastewater a maximum power yield (274 mW/g COD) was reported at OLR of 0.574 kg COD/m<sup>3</sup>.(Winfield et al., 2016) Operation of the MFC at the higher OLR was reported to reduce the CE. While treating leachate, increasing OLR from 0.65 to 5.2 kg COD/m<sup>3</sup>.d resulted in a decrease of overall CE from 14.4% to 1.2% (Zhang et al., 2008a). Hence, it can be said that depending on the configuration of the MFC used and the wastewater being treated there is an optimum range of OLR to obtain maximum COD removal efficiency and harvest maximum power. The hydraulic retention time, which is related to the loading rate, is a key factor in the design of a wastewater treatment plant. The HRT can affect the opportunity for contact between the substrates and microorganisms. From the literature it is evident that higher HRT in the anode chamber favors higher treatment efficiency and higher power production. Increase in power density with increase in HRT up to 15.5 h and with further increase in HRT reduction in power density is reported (You et al., 2019). The optimum HRT depends on the type of organic matter being treated apart from the reactor geometry and strength of the wastewater. The favourable electrolysis times reported in the literature are a little higher than the electrolysis time generally adopted for established wastewater treatment systems such as the UASB reactor. Hence, in order to make volume of MFC comparable with other already

established treatment processes, it is required to modify configuration of MFC to handle more OLR at lower electrolysis time.

#### **2.3.3.6.Nutrient requirement**

The COD/N ratio required for the MFC type wastewater treatment system is reported to be lower than the conventional treatment (Indexed & Author, 2018). The effect of different forms of nitrogen on MFC performance was evaluated, and it is reported that nitrogen can be supplemented in the form of urea, ammonium sulphate, and ammonium nitrate producing similar power (Tiwari et al., 2008). However, removal of nitrogen from the feed is reported to adversely affect power production. The effect of phosphorous concentration in the feed on performance of MFC is not available so far. In general it appears that the 'P' requirement similar to anaerobic processes satisfies the requirement of electrogenic bacteria. However, detailed study is required to ascertain the exact requirement. Successful operation of MFC is reported without addition of trace metals (Min et al., 2005; Min et al., 2008). Most MFC researchers, using pure culture and synthetic wastewater, have not reported any trace metal addition as considered to be essential for methanogens in high rate anaerobic processes. The exact nutrient requirement of the MFC will depend on the type of microorganisms used in the anode for specific organic matter removal. Studies are required to establish exact macro-nutrient and micro- nutrient requirements of the electrogens to sustain their growth in the MFC.

#### **2.3.3.7.Oxidants used in cathode**

##### **2.3.3.7.1.Catholyte properties**

Several catholyte properties have been found to limit the MFC performance due to high ohmic resistance. These restrictions include low proton concentrations at neutral pH range and low ionic conductivity of catholyte solutions in dual chamber MFCs(Jang et al., 2004). Optimization of catholyte composition and concentration can, therefore, enhance the performance of MFCs (Zhao et al., 2006). Increasing the ionic strength of the catholyte improves the cathodic proton transfer rate and results in increased current output (Gil et al., 2003; Jang et al., 2004). Extreme pH and ionic strength of the catholyte can affect cathodic performance adversely, due to inactivation of metal-based catalysts and biocatalysts. The most popular oxidant employed in MFC studies due to its free availability in the air (Erable et al., 2009a; Liu et al., 2005; Logan et al., 2005; Min and Logan, 2004) is oxygen. Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), an intermediate product obtained from the oxygen reduction, has also been studied as oxidant in cathodic compartment (Park et al., 2004). Hexacyanoferrate [Fe(CN)<sub>6</sub>]<sup>3-</sup>

is commonly considered as artificial electron mediator in cathodic chamber emphasised Rabaey and colleagues in their study in 2003, but it often acts as a real final electron acceptor because the reoxidation reaction rate of the product  $[\text{Fe}(\text{CN})_6]^{4-}$  by oxygen is really slow (Rabaey et al., 2005). Permanganate can also be used as an effective cathodic electron acceptor in MFCs, e.g. five-fold more power density can be achieved using permanganate than with other electron acceptors such as hexacyanoferrate and oxygen (You et al., 2006a). Despite their excellent performances in MFCs, hexacyanoferrate and permanganate are not considered as sustainable oxidants because they still require continuous replacement. Instead of oxygen, ferric iron can be reduced to ferrous iron ( $\text{Fe}^{2+}$ ) at the cathode. Ferric iron reduction at the cathode and simultaneous biological ferrous iron oxidation of the catholyte was demonstrated using bipolar membrane separating the anode and cathode in the MFC (Ter Heijne et al., 2007). The immobilized microorganism *Acidithiobacillus ferrooxidans* is showed to be capable of oxidizing ferrous iron to ferric iron at a rate high enough to ensure power output of 1.2 W/m<sup>2</sup> and a current of 4.4 A/m<sup>2</sup>.

#### **2.3.3.7.2. Catholyte concentration**

Power output has been shown to be proportional to the oxidant concentration in the catholyte of dual chamber MFCs (Gil et al., 2003). For example, increase in power output is reported with increasing loading rate of  $\text{H}_2\text{O}_2$  (Tartakovsky and Guiot, 2006). Increasing the dissolved  $\text{O}_2$  concentration is limited by its solubility in water. Stirring and flushing the catholyte with air or pure  $\text{O}_2$  and recirculation of the catholyte have been tested in attempts to enhance the oxygen flux to the cathode (Jong et al., 2006). When permanganate concentration was increased from 0.02 to 0.2 g/L in the cathodic compartment, 3-times improvement in the performance of MFC was reported (Maqsood et al., 2022). Similar trend of increasing power output with increase in nitrate concentration in the cathodic chamber is reported (Clauwaert et al., 2007a).

#### **2.3.3.8. Effect of external load**

Some microbial fuel cells can use direct current to enhance the degradation of pollutants but unlike the ordinary MFC this kind of MFC does not generate bioelectricity and are called microbial electrochemical snorkels (MES). They continued to explain that graphite electrode MES enhanced the removal of hydrocarbons by 12% in 200 days from contaminated soil. On top of that explained main phenomena of using external load to help in degrading waste are called bioelectrokinetics. The weak current is said to increase the temperatures of the

soil or waste water as a result inducing the microbes to produce natural enzymes and other biological substances like surfactants which help in the efficient interaction and degradation of the substance(Lan et al., 2023). To verify this Lan and colleagues in 2023 stated that there was a positive correlation relationship between the voltage and the pollutant removal efficiency of the MES. In other words this means an increase in external voltage lead to an increase in the degradation of waste in the polluted soil through the MFC.As way of enhancing the efficiency of the MFC in the removal of toxins, reverse polarization can also be used which involves reversing the harvested current back in to the substrates through electrodes. This was shown through a study where the MFC was polar reversed every 1.5 hours and 67% removal efficiency of the dichlorophenols was achieved. The studies performed until now on MFCs reveal that the anode potential represents the determining element for the energy gain in case of microorganisms used as a catalyst in the anodic chamber (Sonawane et al., 2022; M. Zhou et al., 2013)). The external load used during the start-up period controls the bacterial growth rate, bacteria adhesion, and also determines the electrical energy output (Aelterman et al., 2008b). In fact, the external resistance is used in order to control and impose the anode potential and also to adjust the electrons flow between anode and cathode. The value of external resistance should be linked with substrate loading in order to enable the biocatalyst to take advantage of higher substrate concentration and to convert it in electrical energy (Aelterman et al., 2008a). When the substrate concentration is increasing, in order to improve the current generation, the external load must decrease. Gil et al. (2003) showed that external resistance was the rate-determining factor at over 500  $\Omega$  by limiting the electron flow between anode and cathode. Also, they proved that at external load lower than 200  $\Omega$ , proton flow between anode and cathode was the main limiting factor. Slightly higher COD removal efficiency was reported in MFC when the external load was lowered (Sabrina et al., 2018).A similar trend was reported by Gunawardena et al. (2008). Thus, the data presented in literature so far suggests that it is very important to determine and choose the right external load for each practical usage of MFC. This right load will depends on the configuration of the MFC, substrate concentration and OLR used. Even though the bioelectrokinetics have a positive impact in the efficiency of the MES bioremediation it is affected by various factors which need to be optimized to achieve maximum pollutant removal. This factors includes type of material used to make electrodes, the electric field, microbial community and soil or substrate properties(Lan et al., 2023).Selection of the type of electrode is deemed as one of the most significant decision when the aim is to remove the waste by bioelectrokinetics. There the type of material selected should

have high electrical conductivity, corrosion, resistance, affordability of the material. Some materials such as aluminium and titanium when used as electrodes they tend to form an oxide film on their surfaces which reduce the efficiency of bioremediation through the MFC. (Lan et al., 2023). But some material like activated carbon and graphite interact well with both the chemical and biological environment and lead to maximum degradation of pollutants. Microbial community also have an impact in the removal efficiency of pollutants through electrokinetics, this is because this process is main carried out by microbe either in the soil or wastewater or any selected feedstock. As a result studies showed that mixed communities of microbes have a high efficiency in degrading petroleum wastewater due to a vast and wider variety of enzymes they produce which have the ability to target and degrade wider range of pollutants as compared to pure cultures which only targets a specific substrate. And lastly substrate properties include concentration and pH which affect the bioelectrokinetics because MFC are mainly bioelectrochemical systems which are very reliant on microbial activities. Microorganisms are different and require different condition to function at optimal, some microbe are anoxic, facultative, aerobic and anaerobic. Some prefer acidic, saline or basic conditions to function at optimal there based of the microbial community suitable conditions should be maintained to favour the microbes metabolism.

#### **2.3.3.9. Electrode materials**

Selecting the right anode material is crucial for the performance and efficiency of microbial fuel cells (MFCs). The anode, where oxidation occurs and current enters the external circuit, significantly impacts the MFC's effectiveness. Recent research has focused on designing and fabricating anode materials that enhance MFC performance (Tsekouras et al., 2022). Key aspects include the anode's ability to support stable biofilm formation, which is essential for efficient electron transfer. The anode must be biocompatible with electroactive microbes and exhibit high electrical conductivity. Additionally, it should resist corrosion, provide mechanical strength, and maintain chemical stability. Increasing the surface roughness of anodes helps bacteria adhere better, facilitating stable biofilm development and improving power density (Roy et al., 2023). However, excessive roughness can lead to increased fouling, which might affect long-term performance. Therefore, anode materials should be durable and stable under varying operating conditions and pH levels. Enhanced porosity and surface area also contribute to effective biofilm formation (Tan et al., 2020).

When selecting and designing an anode, several factors must be considered, including mass transfer efficiency, biofilm growth, ohmic and activation losses, and redox reactions. The biofilm formation and growth are influenced by the anode's surface properties, while ohmic loss relates to the material's internal resistance. As a result, research has aimed at optimizing anode materials to improve MFC efficiency. Various materials, including different carbon-based options, metals, and metal oxides, have been used to manufacture anodes, each offering unique designs and properties. The specific attributes and potential applications of these anode materials are discussed in the following section.

#### **2.3.3.9.1. Carbonaceous electrodes**

Carbon is the most widely used material for anodes in microbial fuel cells (MFCs) due to its exceptional biocompatibility, chemical and thermal stability, high conductivity, mechanical strength, and cost-effectiveness (Salar-García et al., 2021). The large surface area and rough texture of carbonaceous anodes create favorable conditions for bacterial growth, enhancing the performance of MFCs. Carbon anodes offer desirable features such as being biocompatible and non-corrosive, which makes them preferable over metal or metal-oxide-based anodes. Research over the past few decades has focused on the development and application of carbon-based anodes (Najihah et al., 2024). Various forms of carbon materials, such as carbon cloth, graphite rods, and carbon plates, have been employed as anodes. These can be categorized into three structures: flat, packed, and brush.

**Flat Structures:** Common flat carbon-based anode materials include carbon paper, carbon cloth, graphite sheets, and plates. Carbon paper, while thin and somewhat fragile, is advantageous for easy wire connections. Graphene sheets are more durable than carbon paper, while graphite electrodes perform better with a roughened surface, though they are costly for large-scale use (Wadajo et al., 2021). Carbon fabrics are advantageous due to their porosity and large surface area but are expensive. Carbon mesh offers a cost-effective option and produces higher power density compared to sheets or cloths (Tan et al., 2020). Graphite rods, felt, and foams are also used; graphite rods are inexpensive and stable but difficult to enhance in surface area. Studies, such as those by Chaudhuri et al. (2003), have shown that graphite foam provides better power density and cell biomass than other anode materials.

**Packed Structures:** Carbon-based electrodes can be used in a packing form to increase the surface area for bacterial growth. Granular graphite and other materials like granular activated

carbon, carbon felt, and small graphite cubes can be used to enhance the anode's surface area and conductivity by stacking them closely together (Kalathil et al., 2018).

**Brush Structures:** Carbon brush electrodes, made from carbon fiber wound around non-conductive titanium wires, offer a large surface area and high current density. Logan et al. (2007) first utilized these brush anodes in MFC operations, finding them more efficient than conventional carbon paper anodes.

#### **2.3.3.9.2 Carbon Nanotubes (CNTs)**

Carbon nanotubes (CNTs) exemplify the application of nanotechnology, showing great promise as anode materials due to their high specific surface area, mechanical strength, stability, and conductivity (Tan et al., 2020). Recently, the combination of conductive polymers with CNTs has gained significant interest. CNTs are known to improve electron transfer at the anode and increase the effective surface area. Conductive polymers like polyaniline not only protect microorganisms but also enhance electrocatalytic activity. Zou et al. (2008) developed a microbial fuel cell (MFC) using multi-walled CNTs combined with carbon paper as the anode. This configuration demonstrated a higher power density compared to a graphite anode, attributed to the carboxyl groups on the CNT surface. The large effective surface area and specific surface properties of CNTs contribute to their superior efficiency over traditional microporous adsorbent media. Despite their advantages, CNTs face challenges in large-scale applications, such as potential clogging issues, higher operational costs, and complex production processes (Roy et al., 2023).

#### **2.3.3.9.3 Graphene**

Graphene, a single-layer crystalline material with carbon atoms arranged in a hexagonal lattice, shows considerable promise as an anode material in microbial fuel cells (MFCs) due to its exceptional electrical conductivity, high mechanical strength, large surface area, and biocompatibility (Schneider et al., 2023). Its electron mobility surpasses that of other carbon-based materials, making it highly effective. Various forms of graphene, including graphene oxide, reduced graphene oxide, and modified graphene, are utilized for their suitability in enhancing MFC performance. Zhang et al. (2011) were the first to develop a graphene-based bioanode system for MFCs, which outperformed conventional steel mesh anodes. This improvement is attributed to the high surface area of graphene, which supports better biofilm formation and enhances mass transfer within the biofilm.

#### **2.3.3.9.4. Conductive Polymers**

Conductive polymers have attracted attention for their ability to improve MFC performance through excellent conductivity, enhanced bacterial adhesion, and increased biochemical activity. Polypyrrole (PPy) and Polyaniline (PANI) are commonly used in MFCs due to their strong conductivity and stability (You et al., 2019). PPy is favored for its robustness and ease of synthesis, while PANI is noted for its biocompatibility, environmental durability, and low cost (Gajda et al., 2017). However, challenges with conductive polymers include structural instability, cathodic overpotential, and proton accumulation in the biofilm, which need to be addressed to optimize cost and efficiency in MFCs.

#### **2.3.3.9.5. Metal and Metal Oxide Anodes**

Although metals offer higher conductivity compared to carbon-based anodes, their use in MFCs is limited primarily due to issues of corrosion and poor biocompatibility. Metals such as stainless steel, titanium, and platinum are used as anode materials, with stainless steel exhibiting challenges due to its poor bacterial adherence and limited power density (Satyam B et al., 2011). Noble metals like platinum and gold can enhance performance and act as catalysts for electron transfer but are expensive, which restricts their large-scale use (Roy et al., 2023). Metal-oxide-based anodes offer a solution by reducing internal resistance and improving biofilm stability through reduced toxicity to microbes. Combining metal-oxide nanoparticles with carbon-based materials or conductive polymers can further enhance MFC efficiency. Table 2 shows in details the advantages and disadvantages of different electrodes in the MFC.

Different electrode materials have been used in MFCs overtime leading to different results in both treatment efficiency and power generation. In one study, carbon cloth was used for both the anode and cathode with an anode size of 2 cm by 2 cm and a surface area of 4 cm<sup>2</sup>. This setup, which did not include a catalyst, utilized *S. putrefaciens* CN32 as the inoculum source and achieved a power density of 679.7 mW/m<sup>2</sup> (Moradian et al., 2021). Another study explored composites, specifically reduced graphene oxide combined with polypyrrole (rGO/PPy) for the anode and carbon paper for the cathode. The anode measured 1 cm by 1.5 cm with a surface area of 1.5 cm<sup>2</sup> and employed platinum (Pt) as the catalyst. *E. coli* was used as the bacterial inoculum, resulting in a power density of 1068 mW/m<sup>2</sup> (Silva et al., 2022). A third study involved carbon paper modified with graphene oxide for the anode and carbon paper for the cathode. The anode dimensions were 5 cm by 3 cm, but the surface area was not specified, and no catalyst was used. Anaerobic sludge served as the inoculum, and this configuration achieved a power density of 368 mW/m<sup>2</sup> (Najihah et al., 2024). In contrast, a study using stainless steel

electrodes for both the anode and cathode, with an anode size of 20 cm by 30 cm and a surface area of 0.12 cm<sup>2</sup>, employed platinum (Pt) as the catalyst and marine sediments as the inoculum. This setup yielded a much lower power density of 23 mW/m<sup>2</sup> (Kalathil et al., 2018). Finally, another instance used carbon cloth for both electrodes with the same dimensions and surface area as the first case, no catalyst, and *S. putrefaciens* CN32 as the inoculum, achieving a power density of 679.7 mW/m<sup>2</sup> (Wang et al., 2022). The table 3 provides a summary of various types of materials used as electrodes in microbial fuel cells (MFCs), including their characteristics and performance.

Table 2 Shows the advantages and disadvantages of different electrodes in the MFC application

Anode Materials	Advantages	Disadvantages
Carbon nanotube (CNT)	<ul style="list-style-type: none"> <li>• Large surface area</li> <li>• High mechanical strength</li> <li>• Electrical conductivity</li> </ul>	<ul style="list-style-type: none"> <li>• Clogging</li> <li>• High operational cost</li> <li>• Complex synthesis procedure</li> </ul>
Carbonaceous anode	<ul style="list-style-type: none"> <li>• High conductivity</li> <li>• High stability</li> <li>• Biocompatibility</li> </ul>	<ul style="list-style-type: none"> <li>• Low power density</li> <li>• Limited electro catalytic activity.</li> </ul>
Graphene	<ul style="list-style-type: none"> <li>• Excellent electrical conductivity</li> <li>• High mechanical strength</li> <li>• Large surface area</li> <li>• Biocompatibility</li> <li>• High electron mobility</li> </ul>	Complex synthesis procedure
Conductive polymer	<ul style="list-style-type: none"> <li>• Excellent conductivity</li> <li>• Better bacterial adhesion</li> <li>• Enhanced biochemical activity</li> </ul>	<ul style="list-style-type: none"> <li>• Accumulation of proton biofilm</li> <li>• Cathodic overpotential</li> </ul>

		• Structural instability especially at high temperatures.
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Table 3. Shows the performance of MFCs with different electrode materials and inoculations

Type of Material	Electrodes		Size of Anode	Surface Area of Anode	Catalyst	Inoculum Source/ Bacteria	Power Density	Reference
	Anode	Cathode						
Carbon-based	Carbon cloth	Carbon cloth	2 cm × 2 cm	4 cm <sup>2</sup>	Without catalyst	<i>S. putrefaciens</i> CN32	679.7 mW/m <sup>2</sup>	(Moradian et al., 2021)
Composites	r GO/PPy	Carbon paper	1 cm × 1.5 cm	1.5 cm <sup>2</sup>	Pt	<i>E. coli</i>	1068 mW/m <sup>2</sup>	(Silva et al., 2022)
Carbon-based	Graphene oxide modification with carbon paper	Carbon paper	5 × 3 cm <sup>2</sup>	-	-	Anaerobic Sludge	368 mW/m <sup>2</sup>	(Najihah et al., 2024)
Metal	Stainless steel	Stainless steel	20 × 30 cm <sup>2</sup>	0.12 cm <sup>2</sup>	Pt	Marine sediments	23 mW/m <sup>2</sup>	(Kalathil et al., 2018)
Carbon-based	Carbon cloth	Carbon cloth	2 cm × 2 cm	4 cm <sup>2</sup>	Without catalyst	<i>S. putrefaciens</i> CN32	679.7 mW/m <sup>2</sup>	(Wang et al., 2022)

### 2.3.3.10. Membranes in Microbial Fuel Cells (MFCs)

In microbial fuel cells (MFCs), membranes are critical components that separate the anodic and cathodic chambers (Adesiji et al., 2020). They are essential for MFC operation as they facilitate proton transfer while preventing the diffusion of oxygen between the chambers. This

separation is crucial for maintaining the chemical and ionic balance necessary for efficient MFC function. Although a single-chamber MFC without a membrane might show higher current density, the lack of separation can lead to increased oxygen and substrate diffusion, which reduces coulombic efficiency (CE)(Roy et al., 2023). Thus, membranes play a key role in sustaining the long-term efficiency of MFCs. However, membranes come with their own set of challenges, including high costs, susceptibility to fouling, and increased internal resistance(Niju, 2023). Researchers have focused on finding the optimal membrane for MFCs that balances cost, internal resistance, selectivity, chemical and mechanical stability, and resistance to fouling.

#### **2.3.3.10.1. Ion Exchange Membranes**

Ion exchange membranes (IEMs) are designed to facilitate ion transport and were first developed by Ostwald in 1890. These membranes have undergone significant advancements and are now used in various industrial applications(Tremouli et al., 2018). IEMs face challenges such as higher internal resistance when used in MFCs(Wang et al., 2022). They are classified into three main types based on the type of ions they transport: Cation Exchange Membranes (CEMs), Anion Exchange Membranes (AEMs), and Bipolar Membranes (BPMs)(Tremouli et al., 2018).

#### **2.3.3.10.2. Cation Exchange Membranes (CEMs):**

These membranes allow the transport of protons and contain fixed negative charges. Examples include Nafion, Zifrons, and Hyflon. Nafion is particularly popular due to its high proton conductivity, attributed to its sulphonate groups(Sabrina et al., 2018).

#### **2.3.3.10.3. Anion Exchange Membranes (AEMs):**

These membranes facilitate the transport of negatively charged ions and contain positively charged ions(Majumder et al., 2014). They can generate a higher current compared to CEMs by consuming protons with hydroxyl ions, preventing an acidic environment in the anode chamber. This increases ion transport at the anode and reduces resistance at the cathode(Li et al., n.d.). However, AEMs generally have lower conductivity than CEMs due to the larger size of anions(Cell, 2016). Improvements in conductivity, water retention, and stability are achieved through the use of conductive polymers and inorganic fillers like TiO<sub>2</sub>, SiO<sub>2</sub>, and graphene.

**2.3.3.10.4. Bipolar Membranes (BPMs):** BPMs combine CEMs and AEMs to facilitate the transport of both  $H^+$  and  $OH^-$  ions across the water-splitting interface (Beni et al., n.d.). Although BPMs can achieve high power densities, they face issues related to maintaining a stable pH gradient (Jabbar et al., 2023).

#### **2.3.3.11. Porous Membranes**

Porous membranes operate based on pore size and include materials like glass wool, ultra-filtration membranes (UFMs), and micro-filtration membranes (MFMs). These can serve as cost-effective alternatives to traditional membranes. For example, glass wool is a low-cost option suitable for single-chamber MFCs (Duteanu & Ghangrekar, 2010). UFMs are commonly used in MFCs due to their permeability to both positively and negatively charged ions. However, porous membranes share some of the issues faced by membrane-free technologies. They can allow larger molecules, including oxygen, to pass through, which might affect performance (Sabrina et al., 2018). While they offer reduced internal resistance, this advantage can be diminished by biofouling. Addressing these challenges is crucial to improve the efficiency and practicality of porous membranes in MFC applications (Tsekouras et al., 2022).

### **2.4 Performance of Microbial Fuel Cell in treatment of petroleum wastewater**

#### **2.4.1. Analysis and evaluating the use of Microbial Fuel Cell in removal of pollutants from petroleum wastewater**

When Jabbar and co-workers in 2023 performed, a study aimed at using a continuous Microbial Fuel Cell to treat wastewater from an oil refinery in Baghdad City, Iraq. A dual chamber MFC was adopted with graphite electrodes' and a cation exchange membrane called type: CMI-700 from the USA membrane International. The samples collected were initially analysed for conductivity, Chemical Oxygen Demand, Total Dissolved Solids and pH. Furfural which is a furan derived heterocyclic aldehyde and phenols quantities were determined through the use of an ultraviolet spectrophotometer. A fresh activated sludge was obtained from the outlet of biotreatment in Al-Ruatumia wastewater plant, which was upon characterisation showed that that it has *Bacillus* sp. as the dominant type in the mixed culture. The wastewater was treated continuously for 30 days and the results revealed that COD was removed to up to 95.7% ,which is higher than that from the study (Luo et al., 2010) in which only 68% of the COD was removed when using an MFC in a batch mode for 30 hours. The possible reason behind this difference might have been the continuous stirring of the wastewater in a continuous system increase the

microbes and substrates interaction which lead to more degradation unlike in a batch system where particle motion is very negligible.

Upon 30<sup>th</sup> day of the experiment ,the removal efficiency of furfural was reveal to be 99.04%,As for phenol degradation the removal efficiency of 99% was obtained from using the MFC which is higher than the removal efficiency which was recorded by Morris and Jin in 2004, when they used an anaerobic digester to remove phenols from waste water. The possible reason for this is the proton and electron exchange in the MFC that doesn't occur in an anaerobic digester as a result more degradation was experienced in the MFC. The electricity generated was lowest at during the 1<sup>st</sup> day but continued to increase until it reached a steady state, which was corresponding to the COD profile. Majumder and coworkers in 2014 also experienced corresponding findings during the initial days of the operation and to justify this phenomena they stated that the COD removal was low due to the Columbic efficiency of the system which increased as the cycle proceed, in conclusion they established that COD removal efficiency is directly proposal with columbic efficiency which refers to how well the system carries and accumulate charge. Another justification for low COD removal and Coloumbic efficiency at initial stages maybe that during the initial days the microorganism where still being acclimatized to the environmental conditions such as pH, temperature and substrate in the anode chamber. After this phase the microorganism were well adjusted to the environment and stated acting on the substrate, which in this case was petroleum wastewater to produce more electron and protons, which lead to the generation of electic current. This study successfully covered the research topic objectives but they was no information mentioned about the pH of the substrate and the temperature at which the experiment took place at ,as this are some factor that can affect the microbial metabolism processes in the MFC and may even affect the efficiency of the system. The other shortcoming from this study was that they did not mention the reason why they chose membrane type: CMI-700 as their proton exchange membrane was it related to the ion exchange prowess or its interaction with the microbes. In contrary to this study instead of using a mixed culture, Majumder and colleagues in 2014 used a specific kind of exoelectrogens called *Pseudomonas Putida* to degrade the petroleum wastewater in the MFC. The findings showed that the lower COD removal efficiency and other pollutants removal was observed in such a system as compare the high level of removal efficiency, which was experienced by Jabbar in 2023, when they used a mixed culture. The reason to this is that in a mixed culture there are different kind of microorganism which produces different enzymes and have different metabolic process which are able to degrade a

wide variety of pollutants unlike when one specific micro-organism is used .A single micro-organism type will have a specific preference or produce specific substrates which act on specific substrates so some of the pollutants may not fall with that band targeted by the enzymes produced by the organism as a result they will be negligible degradation of such enzymes, leading to the pollutant removal of the system to be lower than in mixed cultures.

In another research Yuan and colleagues in 2015 argued that biofilm build up on the anode have a positive impact on the performance of the MFC both in contaminant removal and power generation. They further explained bacteria strategically position themselves on to the anode surface to form a bacteria community called a biofilm. To support this argument Yaun and colleagues in 2015 used dual chamber MFC with Nafion 177 proton exchange membrane and PACF as electrodes. The electrodes were washed in water and nafion 177 was drenched in HCL overnight as a way of avoiding any contamination. All parts of the MFC were assembled beneath distilled water to further avoid contamination in order to make the experiment fair. The anode chamber was filled with an anaerobic sludge from oil treatment plant in Felda, Malaysia. The cathodic chamber was filled with potassium permanganate solution. The voltage and current were measured every 15 minutes and polarised curves were obtained from different external resistance. The results showed that the microorganisms which were predominant. In the anaerobic sludge inoculum were *Pseudomonas menduona*, *Pseudomonas viridiivida* ,*Acinetobacter schindler* ,*Actinobacillos capsulatus* and *Brevibactrium* which were all detected through using the BIOLOG GENE III test. The MFC was operated for 20 days and on the 20<sup>th</sup> day the operation showed lag, stationery and decline phase .But of the 14<sup>th</sup> day the effluent showed COD removal efficiency of 70% and columbic efficiency of 24%,the low columbic efficiency can possibly be due to build-up of organic constituents ,methane etc. in the anode chamber .Anode biofilm formation was shown to enhance the density and charge transfer. This is because in the 3<sup>rd</sup> day to the 14<sup>th</sup> day anode resistance  $R_c$  values reduced by 16.9%,the biofilm grow on the anode polarization resisting with resulting higher power generation than in the early operation days where there was less growth of microorganism on the surface of the anode. A similar approach was used by Jabbar and colleagues in 2023 where they successfully analysed the current and voltage generated by the MFC. This study was carried with delicacy especially the thorough sterilization steps which were taken makes the results compellingly reliable. Although the experiment covered and explained the topic well, they did not mention anything about the pH ,whether it contributed to the biofilm build up or had no effect and also

did not say anything about the temperature at which the experiment was carried out as a result make it very difficult for someone else to replicate their experiment and verify their findings.

Heavy metals make part of the pollutants that are found in petroleum waste water. Heavy metals pollutants, such as chromium, copper, vanadium and mercury, have been removed using two chambered MFC cells. Heavy metals in MFCs are removed through the reduction of the cathode metal in the anaerobic cathodic chamber, while in the anodic chamber; organic matters are used as sources of protons and electron donors. It has been demonstrated that such processes as biosorption and precipitation reactions (i.e., sulfides and hydroxides) greatly aided in the removal of heavy metals from wastewater in the MFC system. Al-asheh & Aidan in the year 2022 carried out a study aimed at examining the efficiency of the MFC in the removal of gold as a heavy metal from a pure tetrachloroaurate solution. This experimental study was also focused in the recovery of precious metals by using the MFC technology. The results showed that the percentage removal and recovery of gold is directly proportional to the concentration of the substrate. The study also revealed that at very acidic conditions of pH 1 and 2 the highest removal efficiency of 100% to 99% was experienced for gold which is different from some metals such as Cu(II) which had the higher removal efficiency at higher pH values. This study proved that the pH of the substrate plays an important role in the efficiency of the MFC in the removal of heavy metals and also showed that for the MFC to remove a variety of metals, there must be a pH variation. This experiment managed to cover all the aims and the objective completely and thoroughly. They were also specific in how most of the factors like concentration, pH and microorganism affect the MFC, by so doing make the paper unbiased and simple to replicate in order to verify their findings.

According to Tao and colleagues (2011), from using a single chambered MFC with carbon brush as an anode and carbon cloth reacted with Platinum, managed to recover 98.3% of Cu (II) from industrial wastewater sludge and generated a maximum power of 10.2W/m<sup>3</sup>. Heijne colleagues in 2010 attempted to remove the same metal (Cu(II)) using a double chamber MFC with graphite electrodes. The anode chamber was kept under anaerobic conditions in this study and Acetate was used as the substrate. The results showed that a higher recovery removal of Cu(II) was experienced than from the single MFC that was used by (Tau et al., 2011). The possible reason for this may be due to mixed culture microbe in the sludge, because a mixed culture contains different microbes it means there is a wider range of substrates in this culture therefore being able to effectively remove most pollutants from the wastewater. The other possible reason may be the presence of the ion exchange membrane that protects the anode

chamber from being contaminated with Oxygen as the microbes in that chamber most require anaerobic conditions to function effectively and exposure to oxygen may cause microbial shock or toxicity to microorganisms responsible for breaking down the substrate. The use of the ion exchange membrane also increases the efficiency by increase the rate of proton movement to the cathode hence encourages more current generation.

In another study Li and colleagues in 2018 carried out a study using the two chamber MFC to remove Chromium from domestic wastewater .In this experiment the anode was made of graphite and the different cathode were used in three different MFC set ups i)Carbon felt,ii)Carbon cloth and ii) carbon brush. Acetate was used as a substrate and the result showed that there was 100% removal recovery was obtained from using carbon cloth as a cathode in the MFC which is higher than the other two set ups .The reason why carbon cloth removed higher chromium was because of its large surface area which leads to maximum ion interaction with the cathode as compared to other systems which were understudy .This also show that the power generated is proportion the how well the MFC is able to remove pollutants from the waste water because the highest power of  $122,91\text{mW/m}^2$  generated was observe in the MFC that used carbon cloth as a cathode. Eijne and co-workers in 2010 used a dual chamber MFC to remove chromium but using graphite electrodes and recorded lower power and Chromium removal efficiency. From these studies, it is evident that carbon cloth is a more suitable material to be used in MFC electrode to ensure maximum power generation and metal removal efficiency. Wang in 2010, also carried out a test to prove that heavy metals can be removed by using the MFC .In this study a dual chamber MFC with carbon felt anode and carbon paper cathode was used to remove Hg (II) from a mixture of wastewater sludge and artificial wastewater. The results showed that 99.54% of Hg(II) was removed from the wastewater and a power generation of  $433.1\text{mW/m}^2$  was recovered. The same set up was used by Choi and colleagues in 2012 to remove Ag(I) and 99.91% was the maximum removal recovery and a power of  $6.58\text{W/m}^2$  was generated which was the lowest in all the previous experiments of the same set up. The power generation may be due to the use of an unsuitable ion exchange membrane that can easily experience bio fouling thus not serving its purpose well and reducing the power generated, as fewer protons will be transported through the PEM to the cathode chamber to complete the circuit. The other reason may be the build-up of products of metabolism processes in the anode chamber that can end up being toxic to microorganisms and low substrate concentration can be another reason for lower power generation. In conclusion, findings from the previous literature show that the MFC can be used to remove the heavy metals

and generate relatively high power from the process especially if carbon cloth is used as electrodes. The other important factor noted from the findings is that when it comes to metal removal and power generation the double chamber MFC is more effective in removal of metal pollutants than a single type of MFC.

#### **2.4.2. Comparison of Microbial Fuel Cell with conventional methods used to treat petroleum wastewater.**

Petroleum wastewater poses significant challenges due to its high complexity and pollution potential (Aspects et al., n.d.; Faustine, 2008; Joseph et al., 2021). As discussed earlier in this paper the petroleum wastewater poses health threats to human beings due to some of the constituents such as the PAHs, benzene and cyanide are carcinogenic. This means that they can cause abnormal multiplication of body cells leading to neoplastic diseases like cancer, skin conditions and even blindness in situations where there is optical contact with the polluted water. Studies from (Chouksey, 2004) showed that not only human life is in danger due to the water pollution caused by contamination from petroleum industries but even the marine and inland organisms are affected by this phenomenon. As a result it was estimated that 100,000-300,000 birds died from the exposure to petroleum waste (Paine et al., 1996). Pre and post spill analysis showed that the sea otter (*Enhydra lutris*) population decreases in the oil polluted portion of the Prince William Sound (PWS) in years following the spillages. Some reviews also stated that the petrochemical contaminations also lead to contamination in the whole tropic level as a result even affect inland organisms through bioaccumulation and biomagnification. As a way of reducing these implications several conventional wastewater treatment methods have been used over the years as an attempt to remove toxic petroleum contaminants from the petroleum wastewater. This includes physical methods such as sedimentation, filtration, and flotation. Chemical methods involving coagulation, flocculation and chemical oxidation and biological means of treatment including aerobic and anaerobic digestion. Even though some efforts have been made to reduce the implications of petroleum waste to the environment especially aquatic, some petroleum industries still dispose their wastewater untreated to the environment which leads to more pollution of water resources (Bryan & Ejumudo, 2019). In this chapter a comparison of the conventional methods and MFC in treatment of petroleum wastewater will be made in order to scrutinize and draw conclusions on the benefits and shortcomings of each approach.

Generally, when it comes to the removal of heavy metal from the petroleum wastewater, the microbial fuel cell was proved superior to the conventional methods. This is because from the

studies literatures from (Al-asheh & Aidan, 2022) showed that the averages removal efficiency of heavy metal from petroleum waste water was 98% which is very high as compared to the removal efficiency incurred from using the conventional methods. To achieve the same removal efficiency through using the conventional methods different methods have to be combined to treat the petroleum wastewater leading to the process being more costly. Studies also revealed that the highest removal of petroleum contaminants was through the use of the ozonation process among the conventional methods which is a very complex methods requiring high levels of expertise and advance equipment as compared the MFC .Most conventional methods leads to sludge generation creates a problem of sludge management, which is still an area of concern in wastewater management but the MFC does not produce any sludge from treatment of the wastewater.

Due to the fact that this wastewater contains large amounts of salt, due to the increase in osmotic pressure, it destroys the cell wall of the normal microorganisms in the MBR system. In this study, the purified bacteria that were obtained from the areas of oil deposits in the sea, after exposure to the main sewage, were able to decompose 50% of phenanthrene, which is a complex and difficult-to-decompose aromatic compound with three benzene rings, after 45 days. Based on these results, these bacteria can break down other compounds in crude oil. By reducing the salt concentration in this experiment, contrary to expectation, the performance of bacteria purified from the environment with high salt concentration did not decrease. This confirms that these bacteria belong to the halotolerant group. In a similar study, Xianling and coworkers in 2018, studied the purification of petroleum hydro-carbons in a membrane bioreactor by purifying different bacteria from oil-contaminated areas. In this system, it was found that COD removal efficiency was different in steady state, and despite the gradual increase of COD, the efficiency increased from 93 to 96.5%. The reason for this can be seen in the increase in Multiliquor Suspended Solids (MLSS) concentration in these types of systems, which reached 16.2 g /L in this system.

Jabbar in his study in 2023 explained that the profile of phenol removal in the MFC also consisted of two successive phases. The first phase revealed substantial variation in the first 10 days. Afterward, a steady state appeared in the second phase after several days. The profile showed the significant removal of phenol with a high removal efficiency of 99% in the last ten days. The results of this study revealed that the MFC can improve phenol degradation compared with other techniques dominated by normal anaerobic metabolism. Morris and Jin (2007) attributed this improvement to the transfer of electrons to the terminal electron acceptor

of oxygen in the cathodic chamber rather than to the electrons acceptors, such as metals and sulfate, in the anaerobic anodic section, causing indirect aerobic degradation. Although these methods have shown effectiveness in the wastewater treatment they often suffer several shortcomings such as high energy consumption, operational complexities and generation of secondary waste (Sludge production).

Microbial fuel cell (MFC) would replace the secondary treatment system and tertiary treatment (removal of nutrients, ammoniacal nitrogen, phosphorus and organics components)(Yokoyama et al., 2006). In addition, multi-stage treatment combining anaerobic digestion and/or hydrogen fermentation and MFC technologies may result in reduced accumulation of inhibitory by-products and allow effluent polishing to more stringent discharge standards (Kim et al., 2010, Logan and Regan, 2006b; Pham et al., 2006). Combining an MFC with AD and Bio-hydrogen would therefore maximise total energy recovery and consequently increase the sustainability of wastewater treatment. The additional heating system to maintain temperature may not be necessary for energy recovery or wastewater treatment using MFC technology (Alzate-gaviria, n.d.)

Switching wastewater treatment from an aerobic to anaerobic process would dramatically cut energy consumption by obviating the need to aerate the sludge. However, conventional anaerobic treatment technologies are often thought of as being slow, need concentrated waste and high temperatures to operate reliably, the effluent often requires further treatment before it can be discharged and sludge disposal is still required. Microbial fuel cells appear to operate at lower temperatures and yield less biomass.

MFCs have advantages over other technologies used for generating energy from organic matter. First, the direct conversion of substrate into electricity permits high conversion ambient at efficiencies. Second, they operate efficiently temperature, including low temperatures. Third, they do not require the treatment of biogas generated in the cell. Fourth, they do not require additional energy to aerate the cathode, given that it can be aerated passively. Fifth, they have the potential for application in remote areas without electrical Infrastructure, making them an additional renewable energy option to meet global energy requirements. Finally, MFCs involve an anaerobic process, and bacterial biomass production will therefore be reduced compared to that of an aerobic system. On top of that, the MFC reduces the waste aeration, in an MFC of one chamber with an air cathode; the transfer of the necessary oxygen is achieved passively meaning it does not require any energy or mechanical pumps to function. In any case,

the air supply is considerably reduced compared to a secondary AS treatment system that requires about 50% of the electric power consumed by the waste treatment plant (Tsekouras et al., 2022). Reduction of solids production is also another benefit of using the MFC. This is because the MFC functions anaerobically, therefore the bacterial biomass production will be more limited than in the aerobic operation of an AS system or a biological filter. This has the effect of reducing the cost of expensive sewage sludge treatment installations. Lastly, the MFC produces less or no odour as compared to some conventional methods that polluted the air as they are used to treat wastewater which can be life threatening to the workers and settlement close to the treatment plants. This is because of the anaerobic operation of the MFCs, it is possible to avoid the extensive open to the air areas of the biological filters, and the large quantities of air flow in the aeration basins, hence considerably reducing the release of disturbing odor to the surroundings (Ma et al., 2021).

**Table 4: Comparison summary of the Microbial Fuel Cell and conventional treatment methods**

<b>ASPECT</b>	<b>MICROBIAL FUEL CELL</b>	<b>CONVENTIONAL METHODS</b>	<b>REFERENCES</b>
Treatment efficiency	High	Variable	(Emmanuel et al., 2016)
Energy Generation Potential	Generates Electricity	No energy generation	(Agüero-quiñones et al., 2022; He, 2013; Passos et al., 2016)
Organic Removal Efficiency	High, especially hydrocarbons	Efficient but limited to some hydrocarbons	(Bahan et al., 2011)
Treatment time	Relatively longer	Relatively short	(Ray et al., 2005)
Scale up potential	Still challenges being faced	Easy	(Oh et al., 2010)

Secondary Pollution	No secondary pollution	High secondary pollution from running the mechanical equipments	(He, 2013)
Removal of heavy metals	High removal efficiency	Require a combination of different treatment methods to achieve high efficiency	(Widani & Yanti, 2023)
Sludge generation	No sludge generation	Sludge generation is an area of concern in using conventional methods	(Wang et al., 2022)
Operational cost	Relatively cheaper than conventional methods	High cost to run and install the equipments.	(A. Ali et al., 2021)

Table 4 shows that Microbial fuel cells (MFCs) exhibit high treatment efficiency and can generate electricity, particularly excelling in removing hydrocarbons. However, they require longer treatment times and face scaling challenges. In contrast, conventional wastewater treatment methods are easier to scale but have variable efficiency, generate secondary pollution from mechanical equipment, and produce sludge. Additionally, conventional methods are generally more expensive to install and operate. MFCs also demonstrate high efficiency in heavy metal removal without generating sludge, making them a more environmentally friendly option. Overall, while MFCs present innovative advantages, conventional methods still dominate in practicality for large-scale application

**Table 5: summary of the shortcoming in using conventional methods to treat petroleum wastewater**

Method	Shortcoming/Gap	References
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Chemical methods	The can be expensive ,require other treatment methods and generate a lot of sludge	(Varjani, 2020)
Biological methods	Due to the complexity and toxicity of petroleum wastewater some microorganisms can be inhibited.	(Nikolopoulou et al., 2010)
Physical methods	Not effective in the removal of dissolved organic compounds	(Zueva et al., 2020)

Although there are some methods currently used to treat the petroleum wastewater like those shown in table 5 their effectiveness in the waste water treatment they often suffer several shortcomings such as high energy consumption, operational complexities and generation of secondary waste (Sludge production).On top of that the efficiency of the methods in the removal of pollutants such as heavy metals is very lower as compared to when the MFC is used. This was shown by Zueva in 2020 in the study, which revealed that a 99% removal efficiency of heavy metals were removed from the wastewater. This makes the MFC superior because it achieves a high removal efficiency without the combination of any other methods prior treatment. Most of the former mentioned methods require a subsequent treatment of water using different techniques inorder to achieve such a higher removal efficiency. Therefore making the conventional methods to be relatively more expensive than the MFC. According to Zueva in 2020,some physical treatment methods such as Microfiltration (MF) and ultrafiltration (UF) are typically used to petroleum wastewater as pre-treatment for other methods like reverse osmosis however there are some shortcomings experienced as conventional flotation techniques are not efficient in removing the oil and grease content from the petroleum wastewater without any chemical intervention. One of the important benefits of the MFC is its ability to generate green energy. According to (Aljuboury et al., 2017) conventional methods such as anaerobic treatment also give biomass which sometimes is very difficult to use due to the variable quality and on top of that the production of sludge is an area of concern which leads to secondary pollution. Some methods such as Ozonation require

specialised personnel and equipment to operate which means that they are limited to only experts unlike the MFC, which can be used to treat and generate energy even in the remote areas. On the other hand, studies show that the catholyte that is created during the operation of the ceramic MFC can be used as a coagulant in treatment of wastewater (Merino Jimenez et al., 2017).

## **CHAPTER THREE**

### **3. MATERIALS AND METHODS**

#### **3.1. Research Approach**

The research approach utilized in this study was quantitative methods. This is because an experimental setup of the clay based microbial fuel cell was used to treat petroleum wastewater and generate energy. Furthermore, the experimental procedures were also used to extract and culture the bacteria from oil-contaminated samples. This research approach also involved the synthesis of petroleum wastewater in the laboratory and the subsequent treatment of the petroleum wastewater using clay based MFC system. This research approach allowed for the controlled conditions and accurate measurements, enabling the evaluation of the systems performance. In this experiment, the independent variables were concentration of the wastewater, pH, temperature, hydraulic retention time, inoculation ratio. The MFC was operated at a pH of 7 based on the pH of the petroleum wastewater, at room temperature and for a retention time of 30 days. The reasons for these conditions were to simplify the MFC and make it in such a manner that it is economically friendly and easily applicable operational parameters. The dependent variables were the pollutant removal efficiency in terms of (BOD, COD) and power generation.

The design for this study was experimental design, which included single group pre-test post-test design. This design involved the treatment of the petroleum wastewater using the clay based MFC system and measuring the changes in the wastewater quality parameters before and after treatment. The design allows for the assessment of the systems effectiveness in wastewater treatment and energy generation. According to Bacon-shone (2020) experimental design is used when the researcher wants to examine the interaction of variables by manipulation some and keeping some constant as control. The manipulated variables are called independent variables and the effects of the manipulation will be implicated in the dependent variables. So in this experimental design the independent variable were temperature, pH, concentration of the wastewater and retention time and the two main dependents variables that were observed was energy generated in terms of voltage and lastly the pollutant removal

efficiency of the wastewater (COD and BOD removal efficiency) through the clay-based MFC. Therefore, the experimental design was deemed superior in exposing the objectives of the study and the ability for replication of the study to verify findings can be made at ease. The experimental design method described above offers several advantages. Some of the advantages are that it allows for the examination of various parameters and a condition, providing a comprehensive understanding of clay-based MFC system performance in treating wastewater and energy generation.

### **3.2. Materials**

In order to construct an experimental set up of the clay-based microbial fuel cell and the following materials and chemicals were used. 2L plastic buckets, cylindrical clay membranes of 9cm diameter and 15cm height, copper wires (0.1mm diameter), (100cm\*1000cm) non-woven carbon cloth from Easy Composite Pty Ltd(United Kingdom), glue, anaerobic jar ,steam autoclave, water proofing membrane, petri dishes ,volumetric pipette,1 $\Omega$  -10 k $\Omega$  resistor's box, beaker, stirring rod, oven, laminar airflow, colony counter, digital pH meter. The chemicals used included sodium sulphide (Na<sub>2</sub>S), hydrochloric acid (HCL), sodium hydroxide(NaOH), phenol, engine oil, potassium permanganate (KMnO<sub>4</sub>), sodium chloride (NaCl), potassium ferricyanide (K<sub>3</sub>[Fe(CN)<sub>6</sub>]), crystal violate (C<sub>25</sub>N<sub>3</sub>H<sub>30</sub>Cl), gram's iodine, ethanol (95%), nutrient agar, distilled water, kaolin clay, ,sodium carbonate(Na<sub>2</sub>CO<sub>3</sub>), Potassium monobasic phosphate(KH<sub>2</sub>PO<sub>4</sub>) and potassium dibasic phosphate(K<sub>2</sub>HPO<sub>4</sub>)(Phosphate buffer solution). For investigation of the morphology of microorganisms, a light microscope was used at a magnification of 100X .The anaerobic glass jar was used for culturing of the anaerobes container. Autoclave was used for disinfecting the apparatus prior bacteria culturing and lastly the digital multimeter was used for measuring voltage and current generated by organisms (DL MULT1999DET).

### **3.3. Sampling**

Through scrutinizing different research papers the most suitable source of inoculum was found to be diesel oil-contaminatedsoil that has about a year being contaminated (Hazaimah & Ahmed, 2021). Due to this reason, the soil samples were collected from a garage in Arat Kilo area of Addis Abba city in Ethiopia. The samples were collected and sealed in sterilized plastic 50ml centrifugal tubes. Three different samples were collected from different depths of 50cm, 100cm and 150cm from an area where oil was being disposed within the workshop. Samples were put

in a zipper bag in which they were then transported to Addis Ababa University where they were stored in a refrigerator at temperatures of 4°C for further analysis.

### **3.4. Synthesis of petroleum wastewater**

In order to create the synthetic wastewater treatment different articles were studied to find out the different petroleum wastewater parameters, which were identified by other researchers. To create a 1L sample of the synthetic petroleum wastewater the following chemicals were used. For pH adjustment 0.1M HCL, from 97% HCL and 0.1M of NaOH were prepared for adjustment of the pH to the desired level of 7.1-7.4. The 1L synthetic petroleum wastewater was prepared by mixing 0.5g potassium permanganate, 0.3g of kaolin for the Total suspended solids (TSS), 3g of NaCl,  $1.5 \times 10^{-5}$ g of sodium sulphide, 10ml engine oil and 5ml of Phenol was added. 1M HCL and 0.1M of NaOH solutions were used to adjust the pH of the mixed solution to the desired level of pH 7.1-7.4 (Majumder et al., 2014). The solution was then stirred using a magnetic stirrer at 160 rpm for an hour to mix it well. The solution was stored in a labelled 1L volumetric flask. The synthesized wastewater comes with various advantages as compared to the real wastewater from the field because synthetic wastewater was precise control over the composition and concentration of different contaminations in petroleum wastewater. Therefore, synthetic wastewater can be easily produced, ensuring that experiments can be repeated with the same conditions and also this allows for the creation of petroleum wastewater of different strengths, which will give a high degree of how effective and efficient is the clay based MFC in the treatment of this kind of wastewater.

### **3.5. Characterisation of the synthetic wastewater**

The water quality parameter were determined through the use of the APHA (American Public Health Association) standard methods. The water quality parameters that were determined were the pH, Chemical Oxygen Demand (COD), and Biological Oxygen Demand (BOD). The pH was measured using a digital pH meter, Chemical Oxygen Demand (COD) was determined through using closed flux colorimetric method (APHA 5220 D), and Biological Oxygen Demand (BOD) was determined through the Winkler (APHA 2021).

### **3.6. Microbial extraction and culturing**

Bacterial strains resistant to complex petroleum environments extreme, were extracted from a contaminated soil (50–150 cm depth) collected from a long-term garage diesel oil waste disposal site in Arat Kilo, Addis Ababa, Ethiopia, coordinates 9°02'00.9''N 38°46'01.9''E. The bacterial strains were grown and isolated using 1 litre selective medium made of 5g, NaCl, 2g

NaNO<sub>3</sub>, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 1g, K<sub>2</sub>HPO<sub>4</sub> 0.27g, KH<sub>2</sub>PO<sub>4</sub> 0.35g, 0.2g MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.1g CaCl<sub>2</sub>.2H<sub>2</sub>O and supplemented by 10ml of trace metals stock 30gH<sub>3</sub>BO<sub>3</sub>, ZnSO<sub>4</sub>.7H<sub>2</sub>O 10mg, NaMoO<sub>4</sub>.2H<sub>2</sub>O 3.0 mg, MnCl<sub>2</sub>.4H<sub>2</sub>O 3.0mg, NiCl<sub>2</sub>.6H<sub>2</sub>O 2.0mg, CoCl<sub>2</sub>.6H<sub>2</sub>O 10mg, CaCl<sub>2</sub>.6H<sub>2</sub>O 1.0mg ) which was sterilized through filtering with a 0.20 micron pore size filter paper and and lastly 10ml of diesel oil was added (Maqsood et al., 2022). 0.1M NaOH and 0.1 M of HCL were used to adjust the pH of the media to 7.1-7.4. To make this media diesel oil was used as the sole carbon and nitrogen source to enrich selectivity of petroleum wastewater degrading microbial consortia. A selectively medium was used because only the anaerobic microorganisms that can survive on degrading the petroleum waste were necessary for this experiment, therefore the conditions were kept this way to only encourage the growth of such organisms. The selective medium was made in a manner than it mimics the petroleum wastewater as a result this ensured more confidence that the microorganisms extracted will be able to survive in the complex petroleum environment ,under anaerobic condition at a pH of 7.1-7.4 as these will be the operating condition of the MFC. 100ml of nitrogen supplement was added which consisted of 10g NaNO<sub>3</sub>, 2g (NH<sub>4</sub>)SO<sub>4</sub>, 0.54g K<sub>2</sub>HPO<sub>4</sub>, 0.7 KH<sub>2</sub>PO<sub>4</sub>, 0.4g MgSO<sub>4</sub>.7H<sub>2</sub>O (Nawaz et al., 2022). The media was autoclaved for 2 hours. The autoclaved media was taken to septic hoover and under septic conditions 1ml of trace metal and 1g of the oil-contaminated soil added to the medium in a 150ml glass bottle, filled to maximum to ensure that there is anaerobic conditions, and was sealed with parafilm to ensure an extra protection against oxygen contamination. The inoculate was incubated at a temperature of 30°C under anaerobic conditions together with the control which had no contaminated soil. The main aim of ths procedure was to extract and enrich mixed consortia of organisms from the oil-contaminated soil, which will then be used in the clay, based MFC as inoculum. The reason why a mixed consortia was chosen instead of using pure isolates was that according to (Varjani, 2020) mixed consortia of microorganisms tend to outperform the pure isolates in terms of efficiency and power generation in the MFC. The reason for this phenomenon was attributed to a mixed consortium having a variety of bacteria species, which have the ability to degrade a vast umbrella of substrates as compared to pure cultures, which tend to produce enzymes specific to a certain types of substrates. The other reason for the superior performance of the mixed consortia was that, a mixed consortia of microbes tend to form a synergic relationship among themselves in which the end products of one microorganisms are used as a substrate by other microorganism, therefore due to this phenomena more organic compound will be degraded and more production of electrons. As a

result, this makes it very advantageous for mixed consortia to be used in the MFC than pure isolates.

### **3.7. Fabrication and preparation of the clay tubular membrane separator**

According to (Me & Bakar, 2019) a cylindrical design of the MFC membrane limits dead liquid space compared to planar designs, which means there is continuous interaction of the liquid and the periphery of the walls of the membrane as a result of this there is enhanced mixing and continuous flow of the liquid in the system thus more frequent electron and ion transfers. The aspect of the tubular design is essential because the flow of mass at the electrode must not be limited. On top of that (Liu et al., 2018) also stipulated that a high pollutant removal efficiency and power generation was experienced when using a tubular ceramic membrane as compared to a planar. As a result of these, finding a tubular design was adapted in this experiment because of the superiority exposed by other studies over planar membranes. The double chamber clay based MFC was constructed for this experiment, this clay based MFC was classified as double MFC configuration because the anode chamber and cathode chambers were separated by the clay based membrane. This double chamber membrane configuration was selected over the single chamber because the double chamber MFC is often preferred for its ability to minimize cross-contamination, enhance control over environmental conditions, improve electron transfer efficiency, and facilitate scalability in diverse environmental and energy-related applications. The clay based membrane of the following specifications were assembled in (Ensira Pottery, Ketchane, Ethiopia), 11.5cm height, 15cm diameter and thickness of 1.2cm and the tubular ceramic membrane was sealed one end. The thickness of the MFC was made as small as possible because according to (Winfield et al., 2016) the thicker the walls of the ceramic membrane the less efficient the MFC was in pollutant removal and energy recover, therefore in basic term the thickness of the membrane is inversely proportional to the efficiency of the MFC.

### **3.10. Experimental set up and MFC operation**

A double chamber MFC was assembled using 11.5 cm height, 15 cm diameter and 1.2cm thick tubular clay membrane fabricated in local pottery workshop in Addis Ababa, Ethiopia sealed at one end forming a hollow chamber. This design decision to use a tubular membrane was

made because according to (Me & Bakar, 2019) tubular design limits dead liquid space compared to planar designs, allowing maximized mixing and continuous flow of liquid in the system. The tubular design is also essential because unlike the planar membrane designs it enables for an unlimited the flow of mass at the electrode therefore it is an optimal design for the MFC. Apart from the abundance in availability this kind of clay is the best candidate for the clay minerals such as montmorillonite, kaolinite and bentonite have a layered structure with a high surface area and a negative overall charge. The negative charge is the main reason, which makes the clay to be able to adsorb the metal ions. This property makes it possible for the clay to also act as membrane separators, which further degrade heavy metal in the MFC technology (Salar-García et al., 2021). A carbon cloth 1 from (Easycomposite,UK) was used as both the anode and cathode. To form the anode, 546cm<sup>2</sup> of carbon cloth was wrapped around the ceramic cylinder and 455.2 cm<sup>2</sup> of carbon cloth was attached to the internal periphery of the ceramic membrane to form the anode. Copper wires of diameter of 0.1 mm diameter were then used to connect the cathode and the anode. The opening of the clay membrane was covered by a polyethylene waterproof membrane with an opening for feeding anode chamber with water sample and microbial inoculum obtained from soil contaminated with oil. From there the set-up was placed inside a 2L plastic bucket, which was then be filled with a mixture of 0.1M Potassium ferricyanide in 0.1M Phosphate Buffer solution, 480ml and 220mL respectively. The reason for using potassium ferricyanide and a phosphate buffer in the cathode is that Potassium ferricyanide  $K_3Fe(CN)_6$  serves as an electron acceptor in the cathode chamber. The phosphate buffer on the other hand helps to neutralize any acids or bases that might be generated during the electrochemical reactions. This buffering action prevents the development of highly acidic or alkaline conditions, which could otherwise inhibit the performance of the MFC and potentially damage the cell components. During the operation of the MFC, electrons are transferred from the anode to the cathode. Potassium ferricyanide acts as a redox mediator, accepting electrons from the electrode and undergoing a reduction reaction. This process facilitates the overall electron transfer, enhancing the performance and efficiency of the MFC. The anode chamber was filled with 750mL of synthetic petroleum wastewater, 100ml of nitrogen supplement which consisted of 10g of NaNO<sub>3</sub>, 2g of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.54g of K<sub>2</sub>HPO<sub>4</sub>, 0.7 of KH<sub>2</sub>PO<sub>4</sub> and of MgSO<sub>4</sub>.7H<sub>2</sub>O. For duplicate MFCs, MFC1 and MFC 2 75mL of microbial inoculum as a source of microbial consortia .For MFC control there was no microbial inoculation added. All MFC systems were operated simultaneously at room temperature and batch mode with the inoculation time of 30 days.



**Figure 4a: Fabrication of Clay-based Microbial Fuel Cells, Fig 4b: final experimental MFC set ups in petroleum wastewater degradation and energy generation**

Figure 7a and 7b shows MFC-1, MFC-2, MFC-c(control) which had no inoculation added in the anode chamber, was operated in fed batch mode for a total of 30 days at cycle time of 12 hours, with the pH of 7.1-7.4, and was operated at room temperature. The readings were taken for every hour for 12 hours a day. After every 7 days of operation, the samples were drawn from each MFC for testing the COD and the BOD, which was used to determine the COD and BOD removal efficiency of the MFC.

### **3.11. Electricity generation measurements of the Microbial Fuel Cell**

The open circuit voltage generation data was measured using digital multimeter and, readings were taken after every hour from 08:00hours for 12hours over a period of 30 days . During each measurement time, three voltage readings were taken and the average voltage was reported. For the closed circuit voltage, external resistance load of 10-1000 Ohms were varied for a period of 13 days and readings were taken in the same manner as OCV readings. For every hour, three readings were taken and then averaged to make a single value for that particular hour. The following equations were used to determine current, power density, and COD removal efficiency.

Eq. 1:  $V = IR$ (Ohms Equation)

Where V is the voltage in Volts,I is the current in Amperes and R is resistance in Ohms

$$\text{Eq. 2: } P = \frac{VI}{a}$$

Where, P is the power density in W/cm<sup>2</sup>, V is the potential in Volts, I is the current in Amperes and a is the area of anode chamber in cm<sup>2</sup>.

**COD removal efficiency:** COD of the analyte was measured at different days of operation using was determined using closed flux colorimetric method (APHA 5220 D), ((APHA2021). The COD removal efficiency ( $\eta$ ) was calculated using

$$\text{Eq. 3. :} \eta = (\text{COD}_i - \text{COD}_t) / \text{COD}_i \times 100\%$$

Where,  $\text{COD}_i$  = initial COD (mg/L) and  $\text{COD}_t$ = COD at specified time (mg/L).

## **CHAPTER FOUR**

### **RESULTS AND DISCUSSIONS**

#### **4.1. Synthesis and characterisation of petroleum wastewater**

In the study of wastewater treatment, particularly in the context of petroleum refinery effluents, the preparation of synthetic wastewater is a crucial step that allows for controlled experimentation and assessment of treatment technologies. The synthetic wastewater used in this study was designed to mimic the characteristics of actual petroleum refinery wastewater, ensuring that the results obtained are relevant and applicable to real-world scenarios.

The synthetic wastewater was prepared using a combination of organic and inorganic compounds that are commonly found in refinery effluents. This mixture aimed to replicate the high Chemical Oxygen Demand (COD) and Biological Oxygen Demand (BOD) typically associated with such wastewaters, providing a suitable substrate for microbial activity in the microbial fuel cells (MFCs). The pH of the synthetic wastewater was carefully monitored and maintained within the range of 7.1 to 7.4, which aligns with the typical pH values observed in actual petroleum refinery wastewater (see Table 1). This pH range is essential for optimizing microbial growth and activity, as it falls within the preferred range for most electroactive bacteria.

Upon preparation, the synthetic wastewater exhibited a COD of 2128 mg/L and a BOD of 1476 mg/L. These values reflect a high concentration of organic material, providing an ideal environment for microbial communities to thrive. COD serves as an indicator of the total amount of oxygen required to chemically oxidize organic and inorganic matter in the wastewater, while BOD measures the oxygen consumed by microorganisms during the decomposition of organic matter. The significant disparity between COD and BOD values suggests the presence of a substantial amount of readily degradable organic material, which is crucial for the performance of MFCs.

#### **4.2. Extraction of exoelectrogens from the soil**

After seven days of incubation, there was a clear turbidity and suspension seen in the anaerobic inoculated media and had a clear distinction from the control, indicating successful microbial growth as shown in figure 8. This shows that the selective media that was made to mimic the petroleum wastewater was a suitable substrate for the microbes and most importantly, these results show that only microbes that can survive in the complex chemical environment of

petroleum wastewater survived. According to (Aelterman et al., 2006) it is very advantageous to use mixed culture than a pure culture because a mixed consortium consists of multiple microbial species with diverse metabolic capabilities. This diversity allows them to collectively utilize a wider range of substrates present in the wastewater, including complex organic compounds like those found in petroleum wastewater (Jabbar et al., 2023a). The other reason for a mixed consortia being preferable is that different microbial species within the consortium can engage in synergistic interactions. For example, one species may produce metabolites or enzymes that facilitate the degradation of complex organic molecules, which are then utilized by another species for further breakdown. This cooperative behaviour enhances overall substrate utilization efficiency and degradation rates compared to a single species (Jabbar et al., 2023b). Therefore, the inoculum was used directly in to the MFC for this reason instead of isolating and pure culturing.



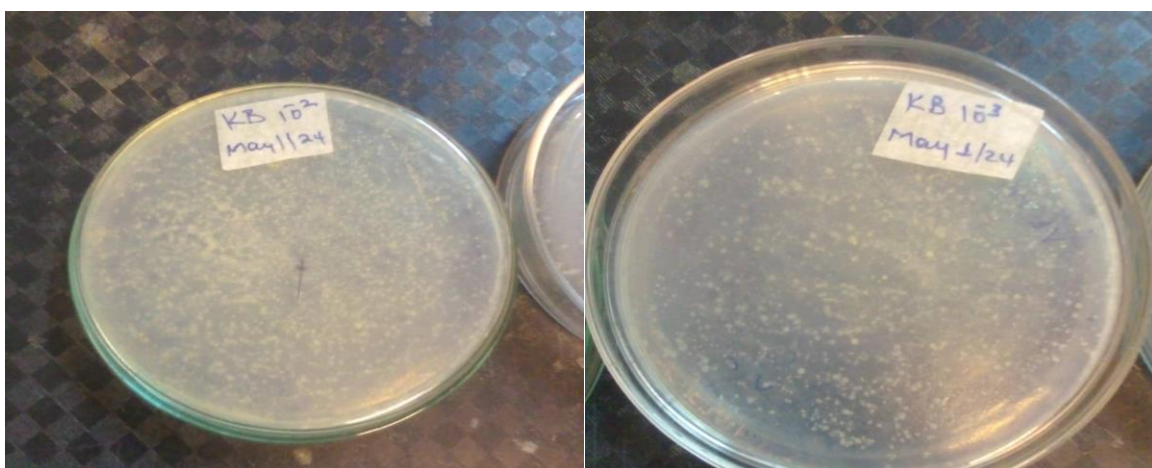
**Figure 5: Anaerobic culture Vs Control after 7 days of incubation**

As shown in figure 5 there is a clear turbidity and suspension seen in the anaerobic inoculated media and had a clear distinction from the control, indicating successful microbial growth from the oil contaminated soil. This shows that there are some microbes, which managed to survive

in the complex environment of the petroleum wastewater, and anaerobic conditions, which will be the same operating conditions for the microbial fuel cell.

### 4.3. Characterisation of the microbes

Growth was shown by the development of white mass in form of a circular shape as shown in figure 6. This shows that microorganisms from the soil managed to survive or grow in conditions of the selective medium, which imitated the petroleum wastewater. Therefore, because of these results there is confidence that the microorganisms will be able to survive by degrading the organic compound in the petroleum wastewater and thus making them applicable for the clay-based microbial fuel cell experiment. The used of a selective medium led to a restriction in a lot of microorganism growing, therefore three distinctive colonies were identified and taken for screening and evaluation using staining method. From this procedure the results showed that there is a consortia of microorganisms in the inoculum some show a circular shape and clustered together and some showed a rod shape. These are desirable results because according to (Fang & Achal, 2019) a mixed consortia of organisms have a high efficiency in degrading organic matter in the wastewater and have a higher potential in producing electricity that when pure cultures are use. The reason for this is that a community of different organisms produce different enzymes to degrade different substrates or materials in the wastewater as a result of this variation there is a wide umbrella of organic materials that can be degraded unlike pure cultures, which produce one kind of enzymes to degrade a specific substrate.



**Figure 6: Anaerobic microbial culture on a petroleum wastewater**

Figure 6 shows the growth of microorganisms on the solid nutrient agar, which contains the petroleum wastewater. These results showed that there are different colonies of microorganisms

in the inoculum and most of which through the visual observations seems to be of a similar colour and shape. The possible reason for this might be the usage of a selective medium under anaerobic conditions, which inhibited the growth of other microbes that cannot grow in such conditions. As a result colonies that can survive from degrading or metabolising on petroleum wastewater were the ones that managed to survive. This is also evidence that there is a mixed consortium of microorganism in the inoculum, which is desirable for the microbial fuel cell because according to (Jabbar et al., 2023b) mixed consortia of microorganisms tend to outperform pure cultures in the microbial fuel cell in terms of both the treatment and energy generation efficiency. In a mixed consortia of microorganisms a variety of microbes produces different substrates which results in a vast degradation of different organic compound in the wastewater and sometimes microbial communities have forms a synergy relationship which involves microorganisms further using other microbes products in their metabolic process thus further degrading the wastewater and producing more electrons in the microbial fuel cell.

#### 4.4. Colony characteristics

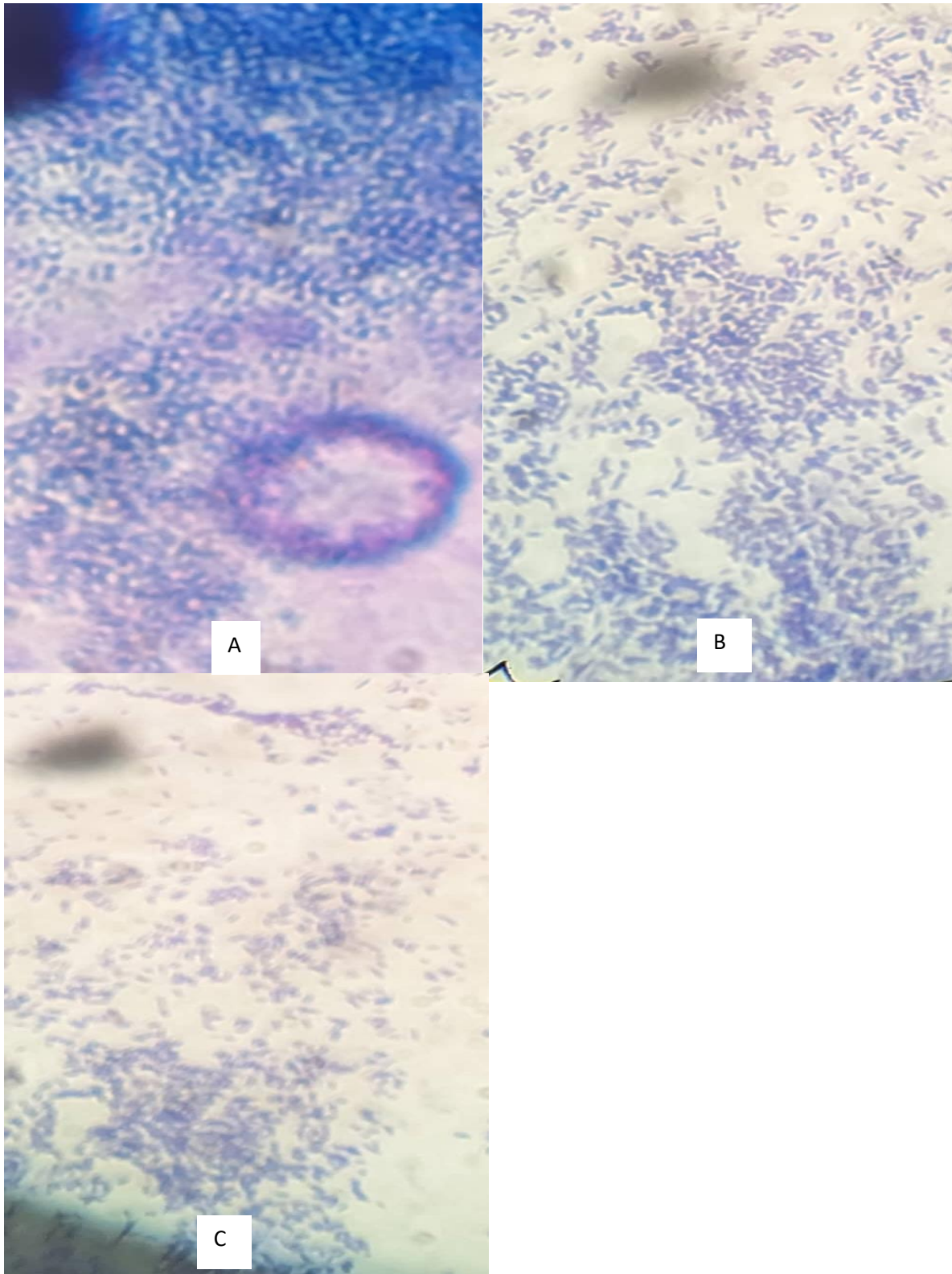
Different bacterial colonies with their varied characteristics like growth pattern, pigmentation, size, elevation, and margin on agar plate were observed on petri plates.

**Table 6: Colony characteristics observed in the plates after incubation in the anaerobic jar**

S no.	Plate	Growth	Pigment	Size	Elevation
1	10 <sup>-2</sup>	Large	Yellow	Large	Raised
2	10 <sup>-3</sup>	Large	Yellow	Large	Flat
3	10 <sup>-4</sup>	Slight growth	White	Moderate	Flat
4	10 <sup>-5</sup>	Slight growth	Yellow	Moderate	Raised
5	10 <sup>-6</sup>	Slight growth	Yellow	Moderate	Raised
6	10 <sup>-7</sup>	No growth	No growth	No growth	No growth
7	10 <sup>-8</sup>	No growth	No growth	No growth	No growth
8	10 <sup>-9</sup>	No growth	No growth	No growth	No growth
9	Control	No growth	No growth	No growth	No growth

Table 6 shows different characteristics of colonies that grew anaerobically in the anaerobic jar. The characterisation were based on the visual characteristics such as the colour, growth size and elevation. As mentioned in the previous chapter each plate was streaked with a sample as per the serial diluent. Therefore results show that there was a higher growth in factor 10<sup>-2</sup> and

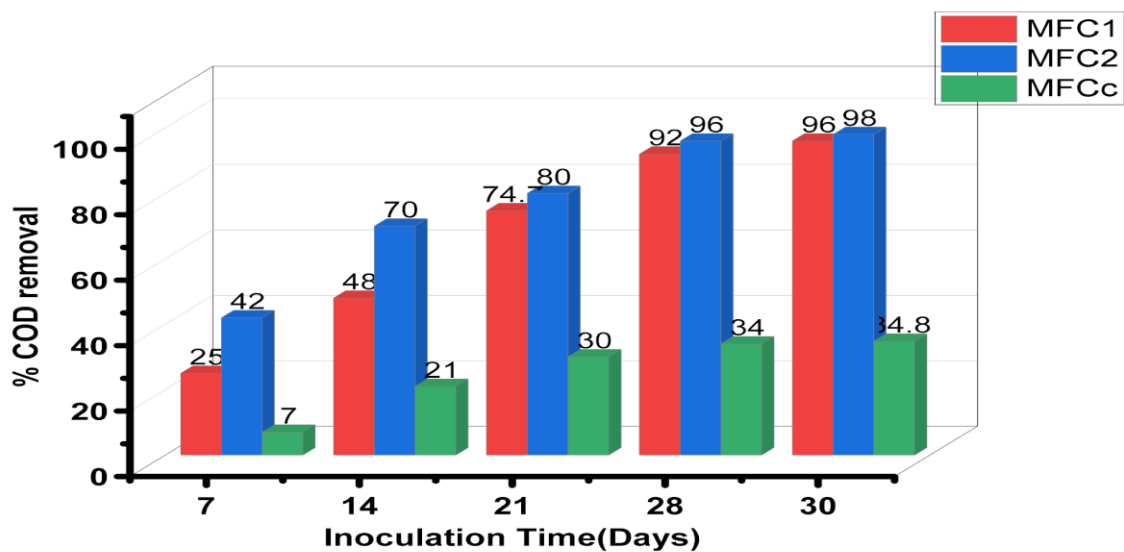
10<sup>-3</sup> which makes a lot of sense because the purpose of serial dilution is to reduce the concentration of micro-organisms so at these dilution factors there was still a very high concentration of microorganisms from the inoculum which is signified by high microbial growth on the plates. As the dilution factor increases the microbial concentration decreases, which was justified by these results because high dilution factors resulted in low to no microbial growth.



**Figure 7: Images of cultured microorganisms (A:Rod shaped , B:circular and C:oval shape)**

Figure 7 shows images of three distinctive colonies through the light microscope after the staining process. From the images the first conclusion that can be drawn is that, there is a mixture of different microorganism in the inoculum, which is advantageous in the microbial fuel cell application. The other observation is that the shape and the morphology of the microorganisms are distinctive as shown in figure 7, some micro-organisms depict a circular shape and are clustered together and some are rod shaped and appear to be both scattered and clustered community.. Therefore this is a clear indication that a consortia anaerobes that can survive in the anodic environment of the clay based MFC were successfully extracted from the oil contaminated soil for the purpose of application in the clay based microbial fuel cell. The results also shows the presence of mixed consortia of organisms in the inoculum, which enhances the efficiency of the MFC.

#### 4.5. Efficiency of the clay based Microbial Fuel Cell in treatment of synthetic petroleum wastewater

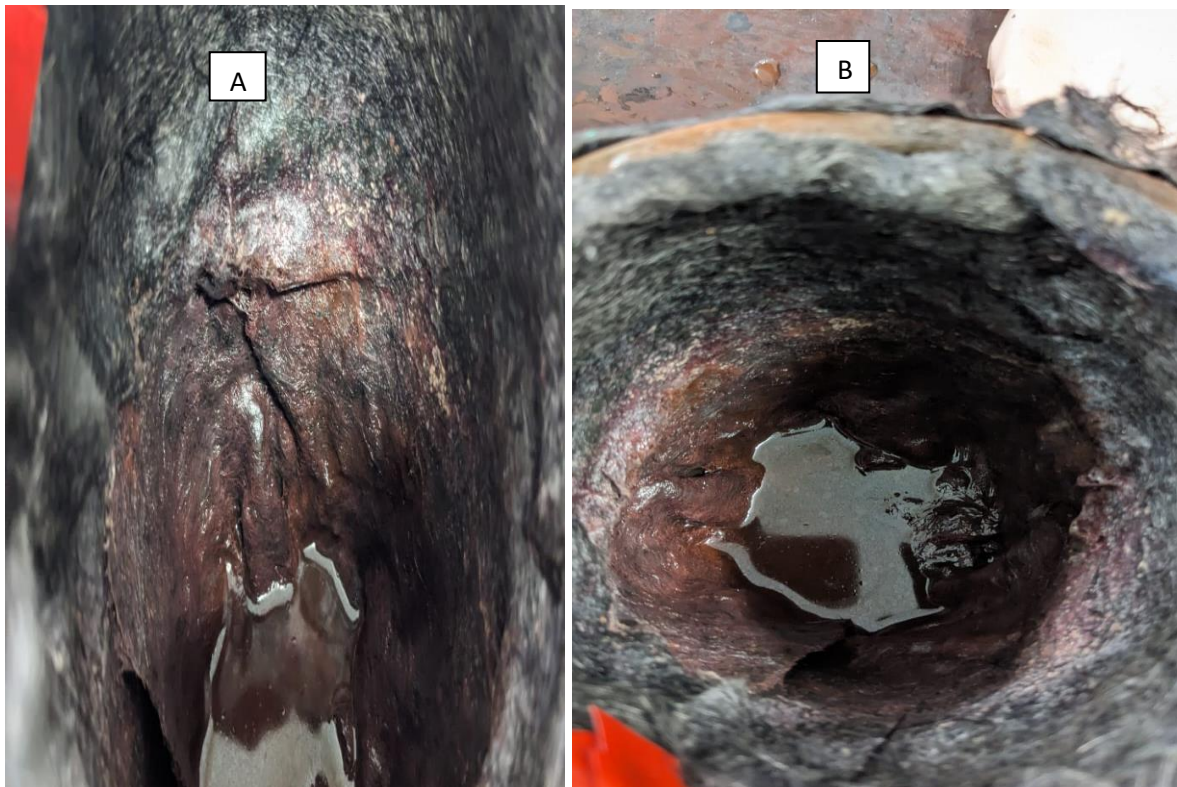


**Figure 8: Chemical Oxygen Demand removal efficiency of Microbial Fuel Cell reactors inoculated with extracted microorganisms**

The clay based membrane microbial fuel cells, designated as MFC1, MFC2 and MFCcontrol demonstrated distinct capacities in removing chemical oxygen demand over a period of 30 days. As shown in figure 8, MFC1 exhibited a gradual increase in COD removal efficiency, reaching 25% after seven days, 48% after 14 days, 75% after 21 days, 92% after 28 days and achieving a maximum efficiency of 95% after 30 days. In contrast, MFC2 showed more rapid COD removal, with efficiencies of 47%, 70%, 80%, 95% and 98% at the corresponding time points. The results obtained from MFC1 and MFC2 as the duplicate clay based microbial fuel

cells, which were both inoculated by anaerobes obtained from the soil showed a slow rate of COD removal in the first 14 days, a gradually increasing phase after 21 days and steady state within the last 30 days of operation. This highlighted that time has significance influence in the COD removal efficiency of the MFC as other conditions like pH, temperature and inoculation volume were kept constant. The reason for this is because during the initial inoculation of the MFC, the microorganisms have to first acclimatize to the anode environment before they start degrading the organic compounds in the wastewater. As shown in figure 4 there is a biofilm formation, which is more profoundly visible in MFC1 and MFC2 as compared to the MFC control. Therefore the more time the microorganisms spend in the anode the more acclimated they become to the anodic environment thus there is an exponential growth and vast metabolic activity by the microorganisms. As a result more substrate is degraded and subsequently more COD is removed from the petroleum wastewater as observed in this study. The significantly high COD removal efficiency showed that the clay based MFC is highly effective in removing the organic constituents of the synthetic petroleum wastewater. This study pioneers the use of clay based MFC in the treatment of petroleum wastewater and generation of energy. Therefore looking at the COD removal efficiency of the clay based MFC1 was 96% and 98% for MFC2. These results show that the clay based microbial fuel cell was superior in removing organic contents from the petroleum wastewater to add emphasis on this (Jabbar et al., 2023a) conducted a study using the MFC with (type: CMI-7000, Membrane International INC., NJ, USA) as an ion exchange membrane and aeration to induce reduction in the cathode the achieved COD removal efficiency was 95% which is lower than of both duplicate clay based MFCs. As a result, this proves that the clay based MFC is effective in treating the petroleum wastewater even more effective than artificial ion exchange membranes and without any mechanical intervention like aeration mechanisms. Therefore, with minimal input parameters and cost the clay based MFC outperformed the traditional MFC in treatment of the petroleum wastewater. To add emphasis on superiority of clay based MFC in the removal organic compounds from petroleum wastewater, at 2010 Luo and colleagues in their study of using a continuous inflow cyclic bio-reactor containing a stirring mechanism obtained a COD removal efficiency of 71% from the oil refinery wastewater which is very lower as compared to the ones compared illustrated by the clay based MFC. This shows that even with less mechanical intervention in the clay based MFC still a higher COD was achieved proving that the clay based MFC is more lucrative method to treating petroleum wastewater.

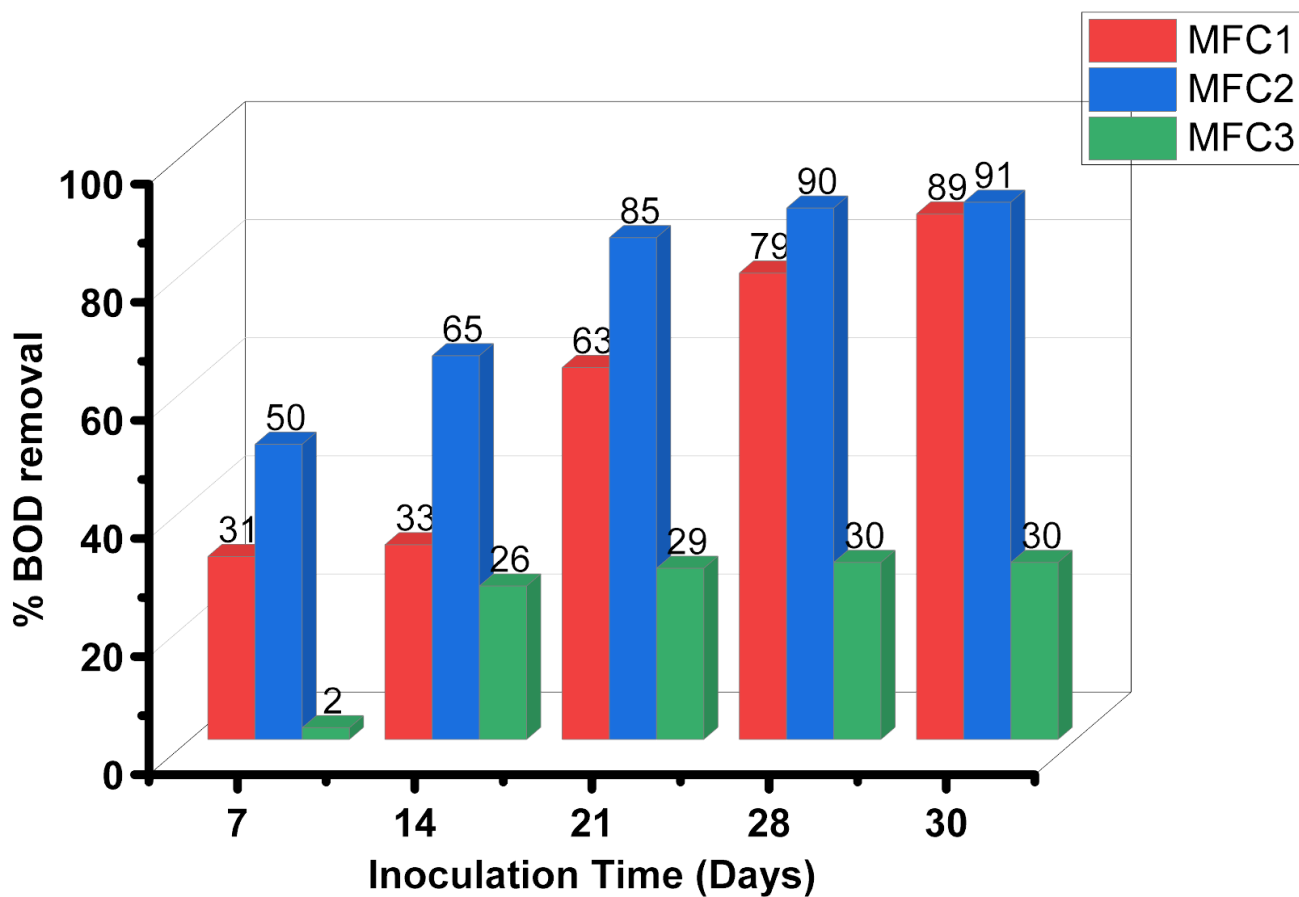
Lastly the control showed to 7%, 21%,30% ,34%,34.8% COD removal efficiency at the same time points at the duplicate MFCs. This was the lowest COD removal efficiency due to the fact that there was no inoculation that was added in this MFC, therefore because of very little activity in this MFC it further proves that the high COD removal efficiency is indeed a contribution of the inoculation that was used in the duplicate MFCs. On top of that, the reason why there might have been some activity in the control even though there was no inoculum added is that, during the manufacturing of the clay based membrane there was no strict regulation and treatment of the clay and the water used therefore due to this reason there may be some traces of exoelectrogens in the clay-based membrane which later contributed in removal of some COD. The other reason for the observed COD removal efficiencies in the control is that clay minerals such as bentonite or kaolinite can lead to the adsorption of organic compounds on the surface of the clay. Therefore because organic compounds contribute to the COD this leads to the COD removal in the wastewater as observed in the control. This reason is justified because Ewis and colleagues in 2022, successfully used clay to remove organic pollutants from wastewater.





**Figure 9 .Biofilm formation on the anode for A: MFC1, B: MFC 2 and C: MFC control**

As shown in figure 9 , there was more profound and visible biofilm formation on MFC1 and MFC2 which were inoculated with the extracted microbes as compared to MFC control which had no inoculation .This may be the reason why high COD removal efficiencies were experienced by these MFCs than the control . On top that the visible growth on the anode can be attributed to the successful growth of microorganism extracted from the soil and also their success in the removal of organic compounds from diesel-oil wastewater. This conclusion is due to the MFCcontrol having very no clear visible growth on the anode thus why it was not as efficient in removing COD than the MFC1 and MFC2. The biofilm on the anode creates a dense cluster of microorganisms that are directly in contact with the electrode. This setup promotes effective degradation of organic substrates in the wastewater. The biofilm matrix ensures that organic matter remains concentrated near the microorganisms, enhancing their ability to break it down. Research indicates that biofilms enhance COD removal efficiency by bringing microbes closer to the substrates (Palaniandy & Feroz, 2018).

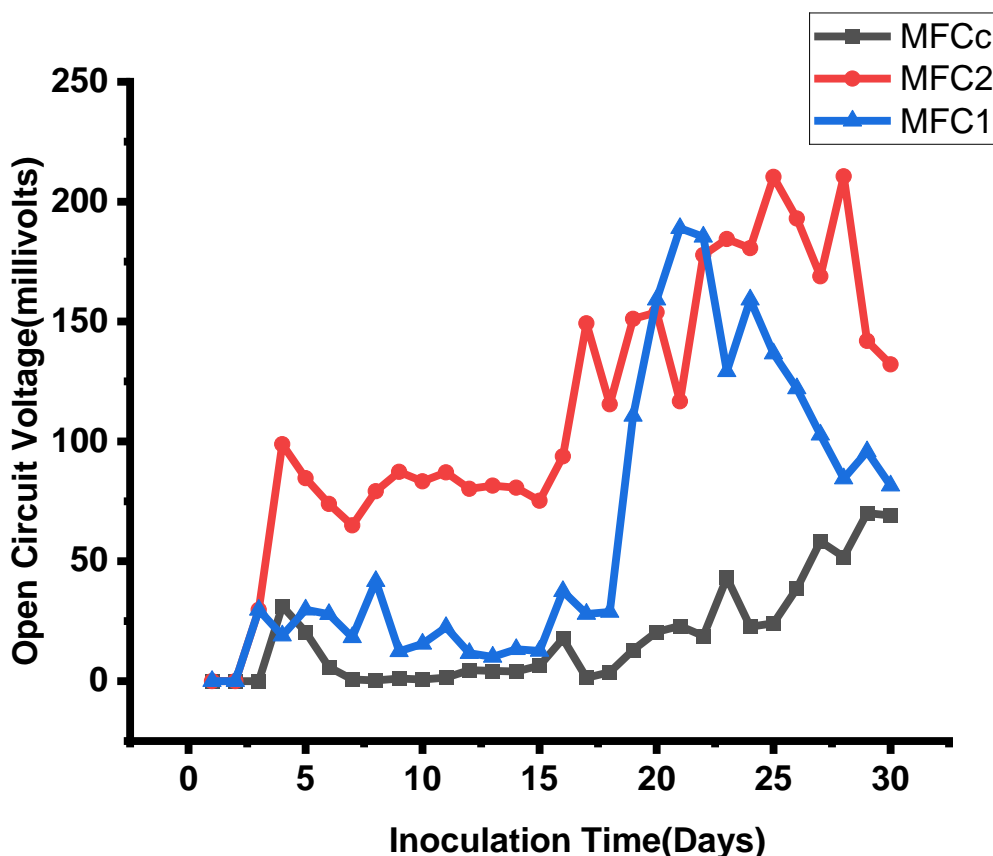


**Figure 10: Biological Oxygen Demand removal efficiency of Microbial Fuel Cells inoculated with extracted microorganisms**

The clay membrane microbial fuel cell (MFCs), MFC1 and MFC2, inoculated with a mixed culture from a mixed culture from oil contaminated soil, demonstrated a significant contribution in the removal of biochemical oxygen demand (BOD) over a 30 day period. MFC1 exhibited gradual improvement in BOD removal efficiency, achieving 31% after 7 days, 33%

after 14 days ,63% after 21 days,79% after 29 days and reaching 89% after 30 days as depicted by fig 10. In comparison, the duplicate MFC2 showed to have higher initial efficiency and faster stabilisation, with removal rates of 50%, 65%, 85%, 90% and 91% at corresponding points in time. The results portrayed by MFC1 and MFC2 exposes the effectiveness of clay membrane microbial fuel cells in biodegrading organic pollutant in the petroleum wastewater, as indicated by their BOD removal efficiencies. Both MFC1 and MFC2 demonstrated a progressive increase in BOD removal efficiency over the 30-day period .This temporal evolution suggests the gradual acclimatisation and proliferation of microbial populations with the MFCs, adapting to the provided substrate and optimising metabolic pathways for more efficient BOD degradation. The higher initial efficiencies observed in the MFC2 may be attributed to a more robust initial microbial community or favourable conditions that promoted rapid activity. The presence of mixed consortia of microbes from oil-contaminated soil in both MFCs is the reason of this enhance degradation. This is because the inoculation introduced a diverse microbial species capable of metabolising complex organic pollutant present in the petroleum wastewater, thereby enhancing the overall biodegradation rates .MFCcontrol, which was not inoculated by micro orgasms showed a markedly lower BOD removal efficiency, highlighting the critical role of microbial communities in driving bioremediation processes with the fuel cell environment. The BOD removal efficiency that was obtained in this experiment were higher than that obtained by Ismail and Jaeel in 2013 were they used a pure strain of *Bacillus Subtilis* in an up flow MFC. They recorded a BOD removal efficiency of 82%, which is lower than the one observed in the clay based MFC experiment. The reasons behind this may be the use of a pure culture in their MFC unlike the mixed consortia, which was used in the clay based MFC. Unlike the pure culture a mixed consortia has a variety of microorganism working together to degrade petroleum wastewater, therefore this means there is a vast excretion of different enzymes, which can target a wide range of pollutants.

#### **4.6. Green energy recovery potential of the clay based MFC**



**Figure 11: The daily generation of open circuit voltage by Microbial Fuel Cell**

Figure 11 reveals the behaviour of the OCV generation over a period of 30 days for three different MFC (MFC1, MFC2, MFCcontrol). MFC1 and MFC 2 were inoculated with 10% inoculum by volume, obtained from the oil contaminated soil and MFCcontrol was not inoculated in order for to see if indeed the microbes from the soil are the ones contributing to the voltage generated by the clay-based MFC. The results showed that for MFC1, no voltage was generated in the initial 2 days of the experiment. MFCs rely on microbial activity to generate electricity through the oxidation of organic matter. In the initial days, the microbial population on the anode (where oxidation occurs) is still establishing and growing. It takes time for the microorganisms to colonize the electrode surface and form a biofilm that can efficiently transfer electrons. Subsequently, from day 4 to 17 the voltage showed a fluctuation behaviour between 29.7mv and 41.7 mv, this indicated the initial microbial colonisation and initiation of microbial activities. This shows that at this stage microorganisms are begin to acclimatise well to the anodic environment and they started the production of the essential enzymes for degradation of organic compounds. The other reason for this behaviour is the fact that the

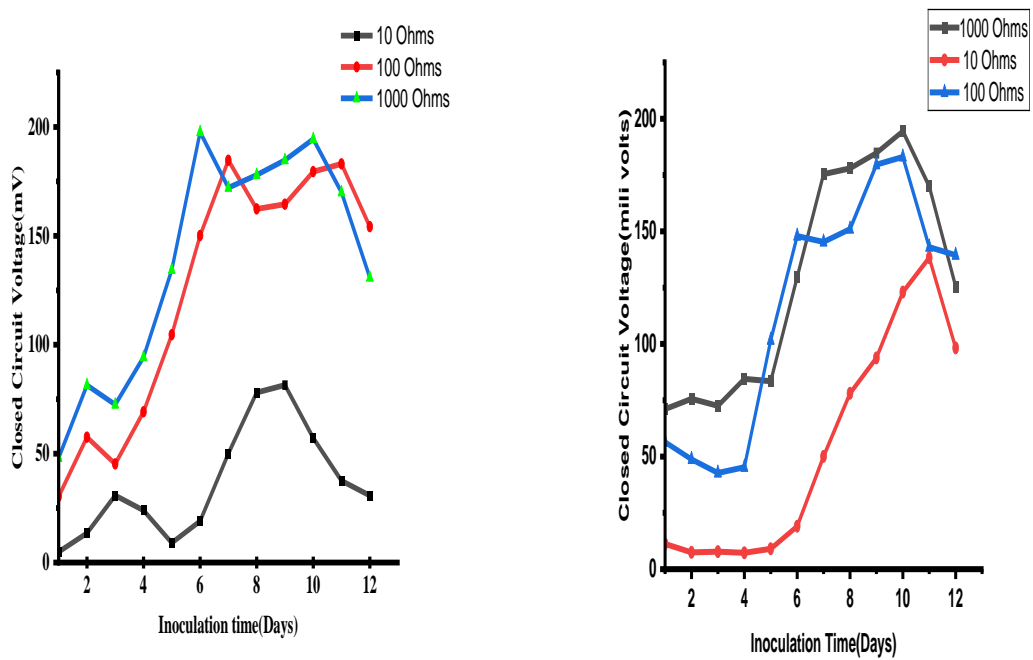
microorganisms are beginning to successfully form a biofilm on the anode and most importantly shows that the microbes from the soil are exoelectrogenic, which means they are electron-producing organisms thus electricity was produced as they degrade the petroleum wastewater. The voltage then gradually increased until it reaches the maximum of 188.92mV around day 20 before declining steadily. This shows the growth phase behaviour of the microbial activity in the MFC, because the increase in the voltage to the maximum shows that the micro-organism have successfully acclimatized to the anodic environment and successfully formed biofilms on most of the anode surface thus there is enhanced direct transfer of electrons in the MFC as microorganisms carryout enzymic reactions for their metabolic processes, see figure 9. The steady increase in the voltage may also be due to some microorganism releasing electrons as a by-product of oxidation reactions. These electrons are usually transferred by electron carriers such as NADH (Nicotinamide adenine dinucleotide) and FADH<sub>2</sub> (Flavin adenine sinucleotide) within the microbial fuel cell.

Similarly, MFC2 showed no voltage output in the first two days .From day 4 to 15 ,the voltage ranged between 98.83mV to 147mV,reflecting microbial adaptation and metabolic activity. The voltage then increased steadily , reaching a maximum of 210.35mV around day 18 before declining steadily. The decline of the OCV may be because of biofouling of the clay membrane,depletion of the substrate and the build-up of products which are toxic to the microbes.In contrast with MFCcontrol ,which remained un-inoculated with any microbes ,exhibited some activity ,with the voltage reaching a maximum of 58.29mV on day 27.This unexpected activity in the control warrants further investigation into potential abiotic factors or even possible biotic factors that lead to voltage being generated though negligible as compared to MFC1 and MFC2 .Some of the possible reason for the activity in the control MFC is that the clay used in the MFC was prefabricated therefore there is no confident knowledge of whether the clay was treated and also the quality of the water used to mould the clay,as a result of this there might be some microbes which exist in the soil which were present in the clay as studies showed that some exoelectrogens such as geobacter can exist naturally in the soil(Moon et al., 2005). Therefore, there is a possibility of clay-based membrane being contaminated by such microorganism leading to them to metabolise as they degrade the petroleum wastewater and as a result produce electrons, which are observed as the voltage. The abiotic factor that might have led to this an activity in the MFC in terms of OCV is that ,the components used in the MFC such as potassium ferricyanide which is a strong oxidising agent can facilitate electron transfer from the anode to cathode via external circuit to the cathode thus

leading to generation of voltage. The other reason may be that some organic compounds contained in the petroleum wastewater can undergo oxidation reactions and release electrons directly to the anode, which are then transferred by external circuit. Last the reason for an activity in the control may have been due to the carbon cloth which was used as electrodes in the MFC has a very high surface area and conductivity, which can facilitate electrochemical reactions even without the presence of microorganisms.

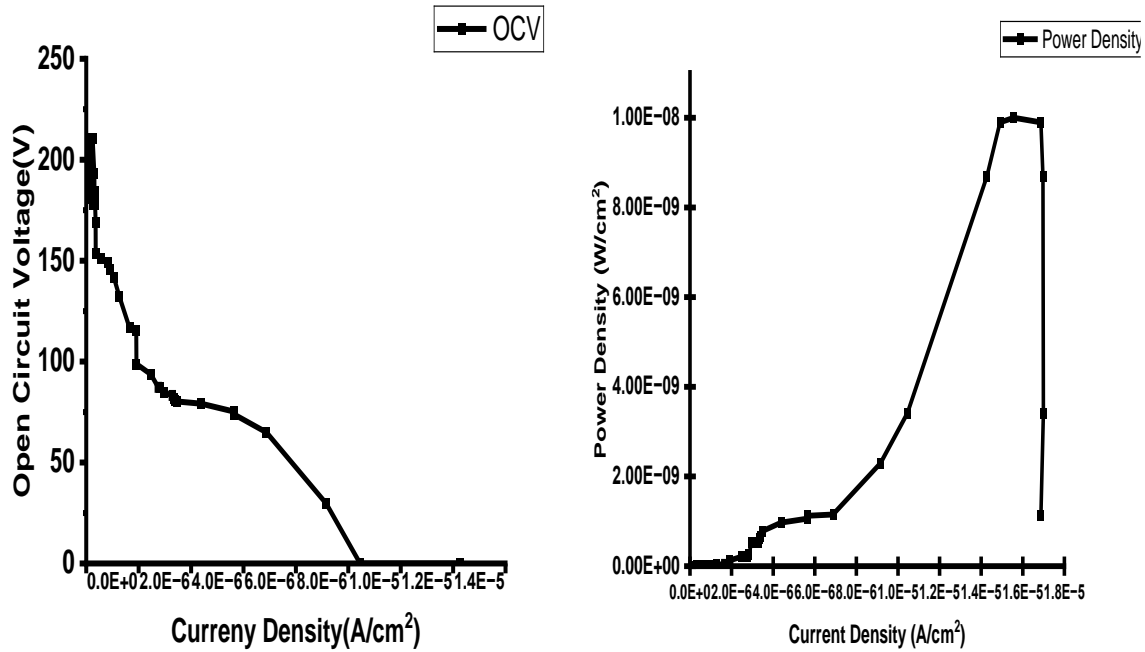
The OCV time profile of MFC1, MFC2 and MFCc provided a valuable insight into the dynamics of the microbial fuel cell operation. Also showed that high OCV generated by MFC1 and MFC 2 was due to the microbial intervention in the MFCs because there is a very huge gap in terms of the OCV generated by these MFCs and the control. The results also showed that there was unexpected activity in the MFCc, which even when there are a lot of possible reasons stated to justify it, further investigations are needed especially in the abiotic factors influencing the electrochemical processes.

Using clay based MFC have never been used before to treat petroleum wastewater therefore there are no similar studies to compare its efficiency with, but looking at other studies where traditional MFC membranes were used in MFC technology to treat petroleum wastewater, Jabbar and colleagues in 2023 achieved 235mV of OCV while using the petroleum wastewater as a substrate. This OCV was higher than the one of my study which was 210mV with a difference of only 25mV. The reason for this difference might be from the pumping of the oxygen they used in the cathode chamber to enhance the oxygen reduction reaction. Looking at this small difference between the OCVs generated through these systems, it shows that the clay based MFC is efficient in producing electricity with less intervention of mechanical intervention and aeration, making it a cheaper and feasible but yet energy efficiency system.



**Figure 12: Closed circuit voltage of Microbial Fuel Cells(Caption A: MFC1 and Caption B: MFC2) respectively across 10,100 and 1000 Ohms resistance**

Initially there was a drop in voltage production when compared to that of open circuit. The maximum voltages of the closed circuit across all variable resistances were measured as less to open circuit due to the potential across the cell decreased immediately when the external circuit was connected to the cell. The influence of external resistance directly affects the flow of electrons from the anode to the cathode through the external circuit. Higher resistances leads to increase voltage drop across the circuit, reducing the net potential difference available for power generation. Therefore the graph above makes sense in illustrating this phenomena because the highest open circuit voltage recorded 207mV for MFC2 and the for MFC1 186mv. On the contrary when the external resistances were loaded the highest voltage for MFC1 was 139 mV from a 10 Ohms resistor on the 11<sup>th</sup> day and for MFC2 the highest CCV was 186 mV on the 6<sup>th</sup> day. On top of that, the reason for this drop is that external resistance affects the rate at which microbes can transfer electrons to the anode. This is because lower resistances typically lead to higher current densities, stimulating microbial activities by facilitating faster electron kinetics (Schneider et al., 2023).



**Figure 13: Polarisation curves for the clay based Microbial Fuel Cell**

The electrochemical properties of Microbial Fuel Cells (MFCs), including overvoltage, can be assessed using polarization curve analysis, which involves plotting current versus voltage under different resistances during MFC operation (Moon et al., 2005). A polarisation experiment was conducted through the use of variable external resistor box and the voltage was measured as closed circuit voltage. From the closed circuit voltage and the resistance values the ohms law was used to calculate the current and subsequently the power generated at any given time of the experiment. Lastly, through dividing the current and power generated with the area of the anode current densities and power densities of different time intervals were determined. As illustrated in Figure 6 the experimental polarization curves showed an optimum current density of 15.5 mA/cm<sup>2</sup>, the power density achieved under a 10 Ω external resistance was 9.99 μW/cm<sup>2</sup> during stable operation. This indicates a favourable internal resistance for an efficient conventional MFC design, encompassing all electrical connections. Comparison with findings from Huang and colleagues in 2021 highlighted higher internal resistances observed with various electrode materials like graphite felt and carbon brush, underscoring the effectiveness of the current MFC design. The maximum OCV of 210.56mV represents the highest voltage output of the MFC when no load was applied. This point on the polarisation curve signifies the peak of the electro chemical potential generated by the MFC. Current density, represented on the x-axis, indicates that the rate at which electrons are transferred through the external circuit per unit area (Aljuboury et al., 2017). From the graph, it is clear

that at low current densities the MFC operates closer to the OCV, this indicates efficient electron transfer and minimal internal resistive losses. As the current density increases, the OCV decreased due to the increased polarisation losses. These losses include ohmic losses, which occurs due to resistances encountered by ions and electrons as they move, activation over potentials, which are losses that arise from the energy required to initiate and sustain electrochemical reactions at the anodes. The reaction involves the transfer of electrons between the electrodes and ions in the electrolyte. Over potential, occur due to kinetic barriers, which needs to be overcome for electrochemical reaction to occurs. Lastly is the diffusion polarisation, which occurs due to the depletion or accumulation of reactants or products at the electrode surfaces. In the Microbial Fuel Cell (MFC), this loss is mostly related to the diffusion of the substrates to the biofilm or the depletion of oxygen in the cathodes.

## CHAPTER FIVE

### **CONCLUSIONS**

Based on the objectives stated at the beginning of this thesis, the study successfully addressed several key aspects related to the treatment of petroleum wastewater using clay-based microbial fuel cells (MFCs). Firstly, the investigation identified and characterized the pollutants present in synthetic petroleum wastewater, focusing on parameters such as BOD and COD. This analysis provided crucial insights into the initial composition and challenges posed by such wastewater. Secondly, the study explored whether microbes extracted from oil-contaminated soil possessed exoelectrogenic capabilities. This aspect was crucial in determining whether these microbial communities could effectively participate in electron transfer processes within the MFCs, hence contributing to electricity generation. Thirdly, the effectiveness of clay-based

MFCs in removing pollutants from petroleum wastewater and generating green energy was evaluated. The research demonstrated varying degrees of efficiency in COD and BOD removal across different MFC configurations (MFC1, MFC2, MFC control). The maximum COD removal efficiency for MFC1, MFC2 and MFC control being 96%, 98% and 34.8% respectively. As for BOD removal efficiency the maximum removal efficiency for MFC1, MFC2 and MFC control was 89%, 91% and 30% respectively. Therefore the conclusion drawn is that clay based MFC are highly effective and lucrative in the removal of organic compounds from the petroleum wastewater . Additionally, the clay-based MFCs showed distinct levels of daily average voltage generation (OCV) and power density potential with a maximum of 210.35mV and a maximum power density of 9.99  $\mu\text{W}/\text{cm}^2$  for the highest performing MFC2, indicating their capacity to convert organic pollutants into usable electrical energy. Throughout the study, unexpected activity observed in the control, which was attributed to the possibility of, influence both biotic and abiotic factors, such as untreated clay during fabrication and the inherent electrochemical properties of carbon cloths as electrodes. In conclusion, the findings of this thesis confirm that the exoelectrogenic microbes extracted from oil-contaminated soil successfully degraded petroleum wastewater within the clay-based MFCs. This degradation not only contributed to pollutant removal but also generated green energy through efficient electron transfer processes. These results highlight the potential of clay-based MFC technology as a sustainable approach for treating petroleum wastewater and harnessing renewable energy from organic contaminants.

## CHAPTER SIX

### 6. RECOMMENDATIONS

1. Future researchers should work on improving the MFC technology and simplifying it to operational parameter, which can be even operated by people in rural areas where there is shortages of energy. Therefore, there should be more emphasis on the applicability of the method at a cheap budget and reasonable operational conditions that will not cripple people from exploring and using this beautiful technology to improve their lives.
2. Governments' officials especially in infrastructure development and planning should consider using microbial fuel cells, especially the clay based MFC as a way of reducing

the utilisation of fossil fuels. For example, garages can use this technology to power up light bulbs in the workshop from the wastewater generate in the workshop.

3. Future researchers should work on optimising the clay based microbial fuel cell especially in the petrochemical industry as this research prove that they are efficient in treatment and producing electricity.
4. Further studies should be made to experiment different methods of modifying the properties clay to increase electricity-generating efficiency of the MFC and its ability to treat petroleum wastewater.
5. Further research should be done to identify exactly which microorganisms extracted from the oil contaminated soils are exoelectrogenic and responsible from treating the petroleum wastewater and how the microbes interact with each other and MFC environment.

## CHAPTER SEVEN

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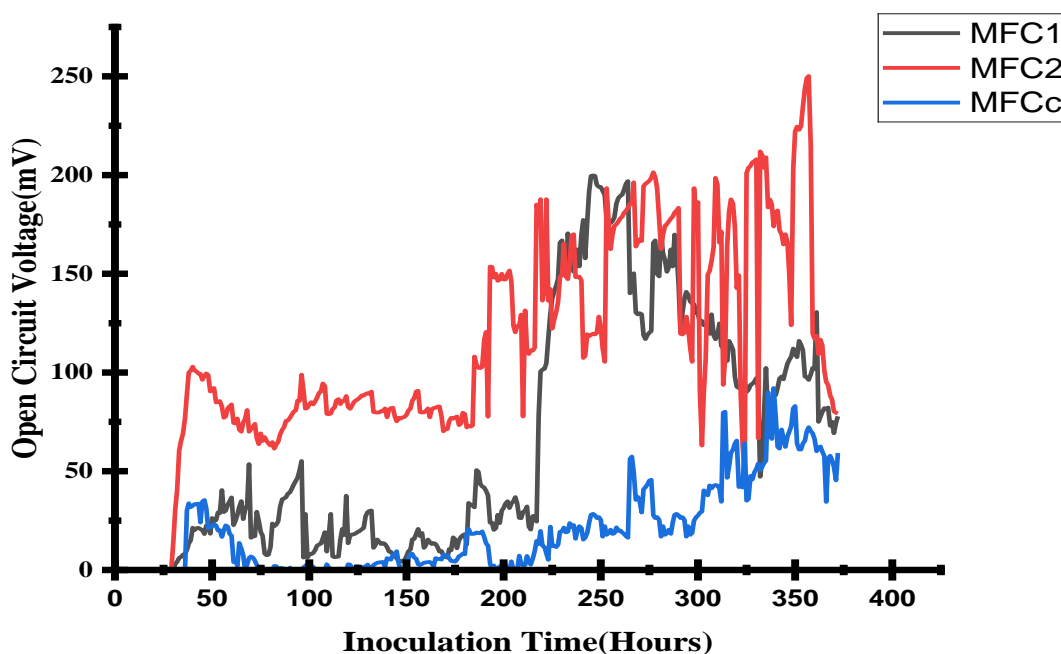
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**Figure 4: Hourly generated open circuit voltage by Microbial Fuel Cell reactors**

The graph above shows the behaviour of the open circuit voltage as a function of time and exhibits a characteristic trend indicative of the electrochemical process occurring within all MFCs. The open circuit voltage, reflects the electrochemical potential generated by microbial metabolism within the MFC. As microorganisms oxidize organic matter, they release electrons that flow through the external circuit, creating a potential difference between the anode and cathode. The higher the OCV, typically the more active the microbial community is in producing electrical potential. As shown in figure 13 MFC1, MFC2 and MFCcontrol, all shows an uptrend with a bit of a consolidation from 48<sup>th</sup> hour to 150<sup>th</sup>hour. The reason for this consolidation may be because of the microorganism taking time to create a sufficient biofilm on the surface of the anode because after this period there was a gradual increase in the OCV in all graphs including the control. The control showing some activity may be due to some micro-organisms being present in the clay which are exoelectrogens but the high difference in the OCV generated in the control and the duplicate MFCs shows that indeed the inoculum from the oil contaminated soil contributed mostly to the bioelectrochemical activities and generation of the electricity by the MFC1 and MFC2. Over the duration of the observation, the highest OCV of 250mV was observed in MFC2 at 310<sup>th</sup> hour, followed by the OCV of 200 mV which occurred in the 250<sup>th</sup>. Lastly the highest OCV of the control was 75 mV at 330<sup>th</sup> hour. These maximum OCV values shows the maximum potential each MFC can produce at the conditions

of pH 7.1 and 10 % anode volume inoculum fed with synthetic petroleum wastewater and operated at room temperature. After reaching these peak voltages, the graphs showed a decline. According to Sato and co-workers in 2023, this decline may be due to bio-fouling which blocks the micropores of the ceramic membrane inhibiting the efficient proton transport, degradation and exhaustion of organic compounds from the petroleum wastewater therefore as a result of this the food for microorganisms becomes scarce and starving microbes end up dying hence the death phase.

## ANNEX

### A.SAMPLING PROCEDURES FOR OIL-CONTAMINATED SOIL INOCULUM

#### 1. Site Selection

A sampling site was identified at a garage in the Arat Kilo area of Addis Ababa, Ethiopia, where oil has been disposed for approximately one year.

#### 2. Sampling Equipment

A plastic 50 mL centrifugal tubes was sterilized for sample collection and a zipper bag was utilized for samples transportation to avoid contamination.

#### 3. Collection of Soil Samples

During samples collection, depth sampling was conducted where sample were collected from three different depths 50cm, 100cm and 150cm and to ensure a representative sample of the contaminated soil, at 50 cm depth, a clean soil auger or trowel was used to collect soil, at 100 cm depth, the same process was repeated, ensuring the tool used is sterilized before each sample and at 150 cm depth, a final sample was collected maintaining sterile conditions throughout. To prevent contamination and moisture loss, each collected soil sample was immediately transferred into a sterilized 50 mL centrifugal tube and tubes were sealed tightly afterwards

#### 4. Transport

For easy and safe transportation, the sealed centrifugal tubes were placed into a zipper bag to protect them. Moreover, it was ensured that the zipper bag is clean and free from contaminants. And the samples were transported to Addis Ababa University promptly

#### 5. Storage

Upon arrival, the samples were stored in a refrigerator at a temperature of 4°C to preserve the microbial community for further analysis.

### B.MICROBIAL FUEL CELL (MFC) DIGESTER CONSTRUCTION PROCEDURE

#### 1. Materials used :

- Tubular clay membrane (11.5 cm height, 15 cm diameter, 1.2 cm thick)
- Carbon cloth (from Easycomposite, UK)
- Copper wire (0.1 mm diameter)

- Polyethylene waterproof membrane
- 2L plastic bucket
- Phosphate buffer solution (0.1M)
- Ferricyanide (0.1M)
- Synthetic petroleum wastewater
- Microbial inoculum (from soil contaminated with oil)
- Nitrogen supplement components:
  - $\text{NaNO}_3$  (10 g)
  - $(\text{NH}_4)_2\text{SO}_4$  (2 g)
  - $\text{K}_2\text{HPO}_4$  (0.54 g)
  - $\text{KH}_2\text{PO}_4$  (0.7 g)
  - $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$

## **2. Construction Steps:**

### **1. Preparation of the Tubular Clay Membrane**

The tubular clay membrane was fabricated in Ketchani, Ethiopia using \*\*\*\*. , one end was sealed to form a hollow chamber suitable for the MFC,

### **2. Assembling the Cathode**

A carbon cloth from Easycomposite, UK was cut into a piece of carbon cloth measuring 546 cm<sup>2</sup> and wrapped around the exterior of the tubular clay cylinder, ensuring it is secured firmly.

### **3. Assembling the Anode**

Then another piece of carbon cloth measuring 455.2 cm<sup>2</sup> was cut and attach to the internal periphery of the tubular clay membrane, making sure it was evenly distributed for optimal contact with the wastewater.

### **4. Wiring Connections**

For the wiring connections, copper wire (0.1 mm diameter) was used to connect the anode and cathode and It is important to ensure that the connections are secure to facilitate efficient electron transfer.

### **5. Sealing the Clay Membrane**

The open end of the clay membrane was covered with a polyethylene waterproof membrane, leaving an opening for feeding the anode chamber with the synthetic wastewater and microbial inoculum

#### **6. Placement in Plastic Bucket**

The assembled MFC setup was placed inside a 2L plastic bucket. The purpose of the bucket was to serve as an external chamber for the catholyte.

#### **7. Preparation of Catholyte**

The bucket was filled with a mixture of 0.1M phosphate buffer solution and 0.1M ferricyanide at a 1:1 ratio and thorough mixing was ensured

#### **8. Filling the Anode Chamber**

750 mL of synthetic petroleum wastewater was introduced into the anode chamber and 75 mL of the microbial inoculum obtained from soil contaminated was added with oil to provide the necessary microbial consortia for degradation.

To prepare the nitrogen supplement the following components were mixed:

6. Dissolve 10 g of  $\text{NaNO}_3$ , 2 g of  $(\text{NH}_4)_2\text{SO}_4$ , 0.54 g of  $\text{K}_2\text{HPO}_4$ , 0.7 g of  $\text{KH}_2\text{PO}_4$ , and the appropriate amount of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ .

Hence a 100 mL of the nitrogen supplement was added to the anode chamber to support microbial growth.

#### **7. Final Setup**

For the final setup, all connections were checked to ensure that they are secure and that there are no leaks in the setup. Then the system was allowed to equilibrate before initiating the experiment.

### **C.PROCEDURE FOR ISOLATION AND ENRICHMENT OF ANAEROBIC BACTERIA**

#### **Materials Needed:**

- Contaminated soil (50–150 cm depth) from Arat Kilo, Addis Ababa
- Selective medium ingredients:
  - $\text{NaCl}$  (5 g)
  - $\text{NaNO}_3$  (2 g)
  - $(\text{NH}_4)_2\text{SO}_4$  (1 g)
  - $\text{K}_2\text{HPO}_4$  (0.27 g)
  - $\text{KH}_2\text{PO}_4$  (0.35 g)

- $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.2 g)
- $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (0.1 g)
- Trace metals stock components:
  - $\text{H}_3\text{BO}_3$  (30 g)
  - $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  (10 mg)
  - $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  (3.0 mg)
  - $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  (3.0 mg)
  - $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$  (2.0 mg)
  - $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  (10 mg)
  - $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$  (1.0 mg)
- 0.1 M NaOH and 0.1 M HCl (for pH adjustment)
- 150 mL glass bottles
- Parafilm (for sealing)
- Nutrient agar (5 g for enrichment)
- Petri dishes (10)

## **Procedure:**

### **1. Preparation of Selective Medium:**

- Measure and combine the following ingredients into a 1-liter sterile container:
  - 5 g NaCl
  - 2 g  $\text{NaNO}_3$
  - 1 g  $(\text{NH}_4)_2\text{SO}_4$
  - 0.27 g  $\text{K}_2\text{HPO}_4$
  - 0.35 g  $\text{KH}_2\text{PO}_4$
  - 0.2 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
  - 0.1 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$
- Add 10 mL of trace metals stock prepared from:

- 30 g  $\text{H}_3\text{BO}_3$
- 10 mg  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$
- 3.0 mg  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$
- 3.0 mg  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$
- 2.0 mg  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$
- 10 mg  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$
- 1.0 mg  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$

## **2. pH Adjustment:**

- Adjust the pH of the medium to 7.1-7.4 using 0.1 M NaOH or 0.1 M HCl as necessary.

## **3. Sterilization:**

- Sterilize the prepared medium by filtering it through a 0.20 micron pore size filter paper.

## **4. Nitrogen Supplement Preparation:**

- Prepare 100 mL of nitrogen supplement by dissolving the following:
  - 10 g  $\text{NaNO}_3$
  - 2 g  $(\text{NH}_4)_2\text{SO}_4$
  - 0.54 g  $\text{K}_2\text{HPO}_4$
  - 0.7 g  $\text{KH}_2\text{PO}_4$
  - 0.4 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$

## **5. Inoculation:**

- Under sterile conditions, add 1 g of oil-contaminated soil to a 150 mL glass bottle containing the sterilized selective medium.
- Add 1 mL of the trace metal solution to the same bottle.
- Fill the bottle to the maximum capacity to minimize oxygen exposure, and seal it with Parafilm to ensure anaerobic conditions.

## **6. Incubation:**

- Incubate the inoculated medium at 30°C under anaerobic conditions.
- Include a control setup without contaminated soil for comparison.

## **7. Monitoring Microbial Growth:**

- Observe the inoculated media for turbidity and signs of microbial growth over the next 7 days.
- After 7 days, a noticeable turbidity indicates successful growth of anaerobic bacteria, distinguishing it from the control.

## **8. Subculturing:**

- Prepare a subculture by transferring a small volume of the inoculated medium to fresh selective medium to reduce microbial concentration for further studies.

## **9. Enrichment Culture Preparation:**

- Prepare nutrient agar by dissolving 5 g of nutrient agar in a suitable volume of distilled water and autoclaving it.
- Pour the prepared agar into 10 sterile Petri dishes.
- Perform serial dilutions of the original inoculum and plate each dilution on the prepared Petri dishes for isolation of anaerobic bacteria.