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Electricity Production from Paddy Soil in Double Chamber Microbial Fuel Cells and Isolation of the Electroactive Bacteria Involved

Nurul Faqihah Binti Che Mohd Nasir

J20A0594

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DECLARATION

I declare that this thesis entitled "Electricity Production from Paddy Soil in Double-Chamber Microbial Fuel Cells and Isolation of the Electroactive Bacteria Involved" is the results of my own research except as cited in the references.

Signature : 

Student's Name : Nurul Faqihah Binti Che Mohd Nasir

Date : 29/2/2024

Verified by:

Signature : _____

Supervisor's Name : _____

Stamp : _____

Date : _____

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Penghasilan Elektrik daripada Tanah Padi Menggunakan Dwi Ruang Sel Bahan Bakar Mikrob (DMFCs) dan Pengasingan Bakteria Elektroaktif Yang Terlibat

ABSTRAK

Sel bahan bakar mikrob (MFC) baru-baru ini mendapat perhatian kerana dapat menghasilkan tenaga mampan. Tanah padi yang tenggelam mewujudkan persekitaran dengan tahap oksigen yang rendah, dikenali sebagai mikroaerofilik, dan mengandungi sejumlah besar bahan organik. Akibatnya, ia menghalang penguraian bahan organik dan melambatkan penguraiannya. Kepelbagaian EAB mungkin berbeza berdasarkan jenis medium yang digunakan. Oleh itu, kajian ini bertujuan untuk membina DMFC yang berfungsi menggunakan reka bentuk jambatan garam dan mengasingkan EAB daripada tanah padi yang diperkaya dalam DMFC. Sampel tanah dikumpul dari sawah tertentu dari Machang, Kelantan, pengayaan tanah padi dalam MFC, pengekstrakan DNA tanah, pengasingan dan pertumbuhan bakteria, dan mengambil tahu mengenai ciri-ciri bakteria menggunakan ujian biokimia. Kegunaan DMFC boleh diperhatikan dengan memerhatikan perubahan warna kalium ferricyanide akibat proses pengurangan. Peningkatan yang luar biasa dalam ketumpatan kuasa semasa eksperimen, terutamanya dalam pengayaan kedua, menunjukkan perbezaan yang sangat ketara dengan kenaikan sebanyak 40 kali ganda. Nilai maksimum ketumpatan kuasa untuk pengayaan pertama ialah 22.300 W/m^2 manakala persediaan pengayaan kedua ialah 1208.830 W/m^2. Kajian ini menemukan tiga jenis bakteria (MC-1, MC-2, dan MC-3) yang telah diasingkan daripada DMFC kedua. Kesimpulannya, EAB daripada tanah padi boleh menjana tenaga elektrik.

Kata kunci: Dwi Ruang Sel Bahan Bakar Mikrob (MFC), Bakteria Elektroaktif (EAB), Tanah Padi

Electricity Production from Paddy Soil in Double Chamber Microbial Fuel Cells and Isolation of the Electroactive Bacteria Involved

ABSTRACT

Microbial fuel cells have recently garnered attention due to the growing interest in producing sustainable energy. Submerged paddy soil creates an environment with low levels of oxygen, known as microaerophilic, and contains a significant amount of organic matter. Consequently, it prevents the decomposition of organic materials and retards their decomposition. Diversification of EAB may differ based on the type of medium used. Hence, this study aimed to construct functional DMFCs using a salt bridge design and isolate EAB from paddy soil enriched in DMFCs. Soil samples were collected from a specific paddy field from Machang, Kelantan, enrichment of the paddy soil in MFCs, soil DNA extraction, isolation and cultivation of bacteria, and characterization of the bacteria using biochemical tests. Functionality of DMFCs could be observed by observing the colour changes of potassium ferricyanide due to the reduction process. Remarkable improvement in power density during the experiment, notably in the second enrichment setup, showed a very significant difference with a 40-fold increment. Maximum value of power density for first enrichment setup was 22.300 W/m^2 while the second enrichment setup was 1208.830 W/m^2. This study reported three bacterial strains (MC-1, MC-2, and MC-3) were isolated from the second DMFCs. In conclusion, EAB from paddy soil can generate electricity and significantly contributes to advancing our comprehension of EAB behavior in paddy soil within the flooded habitat. These findings highlight the potential of paddy soil as a resource for sustainable energy production through microbial fuel cell technology.

Keywords: Double Chamber Microbial Fuel Cells (MFCs), Electroactive Bacteria, Paddy Soil

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LIST OF ABBREVIATIONS

MFC	Microbial Fuel Cells	1
ATP	Adenosine triphosphate	2
PEM	Proton exchange membrane	2
TEA	Terminal electron acceptor	2
EAB	Electroactive bacteria	3
NH_4^+	Ammonium	3
DOC	Dissolved organic carbon	3
DNA	Deoxyribonucleic acid	4
EET	Extracellular electron transfer	6
BES	Bioelectrochemical system	7
KCl	Potassium chloride	12
NaCl	Natrium chloride	12
H_2O_2	Hydrogen peroxide	12
UV	Ultraviolet	15
LB	Luria Bertani	16
Fe(II)	Ferrous ion	17
Fe(III)	Ferric	17

LIST OF SYMBOLS

g	Gram	12
µL	Microlitre	12
mL	Mililitre	12
%	Percentage	13
π	Pi	14
V	Voltage	14
I	Current	14
W	Watts	14
m^2	Square meters	14
mA	Milliampere	15
°C	Celsius	16
+	Positive	23
-	Negative	23

CHAPTER 1

INTRODUCTION

1.1 Background of Study

There has been a fundamental change in the research and study of innovative technologies due to the growing demand for renewable energy sources. Among these, microbial fuel cells (MFCs) are a potentially promising option. These cells generate energy by utilizing the distinctive metabolic processes that bacteria engage in (Logan, 2009). Paddy soil is a resource that possesses special properties due to the flooding conditions present in paddy fields. In this context, our attention is directed towards the diverse and varied microbial communities in paddy soil.

The global pursuit of renewable energy sources has become vital to address the environmental concerns associated with conventional energy production due to its dual capability to treat pollutants and recover energy in electricity. Anaerobic digestion methods are currently being utilized extensively in treating organic wastes. These processes generate biogas, such as methane, which can be turned into either heat or power. Since hydrogen is more valuable than methane, researchers have also tried to figure out how to make hydrogen gas practically through anaerobic digestion (Watanabe, 2008). One notable disparity between an MFC and chemical fuel cells is in their corresponding substrates. In contrast to conventional chemical fuel cells that rely on purified fuels like hydrogen and methanol, microbial fuel cells (MFCs) can utilize a diverse array of substrates, ranging from laboratory bacterial growth media to different types of solid and liquid organic wastes, due to the presence of mixed microbial populations in the electroactive biofilms (You et al., 2021).

MFCs have become a prominent technology in the renewable energy field as they navigate this domain's intricate landscape. Throughout history, the fuel cell has had a long-standing presence, with the credit for the first successful cell often given to Sir William Grove in England in 1839 (Lewis, 1966). The historical evolution of fuel cells, dating back to Sir William Grove's observations in 1839 and Potter's 1911 discovery of

electrical current generation by bacteria, underscores the enduring quest for harnessing energy from unconventional sources (Grove, 1839; Potter, 1911).

MFCs are bioreactors in which specific electroactive microorganisms oxidize organic substrates. These microorganisms can transport electrons to an external anode. After that, the current moves to the cathode, where it may reduce down on oxygen or other ions respectively (Tabassum et al., 2021). A typical MFC design comprises two chambers, particularly anaerobic and aerobic chambers. Bacteria oxidize the substrate in the anaerobic chamber, which results in the production of electrons and protons. There are three ways in which electrons can be transferred to the anode: either through an exogenous electron carrier, through the mediator, or straight from the bacterial enzymes to the electrode directly (Chen et al., 2014). Conventional MFCs comprise anodic and cathodic chambers separated by a proton exchange membrane (PEM) or a salt bridge. In the anodic chamber, microorganisms oxidize the substrate, producing electrons and protons during the reaction. The anode captures electrons and transports them to the cathode through an external circuit. Protons are brought into the cathodic chamber through the PEM or salt bridge. The electrons and the protons then interact with oxygen-producing water (Nawaz et al., 2020).

Microbial Fuel Cell operates as a bioelectrochemical system wherein microorganisms serve as catalysts in the oxidative degradation of organic matter, leading to the production of electrons and subsequent ATP synthesis through an intracellular electron transport chain. After the electrons are released, they are accepted by a terminal electron acceptor (TEA), which undergoes reduction. An example of this would be the reduction of oxygen to water, which can be accomplished through a catalyzed process between electrons and protons. There are a number of TEAs that readily diffuse into the cell, including oxygen, nitrate, sulfate, and others. Once within the cell, these TEAs take electrons and generate products that can diffuse out of the cell. On the other hand, scientists have discovered that certain bacteria can exogenously (outside the cell) transfer electrons to a TEA, such as a metal oxide like iron oxide (Logan, 2007). Bacteria that can transfer electrons from outside sources, known as exoelectrogens, can generate electricity in a microbial fuel cell (MFC).

Due to paddy soil's distinctive microbial composition, it takes our interest in this vast field. Anaerobic environments are created in paddy fields due to the waterlogged circumstances, which encourage the growth of various microorganisms. The flooding circumstances in rice fields provide anaerobic environments, which are low in oxygen,

which help conserve organic matter and slow its decomposition (Bond & Lovley, 2002). A study discovered that high-performing MFCs were made from paddy soils with high concentrations of dissolved organic carbon (DOC) and NH_4^+ in the porewater. These soils were chosen because they were suitable for an active and have a highly electrogenic bacterial community at the anodes. On the other hand, low-performing MFCs were produced from soils that had low concentrations of DOC and NH_4^+ (Wang et al., 2015). Hence, paddy soil was chosen in this research as it is a rich source of electroactive bacteria.

1.2 Problem Statement

Abundance of organic matter can be found in paddy soil as the flooded conditions in paddy soil create an anaerobic (low-oxygen) environment. Hence, it helps to preserve organic matter and slow down its decomposition. This creates an environment beneficial for microorganisms capable of reducing iron(III) (Deng et al., 2015). The distinctive characteristics of paddy soil from other sources allow differences in the diversity, composition, and dynamics of EAB. It is essential to acknowledge this possible difference to maximize the efficiency of paddy soil enriched in double-chamber microbial fuel cells (DMFCs). Therefore, this study aims to isolate EAB from paddy soil.

1.3 Objectives

The objectives of this research are:

1. To construct functional double-chamber microbial fuel cells using salt bridge design.

2. To isolate and characterize electroactive bacteria from microbial fuel cells supplemented with paddy soil.

1.4 Scope of Study

The project aims to further study in the field of microbial fuel cells by utilising paddy soil, which is known for its anaerobic conditions and high organic content. The first stage is soil sampling, where the soil is collected from a paddy field and enriched in

microbial fuel cells (MFCs). The next stage is soil DNA extraction, isolation of bacteria, and biochemical test of bacteria using several methods.

The results will not only enhance techniques for generating electricity, but also broaden our awareness of the potential of electroactive bacteria present in paddy soil.

1.5 Significances of Study

The finding holds relevance as it can enhance our understanding of the behaviour of EAB in paddy soil, which is abundant in organic material and lacks oxygen. The flooded conditions of paddy soil provide a distinctive habitat that conserves organic matter and promotes microorganisms that are capable of reducing iron(III). The research addresses the need to explore alternative sources for electricity production to enhance the utilisation of paddy soil using DMFCs and identification of EAB through the isolation.

CHAPTER 2

LITERATURE REVIEW

2.1 Microbial Fuel Cells (MFCs)

Electrogenic bacteria in microbial fuel cells convert organic materials into electricity. MFCs systems are promising renewable energy sources for researchers and companies due to their high energy conversion efficiency and versatility. MFCs efficiency is based on the design (Figure 2.1), substrate used, and microorganism used. The MFC, or microbial fuel cell, has an anode, cathode, electrolyte medium, PEM as a mediator, anode-cathode materials, and microorganisms (Choudhury et al., 2017). Due to the anaerobic oxidation of pollutants by microorganisms like algae and bacteria, electrons that can generate electricity are generated in MFCs (Roy et al., 2023). Electrons in the external circuit generate electricity. EAB in the anode inhibit organic waste degradation via anaerobic respiration and metabolism. On the other hand, the electron that makes its way through the circuit eventually arrives at the cathode, and there, it is accepted by an electron acceptor in the form of oxygen (O_2).

To put it another way, microorganisms can create electrons and protons for their respiratory systems by degrading a variety of organic and heavy metal complexes while the MFC is in operation. Elctrogenic bacteria can form biofilm on electrodes, which transfers electrons better than insoluble electron acceptors. Electrode-sharing exoelectrogens can also create electricity. This action can occur via redox-active proteins, soluble electron shuttling molecules, conductive pili, or direct electron transfer (Yaqoob et al., 2021).

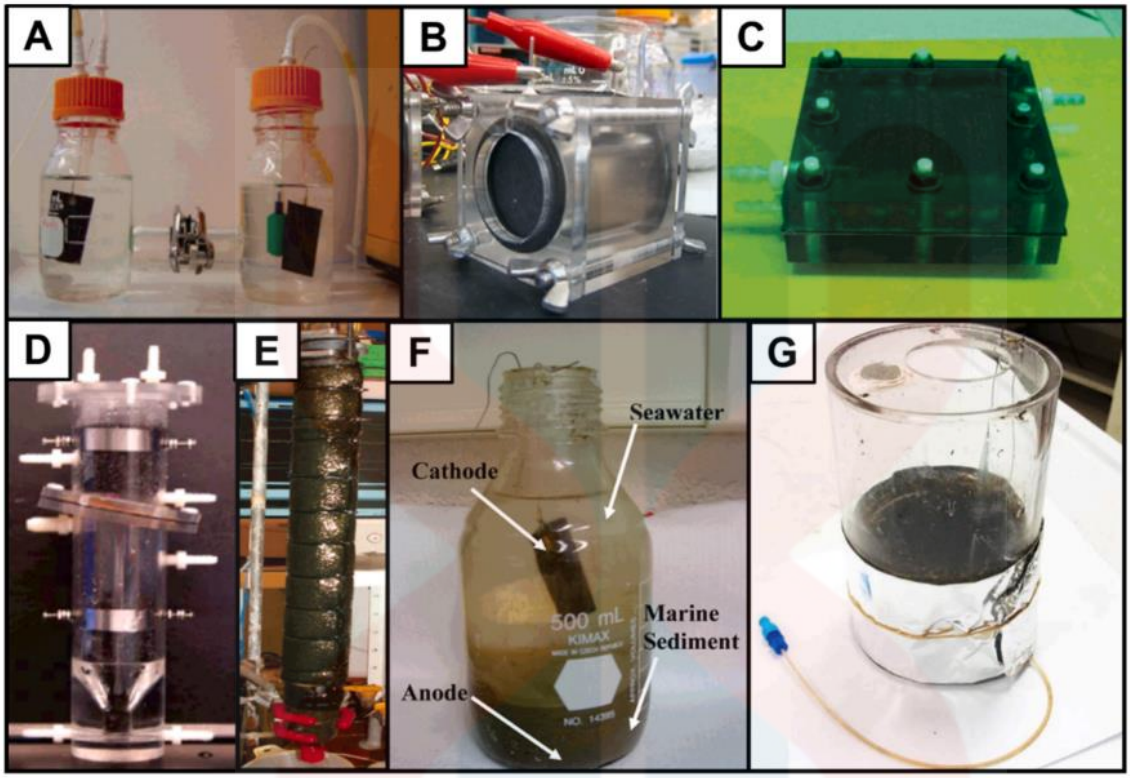


Figure 2.1: Design of microbial fuel cells available. (A) H-type Double Chamber MFC, (B) Single Chamber MFC air cathode system, (C) Flat plate MFC with PEM, (D) Upflow, tubular type

MFC (anode below, cathode above), (E) Upflow, tubular type MFC (inner anode and outer cathode), (F) Sediment MFC, (G) Soil MFC

(Tabassum et al., 2021).

2.1.1 Electrogenic bacteria

Electrogenic bacteria have garnered increasing attention recently due to their potential applications. Electrogenic bacteria are a type of living organism that possesses the ability to transfer electrons to electron acceptors located outside of their cellular structure (Sacco et al., 2017). To sustain their livelihood, the bacteria require a supply of energy. This energy acquisition process is a two-fold mechanism involving oxidation, which consists of removing electrons from an organic substrate, and reduction, which involves the transfer of electrons to the ultimate electron acceptor, namely oxygen (Shaikh et al., 2016). Extracellular Electron Transfer (EET) applies to microorganisms' bioelectrochemical mechanisms, wherein electrons transfer from the intracellular

space to the extracellular environment (Light et al., 2018). *Shewanella, Geobacter*, and *Desulfuromonas* are among the electrogenic bacteria that have been extensively researched (Logan, 2009). These bacteria are frequently detected in various settings, such as marine and freshwater sediments and soil.

Additionally, the study will be achieved through a sample preparation process to facilitate the breakdown of larger biomolecules and the identification of the microorganism through metagenomics analysis. These microorganisms can be utilized in various applications, including bioenergy, bioremediation, and bioelectrochemical systems (BES), to generate electrical energy. In this research, the bacteria expected to appear are *Geobacter sulfurreducens* D8 and *Geobacter sulfurreducens* subsp. ethano-licusCL-1, *Citrobacter sp.*strain ND-2 and *Geobacter sp*. strain RPFA-12G-1 (Yee et al., 2020).

2.1.2 Bioelectricity generation

A microbial fuel cell is a tool that utilizes compost, sewage, sludge, or soil as a substrate for the biodegradation of organic matter to generate bioelectricity with the help of microorganisms as a catalyst. A conventional microbial fuel cell is comprised of two compartments, namely the anode and cathode, which are partitioned by a membrane known as a cation-specific membrane. Within the anode compartment, microorganisms facilitate the oxidation of organic matter, resulting in the production of both electrons and protons. A biofilm will be produced at the anode surface (Moqsud et al., 2013). MFCs utilize bacterial catalysts to enable the oxidation of inorganic substances, such as sulfur compounds, and organic matter, such as mud, food, vegetable, fruit, plant leaves, and grass pieces, to produce electrical energy. Several researchers have demonstrated that MFCs can produce hydrogen efficiently. MFCs serve the dual purpose of generating electricity and purifying water within the system. (Kasipandian et al., 2020).

2.1.3 Electrochemical activity

The term "electrochemical activity" applies to the capability of a substance or material to engage in or promote reactions involving the transfer of electrons. In MFCs, the electrochemical reaction occurring at the electrode surface consists of the transfer of electrons between an electron

donor, also known as a reducing agent, and an electron acceptor, also known as an oxidizing agent. Electrochemical activity is essential in many important events, including electrocatalysis, energy storage and conversion, and corrosion prevention.

There are several electrochemical methods to measure electrochemical activity, such as cyclic voltammetry, electrochemical impedance spectroscopy, and chronoamperometry (Kashyap et al., 2014). These methods provide an understanding of the substances' redox properties, the electron transfer kinetics, and the magnitude of the surface area amenable to electrochemical reactions.

Next, the electrochemical activity of MFCs is affected by many aspects, including material composition and morphology, microorganisms used, MFC design, and operating parameters, including temperature, pH, and electrolyte concentration. Like other microbial systems, optimal pH and temperature settings boost bacterial growth and MFC performance. In addition, although salt has been found to have an unfavorable impact on microbial growth, it has been observed that elevated salinity and ionic strength can lead to an increase in substrate conductivity and electrochemical activity, thereby improving the performance of MFCs (Shanmuganathan et al., 2018).

MFCs have electrochemical activity, which presents promising opportunities for various real-world uses, including wastewater treatment, biosensor development, and bioenergy production. MFCs have demonstrated efficacy in treating different kinds of wastewater streams, including domestic, industrial, and agricultural, like bamboo effluent (Dai et al., 2021), simultaneously producing electrical energy.

2.2 Paddy

Rice, scientifically known as *Oryza sativa*, is a consumable starchy cereal grain derived from a grass plant belonging to the Poaceae family (Britannica, 2023). "Paddy," also known as a rice paddy, refers to a small, flat, and waterlogged agricultural field primarily used for cultivating rice in southern and eastern Asia. In the Far East, wet rice cultivation is the most common form of farming. Despite only requiring a small portion of the available land, it is responsible for feeding the vast majority of the population living in rural areas (Britannica, 2016). About three billion

people rely on rice as a daily staple diet, with 90 percent of all rice eaten worldwide coming from Asia. In many Asian countries, rice provides most of the daily calories and nutrients consumed. The 23 species of *Oryza* include the Asian staple *Oryza sativa* L. and the West African staple *O. glaberrima Steud* (Nadaf, 2016).

In 2022/2023, the rice yield in Malaysia will be 4.0 tons/hectare, while in 2023/2024, the rice yield in Malaysia is expected to increase to 4.1 tons/hectare (FAS, 2023). Kedah was the major producer of rice in Malaysia during those 3-year averages (Figure 2.2), which produced 37% of rice, according to the Foreign Agricultural Service, U.S. Department of Agriculture.

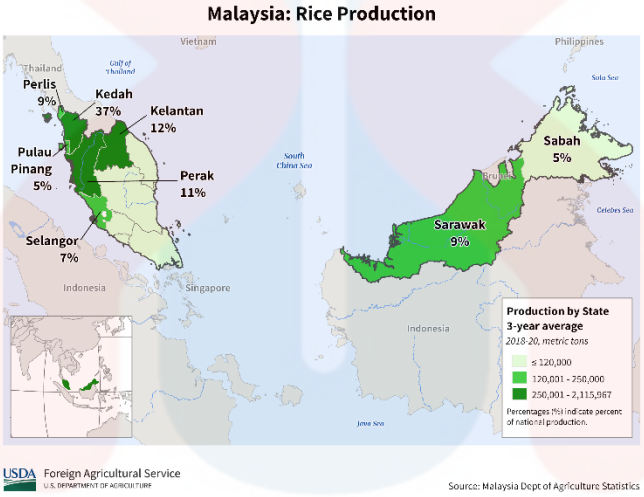


Figure 2.2: Production of rice by state in Malaysia 3-year average in 2019/2020 (FAS, 2023)

2.3 Microbial functions and diversity in bacterial communities

A bacterial community is a collection of bacteria that coexist within a shared habitat, showing variation in their species composition, occupied niches, and impact on various surroundings (Stubbendieck et al., 2016). Bacterial populations can thrive in diverse ecological niches, including terrestrial, aquatic, and human biological systems. A range of factors, such as temperature, pH, nutrients, and interactions with other microorganisms, can impact bacterial communities' composition and diversity.

Bacterial communities are significant contributors to various ecological processes, as they are involved in multiple functions such as nutrient cycling, biodegradation, bioremediation, electricity generation, and interactions between plants and microbes. Beneficial microorganisms also have been found to engage in various activities that promote plant growth in agriculture. These activities include nutrient fixation, mineralization, solubilization, mobilization, and the production of siderophores, antagonistic substances, antibiotics, and plant growth-promoting substances (Suman et al., 2022). In addition, the impact of bacterial communities extends to human health, as changes in the bacterial composition within the gastrointestinal tract have been associated with various illnesses.

Modern sequencing technologies have facilitated the research of bacterial communities with a higher degree of precision, thereby enabling the identification of distinct bacterial species and their respective roles within the community. The abovementioned phenomenon has increased interest in exploring microbial ecology and the importance of bacterial communities in ecosystems.

2.4 Ecological resilience and the role of biodiversity conservation

Alternatively, biological diversity or biodiversity is the variety of life and refers to the variation at all biological organization levels. It includes the number of species, the genetic diversity within those species, and the diversity of ecosystems and habitats (Gaston, 2013). There are several reasons why biodiversity is essential. Primarily, it offers various crucial ecosystem services, including sustenance, potable water, and atmospheric cleansing. Furthermore, preserving biodiversity plays a vital role in sustaining the vitality and adaptability of ecological systems, ultimately yielding positive outcomes for the well-being of people. Biodiversity is intrinsic and significant for cultural, ethical, and aesthetic rationales (Díaz et al., 2006). However, human activities threaten diversity, including habitat destruction, pollution, and climate change. The depletion of biodiversity can significantly affect the operational mechanisms of ecosystems and the welfare of humanity (Cardinale et al., 2012). In conclusion. the variety of living things in a particular ecosystem, geographic area, or globe is known as biodiversity. It is crucial for several reasons, including sustaining ecosystem health and resilience and offering essential ecological services and cultural, ethical, and aesthetic considerations. Conservation efforts aim to preserve

biodiversity and minimize its threats from human activities, including habitat destruction, pollution, and climate change.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Several chemical reagents have been used in this study for constructing the salt bridge, preparing the catholyte for the cathode chamber, conducting DNA extraction, and performing biochemical tests.

Table 3.1: List of raw materials and chemical reagents

Materials (Chemical reagent)	
Sample collection	Soil sample
Salt bridge	20g of agar powder, 75.5g of potassium chloride (KCl)
Catholyte	1g of potassium ferricyanide ($K_3[Fe(CN)_6]$)
Soil DNA extraction	Soil DNA extraction kit from NucleoSpin Soil from Macherey-Nagel brand
Gel electrophoresis	1 µL Hindl marker, 1 µL loading dye, 4 µL deionized water, 0.3g agarose gel, 39.5 mL TAE buffer
Isolation of bacteria	5g of tryptone, 5g of sodium chloride (NaCl), 2.5g of yeast extract, 8g of agar powder, ethanol
Characterization of bacteria	Crystal violet, saffranine, acetone, iodine, hydrogen peroxide (H_2O_2), and tetramethyl-p-phenylenediamine dihydrochloride

3.2 Methods

3.2.1 Sample collection

The soil sample used in this research was collected from a paddy field located at Lorong Kubor, Pekan Machang, at coordinate 5°45'32.9"N 102°13'05.2"E. The soil was collected in a sterilized beaker.

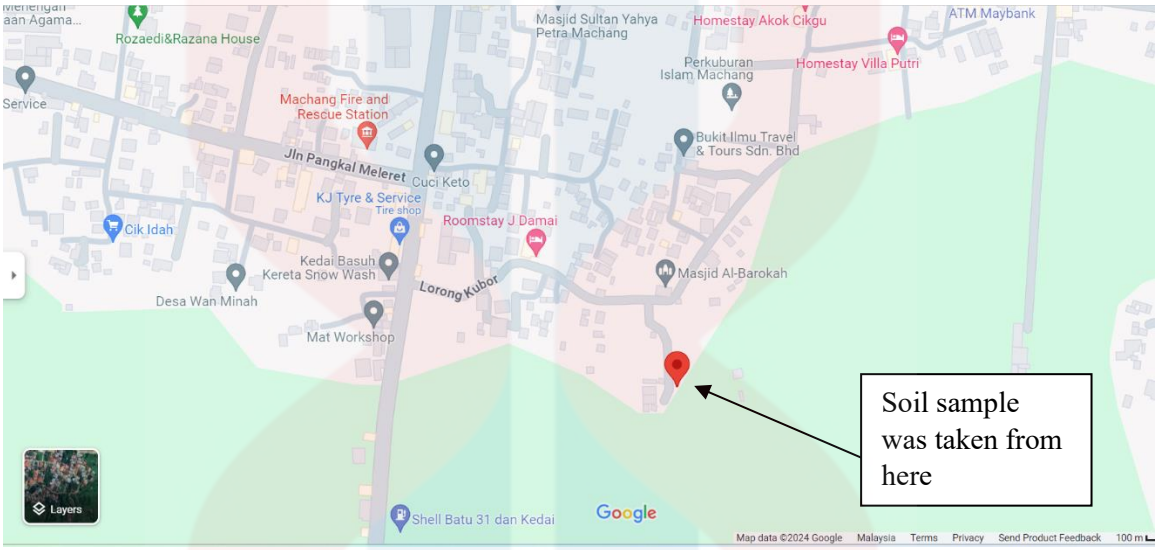


Figure 3.2.1: Map where soil sample was collected.

3.2.2 Double-chamber microbial fuel cells (DMFCs) setup

DMFCs were constructed using two 150 mL conical flasks, one set of graphite electrodes with 10 mm diameter and 100 mm length, and a salt bridge. The salt bridge was formed using 7% KCl in 20 g agar (Ogbulie, 2017). The working volume for the anode and cathode chambers was 100 mL. The catholyte in the cathode chamber was a potassium ferricyanide solution where 1 g of potassium ferricyanide was diluted in 100 mL of distilled water (Aye et al., 2018). The electrodes were connected to crocodile wires and to an EXTECH 540 Digital Multimeter. The exposed electrodes were sealed using parafilm tape for microaerophilic condition.

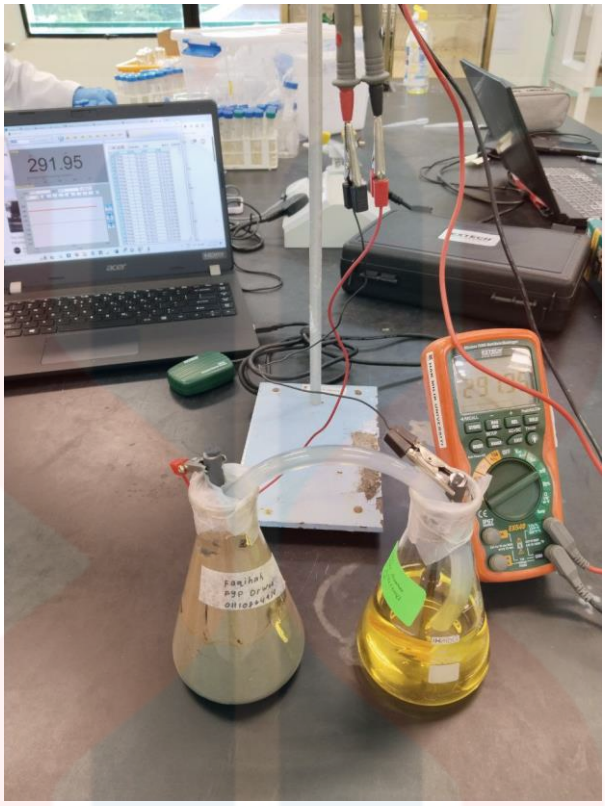


Figure 3.2.2: Double chamber microbial fuel cells setup (DMFCs).

3.2.3 Enrichments

The first enrichment setup was solely paddy soil (150 mL) without any addition of nutrient medium in the anode chamber. After a week, a second enrichment setup was constructed which in the anode chamber contained 140 mL of sterilized paddy soil with the addition of 10 mL of paddy soil from the first enrichment setup.

3.2.4 Voltage, current, and power density measurement.

The voltage and current were recorded for every 20 min every day. The power was calculated using Eq 1

$$P(W) = Voltage, V\ (V) \times Current, I\ (A)$$

Next, the surface area of the electrode was calculated using Eq 2

$$A\ (m^2) = \pi r^2$$

Lastly, the power density was calculated using Eq 3

$$Power\ density = \frac{Power\ (mW)}{Area(m^2)}$$

3.2.5 Soil DNA extraction

Soil DNA extraction was conducted using a NucleoSpin DNA extraction kit by Macherey-Nagel according to the protocol provided.

3.2.6 Gel electrophoresis

0.3 g of agarose gel was prepared with 39.5 mL of TAE buffer, heated, and cooled down before adding 1 µL of FloroSafe DNA stain. Next, the mixture was poured into the gel tray, placed the comb and let it harden for 30 minutes. Then, the DNA marker was loaded into the first well, while the rest of the well was loaded with DNA samples that had been extracted. Run the gel at 80V, 200 mA for 45 minutes. Lastly, the gel was observed under UV light.

3.2.7 Screening of bacteria strains

Seven test tubes containing 9 mL of distilled water each were prepared and sterilized. 1 g of soil sample was added into 10^0 labeled test tubes and swirled. Next, 1 mL of 10^0 was transferred to 10^{-1} until the last dilution. The dilutions were done from 10^0,10^{-1},10^{-2},10^{-3},10^{-4},10^{-5} and 10^{-6}. Then, a spread plate procedure was conducted where 1 mL of 10^{-2},10^{-4} and 10^{-6} was poured on the Luria Bertani (LB) agar plate, where a hockey stick was used to spread the bacteria to the agar plate. Lastly, the plates were incubated for a day at 30°C.

3.2.8 Characterization of bacteria

A single colony from the spread plate was isolated using an inoculation loop on LB agar plates and incubated again for a day at 30°C.

3.2.6.1 Gram staining

The sample was transferred to the microscope slide using an inoculation loop. Then, saline water was looped and rubbed with the sample before slightly drying it. Crystal violet stain was added over the culture for about 1 minute before rinsing with water. Next, iodine solution was added onto the slides for another 1 minute before rinsing again under running water. The decolorizer, acetone, was added onto the slide, rinsed for 5 seconds, and removed quickly with water. Lastly, a few drops of safranin were added for 45 seconds. The slide was washed off with

water and left to air dry. The slide was observed under a microscope to determine whether the bacteria sample was categorized as Gram-positive or Gram-negative bacteria.

3.2.6.2 Catalase test

A single bacterial colony was transferred onto a glass slide, and 3% hydrogen peroxide (H_2O_2) was dropped onto the bacterial specimen.

3.2.6.3 Oxidase test

The sample was spread on the filter paper using an inoculation loop. Then, a drop of tetramethyl-p-phenylenediamine was dropped on the sample.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Results and Discussion

4.1.1 Potassium ferricyanide reduction

In this research, while analyzing the power density and voltage for the DMFCs, potassium ferricyanide reduction can be observed where the catholyte containing potassium ferricyanide reduced and changed color from yellow to dark green and blue.

Figure 4.1.1 (a): The colour of potassium ferricyanide solution.

Potassium ferricyanide, with its red color and octahedral-shaped $[Fe(CN)_6]^{3-}$ ions, is used in an experiment to convert Fe(III) into Fe(II). This conversion involves a reduction process, where iron gains electrons. The reduced Fe(II) is read at 700 nm, which means that a photon with a

wavelength of 700 nm is received. This promotes an electron, which makes the Fe(II) excited and unstable. The energy emitted by an electron when it goes back to its ground state (steady) is the same as the energy in the red area of a color wheel (Bibi Sadeer et al., 2020). This is why the reduced form of potassium ferricyanide-Fe(II) is a dark blue color called Prussian blue.

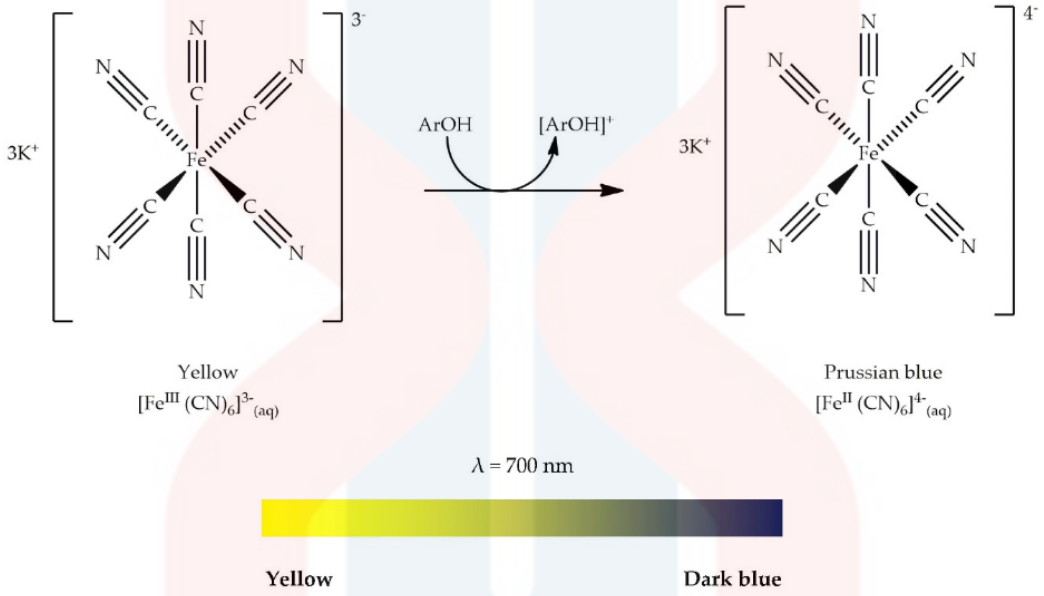


Figure 4.1.1 (b): Mechanism of reaction for potassium ferricyanide

(Bibi Sadeer et al., 2020)

4.1.2 Voltage and power density

Voltage, also known as electric potential difference, measures the amount of potential energy per unit charge in an electrical system. Power density quantifies the amount of electrical power generated by the MFC relative to the surface area of the electrodes. Analysis for these two DMFCs setup was performed every day for ten days. The readings were recorded for 20 minutes daily.

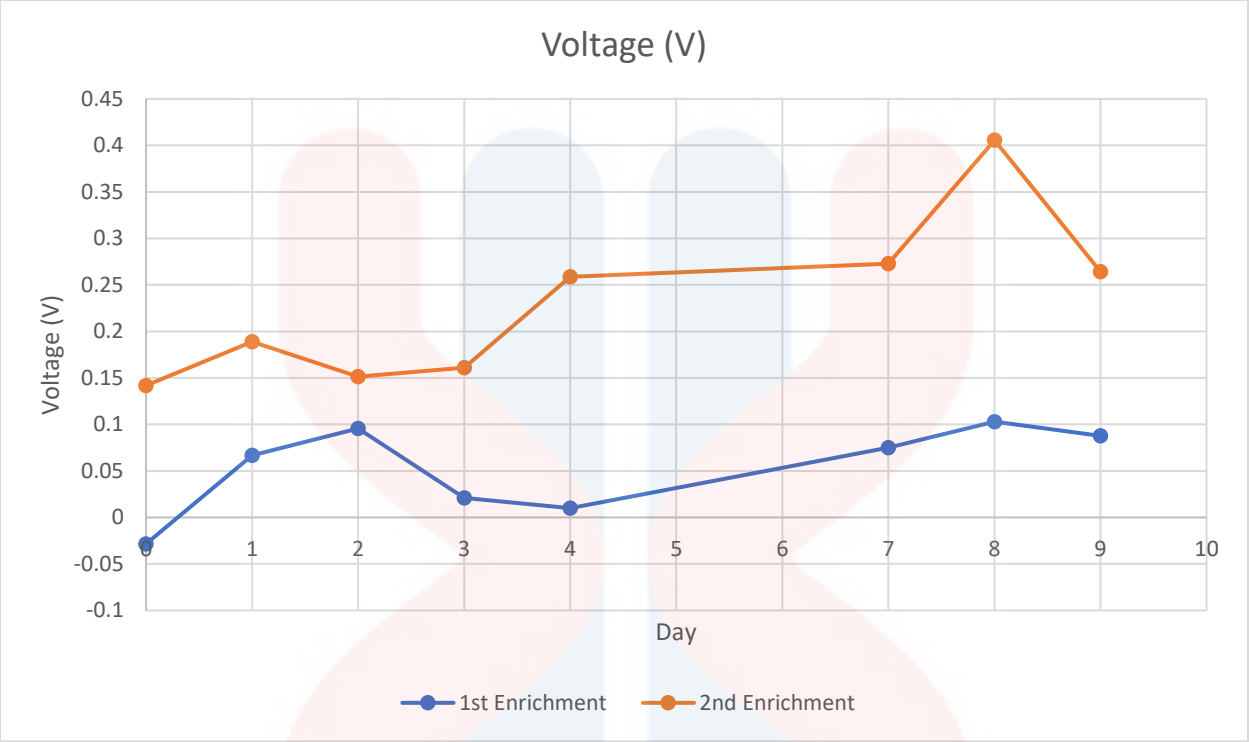


Figure 4.1.2 (a): The differences in voltage between 1st enrichment and 2nd enrichment for nine days.

For the first enrichment, where the paddy soil was enriched in DMFCs without adding any nutrient media, the voltage on day 0 was recorded -0.028 V. For the second enrichment setup, 20 mL of soil from the first enrichment were acclimatized in sterilized paddy soil as we wanted to identify which bacteria resided in the paddy that helps generate power. On day 0, the voltage recorded was 0.142 V. There was a four times fold increment from the first enrichment. The maximum voltage for the first enrichment setup only reached 0.103 V, while the maximum voltage for the second enrichment reached 0.406 V on day 8. The enrichment culture technique involves designing a highly conducive growth environment for a specific organism of interest. Moreover, this enrichment method can be accomplished by providing particular environmental parameters, such as incorporating nutrients or modifying temperature, pH, or other variables, that promote the development of the desired microorganisms. Enrichment culture methodologies are employed to amplify a small population of the targeted microorganisms to different quantities (Madhuri et al., 2019).

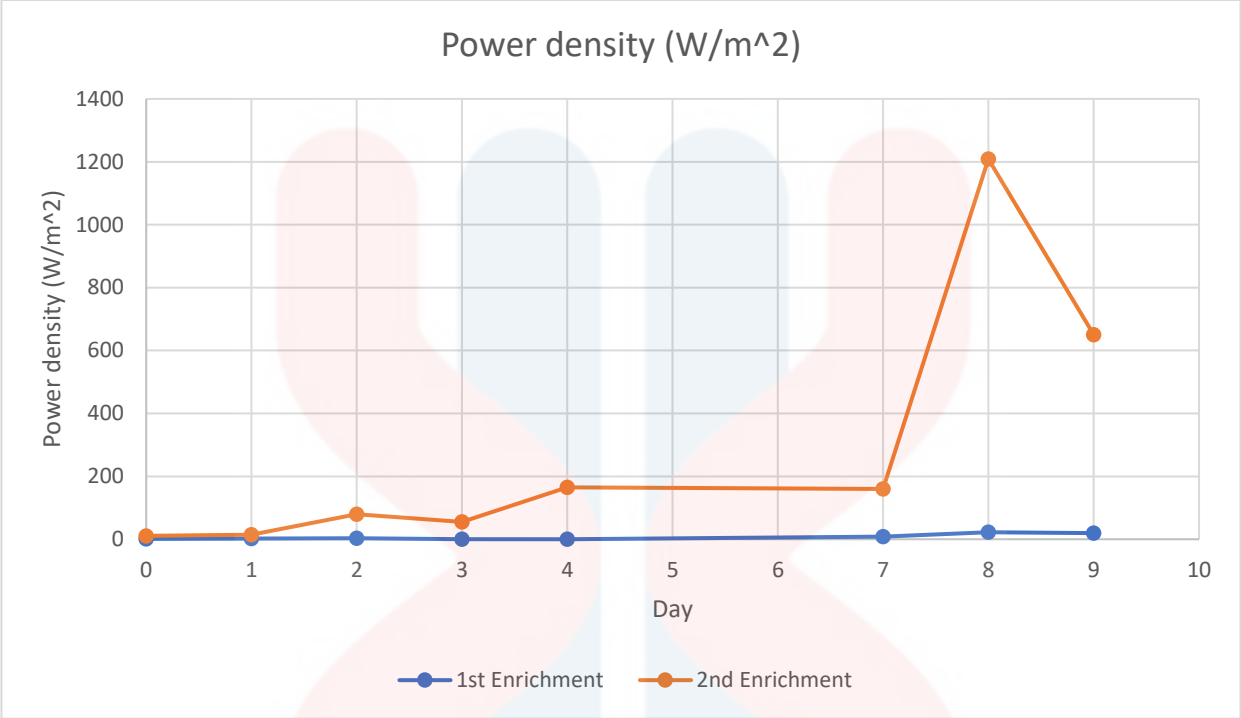


Figure 4.1.2 (b): The differences in power density between 1st enrichment and 2nd enrichment for nine days.

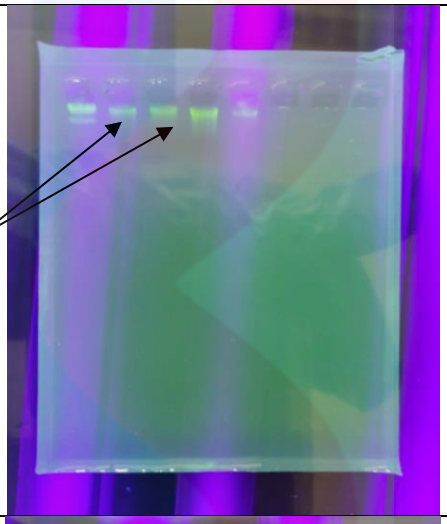
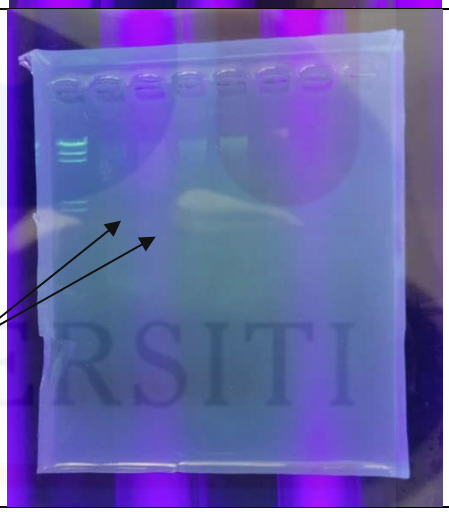
Throughout the nine days, the second enrichment consistently achieves higher power density than the first enrichment with 40-fold augmentation. These findings indicate that the second enrichment technique helps stimulate electrogenic bacteria responsible for producing electricity in the MFC. The second enrichment shows significant differences in power density, specifically on days 2 and 3, 79.081 W/m^2 and 55.430 W/m^2, respectively. This situation may arise from various circumstances, such as alterations in the availability of substrates, nutritional conditions, or microbial community dynamics. The maximum for both enrichment setups on the eighth day shows a significant increase in power density for the second enrichment, surpassing 1208.830 W/m². In contrast, the maximum value for the first enrichment was 22.300 W/m^2. This result shows a critical time in microbial activity and power production. The first enrichment also indicates periods of higher power density, but they are less frequent and noticeable than the second enrichment. This further highlights the efficacy of the second enrichment. From days four until 7, the bacteria were in the stationary phase for both MFCs setups. This might be because the bacteria that generate power are still adapting to the new environment. In another study, different substrates were used as anolytes in the double chamber microbial fuel cells which were glucose, acetate and

sucrose. The maximum power density recorded were 31, 53.4 and 52.3 mW/m^2 respectively (Ullah & Zeshan, 2020). Another research using double-chamber microbial fuel cells and domestic wastewater with the addition of acetate as substrate resulted 70 mW/m^{-2} in power density (Jatoi et al., 2021) which was higher than using only glucose, acetate and sucrose as substrates. In comparison, using paddy soil as one of the substrates might be more efficient than other substrates and more environmentally friendly.

4.1.3 Soil DNA extraction

Gel elctrophoresis with band size 2027 bp for HindI brand and 1% of agarose was conducted.

Table 4.1.3: a) Gel electrophoresis results for the 1st enrichment, while b) shows the result of gel electrophoresis for the 2nd enrichment in MFCs after soil DNA was extracted.

a)	1st Enrichment	
b)	2nd Enrichment	

4.1.4 Screening and isolation of bacteria

Three distinct colonies appeared after a day. The single colonies were then isolated on a new LB agar plate labeled with MC-1, MC-2 and MC-3.

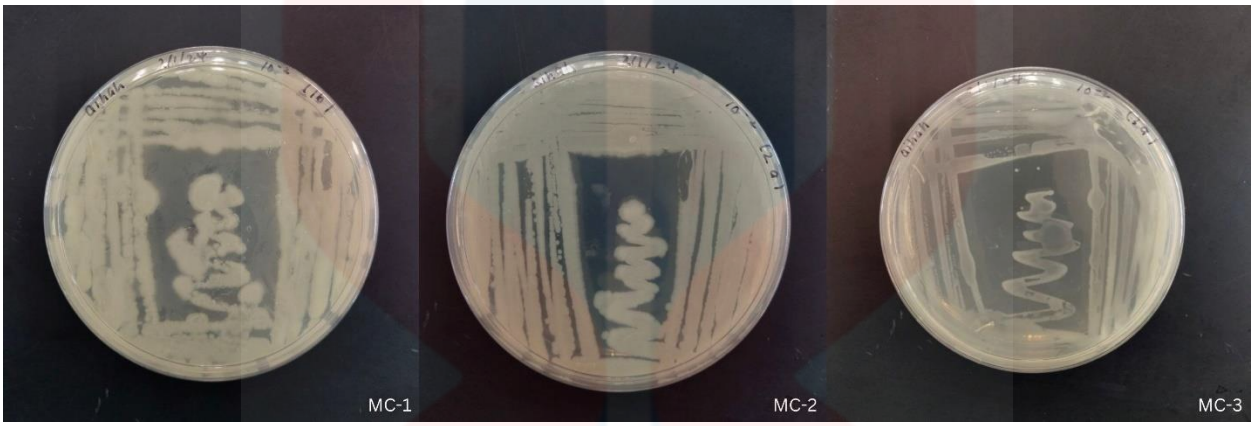


Figure 4.1.4: Colonies appeared after a day of incubation.

Table 4.1.4: Morphology of the bacteria.

Colony	Color	Texture	Edge	Elevation	Surface	Whole colony
MC-1	Milky yellow	Slimy	Lobate	Raised	Smooth	Irregular
MC-2	Milky yellow	Sticky	Undulate	Flat	Smooth	Irregular
MC-3	Transparent	Slimy	Entire	Flat	Smooth	Punctiform

The morphology of the colonies can be referred to in Table 4.1.4, where the colour for samples MC-1 and MC-2 was the same, which were milky yellow, while MC-3 was transparent. The edges for those three samples were different, which were lobate, undulate, and entire, respectively. Next, the texture for MC-1 and 3 MC-3 were the same, which was slimy, while the texture for MC-2 was a bit sticky than the others. The elevation of the colonies was also observed, with 1 (b) elevation being a bit raised than the other two samples that were flat. Lastly, the whole colony for morphology for MC-1 and MC-2 was irregular, while MC-3 was punctiform.

4.1.5 Biochemical test

Table 4.1.5: Biochemical test

Colony	Catalase test	Oxidase test	Gram staining	Shape
MC-1	+	-	Gram-negative	Bacillus
MC-2	+	-	Gram-negative	Bacillus
MC-3	+	-	Gram-negative	Coccus

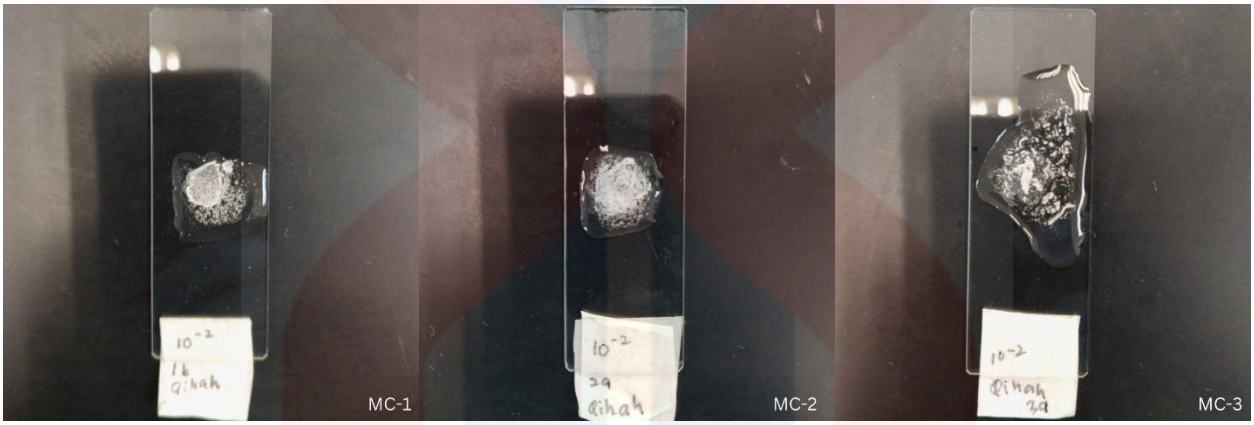


Figure 4.1.5 (a): Catalase test

Catalase test identifies the presence of bacteria that produce catalase enzyme. The bacteria in question produce catalase, an enzyme that counteracts the effects of hydrogen peroxide by producing bubbles, thereby showing a favorable outcome in the test. Most catalase is produced by obligate aerobes and facultative anaerobic bacteria (AL-Joda & Jasim, 2021). Figure 4.1.5 (a) indicates that all samples were catalase-positive, meaning the bacteria might be aerobes or facultatively anaerobic. This was possible as the DMFCs setups were only micro anaerobic not fully anaerobic.

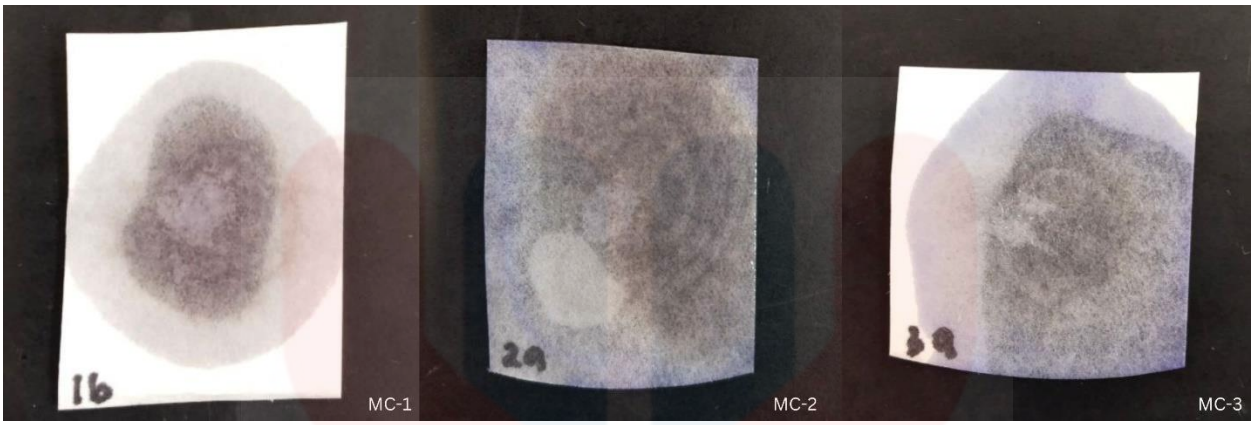


Figure 4.1.5 (b): Oxidase test

For the oxidase test, this test can detect microorganisms that produce cytochrome oxidase enzymes. Cytochrome oxidase transfers electrons from the donor (electron transport chain) to the final acceptor (oxygen), mainly water (AL-Joda & Jasim, 2021). A dark purple color will result from cytochrome oxidase oxidizing the electron donor. An artificial electron donor used in this study was tetramethyl-p-phenylenediamine dihydrochloride. If a bacteria is oxidase-positive, it is aerobic, which means it can use oxygen as a terminal electron acceptor during respiration. This, however, does not prove that they were strictly aerobes. Oxidase-negative bacteria might be facultative, aerobic, or anaerobic; the oxidase-negative result indicates that the organisms lack the cytochrome c oxidase necessary to oxidize the test reagent. Based on Figure 4.1.5 (b), all samples were oxidase negative, meaning the microorganisms lacked the cytochrome c oxidase required to oxidize the test reagent. This also suggests that the bacteria might be a facultative anaerobe.

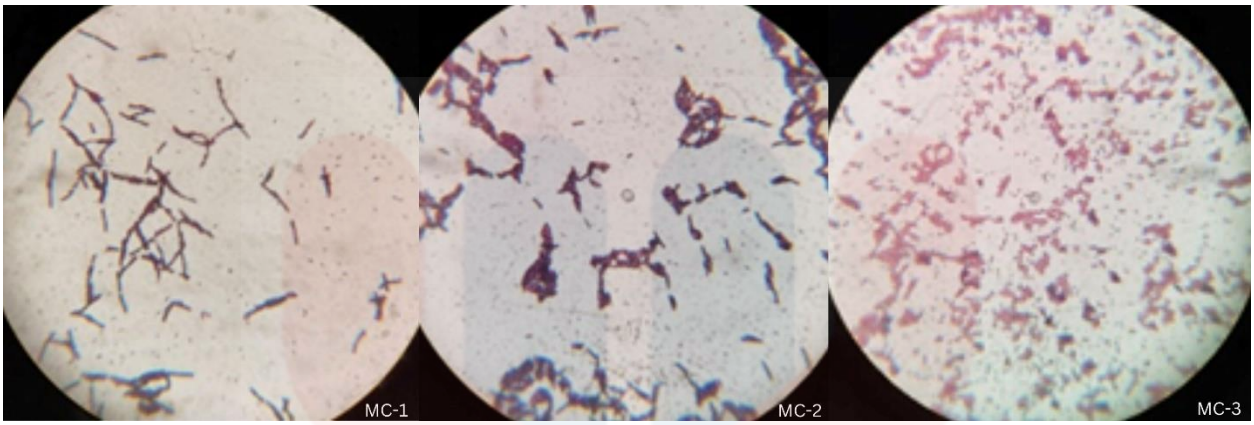


Figure 4.1.5 (c): Gram-staining.

Lastly, all samples were observed under a microscope at 100× magnification for the gram staining procedure. The gram-staining procedures indicate that the bacteria were all gram-negative based on Figure 4.1.5 (c), which showed the pink stained color. Even so, the morphologies for the bacteria were different. The shapes for samples MC-1 and MC-2 were bacillus, which was rod-shaped, while sample MC-3 was coccus, which was circular-shaped bacteria.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

From this result, the design for DMFCs using salt bridge successfully utilizes the metabolic activity of the microorganism to generate electricity by reducing the potassium ferricyanide. Next, the bacterial strains isolated are capable of generating electricity from paddy soil collected in Machang. Enriched microbial communities in paddy soil can generate electricity with 40-fold increment in power density. The results indicate the possibility of utilizing electroactive bacteria found in paddy soil using dual chamber microbial fuel cells.

5.2 Recommendations

Further studies into the ability of paddy soil to serve as a plentiful reservoir of electroactive bacteria for utilization in microbial fuel cells (MFCs) are highly encouraged based on the data obtained from this research. The primary focus of next-generation research should be improving the enrichment process and identifying the specific bacterial species that significantly affect the enhancement of power density. This study clearly shows that more research needs to be done on the interactions and complexity of microbes in paddy soil so that paddy field microbial fuel cells can be fully utilized. The implementation of these rules has the potential to maximize the utilization of paddy soil as a source of renewable energy that is both dependable and efficient:

5.2.1 Confirming the electroactivity of the bacteria isolated

Develop DMFCs using the same design, and catholyte. For anolyte, use sterilized paddy soil or other substrate with the addition of single bacteria that have been successfully isolated to confirm the electroactivity of the bacteria.

5.2.2 16S DNA Sequence and metagenomic analysis

By employing cutting-edge methods such as 16S DNA sequence analysis and metagenomic investigations, one can acquire a more comprehensive understanding of microbial community dynamics.

5.2.3 Continuous readings measurement

In order to ensure reliable and accurate measurements, future research should include a method of constantly recording the voltage and current for an entire day, which is 24 hours. This will make it possible to observe readings that have been stabilized and will make it easier to conduct an in-depth study of the data. In addition, the utilization of new analytical techniques, such as metagenomic analysis, may be of benefit in interpreting the complex interactions within the communities of microorganisms.

5.2.4 Develop a fully anaerobic MFCs setup

To maintain anaerobic conditions and facilitate efficient electron transfer, it is necessary to use an anaerobic microbial fuel cell system that has been thoughtfully designed with a proton exchange membrane (PEM) like Nafion as a mediator.

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APPENDIX A

Power formula:

$$P(W) = Voltage, V\ (V) \times Current, I\ (A)$$

Surface area of the electrode:

$$A\ (m^2) = \pi r^2$$

Power density (W/m^2):

$$Power\ density = \frac{Power\ (W)}{Area(m^2)}$$

APPENDIX B

Raw data for first enrichment setup:

Day	Voltage (mV)	Current (mA)
0	-28.397	-0.004
1	66.894	0.003
2	95.801	0.003
3	20.943	0.001
4	10.004	0.000339
7	74.942	0.009
8	102.971	0.017
9	87.878	0.017

Raw data for second enrichment setup:

Day	Voltage (mV)	Current (mA)
0	141.874	0.006
1	188.857	0.006
2	151.411	0.041
3	161.157	0.027
4	258.851	0.05
7	272.674	0.046
8	405.527	0.234
9	264.389	0.193