DEVELOPMENT AND ELECTROCHEMICAL CHARACTERIZATION OF A *PSEUDOMONAS AERUGINOSA*-BASED PURE CULTURE MICROBIAL FUEL CELL

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DEVELOPMENT AND ELECTROCHEMICAL CHARACTERIZATION OF A *PSEUDOMONAS AERUGINOSA*-BASED PURE CULTURE MICROBIAL FUEL CELL

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ABSTRACT

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Microbial fuel cells (MFC) are fuels cells that utilize microorganisms as catalysts for the production of electricity. MFCs have important potential for power generation in remote locations and to complement waste water treatment facilities with waste removal and power generation. In order to achieve these goals, power output needs to be increased as well as an understanding the effect that varying electron donors has on electricity production. In order to move the field forward, the research presented herein utilizes the γ-proteobacteria *Pseudomonas areuginosa* in pure culture for electricity production. Most research for MFCs utilizing *P. aeruginosa* have been run using mix cultures and taking advantage of electron mediators produced by *P. aeruginosa* to increase power output of MFC. While this tends to improve power density for the MFC, it prevents studies that elucidate the effects of individual species.

These studies utilized *P. aeruginosa* as the sole biological catalyst to oxidize organic substrates and produce electrical power. A two-chamber H-style MFC constructed from polypropylene bottles and using a 1 mil Nafion® membrane was utilized for these experiments. Using this setup, several issues were investigated including the

effect of the growth substrates glucose, formate, succinate, lactose, and cellobiose on cell growth and electricity production and the effect of glucose and succinate concentration on cell growth and electricity production. It was found that *P. aeruginosa* is capable of oxidizing glucose and succinate to generate electricity. When using glucose as electron donor, the maximum power density was 46 mW/m^2 with the current of approximately 0.35mA – 0.45mA (peak value 0.7 mA) and the electrical potential of approximately 0.05 to 0.15 V. When using succinate as electron donor, the power density was nearly 40.6 mW/m^2 with current of 0.39-0.41mA and the electrical potential of approximately 0.1-0.13 V.

The most suitable substrates for growth and electricity production were glucose and succinate; which are also the important carbon sources/intermediate substrates in glycolysis/tricarboxylic acid cycle. The optimum concentration of substrates in these studies was found to be 0.7% (w/v) each of glucose or succinate in mineral salt medium. At this level, the batch MFC model can maintain the maximum power output more than three days. Higher concentrations of substrate did not increase net power and, in fact, led to a reduced power density.

Other common substrates, such as acetate and formate, which had been utilized as electron donors in many MFCs, were not suitable for MFC solely using *P. aeruginosa*. Not only was there no net power output, but also the bacteria could not survive and grow in medium with these substrates as the sole carbon source. These findings confirm that *P. aeruginosa*m, and more generally other individual bacteria species, can only utilize specific substrates. This suggests the mixed cultures would be more efficient in processes

such as wastewater treatment, which contains many types of electron donors at comparatively low concentrations.

Dedicated to my parents and motherland

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$\boldsymbol{I}$	Current
$\boldsymbol{E}$	Potential
$\boldsymbol{P}$	Power
$\boldsymbol{P_{density}}$	Power Density
$\boldsymbol{S}$	Surface area

CHAPTER 1

INTRODUCTION AND OVERVIEWS

For the second time in less than a decade, the price of petroleum has exceeded $100 per barrel. Along with the increase in petroleum prices has been a resurgent interest in alternative energy, there is currently research in many areas of alternative energy including methanol and hydrogen fuel cells, wind power, cellulose derived fuels and solar power. Another sustainable energy field that has started to receive attention in the last decade is microbial fuel cells. This paper presents research performed on microbial fuel cells to understand the role of electron donors on system performance.

In general, a microbial fuel cell (MFC) is a device that generates electric power by utilizing bacteria. Compared to other present alternative energy with many respective shortcomings, such as solar power (low efficiency), wind power (limited by location) and nuclear power (lack of safety and high investment), which restrict their wide application like fossil fuel, MFCs reserve common advantages of normal fuel cells which are high fuel conversion efficiency and simplicity of design; moreover, MFCs are more environmentally friendly in that they are capable of utilizing a wide range of renewable fuels [1]. Actually, one attractive advantage of MFC is capacity of generating power when fed with wastewater; that requires the bacteria can consume different kinds of substrates in anode medium from inorganic compounds to organic compounds.

The microbial fuel cells have similar structure as methanol fuel cell (DMFC) with electrochemical processes that take place in the electro-catalytic anode and cathode [4]. The differences between them are that a MFC utilizes anaerobic bacteria not a chemical reaction to oxidize substrates (e.g. glucose, methanol or industrial wastewater) and produce electron and proton. And then it uses anode as a solid-state intermediate electron acceptor. Electrons then flow through a circuit to the cathode, where they reduce oxygen (terminal electron acceptor) with proton and the cathode process can also be catalyzed by microorganism.

A typical design of a microbial fuel cell consists of two chambers with anode and cathode electrodes separated by proton exchange membrane [6]. This membrane functions as a permeable way for transport of protons across the membrane to the cathode where they combine with oxygen and electrons to form water. This typical reaction at the cathode needs a metal catalyst, such as platinum, but many other options have been explored recently including use of microorganisms in the cathode chamber [6].

Reactions 1 and 2 show the two simplified reactions in MFCs. Initially, bacteria oxidize the substrates and generate electrons (e^-) and protons (H^+). To complete the circuit, the electrons flow through the circuit to the cathode and protons pass through the membrane to the cathode chamber where they react to reduce oxygen, producing water at the cathode.

Anode Chamber $$\text{Glucose} \xrightarrow{\text{Bacteria}} \text{Oxidated Product} + H^+ + e^- \quad (1)$$

Cathode Chamber $$H^+ + e^- + O_2 \longrightarrow H_2O \quad (2)$$

The theory of MFCs originated from bioelectric phenomena observed by Luigi Galvani in 1790 [2, 4]. In 1912, Potter invented the first MFC model with *Eschericha coli* and *Saccharomyces*. Then Cohen produced another MFC model with potential of 35 V [5]. MFCs, however, did not get much attention and improvement before 1960s since the lack of understanding of MFCs' theory and regular engineering technique restricted setting regular MFC models up. After NASA attempted to find an alternative energy source for its spacecraft, MFCs had begun to catch the scientists' interests [5].

In the 1990's, Bruce Logan and his colleagues [6] showed that electricity could be generated when the substrates, such as glucose or wastewater contained organic compounds, were fed to the microorganism in the anode chamber of MFC [7]. Over the past decade, the power of MFCs has increased very much along with the deeper understanding of the mechanism of electron generation and transport in the MFCs, which has nearly reached the lower limit of commercial fuel cell (near 10W level) [5]. However, due to challenges with technical feasibility and high costs, current MFCs still cannot compete with conventional fuel cells. For example, to increase efficiency of cathode, many researchers prefer to utilize Pt-catalyzed cathode or ferricyanide as continual-fed catholyte. These methods increase the cost of MFCs and reduce sustainability [3, 5].

Based on the sources of fuel, microbial fuel cells could be divided into two basic types: 1. Cells which use a primary fuel (usually an organic waste such as corn husks) and generate a material such as hydrogen, which is then used as a secondary fuel within a conventional hydrogen/oxygen fuel cell. 2. Cells which generate electricity directly from an organic fuel such as glucose, using either enzymes or bacteria. These MFCs look likes a combination of a bioreactor and a common fuel cell, the most attractive point is that the

MFCs are able to consume large quantity and wide range of organic wastes from industry while they are generating electric power [1]. Therefore, we receive "double" benefits, including energy and environment, from operation of MFCs.

Power calculations

The power output by an MFC is calculated from the measured voltage, E_{MFC}, across the load and the current as

$$P = I \times E_{MFC} \tag{3}$$

And the current can be expressed by $I = E_{MFC}/R_{ext}$, Equation 3 is transformed to (when $R_{ext} >> R_{int}$)

$$P = \frac{E_{MFC}^2}{R_{ext}} \tag{4}$$

For comparing the efficiency of different kinds of MFCs with different electrodes (sizes, shapes and materials) is necessary to introduce a new concept, Power density, as shown in Equation 5.

$$P_{Density} = \frac{E_{MFC}^2}{R_{ext} \times A_{electrode}} \tag{5}$$

Where $A_{electrode}$ is the surface area of electrode

Different sizes of MFCs have varied net power output, it is necessary to find a united criterion to compare power of MFCs. Usually the cathode reaction could be the controlling step in the whole process of MFCs, and the surface area of cathode often be affected by the varied designs of MFCs [3]. Therefore, it is reasonable to divide power generation by the cathode surface area, A_{cat}, to eliminate effects from volumes, shapes and structures of different MFCs (Equation 6).

$$P_{cat} = \frac{E_{MFC}^2}{R_{ext} \times A_{cat}} \tag{6}$$

The power in Equation 6 is the theoretical value, however, the actual power generation of MFC is also affected by internal resistance R_{int}, which can be viewed as the efficiency of electron transfer in the MFCs. To account for this, open circuit voltage (OCV) is used in place of E_{MFC} [3]

$$P_{OCV} = \frac{OCV^2}{(R_{int}+R_{ext})\times A_{cat}} \tag{7}$$

The maximum power output possible, P_{max}, for a calculated cell electromotive force (3), is

$$P_{max} = \frac{OCV^2}{(R_{int}+R_{ext})\times A_{cat}} \times \frac{R_{ext}}{(R_{int}+R_{ext})} = \frac{OCV^2\times R_{ext}}{(R_{int}+R_{ext})^2\times A_{cat}} \tag{8}$$

This equation is used to calculate the theoretical power output of MFCs (3).

According to Equation (8), the internal resistance is essentially the efficiency of electron transport from anode to cathode. Hence, one important method of improving net power is decreasing internal resistance which reduces the power generated by MFCs lost to the internal resistance. In other words, increase the efficiency of electron transfer in the MFCs. There have been many methods reported to reduce internal resistance of MFCs from 1960's [1, 8], including transfer intermediate or catalyst, biocathode, modification of electrode and utilizing particular bacteria.

Composition of MFC system

As previously mentioned, the MFC consists of anode, bacteria, cathode, possibly a PEM membrane, etc. These components will be discussed below.

Anode

Anode Bacteria

The bacteria in the anode chamber of MFCs are maintained in an anaerobic environment. The first bacteria applied in MFCs were *E. coli* which utilized methylene

blue as an electron mediator [8]. Since then, many species were reported to be utilized in the MFCs with presence of artificial mediator, such as *Proteus vulgaris* (thionine as mediator) [9], and *Gluconobacter oxydans* (osmium containing polymer as mediator) [10]. Among these anaerobic bacteria, *P. aeruginosa* is extremely attractive for its potential capacity of not only oxidizing substrates to generate electrons, but also transferring electrons to the electrode via self-produced phenazine-based mediators [11, 12]. *P. aeruginosa* could enhance electron transfer rates by the presence of the produced mediators in mixed microbial systems [13]; it let *P. aeruginosa* become a good option in recent MFCs research.

Transfer mediators

For the mechanism of electron transfer in the anode, Rabaey and Boon [13] present four possible paths for electron passing from cell to the electrode in pure bacteria culture or mixed microbial system: i) Mediator producing bacteria use mediators to transfer electron to the anode surface; ii) Non-mediator producing bacteria utilize artificial mediators in the solution to transfer electron; iii) Non-mediator producing bacteria utilize mediators produced by other cells to achieve electron transfer; iv) The bacteria form a biofilm on the surface of anode and transfer electron directly [9]; therefore, there are two main mechanisms of electron transfer from bacteria to the electrode.

The first one is mediated transfer which utilizes exogenous or endogenous electron “shuttles” to transport electron from bacteria membrane to electrode surface. Methylene blue and thionine were both very popular artificial (exogenous) mediators in previous research [14, 15]. However, since artificial mediators cannot refresh by

themselves and need to be supplied continuously during long-term MFCs experiments, the cost becomes a serious limitation for extended operation. Natural (endogenous) mediators are usually secondary metabolism products of microorganisms [11]. They can be continuously produced and refreshed continuously by microorganisms in MFC, so the cost will decrease. One kind of natural mediator in the MFC experiments is pyocyanin (Figure 1), which is produced by *P. aeruginosa* (though it can be synthesized artificially) [11, 12]. Pyocyanin is a redox-active antibiotic pigment which allows *P. aeruginosa* to kill other hazardous cells. Its redox-activity can also be utilized to accept or donor electrons and therefore support electron transport. Rabaey and Boon [13] found that the power output of KRP1- phzM was positively stimulated upon addition of pyocyanin to the MFCs. The peak power output increased from 77 to 409μW/m^2, which resulted in an average power increase over the 145 hour experimental period from 40 to 95μW/m^2. The ability of pyocyanin to improve power output has brought a number of research efforts to evaluate its use in MFCs [5, 11, 12, 13]. Nevertheless, producing these endogenous mediators could increase requirement for anabolism energy ($\Delta G_{0\ biol}$), which would reduce electrical energy generation, so that it is necessary to make an optimum balance for distribution of total energy between electricity generation and secondary metabolism [5].

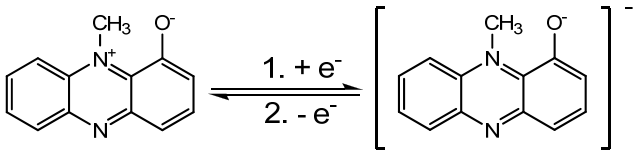
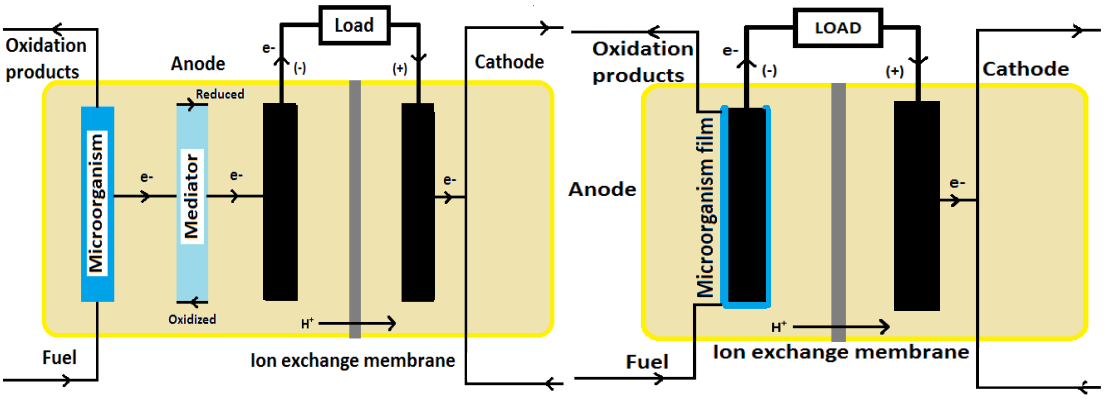


Figure 1: Redox reaction of pyocyanin

Direct transfer and biofilm

As an alternative to suspended microorganism in the anode medium, the bacteria can attach on the surface of electrode with a physical/electrical contact, then they can transport electron with "direct transfer" mechanism (Figure 2a). In this mechanism, bacteria utilize the outer membrane redox macromolecules to connect electrode with electric charge bonds, such as cytochrome c proteins [16, 17], then they can transfer electron to electrode follow the electron transport chain from *in vivo* to electrode. Besides redox macromolecules, recent research found that some microbial strains can build up electronically conducting molecular nanowires to create physical electrical connections with electrode without direct contact [18]. By either redox macromolecules or conductive nanowires, bacteria are able to not only attach but also grow and metabolize the substrates (e.g. glucose) on the surface of electrode just like a "film" formed on the outside of electrode (Figure 2b). Many articles reported that this biofilm can increase the efficiency of electron transfer which may be due to reduction of distance and intermediate steps. For example, Allen and Bennetto found that the immobilization of *P. vulgaris* onto graphite electrode led to faster response to substrate addition [19]. *P. aeruginosa* has been shown to attach to the surface of electrodes, which would promote in electricity generation. Present biofilms are still mobile, or semi-mobile at least, so that their performance may be influenced by fluid flow in the MFCs. If it is possible to immobilize bacteria on the surface of electrode like immobilization of enzyme applied in bioengineering industry, the efficiency is expected to be further enhanced.



(a) (b)

Figure 2: Sketch of mediated transfer and biofilm transfer in MFCs.

Cathode

Biocathode

Usually MFCs have abiotic (microorganism free) cathode which is of simple construction since it only transfers electrons to the terminal electron acceptor. Because of slow oxygen reduction rates on the surface of graphite electrodes, catalysts or artificial electron mediators are necessary. Platinum is the most popular catalyst for oxygen reduction in MFCs due to its excellent catalytic ability. But its application is limited by the excessive cost and possible poisoning by components in the substrate solution [20]. Potassium ferricyanide is a commonly-used artificial electron mediator; however, despite its excellent performance, ferricyanide is not considered a sustainable chemical, since it requires continuous replenishment [21].

Sometimes the anode bacteria could go through the PEM membrane and into cathode chamber or be intentionally inoculated into the cathode. These have been shown, similarly to anodic microorganisms, to be present either in suspension or can develop a biofilm on the surface of cathode electrode. According to some research, rather than

preventing microbes from establishing themselves on cathode, bacteria could be used as biocatalysts to accept electrons from the cathode electrode [1]. Rhoads had set up a MFC whose cathode was porous graphite with *Leptothrix discophora* suspension. The suspension contains manganese which is oxidized to MnO_2 by the bacteria in the presence of oxygen. The resultant biomineralized MnO_2 was then reduced at the cathode surface without an electron mediator. It was found in this case that power densities of 12.7 $\mu W/cm^2$ could be obtained from these cells [21]. In addition to bacteria, enzymes have also been utilized to catalyze the reduction of oxygen at the cathode as an alternative to platinum. Willner [22] showed that microperoxidase can be immobilized onto a gold cathode and along with a quinone modified cathode and utilized in a biofuel cell fuelled by NADH and H_2O_2. He and Angenent [20] also reported that similar cathodes were used along with apoglucose oxidase/quinone/flavin adenine dinucleotide phosphate modified anode to build a glucose/H_2O_2 fuel cell with a power output of 32 μW.

In general, biocathode MFCs have microorganism both in the anode and cathode, and cathode microorganism can function as catalysts to assist the electron transfer or even produce oxygen under special condition (algae) without application of platinum or potassium ferricyanide, which means it avoids the disadvantage of high cost in abiotic cathode MFCs [20].

Anaerobic cathode

In spite of other improvement of MFCs, anaerobic cathode focuses on not only increasing efficiency of electron transfer, but also enhancing its capacity of treating waste organic compounds. In most cases of microbial fuel cells, the cathode chambers are

aerobic, even open air, which encourage oxygen going into cathode chambers. The aerobic cathode MFCs take oxygen as electron acceptors, then the oxygen anion reacts with protons which come from the anode to produce water. The anaerobic cathode MFC systems, however, use other chemical substrates, such as nitrates, and usually utilize anaerobic microorganism (different from anode bacteria) as catalyst. Although anaerobic MFCs have lower power output since half cell potential of NO_3^-/NO_2^- (+0.43V) is much lower than O_2/H_2O (+0.82V) [23], the anaerobic cathode could be applied to enhance subsurface bioremediation of contaminants, which could not be completed by aerobic cathode MFCs.[25] Even if the aerobic electron pathway is more thermodynamically favorable than anaerobic terminal electron acceptors such as nitrate or sulfate, it is possible to increase the metabolic rate of anaerobic bacteria in cathode chamber with sufficient anaerobic terminal electron acceptor, instead, which would result in faster degradation of contaminant substrates in cathode. Morris reported that anaerobic biodegradation of diesel range organics (compounds eluting with n-alkane markers ranging in size from C-8 to C-25) was significantly enhanced in an anaerobic cathode MFC (82% removal) as compared to an anaerobically incubated control cell (31% removal) over 21 days at 30 °C, and 31 mW/m^2 power was generated during diesel degradation [23].

Beyond Power Generation: Other Uses of Microbial Fuel Cells

Wastewater treatment

One area where MFCs have been hypothesized to have an obvious advantage is the use of industrial wastewater for the substrate or "fuel" of MFCs. MFCs are capable of a completely different approach into wastewater treatment since the treatment process can

become a process of generating electrical energy rather than a drain on electrical energy. The integration of MFCs to water infrastructure would bring both economic as well as environmental benefit to the process. Logan calculates that the wastewater from a town of 150,000 people could potentially be used to generate up to 2.3 MW of power (assuming 100% efficiency), although a power of 0.5 MW might be more realistic [24]. This research also showed that the microbial fuel cell is able to remove nearly 80% of the chemical oxygen demand (COD) of wastewater and the electric power generated in this process could be used on site to power further treatment of the wastewater. An economic study within the review of Logan shows the potential for this application, though this is highly dependent on local power costs [24].

Other applications

The operation of MFCs needs oxygen and fuel (usually glucose), which can conceivably be taken from their immediate environment *in vivo*, so that it is possible to utilize MFCs as potential power sources to support a range of possible implantable medical devices. Indeed, it must be strict to choose the regular microorganism and medium which are no dangers to human body; and it also need to redesign the structure of MFCs, included input and output pattern of material, to make it be more suitable for working *in vivo*. Actually, in 2001, Katz [26] had set up a fuel cell with a glucose oxidase-based anode and cytochrome c cathode; it could be utilized to supply power to a biosensor for glucose in the human body. Although it was an enzyme-based fuel cell, it still showed that the microbial fuel cell also had similar potential in implantable power sources with the regular structure.

Challenges for MFCs

There are two significant challenges facing microbial fuel cells: i) fully exploit the maximum power generation by MFCs; ii) find ways to make MFC systems economical. In the past 10 years, though the maximum power production has a nearly logarithmic increase from 0.01 mW/m^2 in 2000 to more than 1000 mW/m^2 in 2008 [27, 28, 29, 30, 31], the cost of MFCs has also increased sharply due to the inclusion of expensive anode and cathode materials. For example, platinum, a popular material as chemical catalyst in cathode, has the outstanding performance for increasing power generation [32]; however, the platinum electrode is much more expensive compared to graphite electrode which would limit its application value in future commercial MFCs. For the cathode, maintaining an aerobic environment through pumping air/oxygen into the system utilizes power generated by the MFC [33]. Additionally, it is less efficient and expensive to use ferricyanide to mediate electron transfer for increasing power generation [34]. Consideration of the trade-off between system performance and material cost must be made in any economic analysis of microbial fuel cells [24].

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CHAPTER 2

EFFECTS OF ELECTRON DONORS ON ELECTRICITY PRODUCTION BY THE BACTERIUM *PSEUDOMONAS AERUGINOSA* IN A MICROBIAL FUEL CELL

Introduction

Microbial fuel cells are fuel cells that utilize microorganisms to produce electricity. A typical design of a microbial fuel cell consists of two chambers with anode and cathode electrodes separated by proton exchange membrane (PEM) [1]. The PEM functions as a permeable way to transport protons through the solution across the membrane to the cathode where they combine with oxygen and electrons to form water. This reaction at the cathode typically requires a metal catalyst, such as platinum, but many other options have been explored recently included use of microorganisms in the cathode chamber [2]. Electrons are the result of oxidation reaction of the substrates with the microorganisms in the anode chamber. These electrons travel to the cathode through the external electrical connection producing electrical current.

There has been a wide variety of organisms used for MFC including *Escherichia coli, Proteus vulgaris* and *Rhodoferax ferrireducens* [3]. In the studies presented herein, *Pseudomonas aeruginosa* was selected for use in microbial fuel cells (MFC). *P. aeruginosa* has been used in a number of published MFC systems [4, 5] and has been utilized because of its ability to produce electron mediators, such as pyocyanin, to

facilitate electron transfer from planktonic cells to the anode [5, 6, 7]. However, few papers describe the experiments directly utilizing *P. aeruginosa* to oxidize substrates and produce electric power in a MFC system [1].

The MFC model in our experiment had an "H-type" structure which has been used extensively by other groups [8, 9, 10]. This design consists of two chambers with a tube connector between them. A Nafion proton exchange membrane was utilized to separate the anode and cathode chambers. The relatively large volume provides for greater amounts of medium and substrates for bacterial growth and makes the apparatus more versatile. Furthermore, larger chamber volume offers more room to fit different shape electrodes and other useful apparatuses, such as oxygen supplement and stir bars.

In this project, multiple media were considered for growth of *P. aeruginosa*, specifically, Luria-Bertani (LB) medium and mineral salts medium (MS) [10]. LB medium is a complex medium which contains undefined organic substrates (in the form of typtone and yeast extract). LB is an ideal medium for *P. aeruginosa*, and is often supplemented with glucose for growth of *P. aeruginosa*. As will be discussed later, the LB medium resulted in an accelerated rate of corrosion of the wire connectors in the anode chamber and high levels of background current. Mineral salts (MS) medium should be an alternate option, which not only provides a suitable environment for the bacteria, but also lacks the complex, undefined organic compounds. It actually acts as a buffering solution utilizing HPO_4^{2-} and $H_2PO_4^-$ for buffering. One attractive advantage of MFC is capacity of generating power when fed with wastewater; that requires the bacteria to consume different kinds of substrates in anode medium from inorganic compounds to organic compounds. Hence, it is reasonable to investigate a range of

electron donor substrates for present MFC model with *P. aeruginosa*. This experiment also explored the suitable concentration of glucose in the working MFC, which is necessary for optimizing the operation of MFC model and avoid waste of materials. Additionally, pure *P. aeruginosa* were cultured in five kinds of culture medium with different substrates and varied concentration glucose, then checked for biomass over different periods. Those experiments were expected to provide indirect support or examine for the results from MFC experiments.

In general, the experiments described herein evaluated electric power generation using an H-type two-chamber MFC with *P. aeruginosa*. The experiments focused on the effects of different medium, substrates and concentrations on net power output.

Methods and materials

H-type MFC design

The major MFC model used for these experiments consisted of two 175 ml plastic bottle with 25 ml head space. Two plastic connectors were fixed on the side of chambers with 1-1/2" sanitary connectors. The sanitary connectors were used to hold 1 μm thick Nafion membrane (incubated in DI water in 37°C before used) and sealed by clamp (Figure 3). The anode electrode was 5cm×2.5cm carbon cloth (Graphtek LLC, Buffalo Grove, IL) and the cathode electrode was 2.5 cm×2.5 cm Pt foil (Sigma-Aldrich, St. Louis, MO). The electrodes were connected by steel clips to copper wires attached to an Agilent A3700 data logger for data monitoring. A bubble stone fed with air by a peristaltic pump was placed in the cathode chamber. The anode chamber was agitated by magnetically driven stirring bar to ensure the medium was well mixed during the experiments.

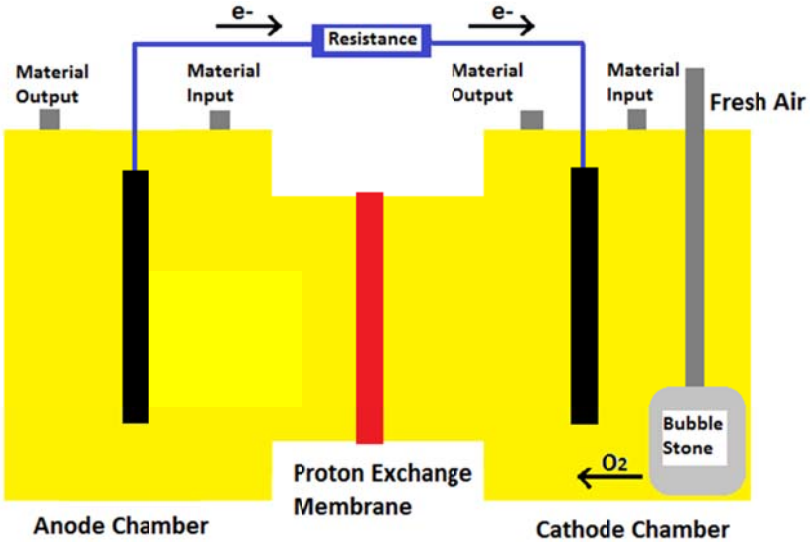

Figure 3: Sketch of bottle MFC model. The black rectangles represent electrodes; the red one is proton exchange (Nafion) membrane.

P. aeruginosa was grown in LB medium (10 g/L typton, 10 g/L NaCl, 5 g/L Yeast extract) supplemented with 20 g/L glucose and 100 µg/L ampicillin. *P. aeruginosa* was also grown in mineral salt medium with glucose (MSG, 0.7% K_2HPO_4, 0.3% KH_2PO_4, 0.1% $(NH4)_2SO4$, 0.005% $MgSO_4 \cdot 7H_2O$ and 0.00025% $FeCl_3 \cdot 6H_2O$ plus 20g/L glucose and 100 µg/L ampicillin. The catholyte medium was 100 mM K_2HPO_4 buffer.

Characterization of MFC internal resistance

A similar MFC design was used to determine the internal resistance of the MFC, except that graphite rods were used instead of carbon cloth. The anode position was varied in proximity to the PEM ranging from 0.5 to 5.0 cm to the PEM, as shown in Figure 2. LB medium with 2% glucose was used in these studies and the cathode medium was 100 mM K_2HPO_4 buffer plus 50 mM $K_3Fe(CN)_6$ solution as electron transfer mediates to improve catalytic efficiency because graphite rod was used as the cathode.

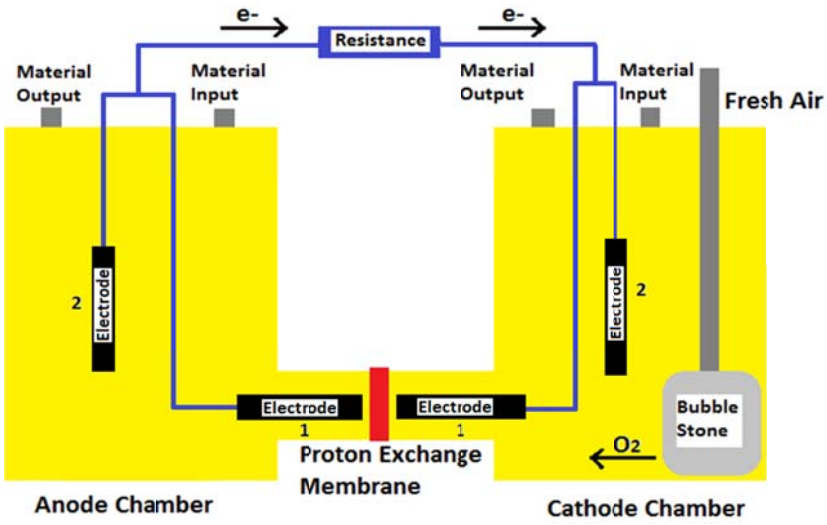

Figure 4: Sketch of MFC model with electrodes in different positions. Electrical properties were monitored when the electrode pairs were positioned at matched positions 1 or positions 2.

Bacterial culture

In preparation for use in the MFC, *P. aeruginosa* was grown on LB/carbenicillin plates; a colony was used to inoculate a culture tube, which was then transferred to a 500ml flask containing the same medium. When anaerobic growth was desired, the medium was supplemented with 0.5% $NaNO_2$ as electron acceptor for anaerobic growth. The culture was batch cultivated at 37°C until reaching stationary phase and then centrifuge (10000 x g for 10min) and resuspended in 10 ml of medium, which was used to inoculate the MFC. Prior to MFC inoculation, the anode chamber was flushed with nitrogen for 30 minutes to remove oxygen.

MFC operation

The MFC was run without bacteria for approximate 24 h to obtain a stable baseline of current and potential. Once this was achieved, the MFC was inoculated with 5 ml of *P. aeruginosa* culture as prepared above. All of the experiments were semi-batch

mode with fresh anode medium (varied concentration and supplement) added to the anode chamber with regular interval duration. No cathode medium refreshment occurred during the experiment.

In the experiments with varied substrates, the four kinds of substrates chosen were: succinate, cellobiose, acetate and formate. The MFC was run without bacteria during the first 24 h while the background current stabilized, then a 10 ml inoculum was added into anode chamber along with medium supplement. The medium was replenished approximately every 24 h.

For experiments varying the concentration of glucose, both LB medium and MS medium were utilized. Operation was as previously described for background stabilization and inoculation. During MFC operation, the concentration of glucose was progressively increased from 0.2% up to 8%. The power output was monitored at each concentration until a stable level was achieved and then the concentration stepped up until no noticeable increase in power was observed.

In the test of biomass with varied substrates, six kinds of culture were prepared in 10 ml culture tubes. The culture consisted of 8ml MS medium with 100 μg/mL ampicillin plus a single substrate: glucose, succinate, acetate, formate and cellobiose, respectively. Each substrate had a concentration of 0.5% and one was a control culture without any substrate. *P. aeruginosa* were grown in 10 ml MSG medium at 37°C overnight in advance. Then transferred 1 ml to each prepared tube with different culture, and incubated and shake at 37°C. Cell density was monitored by UV absorption at 600 nm at 2, 4, 8, 10, 24, 32, 48 and 72 hours. A similar experiment as performed at 0.1%, 0.5%, 1%

and 2% glucose to determine the growth effects of the concentration of glucose in MS medium.

Electrochemical monitoring

Open circuit voltage (OCV) and short circuit current was monitored with Aligent A3700 Data logger. The theoretical maximum power density was calculated by following equations described in introduction chapter.

$$P_{max} = \frac{OCV^2 \times R_{ext}}{(R_{int} + R_{ext})^2 \times S_{Cathode}} \quad 1$$

When the circuit was connected with an external resistance, the actual power density was directly calculated by basic electric power equation (Equation 2):

$$P = \frac{I \times U}{S_{Cathode}} \quad 2$$

Results and discussion

Power and current generation of H-type MFC

When the MFC consisted of carbon cloth and platinum foil as anode and cathode, respectively, the current with was approximately 0.35mA – 0.45mA (peak value 0.7 mA) and the electrical potential was approximately 0.05 - 0.15 V with glucose as substrates and LB as anode medium. The power generated by the MFC was approximately 113 μW (power density 46 mW/m^2). Because the surface area of electrodes were greater than before, the net current (power) output could maintain a stable value for more than 24 h [16], especially when platinum foil was used as the cathode, which not only increased area, but also improved catalytic ability.

Effects of background current in two kinds of anode media

One significant issue in these studies was the low signal relative to the background current. In the MFC with LB as anode medium, the background current was approximately 2/3 of total current output during operation. The low net power generated by bacteria provided low confidence for the power analysis. A search for alternative media identified MSG as a likely candidate for reducing the background current due to its defined nature. *P. aeruginosa* has also been shown to grow well on MSG medium [10]. Compared to corresponding data of LB medium, the maximum net current of MFC with MSG medium was 0.24 mA with background current of 0.017-0.019 mA (compared to a maximum net current was 0.34 mA with background current of 1.25 mA). Although the net current was slightly lower than when grown on LB, the signal-to-noise value increased significantly allowing for greater confidence in the resulting analysis. The majority of experiments were therefore performed in MSG medium. Utilization of MSG resulted in slower growth of the bacterial culture. Thus LB medium was more suitable to practical applications, while MSG medium was best for scientific studies which emphasized accuracy.

Power generation with varied substrates

When *P. aeruginosa* was grown on various substrates, the peak power density was approximately the same for succinate and glucose, about 40 mW/m^2 (Table 1). For succinate this peak power occurred at a concentration of 0.5-0.7% with a peak potential of 0.13 V and peak current of 0.41 mA compared to glucose where the peak potential occurred at 0.7-0.9% glucose and had a power density of 37 mW/m^2 with a peak potential of 0.12 V and peak current of 0.4 mA. The stable working power density was 13 mW/m^2

for glucose and 17 mW/m^2 for succinate. The medium with cellobiose, formate and acetate had no obvious net power in spite of concentration during the experiments. Because MS medium only provides an suitable environment (pH, trace elements) without any nutrient, *P. aeruginosa* is not able to survive in this inorganic medium; it still needs regular organic compounds to maintain the metabolize activity as other heterotrophic bacteria. Actually, there was no obvious growth of *P. aeruginosa* in the anode with cellobiose, formate or acetate. One possible explanation for this is that the glucose dehydrogenase (GDH) activity of *P. aeruginosa* is very low for cellobiose compared with glucose (17). If the cellobiose (β-glucoside disaccharide) is first hydrolyzed to glucose it could be readily consumed by the cell and utilized for energy production. In pure culture, however, there was no microorganism or enzyme was capable of breaking cellobiose down, so *P. aeruginosa* could not oxidize cellobiose to produce electricity.

Table 1 Results of varied substrates experiments

Substrate	Medium	Peak Current (mA)	Peak Potential (V)	External Resistance (Ω)
Glucose	MS	0.4	0.12	45
Succinate	MS	0.41	0.13	45

Succinate is a kind of carboxylate anion and a key component in the citric acid cycle. It can be oxidized to donate electrons to electron transport chain in the cells [13, 14]. It has the similar role as glucose in biochemical reactions, so that it is not surprising that succinate can be utilized by *P. aeruginosa* to produce electricity. This hints that those organic substrates in citric acid cycle or similar processes should also have potential in

application of MFC with *P. aeruginosa*. In fact appears to have evolved into a very efficient organism and is capable of utilizing both glucose and succinate at very similar efficiencies as determined by the comparable level of power density.

The indirect proof was also shown in biomass experiments (Figure 5). This experiment determines whether *P. aeruginosa* is capable of utilizing the various substrates in the medium for growth. Despite the lack of growth of formate, acetate, and cellobiose, it was decided to confirm that growth would not occur in the MFC when power was being produced. As expected, the previously presented results show that only glucose and succinate were efficiently utilized by *P. aeruginosa* for cell growth and power production.

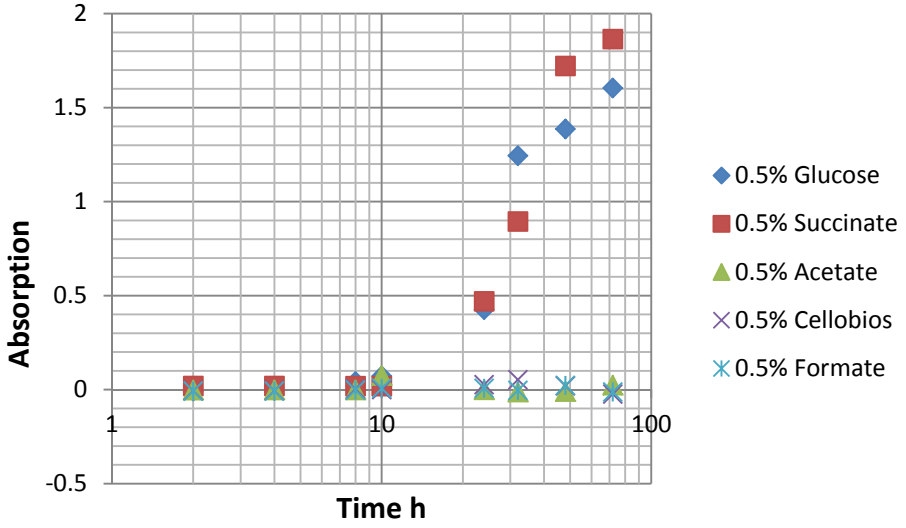

Figure 5: Curves of UV absorption relative to time for *P. aeruginosa* in different substrates

The curves of absorption in succinate and glucose medium showed the typical characters of bacteria growth; it contained lag phase (0 - 10 h), log phase (10 - 40 h) and stationary phase (after 40 – 45 h). The log phase and beginning of stationary phase is the

main period for secondary metabolism of bacteria and also the main stage for electric power producing (18). The higher biomass in this phase usually leads to higher power output. The succinate medium had slightly higher cell density in stationary phase as determined by UV absorption than glucose medium which mean higher biomass, so that the electric power of succinate medium should be a little higher than glucose. It is also possible that succinate is a more direct substrate in catabolism and can be utilized by bacteria more easily.

The relationship between current output and concentration of substrate

The relationship between current produced and glucose concentration was investigated for glucose in LB medium. As summarized in Table 2, the current output increased very quickly with increasing amounts of glucose. A characteristic step response is seen in Figure 6 upon increased addition of glucose to the medium.

This increase continued until a concentration of about 1.5% glucose was achieved, at which point it leveled off but eventually dropped at a much higher concentration of 8%. During operation, when the supply of glucose was stopped and the MFC was operated in batch mode, the current produced by the MFC decreased significantly and then reached original maximum value soon after glucose was restored supply. Although the curves from LB medium could simply show variation trend of current with change of amount of substrates, it was difficult to find more accurate value of maximum current and relevant concentration of substrates since the background current in LB medium was extremely high (nearly 200% of net current value) which overwhelmed the net current signal at low concentration of glucose.

Table 2 Result of experiments of varied concentration of glucose in LB medium.

Time after Test Beginning (h)	Glucose Concentration (%)	Max. Current Output (mA)
14	1	0.14
41	1.5	0.34
72	2	0.36
138	8	0.32

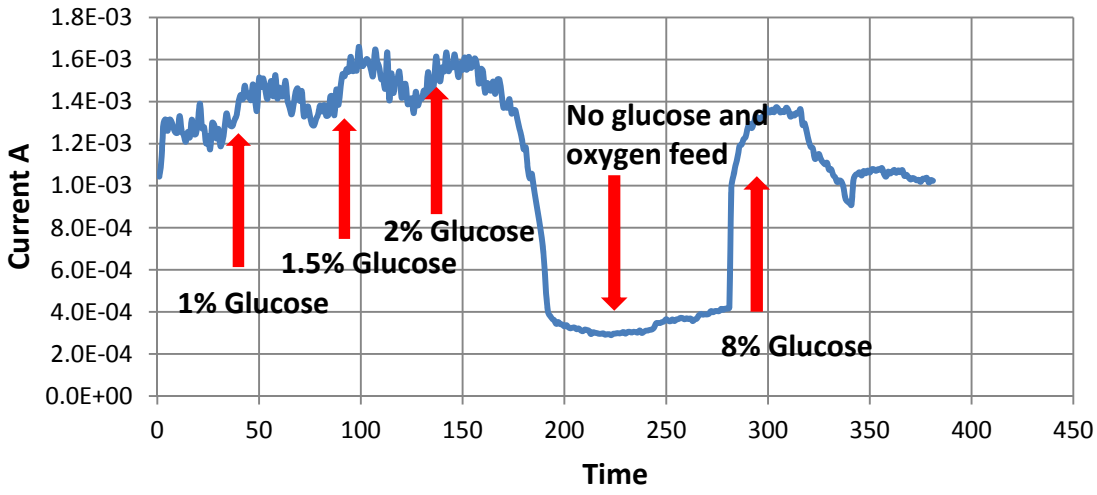

Figure 6: Curves of current output relative to time for experiments of varied concentration of glucose in LB medium.

A repeat experiment in MSG medium resulted in obvious shift in the current when varied concentration. The net current reached an upper limit about 0.7 g/L The net current reached an upper limit and had risen extremely slowly when glucose was approximately 0.8% glucose (see Table 3). Compared to experiment in LB medium, the

net current was sharper without interference of background noise; so that the data gained in this experiment was more credible than experiment in LB medium. When concentration was increased 0.9%, the current and power density sharply decreased to approximate half of maximum value; it hinted that excess substrates may inhibit bacterial metabolism and electric power generation of MFC, however, the real cause still needs additional investigation in the future.

Table 3 Result of experiments of varied concentration of glucose in MS medium.

Glucose Concentration (%)	Peak Current (A)	Peak Potential (V)	Peak Power Density (mW/m^2)
0.23	4.22E-05	2.16E-02	0.73
0.46	2.33E-04	7.35E-02	13.67
0.7	3.15E-04	9.44E-02	23.75
0.93	1.76E-04	5.60E-02	7.86
1.2	2.00E-04	6.48E-02	10.34

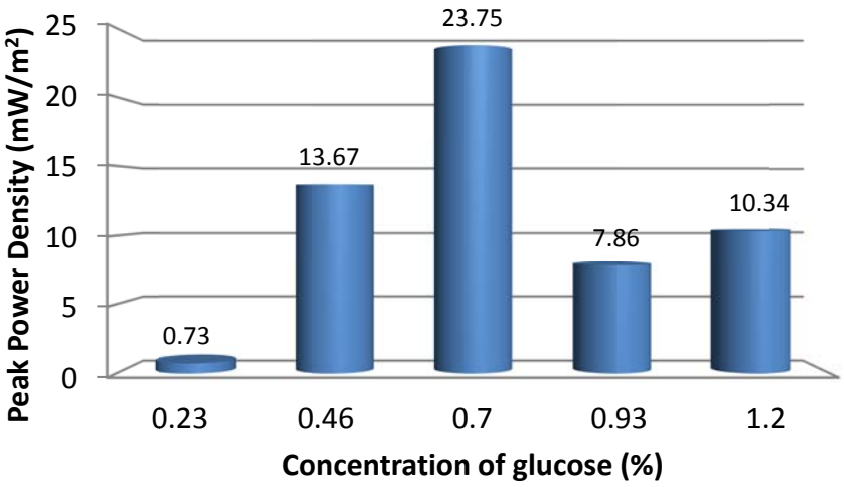

Figure 7: Curve of maximum current outputs relative to concentration of glucose in MS medium.

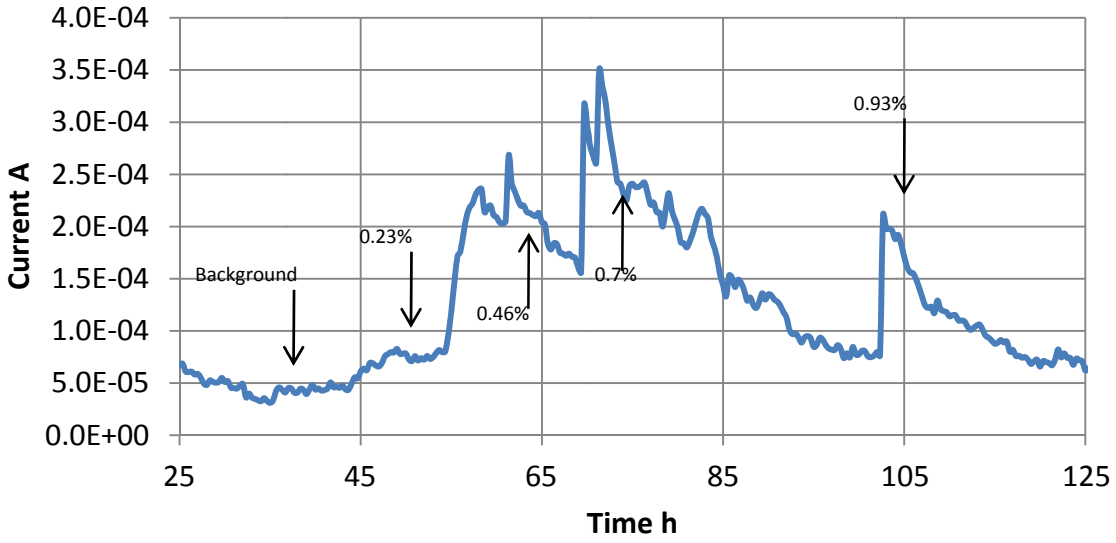

Figure 8: Curves of current outputs relative to time for experiments of varied concentration of glucose in MS medium.

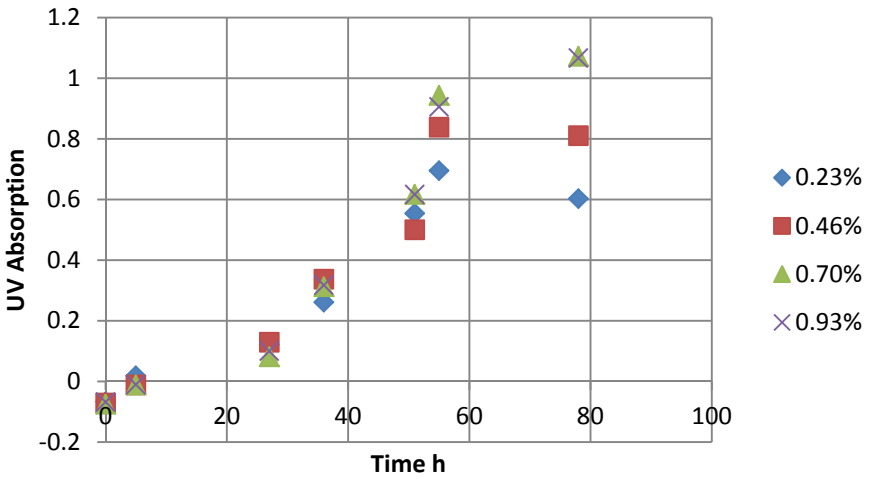

Figure 9: Results of culture experiments with varied concentration of glucose.

According to the growth rate experiment, *P. aeruginosa* cell density would reach a maximum value for glucose concentrations starting around 0.70%, as shown in Figure 9, consistent with results from the MFC peak power density.

H-type MFC with graphite rods as electrodes

The MFC was operated utilizing graphite rods as electrodes to determine the internal resistance of the system. Rods were allowed insertion into the neck for closer placement to membrane, but it lack catalyst activity for cathode reaction compared with platinum foil. This resulted in low current production due to the smaller surface area and reduced robustness of the system. The current generated by MFC was 0.05 mA (peak value) after 24 h and the potential was 0.43 V on average, which resulted in a power density of approximately 3.1 mW/m^2. Two different distances from the PEM were examined, 0.25 cm and 5.0 cm for both the anode and cathode, as shown in Figure 2. Bringing the electrodes closer to the PEM resulted in almost an order of magnitude improvement in the electric current, as shown in Table 4. The potential had been slightly affected by change position of electrodes, which were 0.32V and 0.25V, respectively and

resulted in power densities of 2.326 mW/cm^2 and 0.267 mW/cm^2, respectively. It was obvious that the shapes of electrodes could affect the power output of MFC very much. The larger surface area of electrodes provided extra space for more bacteria to attach and grow on it, which could raise the efficiency of electron transport from cells to anode. This supported the rationale for using materials with higher ratio between surface area [12] and volume such as carbon cloth or platinum foil, to increase power generation by the MFC. Conversely, an electrode with a lower surface area to volume ratio could become a bottleneck for electron transport in the MFC which would lead to lower current and power. By calculating the MFCs' internal resistance, we found that the internal resistance could be reduced sharply if the distance of electrodes decreased, which was considered as main reason for noticeable improvement of the current output.

Table 4 The power density, current and potential output for different positions of electrodes

Position	Distance from membrane (cm)	Potential (V)	Current (mA)	Internal resistance (kΩ)	Power density (mW/cm^2)
1	0.25	0.32	0.04	8	2.326
2	5	0.245	0.006	40	0.267

Conclusions

It is obvious that *P. aeruginosa* is capable of not only producing electron mediators to facilitate electron transport, but also oxidizing organic substrates to generate electricity. In MS medium, the electric power output of MFC had a direct relationship to concentration of substrate (glucose or succinate) up to approximately 0.7%. The potential for producing power is limited by the volume of the MFC and biomass in anode.

Furthermore, the concentration of substrates was saturation limiting and decreased operational efficiency of the MFC above the optimal concentration. This limitation, however, is mitigated in batch MFC operation since excess substrates would be utilized by *P. aeruginosa* and should actually extend duration of maximum power output. During continuous fed MFC operation, however, over charging the substrates would be detrimental to MFC performance.

For large-scale MFCs, the shape or position of electrodes needs to be considered to optimize MFC performance. This is less of a consideration in smaller MFCs because the electrodes will, by necessity, be closer to the PEM reducing the internal resistance of the system. In smaller MFC systems, this may be difficult to achieve when also trying to use electrodes with high surface area to volume ratios as these may not be as accommodating of a material. If both of these improvements are achieved more easily in the narrow space, there will be observable improvement to the power generation by this MFC.

It is well known that platinum has an extremely high activity for catalyzing the reaction between electrons and oxygen in presence of protons at the cathode. The high cost of platinum, however, restricts its application in large system. An extensive amount of recent research has focused on how to modify cathode electrode with a small quantity of platinum [6, 15]. It is reasonable to utilize platinum as the cathode electrode in this MFC system because the capacity of *P. aeruginosa* to produce electricity is being investigated without restriction by cost.

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CHAPTER 3:

PRELIMINARY STUDIES OF THE HYPERTHERMOPHILIC ARCHAEON *SULFOLOBUS SOLFATARICUS* IN A MICROBIAL FUEL CELL

Introduction

Microbial fuel cells have been shown to work with a wide range of microorganisms [1]. To date, most of the MFC characterization has been performed in mesophilic microorganisms, those organisms which grow around 37°C. There have been a handful of studies utilizing microorganisms that grow at elevated temperatures, up to 57°C [2, 3], however, none have looked at temperatures exceeding 60°C. This chapter details experiments performed in a MFC utilizing the hyperthermophilic, acidophilic, archaeon *Sulfolobus solfataricus*, which grows optimally about 80°C. *Sulfolobus* is a member of the archaea domain and belongs to the family *sulfolobaceae*. It can grow in volcanic springs with optimal growth occurring at pH 2-3 and temperatures of 75-80 °C [4].

Materials and methods

MFC setup

Briefly, the MFC system was a two-chamber H-type MFC utilizing platinum foil as the cathode and carbon cloth as the anode. It consisted of two 175 ml plastic bottle with 25 ml head space. Two plastic connectors were fixed on the side of chambers with 1-1/2" sanitary connectors. The sanitary connectors were used to hold 1 μm thick Nafion

membrane (incubated in DI water in 37°C before used) and sealed by clamp (Figure 10). The anode electrode was 5cm×2.5cm carbon cloth (Graphtek LLC, Buffalo Grove, IL) and the cathode electrode was 2.5 cm×2.5 cm Pt foil (Sigma-Aldrich, St. Louis, MO). The electrodes were connected by steel clips to copper wires attached to an Agilent A3700 data logger for data monitoring. A bubble stone fed with air by a peristaltic pump was placed in the cathode chamber. The anode chamber was agitated by magnetically driven stirring bar to ensure the medium was well mixed during the experiments.

The anode medium was DSMZ 182 solution suggested by company. It consisted of 0.1% yeast extract, 0.1% casamino acid, 0.3% KH_2PO_4, 0.25% $(NH_4)_2SO_4$, 0.02% $MgSO_4 \cdot 7H_2O$, 0.025% $CaCl_2 \cdot 2H_2O$ and 1:100 diluted trace element solution (SL-10), 2% glucose and 2% Oxyrase. The cathode medium was 100 mM K_2HPO_4 (pH 7.0).

Microorganism culture

The frozen-dried bacteria were incubated in 5 ml DSMZ 182 solution at 80°C overnight; then transferred into 50ml flask contained 25 ml MSDZ 182 solution and continued incubating at 80°C for two days. A 10 mL inoculum was transferred from the previous culture to initiate growth in new cultures. Between use, cultures were stored at 2-8°C.

MFC operation

The MFC was running in shaken incubator without bacteria at 80°C for the first 24 h while the background current decreased to a stable level. Then a 25 mL inoculum of *S. solfataricus* was injected into the MFC. This experiment was performed in a batch reaction, and refreshed with 75 ml and 100 ml anode medium (contained 2% glucose) in

12^{th} and 72^{nd} hours, respectively. No fresh cathode medium was added after experiment began.

Results and discussion

The maximum net electric power was 70.9 mW/m^2, and the stable value from 24 h to 72 h was 67.7mW/m^2. This was nearly twice the net power of MFCs operated with *Pseudomonas aeruginosa*. This high power output, however, could not maintain a long period; both potential and current output decreased to background level after 72 h even refreshing the anode medium. This duration of stability was much shorter compared to the *P. aeruginosa* MFC (Figures 10 and 11).

The MFC was operated at the conditions that were conducive to optimal growth of *S. solfataricus*, 80°C pH 2.5. The present MFC system, however, was not designed for these extreme conditions. During the experiment, the connections to the anode (clip and wire) were corroded by these conditions and resulted in an approximately 2 mm layer of blue sludge-like products. More importantly, the Nafion membrane performed poorly under these conditions and quickly aged becoming brittle in the high temperature environment and eventually catastrophically failing.

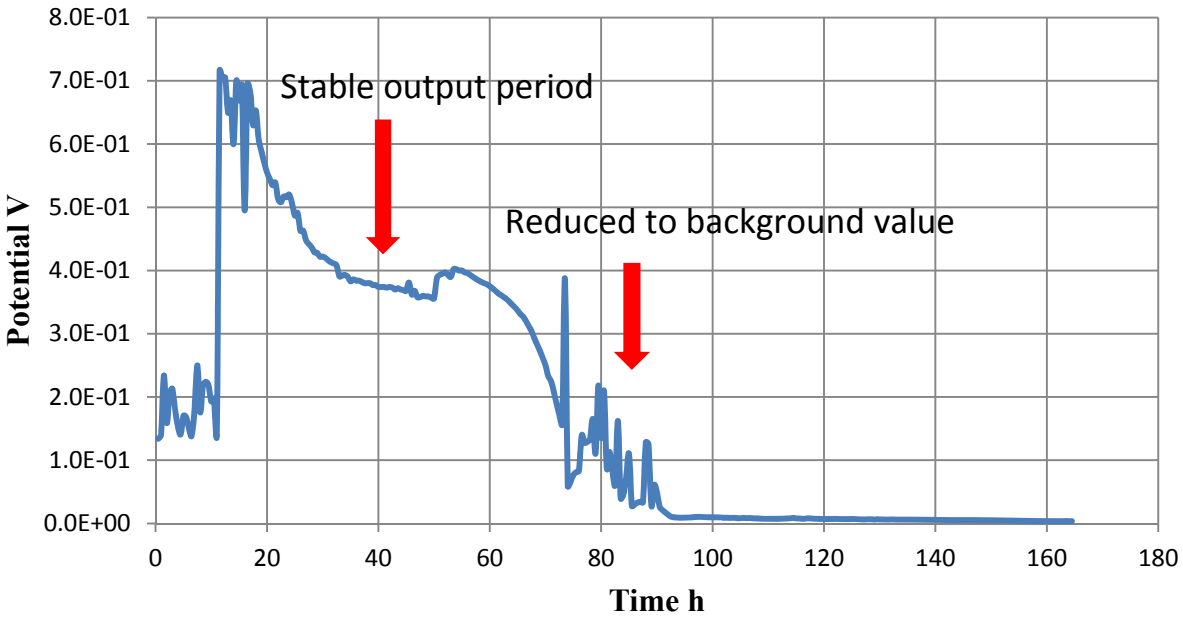

Figure 10: Curve of potential output vs. time in experiment of *Sulfolobus*.

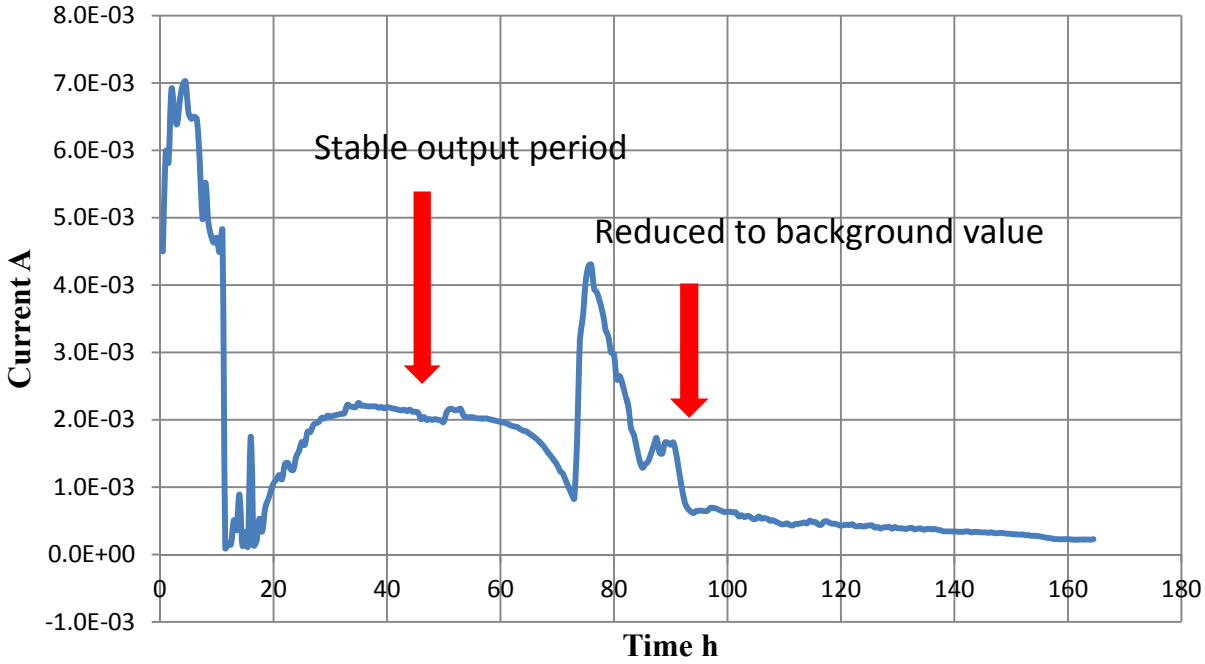

Figure 11: Curve of current output vs. time in experiment of *Sulfolobus*.

Conclusions

This experiment shows that *Sulfolobus* has great potential of electric power generation in MFC. Nevertheless, since its extremer growth surrounding, the MFC model need new materials, such as high-temperature proton exchange membrane, to keep reliability in hyper-thermal and highly-acid condition.

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CHAPTER 4

SUMMARIES AND CONCLUSIONS

In general, *P. aeruginosa* is capable of not only excreting endogenous mediator to promote net power in mix culture of MFCs' anode, but also oxidizing regular substrates and generating electric power individually.

The most suitable substrates in the program are glucose and succinate, which are also the important material or intermediate substrates in actual biological metabolism. This hints that those metabolism intermediate products may be an efficient energy source for MFC. However, other common substrates, such as acetate and formate, which had been utilized as electron donors in many MFCs, are not suitable for MFC with *P. aeruginosa*. Not only there was no net power output, but also the bacteria could not survive and grow in medium with these substrates as the sole carbon source. Specific bacteria can only utilize several specific substrates; that suggests the mixed cultures would be more efficiency in treatment of wastewater which contain varied kinds and amount of substrates in different periods.

The optimum concentration of substrates in this program is nearly 0.7% glucose and succinate in mineral salt medium. At this level, the batch MFC model can maintain the maximum power output more than three days. Higher concentrations of substrate do not increase net power and, in fact, leads to a reduced power density. This optimum, or saturated concentration, usually depends on substrate and microorganism of MFC. For

wastewater treatment, it is not practical to have a single pure substrate at high concentrations; therefore, use of *P. aeruginosa* within consortia is more practical than in pure culture for wastewater treatment.

Although the net power of MFC with *Pseudomonas aeruginosa* still seems low, this may be limited by MFC's configuration. It has been documented that single chamber MFCs have improved efficiency and performance compared to the two-chamber system used in these experiments. A redesign of the MFC system should improve system power and performance. Additionally, the materials of construction for the anode and cathode have been shown to play an important role in improving electricity production and could further be explored in the future. Finally, use of genetic engineering to modify the organism in ways that improve exoelectrogenicity has been shown to have a great impact on MFC performance [1, 2, 3] and similar modification to *P. aeruginosa* could be performed that might impact free electrons and electron transfer to the anode. These represent future areas that can be explored in the study of MFCs utilizing *P. aeruginosa* in pure culture.

References

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2. N. Boon, K. De Maeyer, M. Höfte. **Metabolites produced by Pseudomonas sp. enable a Grampositive bacterium to achieve extracellular electron transfer.** *Appl. Microbiol. Biotechnol.* 77 (2008), 1119–1129.
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