MEDIATOR COMBINED GASEOUS SUBSTRATE FOR ELECTRICITY GENERATION IN MICROBIAL FUEL CELLS (MFC_S) AND POTENTIAL INTEGRATION OF A MFC INTO AN ANAEROBIC BIOFILTRATION SYSTEM

A thesis submitted in the partial fulfilment of the requirements for the Degree of Master of Engineering in Chemical and Process Engineering in the University of Canterbury

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2012

ABSTRACT

Microbial fuel cells (MFCs) are emerging energy production technology which converts the chemical energy stored in biologically degradable compounds to electricity at high efficiencies. Microbial fuel cells have some advantages such as use of an inexpensive catalyst, operate under mild reaction conditions (i.e. ambient temperature, normal pressure and neutral pH), and generate power from a wide range and cheap raw materials. These make microbial fuel cell as an attractive alternative over other electricity generating devices. However, so far the major problem posses by this technology is the low power outputs of the microbial fuel cells that hinder its commercialization. Restriction in the electron transfer from bacteria to the anode electrode of a MFC is thought to be one cause for the low power output.

Most recent MFC research is focused on using contaminants present in industrial, agricultural, and municipal wastewater as the energy source, with very few studies utilising gaseous substrates. Mediators can be added to MFCs to enhance the electron transfer from the microbe to the anode, but have limited practical applicability in wastewater applications because of the difficulty in recovering the expensive and potentially toxic compound. This thesis describes an investigation of electricity generation in a microbial fuel cell by combining a gaseous substrate with a mediator in the anode compartment. The emphasis being placed on the selection of a mediator to improve the electron transfer process for electricity production in an MFC. Subsequently, methods to improve the performance of a mediator MFC in respect of power and current density were discussed. This type of MFC is purposely aimed to be applied for treating gaseous contaminants in an anaerobic biofilter while simultaneously produce electricity.

In this study, ethanol was the first gaseous substrate tested for the possibility to generate electricity in the MFC. Various mediators were previously compared in their reversibility of redox reactions and in the current production, and three best mediators were then selected for the power production. The highest electrical current production i.e. 12 $\mu A/cm^2$ was obtained and sustained for 24 hrs with *N,N,N',N'*-tetramethyl-1,4-phenylendiamine TMPD (N-TMPD) as the mediator using glassy carbon (GC) electrode. The maximum power density reached 0.16 mW/cm^2 by using carbon cloth (CC) anode. The absorption of these mediators by the bacterial cells was shown to correlate with the obtained energy production, with no N-TMPD was absorbed by the bacterial cells. The 24-

hr current production was shown to be accompanied by the decrease in the ethanol concentration (i.e. 1.82 g/L), however ethanol crossover through the proton exchange membrane and ethanol evaporation around the electrodes were most likely to be the major cause of the decrease in the ethanol concentration. A theoretical coulombic efficiency of 0.005% was calculated for this system.

The electrokinetics of microbial reduced mediator in the ethanol-mediator MFCs was also examined. Two methods i.e. linear sweep voltammetry (LSV) and cyclic voltammetry (CV) were used to obtained the kinetic parameters. CV method gave a better estimation of the kinetic parameters than LSV method due to the low concentration of the mediators used, affecting the Tafel behaviors. All CVs showed quasi-reversible behaviors compared to the CVs in the absence of the bacteria, which is thought due to the bacteria decreased the amount of the reduced and the oxidised mediator available at the surface of GC electrode. The highest exchange current density (i_o) was obtained by using N-TMPD as the mediator with the same concentration of the mediator used i.e. 0.13±0.01 mA/cm^2. The power output achieved also the highest (0.008 mW/cm^2) with N-TMPD as the mediator. The power density was improved to 0.03 mW/cm^2 by using CC electrode.

Another main objective of this thesis is to prove anoxic methane oxidation which was believed to occur only in marine sediments, and applies this for power generation in microbial fuel cells. Ferricyanide looked promising when it was used as the electron acceptor (thus as the mediator for the MFC). It was shown that ferricyanide was fully reduced by methanotrophs bacteria with methane as the substrate (versus abiotic and nitrogen control). The highest reduction rate achieved was 3 x10^{-3} mM/min.g. This finding was supported by ferricyanide peak heights disappearance (spectrophotometry at 420 nm), CO_2 production (sensor readings), ferrocyanide formation (cyclic voltammetry), and no other alternate electron acceptor was present. The total CO_2 produced was equal to 0.015 mmoles of CO_2 from starting concentration ferricyanide of 0.2 mmoles (after substraction with an offset value). CV results show 2.4 mM of ferrocyanide was produced after a total addition of 3 mM ferricyanide into the anoxic methanotrophic suspension. The current and voltage generation in microbial fuel cell reactor from the reduced ferricyanide confirmed that ferricyanide received electrons from the bacterial metabolism. The maximum power density of 0.02 mW/cm^2 and OCV of 0.6 V were obtained with 3 mM ferricyanide using LSV method.

ACKNOWLEDGEMENT

I wish to thank Dr. Peter Gostomski and Dr. Aaron Marshall for their guidance and support to complete this thesis. The author also would like to express gratitude to all technicians and staffs of the Chemical and Process Engineering Department University of Canterbury. My acknowledgement also goes to the Directorate General of Higher Education Ministry of Education and Culture of the Republic of Indonesia for their financial support. Finally, my deepest thank to my family, in particular my husband Muhamad Yusa, my daughter Shavina Alifia, and my mother for their continued love and support.

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1 INTRODUCTION

1.1 Energy and population

According to BP (2011), the growth of population and income are the two most important factors that drives the increase in energy use across the world. The world's population has grown to 7 billion people in 2011, and could be more than 8.9 billion by 2050 (BP, 2011; UN, 2011). This increase will likely require 50% more energy by the year 2050 (UN, 2011). Meanwhile, GDP (the gross domestic product as an indicator of the world's income) has increased from 7 to 43 trillion dollars in the past two decades, and is projected to double in 2030 (Figure 1-1c) (BP, 2011). The primary energy consumption has grown by 77% since 1900 (Figure 1-1a) (BP, 2011). Non-OECD (organization for economic co-operation and development) nations share nearly all of future global energy production to 2030.

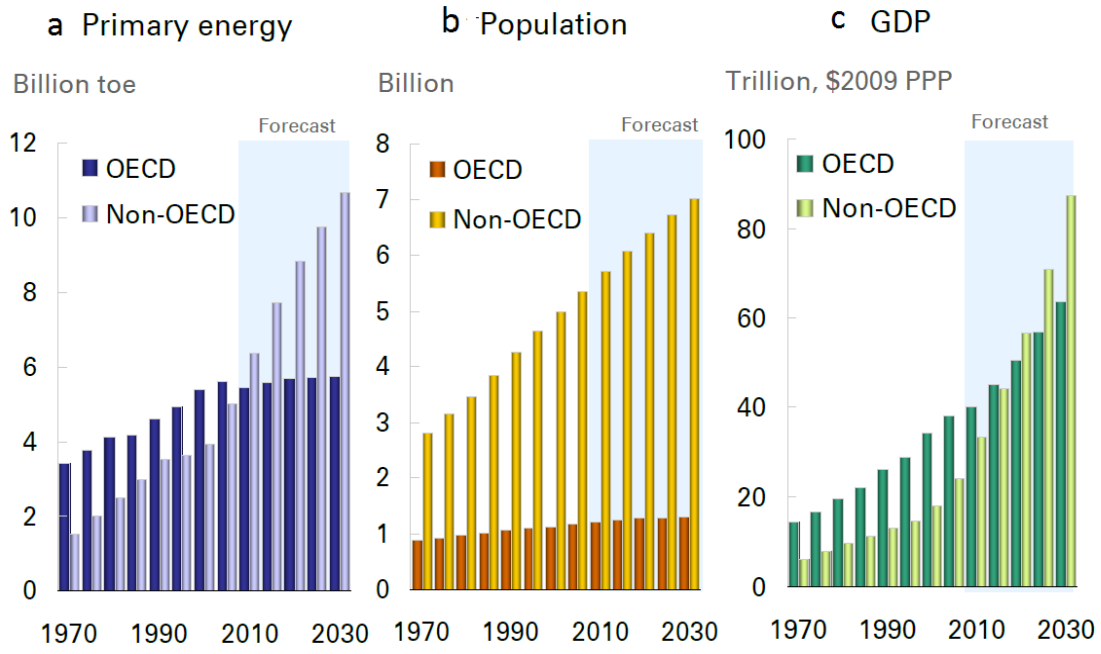

Figure 1-1 Global trends of: primary energy consumption (in tonnes of oil equivalent or toe) (a), population growth (b), and GDP (c) occurs in OEDC and non-OEDC regions (BP, 2011).

The result of the world's energy exploitation has led to a shift of the dominant fuel source from time to time. Fossil fuels i.e. coal, oil and natural gases serve as the most important energy sources today and they have successfully replaced traditional renewable energies in the 1600s such as firewood and dung. This source of fuels will still stay as the dominant fuels over the next two decades and beyond (Figure 1-2). As the chart shows, there will be a moderate share from hydro, nuclear, and other renewable energy in the future. Increasing constraints from the prolonged use of fossil fuels such as emission of greenhouse gases and resource depletion has triggered enormous interest in new renewable energy technologies.

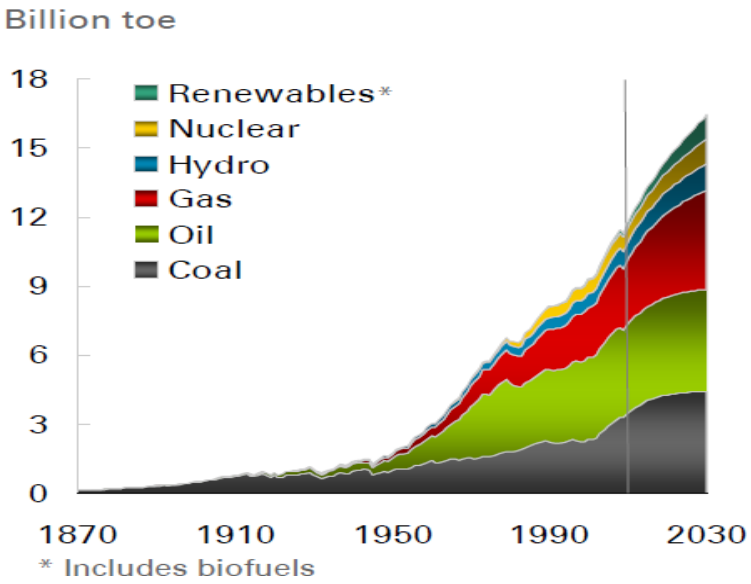

Figure 1-2 Trends and forecast of world energy use by fuel (BP, 2011).

1.2 Renewable energy and biomass

Renewable energy is energy which utilises renewable resources such as sunlight, wind, and geothermal heat. Other than the fact that renewable energy will never be depleted, its major advantage is ideally they do not emit CO_2 or other greenhouse gases (GHGs), thus are regarded carbon neutral. The reason biomass and biofuels, which emit CO_2, are classified as carbon neutral is because it is considered to have a zero balance between the carbon dioxide released during the generation of energy and the carbon dioxide absorbed by plants (RENET, 2012). However, it is important to evaluate each source of

renewable material for its neutrality in comparison to fossil fuels source before it is claimed to be carbon neutral (Heijne, 2010).

Biomass i.e. plants as well as animals, is all organic matter generated through photosynthesis and the other biological process. It is a renewable energy source because the energy it contains ultimately comes from the sun. Examples of biomass sources include wood and wood waste, straw, corn cobs, poultry litter, algae, saw dust, and other waste such as garbage, sewage solid, industrial refuse etc. It can be made to energy by burning or digesting these materials, and energy can be recovered in the form of heat, electricity, or a combination of both.

In 2010, renewable materials shared 16.7 % of global energy consumption, in which 8.5 % originated from traditional biomass (mostly used for cooking and heating), and 8.2 % came from modern renewables (REN21, 2012). The modern renewables include geothermal and solar (3.3 %), hydropower (3.3 %), wind (0.9 %), and biofuels (0.7 %) (REN21, 2012). About 20 % of the renewables were used in electricity generation, with 15.3 % was supplied by hydropower and the remainders were from other renewables (REN21, 2012).

1.3 Advantages and principles of microbial fuel cells as renewable energy generation

Electricity will become an important form of energy in the future. Microbial fuel cell (MFC) represents an innovative technology for electricity generation from biomass-based renewable materials. It is a bioelectrochemical device in which power is supplied from biological redox reaction mediated by micrrorganisms. MFC is believed could provide partial answer to energy demand as well as environmental issues. Although the major research area is to convert waste/wastewater into electrical energy, MFC has many other potential applications in remote sensing (sediment MFCs), bioremediation of pollutants, implanted biomedical devices, and biological hydrogen production (Angenent et al., 2004; Logan & Regan, 2006; Mohan et al., 2010).

Other than having broad of applications, microbial fuel cell technology has many advantages over other energy generation technologies. It can convert the biochemical

energy contained in the biomass directly into electricity. Hence theoretically MFC has the advantage of high overall conversion efficiency compared to other conventional energy generation technologies such as biogas and bioethanol which needs further stages after fermentation to release the energy (Dalvi et al., 2011). Another advantage of MFC is it can be operated at mild reaction conditions due the nature of the biological system. According to Rabaey et al. (2005), it has less energy input requirement if a single chamber air cathode is used (thus low operational cost and ease of operation). Last but equally important, MFC also has opportunities to use a wide variety of fuels. Therefore MFC will likely to become one of the most important sources of electricity in the future.

Basically, an MFC consists of two chambers: an anode and a cathode. Those chambers are separated by a cation/proton exchange membrane (PEM), as depicted in Figure 1-3 (Erable et al., 2010). An external wire usually connects the anode and the cathode to flow electrons (electric current). As microorganisms utilize organic substrate under anaerobic conditions in the anode compartment, electrons and protons are generated through their metabolism. Electrons flow from the microbes to the anode directly or via a redox mediator, and then flow through the electrical circuit to power a device (for example, a light bulb), and finally to a high potential (terminal) electron acceptor in the cathode chamber. Protons diffuse through the solutions in the anode and penetrate the PEM to the cathode where they are combined with electrons and oxygen (widely accepted electron acceptor) to form water (Allen & Bennetto, 1993).

Many electricigens (term for microorganisms that degrade organic compounds and use a solid electrode as an electron acceptor) either pure cultures or mixed cultures have been tested in MFCs to investigate their ability to generate electricity (Lovley, 2006). These microorganisms ranging from pure cultures of obligate and facultative anaerobic bacteria to consortium microorganisms present in wastewater, soil, marine sediment, and activated sludge (Liu et al., 2011; Min et al., 2005; Scott et al., 2008). The pure cultures have been reported to produce less power from organic and/or inorganic waste and have less potential in real practical MFC applications compared to naturally occurring microbial communities (Angenent & Wrenn, 2008). In a few MFC studies, serial enrichments of electricigens from microbial consortia present in a wide range of

environmental samples (Kim et al., 2005) or enrichments of anodic microbes into a new fuel cell have been done (Rabaey et al., 2004), and they have resulted in enhanced power output and conversion efficiency. Several reports have also appeared on identification, screening, and making a chromosome-specific library for electricigens (Logan et al., 2005; Niessen et al., 2004).

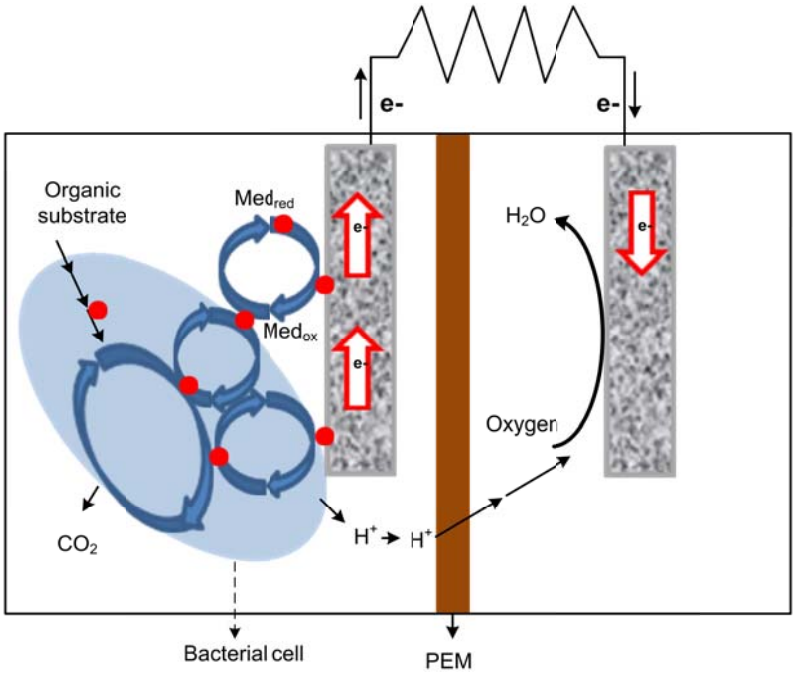

Figure 1-3 Operating principle of a microbial fuel cell, where the oxidation of substrate in the anode releases CO_2, electrons and protons. Electrons pass to the anode directly or via a redox mediator and flow through the electrical circuit. Protons combine with electrons and reduce oxygen to water in the cathode.

There have been considerable efforts over the last twenty years to improve the performance of MFCs, but further research is still needed to overcome several limitations and bottlenecks and to optimise the power outputs in this technology. The optimization of MFCs will require a fundamental understanding of all aspects in a MFC such as architecture, electrochemical and biological processes. Some attempts for the optimization have been reported, these include changing reactor types and electrode materials (Gregory et al., 2004; Schröder et al., 2003), using different types of PEM or without the use of PEM (Kim et al., 2007), investigating various substrates (Kim et al.,

2000), selecting effective redox mediators (Park & Zeikus, 2000; Sund et al., 2007) and the enrichments of anode-colonizing bacteria (Kim et al., 2005).

1.4 Mediator combined gaseous substrates for energy generation in microbial fuel cells (MFCs)

As previously discussed, several factors such as global energy supply security and the need for generating efficient and clean energy have increased the interest in research related to alternative fuel and energy systems. Among these alternative systems, microbial fuel cell technology has been identified as one of the key energy technologies for the future since it can make electricity using any biodegradable material and it can also be modified to produce molecular hydrogen (Kim et al., 2008).

In an MFC, an electron donor (substrate) is a crucial factor determining electricity generation (Liu et al., 2009). The amount of power produced from an MFC can vary with substrate (Choi et al., 2007). For example, the maximum power density of 506 mW/m^2, 305 mW/m^2, and 494 mW/m^2 were reported using acetate, butyrate, and glucose as the substrate respectively (Liu et al., 2005; Liu & Logan, 2004; Min et al., 2005). However, the maximum power density of only 261 mW/m^2 and 146 mW/m^2 were produced when using swine and domestic wastewater as the degraded substrates (Liu et al., 2004; Min et al., 2005). All of those were reported in the same designs of MFC.

The microbes in MFCs produce electricity from degrading organic compounds as the electron donors. These electron donors have been restricted to simple and easily biodegradable substrates such as glucose, acetate, amino and organic acids (Kim et al., 2000; Liu et al., 2005; Logan et al., 2005; Niessen et al., 2004; Rabaey et al., 2004). Nearly all of them have been recognised as valuable compounds for uses as chemical raw materials in industry or for food production. In a few cases, some biorefractory organics, such as cellulose and petroleum contaminants, were also used as the substrate in MFCs (Morris & Jin, 2008; Ren et al., 2007). Some inorganic and other substrates have also been explored, such as phenol (Luo et al., 2009), furfural (Luo et al., 2010), 1,2-dichloroethane (Pham et al., 2009), and ferrous iron (Logan & Regan, 2006a).

Researchers have attempted to search for alternative electron donors in order to make MFC technology more feasible for large scale applications. These are resulted in the use of low-value nutrients such organic compound contained in municipal and industrial wastewater, or in marine sediment (Aelterman et al., 2006; Kumlanghan et al., 2007; Logan & Regan, 2006; Lovley, 2006). Microbial fuel cells utilise low-value organic compounds or waste streams are highly promising because it allows a combination between the recovery of energy and the treatment of the waste stream.

Gaseous pollutants are also a potential source of electron donors for MFCs. Major primary air pollutant gases (e.g. CO, VOCs) are released to the atmosphere each year which can cause harm to humans and environment. Pollution control technique involving application of biological methods such as biofiltration is an alternative to control many gaseous pollutants (Kennes et al., 2009), but normally the process is aerobic (if the gas stream lacks oxygen, air is normally added). An anerobic gas feed can be applied in a MFC. Some recent studies have demonstrated using anaerobic gas feeds such as methane and syngas to produce power in MFCs (Girguis & Reimer, 2009; Mehta et al., 2010).

Much of the recent work with MFCs using wastewater involves biofilms growing directly on the anode to facilitate electron transfer or using organisms that produce soluble electron carriers. Externally supplied (exogeneous) mediators can be employed to enhance the electron shuttle from the bacteria to the anode. However, in a continuous or a fed-batch wastewater system, soluble mediators can accumulate to high concentrations and separating these mediators from the solution is difficult (Logan, 2007). As a result, mediators have limited practical applicability in wastewater applications because of the difficulty in recovering the expensive and the potentially toxic compound. Combining an anaerobic gas feed with mediator in a MFC has not been investigated, and the value of gaseous pollutants will increase if its degradation can be linked to the electricity generation through MFCs. These considerations prompted this study to focus on the development of a microbial fuel cell for direct conversion of gaseous substrates into electricity with mediator enhancing the electron transfer in the anode.

The proposed integration of an anaerobic gas feed with a mediator means the process could be operated in two stages; with the first stage removing the gaseous contaminant by the anaerobic biofilter and producing a stream of reduced mediator, and the second stage generating electricity and producing a stream of oxidised mediator to be recycled to the biofilter (Figure 1-4). The reactions at the biofilter, the anode and the cathode of the microbial fuel cell are as follows (Equation 1-3):

Biofilter : $\mathbf{S + M_{ox} \longrightarrow CO_2 + M_{red} + H^+}$ [1]

Anode : $\mathbf{M_{red} \longrightarrow M_{ox} + e^-}$ [2]

Cathode : $\mathbf{O_2 + 4H^+ + 4e^- \longrightarrow 2H_2O}$ [3]

S, M_{ox} and M_{red} are the gaseous pollutant, the oxidised and the reduced mediator respectively.

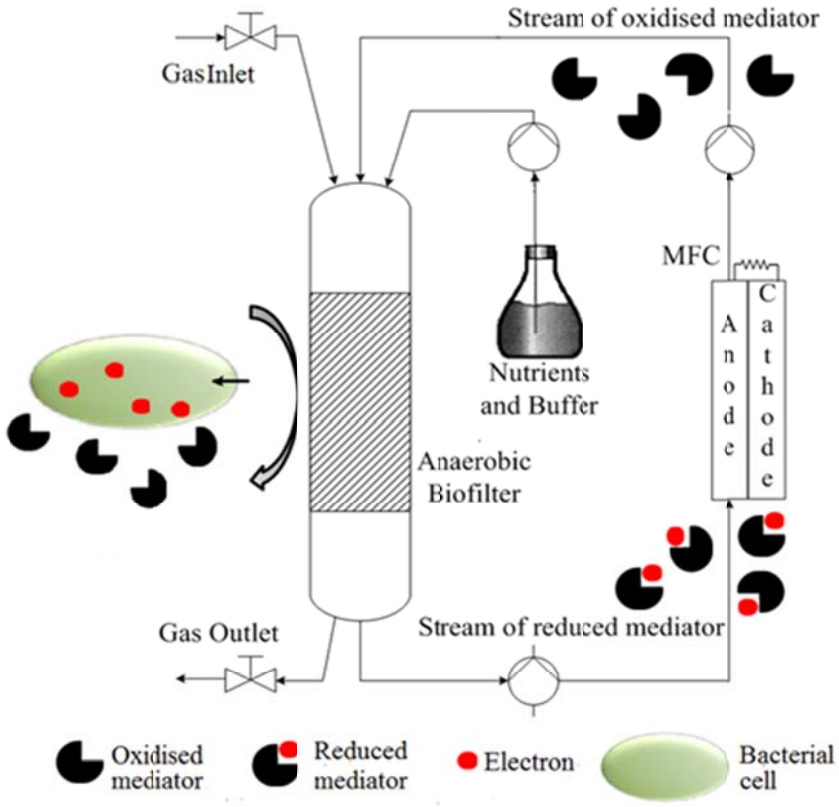

Figure 1-4 A flow diagram of proposed integration of a MFC into a biofilter.

1.5 Research objectives

The objectives of this study were to prove the concept of:

1). Possibility of electricity generation in microbial fuel cell (MFC) with gaseous substrate as the potential electron donor and various mediators for enhancing the electron transfer from microorganisms to the anode.

2). Anoxic methane oxidation using potassium ferricyanide as an electron acceptor (or mediator) for its further use in microbial fuel cell (MFC).

1.6 Thesis organization

The contents of this thesis are structured as follows:

Chapter 1: An introduction to the subject and aims of the research.
Chapter 2: Literature review on electron transfer and microbial fuel cells.
Chapter 3: Ethanol oxidation in microbial fuel cell utilising various mediators.
Chapter 4: Kinetics of oxidation of microbially reduced mediator in ethanol fed MFC.
Chapter 5: Ferricyanide driven anoxic methane oxidation.
Chapter 6: Anoxic methane oxidation coupled with ferricyanide reduction for energy production in microbial fuel cells.
Chapter 7: General conclusions and further works.

1.7 Contribution

The result in Chapter 3 is in the form of a proceeding and has been presented orally in Chemeca 2012 (annual conference of the Australian and New Zealand community of chemical engineers and industrial chemists).

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2 LITERATURE REVIEW

2.1 Electroactive bacteria

The study of electricity generation in a microbial fuel cell (MFC) was initiated by Potter (1912). This study was followed by Cohen (1931) after developing a series of MFCs producing over 35 volts. More than thirty years later, NASA reported the microbial electricity generation in effort to recycle waste into power during spaceflight (Canfield et al., 1963). In the same year, DelDuca et al. used a MFC to produce electricity using hydrogen producing microbes (DelDuca et al., 1963), and this was restudied by Suzuki in attempt to improve the system (Suzuki et al., 1977). The mechanism of electron transfer was still not clearly understood until in the 1980s. Benetto (1981) demonstrated that a chemical compound described as a mediator could improve the transfer of electrons from the microorganisms to the anode. However, commercial applications involves an addition of this artificial mediators was limited because of the high cost and poisoning effect of the mediator (Benneto et al., 1981). The discovery of specific species of bacteria such as *Geobacter* and *Rhodoferax* that could exchange electrons directly to the anode of an MFC via electrically conductive pili in the early of 2000s has prompted a great interest of further research in microbial fuel cells (MFC) (Bond & Lovley, 2003; Reguera et al., 2005). Electroactive bacteria are now defined as bacteria capable of exchanging electrons with an electrode from breakingdown of organic matter, and participating in the generation of current.

Different classifications of electroactive bacteria capable of generating electricity in a microbial fuel cell have been tested, these includes: microbes which can give the highest conversion of substrate; microorganisms that capable to capture light for an energy generation or transfer electron directly to an electrode; microbes that operate at high temperatures; and whether electroactive bacteria that are derived from a consortium of microorganism (i.e. waste water) (Allen & Bennetto, 1993; Bond & Lovley, 2003; Liu et al., 2004; Tsujimura et al., 2001).

2.2 Electron transfer mechanism

Microorganisms live, grow and reproduce due to the metabolic energy they obtain by receiving electrons from a donor substrate, for example glucose, and transferring electrons to a final electron acceptor such as oxygen. This is commonly referred to as aerobic respiration. If the electron acceptor is something other than oxygen (nitrate, sulphate or sulphur can act as the final electron acceptor), the pathway is referred as anaerobic respiration.

The entire respiration process is split into three parts: Glycolysis, the Citric acid (TCA or Kreb's) cycle and Oxidative Phosphorylation (Figure 2-1). Glycolysis occurs in cytosol as the first step in cell respiration. One molecule of glucose is reduced through a series of stages where the potential energy is converted into energy for the cell in the form of ATP or added to the electron transport chain (Figure 2-2). A process that is used to synthesize ATP during glycolysis is known as substrate level phosphorylation. At the same time, glucose is oxidised by NAD^+ molecules which are reduced to NADH. The end product of glycolysis, pyruvate, is converted into acetyl coA (the starting molecule for the TCA cycle) as it enters mitochondrion. Pyruvate also is oxidised by NAD^+ before attaching to acetyl coA. In the absence of oxygen, pyruvate is not generated by cellular respiration but creates waste product i.e. lactic acid and ethanol (in yeast), and the process is called fermentation.

For every one molecule of glucose, two pyruvates and thus two molecules of acetyl coA are synthesized. For every acetyl coA introduces into the Kreb's cycle two CO_2 molecules are produced. As in glycolysis, the generated NAD^+ and FAD^+ in the redox reactions accept electrons and are reduced to their electron carrier forms (NADH or $FADH_2$) and ATP molecule is also produced. The Krebs cycle gives 1 ATP, 3 NADH, 1 $FADH_2$, and 2 CO_2 per pyruvate. The final stage in aerobic respiration is electron transport chain and oxidative phosphorylation where the NADH and $FADH_2$ are taken to produce more ATP.

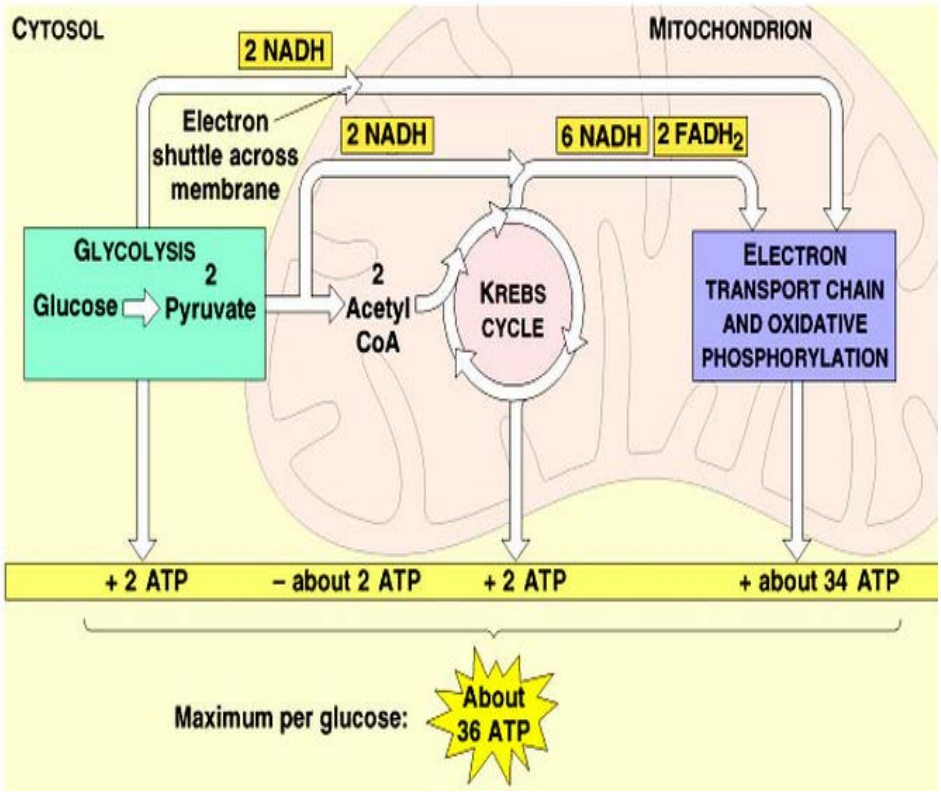

Figure 2-1 Cellular respiration through Glycolysis, Citric acid (TCA) cycle and Oxidative Phosphorylation (Campbell, 1996).

The electron transport chains contains a series of redox enzymes (i.e. NADH dehydrogenase, ubiquinone, coenzyme Q or cytochrome) that function to pass electrons until they are finally pass to oxygen. Energy produced by this process allows protons to move across an internal membrane to create a proton gradient. Thus the proton motive force is generated, enabling activity of ATP synthase and hence the formation of ATP from ADP (therefore it is called oxidative phosphorylation). The aerobic respiration in total theoretically yields 36 molecules of ATP when one glucose molecule is fully oxidised to CO_2.

The difference in redox potential between low potential substrate (or electron donor) and terminal electron acceptor determines how much energy that bacteria could obtain per mole of organic carbon respired. Bacteria undergoes aerobic respiration will make more ATP compared to bacteria from anoxic sediments that reduce sulfate from similar reducing equivalent (for example, NADH). This is because the redox potential difference between NADH (-0.32 V) and oxygen (+0.82 V) is high [0.82 – (–0.32) =

1.14 V]. While with sulphate (-0.22 V), the difference in the potential from NADH is only 0.1 V per mole of organic carbon respired (Rabaey & Verstraete, 2005).

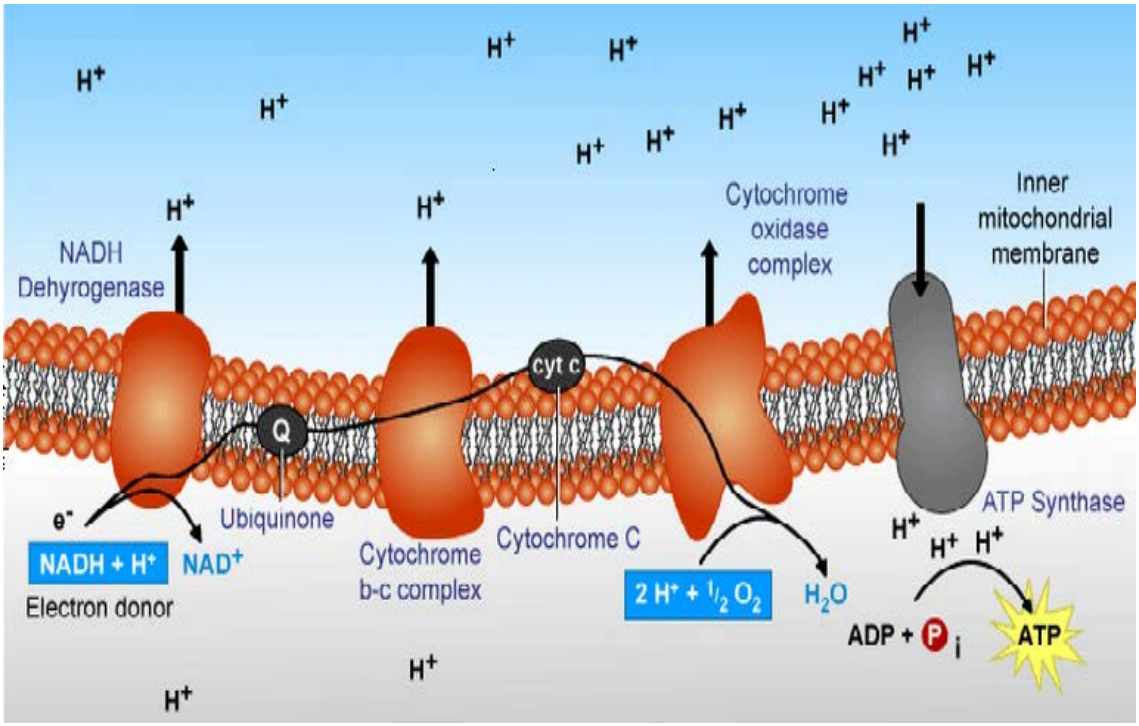

Figure 2-2 The electron transport chain and oxidative phoshorylation in cellular respiration (Woodward, 2012).

In bioelectrochemical system such as MFC, electroactive bacteria rely on conductive electrodes to facilitate respiratory processes. The MFC provides an electrical circuit to take electrons from the bacterial sites to the terminal electron acceptor. The maximum potential obtained in an MFC requires an understanding of the potentials at which electrons may be transferred to the electrode. The link between electron transfer chain enzyme within the membrane and the terminal electron acceptor differs from one organism to another. Electron transfer to an electrode depends on where the enzyme is located in the membrane structures of the cell and if it is able to shuttle electrons out of the cell. Figure 2-3 illustrates the potential difference which could be realised in a MFC. For instance, if an electron is transferred from bacterial cytochrome c to oxygen in the cathode, the maximum potential obtained in the MFC would be 0.6 V (0.82 V - 0.22 V).

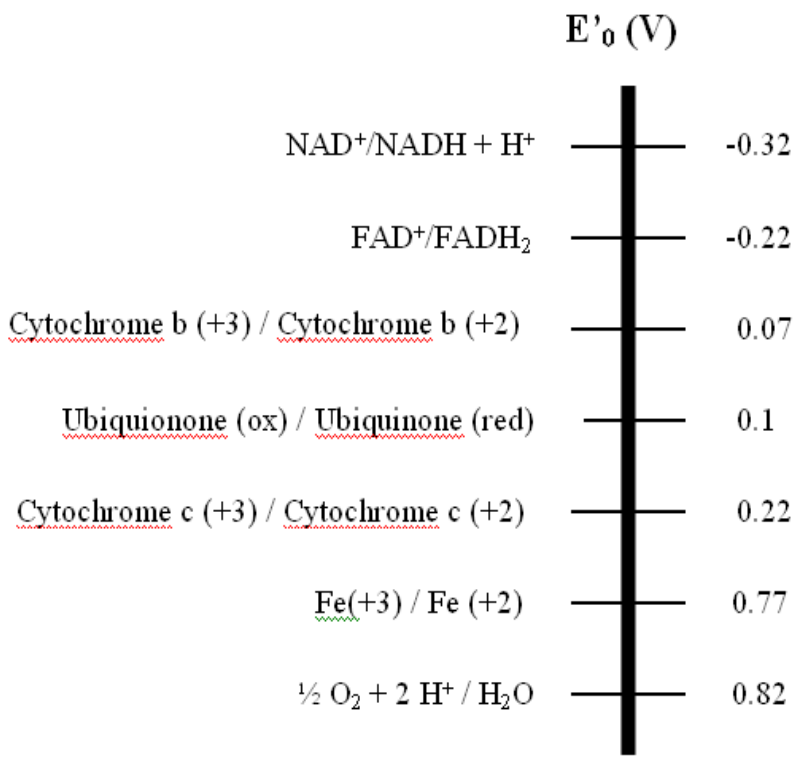

Figure 2-3 Standard redox potential (pH 7, 25°C, vs NHE) of some electron transport chain molecules (Schaetzle et al., 2008).

Mechanisms for transferring of electrons from microbes to the surface of the electrode have been widely studied in the MFC due to the use of bacteria in this technology. Using either soluble electron carriers (indirect mechanism) or electrically conductive pili (direct mechanisms), they can transfer an electron to the anode. The indirect mechanism via electron shuttles or often called electron mediators (whether natural or artificial mediators) has been known for several decades. Electron mediators are defined as chemical compounds which can enter the bacterial cell, and get reduced (because of accepting an electron) before being reoxidised at an anode. In this way, electrons are transferred via this chemical from the cell in the anode i.e. the chemical mediates the transfer of an electron. The following sections are intended to present these various mechanisms of the electron transfer.

2.2.1 Direct electron transfer (DET): Mediatorless transfer system

Some examples of microbial species capable of generating electricity in mediatorless MFC systems are *Geobacter metallicreducens* (Chaudhuri & Lovley, 2003), *Rhodoferax ferrireducens* (Reguera et al., 2005), *Geobacter sulfurreducens* (Bond &

Lovley, 2003), and *Shewanella putrefaciens* (Kim et al., 1999),. Two ways are identified for the direct electron transfer from bacterial cells to the anode electrode (the intermediary electron acceptor):

(A) The electron transfer via *Cytochromes* on the outer-cell membrane onto the anode of an MFC (Myers & Myers, 1992; Xiong et al., 2006).
(B) The production of conductive nanowires (*pili*) in some bacteria (*Geobacter* and *Shewanella)* under biofilm formation that link the microorganisms and the electrode surface (Figure 2-4) (Bond & Lovley, 2003; Reguera et al., 2005).

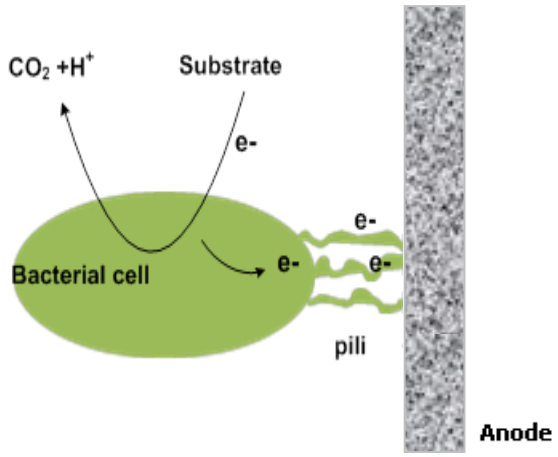

Figure 2-4 Schematic diagram for a direct electron transfer to the anode of an MFC using conductive nanowires (*pili*) in some bacteria

2.2.2 Indirect electron transfer: Mediated electron transfer

Mediators are redox species that can accept electrons from the reduced species in electron transport chain and release them to the solid electrode. There are two methods of the mediated electron transfer:

(A) Self-mediated system;

Several microbial species have been shown their ability of self-mediating electron transfer by producing *endogenous* chemical mediators. This type mediators are reduced inside the cells before get reoxidised on the electrode surface (Figure 2-5a). In this case, examples are *Pseudomonas* species (by producing pyocyanin and phenazines), and *Shewanella* species (by producing flavins) (Marsili et al., 2008; Rabaey et al., 2005).

(B) Artificial-mediated system;

According to Bennetto et al. (1983), the electron transfer across the cell membrane can be enhanced using the addition of artificial mediators (Figure 2-5b). These mediators could be in the form of organic dyes e.g. methylene blue, neutral red, resazurin, *N,N,N',N'*-Tetramethyl-p-Phenylenediamine, and thionine, inorganic complexes e.g. ferricyanide or organometallics (osmium polymers) (Bennetto et al., 1985; Davis & Higson, 2007; Delaney et al., 1984; Emde et al., 1989; Gunawardena et al., 2008; Thurston et al., 1985; Zhang, 2006).

There are some important characteristics that a mediator should have for an efficient electron transport from the microbial intracellular part to the electrode surface (Katz et al., 2003; Rabaey & Verstraete, 2005; Wilkinson, 2000):

(a) The oxidised form of mediator should easily enter the cell membrane to collect the electrons from the reductive species inside microorganisms. On the other hand, the reduced form should easily diffuse out of the membrane to pass the electrons onto the anode (should not be adsorbed on bacterial cells and also electrode surface).

(b) The redox potential of the mediator should be near (and slighly more positive than) the redox enzyme-active site in order to maximize the cell potential (thus the power production). This is because the maximum MFC cell potential will be the difference between the mediator's redox potential and the terminal electron acceptor at the cathode. Barriere (2010) suggested the potential difference of 0.05 to 0.1 V between the mediator and the redox metabolite to provide a fast electron transfer. For example, if the bacterial last redox enzyme in the electron transport chain is cytochrome c (+0.22 V) (from Figure 2-3), therefore N-TMPD (+0.278 V) is the more potential mediator rather than potassium ferricyanide (+0.36 V) and prussian blue (+0.38 V). The reason is because it will be reduced quite fast by bacteria and will yield the MFC cell voltage of 0.54 V compared to 0.46 V and 0.44 V for N-TMPD, potassium ferricyanide and prussian blue respectively (if all using oxygen as the terminal electron acceptor).

(c) The mediator should have high solubility and long-term chemical stability in the electrolyte solution.

(d) The oxidised and reduced form of the mediator should not interact with other metabolic processes (inhibit or decompose).

(e) The oxidation kinetics of the microbially reduced mediator at the electrode surface should be fast (high reversibility).

As most of microbes do not exchange electrons directly with electrodes, many types of chemical compounds have been investigated for their use as mediators, which facilitate the transfer of electrons from the last terminal enzymes of microorganism to the electrode. According to Marcus' theory (1965) apart from those characteristic explained above, another important requirement for a mediator is to provide a high electron transfer rate constant (k_{ET}) with the redox enzyme, for high currents to achieve. Accessibility (e.g. steric effects, orientation and distance dependence) plays a role for electron transfer. The participated reactants should increasingly close to each other to facilitate electron coupling for an electron transfer reaction to take place. Marcus' equation below describes the parameters that affect the electron transfer rate constant (or a decay of the electron transfer constant with distance):

$$k_{ET} = 10^{13} \exp(-\beta(r - r_0)) \exp - \left(\frac{(\Delta G^0 + \lambda)^2)}{4RT\lambda} \right) \quad [1]$$

where β is the distance decay constant in $Å^{-1}$, r is the distance between donor and acceptor in Å, r_o is the value of r at which the frequency of motion of the nuclei equals 10^{13}/s in Å, λ is the Marcus reorganisation energy, and ΔG^0 is the free energy of the reaction, both energies in eV. This equation suggests three things in order to obtain a high electron transfer rate: by decreasing r using a soluble-low molecular weight electron mediator; by increasing the distance of potential between the mediator and the redox enzyme to minimise ΔG^0; and by utilising a fast self-exchange mediator to decrease λ. However, some deviations from this equation have been observed in enzyme-electrode model interactions due influence of other factors such as pH and the ionic strength of the media (Casimiro et al., 1993; Monica, 2002).

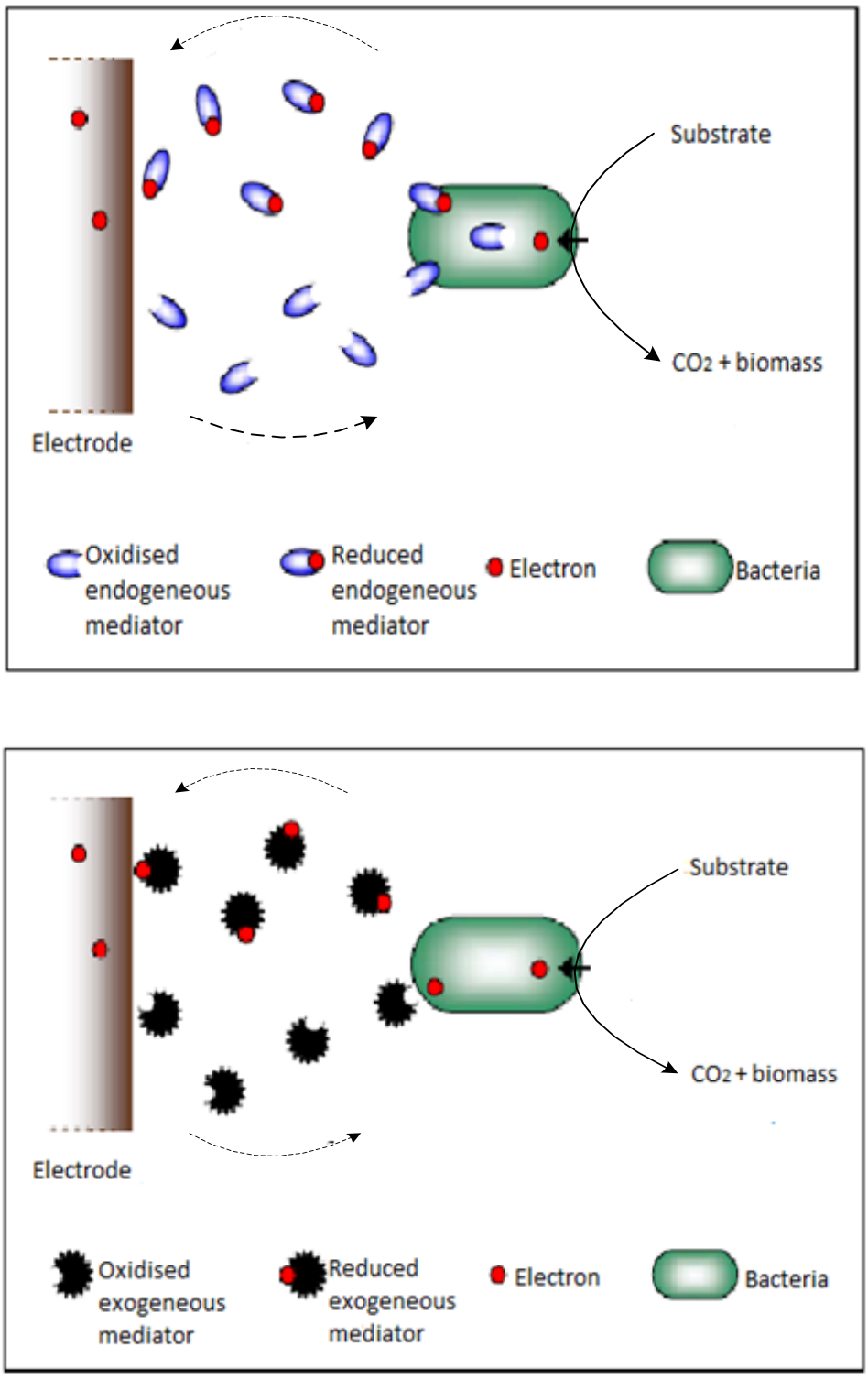

Figure 2-5 Schematic representation of electron transfer mechanism between bacteria and an electrode via: (a). endogeneous mediators, and; (b) exogeneous mediators (Sabatier, 2010).

2.3 Electrochemical approach for evaluating (mediated) electron transfer performance

Figure 2-6 summarizes the process and the electrochemical approach to evaluate the electrons transfer performance. As was previously discussed in Section 2.2, basically, the process starts from the oxidation reduction reactions in the biological system (i.e. oxidation of organic substrate through glycolysis and electron transfer in the electron transport chain) by which biological cells capture and use energy. These electrons can be also transferred to electrodes via the artificial redox mediator (leading to the generation of an electrical current in the microbial fuel cell). The amount of electron transferred to the redox mediator (the current produced) can be analysed using voltammetric and chronoamperometric methods.

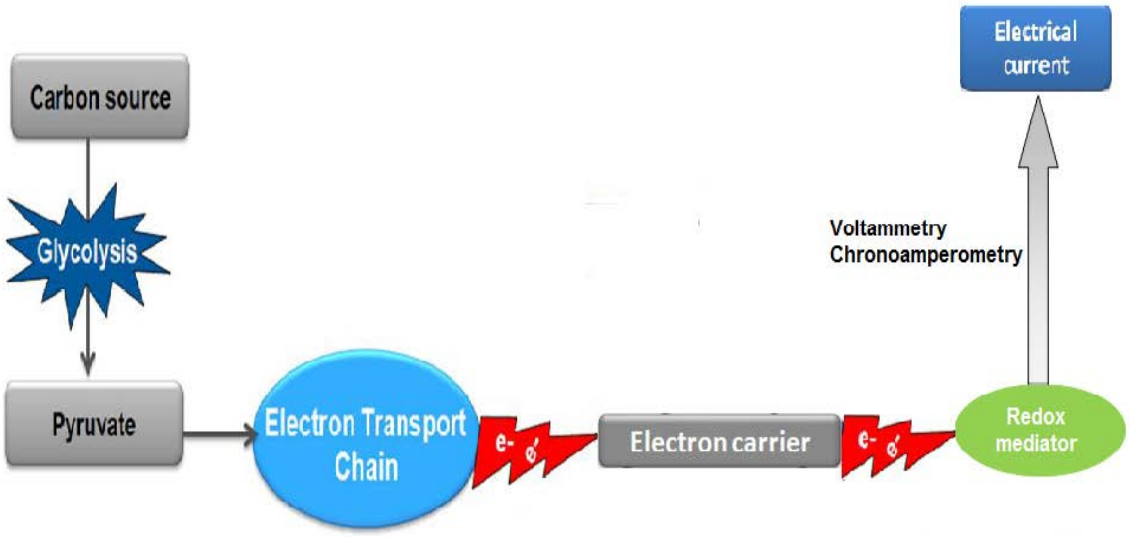

Figure 2-6 Process of electrons flow to obtain the electrical current via artificial mediator using voltammetry and amperometry method in this study.

2.3.1 Cyclic and linear sweep voltammetry (CV and LSV)

Voltammetry is a versatile analytical method based on the measurement of current flowing to/from an electrode immersed in a solution containing electro-active species (analyte). This technique involves three electrodes in which the potential of working electrode relative to the reference is controlled, and the current flows between the working electrode and the auxiliary or counter electrode (Wang, 2000) (Figure 2-7).

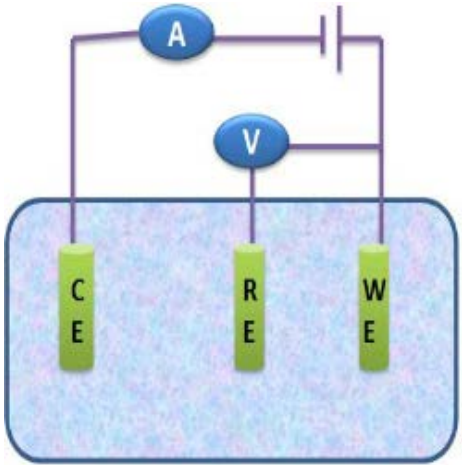

Figure 2-7 Diagram of the three electrode setup, where WE is a working electrode, RE is a reference electrode, and CE is a counter electrode.

In cyclic voltammetry, the flow of current through the working electrode as a function of the applied potential is recorded, creating a voltammogram (Figure 2-8) (Compton & Banks, 2007). The voltammogram shows the occurrence of an analyte's oxidation-reduction process between two potential values of interest (E_1 to E_2) when the potential of the working electrode is changed linearly within these potentials. The term cyclic in cyclic voltammetry indicates the direction of potential scan (the scan is reversed at E_2 to commonly its original value, E_1).

The cyclic voltammetry data can give useful data on the kinetics of electron transfer reactions and thermodynamics of redox processes. Specifically, it offers rapid determination of redox potential (E^o) of the electroactive species, in which it gives information about the cathodic and anodic potential (Ep_c; Ep_a) and the cathodic and the anodic peak current Ip_c/Ip_a. Therefore it can be used to determine the electrode potential required for the oxidation or reduction of the redox species or the mediators e.g. during amperometric measurements. This involves cycling the potential of an electrode (in a dilute mediator solution) between two fixed potential against a reference electrode such as SCE at a desired scan rate. The oxidation and reduction of the mediator results in the flow of current and is seen as a peak on the cyclic voltammogram.

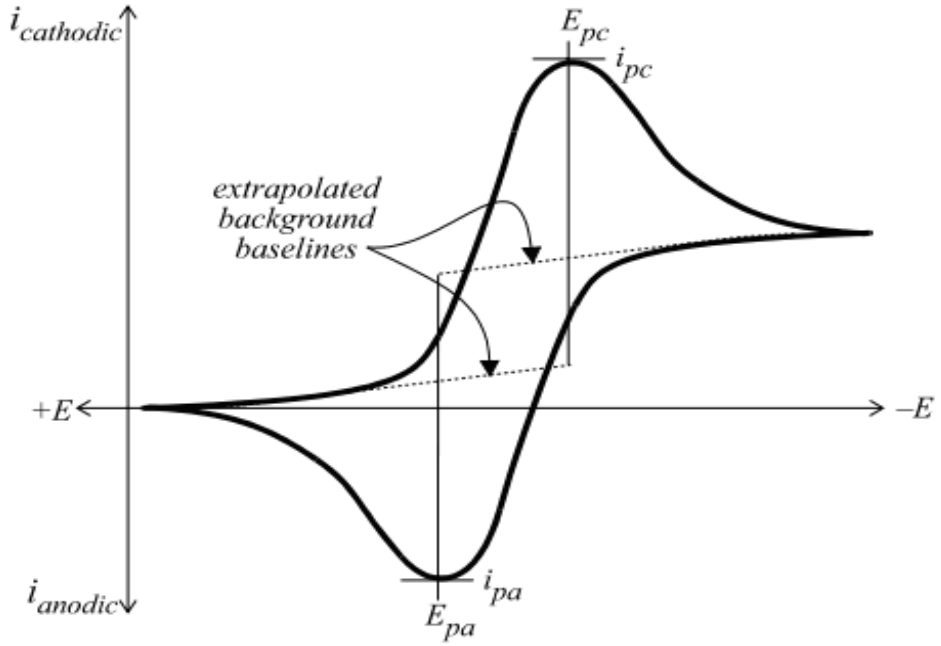

Figure 2-8 A typical cyclic voltammogram.

For all reversible reactions (i.e. the electron transfer is fast), the voltammograms give a typical shape that is shown in Figure 2-8, and the peak currents are given by Randles-Sevcik equation:

$$I_p = (2.69x10^5)n^{3/2}AD^{1/2}Cv^{1/2} \quad [2]$$

where:

n is the number of electron transferred

A is the electrode area (cm^2)

D is the diffusion coefficient (cm^2/s)

C is the concentration of the electroactive species (mol/cm^3)

v is the scan rate (V/s)

Ip is the peak current (A)

This equation can be used to establish the total mediator concentrations available in the solution. The CV parameters below are usually used to identify a reversible process (Kissinger & Heineman, 1983):

a. ΔE_p ($=Ep_c - Ep_a$) or the peak potential separation is 59/n mV at 25 ^oC.

b. Ip_a/Ip_c or the peak current ratio is 1. Both point a and b are for all scan rates.

c. Ip or the peak current is a function $v^{1/2}$ (scan rate), and is independent of v.

The mean of peak potentials provides the redox potential value (E^o) for a reversible process. Variations from the described parameter values characterise a quasi-reversible or an irreversible behavior for a redox process.

Linear sweep voltammetry (LSV) is another voltammetric method which used in this study, and basically the same as cyclic voltammetry (CV). The difference between these two methods is that in LSV the potential of the working electrode is scanned in one direction only (i.e. oxidation or reduction only).

2.3.2 Chronoamperometry

Chronoamperometry is a technique for quantifying concentration of the electrochemically active species in the usual three electrode set up. By applying an analyte-specific electrochemical potential on a working electrode dipped in analyte solution, a redox analyte is either oxidised or reduced. This method records currents as a function of time as the results of the Faradaic (oxidation-reduction) process taking place at the electrodes (due to the potential step).

Chronoamperometry experiments can be in *single potential step* or *double potential step*. In regards to this study (in which potential step is used to determine the current generation from the microbially reduced mediator), only the single step amperometry is discussed. The technique involves stepping away the potential of the working electrode from the open circuit (or E_{rev}) potential of the mediator to a value at which the mediator is either oxidised or reduced in order to satisfy the requirements of the Nernst equation.

The rate of oxidation and reduction of an electroactive species in all electrochemical techniques, will depend on (Ryan, 2004):

1) redox reaction rate and mass transport

2) electrode kinetics (electron transfer at the electrode) which is influenced by:

a. characteristics of the reaction

b. characteristics of electrode surface

c. temperature

The following describes the mechanism of mass transport:

1) Migration – movement of a charged particle due to an applied potential.
2) Diffusion – mass transfer being driven by a concentration gradient.
3) Convection – mass transfer caused by mechanical movement i.e. stirs solution, rotate or vibrate electrode.

In the chronoamperometry, when the current measured depends on the rate at which the analyte diffuses to the electrode, it is said to be diffusion controlled (mass transfer controlled), on can be described by the Cottrell equation for a planar electrode (linear diffusion) (Bard & Faulkner, 2001):

$$I = \frac{nFACD^{1/2}}{\pi^{1/2}t^{1/2}} \quad [3]$$

where,

I is the current (A)

F is Faraday constant (96,485 C/mol)

T is the time (s)

For a microelectrode, the diffusion limited current exponentially approaches a steady state value and this occurs in ms-time scale depending on the type of solution and the size of the microelectrode. This is because the characteristic dimensions of the microelectrode smaller than the dimensions of the diffusion layer within the time scale of the experiment (and so has the hemisperical diffusion layer and expands further into the bulk solution, rather than a plane projecting into the solution as for a planar electrode), therefore the steady state electrochemistry is rapidly established (Wang, 2000). The value of the steady-state, diffusion limited current, $I_{\lim}$ is given by the following relationship (Equation 4), for a microdisk geometry (Pletcher, 1990):

$$I_{\lim} = 4nFrDC \quad [4]$$

where r is the radius of the microelectrode (in cm). Equation 4 quantitatively describes how the diffusion limiting current (I_{lim}) proportional to the concentration of the electro-active species (used to determine the concentration of the oxidized mediator after reduced by the bacteria in this study).

2.4 Past research on mediator microbial fuel cells (MFCs)

Table 2-1 summarizes the development of microbial fuel cells employing different redox mediators and in combination with pure culture of bacteria from the 1960s to the present. The cell voltage and current (or current density) of the mediated MFCs varies with the substrate, inoculum, mediator, and the type of anode used. Based on the substrate used, the highest to the lowest current density generated in the MFCs were with carbohydrate, glucose, methane, and acetate as the substrate respectively (by considering only the current density that have been normalised to the surface area of the electrode). Among them, methane-powered MFC by Van Hees (1965) produced the highest open circuit potential (OCP) at 0.5-0.6 V, but at a low current density (i.e. 0.003 mA/cm^2). It appears that the research by Van Hees (1965) is the only study on combining a gaseous substrate and a mediator in MFC, but the current generated was very low, no effect on the voltage after the addition of an electron mediator, and the methods used was not explicitly explained.

This study is devoted to proof the electricity generation by combining a gaseous substrate with a mediator in an MFC. The advantages such as the improvement of the electron transfer onto the anode, and the ability to use a redox mediator for many times (recyclability) thus does not create environmental problem and reduce cost, allowing us to propose a possible integration of MFC into an anaerobic biofiltration system.

2.4.1 Introduction to fuel cells

Fuel cells are considered as an appropriate alternative over conventional power generation equipments and storage devices. Fuel cells have wide fields of promising applications, extending from small electronic devices such as mobile phones and laptops, to large fuel cell systems connected the electric grid. They also attract great attention due to the direct conversion of chemical energy contained in the fuels to electrical energy. On the contrary, the conventional power plants convert chemical energy in the fuels into mechanical energy (in the heat engine), and then to electrical energy (in the generator). Additionally, fuel cells give the possibility of reducing emissions and less-noise operation than conventional alternatives. Unlike batteries, fuel cells will perform a continuous operation as long as all reactants are supplied and do not need to be replaced or recharged after exhaustion of the reactants.

Table 2-1 MFCs utilizing mediators for coupling electron transfer process at the anode(Katz et al., 2003 with modification)[a].

Microorganism	Nutritional substrate and Anode[c]	Mediator	Cell voltage	Current or current density	Reference
Pseudomonas methanica	CH_4 Pt black, 12.6 cm^2	1-naphtol 2-sulphonate indo 2, 6-dichlorophenol	0.5-0.6V (oc)[d]	0.003mA/cm^2(at 0.35V)	(Van Hees, 1965)
Escherichia coli	Glucose Pt, 390 cm^2	Methylene blue	0.625V (oc)	-	(Davis & Yarbrough, 1962)
Proteus vulgaris *Bacillus subtilis* *Escherichia coli*	Glucose Reticulated vitreous carbon 800 cm^2	Thionine	0.64V (oc)	0.8 mA(at 560Ω)	(Delaney et al., 1984)
Proteus vulgaris	Glucose Reticulated vitreous carbon 800 cm^2	Thionine	0.35V (at 100Ω)[b]	3.5 mA(at 100Ω)	(Thurston et al., 1985)
Proteus vulgaris	Sucrose Carbon	Thionine	0.35V (at 100Ω)[b]	350 mA(at 100Ω)	(Bennetto et al., 1985)
Escherichia coli	Glucose -	Thionine	0.39V (at 560Ω)[b]	0.7 mA(at 560Ω)	(Lithgow et al., 1986)
Lactobacillus plantarum	Glucose	Fe(Tender et al.) EDTA	0.2V (oc)	0.09mA(at 560Ω)[b]	(Vega & Fernández, 1987)

Erwinia dissolvens	Glucose Graphite felt 1 g (0.47 m^2 g^{-1})	Fe(Tender et al.) EDTA	0.5V (oc)	0.7 mA(at 560Ω)	(Vega & Fernández, 1987)-
Proteus vulgaris	Glucose	2-hydroxy- 1,4-naphtoquinone	0.75V (oc)	0.45 mA(at 1kΩ)	(Park et al., 1997)
Escherichia coli	Acetate Graphite 100 cm^2	Neutral red	0.25V (oc)	0.001mA/cm^2(sc)[c]	(Park & Zeikus, 2000)
Escherichia coli	Glucose Graphite felt 1 g (0.47 m^2 g^{-1})	Neutral red	0.85V (oc)	17.7 mA (sc)	(Park & Zeikus, 2000)
Escherichia coli	Glucose Glassy carbon 12.5 cm^2	2-hydroxy- 1,4-naphtoquinone	0.53V (at 10kΩ)	0.18 mA/cm^2(sc)	(Park et al., 2000)
Escherichia coli	Carbohydrate Carbon cloth	Methylene blue	0.3V (oc)	2.1mA/cm^2(sc)	(Scott & Murano, 2007)
Escherichia coli	Carbohydrate Carbon cloth	2-hydroxy- 1,4-naphtoquinone	0.3V (oc)	0.4mA/cm^2(sc)	(Scott & Murano, 2007)
Enterobacter cloacae	Glucose graphite rod and plate	Methylene blue Methyl viologen	0.37V (oc) 0.4V (oc)	0.003mA/cm^2 -	(Mohan et al., 2008)
Escherichia coli	Glucose plain graphite anode	Methylene blue Neutral red	0.48V (oc) 0.6V (oc)	-	(Sharma, 2008)

aIn most studies the biofuel anode was conjugated with an O_2 cathode.
bThe value calculated from other data using Ohm's law.
cThe anode surface is given as a geometrical surface.
dOpen-circuit measurements.
eShort-circuit measurements.

The most employed fuel today in fuel cells is hydrogen, with occasionally used hydrocarbon and alcohols as the fuel. In a polymer electrolyte membrane fuel cell (PEMFC), two protons and two electrons released at the anode are resulted from the oxidation of one molecule of hydrogen. These protons diffuse through the membrane to the cathode, and an external electric circuit connecting the anode and the cathode are transported the generated electrons due to restricted travel through the electrically insulating membrane. The final product of a hydrogen-fueled PEMFC at the cathode is water, which is produced from the reaction between the terminal oxidant i.e. oxygen, and protons and electrons from the anode (Figure 2-9).The reactions at the anode, the cathode, and the total reaction are as follows:

Anode : $\mathbf{H_2 \rightarrow 2\ H^+ + 2e^-}$ [5]

Cathode : $\mathbf{O_2 + 4\ H^+ + 4e^- \rightarrow 2\ H_2O}$ [6]

Total cell reaction : $\mathbf{H_2 + ½\ O_2 \rightarrow H_2O}$ [7]

This process produces a steady current through the wire connecting the anode and cathode. This electrical energy can be harnessed and made to do work.

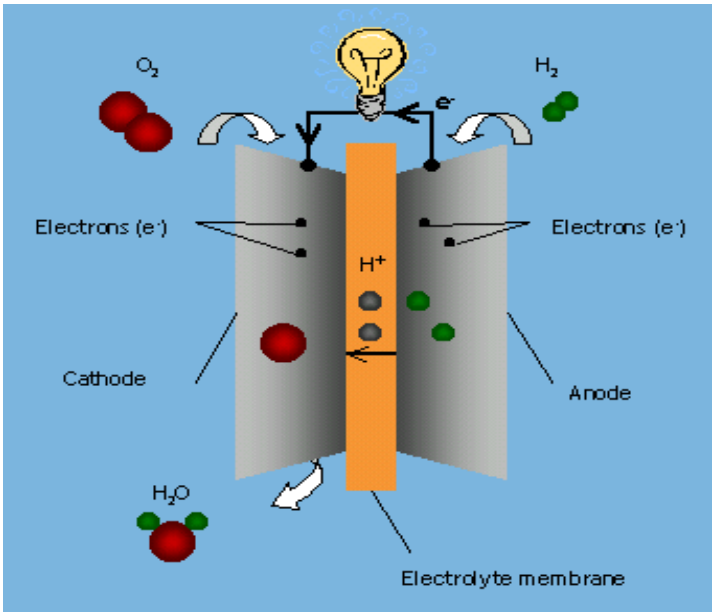

Figure 2-9 Principles of electron transfer in PEMFC(“Fuel cells principle”, 2006)

There are several categories of (chemical) fuel cells, and each of them has its own operational characteristics and applications (Table 2-2). Polymer electrolyte membrane fuel cells (PEMFC), alkaline fuel cells (AFC), phosphoric acid fuel cells (PAFC), molten carbonate fuel cells (MCFC), and solid oxide fuel cells (SOFC) are examples of the most commonly used fuel cells. Two key differences that distinguish each technology to the other are the electrolyte and the operating temperature. This means that there are different ions flowing through the electrolyte. However, for the purpose of this study, low temperature fuel cells are more attractive and relevant because the high temperature energy generation in natural biological system does not take place.

The hydrogen-operated proton exchange membrane fuel cell is quite famous because the operating temperature is below 100 ^{o}C. This type of fuel cell usually uses a platinum electrode/catalyst, and it has a high energy density. A power density of more than 3.5 kW/m^2 has been achieved by Ballard's developed Mark 5E cells (Hoogers, 2003). However, platinum is a very expensive metal, and the catalytic site can be permanently blocked by CO which adds problems in using fuel-reformed hydrogen (Hoogers, 2003). Another problem identified is the extensive machining of graphite bipolar plate to optimise the diffusion of reactants and products, leading to a high manufacturing cost. Increasing concerns about safety issues such as hydrogen lower flash point (i.e. –253 ^{o}C), and a low volumetric energy density (causing an ineffective hydrogen storage), which make it still incomparable to gasoline. For a similar energy content as gasoline, it needs a much larger/heavier vessel volume (Wallace & Ward, 1983).

Table 2-2 Common types of fuel cells (Larminie & Dicks, 2000).

Fuel Cell Type	Mobile ion	Operating Temperature
Polymer Electrolyte (PEMFC)	H^+	30-100^{o}C
Direct Methanol (DMFC)	H^+	20-90^{o}C
Alkaline (AFC)	OH^-	50-200^{o}C
Phosphoric Acid (PAFC)	H^+	~220^{o}C
Molten Carbonate (MCFC)	CO_3^{2-}	~650^{o}C
Solid Oxide (SOFC)	O^{2-}	500-1000^{o}C

Due to the difficulties with hydrogen fuel cells, direct alcohols fuel cells (i.e. methanol, ethanol) have attracted intensive research worldwide (Antolini, 2007; Baglio et al., 2006; Wasmus & Kuver, 1999). However, although they have been used for portable power supplies, there are still some problems. For example, methanol is highly toxic and could lead to long-term environmental problems because methanol is so miscible in water (Lamy et al., 2004). Other alcoholic fuel cells i.e. ethanol, have also been investigated. The problem encountered with ethanol fuel cells (in comparison to methanol fuel cells) is that the cleavage of the C–C bond is difficult with traditional Pt-based catalysts. As a result, there is an incomplete oxidation of ethanol, due to inefficient catalysation of the oxidation of ethanol by precious Pt-based catalysts.

Living organisms are capable to efficiently catalyse the oxidation of many organic substrates (such as alcohols) at ambient temperatures. Over more than four decades, living organisms and enzymes have been shown to generate electrical energy in fuel cells. This type of energy conversion in a fuel cell is referred to as a biofuel cell. There are two types of biofuel cells (biological fuel cells): enzymatic biofuel cell (EFC), and microbial fuel cell (MFC) which is focus of this study. Basically, biofuel cells share many similarities with chemical fuel cells. The fuel is oxidized in an oxygen-free chamber, but rather than a metal catalyst, biocatalysts such as enzymes for EFC or living cells for MFC are used to catalyse fuel oxidation. The advantages of using biocatalysts in a fuel cell are that they can be operated in mild conditions i.e. ambient temperature and pressure, are considered inexpensive, and offer a broad choice of fuels.

To date, the power density (power per unit electrode area) of biofuel cells is still much lower compared to chemical fuel cells. The power density achieved in MFC from a maximum only 12.2 mW/m^2 (Liu et al., 2004) to as high as 1640 mW/m^2 (Cheng & Logan, 2007) has been reported. For EFC, the highest reported power density is $5x10^4$ mW/m^2. Similar research group reported a power density of $1.6\text{-}2x10^4$ mW/m^2 (Akers et al., 2005). Hydrogen fuel cells have produced 2.26 kW/m^2 and 1.00 kW/m^2 for that operates on steam-reformed methanol and ethanol respectively (Uda et al., 2006). However, like other electrochemical cells, there are various factors which can be optimised and thus can affect the power output. These can range from the types of substrate to the design of apparatuses used.

EFC has been applied largely as electrochemical biosensors (Palecek et al., 2006). The most commonly used enzymes in EFC are glucose-oxidase and dehydrogenases (Palmore et al., 1998; Pizzariello et al., 2002). A typical EFC has a lifetime ranged from 8 hours to 7 days compared to a range of 20 hours to 3 – 5 years in MFC (Topcagic & Minteer, 2006). Chaudhuri and Lovly (2003) have demonstrated an EFC of greater than 40 days lifetime. Therefore due to the short lifetime of the biocatalyst, and also the high costs involved in enzyme production, EFCs are only suitable to miniaturise small-scale applications. For these considerations, MFC technology favored over EFCsto generate electricity from biodegradation organic materials.

2.4.2 Microbial fuel cells (MFCs)

Several types, construction materials and evaluation of the MFC performance are described below.

2.4.2.1 Physical construction of microbial fuel cell

Until now, several different designs of MFCs have been constructed including the common single chambered or double chambered MFCs.

A). **Double chambered MFC** (H type) has two compartments, the anode and the cathode (Figure 2-10a). An anode, containing the biocatalyst, growth medium and organic substrate is placed in one compartment. A cathode is located in the other compartment, which usually contains an oxidant. A proton exchange membrane (PEM) or a salt bridge is commonly placed between these two compartments for the transfer of protons (Oh et al., 2004).

B). **Single chambered MFC,** which is presented in Figure 2-10b, only has one compartment for both the anode and cathode, with the cathode direcly expose to the air which omits the requirement of very intensive energy air sparging of the liquid (Liu & Logan, 2004). The anode can be located away from the cathode or close to the cathode with the PEM between them. The same electrolytes as in the two chambered systems are also contained in the anode compartment. The single chambered MFC usually produces higher power density than the double chambered MFC, and also does not require aeration of the cathode chamber as in the double chambered type.

C). Other most popular types of MFCs that have been designed including **upflow** (He et al., 2005), **tubular** (Rabaey et al., 2005), and **flat** (Min & Logan, 2004) designs (Figure 2-10c, 2-10d, and 2-10e).

Literatures showed that researchers have well-tested nearly all of these MFC configurations in a laboratory scale utilising few different variables e.g. high substrate concentration (Liu et al., 2005; Park & Zeikus, 2002). In some studies, connecting the MFC reactor in series to improve the generation of voltage has also been reported (Aelterman et al., 2006).

A microbial fuel cell can be designed with a PEM or without PEM (PEM-less). The PEM in a MFC is not only aimed to provide a way for protons to move into the cathode, but also to avoid oxygen from entering the anode compartment which was proved lowering the coulombic effiency of the system (Wen et al., 2010). Mixing of the MFC contents of the both compartments could also be prevented by using a PEM. In a PEM-less MFCs (such as sediment MFCs and air-cathode MFCs), a PEM is not required to separate the catholyte from the anolyte. For example in sediment MFCs, the anaerobic anode is located in sea sediment and the cathode relies on oxygen above the water surface (Figure 2-11). The anode colonizing microbes oxidize organic compounds in the sediment, and then electrons travel through a circuit to the cathode where they are combined with protons that carry out the oxygen reduction reaction. This design is simple and does not need a PEM due to decreasing oxygen content from the surface to the sediment.

Most of the reported literatures use Nafion ® (sulphonated fluoro-polymer cation exchange membrane) as the PEM, but this membrane is very expensive. Several studies have compared cation-exchange, anion-exchange, and ultrafiltration membranes to determine their effects on MFCs performance (Kim *et al*. (2007). In one study, a significant increase in power density observed with the removal of PEM, but with low coulombic efficiency (Liu & Logan, 2004).

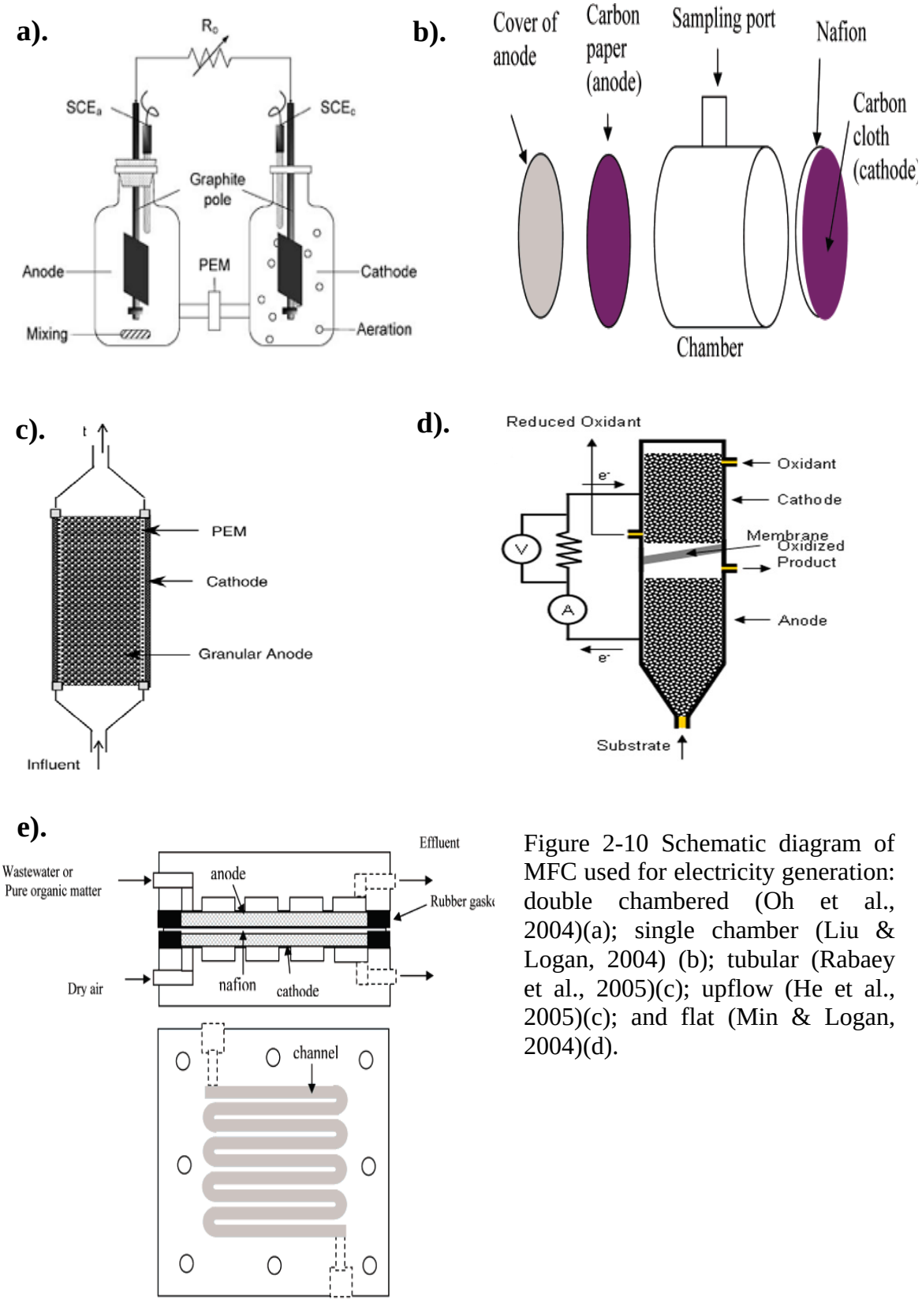

Figure 2-10 Schematic diagram of MFC used for electricity generation: double chambered (Oh et al., 2004)(a); single chamber (Liu & Logan, 2004) (b); tubular (Rabaey et al., 2005)(c); upflow (He et al., 2005)(c); and flat (Min & Logan, 2004)(d).

For this study, the focus was on proving the concept of electricity generation using the combination of gaseous substrates with mediators in MFC. To achieve this, the fuel cell configuration chosen was the simple double chambered MFC as it is widely used and inexpensive for fundamental studies, for example to study new substrates or new microbial species for generating power (Singh et al., 2010). Additionally, since the work was only carried out in a laboratory scale, therefore Nafion was used to pass through the protons to the cathode in this study.

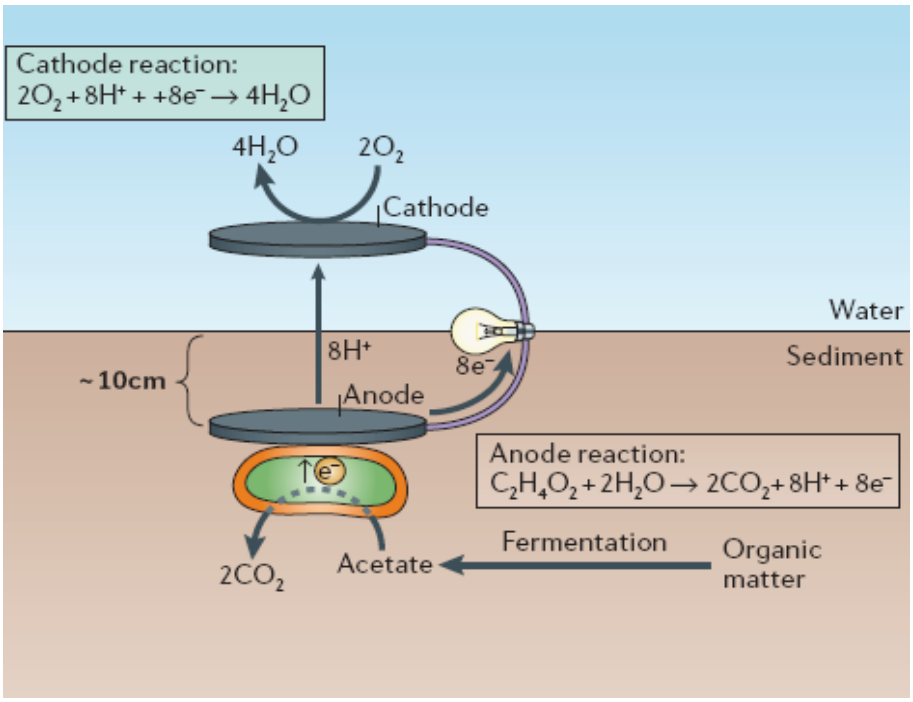

Figure 2-11 A schematic of a sediment microbial fuel cell (Lovley, 2006).

2.4.2.2 Anode and cathode materials and catalysts

Choice of construction materials, the structure of anodes and cathodes and PEM can affect MFC's performance. Furthermore, there are some requirements for the electrodes in order to achieve a successful application of MFC technology at large scales, such as they should have high conductivity and high active surface area, good chemical stability and biocompatibility, and more importantly low cost (Chaudhuri & Lovley, 2003; Logan et al., 2006). Materials made of carbon i.e. graphite, carbon paper, carbon cloth, and reticulated vitreous carbon (RVC) are normally used for the anode and cathode, with graphites are the most common (Kim et al., 2007; Logan & Regan, 2006). One

additional feature for the cathodes is that precious metals (i.e. Pt) is also added in the materials to catalyse oxygen reduction reaction (Cheng et al., 2006). Freguia et al. (2007) have shown that pyrolyzed iron phthalocyanine (FePc) and cobalt tetramethoxyphenylporphyrin (CoTMPP) have a comparable performance with Pt-based catalyst. Using microorganisms in the cathode (biocathodes) has also been reported (He & Angenent, 2006). Several chemicals such as potassium ferricyanide (FR) or permanganate ($KmnO_4$) have been used as the terminal electron acceptors to replace Pt-based oxygen cathode, and it has been shown to enhance the power output by more than 1.5 times (Oh & Logan, 2006; Timmers et al., 2010). However, using FR or $KmnO_4$ is not sustainable because it need to be replaced after exhaustion (He & Angenent, 2006).

2.4.3 Evaluation of microbial fuel cells performance

Polarisation curves, electrode potentials and overpotentials, and coulombic and voltage efficiency are important characteristics to describe the performance of a microbial fuel cell.

2.4.3.1 Polarization curves

In the microbial fuel cell research, polarisation curves are diagrams which are commonly drawn to present the measured cell voltage as the currents (or current density) are produced (Figure 2-12). The current, *I* (A), can be calculated using Equation 8 from known external resistance, and then power, P (W), can be obtained using Equations 9 or 10. Power density (W/m^2) is the power normalised by anode volume or anode/cathode surface area, and is used for comparing different sized systems. However, care should be taken when comparing polarization curves since there are many different methods that can be used by researchers to obtain polarisation curves for a MFC.

$$I = E/R \quad [8]$$

$$P = IE \quad [9]$$

$$P = E^2/R \quad [10]$$

There are other useful values to report the performance of an MFC: open circuit potential (OCP), which is the cell voltage that is measured in the absence of current (infinite external resistance); short circuit current (SCC), which is the generated current when the external resistance is zero; and R_{int} or internal resistance of the system, which reflects the ability of ions to move through the solution from the anode to cathode and it can be obtained by estimating the slope of the polarisation curve. An MFC achieves a maximum power density when the external resistance is equal to the internal resistance.

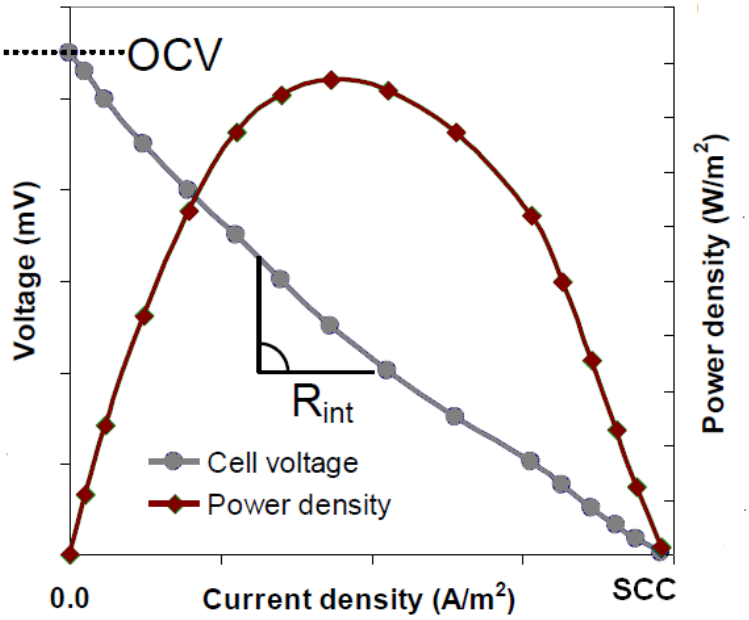

Figure 2-12 A typical polarisation and power curves used for evaluating electrochemical performance of an MFC.

A potentiostat or a variable external resistor is a common tool to obtain a polarisation curve. With a potentiostat, the polarisation curve obtained by sweeping the cell potential using LSV at a scan rate of 0.1 mV/s was an adequate method and similar to the values obtained using the external resistance method (Velasquez-Orta et al., 2009). Although in the beginning a scan rate of 1 mV/s was believed to be more accurate in determining the LSV polarisation curves (Logan et al., 2006), however it resulted in the overestimation of power from OCV to 0.1 V (Velasquez-Orta et al., 2009; Watson et al., 2011). This was thought due to there was not enough time for the bacteria to adjust to the environmental changes they experienced when the obtained polarisation curve

was completed in 30 minutes when using the 1 mV/s scan rate (Velasquez-Orta et al., 2009). The polarisation curve produced by CV method has been reported, but the effectiveness of this method for producing a polarisation curve is still questionable since no other methods were compared (Duteanu et al., 2010).

There are two types of measuring polarisation curves using variable resistances i.e. single cycle and multiple cycle method. In the single cycle, the resistance is varied from OCP in one batch cycle over a short period of time (Heilmann and Logan, 2006). This type has been reported using a range of external resistances from 5-5000 Ω with 15 minutes at each resistance (Zhuang et al., 2010), and using a range from infinite resistance (OCP) to 25 Ω with 20-minute intervals (Watson et al., 2011). The phenomena of power overshoot (a doubling back of the power density curve which is showed by the rapid fall in the cell voltage and current) was also reported in the latter study at resistances lower than 250 Ω. The former study, in which the power density curves produced from brewery wastewater as the substrate did not report this behavior (Zhuang et al., 2010). It was assumed that power overshoot took place since not all of the curves were shown. However, different curve shapes can be caused by a number of different factors such as different cathode construction and materials used.

The multiple cycle method is the single cycle procedure left at a fixed external resistance for a long enough time that steady state behaviour is found before the polarisation curve is taken, and different external resistance is applied for each new feed cycle (Fan et al., 2007; Watson et al., 2011; Zhang et al., 2010). This method is considered more accurate than the single cycle method (Watson et al., 2011). Researchers conclude that the disappearance of power overshoot in the MFC polarisation curves by using this method was due to sufficient time available (approximately 1-2 days) for the bacteria to adjust to a new resistance (Watson et al., 2011). One disadvantage of the multiple-cycle method in biofilm-growth MFCs is that there is a possibility of changes in the bacterial community over time due to the longer time requirement and due to an introduction of a new feed. As a result, there could be changes in the measured power production especially when using complex substrates (i.e. wastewater). Such behaviors should not be found in the combined gaseous

substrate-mediator MFC systems like in this study, therefore this would add one advantage.

2.4.3.2 Electrode kinetics and Butler-Volmer equation

The performance of an electrode reaction can be analysed using Tafel slope (Figure 2-13), in which the polarisation plots are corrected with $IR\Omega$ loss values *i.e.* *IR* - corrected voltages $(= E_{cell} + IR\Omega)$ and can be plotted against $\log_{10} i$ as described by:

$$\eta_{act} = b \log_{10}\left(\frac{i}{i_o}\right) \qquad [11]$$

where i is the current density and η_{act} is the activation overpotential. The term b is the Tafel slope (V), and i_o is the rate of oxidation or reduction of an electroactive species at an electrode at equilibrium, and is usually determined by linear regression at overpotential between 60 mV to 100 mV (Bard & Faulkner, 2001).

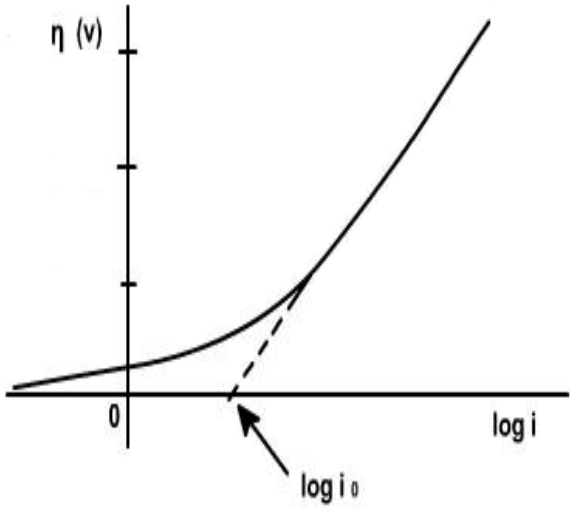

Figure 2-13 A typical Tafel plot for an electrode reaction with β = 0.5 (Zhao et al., 2009).

A high i_o means a fast reaction rate and low activation barrier, in contrast to small i_o for a slow reaction rate. Equation 11 is a simplification of butler-volmer equation in

Equation 12 which is a general representation of the polarisation of an electrode supporting one redox system (Roberge, 1999):

$$I = Ai_0 \left\{ e^{\left(-\frac{\beta nF\eta_{act,c}}{RT} \right)} - e^{\left(\frac{(1-\beta)nF_{act,a}}{RT} \right)} \right\} \quad [12]$$

where A is the surface active area of the electrode, β is the charge transfer barrier, n is the number of electrons participated in the reaction. The Butler-Volmer equation yields the Tafel equation since the first term in that equation becomes negligible in the high overpotential region (>118/n mV).

2.4.3.3 Electrode potentials and losses

The spontaneity of an electrochemical reaction can be calculated based on Gibbs free energy (Bard et al., 1985). This Gibbs free energy is proportional to the maximal work obtained from the reaction, which is equal to the change in Gibbs free energy (ΔG) or can be expressed as:

$$W_{\max} = E_{emf} Q = -nFE_{emf} = \Delta G \quad [13]$$

where W is maximum theoretical work, Q is charge transferred in the reaction I, F is Faraday constant (96,485 C/mol), n is number of electrons per mole product, and E_{emf} (V) is given by the difference of potential between the anode and the cathode (the overall cell electromotive force):

$$E_{emf} = E_{cathode} - E_{anode} \quad [14]$$

Rearranging Equation 13 gives:

$$E_{emf} = -\frac{\Delta G}{nF} \quad [15]$$

Under standard conditions, i.e. pH=0, all concentrations are 1 M and all gas pressures are 1 atm), the equation becomes:

$$E^o_{emf} = \frac{\Delta G^o}{nF} \quad [16]$$

where E^{o}_{emf} is standard cell electromotive force. The actual cell electromotive force of one reaction can be calculated from the standard cell electromotive force (based on Nernst equation):

$$\Delta E_{emf} = \Delta E^{o}_{emf} - \frac{RT}{nF}\ln(\Pi) \qquad [17]$$

where T (K) is the absolute temperature, R (8.314 kJ/mol.K) is the universal gas constant, Π (unitless) is described as the division of the product activities over the reactant activities. In a MFC, a positive value of ΔE_{emf} in Equation 17 shows that the reaction is thermodynamically possible and the electricity can be generated.

The E_{cell} is a maximum achievable MFC voltage lowered by various potential losses as described in Equation 18.

$$E_{cell} = E_{emf} - (\Sigma \eta_{\downarrow} a + \left|\Sigma \eta_{\downarrow} c\right| + IR_{\downarrow}\Omega) \qquad [18]$$

$\Sigma \eta a$ and $\left|\Sigma \eta c\right|$ are the losses associated to the anode and the cathode, and the summation of all ohmic losses related to the generated current (I) and ohmic resistance ($R\Omega$) is shown in $IR\Omega$.

Ohmic losses are the potential losses arise from resistivity of electrode materials, cation exchange membrane, and current collecting materials. The electrolyte also gives resistance and thus the loss. Minimal spacing of the electrodes, using low resistivity membranes, and improving the electrical conductivity of the electrode and the ionic conductiviy of the electrolyte are examples to reduce the ohmic losses.

The overpotentials at the anode and cathode (Figure 2-14) reflect the amount of energy lost at these electrodes and are strongly depend on current. In an MFC they can be classified as (Logan et al., 2006):

- **Activation overpotentials**: described as potential losses due to the activation energy of the electron transfer reaction on the electrode. Enlarging the surface area of electrode, enhancing catalytic activity of the electrode, the establishment of an enriched biofilm,and increasing the operating temperature may reduce the

activation losses (Singh et al., 2010). Addition of a mediator may also improve the electrode catalysis.

- **Concentration polarization**: These losses are also called mass transport losses, which are caused by the limitation of flux of reactants and products at electrode surface (Hoogers, 2003; Larminie & Dicks, 2000). This behaviors can be seen at high current densities, and can be reduced by increasing the electroactive species (such as a mediator) concentration and using high surface area electrode (more reaction sites).

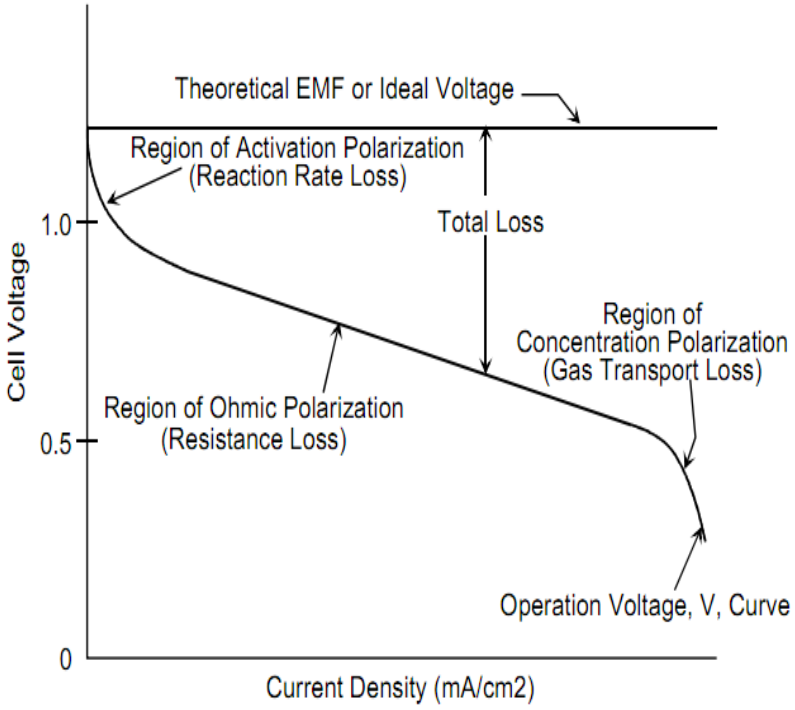

Figure 2-14 Polarization curve of a fuel cell (EG&G, 2000).

2.4.3.4 Coulombic and energy efficiency

These two parameters measure how efficiently the available fuel or substrate has been converted to electricity in the MFC. Coulombic efficiency is calculated as total charge produced from a substrate divided by the maximum possible charge production from the same substrate according to:

$$\varepsilon_c = \frac{\int_0^t Idt}{Fn\frac{\Delta S}{M}} \quad [19]$$

where ε_c is the coulombic efficiency of a MFC, I is the current flow during time 0 to t, ΔS is the changes of substrate concentration, and M is the molecular weight of substrate used.

Energy efficiency is defined as the ratio of the power that can actually be produced to the total energy that could be produced if the substrate were combusted:

$$\varepsilon_E = \frac{\int_0^t E_{cell} Idt}{\Delta H_c m_{in}} \quad [20]$$

where ε_E is the energy efficiency of a MFC, E is the cell voltage, m_{in} is the amount substrate used during time 0 to t, and ΔH_c is the heat of combustion of substrate.

2.4.4 Application of microbial fuel cell

2.4.4.1 Electricity production and biohydrogen

Clearly, the first application of MFCs is in the area of electricity generation. It has been shown that MFCs can produce electricity from the oxidation a wide variety of organic compounds (Hussain et al., 2011; Min et al., 2005; Ren et al., 2008); utilising different types of biocatalysts (Lovley, 2006; Zhao et al., 2009), employing various types of fuel cell designs (Liu & Logan, 2004), and using different kind of oxidants (Chen et al., 2008; Liu & Logan, 2004; You et al., 2006). Energy efficiencies of up to 97% have been achieved in MFCs, compared to only 70 % in small scale chemical fuel cells (Chaudhuri & Lovley, 2003; Rabaey et al., 2003).

Other than electricity, MFCs can also be made to produce biohydrogen. Under this condition, oxygen is not required at the cathode chamber as in usual MFCs and this reduces the possibility of oxygen penetration to the anode lowering the coulombic efficiency of the system. In this modified type of system, hydrogen is the only product

which is produced in the cathodic compartment as the result of the combination of protons and electrons in anaerobic conditions. However, a thermodynamic barrier was still reported and was thought to be overcome by adding external potentials (Sun et al., 2008). These systems have been achieved about two times higher of hydrogen production per mole degraded glucose than in conventional fermentation (Liu et al., 2008). According to Du et al. (2007), hydrogen generated also can be saved for future use to solve the low power outputs of the MFCs.

2.4.4.2 MFCs for wastewater treatment

In the past years, most of MFC research has focussed on the production of power from wastewaters. High organic contents in waste water such as derived from food processing, sanitary wastes, brewery wastes and corn stover, have made them as the potential organic sources for MFCs (Mathuriya & Sharma, 2010; Oh & Logan, 2005; Zuo et al., 2006). Liu et al. (2004) reported the capability of MFCs to remove chemical oxygen demand (COD) from wastewater with an efficiency value of 80%. Other than electricity generation and treatment of organic or inorganic compounds contained in wastewaters, the obvious benefits of using MFCs for wastewater treatment that it can have large savings on intensive aeration (if compared to aerobic treatment of wastewater), and less biomass handling (Logan, 2007).This is also combined with the possibility of methane recovery, although for this one does not draw much attention and is often flared (Ghangrekar & Shinde, 2006).

2.4.4.3 Sediment electricity

The first concept of sediment MFC was introduced by Reimers et al. (2001), with the preliminary test produced 15 mW/m^2. Sediment MFCs rely on the degradation of organic compounds by microbes in anoxic marine sediments combined with an oxygen reduction above the sea surface (Lowy & Tender, 2008). In 2002, large scale sediment MFCs, referred to as Benthic Unattended Generator or BUG MFCs was first reported to power electrical detectors (Tender et al., 2002). Six years later, a similar group reported that sediment MFCs has powered meteorological buoys, enabling the important measurements such as relative humidity and water temperature (Tender et al., 2008). These types of MFCs were able to run for few years without any drop in power outputs,

and have achieved maximum current and power densities up to 135 mA/m^2 and 32 mW/m^2, respectively (Bond & Lovley, 2002; Holmes et al., 2004; Lowy & Tender, 2008; Tender et al., 2002).

2.4.4.4 Biosensors

Biosensors have been suggested as one potential application of MFC technology. Biosensors have the advantage that bacteria could be easily immobilized. Thus makes this possible to test the presence of toxic compounds in river or wastewater, to detect biological oxygen demand (BOD) contents, and to be used as monitoring and controlling device (Lee et al., 2012; Peixoto et al., 2011). It has been reported that MFC-based BOD sensor had a stable operation for more than 5 years with minimal maintenance (Kim et al., 2003).

2.4.4.5 Other emerging opportunities

There is emerging interest for MFC use as power implanted medical devices due to the availability of glucose in the human body as the organic compound and a close relationship between human and microorganisms (Bettin, 2006). An implanted MFC device can have a long lasting operation since it relies on blood of a patient, and eliminate surgery needs for batteries (Franks & Nevin, 2010). An example of this type application of MFC is as an implantable device in large intestine which has been reported to give the maximum power density of 240 mW/m^2 (Du et al., 2011).

The possibilities to generate electricity in an MFC using carbohydrate from plants have also achieved attention among researchers. In microbial fuel cells with plants, plant rhizodeposites from plant roots (i.e. rich-organic compounds such as sugars and organic acids) can be fed to the microorganisms in MFC (Strik et al., 2011). An investigation on the plant saps as the carbon source has produced the highest efficiency of electrical conversion of 50% (Rabaey et al., 2005). All of these application opportunities reflect that MFC could have diverse possible applications for a simultaneous electricity generation as long as there is organic rich substrate as a source of food for microorganisms.

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3 ETHANOL OXIDATION IN MICROBIAL FUEL CELLS: COMPARISON OF VARIOUS MEDIATORS

3.1 Introduction

Ethanol is an alcohol that is produced by fermentation of a wide variety of biological materials such as corn, wheat, sugar beet and cane, barley, and wood. Ethanol production and use has increased dramatically since 1975 reaching 70,000 million litres in 2010 (Figure 3-1). 70% of world ethanol production is consumed in the fuel sector, while the remainder is used in industrial and beverage sectors (Tait, 2005).

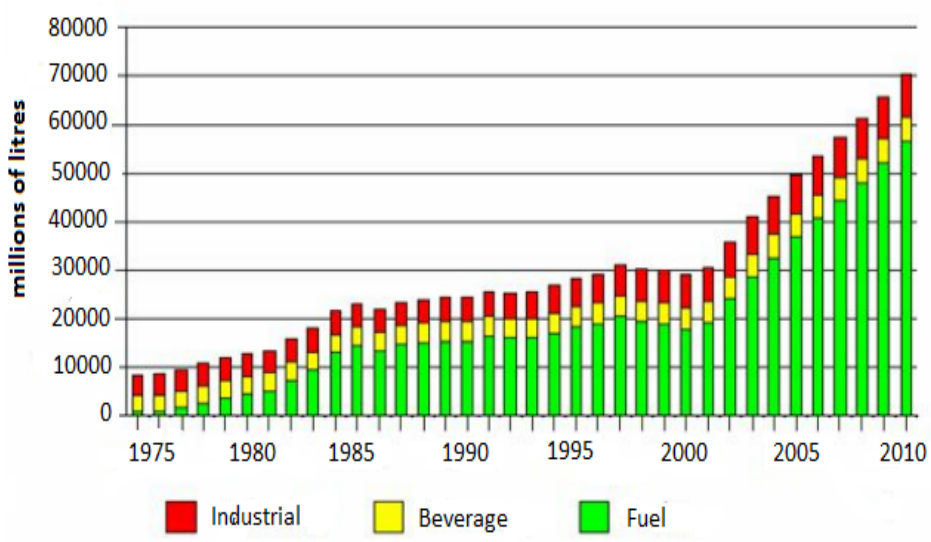

Figure 3-1 Worldwide ethanol production 1975-2010 (Tait, 2005).

One of the negative impacts of the huge increase in ethanol production and consumption is the release of ethanol emissions to the atmosphere. Ethanol and ethanol exhaust have been categorised as volatile organic compounds (VOCs) and they can create ground-level ozone (smog). The impacts of ground level ozone include inhibition to vegetation growth, and major cause of respiratory problems in humans. Moreover, ethanol plants are considered by USEPA as the major source of toxic hazardous air pollutants (HAPs). Exposure to these HAPs has resulted in a variety of health problems, such as cancer, throat irritation, and central nervous system damage (EPA, 2011).

According to NPI (2011), the five main sectors of ethanol emission are: industrial sector (e.g. bakery products and industrial machinery); diffusive sector (e.g. commercial solvents and aerosols), transport sector (evaporation of vehicle fuels or ethanol in the vehicle exhaust); natural sector (ethanol production from fungi and bacteria); and consumer sector (e.g. alcoholic beverages, household cleaners, and paint). The diverse range of ethanol emission sources and the negative impacts of ethanol on human health will inevitably require a breakthrough in technology to reduce these emissions.

Microbial fuel cell technology offers significant promise as an alternative power source due to its ability to generate electricity using microorganisms while reducing pollution. Microbial fuel cells can convert a great variety of organic substrates (such as ethanol) directly into electricity. Although the power density in microbial fuel cells is still limited, it has increased almost six orders of magnitude in the last ten years (Logan, 2010; Pant et al., 2010).

A redox mediator is a soluble molecule that enhances the electron shuttle from the bacteria to the anode in a MFC. The current production in MFCs (in the presence of a mediator):

[1] depend on the the mediator capability to approach the source of electron in the bacterial site and collect the electrons (Sund et al., 2007);

[2] depend on the mediator concentration to give high current density; and

[3] can be affected by the absorption reversibility of the mediator into the cells.

The choice of an appropriate mediator and its combination with other components in the microbial fuel cell may serve as the efficient microbial fuel cell operations with microorganism suspensions (Delaney et al., 1984).

As was discussed in Chapter 1, an externally supplied mediator cannot be easily applied in wastewater applications. This is because the toxic and the expensive mediator the mediator could accumulate in the discharged water, and separating the mediator from the solution is difficult. Based on those considerations, therefore MFC applications in

gaseous pollutant (e.g. ethanol) treatments with the addition of mediators look promising.

The electricity generation based on ethanol have been tested in enzyme-based fuel cells. However, enzymes have been long known for their instability in long term period (denaturation properties). Additionally, acetaldehyde and acetate were produced as a result of an incomplete oxidation of ethanol. There were several publications on pure ethanol or ethanol present in wastewater as the substrate in microbial fuel cells (MFC) (Cai et al., 2010; Kim et al., 2007) but not combined with a mediator. To the author's knowledge, the combination of ethanol and mediator in a MFC has not been investigated.

Acetic acid bacteria (commonly called AAB) usually metabolize sugar and alcoholised compounds (like ethanol), and produce acetic acid (Figure 3-2). Only *Acetobacter* strains such as *Acetobacter aceti*, *Acetobacter peroxydans* and *Acetobacter pasteurianum* which further metabolize this acetic acid through the tricarboxylic acid cycle to produce CO_2 and water (Asai, 1968; De Ley & Schell, 1959; Rao, 1957). AAB are Gram negative, having an ellips or a rod-shaped which appears as single, in pairs or in chains. They have flagella and vary between 0.4-1 μm long. Their optimum growth pH varies between pH 5 and pH 6.5, however these bacteria can also grow at lower pH values between pH 3 and pH 4 (Holt, 1994).

In Chapter 3 and 4 of this study, electricity generation in a MFC from ethanol as the biodegraded substrate is presented, utilising acetic acid bacteria (AAB) as the biocatalyst and various mediators in the anode. MFC parameters such as power and current density were investigated (Chapter 3). Previously, the isolation of the bacterial cells was done to select the bacteria that grew solely on ethanol as the substrate. This was followed by testing different mediators as the electron acceptor. Subsequently, the bacteria and the selected mediators were investigated for their ability in generating electrical current in the MFC. Kinetics of the microbial reduced mediator oxidation on the surface of two different anode electrodes were also studied and discussed in order to obtain a better understanding of the selected mediators (Chapter 4).

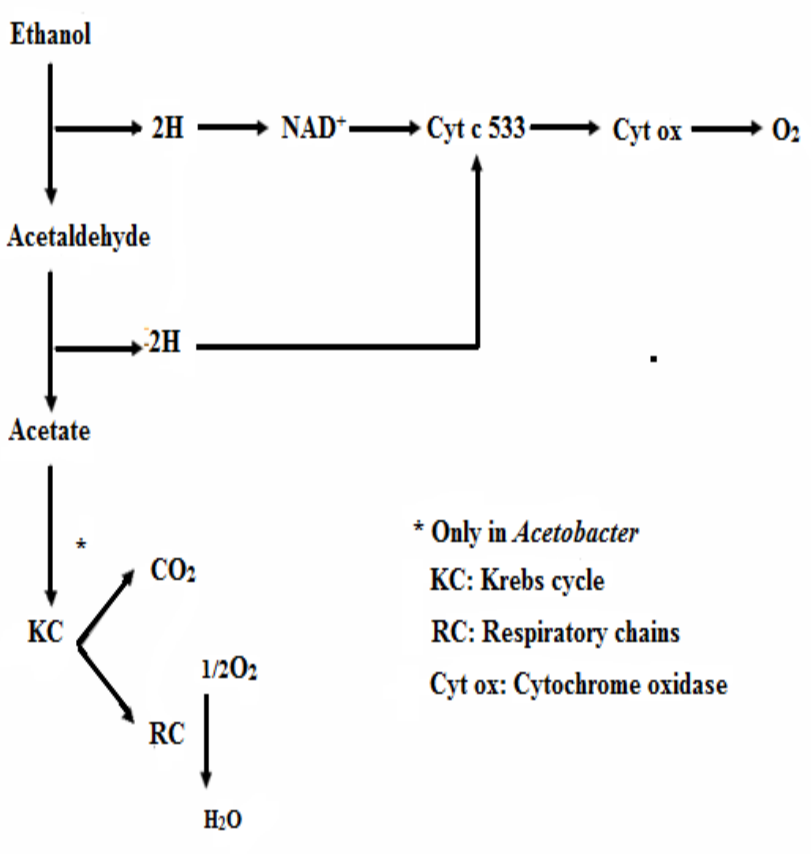

Figure 3-2 Ethanol oxidation by AAB (Benito, 2005).

3.2 Strategies to achieve a high performance in a mediated MFC

The open circuit voltage (OCV) in a MFC is the difference between the equilibrium potential of cathode and anode (E_o cathode and E_o anode) (Figure 3-3b). E_o anode in a mediated MFC is defined initially by the equilibrium potential of the oxidised mediator used (but finally by the equilibrium potential of the reduced mediator in the solution). At OCV, when no current being drawn from the MFC, the cell voltage is at a maximum. As current is produced, the cell voltage (ΔE) is determined by OCV lowered with overpotentials of the anode and cathode (activation and concentration), and ohmic losses (IR) of the fuel cell (Figure 3-3a).

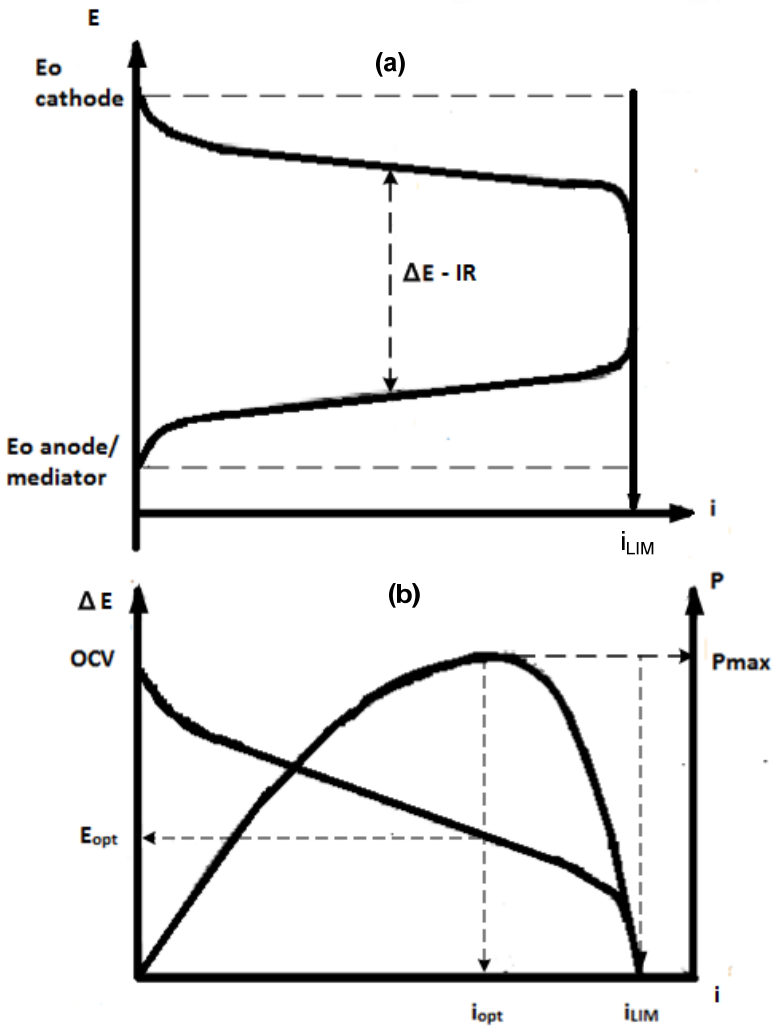

Figure 3-3 A schematic diagram of fuel cell performance obtained from a mediated MFC.

A polarization curve is drawn by plotting the cell voltage and power density as the function of the current density produced (Figure 3-3b). The power density is obtained by multiplying the cell voltage and the generated electrical current. The optimal cell voltage and current were derived from the point of maximum power density.

In a mediated MFCs, the power density decreases to a zero value because of two causes: first when mediator reaches its limited mass transport current ($i_{LIM\ A}$) at the anode surface; or second when catholyte also reaches the mass transfer limitation ($i_{LIM\ C}$) at the

cathode. Both can be seen by a steep increase of the anode potential or a steep decrease of the cathode potential in (Figure 3-3a) and both lead to concentration overpotentials.

To ensure a high performance of a mediated MFC, therefore one must:

1. Maximize cell voltage (ΔE), this can be obtained by:
   - choosing a mediator with a low redox potential (mediator b in Figure 3-4), and it has at least 50 mV potential difference between the mediator and the last redox enzyme inside bacterial cells (Barriere, 2010), but not too low that makes it hard to be reduced by bacteria.
   - choosing an oxidant which has a high redox potential value such as permanganate. However, in real applications, using permanganate is not sustainable and practical instead of oxygen cathode, therefore selection of a mediator plays a vital role in a mediated MFCs.
2. Maximize mass transport limiting current of the mediator (assuming cathode is not a limiting factor), this can be achieved by:
   - by using a high (and optimal) concentration of mediator which will give a high (limited) mass transport current, $i_{LIM\ A}$ (mediator c in Figure 3-4). Mediator a (Figure 3-4) has a lower redox potential compared to mediator c, but it reaches $i_{LIM\ A}$ faster.
3. Maximize exchange current (i_o) between mediator and the anode, thus minimize Tafel slope or charge resistance (generally is determined by η≥70 mV).
   - mediator reoxidation at the anode surface should be fast (high i_o), and this can be achieved by increasing the concentration of mediator and by improving the roughness (active site) of the anode electrode.

In practice, not all of these requirements could be achieved. Hence there is an inevitable trade-off between these factors in choosing the right mediator to obtain an enhanced performance in a mediated MFC.

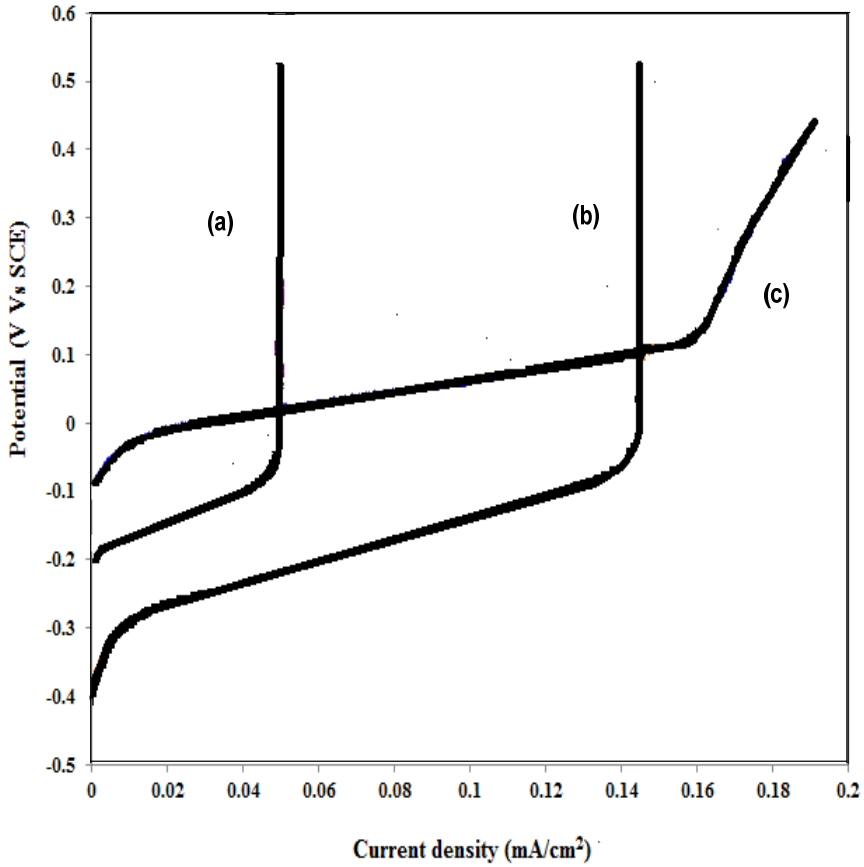

Figure 3-4 Anodic behaviors of several redox mediators as a function of current density (a, b, and c represent diffferent mediators).

3.3 Materials and methods

One test in this study was done in collaboration with another student, Yan Li (Figure 3-5).

3.3.1 Microorganisms and culturing

An AAB, which was identified as *Acinetobacter calcoaceticus* (Macrogen, Korea), was isolated from compost using medium enriched with ethanol as a sole carbon source. The medium had the following composition (per litre of deionised water): $NaNO_3$, 2 g; $MgSO_4 \cdot 7H_2O$, 0.4 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; $FeSO_4 \cdot 7H_20$, 0.003 g; KCl, 0.12 g; KH_2PO_4, 0.48 g, $Na_2HPO_4 \cdot 12H_2O$, 0.55 g, and EDTA disodium salt, 0.00186 g. 1 mL trace elements were also added (per litre): $MnCl_2 \cdot 4H_2O$, 0.02 mg; $ZnSO4 \cdot 7H_2O$, 0.07 mg; $NiCl_2 \cdot 6H_2O$, 0.02 mg; $CoCl_2 \cdot 6H_2O$, 0.1 mg; $CuCl_2$, 0.01 mg; $NaMoO_4 \cdot 2H_2O$, 0.03 mg;

H_3BO_3, 0.02 mg. The medium was sterilized by autoclaving. After cooling, 2.6% (v/v) of ethanol solution and 0.1 g of cycloheximide (to prevent the growth of protozoa) was added into the 1 L sterile medium, and the pH of medium was adjusted to pH 6.5 in all growth experiments.

A 250 mL Erlenmeyer flask was used to grow the aerobic batch cultures at room temperature. The incoming air passed through an initial flask containing a similar amount of ethanol to minimise ethanol evaporation in the growth flask (Eysseric et al., 1997). As the cultures became became visibly turbid for about 4 days, they were plated on Petri dishes and sub-culturing was done regularly until a pure culture was obtained (by observing visually and under microscope that gathered just single bacteria cells grew into a colony). Cell density was measured by optical density (600 nm) and dry cell weight. An optical density above 2 or a dry cell weight of 0.8-1 g/L was used for all MFC experiments.

3.3.2 Redox mediators

Some of the mediators i.e. methylene blue in the chloride salt form [MB], thionine acetate [TH], potassium ferricyanide [FR], 2,3,5,6-TMPD, and neutral red [NR] were purchased from Sigma, and some of them i.e. resorufin [RS], *N'N'N'N'*-TMPD [N-TMPD], toluidine-O blue [TOB], Prussian blue [PB], dichlorophenol indophenol [DCP], safranin_0 [SF], 9,10-anthra-quinone-2,6-disulfonic acid disodium salt [AQDS], and 2 hydroxy 1,4-naphtaquinone [HNQ] were kindly supplied by the Chemistry Department at the University of Canterbury. The stock solutions of the mediators were prepared by completely dissolving in deionized water, and the required mediator concentration were made by diluting the stock solution into the growth medium.

3.3.3 MFC system construction

A conventional two chamber MFCs separated by a proton exchange membrane (Nafion 212) was built (Oh & Logan, 2006). Short tests of current production (1 hr) were done in the MFC reactors with a chamber working volume and PEM cross-sectional area of 10 mL and 2 cm^2, respectively. A 24 hr current production test was done in reactor

chambers of 25 ml and PEM surface area of 4.15 cm^2. The PEM was pretreated by boiling in a 30% H_2O_2 solution and followed by boiling in 0.5 M H_2SO_4, each for 1 hr, and then stored in DI water prior to being used (Chae et al., 2008).

Glassy carbon (0.071 cm^2) and platinum were used as the working (WE) and the counter electrode (CE) respectively for the current production test. A graphite rod (1.1 cm^2) was also used as the WE and CE to compare the performance of the glassy carbon (GC) electrode. For MFC characterization using the linear sweep voltammetry (LSV) method, a carbon cloth electrode (CC) with surface area of 2 cm^2 was used as the WE. The CC electrode was bound to a graphite rod as the connector to a potentiostat. The distance between the electrodes in the anode and cathode chamber was approximately 7 cm. All the data reported in this study is with respect to a saturated calomel electrode (SCE).

3.3.4 MFC performance with mass transport limited current

In this step, the anode potential was set at 0.3 V more positive from the redox potential of the mediator by using a potentiostat (DY2100, Digi-Ivy. Inc) in the MFC experiment (Wagner et al., 2010). By setting the anode potential, the electron transfer rate was sufficiently enhanced that the net current was limited by the mass transfer of the reduced mediator to the electrode. Previously, the anode chamber was filled with the medium which was inoculated with a suspension of AAB bacteria, and then the mediator was added. A 900 mM phosphate buffer (pH 7) was used as the catholyte in the cathode compartment. The anode chamber was maintained anaerobic by gassing the compartment with 100% argon and a control (without mediator) for the MFC was performed. The same protocol was applied when the other mediators were tested.

A test of mediator dye absorption by the bacteria cells was further carried out after the three best performing mediators were identified. The absorption was investigated using a method described by Ganguli and Dunn (2009), in which the absorption was measured using spectrophotometer (Shimadzu type) and was correlated with cyclic voltammetry measurements. A theoretical coulombic efficiency of the MFC was calculated using Equation 19 in Chapter 2.

3.4 MFC characterization using LSV and VR

3.4.1 Polarization by linear sweep voltammetry (LSV)

A 50 mM FR in 1 M phosphate buffer (PBS) solution was used as the catholyte for the both methods, to minimise the differences in cathode polarisation (Oh et al., 2004). For LSV measurements, the anodic current (rate of the reduced mediator oxidation) was measured while the potential was increased at 0.1 mV/s from the measured open circuit potential (OCP) until the mass transfer limited current was reached using the potentiostat (Rabaey et al., 2006). The cathodic current was measured in similar way but in opposite directions. The difference in the anodic and cathodic polarization was used to generate the voltage and the power density curves.

3.4.2 Polarization by varied resistance (VR)

The voltage across the resistor was recorded using 20 min intervals (Heilmann & Logan, 2006). The MFCs were initially allowed to equilibrate after they were fed mediator, while monitoring the open circuit voltage (OCV) for one hour (minimal). Once the OCV stabilized, various external resistances (47 Ω - 9780 Ω) were applied across the cell and the voltage was measured and recorded using a digital multimeter. The current was then calculated from $i=E/R$, where E is the measured voltage across the cell, and R the external resistance. Power densities were calculated using $P= IE = E^2R$, and both were normalised to the projected anode surface area. By varying the external resistance (R) and calculating the power density, the internal resistance of the cells was determined, as was described elsewhere (Clauwaert et al., 2008; Ieropoulos et al., 2010).

3.4.3 Cyclic Voltammetry (CV)

In this study, CV was purposed to observe reversibility behaviour of the mediators, to investigate absorption of the dyes by the bacterial cell, and to find the diffusion coefficient of the mediator. A potentiostat and a 10 ml electrochemical cell containing a glassy carbon (GC) electrode (3 mm OD), a saturated calomel reference electrode, and a platinum counter electrode were used to perform the CV scans. The GC electrode was

activated by polishing with alumina slurry (0.3 μm and then 0.05 μm), and was subsequently sonicated in deionized water for 3 minutes prior to each use.

Standard curves for the CV experiments were generated by scanning the specific electrochemical window of the mediator versus a calomel reference electrode, where only peaks due to the oxidized and the reduced mediator were observed. A linear relationship was observed between peak currents and square root of scan rate after six scan rates i.e. 10, 20, 50, 100, 200 and 500 mV /s and several cycles were employed. This linear response reflected the diffusion controlled behaviour, and the slope of this line was used to calculate the diffusion coefficient of the mediator based on the Randles-Sevcik equation (Kissinger & Heineman, 1983).

3.5 Results and discussion

3.5.1 Mass transport limited current production

Addition of mediator could greatly enhance current density and power output in microbial fuel cells (Du et al., 2007). One of the ideal properties of mediator is that the mediator should have as high a concentration as possible to be able to give a high mass transport limited current. Figure 3-5a and 3-5b show how the mass transport limited current increased proportionally with TH concentration. The observation suggests that the higher concentration of TH added to the system, the more electrons were able to be generated and to be transferred to the anode (Rahimnejad, 2011). Therefore there is a need to find an optimum mediator concentration to have a high electron transfer rate, although according to Sugiura et al. (2011) there will be an optimum value of the mediator concentration which will be influenced by the organism concentration.

Materials and surface area of electrodes also determine the power output. Figure 3-5a and 3-5b show the difference of mass transport limited current produced with two different electrodes, i.e GC (0.071 cm^2 and a roughness factor of 28) and GR (1.1 cm^2). Generally, with the same concentration of TH used in the MFC (for example, at 0.05 mM and 1 mM), the current density produced using GR as the WE was double compared to the GC as the WE (4 to 8 $\mu M/cm^2$ and 9 to 21 $\mu M/cm^2$, respectively). Based on Randles-Sevcik equation and known quantity of the current generated, the

true surface area of the GC electrode was 2 cm^2, higher than the GR electrode but the current produced was lower. The higher current with GR was probably due to the high adsorption of the mediator onto the surface of the electrode. High adsorption usually occurs with graphite and carbon rod WE due to their microporosity, therefore GR WE is one of the most commonly used electrode to increase the power output in MFC (Kinoshita, 2001). However, according to Swades et al. (2003), the power output with GR was lower than with graphite felt due to the increasing surface area of graphite felt.

Figure 3-6 shows cyclic voltammograms (CVs) of several mediators in the growth medium (at concentration of 0.1 mM) using GC electrode which indicate reversible and irreversible behaviour of the mediators (V vs SCE). For each mediator's voltammogram, the potential was scanned three times between the upper and the lower limiting potential of 0.8 to -0.8 V at a sweep rate of 50 mV/s. Two of the mediators i.e. SF and DCP gave no signal (Figure 3-6c and 3-6d), while with FR (Figure 3-6a) and N-TMPD (Figure 3-6a and 3-6b) showing noticeable and significant peaks of the oxidation and reduction. Furthermore, the reversibility was recorded at about 0.2 V and 0.1 V for FR and N-TMPD respectively (Petrovic, 2000; Rogers et al., 2007). Figure 3-6a the voltammograms of FR mediator with the control (without added mediator).

The three mediators (FR, N-TMPD and TH) with the highest current density among all the mediators tested were selected after the short time electricity production using the various mediators in the anode versus control (Table 3-1). The control was the growth medium containing bacteria without any added mediator. One of the reasons for a lower current obtained for the other mediators was due to the irreversible behaviour of the mediators based on cyclic voltammetry results or an incomplete reduction of the mediators. Another reason for the lower current obtained by specific mediators such as MB, was because of the Gram-negative (the ethanol degrading) bacteria reacting with MB, forming a distinctive metallic green sheen (observed as sediment at the bottom of the MFC reactor) due to the metachromatic properties of the dye (Morata, 2006).

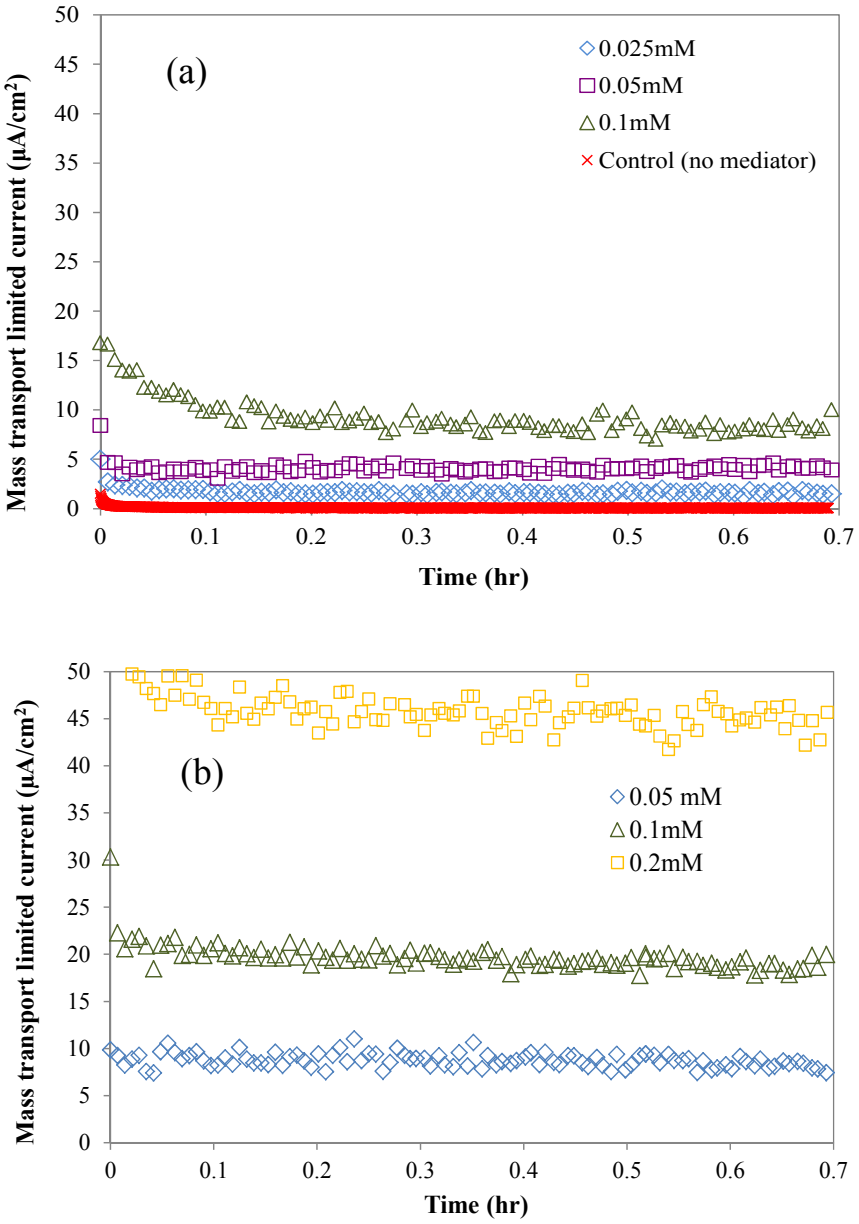

Figure 3-5 Mass transport limited current production with increased microbially reduced TH concentration using: (a) glassy carbon and; (b) graphite rod WE.

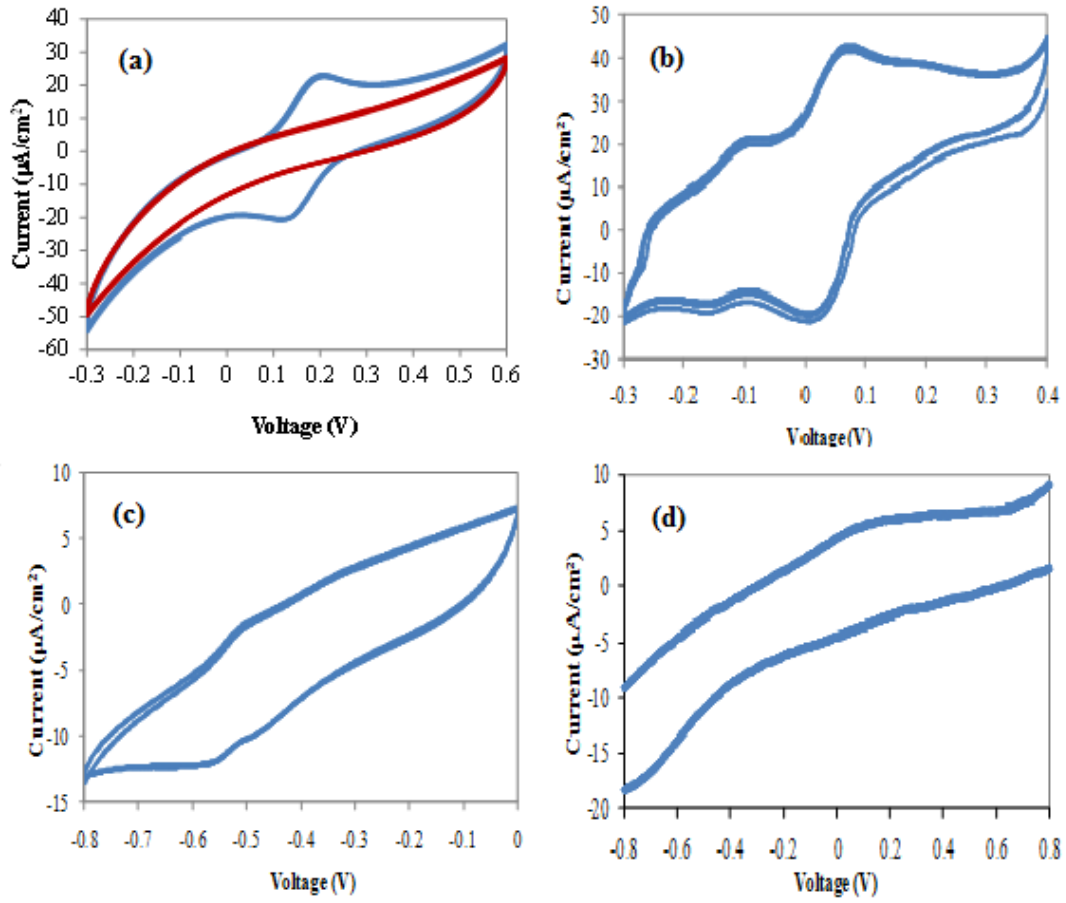

Figure 3-6 Cyclic voltammograms (CVs) of growth medium containing 0.1 mM redox mediator at sweep rate of 50 mV/s: (a) FR; (b) N-TMPD; (c) SF; (d) DCP.

Figure 3-7 shows mass transport limited current production for the three selected mediators over 24 hours. Generally, in all mediator MFC, the rapid decrease of the current in the first few hours was probably due to fouling of the anode by the bacteria decreasing the surface area of the electrode, even though it was then followed by a faradaic current (or a diffusion-controlled reaction) (Bard & Faulkner, 2001). The MFC with N-TMPD as the mediator generated the highest current density of 12 μA/cm^2 (stable after 4 hrs) compared to FR and TH, where the current densities decreased close to zero after 18 hrs of setting the anode potential at 0.3 V more positive than the redox potential of the mediators. The current density value was higher than the value obtained by Park and Zeikus (2000) using NR or TH as the mediators and glucose as the

substrate, which was 8.5 μA/cm^2, but still much lower than the value obtained by Kim et al. (2000) using TH and glucose which was 180 μA/cm^2.

Table 3-1 The electrical current production at half an hour for all the mediators tested (0.1mM of mediator concentration and DCW of 0.8 g/L) using GC electrode.

Mediators	Current production (μA/cm^2)
Control (without added mediator)	0.04
RS	0.9
TH	**7**
FR	15
MB	3.6
N-TMPD	25
TOB	4.4
PB	2.2
2,3,5,6-TMPD	6.9
NR, DCP, SF, AQDS, HNQ	Incomplete reduction or irreversible behaviours

Mediator absorption plays an important role in achieving high power densities in a MFC (Ganguli & Dunn, 2009). The absorption data (Table 3-2) correlated with the obtained current densities, where there was no absorption of the N-TMPD by the bacteria, while 43% and 50% of the TH and FR was absorbed, respectively. The high absorption occurred with FR and TH as the mediators decreasing the reduced mediator concentration thus lowering the currents obtained by the MFCs. The fouling of the anode could also cause the lower currents. Bai et al. (2006) observed FR fouling on a GC electrode surface, and they found a significant FR adsorption below 1 mM FR. In the case of TH, other phenomena observed such as adsorption on the electrode, glass surface and membrane which contributed to TH loss. This observation was supported by Kim et al. (2000) who found a lower cell voltage when using TH concentration

higher than 0.1 mM due to adsorption of TH on the electrode surface and the cell membrane.

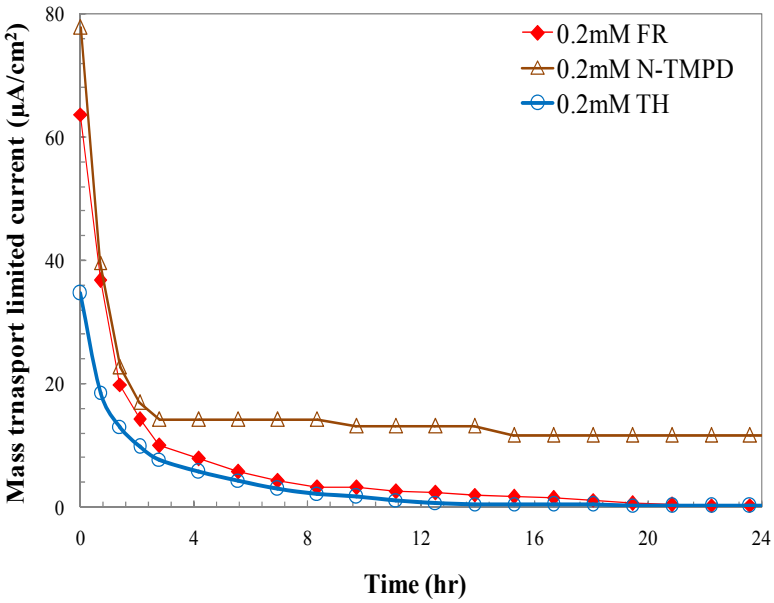

Figure 3-7 Test of current production using a glassly carbon WE for the degradation of ethanol in the presence of selected microbially reduced-mediators.

Table 3-2 Absorption test of 0.2 mM mediator by the bacterial cells (DCW of 1g/L) and current production after 24 hrs for the three selected mediators.

Mediators	Concentration left in supernatant (mM±SD)	Absorption (g/g)	Current production ($\mu A/cm^2$)
TH	0.115±0.003	0.024	0.11
FR	0.101±0.002	0.032	0.26
N-TMPD	0.200±0.004	0.000	12.0

The results in Figure 3-7 show that using N-TMPD as the mediator to generate electricity was more sustainable than the other mediators tested. Zhang et al. (2006) demonstrated N-TMPD as the suitable mediator in methanol biofuel cell because methanol dehydrogenase (MDH) has a PQQ (pyrroloquinolinequinone) as its prosthetic group, in which N-TMPD has a high electron transfer rate constant with the reduced

MDH enzymes (Marcus, 1965; Xia et al., 1996). AAB are known for its ability to oxidise various alcohols and thus can synthesize the enzymes (like MDH) with PQQ as cofactor (Yakushi & Matsushita, 2010). Moreover, the formal standard potential difference between N-TMPD (+0.037 V vs SCE at pH 7) and PQQ (-0.150 V vs SCE at pH 7) is closer if compared to FR (+0.116 V vs SCE at pH 7) (see Appendix 1). According to Marcus (1965), a high electron transfer rate can be obtained by using small redox (mediator) molecules. N-TMPD has lower molecular weight compared to FR and TH as the mediator (i.e. 164 g/mol to 329 and 287 g/mol, respectively).

Theoretically, coulombic efficiency calculated with N-TMPD mediator is very low i.e. 0.005% if all ethanol (starting concentration measured of 2.8 g/L) is consumed for the current production. This is because only a small surface area of electrode (0.071 cm^2) was used and only a short time of the current production. The decrease in the ethanol concentration will be difficult to measure in 24-hr test. However, in this test, ethanol depletion was detected at 1.82 g/L. This suggests that ethanol crossover the proton exchange membrane and/or ethanol evaporation around the lid could be contributed to the ethanol depletion in the system.

3.5.2 Polarization test (VR and LSV)

3.5.2.1 Linear sweep voltammetry (LSV) method

The N-TMPD MFC demonstrated the best performance compared to FR and TH MFCs, with the maximum power density of 0.093 $mW/cm^2 \pm 0.008$ using CC electrodes and LSV method (Figure 3-8a). The highest power density obtained was with 1 mMN-TMPD i.e. 0.16 $mW/cm^2 \pm 0.005$ (Figure 3-8b). In comparison to other MFCs that utilized ethanol as the substrate (but no mediator), the 0.16 mW/cm^2 power density was higher, compared to 0.049 mW/cm^2 and 0.002 mW/cm^2 with carbon paper and carbon felt anode respectively (Table 3-3). However different cathode materials or different oxidants were used in the MFCs making the comparison difficult. Nonetheless, these results look promising.

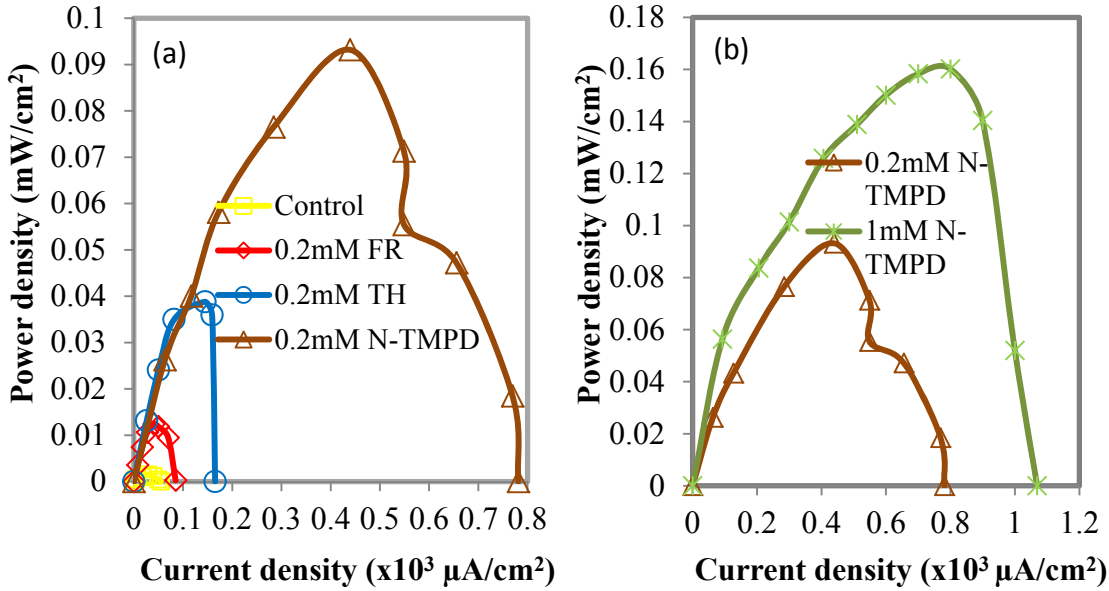

Figure 3-8 Power density curves derived from LSV method at mediator concentration of: a). 0.2 mM TH, FR and N-TMPD, b). 0.2 mM vs 1 mM N-TMPD.

Compared to MFCs that used a similar anode (carbon cloth), whether with or without mediator addition, only one demonstrated comparable power density to this study (i.e. 0.164 mW/cm^2 versus 0.16 mW/cm^2), but a single chamber MFC was used. The two-chambered MFC usually produce less power at a lower density than the one-chambered MFC (Nwogu, 2007). This work was lower than the one obtained by Zhang et al. (2006) which produced a higher power density (0.25 vs.0.16 mW/cm^2), but an enzyme was used as the biocatalyst instead of microorganisms, higher mediator concentration (5 mM), and permanganate in the cathode. Many other mediated MFC results available in the literatures, but since the reported power densities were not normalised by the surface area of the electrode or were reported with the power density normalised by the volume, this made comparing the results difficult.

In a MFC, OCV (a voltage of a MFC under no load condition) and the shape of a polarization curve (due to losses) are often compared (Larminie & Dicks, 2000). An activation polarization can be observed at low currents which are characterized by an sharp drop of the potential. Then, the ohmic behaviour is characterized by a linear fall in voltage as the current increases, and concentration losses are described by high voltage drop at high currents as a result of mass transfer limitations of chemical species

to the electrode (Logan, 2007). The results (Figure 3-9) suggests the highest OCV value (0.81 V) was obtained using 1 mM N-TMPD, almost comparable to the highest reported OCV value in literatures (i.e. 0.85 V) using 0.1 mM NR, graphite felt in the anode and the cathode and similar catholyte (Table 3-4) (Park & Zeikus, 2000). The shapes suggest that by using N-TMPD reduced the ohmic and the concentration losses allowing more electrons to be transferred to the electrode. The 1 mM N-NTMPD was potentially not the optimum concentration tested, therefore the MFC performance could be increased in order to have a high electron transfer rate or high limited mass transport current.

Table 3-3 Production of electricity from ethanol and non-ethanol (mediator or no mediator) MFCs.

MFC	Power density(mW/cm^2)	Cathode	Reference
Ethanol (carbon paper anode, no mediator)	0.049	Carbon paper	(Kim et al., 2007)
Ethanol containing wastewater (carbon felt anode, no mediator)	0.002	Pt in carbon on nickel foam	(Kazemi et al., 2010)
Wastewater (carbon cloth anode, no mediator)	0.077	Carbon cloth, aerated	(Cheng et al., 2006)
All carbon cloth anode: -Waste manure (no mediator) -*E. coli* (methylene blue, 1 M) -*E. coli* (HNQ, 1 M)	 0.0005 0.032 0.018	Carbon cloth, aerated	 (Scott & Murano, 2007) (Scott & Murano, 2007)
Marine sediment (carbon cloth anode, no mediator, 380 Ω)	0.002-0.003	Graphite cloth, oxygen	(Scott et al., 2008)
Domestic wastewater (carbon cloth anode, no mediator, 1 kΩ)	0.164	Air cathode	(Cheng & Logan, 2007)
Domestic wastewater (carbon cloth anode, no mediator)	0.066	-	(Xie et al., 2011)

Table 3-3 (continued)			
E. cloacae (graphite rod and plate): -methylene blue (0.03 mM)	0.00004	Phosphate buffer sol. (PBS)	(Mohan et al., 2008)
E. coli (plain graphite anode): - methylene blue (12 mM) - neutral red (0.1 mM)	0.015 0.039	Plain graphite anode	(Sharma, 2008)
MDH (graphite foil) - 5 mM N-TMPD	0.25	Graphite foil $KMnO_4$	(Zhang et al., 2006)
Ethanol-degrading bacteria All carbon cloth anode (LSV method): -1 mM N-TMPD -0.2mM N-TMPD	0.16±0.005 0.09±0.008	All used carbon cloth, 50mM FR	(This study 2011)

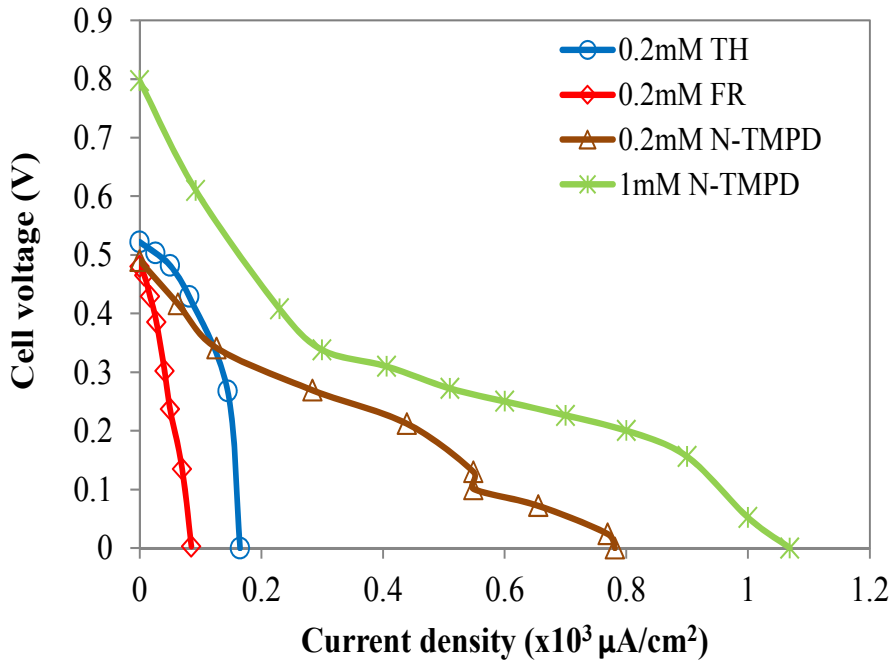

Figure 3-9 Cell voltage curves derived from LSV method.

Table 3-4 Open circuit potential (OCP) values obtained in mediator MFCs.

MFC	OCP (V)	Cathode	Reference
E. cloacae (graphite rod and plate): -methyl viologen (0.1 mM) -methylene blue (0.03 mM)	0.4 0.37	Phosphate buffer sol. (PBS)	(Mohan et al., 2008)
E. coli (graphite felt anode): -neutral red (0.1mM)	0.85	Graphite felt, 50mM FR	(Park & Zeikus, 2000)
All carbon cloth anode: -Waste manure (no mediator) -*E. coli* (methylene blue, 1 M) -*E. coli* (HNQ, 1 M)	0.41 0.3 0.26	Carbon cloth, aerated	(Scott & Murano, 2007) (Scott & Murano, 2007)
E. coli (plain graphite anode): - methylene blue (12 mM) - neutral red (0.1 mM)	0.48 0.6	plain graphite anode	(Sharma, 2008)
MDH (graphite foil): 5mM N-TMPD	1.4	Graphite foil $KMnO_4$	(Zhang et al., 2006)
Ethanol-degraded bacteria from soil, All carbon cloth anode: -LSVmethod (1mMN-TMPD) -LSVmethod (0.2mMN-TMPD) -VR method (0.2mMN-TMPD)	0.81 0.49 0.65 (oc)	All used carbon cloth, 50mM FR	(This study 2011)

3.5.2.2 Variable resistance (VR) method

In VR method, the MFC achieved the maximum power density of 0.078 mW/cm^2 when 0.2 mM N-TMPD was used (Figure 3-10). The LSV method results produced 14-17% greater power density than that obtained using the VR method, except for TH (higher by 30%). The obtained results were similar to previous reports in the literature which were ~20% greater for the LSV method (Kumar et al., 2011; Menicucci, 2005; Velasquez-Orta et al., 2009). However, none of those literatures used a redox mediator in the anode chamber and instead used a growing biofilm to facilitate the electron transfer, and no one has compared the power output using LSV and VR method in the presence of mediator. According to Ieropoulus et al. (2010), the most likely reason of the lower power density in the VR method (in the absence of mediator) was due to a slow

interaction between the microbes and anode as a result of adjustment to the new resistance. In this case, a mediator was used, so when the resistance was varied the voltage of the anode and the cathode changed and this possibly affected the interaction between the mediator and the anode (and thus how the microbes transferred the electrons to the mediator). Therefore, an experiment must be conducted to investigate the interaction of each selected mediator with the anode in order to obtain the best mediator for an MFC (Sund et al., 2007). The poorer interaction between the microbes, the mediator and the anode (in VR method) can be seen in the current density produced, for example when 0.2 mM N-TMPD was used, the current generated was half in VR method compared to LSV method (i.e. 0.23 $\mu A/cm^2$ compared to 0.45 $\mu A/cm^2$ at maximum power density, but higher than without mediator i.e. 0.003 $\mu A/cm^2$) which showed the limitation of the electrons being transferred to the anode. It seemed also that the 20 minutes of changing the resistance did not allow the microbes to obtain a true steady state. From the study, it was concluded that using 0.1 mV/s (LSV method) was more accurate to derive the electrochemical polarization curves.

The power outputs achieved in the MFCs with 0.2 mM TH and FR were less than the N-TMPD (0.027 and 0.011 mW/cm^2), probably with the similar reason mentioned previously for TH (i.e. adsorption on glass surface and cell membrane). Another possible reason (other than the one that previously explained in Section 3.4.1) of a very low power output in FR MFC was due to a high internal resistance measure for the system, i.e. 2170 Ω compare to 825 and 325 Ω in N-TMPD system and TH system, respectively. The maximum power is produced for the smallest internal resistance, therefore minimising the internal resistance is important in MFC construction (Logan 2007).

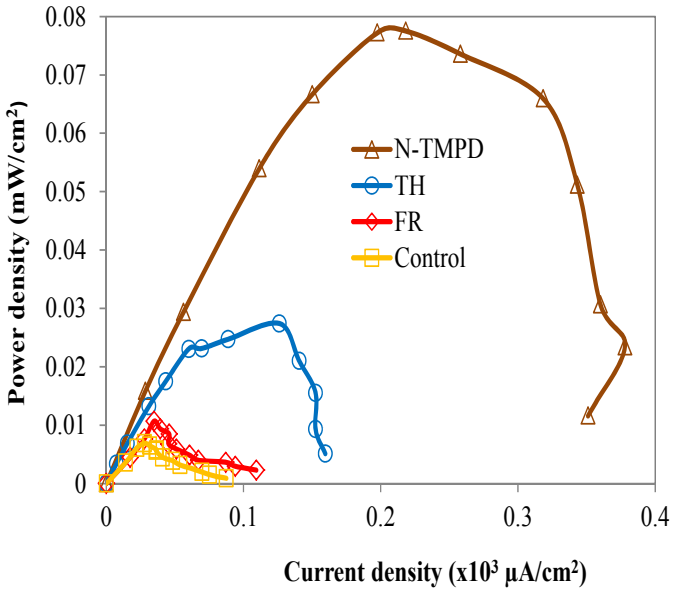

Figure 3-10 Power density curves derived from VR method at mediator concentration of 0.2 mM TH, FR and N-TMPD.

3.6 Conclusions

A conventional H-type microbial fuel cell (MFC) which utilized ethanol as the substrate was demonstrated utilising different electron acceptors (mediators) in the anode chamber. Three best performed mediators (i.e. N-TMPD, FR and TH) were selected prior the electrical current generation (using set the anode potential) and power density production (using linear sweep voltammetry and variable resistance methods) were evaluated.

The highest power production of 0.16 mW/cm^2±0.01 and the highest open circuit potential (OCP) of 0.81 V was obtained using 1mM of *N',N',N',N'*-TMPD and carbon cloth (CC) as the mediator and the electrodes respectively. The reason for the high power output with N-TMPD was probably due to the high electron transfer rate between N-TMPD with the enzymes synthesized with PQQ as cofactor by the ethanol-degrading bacteria (easier to get reduced), had a higher limited mass transport current, and limited absorption of the mediator have been observed. These results are a valuable and highly promising contribution to reduce ethanol contaminant and electricity production.

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4 KINETICS OF OXIDATION OF MICROBIALLY REDUCED MEDIATOR FOR AN ETHANOL FED MICROBIAL FUEL CELLS

4.1 Introduction

Increased population growth and economic development are leading to a rapid increase in energy demand. Fossil fuel-derived energy is limited in supply and will one day be depleted. Most of the current forms of energy production are not sustainable, and increasing potential threat of climate change and global warming indicate a need to develop renewable and carbon-neutral energy production. The development of bio-electrochemical reactors based on microbial fuel cells (MFCs) represents an attractive technology for generating electricity using a wide variety of substrates, varying from pure organic compounds to complex organic waste (Logan, 2004, 2005; Rabaey et al., 2005).

To date, MFCs still produce low power outputs (< 6 W/m^2 and/or ranging from 100-1000 W/m^3) that limit their use in real applications (Xing et al., 2008; Zhao et al., 2009; Zuo et al., 2008). The reason for this is because there are still many limitations imposed by the components involved in the MFC i.e. the microbial type, the anode, the cathode, the electrolyte, and the ion–exchange membrane (if used). Additionally, there are many factors that influence its performance such as temperature, pH, nutrients and fuel cell configuration. The scaling up of an MFC will require a better understanding of all components and conditions to enable detection of the bottlenecks and to improve power outputs.

One of the major limitations of the power output in MFC is the slow electron transfer (ET) from the microbes to the anode of the MFC. The slow electron transfer is due to a certain energy needed to activate the oxidation/reduction reaction on the electrode surface which causes a transfer resistance and hence potential losses (or often called activation polarization). Activation polarization occurs in both anode and cathode of

MFC (the cathode activation polarization will not be further discussed here). Activation polarization in the anode is an obstacle to energy production which is used to be overcome by several ways: by physical contact between the bacterial cell and the electrode surface (or biofilm establishment), by the diffusion of soluble mediator that shuttles electrons between the active site of redox enzymes in the cell and the electrode surface, or by conduction through molecular pili (nanowires) that channel cells to the electrode surface (Xie et al., 2010). In the previous chapter, it has been demonstrated the use of artificial mediators to improve the electron transfer from bacteria in ethanol fed microbial fuel cells. The addition of mediators in the MFC was purposely aimed to investigate the possibility of combining a gaseous substrate and a mediator to generate electricity, and thus investigating the potential integration of MFC into an anerobic biofiltration.

Exchange current density, i_o, (the value of current normalised by the surface area of the electrode at equilibrium) is a useful parameter to assess the kinetics of electrochemical reactions. According to Song & Zhang (2008), the exchange current density depends on several factors: the concentration of the electroactive chemical species at the anode surface; the reaction; and the surface of electrode where the reaction occurs.

Other than exchange current, electrokinetics is often reported in the term of rate constant, k_o (here is described as the heterogeneous rate constant since the exchange current is between the liquid phase to the solid phase or the electrode), and can be estimated from the exchange current value (Beriet & Pletcher, 1993):

$$i_o = k_o nFC \qquad [1]$$

where, i_o is the exchange current density (mA/cm^2)

k_o is the heterogeneous rate constant (cm/s)

n is the number of electrons transferred

F is Faraday constant (C/mol)

C is the concentration of the oxidised or reduced species, in this case the mediator concentration (mol/cm^3).

Glassy carbon (GC) is a carbon-based electrode and is one of the attractive electrodes to study electrokinetics because it is inexpensive, can be used over a wide potential range, and is also inert in most electrolytes (Sturm, 1988). These materials can easily be activated and maintained by carefully abrasion with emery paper, by polishing with alumina, and final cleaning by sonicating in water before each electrochemical measurement (Dekanski et al., 2001). The activation levels of a GC electrode can be seen by observing the difference in the peak potential for a redox couple e.g. usually close to 60 mV for ferri/ferrocyanide redox systems (Ranganathan et al., 1999). This technique is simple and allows the determination of the true surface area of the electrode surface (Bagotsky, 2006).

Carbon cloth (CC) is also a carbon-based electrode with relatively high surface areas. Many research groups have investigated carbon cloth as anode materials in microbial fuel cells to increase the power output per unit volume of reactor (Cheng & Logan, 2007; Liu et al., 2004; Liu et al., 2012; Scott et al., 2008). A few of them have studied the exchange current density between microbe and anode with carbon cloth electrode in order to compare its performance to the other carbon-based electrodes (Lowy & Tender, 2008; Scott et al., 2008; Scott et al., 2008). However, to the authors' knowledge, none of the kinetics-MFC studies have included redox mediator in the anode. This is because the use of redox mediator can create environmental problems thus does not attract considerable interest amongst researchers interested in liquid phase wastewater treatment using MFC technology.

The Butler–Volmer equation is a fundamental relationship in electrochemical kinetics (Equation 2):

$$i = i_o \left[exp \frac{\alpha_A z F \eta}{RT} - exp \frac{(-\alpha_C z F \eta)}{RT} \right] \quad [2]$$

The equation reflects that the exchange current density, the number electrons exchanged in the reaction, and the applied potential affect the generation of electrical current on an electrode. At high overpotential, η (V), the Butler-Volmer equation simplifies to the Tafel equation (Equation 3).

$$\eta_{act} = blog\frac{i}{i_0} \quad [3]$$

where b is the Tafel slope (V), and i is the applied current density (mA/cm^2). The exchange current density, i_0, is usually determined by linear regression at overpotentials, η, between of 60 mV to 100 mV (Bard & Faulkner, 2001; Lowy & Tender, 2008). This overpotential range allows the i_0 values to depend only on the charge transfer controlled electrochemical process, there is no influence of the mass transfer. The value of Tafel slope is given by the following equation:

$$b = \frac{2.303RT}{\alpha_{na}F} \quad [4]$$

where α is electron transfer coefficient, *na* is the number of electrons transferred, *T* is the absolute temperature (K) and *R* is the universal gas constant (J/mol K).

Tafel equation has been previously used to calculate the exchange currents or exchange current densities in anode or cathode of MFCs (Liu et al., 2007; Lowy & Tender, 2008; Manohar et al., 2008). It is used to extract information from reactions which are essentially activation controlled (i.e. charge controlled reactions) by sweeping the potential using LSV method. This technique has allowed the determination of the kinetic parameters such as i_o for individual reaction steps, and consequently, the analysis and comparison of different anode/cathode materials or different catalysts (Freguia et al., 2007; Scott et al., 2008).

Cyclic voltammetry (CV) can also be used to describe thermodynamics of redox processes and the kinetics of electron transfer reactions. It is basically similar to LSV, but further scan of potential (in reverse direction) is taken back to its original value, and the scan can be continued to the forward scan again to make a few repeated cycles. CV Specifically, it gives fast determination of redox potential (E^o) of the analyte, which provides information about the cathodic and anodic potential (E_{pc}; E_{pa}) and the cathodic and the anodic peak current (Ip_a/Ip_c). From these, separation between peak potentials (ΔE_p), the shift of E_{pc}; E_{pa} values during the timescale of experiments; the ratio of peak currents (Ip_a/Ip_c), current as a function of scan rate (Ip vs $\nu^{1/2}$) are obtained, and can be used to determine the electrochemical reversibility of a redox couple (i.e. mediator).

Particularly, ΔE_p can also be used to calculate the heterogeneous rate constant or k^o (thus the exchange current) for the reaction of a redox process.

This work is devoted to the investigation of the rate of mediator reoxidation on GC and CC electrodes in the ethanol-mediator MFC. The estimation of the kinetic parameters of the mediator oxidation along with analysing the power density curves allow us to better understand the behaviour of the selected mediators.

4.2 Experimental

A double-chambered microbial fuel cell was made as previously described (Oh & Logan, 2006). Both of the anode and cathode compartments had a total volume of 25mL and were separated by a proton exchange membrane (Nafion 212) with the surface area of 4.15 cm^2. The anodic compartment was inoculated with suspension of acetic acid bacteria into the medium (previously described in Chapter 3) containing 2.6% (v/v) ethanol. Argon was continuously sparged in the anodic compartment to maintain anoxic conditions. The mediator then was added to the growth medium as the electron acceptor to a concentration of 0.2 and/or 1mM. The LSV and CV scan was started after the mediator was fully reduced (as determined by observing when the coloured mediators became transparent). The cathodic compartment contained 50mM FR in 1M phosphate buffer (PBS) solution to avoid significant polarization at the cathode.

The anodes and the cathodes used in the kinetic study were either glassy carbon (0.071 cm^2) or carbon cloth (2 cm^2). All of the electrodes were pre-treated or were replaced with a new one (for CC) before use. GC was pretreated by polishing with 0.3 and 0.05 μm alumina slurry and successive washing until a mirror-like finish was obtained, followed by sonicating in deionized water for 3 minutes prior to use. A saturated calomel reference electrode (SCE) was used in the compartment of the electrode under investigation, and it is used as the reference electrode for all reported values in this study.

For LSV method, once a stable open circuit voltage was achieved, the potential of the electrode under investigation was scanned at $1x10^{-4}$ V/s in the anodic or cathodic direction until the mass transfer limited current was reached. Tafel parameters were derived from the polarization curves at overpotential regions of <0.2 V when the Tafel behaviours were observed. The power density data were obtained from the difference of the anodic and cathodic polarization curves. Meanwhile, for CV method, the voltammograms of bacterial reduced mediator (after a full reduction or colorless state of bacterial solution was observed), the potential of electrode was scanned at 0.01 V/s, 0.05 V/s and 0.1 V/s in the anodic and cathodic direction within the range of potentials where the redox molecule (mediator) peaks were seen. The important CV parameters were obtained from the analysis of the voltammograms of the mediator as the results of its oxidation and reduction on the electrode surface.

4.3 Results and discussions

4.3.1 Comparison of anode reactions

Tafel parameters for the selected mediators from LSV method for both electrodes were difficult to obtain (only 1 mM N-TMPD with carbon cloth electrode is presented and will be discussed later). This is because the concentration of mediators used in this study were rather low (0.2 and 1 mM) causing mass transfer limitations at fairly low overpotentials (~100 mV). This limits the overpotential range at which Tafel behaviour could be observed. Tafel behaviour should only be observed at overpotentials far enough from equilibrium (in this case OCV), or $\eta > 118$ mV/n, that one of the reaction directions (either anodic or cathodic) dominate the measured current, (Han et al., 1997; Kurasaki, 2004). For example, in the case of TH in which the number of electron transffered is 2, therefore the overpotential should exceed 59 mV and be free from mass transport effects. At higher overpotentials mass transfer will begin to control the current, at which point the current will reach a constant potential-independent value.

Figure 4-1 presents the current potential data of microbially reduced mediator for three selected mediators using GC electrode, which were used to extract CV parameters in

Table 4-1. The voltammograms with N-TMPD gives two reversible oxidation-reduction peaks according to the reactions in Equation 5 and 6 (in this case, the two oxidation peaks were observed at around 0.12 V and 0.47 V vs SCE, respectively) (Evans et al., 2005; Rogers et al., 2007). The first oxidation peak (E_{o_1} = 0.12) was similar to the value reported by Chaka & Bakac (2008). There were slight shifts of the values for FR and TH mediator i.e. around 0.18 V and -0.12 V compared to literatures of 0.19 V and -0.18 V for FR and TH respectively, but this might be due to the difference in the supporting electrolyte (Fox & Dulay, 1996; Kwong, 2004).

$$TMPD \longleftrightarrow TMPD^{+\bullet} + e^{-} \quad [5]$$

$$TMPD^{+\bullet} \longleftrightarrow TMPD^{2+} + e^{-} \quad [6]$$

For all the mediators used, it was obviously shown that the electroactive species i.e. the mediator were electrochemically active by cyclic voltammetry, in which there were oxidation and reduction peaks in the voltammograms observed. However, the peak separation (ΔE_p) values were much higher than 59/n mV FR, N-TMPD and TH, indicating the processes were all quasi reversible. ΔEp values greater than 0.059/n V is the most obvious indication that the process are not completely reversible anymore (quasi reversible) (Bard & Faulkner, 2001). This means that the redox reactions were not fast enough to maintain the concentration of the oxidised and the reduced mediator at the surface of the GC electrode as required by Nernst equation (Nicholson & Shain, 1964).

The presence of ethanol degrading in the solution modified the shape of voltammogram. Initially, with only the mediator presence in the growth solution, all of the three redox voltamograms showed reversibility behaviors e.g. ΔE_p values were around 59 mV and 30 mV for one and two electron transfer, respectively (some graphs are presented in Chapter 3). In this study, in the presence of bacteria, all the voltammograms indicated the quasi-reversible reactions. The voltammograms also show the larger peaks for reduction than for oxidation. During oxidation and reduction by cyclic voltammetry, it seems that the bacteria decreased the amount of the reduced and the oxidised mediator available at the surface of electrode, therefore, the level of the electrons being flowed

from the mediator to the electrode within the timeframe of the applied potential. The same behaviors were observed in the voltammetric interactions of different mediators with *Rhobobacter sphaeroides* (Agostiano et al., 2000).

Table 4-1 The potential and current values of three selected mediators derived from voltammograms in Figure 4-1.

FR (0.2 mM)							
$\nu x10^{-3}$ (V/s)	Ep_a (V)	Ep_c (V)	ΔE_p/n (V)	E_o (V)	$Ip_a x10^{-3}$ (mA/cm^2)	$Ip_c x10^{-3}$ (mA/cm^2)	Ip_a/Ip_c
0.01	0.213	0.140	0.073	0.177	10.2	-14.3	0.71
0.05	0.217	0.135	0.082	0.176	15.8	-22.8	0.69
0.10	0.224	0.120	0.104	0.172	30.8	-40.4	0.70
TH (0.2 mM)							
$\nu x10^{-3}$ (V/s)	Ep_a (V)	Ep_c (V)	ΔE_p/n (V)	E_o (V)	$Ip_a x10^{-3}$ (mA/cm^2)	$Ip_c x10^{-3}$ (mA/cm^2)	Ip_a/Ip_c
0.01	-0.100	-0.149	0.049	-0.122	3.12	-3.55	0.88
0.05	-0.090	-0.140	0.050	-0.115	10.5	-13.2	0.80
0.10	-0.098	-0.158	0.061	-0.128	46	-57	0.81
TMPD (0.2 mM)							
$\nu x10^{-3}$ (V/s)	Ep_a (V)	Ep_c (V)	ΔE_p/n (V)	$E_{o\_1,2}$ (V)	$Ip_{a\_1,2} x10^{-3}$ (mA/cm^2)	$Ip_{c\_1,2} x10^{-3}$ (mA/cm^2)	Ip_a/Ip_c
0.01	0.157	0.080	0.077	0.12	5.1	-6	0.85
	0.498	*	-	-	15.4	-	-
0.05	0.158	0.078	0.080	0.12	14.4	-12.5	1.15
	0.500	*	-	-	26.4	-	-
0.10	0.16	0.068	0.092	0.11	17	-19.2	0.89
	0.503	*	-	-	-	-	-
TMPD (1mM)							
$\nu x10^{-3}$ (V/s)	Ep_a (V)	Ep_c (V)	ΔE_p/n (V)	$E_{o\_1,2}$ (V)	$Ip_{a\_1,2} x10^{-3}$ (mA/cm^2)	$Ip_{c\_1,2} x10^{-3}$ (mA/cm^2)	Ip_a/Ip_c
0.01	*	*	*	*	*	*	*
0.05	0.180	0.077	0.103	0.129	67	-55	1.21
	0.500	0.425	0.075	0.463	85.3	-97	0.88
0.10	0.182	0.075	0.107	0.129	85	-71	1.20
	0.505	0.420	0.085	0.463	130	-149	0.87

** too broad to be determined*

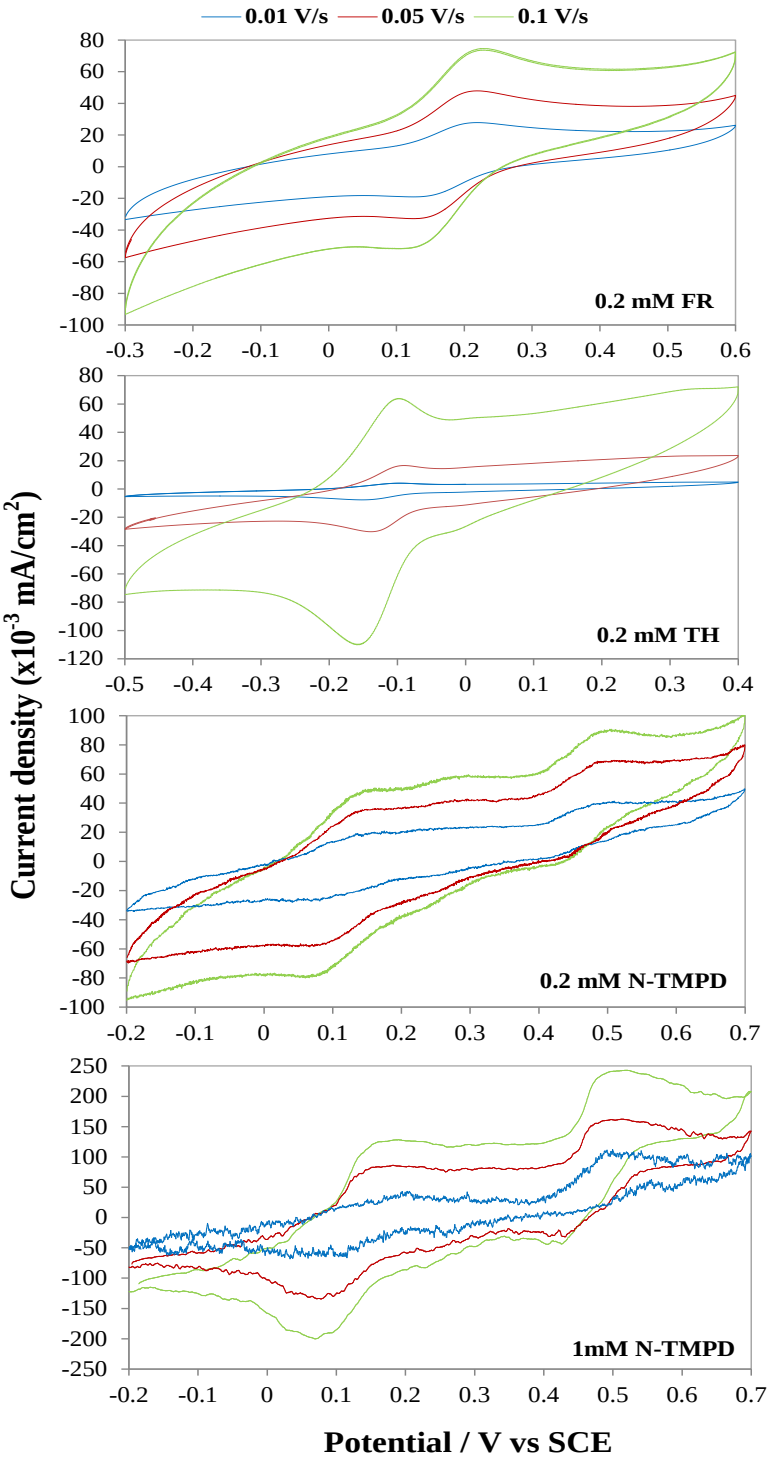

Figure 4-1 Cyclic voltammograms of microbially reduced mediators at three different scan rates using GC electrode: a). 0.2mM FR, b). 0.2mM TH, c). 0.2mM N-TMPD, d). 1mM N-TMPD.

In a quasi reversible process, the electron transfer is influenced by both charge transfer and mass transfer (diffusion). Based on peak currents in Table 4-1, there were increases in the peak currents with the square root of scan rates, but the increases were not quite proportional. This is usually observed for a quasi reversible proces (Vedyadhara, 2011). The ratios of the peak currents (Ip_a/Ip_c) were not completely equal to 1 for all the mediators, indicating not all of the mediators reduced in the forward scan were available to be reoxidised in the backward scan, or reverse. This also indicates that the equilibrium values of the oxidised and the reduced mediator cannot be maintained at the surface of the electrode, or in other words, they were not stable during the time scale of the experiments. The inequality in the peak current ratios are another indicator of a quasi reversible processes (Karthikeyan et al., 2001). Although using N-TMPD as the mediator exhibited broader peaks, due to two steps redox processes, N-TMPD gave the highest oxidation currents. For example, with the same concentration of the mediators used, at the scan rate of 0.1 V/s, N-TMPD produce a total of $48.7x10^{-3}$ mA/cm^2 compared to $30.8x10^{-3}$ mA/cm^2 and $46x10^{-3}$ mA/cm^2 for FR and TH respectively. As the N-TMPD concentration was increased five times, the current also increased more than four times ($215x10^{-3}$ mA/cm^2) (Table 4-1).

The shift of Ep_a values to more positive and Ep_c values to more negative (or increasing values of ΔE_p as a function of an increasing scan rate) is the other characteristic of a quasi reversible process (Zanello, 2003). Previously, Ep_a versus log ν data has been useed to extract kinetic information such as Tafel slope (Raoof et al., 2010). This behaviour was observed for FR and N-TMPD CVs (Table 4-1). In TH system, the Ep_a value shifted to the more negative value at 0.1 V/s (i.e. from -0.09 V to -0.098 V), however ΔE_p kept increasing with the increasing of the scan rate i.e. 0.05 V to 0.061 V. According to Zanello (2003), care should be taken whether the shift of *Ep* values are resulted from uncompensated solution resistance, but this can be distinguished by using different concentration of analyte since slow kinetics does not depend on analyte concentration. By using different concentration of N-TMPD, these behaviours were still clearly seen. Therefore this is another indicator for the quasi reversible nature of the electron transfer, however further investigation is needed such as obtaining the kinetics

information in the higher scan rates (> 0.1 V/s) in order to have a better understanding of the systems.

Several equations have also been proposed to calculate heterogeneous rate constant k_o based on separation of peak potentials, ΔE_p (Gileadi & Eisner, 1970; Killinger & Kochi, 1981; Nicholson & Shain, 1964). In this study, Nicholson and Shain's method was used to determine the k_o using a dimensional parameter value, ψ, which is based on peak separation ΔE_p (see the Appendix 2 for the relationship) according to the following equation:

$$k_o = \psi \left[\frac{\pi a D_{ox}}{\gamma}\right]^{1/2} [v]^{1/2} \quad [7]$$

with $\gamma = \left[\frac{D_{ox}}{D_{red}}\right]^{1/2}$ is the diffusion coefficient ratio of the oxidised and the reduced mediator with $a = \frac{nF}{RT}$, and v is the scan rate. The diffusion coefficient ferricyanide and ferrocyanide obtained and used in this study were 7.3×10^{-6} cm^2/s and 6.7×10^{-6} cm^2/s (thus γ=1.04), whereas the γ values for TH and N-TMPD mediator were assumed to be 1 (with D_{ox} of 3×10^{-6} cm^2/s and 6.43×10^{-6} cm^2/s for TH and N-TMPD, respectively).

Parameters derived from cyclic voltammetry are essential tools to calculate the heterogeneous rate constant (k_o) value of redox reactions (Compton & Banks, 2007). Following to this, Matsuda and Ayabe (1955) have advised the following limits for the reactions to be classified as reversible, quasi reversible and irreversible based on values of k_o and Λ ($\approx \frac{k_o}{\sqrt{\frac{nF\nu D}{RT}}}$, a dimensionless number describing the shape of voltammogram):

*reversible : $\Lambda \geq 15$

*quasi-rev : $15 \geq \Lambda \geq 10^{-2(1+\alpha)}$

*irreversible : $\Lambda \leq 10^{-2(1+\alpha)}$

The electron transfer coefficient, α, can be obtained by using the following equation:

$$E^o = (1-\alpha)E_p^c + \alpha E_p^a \quad [8]$$

Table 4-2 Kinetic parameters of the three selected mediators derived from the voltammograms in Figure 4-1.

FR (0.2 mM)						
νx10^{-3} (V/s)	α	b (V decade^{-1})	ψ	k^ox10^{-3} (cm/s)	i_o (mA/cm^2)	ʌ
0.01	0.51	0.116	1.810	5.2	0.10	3.1
0.05	0.5	0.118	1.052	6.7	0.13	1.8
0.10	0.5	0.118	0.510	4.8	0.09	0.9
TH (0.2 mM)						
νx10^{-3} (V/s)	α	b (V decade^{-1})	ψ	k^{o}x10^{-3} (cm/s)	i_o (mA/cm^2)	ʌ
0.01	0.55	0.054	0.602	1.6	0.06	1.1
0.05	0.5	0.059	0.568	3.4	0.13	1.0
0.10	0.5	0.059	0.312	2.7	0.10	0.6
N-TMPD (0.2 mM)						
νx10^{-3} (V/s)	α	b (V decade^{-1})	ψ	k^{o}x10^{-3} (cm/s)	i_o (mA/cm^2)	ʌ
0.01	0.52	0.114	1.362	3.8	0.07	2.4
0.05	0.53	0.111	1.140	7.0	0.14	2.0
0.10	0.46	0.128	0.723	6.4	0.12	1.3
N-TMPD (1 mM)						
νx10^{-3} (V/s)	α	b (V decade^{-1})	ψ	k^{o}x10^{-3} (cm/s)	i_o (mA/cm^2)	ʌ
0.01	-	-	-	-	-	-
0.05($_{1,2}$)	0.5	0.118	0.525	3.3	0.32	0.9
	0.51	0.116	1.500	9.5	0.91	2.7
0.10($_{1,2}$)	0.51	0.116	0.574	4.2	0.41	0.8
	0.51	0.116	0.920	8.3	0.80	1.6

From the results in Table 4-2, the highest mean k^o value of 6.75x10^{-3}±0.4 cm/s was obtained with N-TMPD mediator compared to 5.5x10^{-3}±0.9 cm/s and 3.05x10^{-3}±0.4 cm/s for FR and TH mediator, respectively (estimated from the values which were in close agreement one to another). Those values were compared with the same concentration of the mediator used. Although further increasing of N-TMPD

concentration lowered the k^o value obtained (i.e. $3.75x10^{-3}$±0.4), however the redox processes increased in the exchange current density (i_o) as expected from Equation 1 and gave the highest value of 0.37±0.05 mA/cm^2. Due to the two steps reversible redox reactions for N-TMPD, further increase by 0.85±0.05 mA/cm^2 in the exchange current were obtained.

The obtained k_o values are within the same order of magnitude to the reported literature values (but in the absence of microbes). For example, the highest k_o value with N-TMPD obtained in this study was $6.75x10^{-3}$ cm/s, which was higher than studies by Rogers *et al.* (2007) and Fernandez and Zon (1990) i.e. 2.6-$7x10^{-3}$ cm/s with 2 mM to 9 mM of N-TMPD concentration used. However, a platinum electrode was used instead of GC electrode that complicates the comparison. Meanwhile, the highest obtained k_o value with FR mediator in this study was $5x10^{-3}$ cm/s, comparable to the values of 4.7-$5.3x10^{-3}$ cm/s for rod GC (Blaedel, 1977), but almost five times higher than the value $1.1x10^{-3}$ cm/s for tubular GC (Blaedel and Engstrom, 1978). Those reported values were in the concentration of FR ranging from 0.01-0.1 mM (lower than was used i.e. 0.2 mM). The variations among k_o literature values are usually observed and most likely to the differences of supporting electrolytes and their concentrations, and also on how the electrodes were pretreated. According to Rice et al. (1989), the rate constants of FR have been reported to vary over three orders of magnitude because of the difference in the activation method.

It is desired for electrode reactions to have a high i_o value as possible, since a higher i_0 means a faster reaction rate and a lower activation barrier). In this study, with the same concentration of the mediator used, N-TMPD mediator demonstrated the highest exchange current density i.e. 0.13±0.01 mA/cm^2, and this did not count the exchange current from the second redox reaction due to the broad peaks thus introducing the difficulty to extract the CV parameters from this second reversible process. Studies on oxidation kinetics based on microbial fuel cells in the presence of mediator have not been found, therefore a comparison to other systems is difficult. However, in comparison to the work by Lowy and Tender (2008) using AQDS modified electrode,

the highest i_o value obtained in this study with 1 mM N-TMPD (0.37 mA/cm^2) was more than six times higher instead of 0.06 mA/cm^2 with 50 mM concentration of AQDS. Compared to i_0 values between 0.003 mA/cm^2 and 0.10 mA/cm^2 whether using pretreated GC, carbon fiber, or graphite ceramic containing Ni^{2+} and Mn^{2+} anode (but all without mediator), this value was also higher (Liu et al., 2007; Wen et al., 2010).

The dimensionless numbers (Λ) values indicate reversibility of a redox process, as the value increases, the process approaches a reversible behaviour thus a fast kinetics (Matsuda & Ayabe, 1955). All the calculated Λ values declined as the scan rate increased (in consistent to the increase in the peak separation), suggesting that the redox processes shifted away from reversible behaviors. The magnitude of those dimensionless numbers imply that the mediator redox on GC electrode were all in the categories of quasi reversible processes (i.e. $15 \geq \Lambda \geq 10^{-3}$, for all α values).

In this study, the calculated Tafel slopes from Equation 4 (based on their α values) were found at around 0.118 V per decade of current with FR and N-TMPD as the mediator, and the values of 0.059 V per decade were found with TH mediator. These are as expected for a single electron transfer (FR and N-TMPD) and two electron transfer reactions (TH) as the rate determining step. The obtained α values were all close to 0.5, suggesting the activation barriers (often called transition states) lies between the products and the reactants as the voltage were applied in the redox reactions (Compton & Banks, 2007).

Since using other mediators and lower concentrations limited the Tafel behaviour observations using LSV method, Figure 4-2 shows only the i_o value of 1 mM N-TMPD reoxidation on the carbon cloth electrode (this value was taken far enough from the equilibrium, higher than $\eta \geq 59$ mV, but before the influence of mass transport was observed). The exchange current density was much lower (0.04 mA/cm^2) than the lowest value obtained from GC electrode (1.03 mA/cm^2). The exchange current density at carbon cloth surface should be higher than glassy carbon due to its high surface area. However, carbon-based electrodes such as carbon cloth contain non uniform pores (with

only a few nanometers in diameter), leading to non uniform ion transport/distribution and further transport limitation near electrode surface (Fellman, 2010). In addition, the true surface area of CC electrodes is hard to be determined, however they are reported to have specific surface areas between 1000 m^2/g to 2000 m^2/g (Fellman, 2010).

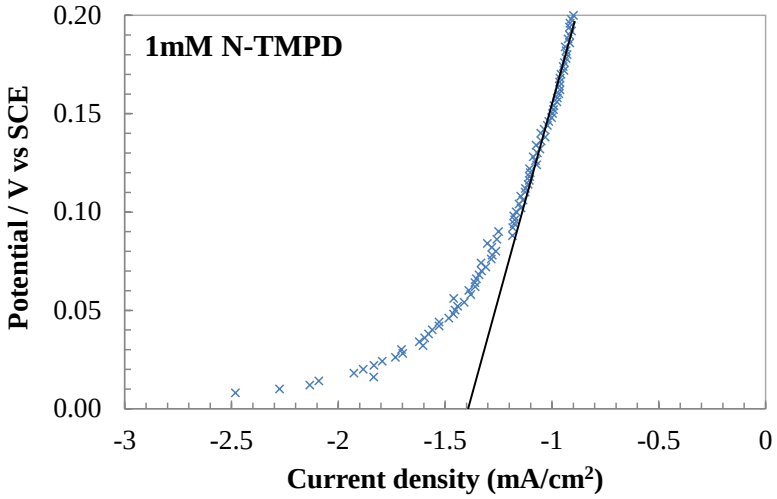

Figure 4-2 Tafel plot estimated for 1mM N-TMPD using LSV method.

4.3.2 Microbial fuel cell performance

Table 4-3 summarizes the performance of the MFCs obtained in this study (with GC and CC electrodes). The data were obtained by using LSV method and were derived from the differences of the cathodic and anodic polarization curves (data for CC electrode were taken from Chapter 3). The results obviously identify N-TMPD as a potential mediator for this ethanol-fed microbial fuel cell whether using GC or CC working electrodes, exhibiting the highest peak power densities of 0.16 mW/cm^2 and 0.03 mW/cm^2 for CC and GC anode respectively (at 1 mM of the concentration). The enhanced of the power output with CC electrode was clearly due to the high surface area of the carbon cloth. Park et al. (2000) reported the short circuit current (sc) and cell voltage values 0.18 mA/cm^2 (sc) and 0.53 V when using 2-hydroxy-1,4-naphthoquinone (HNQ), glucose, and GC as the mediator, the substrate and the electrode. This study produced almost comparable values to that study (Table 4-3), suggesting that the

combination of a gaseous substrate with a mediator in an MFC offers significant promise for electricity generation and gaseous contaminant treatment.

Table 4-3 Microbial fuel cell performance of the ethanol fed MFC with selected mediators (GC and anode electrodes).

Reference	Anode	Power density (mW/cm^2)
This study	1.GC:	
(LSV method)	-without mediator	0.001
	-0.2mM FR	0.001
	-0.2mM TH	0.005
	-0.2mM N-TMPD	0.008
	-1mM N-TMPD	0.030
		0.172 mA/cm^2 (sc)
		0.49 V
Park et al. (2000)	GC	0.18 mA/cm^2 (sc)
	-HNQ	0.53 V
	2. CC:	
	-0.2mM FR	0.013
	-0.2mM TH	0.038
	-0.2mM N-TMPD	0.090
	-1mM N-TMPD	0.160
	-without mediator	0.003

This study confirms our previous results that N-TMPD as the best mediator in the ethanol fed MFC. The results also suggest that it is important to evaluate all aspects of each mediator to obtain a high performance in a mediator MFC. To conclude, N-TMPD was selected as the best performed mediator for several reasons:

(1) it was reduced very rapidly by the bacterial cells (high reactivity between N-TMPD and the last redox enzyme inside the cells), by observing the time required of the coloured mediators to became transparent;

(2) the kinetics of N-TMPD redox reaction was the fastest on GC electrode compared to FR and TH as the mediator, and the two stages reversible oxidation reduction processes further increased the kinetics;

(3) it also provided high mass transfer (oxidation) currents; and it produced high power outputs.

4.4 Conclusions

Potentiodynamic polarization i.e. cyclic voltammetry (CV) and linear sweep voltammetry (LSV) were used to determine the kinetic parameters for the mediator redox reactions occurring at the anode with different anode materials. CV method gave a better estimation of the kinetic parameters data than LSV method due to the low concentration of the mediators used, affecting the Tafel behaviours. The highest exchange current density (i_o) on GC determined by CV method was given by 1 mM N-TMPD as the mediator i.e. 0.37±0.05 mA/cm^2 in the first redox and 0.85±0.05 mA/cm^2 in the second redox, corresponding to k_o value of 3.75±0.5 cm/s and 8.9±0.6 cm/s in the first and second redox reaction respectively. These values also consistent to the highest power output achieved of 0.03 mW/cm^2 by using GC electrode. An improved of the power output was obtained using CC electrode (0.16 mW/cm^2), therefore selection of materials of the electrode is important to enhance the performance of a mediator MFC, while GC electrode is a suitable anode material for studying mediator electrokinetics due to its well defined surface area and easier activation procedures.

The redox reactions of the microbially reduced mediators on GC surface were all classified as the quasi reversible processes based on CV parameters values: the peak separation potentials (ΔE_p) greater than 0.059/n V but below 0.2 V; the inequality in the ratio of peakheight currents (Ip_a/Ip_c); the shift of Ep_a and Ep_c values with the scan rates (to more positive for *Ipa* or to more negative for *Ipc*); the unproportional increase of peak current with increasing scan rate (*Ip* vs $\nu^{1/2}$); and the ratio of heterogenous rate constants (k_o) to $\sqrt{\frac{nF\nu D}{RT}}$ or ($\wedge$). Those values were also compared to the reversible behaviors observed in the voltammograms obtained in the absence of ethanol degrading bacteria. The increase in the kinetics was obtained by increasing the concentration of the mediator used. Therefore, the selection of a mediator for a mediator MFC should also be made in effort to optimise factors such as the kinetics.

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5 FERRICYANIDE-DRIVEN ANOXIC METHANE DEGRADATION

5.1 Methane in the atmosphere

Methane is one of the most potent greenhouse gases playing a major role in atmospheric chemistry. The atmospheric methane concentration has risen by about 158 percent since 1750, starting from 700 ppb in pre-industrial era to 1,750-1,871 ppb in 2010 (EPA, 2012). Although the annual growth rates decreased substantially from about 1% to nearly zero since 1999, it started to increase again in 2007 (IPCC, 2007). Sussmann et al. (2012) confirmed the rise in the global methane budget for 5 years (2007-2011). For example, the level increased by 8 ppb in 2007, and the amount was almost double in 2008 (Sussmann et al., 2012).

Over 70% of atmospheric methane comes from biogenic sources, including natural wetlands, rice agriculture, landfills, termites and oceans. Natural wetlands represent the biggest biogenic source accounting for about 35% of total emissions. Non-biogenic sources involve emissions from the burning of fossil fuel and biomass, waste treatment, and geological sources such as geothermal methane (Rabinowitz et al., 1998). About 60% of the total emission is derived from anthropogenic (human-related) activities. Keppler and co-workers (2005) described an additional new source for methane emission from living vegetation, and estimated a contribution of 10-30% to the global budget. A 10% contribution was confirmed by Intergovernmental Panel on Climate Change or IPCC (2007).

According to IPCC (2007), the largest sink for atmospheric methane, accounting for almost 90% of the total, is the reaction with hydroxyl radicals in the troposphere. A small part is also lost to the stratosphere. Microbial oxidation of methane represents an additional important sink (Rabinowitz et al., 1998). Soils are the major biological sink as a result of microbial methane oxidation and are estimated to remove 26-34 Tg of methane annually (IPCC, 2007). Natural forests and upland soils are the most effective in the oxidation of atmospheric methane (Knief et al., 2003; Kolb et al., 2005). Methane oxidation has also been claimed in other environments such as deserts (Striegl et al., 1992), and the sea surface water (Conrad & Seiler, 1988).

5.2 Biological oxidation of methane

Microbial oxidation of methane is performed by methanotrophic bacteria and archaea (or methanotrophs), a subset of microorganisms known as methylotrophs that utilize methane (or other one-carbon compound) as their sole carbon and energy source (Hanson & Hanson, 1996; Le Mer & Roger; Lieberman & Rosenzweig, 2004). The enzyme methane monooxygenase (MMO) is a specific characteristic of methanotrophs, catalysing the oxidation of methane to methanol (CH_3OH). This step requires oxygen in the first step of the methane oxidation process. Ammonia oxidizers can also oxidize methane to methanol with a similar enzyme to the methane monooxygenase of methanotrophs. However, the specific rate has been reported to be less than 5% of that observed with methanotrophs (Hanson & Hanson, 1996).

The process of aerobic methane oxidation by methanotrophic bacteria has already been known for a long time. Furthermore, their biotechnological potential for production of single cell protein, bioremediation of pollutants, or their use as biocatalysts have been explored (Fuller, 1985; Olah, 2001). The complete pathway for the aerobic methane oxidation to CO_2 by methanotrophs is shown in Figure 5-1. The first step involves the conversion of methane to methanol with the aid of specific enzyme known as methane monooxygenase (MMO), which is used to break the O-O bond in oxygen (Hanson & Hanson, 1996). In the second step, methanol is oxidised to formaldehyde (CHOH) by using methanol dehydrogenase enzymes (MDH), and is further converted to CO_2 through the intermediate formic acid (CHOOH). CHOH metabolism appears to happen by two different pathways in type I and type II methanotrophs. Type I methanotrophs use the ribulose monophosphate pathway (RuMP) whereas in type II methanotrophs, the serine pathway is utilized for the metabolism of formaldehyde.

There are two classifications of methanotrophs that have been described so far (Bowman et al., 1993; Whittenbury et al., 1970). Type I are represented by *Methylococcus*, *Methylomicrobium*, *Methylobacter* and *Methylomonas* which compose the family *Methylococcaceae*. These genera use particulate MMO (pMMO) to oxidize methane. Generally Type I methanotrophs produce cysts and are not able to fix N_2, unless *Methylomonas* and *Methylococcus* species. Type II represented *Methylosinus* and *Methylocystis*. Type II methanotrophs create a branch that distinguish them within the alpha subdivision of the *Proteobacteria.* These types utilize pMMO, and a soluble

enzyme (sMMO) is produced in the absence of copper (in almost type II methanotrophs and in some type I methanotrophs). Some methanotrophs are able to cometabolism methane with other substrates such as TCE and aromatic hydrocarbons due to the presence of sMMO (Hanson & Hanson, 1996; Shukla et al., 2009).

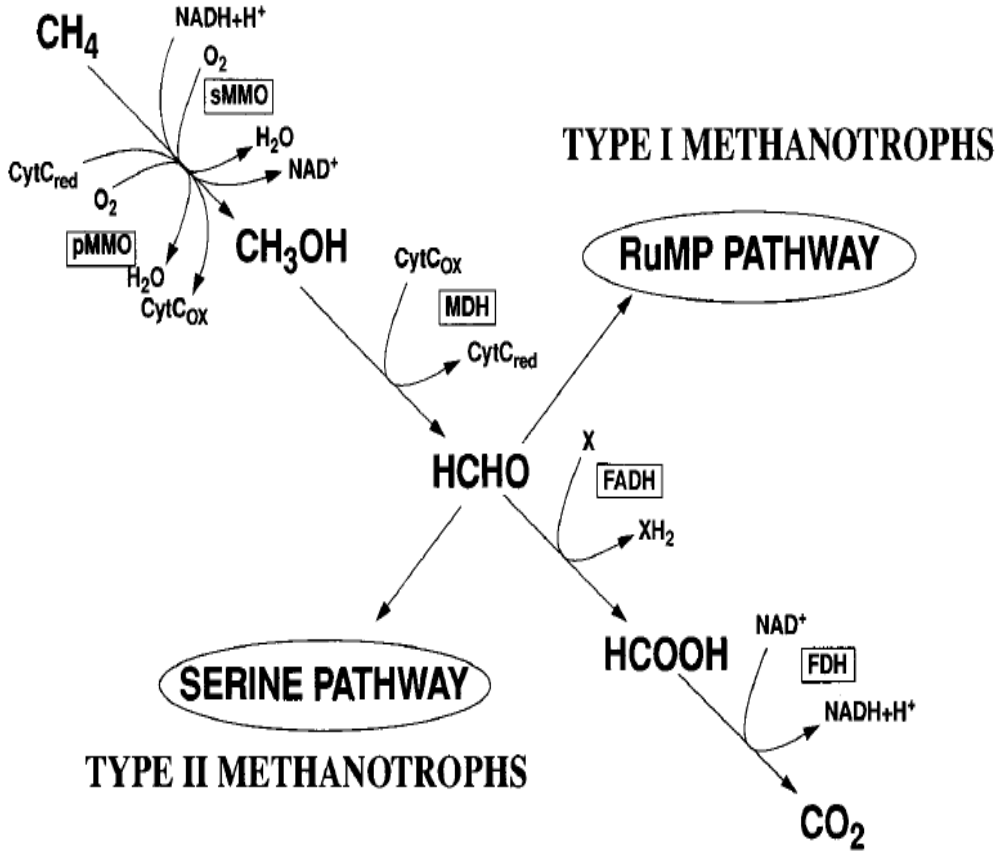

Figure 5-1 The pathway for the oxidation of methane and assimilation of formaldehyde by methanotrophs (Hanson & Hanson, 1996).

Studies by the scientific community have accumulated evidence that methane also is oxidized to CO_2 in anoxic environments, known as anaerobic oxidation of methane or AOM. It was initially believed that AOM is difficult to proceed because methane has the lowest reactivity compared to other alkanes due to the unpolarised C-H bond (Thauer & Shima, 2008). Therefore AOM was viewed as feasible only with electrophilic and superacids reaction (Olah, 2001; Shilov & Shul'phin, 1997). Until 1970s, geochemical observations of anaerobic marine sediments and waters showed that methane diffused upwards from deeper sediments layers and ceased before reaching aerobic zones (Barnes & Goldberg, 1976; Martens & Berner, 1974; Reeburgh, 1976). These observations showed that the disappearance of methane occurred in the layers contained sulphate reducing bacteria.

AOM is a microbially-catalyzed process which is believed to occur mostly in anoxic marine sediments. It is predicted that approximately 90% of all the methane that originates from marine sediments is consumed by this process. Research showed that AOM which proceeds initially only with sulphate as the terminal electron acceptor was carried out by cooperation of methanotrophs with sulfate-reducing bacteria or consortium of microorganisms (Boetius et al., 2000). The free energy change of AOM is very small (ΔG= -21.3 kJ/mol), and thus has to be shared by both partner organisms in the consortium. This also means that it has a low driving force for electrons to flow as shown below by its E^o vs NHE value. The net reaction of the AOM can be formulated as:

$$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O \qquad E^o = 0.02\ V \qquad [1]$$

A study has indicated that some consortia of archaea and bacteria are also able to convert methane with nitrate (Equation 2) instead of sulfate in the complete absence of oxygen (Raghoebarsing et al., 2006). Recent findings by Beal *et al*. (2009) showed that manganese (birnessite) and iron (ferrihydrite) have also been utilised anaerobically by the methanotrophs of a marine methane-seep sediment in the Eel River Basin California, suggesting that marine AOM can use a wider variety of oxidants than previously believed according to Equation 3 and 4. Subsequent evidence also indicates that the AOM is an enzymatic reversal of methanogenesis from carbon dioxide using the nickel containing methyl-coenzyme M reductase (MCR) (Scheller et al., 2010), but MCR operating in the back reaction have been rarely found.

$$5CH_4 + 8NO_3^- + 8H^+ \rightarrow 5CO_2 + 4N_2 + 14H_2O \qquad E^o = 1.0\ V \qquad [2]$$

$$CH_4 + 4MnO_2 + 7H^+ \rightarrow HCO_3^- + 4Mn^{2+} + 5H_2O \qquad E^o = 0.72\ V \qquad [3]$$

$$CH_4 + 8Fe(OH)_3 + 15H^+ \rightarrow HCO_3^- + 8Fe^{2+} + 21H_2O \qquad E^o = 0.25\ V \qquad [4]$$

A recent report shows a mixed cultured of bacteria and archaea that grow slowly on methane and nitrite (Equation 5) as the terminal electron acceptor under strictly anaerobic conditions, as it thermodynamically feasible and has a greater driving force of electrons than with sulfate, nitrate, birnessite and ferrihydrite (Ettwig et al., 2010). '*Candidatus Methylomirabilis oxyfera*' was the name of the bacteria that was

successfully isolated from the cultivations (Ettwig et al., 2010). In this case, 'a new intra aerobic' pathway of nitrite reduction to dinitrogen and oxygen has been found, in which oxygen is still needed as activator of MMO enzymes. However, the catalyzing enzyme of the nitrite reduction has not yet been identified.

$$3CH_4 + 8NO_2^- + 8H^+ \rightarrow 3CO_2 + 4N_2 + 10H_2O \qquad E^o = 1.19\ V \qquad [5]$$

The ability of microorganisms to use ferricyanide (FR) as an alternative electron acceptors for respiratory process has been known for nearly 100 years and now it is a commonly used electron acceptor in biosensors (Ertl et al., 2000; Morris et al., 2005; Tkac et al., 2002). In our previous study (Chapter 3 and 4), some organic dyes including FR molecule have been successfully used as electron acceptors (or mediators) in a microbial fuel cells. Theoretically, methanotrophs should be able to use other oxidants, perhaps FR to oxidize methane anaerobically. The equations below show the anoxic degradation of methane mediated by FR along with the value of the oxidation (6a), reduction (6b), and total reactions (6c). According to the theoretical E^o cell, methanotrophs could use FR as an electron acceptor. The driving force for electron transfer is higher than if using sulphate or ferrihydrite, but lower than if nitrite, birnessite, or nitrate is the electron acceptor.

$$CH_4 + 2H_2O \rightarrow CO_2 + 8H^+ + 8e^- \qquad E^o = -0.24\ V \qquad [6a]$$

$$8FeCN_6^{3-} + 8e^- \rightarrow 8FeCN_6^{4-} \qquad E^o = 0.36\ V \qquad [6b]$$

$$CH_4 + 8FeCN_6^{3-} + 2H_2O \rightarrow 8FeCN_6^{4-} + CO_2 + 8H^+ \qquad E^o = 0.6\ V \qquad [6c]$$

To date, none of the AOM works exhibited pure culture studies and all attempts to the isolation have failed so far. The detailed physiology of anaerobic methane oxidisers, mechanisms of AOM (the metabolic pathways and the enzyme involved) still remains unclear, therefore there is still room for more findings. Therefore, in this study, a preliminary experiment to prove the concept on AOM using pure culture of methanotrophs and FR as the terminal electron acceptor was attempted. The kinetics of reduction of the electron acceptor is reported. This study was done in the presence of other terminal electron acceptor (i.e. nitrate and sulphate). The promising electron acceptor is intended to be applied in the area of electricity generation in mediated microbial fuel cell (MFC). Prior to FR, neutral red or NR (E^o = -0.325 V vs NHE) was

tested as the terminal electron acceptor to see whether it get reduced by methanotrophs in order to maximise the cell voltage in our microbial fuel cell.

5.3 Materials and methods

5.3.1 Microorganism and culture condition

Methanotrophic bacteria (whether mixed or pure culture) were grown in nitrogen mineral salts (NMS) medium (Whitenbury & Dalton 1981) containing per litre of demineralised water: 1 g $NaNO_3$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.02 g $CaCl_2 \cdot 2H_2O$, 0.003 g $FeSO_4 \cdot 7H_2O$, 0.12 g KH_2PO_4, 0.55 g Na_2HPO_4, 0.12 g KCl, and 0.00186 g EDTA disodium salt plus 1 mL trace elements containing per litre of demineralised water: 0.02 g $MnCl_2 \cdot 4H_2O$; 0.07 g $ZnSO_4$; 0.02 g $NiCl_2$; 0.1 g $CoCl_2 \cdot 6H_2O$; 0.01 g $CuCl_2$; 0.03 g $NaMoO_4 \cdot 2H_2O$; 0.02 g H_3BO_3. After sterilizing, cycloheximide was added to each flask to a final concentration of 100 mg/L to prevent the growth of protozoa, and pH of the medium was adjusted to pH 6.5 in all experiments.

Batch cultures were grown in 250 mL flasks which were filled with 200 mL medium, gassed with methane and air at a ratio of 1:1. For the preliminary study with NR, natural gas (82% methane), and soil and compost (25 g of each), were used as the carbon source and the inoculum. The initial inoculum was prepared by suspending the compost or soil (1:10, weight:volume) in the growth medium, which was filtered through a cheesecloth to remove solids and coarse particles. Meanwhile for the investigation with FR, a high purity (100%) methane and dark soil from University of Canterbury area were used as the carbon source and the source of inoculum. The same preparation method for the inoculum as in the former study, but the pure cultures were obtained in this latter study, after several subcultures of the initial inoculum source on NMS agar plates supplied with methanol (0.025% v/v). The flask for FR reduction (kinetic measurements) was also sealed to prevent oxygen leaking and was stirred to maintain homogeneity.

5.3.2 Kinetics of electron acceptor (mediator) reduction

The kinetic measurements were started at the late exponential phase of the batch cultures (vs abiotic and N_2 control). An anoxic condition was created by turning off the air supply to the flask and flowing methane continuously overnight to flush oxygen out

of the flask. During this time, CO_2 level in the system was also monitored continuously. After the CO_2 level reached zero, 1 mM of the electron acceptor was added into the flask, and the reduction was followed using the analytical equipment below. Three cycles of FR injection were employed to confirm whether FR the cycles were repeated (to confirm the electron transfer by the methanotrophs), and whether FR was toxic to the bacteria at higher (3 mM) concentration.

5.3.3 Analytical determinations

A spectrophotometer (Shimadzu), a potentiostat (DY2100, Digi-Ivy. Inc), and a CO_2 analyser (Vaisala GMP343) were used for analytical determinations. In this study, cyclic voltammetry (CV) and amperometric measurements using potentiostat were performed only for the kinetics of FR reduction, while the spectrophotometer was used to obtain the kinetic data (NR at 530 nm and FR at 420 nm) and to measure bacterial cell density at 600 nm. The cell density was also measured in dry cell weights. CO_2 analyser was used to measure CO_2 concentration leaving the reactor from the anoxic oxidation of methane using FR as the electron acceptor.

The amperometric (or the potential step) data were obtained using a microelectrode (25μm OD). The detection of microbially-produced ferrocyanide by using microelectrode was a simple, reliable, and rapid (Morris et al., 2005). A potential step was applied at 0.4 V more positive from redox potential of FR in the growth medium (Bai et al., 2006). Periodic measurements (1 hr period) were done to observe the steady state currents for 20 seconds in a 10 ml sample in parallel to the spectrophotometer (absorbance) measurements, and the values were averaged. The current produced was proportional to the concentration of the ferrocyanide (determined from a generated standard curve). The relationship between the steady state current (or the limiting current, I_L) with concentration was calculated using the equation by Schroder et al. (1990).

A CV scan was done at the end of each cycle of FR reduction, and was used to confirm the amperometric results. A glassy carbon (GC) working electrode (30 mm OD), a saturated calomel reference electrode (SCE), and a platinum counter electrode were used in a 15-ml electrochemical cell. The GC electrode was polished with 0.3 and 0.05 μm alumina paste and was sonicated in deionized water for 3 minutes prior to each use.

Ten milliliter of the biofilm sample was removed from the flask after the FR was fully reduced, and it was immediately centrifuged at 13600 rpm for 3 minutes to obtain the supernatant. The potential was scanned to the GC working electrode submerged in the supernatant from -0.3 to 0.6 V where only peaks due to FR and ferrocyanide (FRO) were observed. Two scan rates of 10, 20 mV /s and several cycles were employed. The resulted currents were used to find the concentration of FRO produced from the kinetics study using a standard curve derived prior to measurements, and this was compared to the amperometric data.

5.4 Results and discussions

5.4.1 Methanotrophs growth curves

Figure 5-2 presents the growth of mixed cultures of methanotrophs from a soil inoculum under 100% and 82% methane atmosphere. The growth with natural gas as the carbon source (82% of methane) was considerably slower (i.e. 360 hr of lag phase) than the growth with 100% of methane. The poorer growth observed was most likely due to a potential inhibitors like acetylene in the natural gas that limited the methane monooxygenase (MMO) activity (Dalton & Whittenbury, 1976). The methanotrophs growth, which used compost as the inoculum and under 82% methane content demonstrated an even longer (i.e. 430 hr) lag phase (graph not shown). To conclude, high purity methane was the carbon source favoured for the growth of methanotrophs compared to natural gas.

Previous studies revealed the difficulties of cultivating methanotrophs at high cell concentrations restricting their use in industrial applications (Han et al., 2009). Usually, the cause of low cell density was attributed to the accumulation of formaldehyde as a toxic intermediate for methanotrophs (Verseveld & Duine, 1987). In this study, the highest OD value of 0.65 and the dry cell weight of 0.5 g/L were achieved after three days under 100% methane. The OD value was higher than the OD value reported by Han *et al*. (2009) i.e. 0.46 for four days of cultivation, but lower than their OD value of 1.59 with 5% (v/v) paraffin oil in the medium.

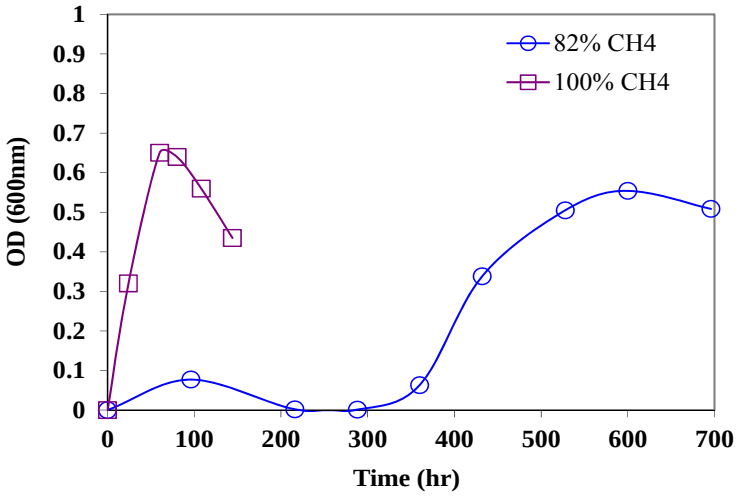

Figure 5-2 The growth curves of (mixed cultures) methanotrophs using 82% methane (natural gas) and 100% (high purity) methane as the substrate.

5.4.2 Neutral red as the electron acceptor

In the absence of oxygen (shut off the air and added NR to the bacterial cultures with pure methane flowing), the observations showed that neutral red (NR) was never completely reduced by the methanotrophic bacteria (data not shown). Park and Zeikus (2000) demonstrated NR was a better electron acceptor (i.e. mediator) than thionin (E^o = 0.064 V vs NHE) in an MFC using *E. coli* or *A. succinogenes* and glucose as the bacteria and the substrate used. However, the mechanism on how NR mediated the electron transfer was not explained. In addition, according to that study, a high NADH concentration was required to drive NR reduction and to produce high current which means that the NR reduction relied solely only on NADH. Since methanotrophs are slow-growing or NADH-limited bacteria that worsened the use of NR as the mediator. McKinlay and Zeikus (2004) also studied the use of NR as mediator for an extracellular iron reduction in *E.coli*. The study concluded *E. coli* has a different mechanism on how electrons were transferred in their respiratory system (Figure 1 in McKinlay and Zeikus, 2004), in which the mediation did not rely on NADH alone but also relied on hydrogenase or formate hydrogenase. In another study, the use of NR as the mediator in *A. succinogenes* only proceeded with the presence of fumarate reductase, hydrogenase or diaphorase, in which fumarate reductase gave the highest activity (Park & Zeikus, 1999). These last two investigations suggested that mediator selection should account for the redox enzymes involves in the respiratory system of microorganisms and their

combination to the substrate used to be able to transfer the electrons to a terminal electron acceptor. The E^o value of NR (-0.325 V vs NHE) is very close to the E^o value of NAD (-0.32 V vs NHE), and the E^o value of methane as the donor substrate (-0.24 vs NHE) is also more positive than the E^o value of NR. These factors contribute to the difficulties in the electron transfer between these compounds.

According to Sund et al. (2007), a mediator is better from the other mediators when it gives a high and appreciable current production in an MFC. A meaningless current production could be resulted from whether no electrons to be donated to the electrode or no electrons are able to be transferred from the bacteria to the mediator (mediator can not access the bacterial electron source), or the mediator inhibits the bacterial metabolism. CV data showed that NR demonstrated inappreciable current production compared to FR (graph not shown), indicating one of criteria above occurred with the NR mediated system.

5.4.3 Ferricyanide as the electron acceptor

The graphs in Figure 5-3 implies that FR was completely reduced (was utilized as the electron acceptor) by the methanotrophic bacteria in anoxic condition. However, the complete reduction of FR could be unrelated to the microbial respiration. For example, there was a possibility that ferricyanide was being consumed by the bacteria, or the reduction could be happened because of the other chemical species present i.e. nitrate in the growth solution (will be explained further in Chapter 6).

There were differences in the reduction rates by the methanotrophs in the two separate tests. The first test gave a higher reduction rate of FR i.e. 2.6 $x10^{-3}$ mM/min.g compared to just 1.6 $x10^{-3}$ mM/min.g in the second test. The most likely reason for the higher reduction rate in the first test than in the second test was because a higher cell density of methanotrophs was used (0.5 g/L compared to 0.4 g/L). This suggests that the higher microorganism concentration thus the higher biocatalyst available to reduce ferricyanide and to degrade the substrate, and those concentrations were not as a limiting factor (FR was the limiting reactant). The highest reduction rate obtained, however, was still four times lower compared to 10 ($x10^{-3}$) mM/min.g in *E. coli* (Ertl, 2000). There has been many reports on *E. coli* as the most well studied bacteria in using FR as an electron acceptor (Boonstra et al., 1976; Emde et al., 1989; Ertl et al., 2000).

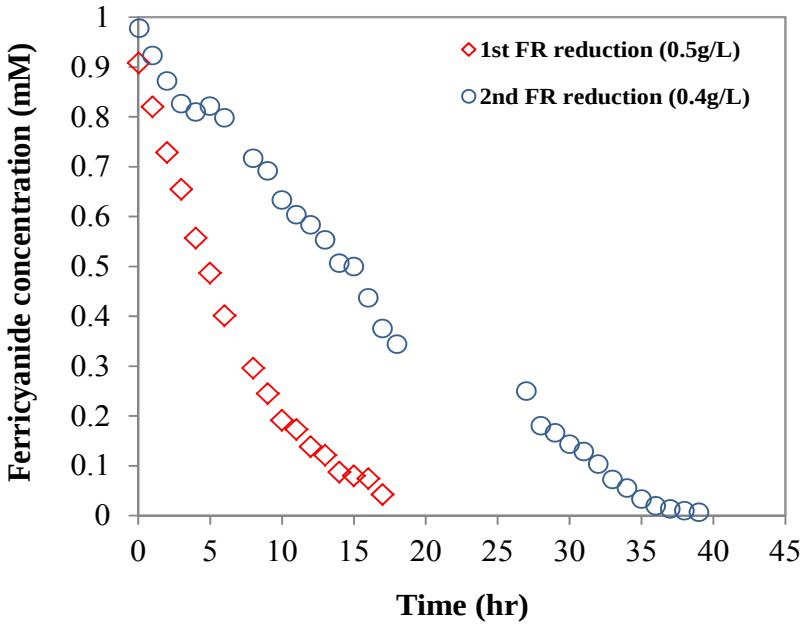

Figure 5-3 Kinetics of 1 mM microbially reduced FR by methanotrophs in the first and second test based on absorbance measurement at 420 nm.

In order to ensure the reduction correlated to microbial processes, two control experiments were performed; one was being abiotic (growth medium with FR and methane), and the other one was without methane (replaced with N_2) in the presences of 1 mm FR. The abiotic control (Figure 5-4) demonstrated stable absorbance values at 1.18±0.05 over 18 hrs, indicating no FR was reduced. For the biological control, when methane was replaced by N_2, it showed similar behaviour (stable at 0.99±0.15). This suggests that there was no electrons obtained from the donors (whether N_2 or NADH). A further test was carried out to test whether there were still living bacteria in the reduction flask methane was re-supplied into the system. As can be seen from the figure, as methane was introduced at 18 hrs, the bacteria started to use FR again. Although the measurements were not continued further, there seemed FR was being reduced by the bacteria even though it was progressing very slowly. Further culturing these bacteria on agar plates, growth was observed indicating there were still living bacteria. However, measuring methane and N_2 uptake is important to know whether the FR reduction was related to substrate degradation. Nonetheless, the reduction in FR concentration is an indication of the utilization of FR as the electron acceptor.

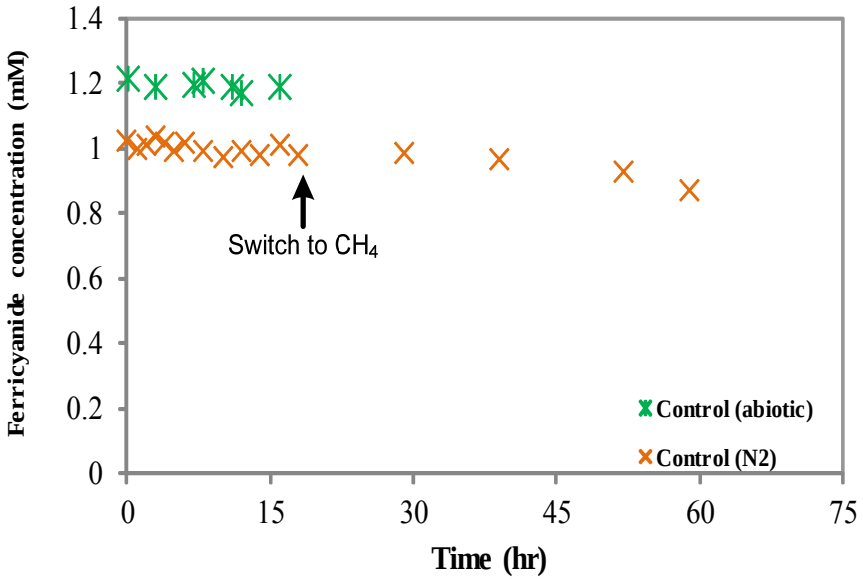

Figure 5-4 Control profiles with 1 mM FR from absorbance measurements: abiotic (without microbes); and N_2 (without methane).

In the second FR reduction test, three cycles were attempted to further prove the ability of the methanotrophs using FR as the electron acceptor in anoxic condition (Figure 5-5). The results suggest the methanotrophs could use FR as the electron acceptor, and also the FRO produced was not toxic to the bacteria up to a concentration of 3 mM (as previously only up to 1 mM FR was applied). These results were supported by microelectrode data in those cycles, confirming that ferrocyanide (FRO) was being produced as a result of FR reduction. Although the reduction rate was reduced by 38% in the third cycle (from $2.6x10^{-3}$ mM/min.g to $1.6x10^{-3}$ mM/min.g), but according to Rabinowitz et al. (1998), the changes in cellular environment such as nutrients and hormones could alter the concentrations of the intracellular redox couples in living cells. They concluded that the observed ferricyanide reduction rate was influenced by the reduction rate of a carrier mediator by the enzymes in the electron transport chain of eukaryotic cells, revealing a regulation of redox enzyme activity. This may also occur in procaryotic cells like methanotrophs, since the flow of metabolites in living cells are controlled by cell needs for survival, and/or change as a response to the cellular environment (Rabinowitz et al., 1998; Stryer, 1995). In this study, the level and activity of intracellular redox couples in the methanotrophs' metabolism such as NAD^+/NADH might contribute to the FR reduction rate, however this was not measured.

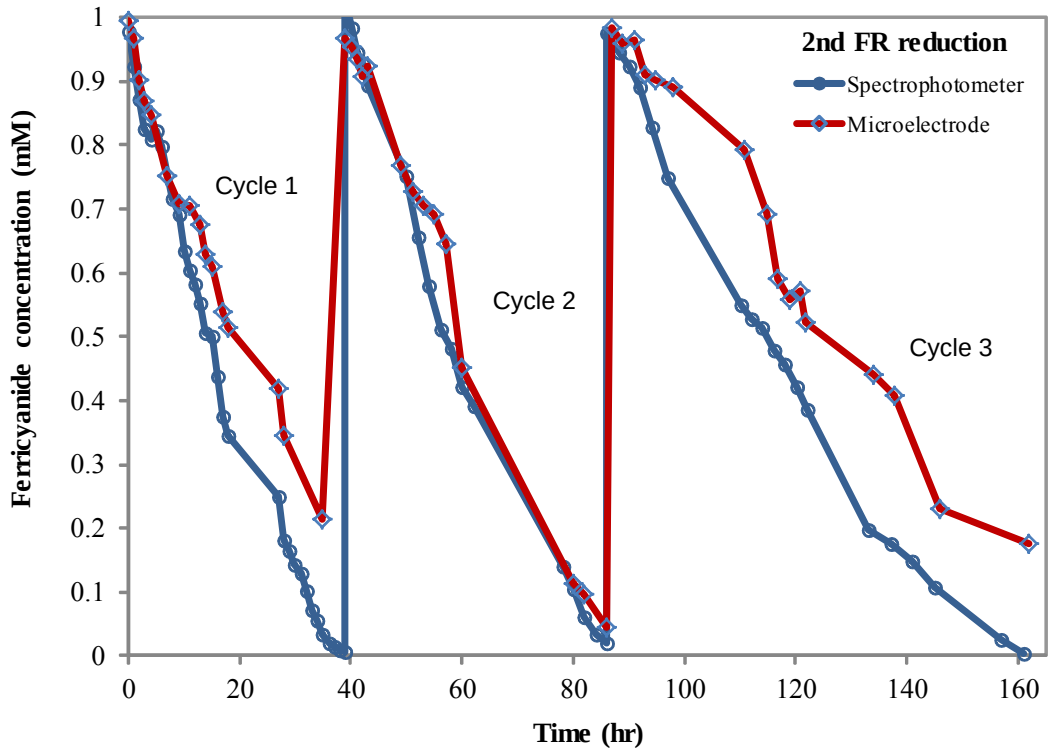

Figure 5-5 Three cycles of microbially reduced FR in the second test (arrows indicating 1 mM FR addition in each cycle).

To give more evidence for the microbial-driven FR reduction (other than spectrophotometric and microelectrode data), cyclic voltammetry (CV) scans were performed at the end of each cycle in the second test. CV results showed that FRO was produced, by determining E^o value (i.e. 0.41 V versus NHE) from the two peaks of the voltammogram (see the Appendix 3). Although it slightly shifted from its E^o value of 0.36 V vs NHE, but a shift of mediator E^o value in the growth medium from reported literature values was also observed by Sund et al. (2007).

Table 5-1 summarizes the initial FR concentration (measured by spectrophotometry) and FRO generation (measured by CV and amperometry) as the result of the FR reduction in the second test. The values of FRO production from CV and amperometry were in close agreement with each other, and suggests that 7 to 9% of FR may have been absorbed by the bacteria in the first and third cycle. However, these values fall within the uncertainty range (for example ± 0.1 for the amperometry), and it can be assumed there was no any absorption occurred since FR is membrane-impermeable (Boonstra et al., 1976).

Table 5-1 Initial FR concentration from UV-vis spectrophotometry and FRO concentration determined from CV and amperometry method.

Second test	FR[a] (mM)	FRO - CV[b] (mM)	FRO - Amperometry[b] (mM)
Cycle 1	0.91	0.82	0.83
Cycle 2	0.94	0.95	0.95
Cycle 3	0.89	0.83	0.82

[a]The initial concentration of FR in each cycle.

[b]FRO production.

All of the FR reduction occurred in the presence of the other electron acceptor in the growth medium, normally nitrate. According to Barret and McBride (2007), the order of preference of electron acceptors is usually due to their redox potentials (E^o) for the half-reactions. Note that O_2 has the highest redox potential, followed by NO_3^- > Mn^{+4} > $FeCN_6^{-3}$ > SO_4^{-2} (see the Appendix 4 for the potential electron acceptors and their redox potentials). The E^o values between nitrate and FR as the electron acceptor is actually only different by 0.07 V (if nitrate was reduced to nitrite). Nonetheless, the methanotrophs utilised FR in the presence of nitrate upon receiving the electrons from the donor (methane). This was probably because FR has the closer E^o value to the last redox enzymes in the bacteria, and they had enough energy for growth or for maintaining themselves. Heterotrophic bacteria mostly found in soil (such as methanotrophs) have been known to be able to use FR as the electron acceptor in their respiratory pathway (Morris et al., 2005). Fe-CN complexes ferri and ferrocyanide, have been long known as pollutants to soil and groundwater originating from anthropogenic sources (Fuller, 1985), although nitrate is also available in soil. Ertl *et al.* (2000) also claimed that all gram-negative organisms like methanotrophs can reduce non-native, hydrophilic oxidants such as FR directly. Some literatures have reported an inhibition of nitrate reduction in bacterial cells in the presence of potassium ferricyanide (Luque-Almagro et al., 2005; Sohaskey, 2005). However, the mechanism on how the bacteria transferred the electrons to FR is still unclear.

No growth was observed during the FR reduction in all three cycles in the second test (stabilised levels in the OD values (±0.05) in Figure 5-6). Although the OD values were low (0.2 to 0.3), as was previously discussed, the growth was observed when further

inoculated the cultures on the plates which means the reduction were not coming from the dead cultures. Similar results were observed by Hadjipetrou (1970) in which the reduction of FR using *E. coli* was also not coupled to growth and ATP formation because FR repressed the synthesis of formate hydrogenase in *E. coli*. In this study, the same phenomena happened but progresses with an unknown mechanism.

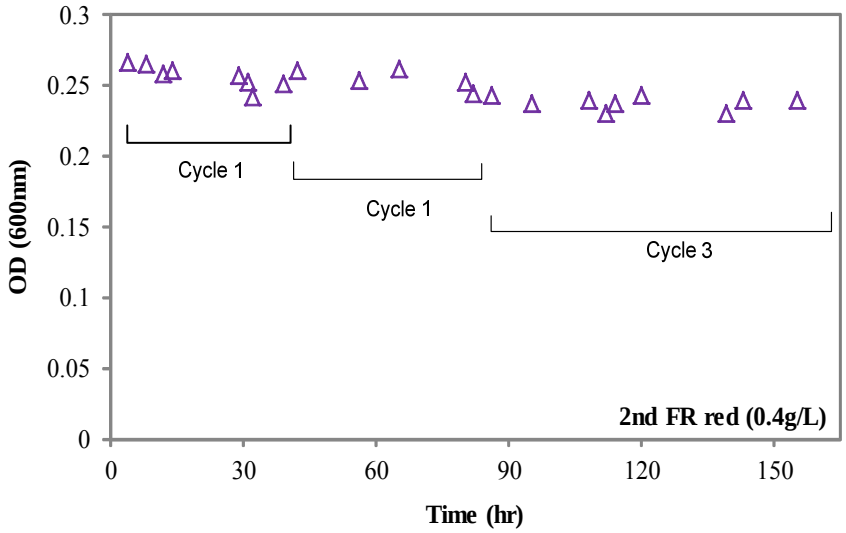

Figure 5-6 The profile of methanotrophs cell densities during the second test of 1 mM FR reduction.

In this study, the CO_2 concentration leaving the reactor was also observed as a result of FR reduction (Table 5-2). Each time when FR was added into the methane-fed cultures in the absence of oxygen, the observations showed that the CO_2 production rose as recorded by a CO_2 analyzer (six tests were attempted in total) (Figure 5-7). Generally, the CO_2 production maintained the same level over the period of the reduction with slight fluctuations (compared to the abiotic control), and on the contrary, it decreased whenever FR started to become colorless i.e. reduced. The values of CO_2 production cannot be determined quantitatively due to the readings in negative regions (cycle 2 and 3). This is because an offset of the analyzer caused by methane absorption.

Although the actual CO_2 values were not determined, the CO_2 concentration being produced as the result of FR reduction was estimated from the amperometry and CV results in Table 5-1, in which the calculations were based on FRO production in Equation 6c (see the Appendix 5 for the example of calculation). The data in Table 5-2 shows that the results from the CO_2 analyser were in agreement with the results from

the amperometry and CV in each cycle within the uncertainty range. This data are valuable thus could support the evidence of AOM in the presence of FR as the electron acceptor.

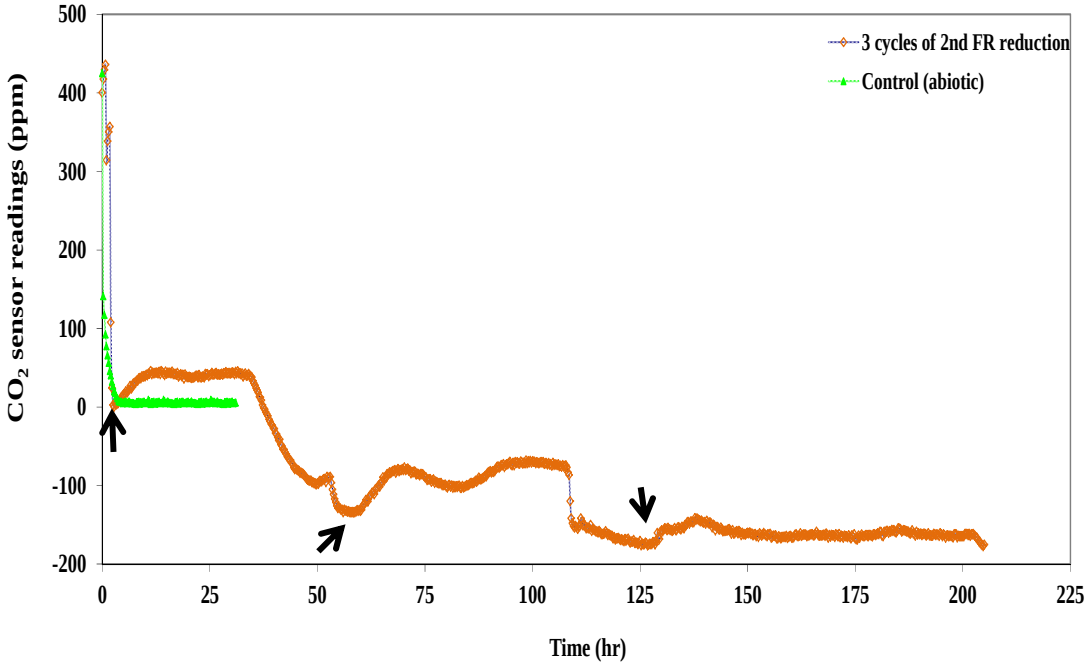

Figure 5-7 CO_2 concentration leaving the reactor in the three cycles of second FR reduction test versus abiotic control.

Finally however, the possibility that methanotrophs could reduce FR in an anoxic environment must be critically evaluated, since methanotrophs need oxygen to activate MMO. The presence or absence of MMO in this methanotrophs is still a major issue which needs to be solved. Only the ones from the deep sea have been identified that can oxidize methane anaerobically. Although all observations showed they can use FR as the electron acceptor, further investigation needs to be carried out. There was still a possibility that the methanotrophs used the 'intra-aerobic' pathway or obtained oxygen from the chemicals supplied in the medium (i.e. nitrate). In addition, there was a possibility of production and consumption of NO (the intermediate compound of denitrification from nitrate) by methanotrophic bacteria, which occurs mostly in a nitrate-containing medium under anaerobic or nearly anaerobic conditions (Ren et al., 2000).

Table 5-2 Comparison of CO_2 production results in the second test.

	Amperometry (ppm)	CV (ppm)	CO_2 analyser (ppm)
Cycle 1	43.5±9.8	43±6.5	41.7±4.9
Cycle 2	41.3±7.3	41.2±4.9	49.1±14.9
Cycle 3	22.3±8.2	22.4±5.5	15.6±7.8

5.5 Conclusions

The investigation on two electron acceptors i.e. neutral red and ferricyanide in the area of anoxic methane oxidation for its later use in methane-fed microbial fuel cell have been carried out. Neutral red cannot be utilised as the electron acceptor for methanotrophs and this is probably because of the very close E^o value to NADH, and methanotrophs is a NADH-limited bacteria.

Ferricyanide looked promising when it was used as the electron acceptor for pure cultures of methane oxidizing bacteria in the presence of other electron acceptors (e.g. nitrate) with the highest reduction rate achieved was 2.6 μM/min.g. However, further investigations must be made in regards to this other electron acceptor and to eliminate the other possibilities. Even though the generated CO_2 was not directly quantified from CO_2 analyzer data, the amperometry and CV results supported its production.

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6 ANOXIC METHANE OXIDATION COUPLED WITH FERRICYANIDE REDUCTION FOR ENERGY GENERATION IN MICROBIAL FUEL CELL - PROOF OF CONCEPT

6.1 Introduction

Biological methane oxidation plays an important role in mitigating of methane into the atmosphere thus reducing the emissions. It can occur in both aerobic and anaerobic conditions. The aerobic methane oxidation has been long known, in which oxygen and methane are combined to form formaldehyde. It is properly understood, and it also has found the applications in some areas such as bioremediation, and production of commercially relevant compounds (e.g., poly-hydroxybutyrate, single cell protein, astaxanthin) (Jiang et al., 2010). Conversely, anaerobic methane oxidation has not yet been fully understood, but it has been identifed in marine sediments, particularly in methane seeps and vents and in anoxic waters (Valentine, 2002). Studies have reported coupling of anaerobic methane oxidation with sulphate reduction (Hinrichs *et al.*, 1999), birnessite and ferrihydrite reduction (Beal et al., 2009), denitrification with nitrate (Raghoebarsing *et al.*, 2006) and nitrite (Ettwig et al., 2010). Nevertheless, the combination of anaerobic and aerobic oxidation of methane may remain as an effective tool for biodegradation this radiatively active compound.

Anaerobic methane oxidation is the interest of this study. To date, no anaerobic methane-oxidizers have been cultivated nor isolated in pure culture. The microorganisms participated in anaerobic oxidation of methane (AOM) have been identified as archaea: ANME-1, which is still related to *Methanosarcinales* and *Methanomicrobiales (*Hinrichs et al., 1999); ANME-2 (Boetius et al., 2000); and ANME-3 (Knittel et al., 2005). A report appears recently on AOM proceed with bacteria '*Methylomirabilis oxyfera'*, but the methane degradation (coupled with nitrite reduction) still involved an intra-aerobic pathway (Ettwig et al., 2010).

In our initial study, it has been shown that in an anoxic condition, ferricyanide could act as an electron acceptor using pure culture of methanotrophs in the presence of other electron acceptor (nitrate), and some data showing to support this finding. The highest reduction rate obtained was 2.6 ($x10^{-3}$) mM/min.g. However, another electron acceptor

i.e. nitrate was present and there was a possibility it was converted to nitrite, and according to Bryan et al. (1994) ferricyanide reduction by nitrite can occur spontaneously. Additionally, E_o (midpoint redox potential) of ferricyanide/ferrocyanide (+0.36 V) is lower than that of nitrate/nitrite (+0.43 V) which makes the reduction is thermodynamically possible. Therefore in this study, a test of ferricyanide reduction was performed without any other alternate electron acceptor.

Microbial fuel cell (MFC) is an engineered bio-electrochemical reactor in which microbes oxidize a variety of fuels derived from carbohydrate and hydrocarbon. It is not new to a MFC with methane as the fuel, but electricity generation with methane in anaerobic environment is thought to be competititive with methane generation since part of the fuel is produced by methanogens (Yang et al., 2011). Furthermore, according to Lewis (1966), the relative electrochemical activity of methane is much lower (or high activation polarization with methane as a fuel) compared to hydrogen or methanol as the fuel (i.e. 2 for methane compared to 100 and 30 for hydrogen and methanol, respectively), thus methane as the fuel in a MFC is rarely reported. An addition of a catalyst to the electrode such as a redox mediator is one of the methods to reduce the activation polarization (Lewis, 1966; Rabaey et al., 2005). A redox mediator in this regards could both function as a mediator and a catalyst.

Process involving electricity generation with methane as electron donor was previously attempted in 1965 by Van Hees, where the aerobic *Pseudomonas methanica* suspension at the bioanode was used. The anode was coupled with air (oxygen) cathode in a device consisted of three compartments made of three cylindrical pieces and two membranes (in which the two membranes arrangement was purposed to restrict severely the diffusion of dissolved oxygen to the anode). The microbial fuel cell developed 0.5-0.6 V on open circuit, but a very low current density of 2.8 $\mu A/cm^2$ was drawn, and the method for obtaining those values was not explicitly explained. The addition of a redox mediator (1-naphtol 2-sulphonate indo 2, 6- dichlorophenol) had no effect on the open circuit voltage of the fuel cell. Therefore until now, there is still no proof on electricity generation by combining methane and a mediator in an MFC.

Girguis and Reimer (2009) also investigated methane powered MFCs but without any mediator. The fuel cell was of a packed bed column where the microorganisms at the anode came from marine sediment. The sediment was claimed to contain both anaerobic and aerobic methanotrophs, and *Methylomonas methanica* was identified as one of the genera. The investigations involved testing the ability of methanotrophs on power production with varying methane and oxygen flow, in which the power generation declined with the reduction of methane and oxygen concentration and led to a discontinuation of the power production. However, it was claimed that 0.1 mW/cm^2 was able to be generated in the MFC, which is comparable to the acetate-powered MFC (Yang *et al*, 2010).

The objective of the present study was to proof the concept of anaerobic methane oxidation (AOM) coupling with a ferricyanide reduction without any other alternate electron acceptor (by supplying ammonium chloride as the N-source) in a laboratory scale reactor. In doing so, therefore a better understanding of the AOM with pure culture, and the identification of other factors which support the concept was obtained. Finally, the investigation on its use to generate electricity in a MFC allows further proof of ferricyanide reduction by methanotrophic bacteria.

6.2 Experimental

6.2.1 Reactor set-up

There were two reactor set-ups: a batch reactor system to study the kinetics of ferricyanide reduction with the pure culture of methanotrophs, and a microbial fuel cell system consisted of an anode and a cathode to study the energy production from the reduced ferricyanide obtained in the kinetics study.

A 250 mL reactor (200 mL working volume) was connected to three gas bottles (i.e. methane, nitrogen and dry air) via mass flow controllers for the growth and kinetics study (Figure 6-1). The reactor was stirred to maintain homogeneity. Neoprene tubing was purposely used as the line connectors to prevent oxygen leaking into the system. The gas outlet from the reactor was flowing through a cold trap to remove the water vapor prior reaching the CO_2 analyzer, and the final gas outlet was released to a fume hood via a deionised water flask.

The kinetic measurement was started after sixty-hour batch culture was obtained (at the late exponential phase). The dry air supply into the reactor was shut off and a high flowrate (was not measured) of nitrogen was used to flush the CO_2 which was previously assumed being produced by the bacteria. Subsequently the methane flowrate was changed to 0.5 mL/min and so did the nitrogen flowrate to 2.5 mL/min to make a ratio of 1:5. The anoxic condition was assumed to be created by the continuous flow of both gases through the reactor (which had previously been confirmed by an Agilent 3000 Micro GC). At the same time, the CO_2 readings were continuously recorded. 1 mM of ferricyanide (FR) was injected into the reactor after a stable (and positive region) of CO_2 level were recorded for seven hours, and its reduction data were collected using the analytical methods below. Three cycles of FR injection were employed as in the previous study, but only in the first cycle, the CO_2 production was able to be measured.

For electricity generation, a similar design of a conventional two chamber MFC described in Chapter 3 was used. The working electrodes used were all carbon cloth ($2cm^2$), but the surface area at the cathode was made four times larger than at the anode to improve the power generation (Cheng & Logan, 2011). A saturated calomel electrode (SCE) was used as the reference electrodes.

6.2.2 Growth medium and inoculum

The nutrient (NMS) solution was prepared according to Whittenbury et al. (1970) and contained (g/L): NH_4Cl, 0.5; KH_2PO_4, 0.272; $Na_2HPO_4 \cdot 2H_2O$, 0.717; $MgSO_4 \cdot 7H_2O$, 1; $CaCl_2 \cdot 2H_2O$, 0.2; ferric ammonium EDTA, 0.005. The medium was supplemented with 0.1% (by volume) of a trace elements stock solution, recommended by Dedysh and Dunfield (2011) containing (mg/L): EDTA, 0.5; $FeSO_4 \cdot 7H_2O$, 0.2; H_3BO_3, 0.03; $ZnSO_4 \cdot 7H_2O$; 0.01; $MnCl_2 \cdot 4H_2O$; 0.003; $CoCl_2 \cdot 6H_2O$; 0.02; $CuSO_4 \cdot 5H_2O$, 0.1; $NiCl_2 \cdot 6H_2O$ 0.002; $NaMoO_4$, 0.002. The medium pH was 6.8. Cycloheximide (100 mg/L) and yeast extract (0.1 g/L) was also added to the medium after sterilizing. For plating, this media was solidified with agar (Difco) and was incubated with methanol 0.025% (v/v).

The inoculum (identified as *Acinetobacter sp*. based on 16S rDNA by Ecogene Auckland) was taken from the fresh grown cultures on the plate (these cultures have

been observed to reduce FR in the previous work). The reactor which was filled with 200 mL of the medium was then inoculated to grow a batch culture, in which methane and dry air was supplied at a ratio of 1:1.

6.2.3 Analytical methods

A spectrophotometer (Shimadzu) and a CO_2 analyser (Vaisala GMP343) were used for the ferricyanide reduction test. While a potentiostat (Reference 3000^{TM}, GAMRY Inc.) and a multimeter was used for electricity generation test. The spectrophotometer measured the decline of ferricyanide peakheight over time, and was also been used for measuring the bacterial growth. The CO_2 analyser was used to record CO_2 profile every five minutes from the AOM test using FR as the electron acceptor. Linear sweep voltammetry (LSV) method by a means of potentiostat was used to obtain polarization and power density curves only for the total 3 mM FR reduced by the bacteria. The multimeter was used to generate voltage curves at a fixed load of 9780 ohm over for 30 hour and the power curves were assessed at 35 hr using a variable resistor box (47 to 9780 ohm) with two different catholytes i.e. 50 mM FR in PBS and 1 g/L $KmnO_4$ (only for 1 mM FR injection). Other than that, to check the presence of nitrite, test strips from Merck were used in the kinetics test.

In this study, cyclic voltammetry (CV) method from the potentiostat was performed to check ferrocyanide (FRO) peakheight at the end of each cycle of FR reduction. The same electrodes and procedures as previously described in Chapter 3 were applied in the CV measurements.

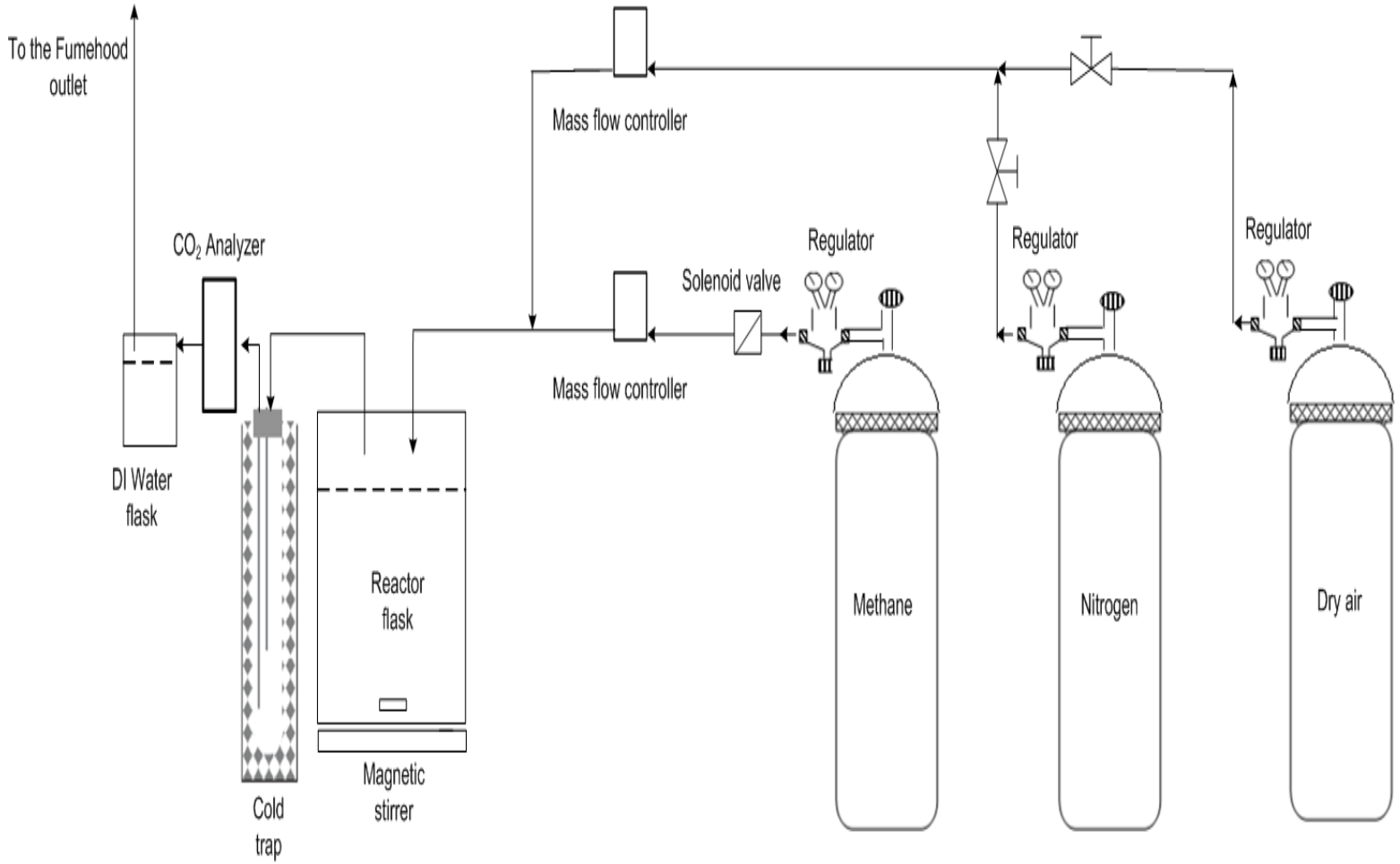

Figure 6-1 Representative diagram of the anoxic methane oxidation set up with ferricyanide as the electron acceptor.

6.3 Results and discussions

6.3.1 Ferricyanide reduction

Following 1 mM FR injection in the first cycle, FR concentration dropped steadily over time versus N_2 control, and its color (also the peakheight based on spectrophotometric measurement) was completely dissappear at 14 hr (Figure 6-2a). Further added FR for the second and third cycle (graphs not shown), the kinetics of FR reduction followed a similar trend and reduction time with the first cycle with the same reduction rate of 3 $x10^{-3}$ mM/min.g. This is the highest value obtained for FR reduction by the pure culture of methanotrophs in this study. The reason for a higher reduction rate than before (compared to $2.6x10^{-3}$ mM/min.g) was probably because more than two times of bacterial density was obtained at late exponential phase or when the kinetics measurements were started i.e. OD of 0.68 compared to OD of 0.3 (Figure 6-2b). It seemed the presence of ammonium chloride as the nitrogen source favored the growth rather than nitrate as the nitrogen source. Ammonium is known could serve as an additional energy source or a nutrient for methane-oxidizer consortium (Nyerges et al., 2010; Schmaljohann, 1991).

The CO_2 production was detected from the CO_2 concentration leaving the reactor at about 500 ppm due to an offset problem of the CO_2 analyzer (the values of CO_2 production was taken by the difference of the observable production values and this offset value) (Figure 6-2c). From the graph, the total CO_2 produced was equal to 0.015 mmoles of CO_2 (from the area under the figure). This is equivalent to the reduction of 0.122 mmoles of ferricyanide (assuming a yield of 100%). The starting concentration of FR added into the reactor at the beginning was 0.2 mmoles of ferricyanide, which means that some of the CO_2 produced, was probably being dissolved in the solution. This value (0.015 mmoles ≈ 148 ppm) was 61% from the calculated theoretical CO_2 production (see Appendix 6 for the calculation). The value was also higher than the previous works (with the highest was 49 ppm due to five times higher of the calculated CO_2 moles fraction in this study). However in the previous work, there was nitrate presence in the growth medium which made difficult to interpret the CO_2 generation. This CO_2 detection is one of the evidence of AOM coupled with FR reduction.

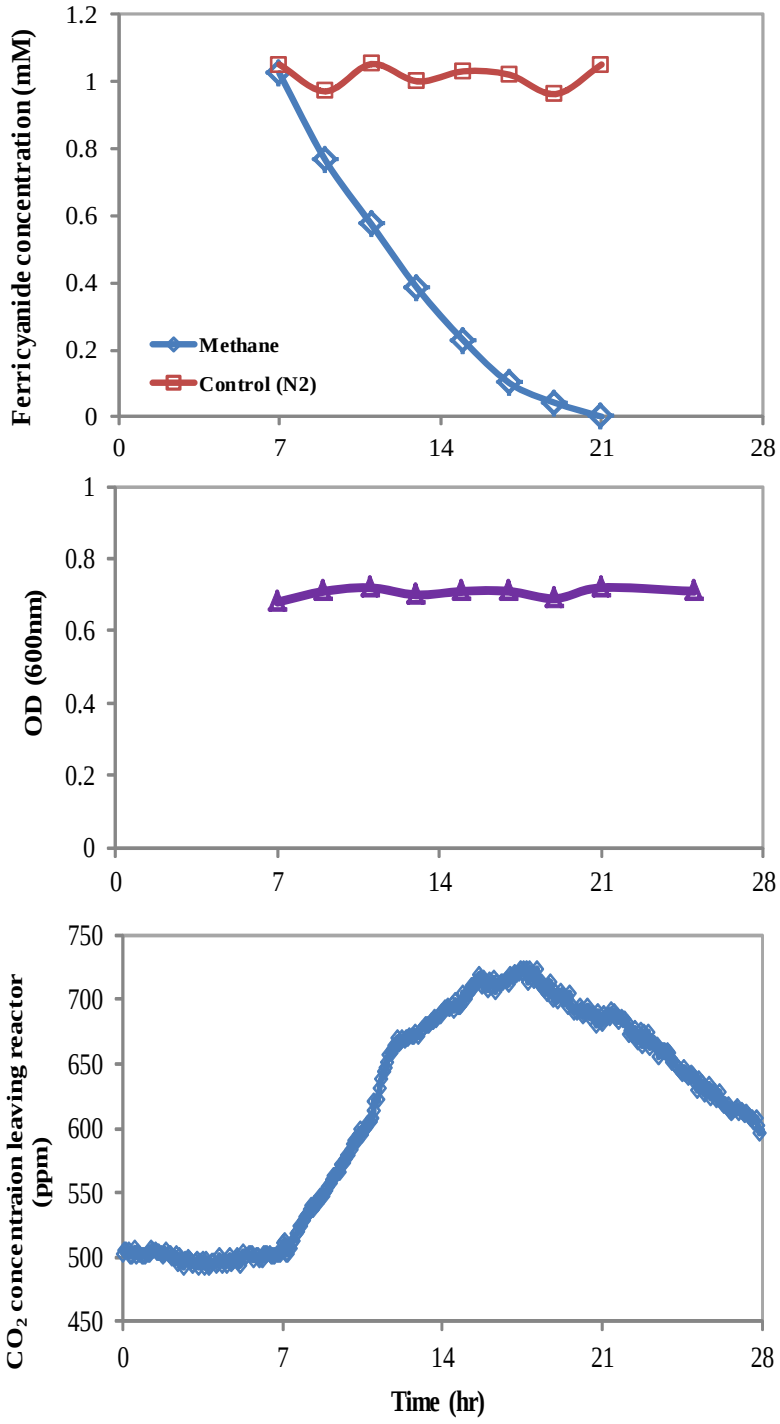

Figure 6-2 The profiles of 1 mM microbially reduced FR in the first cycle: (a). methane versus control; (b). cell densities, and ; (c). CO_2 concentration leaving reactor.

Absorbance measurements show the cell densities levelled off at around 0.7±0.01 during the first cycle (Figure 6-2b). These results correlate with the previous report for FR reduction in the presence of nitrate, in which FR reduction was not coupled with

ATP formation. Hadjipetrou et al. (1970) was not only observed the similar phenomenon with *E.coli* and FR as the microbes and the electron acceptor, but also when nitrite was used as the electron acceptor by *Aerobacter aerogenes* (Hadjipetrou & Stouthamear, 1965). It was claimed that in anaerobic condition, the synthesis of formate hydrogenase (which catalyzes the production of NADH) was repressed if insufficient ferricyanide is present as a result of formate (the electron donor) accumulation, even with other electron acceptor i.e. oxygen, nitrate, and nitrite (Billen, 1951; Gest, 1954; Pinsky & Stokes, 1952). Studies by Futai (1974) and Weiner (1974) in Rose and Tempest (1977) alo showed that no activity of NADH dehydrogenase or L-α-glycerol phosphate dehydrogenase in *E.coli* whole cells with membrane impermeable electron acceptor ferricyanide. Up to 3 mM of FR concentration used, the bacteria did show similar behaviours (maintaining the cell densities). However, it is not yet clear which enzyme that was repressed or was responsible in the reduction of FR. Based on 16S rDNA (Ecogene Auckland), *Acinetobacter calcoaceticus* was identified as the participating methanotrophs. *Acinetobacter sp* is one of methanotrophic genera that related to the phylum gamma proteobacteria (Tambekar et al., 2011). Further test need to be carried out to obtain whether methane monooxygenase involved in the reaction.

From CV data (Figure 6-3a), although no appreciable peak of FR or FRO at the end of the first cycle, there were significant peaks detected (E_o= 0.19 V versus SCE) at the end of the second and the third cycle. Based on the calculation, 2.4 mM of FRO was produced (derived from a calibration curve or Figure 6-3b) after the total addition of 3 mM FR into the methanotrophic suspension. This is another evidence of AOM coupled with FR reduction.

Eventhough several data have shown to accumulate evidence of AOM, ammonium (comes from N-source such as ammonium chloride) oxidation by methanotrophic bacteria is possible. Researchers have demonstrated that ammonium can inhibit methane uptake rates, because ammonium and methane compete for the same active site on MMO (King & Schnell, 1994; Whittenbury et al., 1970). Furthermore, nitrite via hydroxylamine intermediate is reported as the product of ammonium oxidation by many methanotrophs (Nyerges & Stein, 2009; Whittenbury et al., 1970). However the conversion of ammonium to nitrite by methanotrophs is known to happen only in the presence of oxygen (King & Schnell, 1994; Yoshinari, 1985). In this study, the consumption of methane during FR reduction was not measured, but no nitrite was

present (based on test strips measurements) in the system over the period of FR reduction.

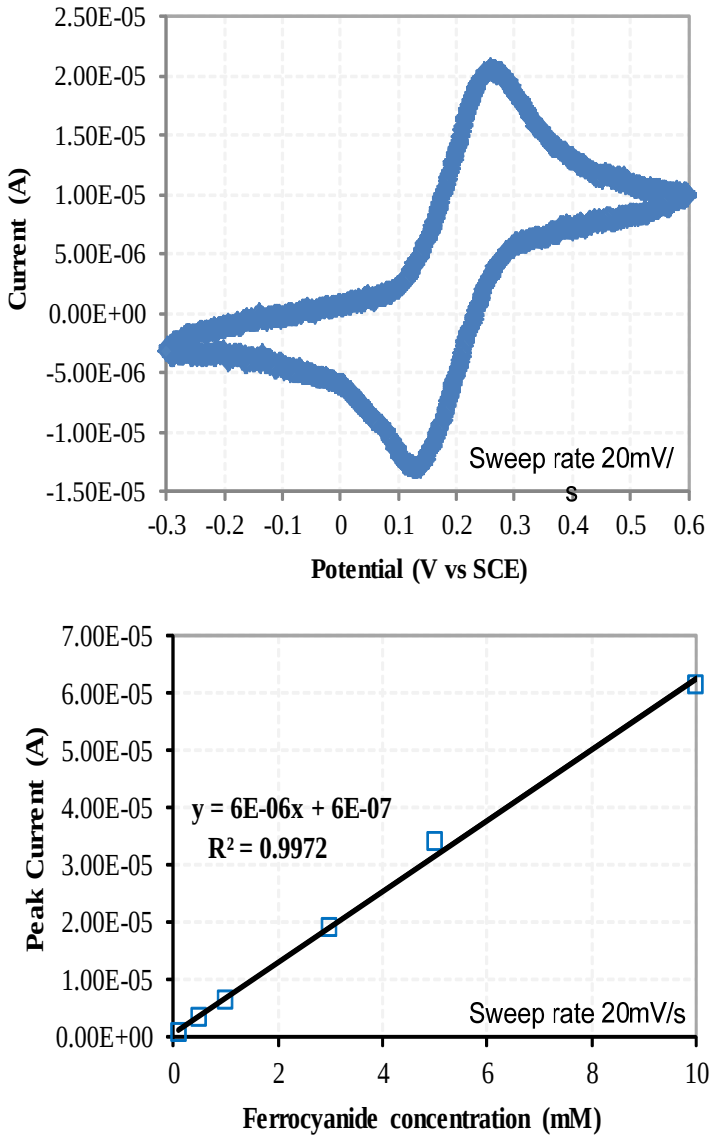

Figure 6-3 A voltammogram at the end of 3 mM of microbially reduced FR (a); and a generated standard curve of FRO using glassy carbon electrode (b).

When the air was re-introduced into the reactor at the end of the completed FR reduction in the third cycle, the color of FR changed from colorless to yellow based on visual observations (as it became re-oxidised if electrons were transferred to oxygen). This gave another evidence of the use of FR as the electron acceptor by the methanotrophic bacteria.

6.3.2 Microbial fuel cell performance

Tests of voltage outputs were performed following achieved a complete reduction of 1 mM FR by the methanotrophic bacteria. FR and $KmnO_4$ catholyte were used in order to improve the power and to avoid high polarization at the cathode (Ter Heijne et al., 2006; You et al., 2006). As shown in Figure 6-4, voltage (thus current) was generated in both MFCs during the start ups or connecting the resistance (no current observed with abiotic control). There was significant difference in voltage observed, the maximum voltage generated by MFC ($KmnO_4$-cat) was 0.31 V at 0.5 hr (Figure 6-4a), which was almost a factor of 1.5 higher than that of MFC (FR-cat) (0.21 V, Figure 6-4b). This was reasonably observed since $KmnO_4$ has a higher redox potential than FR i.e. 0.59 V (alkaline condition) compared to 0.36 V. Nonetheless, this is a proof showing that FR has received the electrons from the respiratory enzymes and transferred them upon reaching the electrode.

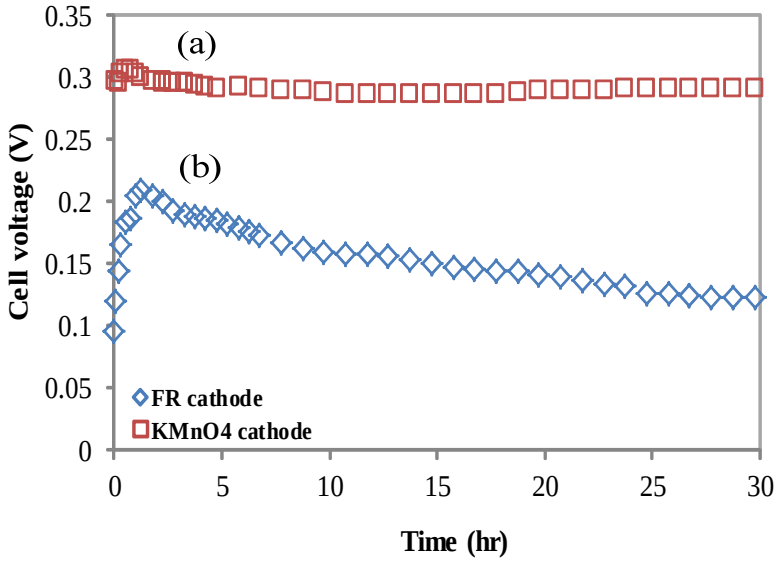

Figure 6-4 Voltage outputs of two MFCs under 9780 ohm resistance with 1 mM FR reduced by the bacteria for 30 hr period: (a). FR cathode; (b). $KmnO_4$ cathode.

The time required to a reach steady state (acclimate to a new resistance) varies in each MFC since different components (such as substrate and microorganisms) are used. One of the most prominent differences between the two MFCs is that MFC ($KmnO_4$-cat) achieved a steady state at 4.25 hr while MFC (FR-cat) reached it after 25.75 hr. It seemed that by using a strong oxidant (i.e. high redox potential) such as $KmnO_4$, allowed the MFC to achieve (six times) faster steady state and a higher (0.31 V)

voltage. The voltage value is almost comparable to mediated MFC works by Benneto et al. (1985) and Thurston et al. (1985) with a value of 0.35 V (both using *Proteus vulgaris* and thionine as the microbes and the mediator at a fixed load of 100ohm).

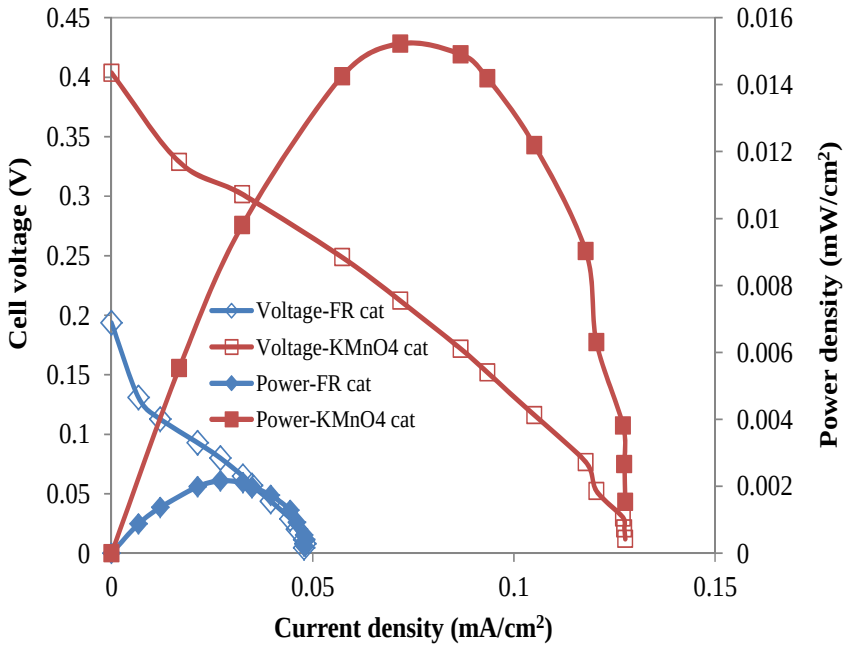

Figure 6-5 Polarization curves and power density curves of two MFCs with 1 mM microbially reduced FR at 35 hr.

Further investigation on the power output difference of the two MFCs, voltages and power densities assessment were performed under various external resistances at 35 hr (Figure 6-5). The $KmnO_4$-cat MFC had a higher both OCV and maximum power density. The OCV of $KmnO_4$-cat MFC was double than FR-cat MFC (0.4 V compared to 0.2 V). The maximum power density of $KmnO_4$-cat MFC was 0.015 mW/cm^2 with a value of more than seven times higher than that obtained in FR-cat MFC (0.002 mW/cm^2). However, in order to ensure a stable and a consistent operation of the energy generation or to have a good evidence of a sustained power by the methanotrophs, it is better to run the MFC over multiple batch cycles (i.e. feeding after substrate, in this case mediator) exhaustion.

The final investigation on the MFC performance using 3 mM FR reduced by the methanotrophs was further performed. This test was carried out using linear sweep voltammetry method and 50 mM FR cathode in PBS solution (graph not shown). At the scan rate 0.1 mV/s, the maximum power density obtained was 0.02 mW/cm^2 with OCV of 0.6 V. The power ouput obtained was still low, and the reason for this maybe due the

inability of bacteria to maintain the current production on the surface of the electrode. This can clearly be seen in a high activation overpotential observed (almost 0.3 V with a generated current of only 0.06 mA/cm^2). However, the OCV value was similar to the methane-MFC work and pure culture study by Van Hees (1965) but the current (scc) obtained was much higher i.e. 0.25 mA/cm^2 compared to just 0.003 mA/cm^2. A control with no mediator generated current density of 0.004 mA/cm^2. The power density achieved was still five times lower than Girguis and Reimer study (2009) i.e. 0.1 mW/cm^2, but it was used a consortium of methanotrophs instead of pure cultures.

6.4 Conclusions

In a laboratory scale reactor, the experiment showed that ferricyanide reduction by pure cultures of methanotrophic bacteria occurred in an anoxic condition and without the presence of any other alternate electron acceptor. CO_2 production, cyclic voltammetry data, no nitrite was present and reoxidising-state after introducing oxygen as other electron acceptor becomes valuable supported evidence of the reduction, although it was not coupled with the growth. Future works need to investigate the bichemistry of the system such as which enzymes that responsible for the reduction, or whether NAD/NADH is playing a role in maintaining the growth.

The current and voltage generation in microbial fuel cell reactor from the reduced ferricyanide confirmed that ferricyanide received electrons from the bacterial metabolism. The maximum power density of 0.02 mW/cm^2 and OCV of 0.6 V were obtained with 3 mM ferricyanide using LSV method.

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7 CONCLUSIONS AND FUTURE WORKS

7.1 Conclusions

A microbial fuel cell (MFC) combining a gaseous substrate and a mediator was developed in this study. The concept of a gaseous substrate mediated-MFC is purposely aimed for the integration of an MFC into an anoxic biofiltration system. The operating principle involves removing the gaseous contaminant by the anoxic biofilter and producing a stream of reduced mediator; and generating electricity and producing a stream of oxidised mediator to be recycled to the biofilter.

Other than the ability of the mediator to approach the bacterial active sites and collect the electrons, there are some important characteristics of a mediator that need to be considered to obtain a high performance in a mediated MFC (for a practical oxygen cathode). These include maximizing mediator concentration to give a high mass transport limiting current (the higher the concentration the higher the current that could be obtained). Equally important is the absorption, because the mediator should not be irreversibly absorbed by the bacterial cells or electrode surface, which could limit the current production. It is necessary for a mediator to have a large enough potential difference between the mediator and the enzyme active site for the electron transfer to occur. On the contrary, the redox potential of mediator should not be too far from the redox potential of the enzyme (or too close to the terminal electron acceptor at the cathode) in order to maximise the cell voltage (consequently the power output) of the fuel cell (note that the open circuit potential in a MFC relies on the potential difference between the mediator and the cathode). Last but also equally important, the kinetics of the microbially reduced mediator oxidation on the electrode surface should be fast, and can be obtained by increasing the concentration of mediator and by increasing the roughness (active site) of the anode electrode.

Among all of the mediators investigated, the best performance was achieved with ethanol using *N,N,N',N'*-tetramethyl-1,4-phenylendiamine TMPD (N-TMPD) as the mediator. The typical MFC characteristics were evaluated using a conventional laboratory scale two chamber MFC with the linear sweep voltammetry method. The OCV achieved with 50 mM ferricyanide catholyte in phosphate buffer solution (PBS)

was 0.81 V. The maximum power output was 0.16 mW/cm^2 with 1 mM N-TMPD (carbon cloth electrode) and improved almost two fold by increasing the concentration of N-TMPD from 0.2 mM to 1 mM). The reason for the high power outputs with N-TMPD was probably due to a high electron transfer rate between N-TMPD and the anode since the experiments were conducted under conditions were the reduced mediator concentration was high (as the mediators were left to become colorless/transparent before the current were measured).

The redox kinetics of the microbially reduced mediators on glassy carbon electrode (determined using CV) all showed quasi reversible behaviors (i.e. the electron transfer were not very fast) when they are compared to the CV obtained in the absence of the bacteria. These characteristics were concluded after observing the peak separation potentials (ΔE_p); the inequality in the ratio of peakheight currents (Ip_a/Ip_c); the shift of Ep_a and Ep_c values with the scan rates (to more positive for *Ipa* or to more negative for *Ipc*); the unproportional increase of peak current with increasing scan rate (Ip vs $\nu^{1/2}$); and the ratio of heterogenous rate constants (k_o) to $\sqrt{\frac{nF\nu D}{RT}}$ or ($\wedge$). These behaviors were thought to be caused by the bacteria decreasing the amount of the reduced and the oxidised mediator available at the surface of electrode, therefore, the level of the electrons being flowed from the mediator to the electrode. However, the oxidation-reduction of microbially reduced N-TMPD has shown to exhibit faster kinetics than FR and TH mediator (i.e. k_o = 6.75x10^{-3}±0.4 cm/s and i_o = 0.13±0.01 mA/cm^2). N-TMPD is well known with its two stages reversible oxidation-reduction reactions, and this was thought to give the contribution to the fastest kinetics observed with N-TMPD. Increasing N-TMPD concentration by five times also contributes to the increase of the exchange current, i_o, by almost three times.

The material and surface area of anode electrodes highly influence the exchange current for the reaction and thus the power output in an MFC. This study showed an improved of the power output by more than five fold when the glassy carbon electrode (0.03 mW/cm^2) was replaced with carbon cloth electrode (0.16 mW/cm^2) using 1 mM N-TMPD, therefore selection of the material and surface area of the electrode is important to enhance the performance of a (mediated) MFC. Glassy carbon electrode is a suitable anode material for studying mediator electrokinetics due to its well defined surface area

and easier activation procedures. While carbon cloth electrode can be used to enhance the power production of a MFC because of the high surface area, however it was difficult to predict electrokinetics on carbon cloth electrodes due to the non-uniform pores structure of the electrodes.

In the presence of a low concentration of mediator, the cyclic voltammetry (CV) method gave a better estimation of the kinetic parameters than linear sweep voltammetry (LSV) method. This is because the mass transfer limited current occurs at fairly low overpotentials (~100 mV) for some mediators which limits the overpotential range at which Tafel behaviour was observed. Whereas estimating the rate of electron transfer using the CV method was more conveniently obtained since the voltammogram shapes and values could easily determine the reversibility of a redox process.

In this study, the observations showed that N-TMPD was the best performed mediator in the ethanol fed MFC. The reasons proposed for this include:

- it was reduced very fast by the bacterial cells (high reactivity between N-TMPD and a redox enzyme inside the cells);
- it had limited absorption by the bacterial cells;
- it gave a high cell voltage;
- it produced a high mass transport limiting current, thus giving a high power ouput;
- it was kinetically faster than FR and comparable with TH (with its two electron transfer) due to its two reversible redox steps, and the kinetic parameters are comparable with the conditions without microbes (literatures).

In this research it was discovered, to my knowledge for the first time, that a pure culture of methanotrophs bacteria that was isolated from soil could couple oxidation of methane in an anoxic condition utilizing ferricyanide as the final electron acceptor. The discovery was supported by ferricyanide peak heights disappearance (spectrophotometry), CO_2 production (sensor readings), ferrocyanide formation (cyclic voltammetry), and by testing that no other alternate electron acceptor was present. Further evidence was made when ferricyanide reduction stopped as oxygen was introduced into the anoxic reactor. This finding was an important breakthrough in the

area of anoxic methane oxidation (AOM) which is believed to occur mainly in deep sea with a consortium of microorganisms (sediments). This also represents soil as an important sink for mitigating methane not only in an aerobic condition but also in an anaerobic condition. The observation have shown that ferricyanide was being used as the electron acceptor by the methane oxidizers, and heterotrophic bacteria mostly found in soil has been known for a long time could use ferricyanide ion instead of oxygen as the electron acceptor in their respiratory pathway. Ferri- and ferrocyanide is recognised as contaminants present in soil and groundwater as a result of human related activities.

This finding was also an important breakthrough in converting methane directly into electrical energy in MFCs. The maximum power output achieved with 3 mM ferricyanide in the methane-mediatored MFCs was still low (0.02 mW/cm^2) using linear sweep voltammetry method (although the OCV obtained was 0.6 V). One hypothesis for the low power ouput obtained was maybe due the inability of bacteria to maintain the current production on the surface of the electrode. This can clearly be seen in a high activation overpotential observed (almost 0.3 V with a generated current of only 0.06 mA/cm^2), and easily achieved a mass transport limiting current (0.25 mA/cm^2).

The concept of combining a gaseous feed (contaminants) with a mediator in a MFC represents a highly promising sustainable and environmentally-friendly source of energy. Also its integration with an anoxic biofilter will increase the value of the gaseous contaminants (while reducing the emissions) since simultaneously it can be made to produce electricity. Additionally, the electron transfer to the anode (thus current generation) is enhanced by the presence of mediator (an increase of more than twenty fold with the poorest mediator tested). These results also mean expanding the probability the range of suitable gaseous substrates used to provide a substantial alternative energy source such as to treat high volatile organic compound (i.e. benzene, toluene, ethylbenzene). Determining a better fuel cell design and operating conditions along with identifying possible constraints could help to improve the performance of the MFCs.

7.2 Suggestions for future research

Recently, MFCs are considered as a promising technology for simultaneous production of renewable electricity and treatment of organic wastes. The intensification of research

has allowed the applications of this technology and the diversity of the fuels that could be utilized, including gaseous substrates. In this study, it was shown that by combining a gaseous substrate with a mediator in a MFC to generate electricity is possible, and a range of utilizable mediators have been tested. However, the application of the gas feed-mediatored MFCs requires further improvements in the power outputs and efficiency of the systems. Such improvements require identification, characterization and optimization that affect the performance of MFCs (i.e. biological, electrochemical and engineering factors).

Until now, there have been no reports on an ethanol-mediatored MFC. Attempts have been made to identify the best performed mediator for an ethanol-fed MFC and a significant power output was obtained (e.g the OCV value was almost comparable with the highest OCV value reported in mediatored MFC). However, efforts that would improve the performance must continue to be made. The example to this is such as finding the optimum concentration in order to ensure a high electron transfer rate (high mass limited currents) and investigating the stability of the mediator over long term experiments. At the same time, increasing the kinetics on the electrode surface or searching for an alternative anode material that gives faster and more predictable kinetics would be two possible solutions. In this study, most of the catholyte used were ferricyanide. It is a good chemical to be utilised as a catholyte in laboratory experiments, but it is not practical for large scale applications. Oxygen is commonly known to have a high overpotential if it is used at the cathode, but it would never run out, and reducing this barrier also give a great potential to increase electricity production in MFCs. Therefore the final goal should be planned to overcome activation, ohmic, and mass transport overpotentials in both of the MFC compartments while designing a better fuel cell configuration.

This work has shown that ferricyanide was used as the electron acceptor by the methanotrophic bacteria in anoxic conditions, and several results have shown valuable insights (as were mentioned previously). However, there are yet many questions unanswered therefore more work is needed to obtain detailed fundamental information such as: which enzyme is responsible for ferricyanide reduction; what is the underlying principle of the electron transfer by the methanotrophs in the presence of ferricyanide; is methane being oxidised and how; how many ferricyanide reduction cycles can the

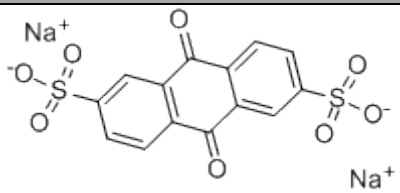
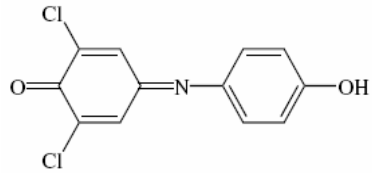
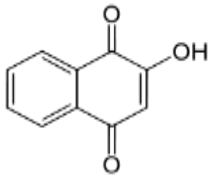
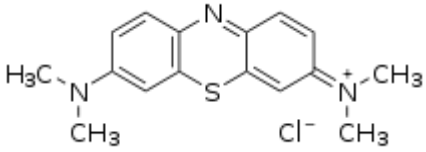
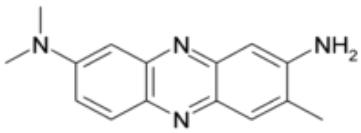
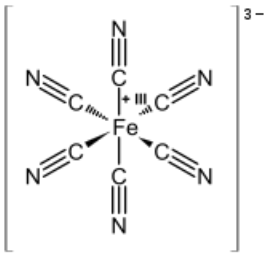
methanotrophs perform (e.g. how much ferricyanide can the microbes reduce under anoxic conditions); are there any parameters or operational conditions that could control or predict the reduction; can other electron acceptors / mediators be used; will current be generated over multiple cycles and how can it be improved? This methane mediatored MFC is far from its potential capacity, but the study has shown the electricity can be produced from the reduced form of ferricyanide. Searching for more suitable mediator to improve the power output in this type MFC is required.

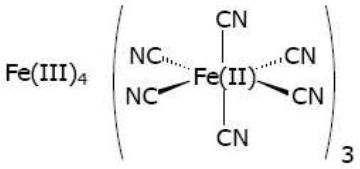
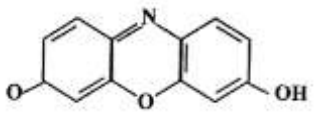
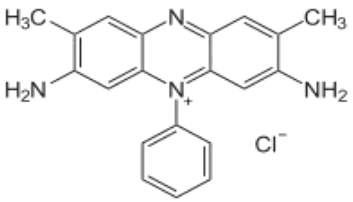
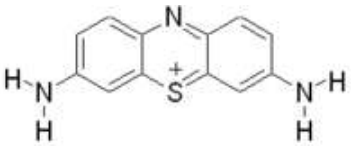
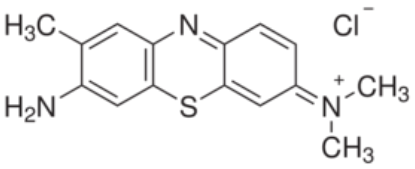
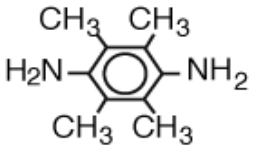
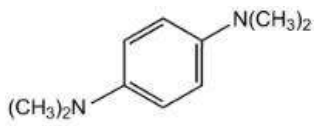
The most important and immediate future work after obtaining an optimised performance of the gaseous substrate mediated MFCs is its integration into an anoxic biofilter. Thus the parameters or the operational conditions that are required for maintaining the electricity generation could be obtained. Right now MFCs have not been put into commercialization, but an integration of MFCs into other existing technology (i.e. biofiltration) should lead the MFCs to be economically viable. Undoubtedly, the inventions in this study, and the advancements in science and technology would be able to solve not only issues in MFCs, but also be able to provide a solution to the environmental and energy crisis issues.

APPENDICES

Appendix 1

Redox potential of mediators used in the microbial fuel cell study.

Mediators	Redox potentials (V vs NHE)	Structural formula
9,10-anthra-quinone-2,6-disulfonic acid disodium salt (AQDS)	-0.184	
2.6-Dichlorophenol Indophenol (DCP)	0.217	
2 hydroxy 1,4-naphtaquinone (HNQ)	-0.137	
Methylene blue (Luvsanjamba et al.)	-0.021	
Neutral red (NR)	-0.325	
Potassium ferricyanide (FR)	0.360	

Prussian blue (Campbell)	0.380	
Resorufin (RS)	-0.051	
Safranine_O (Manohar et al.)	-0.289	
Thionine acetate (TH)	0.064	
Toluidine blue-O (TOB)	0.034	
2,3,5,6-TMPD	0.220	
N',N',N',N'-TMPD	0.278	

Appendix 2

Dimensional parameter, ψ, as a function of peak separation ΔEp in cyclic voltammogram for Nicholson-Shain's method (Nicholson & Shain, 1964).

ψ	**Δ*Ep*** **(298 K)**
19.20	60.0
11.50	61.0
8.40	62.0
6.45	63.0
5.10	64.0
4.30	65.0
3.63	66.0
3.16	67.0
2.81	68.0
2.51	69.0
2.26	70.0
1.51	75.0
1.11	80.0
0.92	85.0
0.77	90.0
0.65	95.0
0.57	100.0
0.50	105.0
0.44	110.0
0.39	115.0
0.36	120.0
0.32	125.0

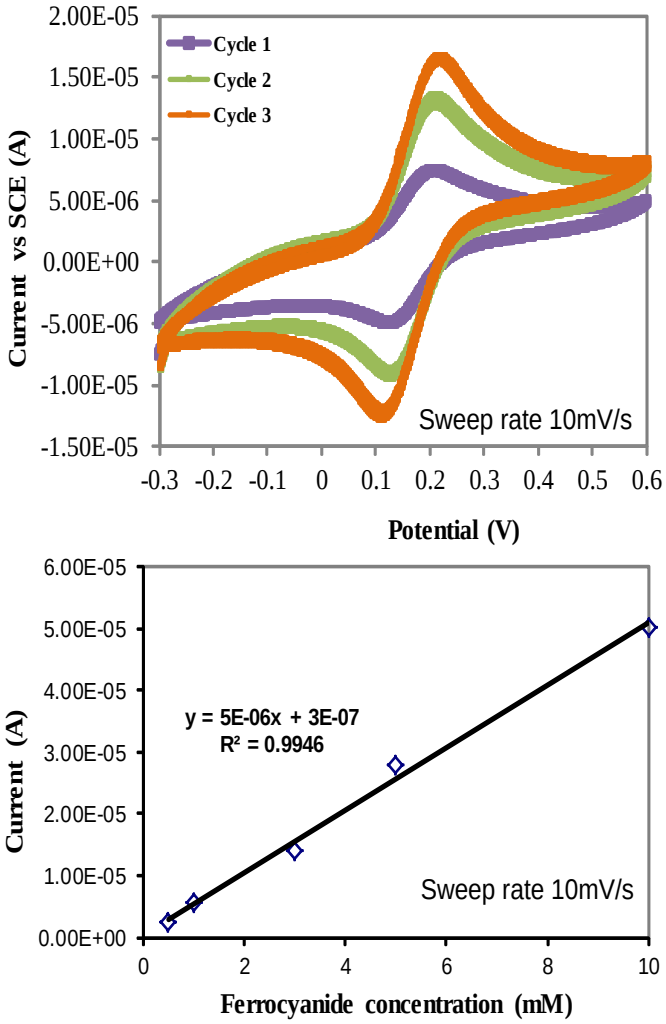
Appendix 3

Redox potentials (E^o) of electron acceptors that could be used by microorganisms for the AOM modification(Thauer & Shima, 2008).

Redox couple	n	E^o vs NHE (V)
CO_2/CH_4	8	-0.24
S^o/H_2S	2	-0.27
SO_4^{2-}/HS^-	8	-0.22
SO_3H^-/HS^-	6	-0.12
APS/SO_3H^-	2	-0.06
CH_3SH/CH_4+H_2S	2	-0.01
$Fe(OH)_3/Fe^{2+}$	1	+0.01
Fumarate/Succinate	2	+0.03
Dimethylsulfoxide/dimethylsulphide	2	+0.13
CH_3OH/CH_4	2	+0.16
NO_2^-/NH_3	1	+0.2
NO_2^-/NH_3	6	+0.33
NO_2^-/NO	1	+0.34
$FeCN_6^{-3}/FeCN_6^{4-}$	1	+0.36
Mn^{4+}/Mn^{2+}	2	+0.41
NO_3^-/NO_2^-	2	+0.43
MnO_2/Mn^{2+}	2	+0.52
$2NO_3^-/N_2$	10	+0.76
O_2/H_2O	4	+0.84
$2NO/N_2O$	2	+1.2
N_2O/N_2	2	+1.36
$CH_3(radical)/CH_4$	1	+2.1

Appendix 4

Voltammogram of ferrocyanide determined at the end of each cycle in the second test (E^o = 0.17 V vs SCE) and a generated standard curve using GC electrode.



Appendix 5

Example calculation for CO_2 production (amperometry cycle 1 Table 5-3).

From Equation 6c:

$CH_4 + 8FeCN_6^{3-} + 2H_2O \rightarrow 8FeCN_6^{4-} + CO_2 + 8H^+ \qquad E^o = 0.6\ V$

Put the actual conc from 5-1 used in this table, can't follow where 0.83 mmol comes from

FRO produced (Table 5-1)	0.83	mmol
In 200 mL solution	0.1668	mmol
	0.00017	mol
mol CO_2 produced	0.000021	mol
P	1	atm
R	0.082	Latm/mole.K
T	298	K
Vol. CO_2 produced	0.00051	L
	0.51	mL
Prod. rate of CO_2		
(39 hrs reduction)	0.00022	mL/min
CH_4 at the outlet	5	mL/min
CO_2 mol fraction	4.35E-05	mL/mL or mol/mol
Convert to ppm($x10^6$)	43.5	ppm

Appendix 6

Theoretical CO_2 production

At inlet:	CH_4	0.5	ml/min		
	N_2	2.5	ml/min		
	total	3	ml/min		
	P	1	atm		
	R	0.082	Latm/mol.K		
	T	298	K		
Equation:	**$CH_4 + 8FeCN_6^{3-} + 2H_2O$**		**⟶**	**$8FeCN_6^{4-} + CO_2 + 8H^+$**	
	to reduce	1mM $FeCN_6$	1	mmoles/L	
		in 200 mL	0.2	mmoles	
	will need		0.025	mmoles	CH_4
	will produce		0.025	mmoles	CO_2
			0.000025	moles	CO_2
	Ideal gas law:	VCO_2	0.00061	L	
			0.61	ml	
Production rate of CO_2 (14 hr reduction)			0.0007	ml/min	
CH_4+N_2 at outlet∞			3	ml/min	
Total flow			3	ml/min	
Concentration CO_2 (fraction mol)			0.00024	ml/ml or mol/mol	
Convert to ppm ($x10^6$)			242	ppm	(theoretical)
From the experiment (area under curve 6-2c)			148	ppm	(∞0.122 mmoles FR)
Experimental/theoretical			61	%	