

**POTENTIAL OF MIXED CONSORTIUM OF *ENTEROBACTERIACEAE* AND
SERRATIA MARCESCENS IN SYNTHETIC WASTEWATER TREATMENT
AND POWER GENERATION IN MICROBIAL FUEL CELL**

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Abstract

Microbial Fuel Cell (MFC) is a promising technology that can treat wastewater and generate electricity simultaneously. Basic principle of an MFC uses microorganism in the form of biofilm that is cultivated on surface of anode that oxidizes the substrate provided. Common challenge faced for the realization of this technology in actual wastewater treating system includes the strict control of specific microbial strain growth throughout the treatment period. To overcome this, the performance of using common water-borne bacteria as the extracellular electron transfer (EET) strain was evaluated. A total of 14 water-borne bacteria, mostly from *Enterobacteriaceae* family, were isolated and subsequently identified from two different water sources, one with a more diverse community in an open system (lake) while the other is a more controlled environment in a closed system (household aquarium). Two dual-chambered MFC were set-up with different anode materials made up of graphite plate and carbon cloth to study the growth of a mixed consortium of bacteria isolated from lake water and pure culture from aquarium on respective electrode surface. Glucose, lactate, acetate, fumarate, and lastly fumarate coupled with lactate were then supplied as synthetic wastewater to determine power generation and wastewater treating ability. Pure culture of *Serratia marcescens* isolated from household aquarium were then used in similar set-up to evaluate their effect on MFC. The power densities generated from two reactors with different anode materials showed that the mixture of waterborne bacteria demonstrated better growth on graphite plate compared with carbon cloth. All reactors were capable in generating low amount of electricity, with highest at 0.90 mW/m^2 through using fumarate coupled with lactate, lowest for all reactors when using glucose. This is due to the position of glucose in the respiratory pathway as compared to acetate. Additionally, MFC was able to utilise substrate inoculated efficiently, with high Chemical Oxygen Demand (COD) removal recorded at 95% when fumarate was supplied with lactate. However, low Coulombic Efficiency (CE) value was obtained which suggests electron loss during the EET process to other respiratory process or pathways such as methanogenesis. While it can generate power and treat synthetic wastewater, efforts such as characterisation, and engineering from the genetic aspects were still needed for these organisms to be applied in an MFC.

List of publications

1. Leung, Dexter Hoi Long; Chong, Siewhui; Lim, Yin Sze; Pan, Guan-Ting, 5th Postgraduate Colloquium for Environmental Research (POCER): Usage of Enterobacteriaceae in Dual-chambered Microbial Fuel Cell, Proceedings of the 5th Postgraduate Colloquium for Environmental Research (POCER 2019), eISBN 978-967-11279-6-4.
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List of abbreviations

ABC	ATP-Binding component
AD	Anaerobic Digestion
ArcA	Aerobic respiration control protein
ATP	Adenosine Triphosphate
ATP	Adenosine triphosphate
BES	Bioelectrochemical system
c-Cyts	C- type cytochromes
c-di-GMP	Cyclic dimeric guanosine monophosphate
ccm	Cytochrome-c biogenesis ATP-binding export protein
ccmAB	Complex of Cytochrome-c ATP-binding export protein ccmA and ccmB
CE	Coulombic efficiency
CEM	Cation exchange membrane
CO ₂	Carbon Dioxide
COD	Chemical oxygen demand
CRISPR	Clustered regularly interspaced short palindromic repeat
CRP	C-reactive protein
CymA	Inner membrane tetrahaem cytochrome
DNA	Deoxyribonucleotide Acid
ds-DNA	Double-stranded Deoxyribonucleic Acid
EET	Extracellular electron transfer
Fcc3	Flavocytochrome c3 fumarate reductase
fdh	FDH formate dehydrogenase
FMN	Flavin mononucleotide
FRB1	Fosfomycin Resistant Big
FRS1	Fosfomycin Resistant Small
GapA2	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green Fluorescence Protein
GMP	Guanosine Monophosphate
H ₂	Hydrogen
H ₂ O	Water
His	Histamine
MCA	MacConkey Agar
mdh	Malate dehydrogenase
msh	Mannose-sensitive hemagglutinin
Met	Methionine
MFC	Microbial Fuel Cell
<i>mxl</i>	Putative carbohydrate containing cell associated component
Na	Nicotinic Acid
NAD	Nicotinamide adenine dinucleotide
nadD	Nicotinate-nucleotide adenylyltransferase

nadE	NH(3)-dependent NAD(+) synthetase
NADH	Nicotinamide adenine dinucleotide
nadM	Nicotinamide-nucleotide adenylyltransferase
nadV	Nm phosphoribosyltransferase
NaMN	Nicotinic acid mononucleotide
Nuo/Nqr1	NADH dehydrogenases
O ₂	Oxygen
OMCs	Outer membrane c-type cytochromes
<i>omcZ</i>	Gene encoding c-Cyts Z
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
PEM	Proton exchange membrane
pflB	Formate acetyltransferase 1
Phe	Phenylalanine
<i>pilA</i>	Gene encoding type-IV pili
PilA	Protein translated from <i>pilA</i>
<i>placI</i>	Lactose promoter
Pnc	Nm mononucleotide deaminase
POME	Palm oil mill effluent
RF	Riboflavin
ribADEHC	Flavin biosynthesis gene cluster
ssDNA	Single-stranded DNA
STC	Small tetrahaem cytochrome
TCA	tricarboxylic acid cycle
TMAO	Trimethylamine N-oxide
Trp	Tryptophan
Tyr	Tyrosine
WT	Wild-type
Ycel	Multidrug resistance protein MdtH
YdeH	Diguanylate cyclase

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Chapter 1: Introduction

Globally, 80% of wastewater will be released back into the environment without going through any treatment. The opportunities to exploit these wastewater as a resource are enormous as they are an affordable and sustainable source of energy, water, and recoverable material (UNESCO, 2017). Malaysia has a population of 32.4 million based on Department of Statistics Malaysia, which had increased by 14.4% since 2010, and the current estimated volume of generated wastewater in the municipal and industrial sectors is projected at around 3.39 billion cubic meters annually (Department of Statistics Malaysia, 2018). This number is going to escalate in reflection to the increasing population size. The dominant wastewater treatment types in Malaysia are categorized into preliminary (removal of oil, grit, grease, rags, and rubbish), primary (removal of materials through sedimentation or flotation), and secondary (removal of organic and suspended solids through biological treatments), with no plans for initiation of a tertiary system (Mat et al., 2013). The major constraints to wastewater treatment faced would be the low sewerage tariff that is unable to support the high operation and maintenance cost that cannot be borne by the populations in Malaysia.

Wastewater consisting of a large amount of biodegradable organic matter contain energy which can be recovered rather than expended. The representative technology which recovers energy from wastewater (and sludge) is the anaerobic digestion (AD) processes. One of the major drawbacks of anaerobic digesters has been the requirement of long solid retention time which is not associated with the increasing volume of sludge produced from industrialization and human activities, long start-up period, impure biogas and incomplete or insufficient removal of organic matters, pathogens and nutrients in the final effluent, thereby failing to comply with the local standards for discharge or reuse (Chong et al., 2012; Mesfin Yeneneh et al., 2013). Anaerobic digestion is a process of converting the biodegradable matters in wastewater sludge to generate biogas consisting of mainly methane and carbon dioxide in the absence of oxygen. However, this technology prefers tropical climate conditions (37°C) and requires long retention times. Apart from the anaerobic digestion processes, most of the wastewater treatment plants use activated-sludge or trickling-filter system to remove the organic matter, nutrients and pathogens in wastewater to the acceptable discharge standards by means of aeration. The requirement of post-stabilization or effluent polishing to meet stringent discharge standards has led to the frequently adopted development of two-step processes and aerobic post-treatment units. However, aeration requires considerable operating and energy costs (Aelterman et al., 2006).

Potter (1911) discovered the generation of electromotive force through the decomposition of organic compounds by the fermentative activities of *Saccharomyces spp.* and other organisms. This soon leads to intensive research and development of microbial fuel cell (MFC), which utilizes biological catalysts at an anaerobic anodic compartment of the fuel cell to generate bioelectricity. Recently, wastewater treatment using MFC technology has emerged intensively due to its good temperature tolerance and requirement of shorter retention times compared to anaerobic digestion. In the past two decades, collaborative efforts of researchers had greatly optimized and improved the magnitude of power generated by MFC through various methods; including supplementation or control of different substrates (Sun et al., 2015; Zhang et al., 2017) , utilization of different materials in the design of the fuel cell (Liu et al., 2004; Zielke, 2005; Deval and Dikshit, 2013), and most recently through genetic modifications of the microorganism that act as biocatalyst (Li et al., 2017; Li et al., 2018a). In comparison to aerobic units such as the most-commonly used activated-sludge and trickling-filter systems, microbial fuel cell technologies offer four main advantages: (1) production of a useful product in the form of electricity (or hydrogen gas in microbial electrolysis cells), (2) no aeration requirement, (3) reduced solids production, which is five times lesser than that of an aerobic process, and (4) potential for odour control (Logan et al., 2008).

Cytoplasmic membranes are present in cell envelopes in most microbial communities that will act as the primary barrier to the extracellular environment. These serves as the center of electron transfer essential for energy generation. Cell envelopes contains external structural components such as peptidoglycan, S-layer, and outer membrane which cannot conduct electricity and is physically impermeable to minerals (Albers and Meyer, 2011; Shi et al., 2016) Microorganisms have evolved and obtained specialized mechanisms to overcome these obstacles which prevent transfer of electrons – these organisms are categorized as exoelectrogens (Melton et al., 2014). The transfer of electrons between redox carriers within the cytoplasmic membrane and extracellular minerals is referred as microbial extracellular electron transfer (EET). MFCs utilize exoelectrogens in the form of biofilms to ferment carbon sources in which electrons are released in the process.

Shewanella spp. and *Geobacter spp.* are the commonly studied exoelectrogens that oxidizes Hydrogen (H_2) and other organic matter and then transfer the released electron to these extracellular minerals for respiration (Kumar et al., 2016). Genes and pathway that is related to EET in *Shewanella spp.* are extensively well-established. Metal reducing (Mtr) pathway are identified in *Shewanella* MR-1 strain that transfer electrons from quinone and quinol pool across the outer membrane to surface of Fe (III) containing mineral via the periplasm and cytochrome-C. This pathway is controlled by ATP-binding

cassette (ABC) transporter complex, ccmAB, which is a complex consist of Cytochrome-c biogenesis ATP-binding export protein ccmA and ccmB (Shi et al., 2016). It is also recently discovered by Yang et al. (2017) that extracellular non-cytochrome C such as proteins with Fe-S cluster contribute to EET in the facilitation of electron mediators.

Previous research had proved the superiority of MFCs in several aspects as compared to other competent conventional technologies used to treat wastewater, namely the improved conversion efficiency due to the direct conversion from the substrate's electrical energy from chemical energy; ability to treat low-strength wastewater that is otherwise unsuitable for anaerobic digestion (Rittmann, 2008; Watanabe, 2008); ability for system conduction in ambient temperature (Mathuriya and Sharma, 2009); and 50-90% less solid waste production to be dealt with (Du et al., 2007). On top of that, power densities generated by MFCs has reached over 4200 mW/m³ in a study by using single chamber MFC. Anode material were of graphite fibre brush and domestic wastewater was used as the inoculum (Luo et al., 2011). In a separate study, removal of chemical oxygen demand (COD) and contaminants by up to 100% were reported by using a dual-chambered MFC with carbon cloth anode, inoculated with furfural (Sharma and Li, 2010). Most importantly, unlike the anaerobic digestion processes in which the biogas produced has to go through scrubbing process (to remove hydrogen sulfide), and the combustion process in a gas motor, microbial fuel cells can simultaneously capture energy in the form of electricity or hydrogen gas (Logan et al., 2008; Rismani-Yazdi et al., 2008) .

Research had also shown that it is feasible to utilize food waste leachate as a fuel for bioelectricity generation in MFCs (Wang and Lim, 2017). It is generally still not feasible to incorporate this technology into real life situation as the studies highlighted previously were all experiments of MFCs conducted at only laboratory scale (less than 1L) (Keller and Rabaey, 2008). There are also a number of limitations and challenges that has not been addressed yet (Janicek et al., 2014). However, it has been foresighted that this technology could potentially be the solution to cope up with the problems that will soon to emerge due to the rapid advancement in various production fields resulting in increased output of wastewater.

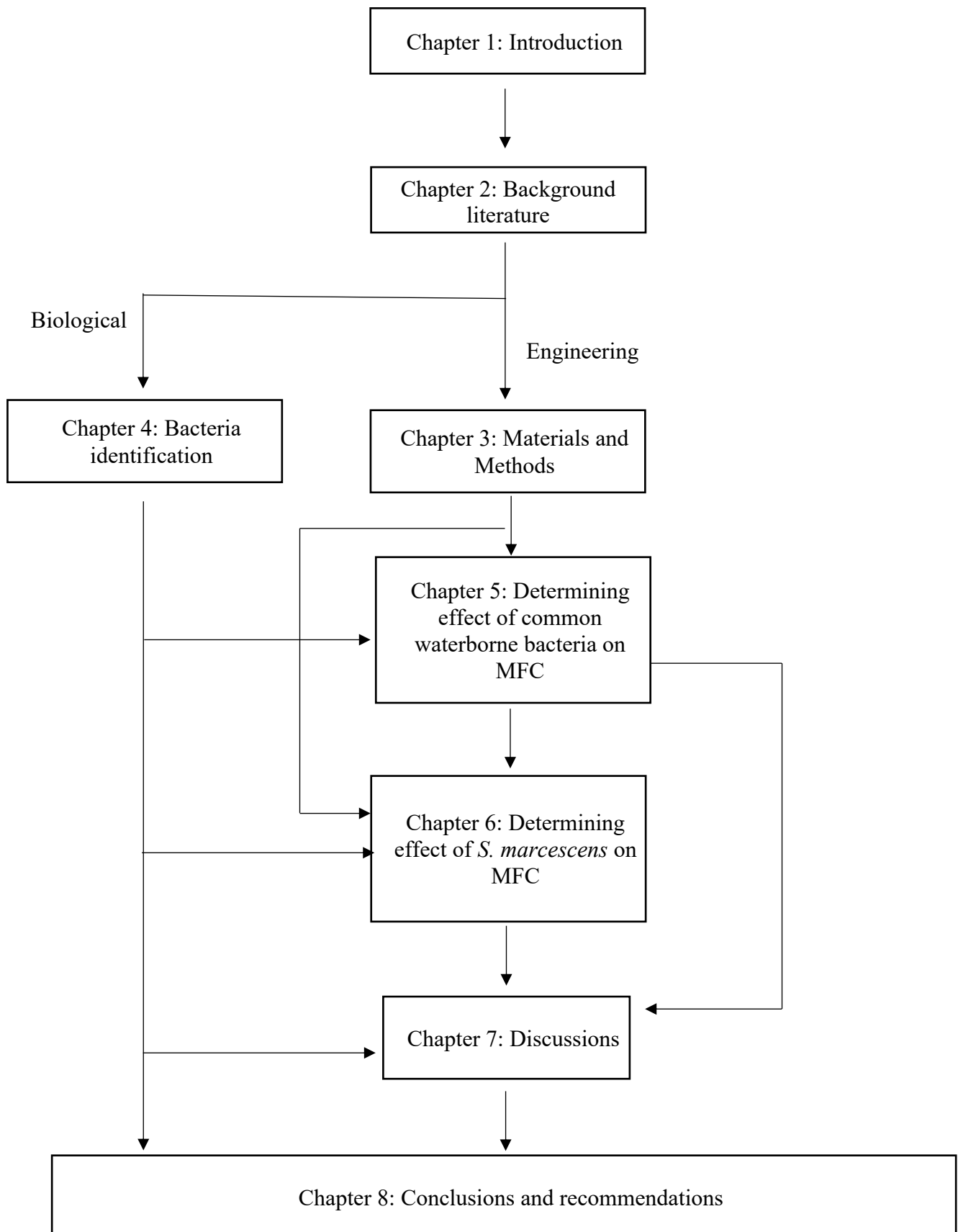
1.1 Problem statement

Where the growth of exoelectrogenic biofilms in concern that is mainly responsible for the generation of bioelectricity, it is still inapplicable in a real-world situation where contamination is very likely to happen which causes a shift in growth of microbial consortia on the biofilm, ultimately affecting the potential generation of bioelectricity. Alternatively, few methods could potentially solve this problem, but they could pose another separate issue instead. Selective enrichment could be utilized to selectively enhance the growth of the exoelectrogenic strains, but it is not effective in controlling contaminants; antibiotic could be utilized to control microbial population effectively, however this causes acquired resistances and increase of antimicrobial resistance; or complete sterilization of the organic waste by chemical treatment prior to MFC treatment that is too meticulous and low cost-efficient as errors that could happen during the procedure would render the process redundant. Therefore, this project aims to tackle the above-mentioned problems by utilizing random microbiota obtained from lake and water as the inoculant and optimize the operation of MFC by taking into consideration the electrode selection and the growth of bacteria biofilms on the surface that allow increased power production through oxidation of simple substrates which is commonly found in foodwaste leachate, and its bio-electrochemistry properties in treating various types of wastewater.

1.2 Objectives and motivation for project

- (i) To isolate and identify organism from accessible water sources to be subsequently inoculated into MFC.
- (ii) To determine the effects of different kinds of electrode materials, carbon and graphite, on the performance of MFC, in terms of the growth of biofilms, and how will this subsequently affect power generation, and removal of organic matters.
- (iii) To determine the potential of MFC inoculated with common waterborne bacteria obtained from lake and water samples in generating electricity and treating synthetic wastewater.
- (iv) To determine potential of MFC inoculated with a single isolate of *S. marcescens* obtained from household aquarium.

1.3 Flow of thesis



Chapter 2: Background literature

2.1 Introduction

Microbial Fuel Cell (MFC), is a part of a bioelectrochemical system (BES). It consists of two chambers that houses an anode and cathode, which are separated by a proton-exchange membrane (PEM), as illustrated in Figure 1. Bacteria will form biofilms on anode surface and utilises the organic matter to produce electrons which will be subsequently transferred to the cathode via an external circuit.

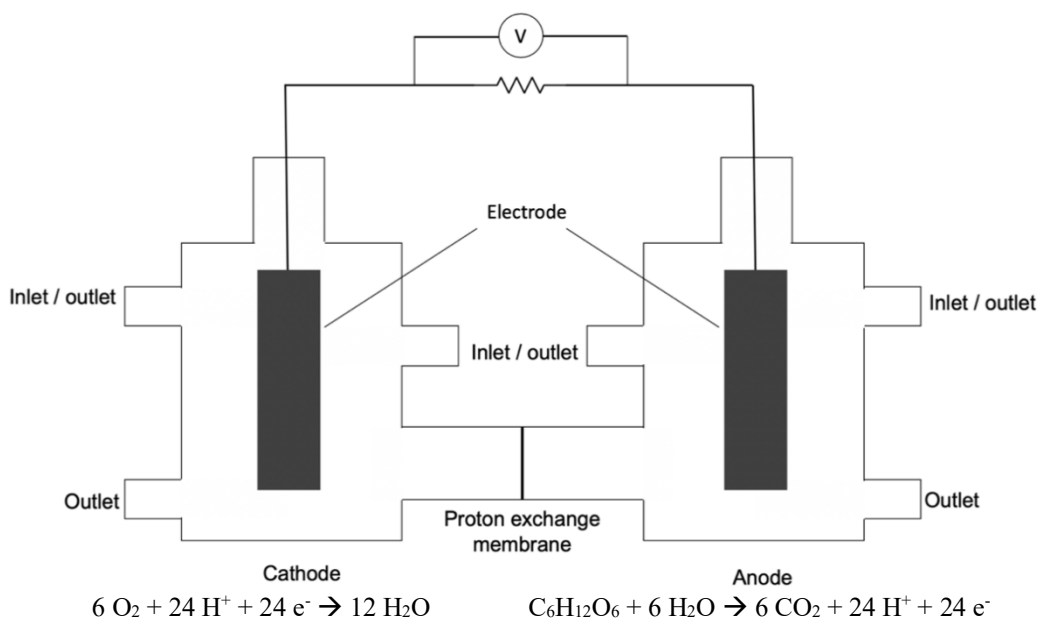


Figure 1: Schematics of a typical dual-chambered MFC set-up. In the cathode chamber, a constant air supply is supplied, where the anode chamber were kept in an anaerobic condition to allow anaerobic respiration of bacteria to produce electrons.

Extensive research had been established over the past decades on optimum materials and other considerations such as cost efficiencies, while determining what material to be utilized in a MFC (Logan, 2007). Improvements and manipulations are being made into parameters that bottlenecks the potential of a MFC such as the cell design (Mathuriya, 2016), electrode materials (Choudhury et al., 2017), environmental conditions (Tkach et al., 2017), and more recently through genetic manipulations from a microbiological perspective. Voeikova et al. (2013) demonstrated that the mutant *S. oneidensis* MR-1 mutants Fosfomycin Resistant Small (FRS1) and Fosfomycin Resistant Big (FRB1) that have enhanced reducing activities exhibited 1.7 times higher current densities as compared with *S. oneidensis* MR-1. Synthetic flavin biosynthesis pathway of *Bacillus subtilis* was expressed heterologously in *S. oneidensis* MR-1 by Yang et al. (2015), which had increased concentration of

secreted flavin at 25.7 times, ultimately increasing power output by 13.2 times as compared to wild type *S. oneidensis* MR-1 (Yang et al., 2015). In a study carried out by Min et al. (2017), flavin biosynthesis gene cluster *ribD-ribC-ribBA-ribE* from *S. oneidensis* MR-1 are co-amplified along with metal-reducing conduit biosynthesis gene cluster *mtrC-mtrA-mtrB*. This co-expression had led to 110% to 87% increase in maximum current density being generated (Min et al., 2017). Ng et al. (2018) demonstrated the potential in expressing exogenous *mtr* genes by using recombinant DNA technology. In the experiment, the Mtr pathway genes *mtrA*, *mtrC*, and *mtrCAB* are successfully expressed in *S. oneidensis* MR-1 by using a lactose promoter (*placI*). This expression had led to higher ferric reductase activity and a 1.53-fold higher enzymatic activity as compared to the wild type.

MFC uses microorganisms, in the form of a biofilm, that are capable to transfer electrons produced during anaerobic respiration extracellularly to an anode, known as extracellular electron transfer (EET), these microorganisms are termed as exoelectrogens. Exoelectrogens that are widely studied with a quite established genome database are *Shewanella spp.* and *Geobacter spp.*, more specifically *Shewanella oneidensis* and *Geobacter sulfurreducens* (Santoro et al., 2017). The former is well known for its bidirectional flow of electrons across the cellular membranes (Jiao et al., 2011; Schuetz et al., 2009; Coursolle et al., 2009; Bretschger et al., 2007; Lies et al., 2005), while the latter exchange electrons through conductive nanowires to various electron acceptors (Reguera et al., 2005). Different substrates can be utilized depending on the strain of microorganism used, where lactate, acetate, and glucose, are the typical substrates used by exoelectrogens for anaerobic respiration (Santoro et al., 2017). Along with the refinement of techniques in molecular biology and the rapid advancement of biotechnology, it is only a matter of time where substrate and biofilms formation metabolic pathways of various organisms are identified and cloned into a strain where it could be ubiquitously utilized as an exoelectrogens for efficient current generation in a MFC.

At the current stage of research, the relationship and interactions among various exoelectrogens in a mixed-cultured biofilm are still not well defined. Additionally, more organisms are being identified that confers exoelectrogenic capabilities (Sharma et al., 2016; Sacco et al., 2017). Recently, Ueoka et al. (2018) demonstrated an electrode plate-culture (EPC) method that allow selective isolation of exoelectrogens specifically through utilizing an electrode plate covered with medium as a sole electron acceptor. The isolation of exoelectrogens in a complex microbiome solely based on their capabilities to undergo EET, and not based to their specific nutrient requirement which is being used now at present is possible. This could potentially lead to more different exoelectrogens being isolated and subsequently identified. A recent study had discovered an eight-gene locus in *L. monocytogenes* that

is capable for EET. The locus encodes specialized NADH dehydrogenase that channels electron produced from aerobic respiration to a discrete membrane-localized quinone pool, segregating EET (Light et al., 2018). These findings had shown that previously undiscovered species of bacteria that are also capable in performing EET.

In this chapter, the mechanisms on how different types of commonly studied exoelectrogens form biofilms at the genetic level, electron transfer mechanisms, general types of MFC, their design and principles, and MFC evaluation techniques are reviewed.

2.2 *Extracellular electron transfer*

2.2.1 *Biofilm formation*

Biofilms are referred to as a complex of embedded microorganisms that stick with each other that could be a pure or a mixed culture within an extracellular matrix composed of self-produced extracellular polymeric substances (Snider et al., 2012; Parameswaran et al., 2013). Electroactive biofilms are imperative for exoelectrogens to carry out EET for bioelectricity generation efficiently. Current generated is directly proportional to biomass of the biofilm and type of electrode surface where negatively charged and hydrophobic anode surface are less favourable for electroactive biofilm formation and will also consequently affects the performance of the fuel cell. Optimum thickness is preferable to achieve substantial current densities as very thick deposition of biofilm are shown to limit electron flow (Wrighton et al., 2011; Parameswaran et al., 2013). Electrons generated during respiration of substrate by the exoelectrogens will go through their respective processes, depending on the type of organisms, and then transported to the surface of the electrode through the biofilm. Additionally, biofilms are also needed for the reduction of organic matter. These process are mediated by the c-type cytochromes (*c*-Cyts), or also known as the redox-active proteins (Brutinel and Gralnick, 2012; Kotloski and Gralnick, 2013). Microbial proteins such as type-IV pili, and electron shuttles like flavins, pyocyanin, *et cetera*, also enhances the conductivity of the biofilm, thus increasing electron transfer (Malvankar and Lovley, 2012; Orellana et al., 2013; Parameswaran et al., 2013).

For *Geobacter sulfurreducens*, morphology of biofilm is dependent on type of electrode material used in the form of pillars or thick homogenous layers) and are found to form a thick homogenous cell layer with slightly pillar structures when carbon cloth is utilized as the anode. When a solid graphite rod is

used instead, a heterogeneous pillar structure are formed in the biofilm. Growth conditions, types of electron acceptor, and the design of reactor itself also play a role in the formation of biofilms (Nevin et al., 2008). It is also worthwhile to note that thicker and more structured biofilms are grown on unsmooth graphite surfaces inoculated with fumarate when it is compared to conditions where graphite is the sole electron acceptor (Nevin et al., 2008). Biologically, gene encoding *c*-Cyts Z, *omcZ*, and gene encoding type-IV pili, *pilA*, are generally responsible for biofilm formation and current generation (Malvankar and Lovley, 2012). Type IV pili are made up of monomers of PilA with two isoforms, short and long PilA (Richter et al., 2012). Long PilA is responsible for the attachment of cells during formation of biofilm, while short PilA is vital in overall conductivity of biofilm as it will mediate the localization of *c*-Cyts, OmcZ on the outer membrane of the organism (Richter et al., 2012). It is also suggested that genes involved in synthesis of cyclopropane fatty acids and exopolysaccharide matrix plays a role in biofilm formation (Cologgi, 2012). Direction of electron flow also affects the thickness of biofilm formed where *G. sulfurreducens* biofilms formed on anode are thicker than to those formed in cathode that serve as electron donors (Gregory et al., 2004; Strycharz et al., 2008).

Those discussed previously are biofilms formed by pure culture of the respective species, which could not be controlled in actual application where multiple consortium of microorganisms exists in the system. In a mixed culture biofilm, observed through studies where *S. oneidensis* and *G. sulfurreducens* are co-cultured for biofilm formation, it is reported that the latter dominated 91% of whole cell number in the biofilm formed. However, it is also reported that number of cells for both organism in the biofilm are improved, indicating that there are a symbiotic effect between the two exoelectrogens (Dolch et al., 2014). In a mixed culture biofilm, the exoelectrogens participate in processes leading to biofilm formation with their pili, leading to a further specific contact between intra and interspecies, and between cell surface and insoluble electron acceptors (Dolch et al., 2014; Gorby et al., 2006; Reguera et al., 2006).

2.2.2 Direct electron transfer – *Geobacter sulfurreducens*

G. sulfurreducens is a typical example of microorganisms that can directly transfer electron to Fe(III) oxides or oxyhydroxides through pili that are found on the cell envelope formed by protein filaments, which are assemblies of the pilin protein PilA (Cologgi et al., 2011; Reguera et al., 2005). These pili are known as nanowires that are capable of conducting electricity. PilA will be matured when the protein's pre-polypeptide is recognised and processed by a dedicated signal peptidase and subsequently assembled by type IVa pilus apparatus (Reguera et al., 2005). The conducive

characteristics of these nanowires are due to the five aromatic amino acids (Trp, Phe, Tyr, His, and Met) that is responsible for the pi-pi orbitals overlaps in the pili structure that allows this conductivity (Vargas et al., 2013).

2.2.3 Indirect electron transfer

Most bacteria use the indirect electron transfer (IET) mechanism for electron transfer that will ferment carbon sources to produce metabolites such as alcohol or hydrogen. These products are of redox species and contains an electron that can act as the electron donor (Pinto, 2016). With help of low molecule, and soluble mediators such as phenazine, pyocyanin, thionine, riboflavin, and humic acid that will allow electron to be transferred to anode without direct contact between cell and the electron acceptor. These mediators could enter the bacteria cells, and carry the electrons produced from metabolic reactions of EAB to be transferred out to the anode subsequently (Cao et al., 2019).

2.3 Microbial fuel cell

2.3.1 Air-cathode MFC

Most air-cathode MFC reported in literature comes in the form of a single-chambered cube reactor (Figure 2A) developed at Penn State (Liu and Logan, 2004), or double-chambered air-cathode cube type MFC. General design of these type of MFC consist of only one chamber that houses both anode and cathode which is either located far apart or separated by a PEM. For this MFC design, the organic substrate along with the bacteria is inoculated into the anodic chamber, where the proton produced will travel to the cathode, reacting with O₂ in the air, forming H₂O. This type of MFC which cathode electrode is exposed to the air have considerably lower operating and construction costs due to the lack of aeration requirement in the cathode. This design also has considerably higher power production due to lower internal resistance compared to dual-chambered MFC with aqueous cathode (Pant et al., 2010). However, this design is limited by several factors such as membrane dehydration, evaporation of anolyte (Fan et al., 2007) and salt deposition which cause the limitation of diffusion of proton from anode to cathode and imbalances in pH (Cheng and Logan, 2011).

2.3.2 Double-chambered MFC with aqueous cathodes

The simplest MFC design that consist of two PEM-separated chambers are shown in Figure 2B. It is generally used to study electrode materials, microbiological processes, and to optimize parameters. As compared to the single-chamber MFC, electrolyte is present in the cathodic chamber which will be discussed further in the next section. This type of MFC are also often operated in batch mode. Many researchers use this design for studies on power production (Parkash, 2015; Ivanov et al., 2017; Ogbulie et al., 2017; Ding et al., 2014; Kumar et al., 2016; Choudhury et al., 2017; Wang et al., 2017; Deng et al., 2017).

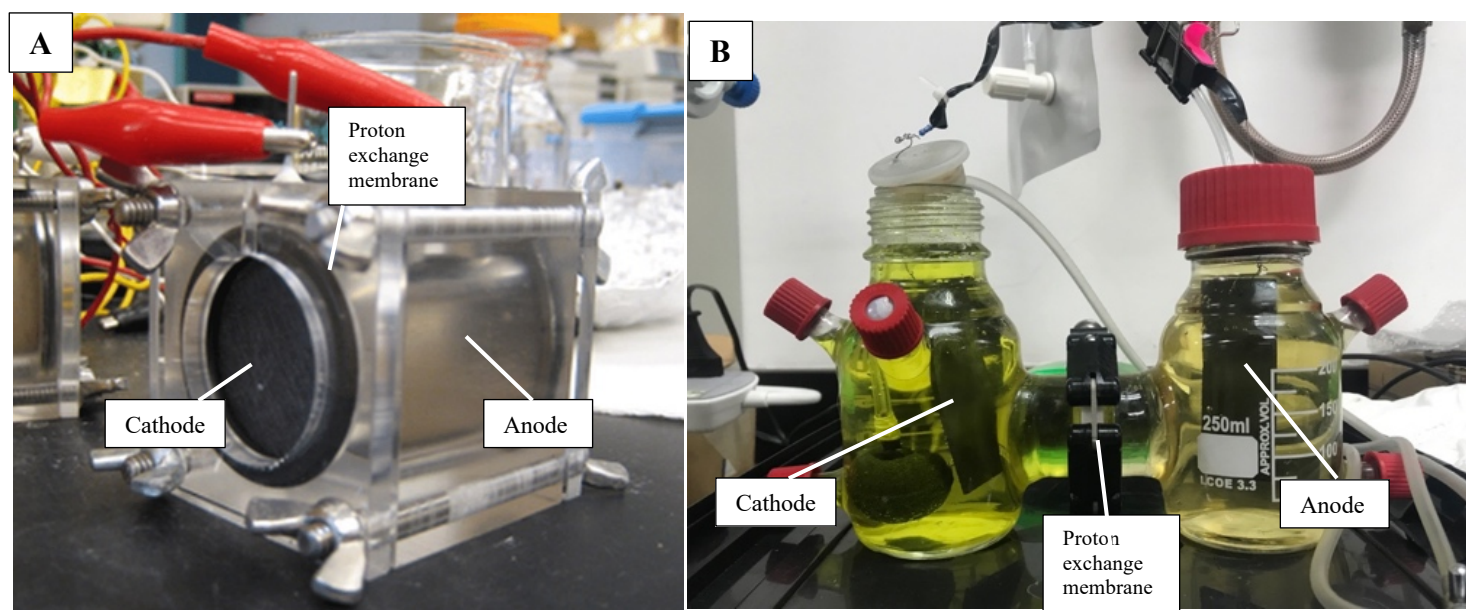


Figure 2: 2A illustrates a typical single chambered air cathode reactor. Photo taken from (Logan, 2007). 2B illustrates a simple dual-chambered MFC set-up.

2.3.3 *The catholyte*

The catholyte is to enhance the flow of protons from the PEM membrane to the cathode electrode, therefore their characteristic could greatly impact the efficiency of the MFC. O₂ gas is the most commonly used electron acceptor at the cathodic compartment due to high oxidation potential and the final product just being H₂O with the reaction $\text{O}_2 + 4\text{H}^+ + 4\text{e}^- \rightarrow 2\text{H}_2\text{O}$. However, it is demonstrated in studies that constant supply of O₂ gas is cost-inefficient (Strik et al., 2010). Additionally, using O₂ as electron receiver also shows issues such as poor contact with electrode surface and slow rate of reduction of oxygen with normal electrode made with carbon materials (Rhoads et al., 2005). Using different catholytes could give beneficial results such as reduce operating costs and increases power generation. Examples of catholytes reported from literatures are ferricyanide, nitrogen species, persulfate, permanganate, manganese dioxide, mercury, iron, copper, hydrogen peroxide, carbon dioxide, as tabulated in Table 1 (Ucar et al., 2017).

Table 1: Lists of electron acceptors used of MFC and their general application and advantages and / or limitations

Catholyte	Chemical equation	Applications	Advantages and / or limitations	References
Ferricyanide	$Fe(CN)_6^{3-} + e^- \rightarrow Fe(CN)_6^{4-}$	Limited to basic laboratory studies	Lower overpotential compared to conventional oxygen, resulting in a faster reaction rate and higher power output. However, it is not sustainable, toxic, and chemical regeneration is difficult.	(Korneel Rabaey et al., 2003; Schröder et al., 2003; Logan et al., 2006)
Nitrogen species	$NO_3^- + 2e^- + 2H^+ \rightarrow NO_2^- + H_2O$ $NO_2^- + e^- + 2H^+ \rightarrow NO + H_2O$ $NO + e^- + H^+ \rightarrow \frac{1}{2}N_2O + \frac{1}{2}H_2O$ $\frac{1}{2}NO_2 + e^- + H^+ \rightarrow \frac{1}{2}N_2 + \frac{1}{2}H_2O$	Simultaneous removal of carbon and nitrogen.	Crossover of ammonia from anode to cathode through CEM causes low nitrogen removal in effluent	(Virdis et al., 2008; Fang et al., 2011; Puig et al., 2011)
Persulfate	$S_2O_8^{2-} + 2e^- \rightarrow 2SO_4^{2-}$	Utilized in the clarification of swimming pool, micro-etching of copper printed circuit boards, and hair bleaching. Have a higher oxidation reduction potential than many catholytes (such as permanganate) used in MFC	Decreased performance of the cell compared with MFC with $K_3Fe(CN)_6$ at medium to high current densities	(Li et al., 2009)

Permanganate	$MnO_4^- + 4H^+ + 3e^- \rightarrow MnO_2 + 2H_2O$ $MnO_4^- + 2H_2O + 3e^- \rightarrow MnO_2 + 4OH^-$	Limited to basic laboratory studies	Does not require catalysis and shows higher power output in acidic conditions. However, continuous liquid replacements are needed during the depletion of permanganate during electrical generation. pH has to be controlled for stable output which is only applicable in small-scale power supplies.	(You et al., 2006)
Manganese dioxide	$MnO_2 \rightarrow H^+ + e^- \rightarrow MnOOH_{(s)}$ $MnOOH + 3H^+ + e^- \rightarrow Mn^{2+} + 2H_2O$ $Mn^{2+} + O_2 + 2e^- \rightarrow MnO_2$	Good cathode material and catalysis for battery and alkaline fuel cells. Can also be utilized as alternative cathode catalyst to platinum due to the lower cost.	More efficient usage of electron mediators between cathode and oxygen as compared with their direct utilization due to their difficulties such as low solubility	(Liew et al., 2015; Zhang et al., 2009)
Mercury	$2Hg^{2+} + 2Cl^- \rightarrow Hg_2Cl_2(s)$ $Hg_2Cl_2(s) + 2e^- \rightarrow 2Hg(l) + 2Cl^-$	Use for removal of mercury from aquatic environment	Mercury is tedious to be handled due to its toxicity	(Wang et al., 2011)

Iron	$Fe^{3+} + e^{-} \rightarrow Fe^{2+}$	Utilized as electron mediator to enhance performance in cathode chamber.	Reversible electron transfer provides faster reaction, increased standard potentials, biodegradability, and release of valuable compounds such as Phosphate. However, MFC with ferric ions requires utilization of a bipolar membrane instead of a standard CEM which is not suitable for pH control.	(ter Heijne et al., 2006; Fischer et al., 2011)
Copper	$4Cu^{2+} + 8e^{-} \rightarrow 4Cu(s)$	Simultaneous copper recovery and energy production	Non cost-effective reactor design currently and still in early stage of development	(ter Heijne et al., 2008)
Hydrogen peroxide	$H_2O_2(aq) + 2H^{+} + 2e^{-} \rightarrow 2H_2O(l)$	Remove contaminants with hydroxyl radicals formed as a result of reaction with fenton	Strong oxidizing properties. Their utilization has been reported that can provide stability in a long-run operation of MFC	(Tartakovsky and Guiot, 2006)
Carbon dioxide	$HCO_3^{-} + 4e^{-} + 5H^{+} \rightarrow CH_2O + 2H_2O$ $CO_2 + 8H^{+} + 8e^{-} \rightarrow CH_4 + 2H_2O$	Methane production from CO ₂ reduction.	For the production of methane, process consumes less energy as there is no heat needed at the cathode to maintain temperature. System is also effective at low substrate concentrations	(Cao et al., 2009)

2.3.4 Electrode materials

An ideal electrode can improve interactions between microbial biofilm and the material surface, improving MFC efficiency. Several characteristics are often evaluated in electrodes: electrical conductivity, mechanical strength, biocompatibility, mechanical strength, inner structure, environmental impact, and cost (Rinaldi et al., 2008; Guo et al., 2015; Mustakeem, 2015). Anaerobic respiration of bacteria using the supplied substrate will result in generation of electron that will be transported through an external circuit to the cathode where reduction of oxidizing agent will happen. Both processes oxidation and reduction will happen simultaneously, causing a continuous flow of electrons, generating current. The selection of materials for electrodes is a critical step to prevent corrosion and to enhance biofilm growth. Carbon and metallic based materials exhibits the positive traits of the mentioned characteristics (Rimboud et al., 2014; Venkataraman et al., 2011). Carbon cloth are very often used as anode material in MFCs (Kipf et al., 2013; Nevin et al., 2008; Larrosa-Guerrero et al., 2010; Liu et al., 2005). This is due to the high surface area, electrical conductivity, flexibility, mechanical strength and porosity which are all the ideal traits needed in a good electrode. However, this comes short in terms of cost as it is generally expensive. Graphite sheet (or plate) is a simple electrode that guarantee high electrical conductivity at a relatively lower cost as compared with carbon cloth. But this comes in short in terms of surface area and surface to volume ratio, resulting in lower output levels as compared to more porous or structured materials (ter Heijne et al., 2008; Coursolle and Gralnick, 2010). Other electrode materials that were used including stainless steel, and ceramic electrodes. However there were limitations as compared to carbon-based electrodes as the former were reported to not able to develop thick and robust biofilm, where the latter has a very high cost which is not suitable in a MFC (Kalathil et al., 2018).

2.4 Evaluation of MFC

2.4.1 Polarization and power density curves

Polarization curve is utilized for current characterization as a function of voltage (Logan, 2007), and is the parameter which could determine the performance of a MFC. The circuit's external resistance (load) is varied to obtain a new voltage value (and current) through formula:

$$V = IR \quad (1)$$

where V = voltage measured across the resistor; I = current; and R = resistance.

Power density curve is then calculated from measured voltage as:

$$P = IV, \quad (2)$$

$$\text{or alternatively } P = I^2 R; \quad (3)$$

where P = Power, I = Current, and R = Resistance

MFC literatures typically use power density curve to determine the maximum power generated. It is also important to note that it is difficult to compare power of reactor using different configurations (such as electrode size), hence researchers typically normalize power output to electrode surface area or volume.

2.4.2 Wastewater removal efficiency and Coulombic efficiency (CE)

Wastewater removal efficiency is obtained through Chemical oxygen demand (COD) tests where initial substrate concentration being supplied is compared with the COD of the substrates after a time period. This change in COD obtained were then used to calculate the CE value. CE is defined by the ratio of the electron number transferred towards the anode the maximise the theoretical number of electrons generated from the complete oxidation of substrate inoculated. It is one of the most important parameters used to evaluate MFC's performance as it indicates the contribution of microbial respiration to EET.

CE values for glucose are obtained as described in Logan (2008) with the formula:

$$C_E = \frac{M_S \int_0^{t_b} I dt}{F b_{es} v_{an} \Delta c} \quad (4)$$

Where Δc is the change in substrate concentration over a time $= t_b$, M_S is the molecular weight of substrate, F denotes the Faraday's constant, $b_{es} = 4$ for the number of electrons exchanged per mole of oxygen, and v_{an} is the volume of liquid in anodic chamber. As for other substrate, the following equation are used:

$$C_E = \frac{8 \int_0^{t_b} I dt}{F v_{an} \Delta COD} \quad (5)$$

Where 8 is the constant used for COD based on $M_{O_2} = 32$ for molecular weight of Oxygen gas.

2.5: Engineering *S. oneidensis* for performance improvement of microbial fuel cell – a mini review

This chapter was submitted to Applied Biochemistry and Biotechnology as a review article.

2.5.1 Abstract

Microbial Fuel Cell (MFC) is a promising technology that can treat wastewater and generate electricity simultaneously. Basic principle of a MFC uses microorganism in the form of biofilm that is cultivated on surface of anode that oxidizes the substrate provided. Different electrode surface will have difference in biofilm growth from different organisms that will ultimately affects Extracellular Electron Transfer (EET). EET are carried out by the microorganisms that produces protons and electrons which will migrate to the cathode chamber via a PEM and external circuit respectively. A model organism that is wide established that are capable to carry out EET are *Shewanella oneidensis*. A metal reducing pathway are utilized by these organisms that are able to transfer electrons obtained from metabolism of substrate from anaerobic respiration outside the cell wall, which then will be accepted by the electrode. *S. oneidensis* are genetically engineered for several aims which include studying the underlying mechanisms in EET, and also to enhance power generation and wastewater treating potential when used in a MFC. Through engineering *S. oneidensis*, genes responsible for EET are identified and efforts are given to enhance the effect through genetic engineering. Examples such as overexpressing genes related to EET which include biofilm formation, mediator biosynthesis, and respiration. This review highlights the strategies used in transformation of *S. oneidensis*, then focuses on the recent development of MFC in terms of intervention through genetic modifications.

2.5.2 Introduction

Exoelectrogens that are widely studied with established genome database are *Shewanella spp.* and *Geobacter spp.*, more specifically *Shewanella oneidensis* and *Geobacter sulfurreducens* (Santoro et al., 2017). The former is well known for its bidirectional flow of electrons across the cellular membranes (Jiao et al., 2011), while the latter exchange electrons through conductive nanowires to various electron acceptors (Reguera et al., 2005). Different substrates can be utilized depending on the strain of microorganism used, where lactate, acetate, and glucose, are the typical substrates used by exoelectrogens for anaerobic respiration (Santoro et al., 2017). Along with the refinement of techniques in molecular biology and the rapid advancement of biotechnology, it is only a matter of

time where substrate and biofilms formation metabolic pathways of various organisms are identified and cloned into a strain where it could be ubiquitously utilized as an exoelectrogens for efficient current generation in a MFC. As different bacteria utilizes different substrate for anaerobic respiration – recent studies had discovered different exoelectrogens that could be used to liberate electrons from cellulose, which is a complex polymer (Toczyłowska-Mamińska et al., 2018). This process is achieved through a syntrophic consortium where interspecies communication leads to decomposition of cellulose into volatile acids through metabolic cooperation. This signifies the importance to look into the metabolic pathway of various exoelectrogens at the DNA level and to also further establish the biological interactions between different species in a biofilm and how they each play a role in substrate oxidation.

2.5.3 Transformation of *S. oneidensis*

Transformation of *S. oneidensis* were mainly carried out to study genes essential for EET by gene repression or overexpression, with the aim of enhancing overall power generation capabilities, and wastewater treating potential of MFC. Factors that have to be considered in a typical gene transformation experiment in *S. oneidensis* include promoter, plasmid, and also transformation method. In most studies, researcher tend to use an IPTG-inducible promoter element *PlacIq-lacIq-Ptac* to induce overexpression of inserted gene in the plasmid vector, which most studies shown reliable results in terms of power densities produced (Table 1). Recently, West et al. (2017) had demonstrated the utilization of trimethylamine N-oxide (TMAO) as an inducer of EET by replacing the native *mtrCAB* promoter, which controls expression of *mtr* pathway responsible with EET, with P_{torF} , which regulates expression of operon which encodes the TMAO respiratory system in *S. oneidensis*. This transformation enables the overexpression *mtr* pathway in *S. oneidensis* to be induced by a range of TMAO concentrations, albeit with comparatively lower current produced than of WT-strain.

Ng et al. (2018) evaluated the performance of various plasmids and promoters through the expression of GFP proteins, and subsequently Mtr pathway genes, in recombinant *S. oneidensis*. They reported the replication origin *repB* along with pLacI promoter are able to drive expression of inserted genes with higher enzymatic activity as compared with controlled strain. Plasmid DNA are delivered into cells most commonly through conjugation from *E. coli* or electroporation, which are utilized in different situations where the latter are more convenient for transfer of both linear and circular DNA. Gene editing tools are recombineering (Lambda-Red, Cre-Lox), and also CRISPR. Lambda Red protein is a protein derived from phage lambda and consisting of three parts: Exo (lambda exonuclease),

Gam and Beta. Exo degrades dsDNA from 5' to 3' ends, leaving a ssDNA at the recessed region; Gam prevents nucleases produced by *E. coli* from degrading linear dsDNA, Beta binds to ssDNA regions produced by Exo and facilitates the recombination process by promoting the incorporation of product to target site (Figure 3) (Sawitzke et al., 2007).

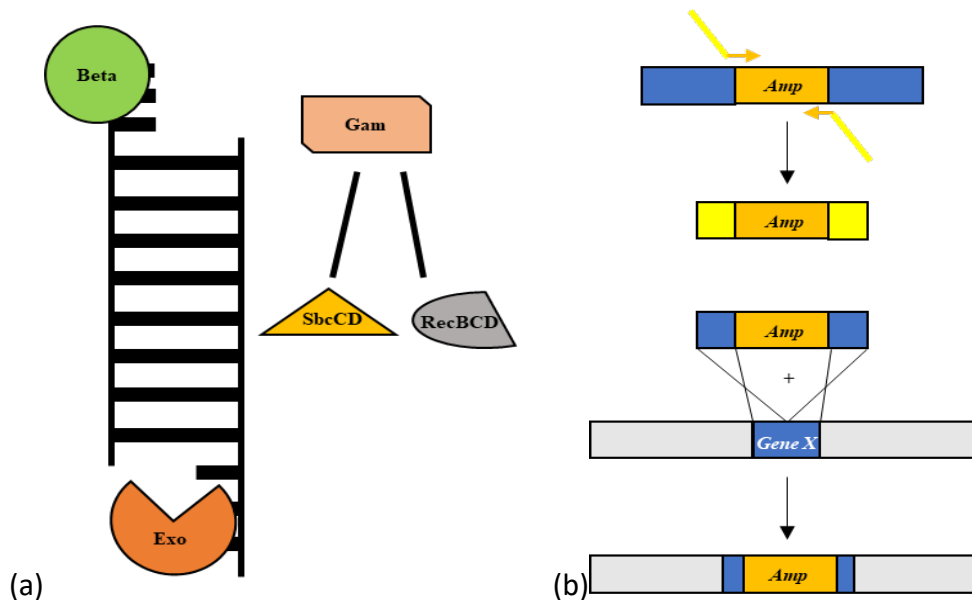


Figure 3: **3a** The three components of Lambda-red recombineering system with Exo, Beta, and Gam. Gam prevents digestion of linear DNA by endogenous RecBCD and SbcCD; Exo degrades linear dsDNA from 5'; and Beta promotes annealing of ssDNA created by Exo to a complementary target in the cell; **3b** An overview of using this system to knockout a gene of interest with an ampicillin resistant cassette

While this recombineering technique are generally employed to programming *E. coli*, Corts et al. (2019) had established the optimum conditions for making electrocompetent cells and had demonstrated the application of lambda red recombineering in *S. oneidensis* by expressing recombinases under an arabinose inducible promoter pBAD and targeted the integrated *lacZ* gene from *E. coli* in the chromosome of *S. oneidensis*, then subsequently the native *rpsL* gene. The Cre/*lox* system is a site-specific recombinase which contain the Cre protein originated from P1 bacteriophage which catalyses the recombination at the *loxP* sequence (Abremski and Hoess, 1984). So far, no research had utilized the Cre/*lox* system for programming *S. oneidensis*. However, Enyeart et al. (2013) had established the system Genome Editing via Targetrons and Recombinases (GETR) where mobile group II introns, termed 'targetrons', works in tandem with Cre/*lox* recombination in *S. oneidensis*. Retargetable mobile group II introns has been developed recently as a tool that can be designed to be inserted into a given DNA site at high efficiency. Intron-encoded protein (IEP) are involved in self-splicing and in the process of 'retrohoming' where the intron site reverse splices into DNA specifically. A targetron which intron contain a *loxP* site that allow gene insertion into ribosomal *rrs* genes in *S.*

oneidensis was designed as well. It was reported that insertion was found in most copies of *rrs* gene in the genome, which proof that the system could be potentially utilized for reprogramming *S. oneidensis*.

The clustered regularly interspaced short palindromic repeat (CRISPR) system allow targeted genome editing in a range of different organisms (Figure 4) (Barrangou et al., 2007). The first usage of CRISPRi (CRISPR-interference) system to repress Mtr and biofilm formation genes *speF* and *uvrY* to establish a CRISPRi-sRNA method that allow transcriptional and translational gene regulation in *S. oneidensis* are reported (Cao et al., 2017). Through CRISPRi, the repression of both genes *speF* which encodes a putrescine biosynthesis gene that produces putrescene as a strategy employed by prokaryotes for active cell dispersal from biofilms, and *uvrY* which is related to synthesis of surface polysaccharide that deters formation of biofilms, had enhanced power generation of MFC by 65% when compared with the control strain. These various tools in engineering *S. oneidensis* could be further exploited for more discoveries on EET related genes and manipulations could be performed to enhance effect of said genes with the objectives to ultimately increase overall potential of an MFC.

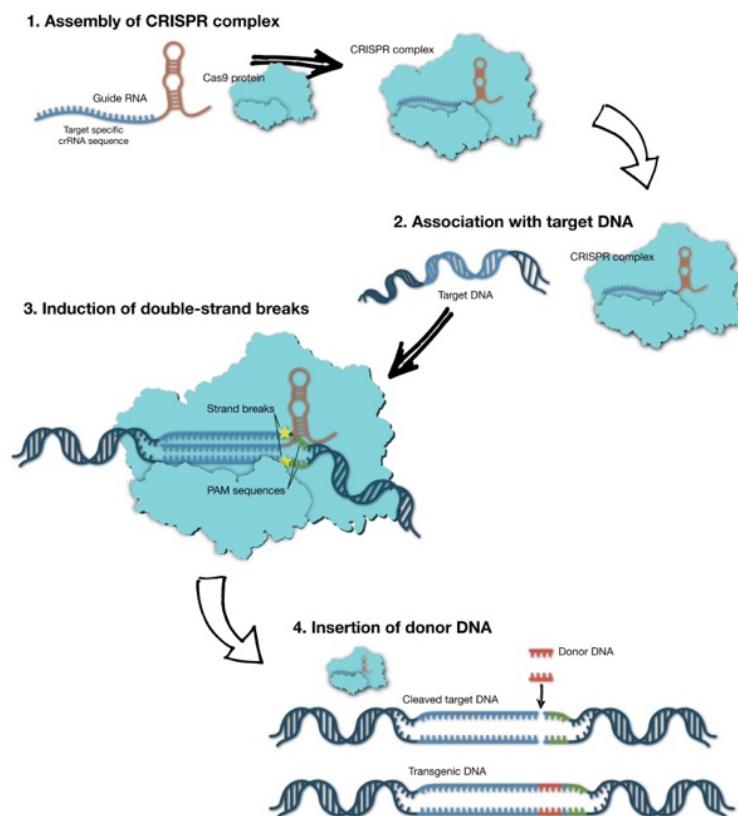


Figure 4: Adapted mechanism of genome editing using CRISPR/Cas9 system (Costa et al., 2004). This editing system requires a single-guide RNA (sgRNA) that directs the Cas9 endonuclease to the specific site of interest in the genomic DNA, resulting in a double strand break. With the presence of a donor DNA in trans, a transgenic DNA can be synthesized; whereas with the absence of a donor DNA, double strand break will be repaired by the host cell, resulting in an insertion or deletion that will disrupt the ORF of a gene

A list of experiments is compiled for comparison for manipulations of genes in *S. oneidensis* and the MFC performance which also be discussed in further sections (Table 2). Manipulations are categorised into gene function which include biofilm forming, respiration, mediators, substrate utilisation, and also EET itself. From the table, it is observed that power produced from *S. oneidensis* is still inferior to other studies where power generation can reach up to 70.8 mW/m³ on different parameters such as organism used, MFC design, which are extensively reviewed (Gude, 2016). Even with lower power generated, developing more approaches in engineering *S. oneidensis* is very important on the future development of MFC as *S. oneidensis* serves as a model organism for EET and more studies on EET should be focus at the molecular level. From studies of gene functions, there were no attempts in overexpression of biofilm formation related genes. While it is proven that biofilm formation plays an important role for optimal MFC performance, manipulations had been attempted from the electrochemical aspects in which treatments were directed at carbonaceous electrode to study the

surface attachment of the organisms (Cornejo et al., 2015). Coupling electrode modification with genetic manipulations can be one potential aspect where future research can venture into. Manipulations in hope to allow a wider range of substrates that is utilisable were performed. Most widely used synthetic substrate used in MFC studies were respectively glucose, lactate, and acetate, where acetate and lactate both shows high power densities generated which glucose lacks behind due to the position in the metabolic pathway (Pant et al., 2010). However, in actual wastewater such as foodwaste leachate, concentration of glucose will be expected to be the highest. One aspect that can be looked into would be to optimise the bacteria in hope for high performance in MFC while fed with glucose that may be possibly achieved through the deactivation of the genes responsible for fermentation pathway when glucose is present.

Table 2: List of experiments performed to engineer *S. oneidensis* for enhanced effect in MFC

Elements	Remarks	Energy output (mV/m ²)	Ref
<i>speF</i>	Gene repression by CRIPSRI	36.8	(Cao et al., 2017)
<i>uvrY</i>	Gene repression by CRIPSRI	30.5	(Cao et al., 2017)
<i>speF-uvrY</i>	Gene repression by CRIPSRI	40.3	(Cao et al., 2017)
<i>arcA</i>	In-frame deletion	n.a.	(Thormann et al., 2005)
<i>crp</i>	In-frame deletion	n.a.	(Thormann et al., 2005)
<i>etrA</i>	In-frame deletion	n.a.	(Thormann et al., 2005)
<i>mtrA</i>	Gene knock-out	4.5	(Cao et al., 2017)
<i>mtrA</i>	Gene repression by CRIPSRI	19.3	(Cao et al., 2017)
<i>mtrB</i>	Gene repression by CRIPSRI	14.1	(Cao et al., 2017)
<i>mtrC</i>	Gene repression by CRIPSRI	12.8	(Cao et al., 2017)
<i>oprF</i>	Porin gene from <i>P. aeruginosa</i> . Mutant <i>S. oneidensis</i> is inoculated into MFC together with <i>S. cerevisiae</i>	123.4	(Lin et al., 2017)

<i>mtrC-mtrA-mtrB</i>	Genes cluster for metal reducing pathway amplified from <i>S. oneidensis</i> and overexpressed	20	(Min et al., 2017)
<i>ribADEHC</i>	Overexpression of flavin biosynthetic pathway from <i>B. subtilis</i>	233.0	(Yang et al., 2015)
<i>ribD- ribC- ribBA- ribE</i>	Gene cluster for flavin biosynthesis amplified from <i>S. oneidensis</i> and overexpressed	15	(Min et al., 2017)
<i>xylT-xylA-xylB</i>	<i>xylT</i> encodes D-xylose-proton symporter from <i>Clostridium acetobutylicum</i> , <i>xylA</i> encodes xylose isomerase and <i>xylB</i> encodes xylulokinase, both from <i>E. coli</i>	64,800	(Li et al., 2017)
<i>Gxf1-xylA-xylB</i>	<i>Gxf1</i> encodes glucose/xylose facilitator 1 from <i>Candida intermedia</i>	88,800	(Li et al., 2017)
<i>xylT-XYL1-XYL2-XKS1</i>	<i>XYL1</i> encodes D-xylose reductase, <i>XYL2</i> encodes xylitol dehydrogenase, <i>XKS1</i> encodes D-xylulokinase, both from <i>Scheffersomyces stipites</i>	101,120	(Li et al., 2017)
<i>Gxf1-XYL1-XYL2-XKS2</i>	Genes expressed as a module	117,440	(Li et al., 2017)
<i>pflB</i>	Native gene encoding pyruvate-formate lyase to formate from pyruvate	389,440	(Li et al., 2018a)
<i>fdh*</i>	<i>fdh*</i> from <i>Candida boidinii</i> encoding NAD ⁺ -dependent formate dehydrogenase to convert CO ₂ from NADH	426,240	(Li et al., 2018a)
<i>mdh</i>	Native gene encoding NAD ⁺ -dependent malate dehydrogenase that converts oxaloacetate from malate	429,440	(Li et al., 2018a)
<i>pflB-fdh*-gapA2-mdh</i>	Plasmid constructed with both <i>pflB</i> and <i>fdh*</i> operating under one module and <i>gapA2</i> and	105.8	(Li et al., 2018a)

	<i>mdh</i> under another module, both with individual <i>placI</i>		
<i>ycel</i>	Gene originated from <i>B. subtilis</i> encoding a bifunctional <i>niaP</i> transporter constructed for Na utilization	493,120	(Li et al., 2018b)
<i>ycel-nadV-pncC</i>	<i>nadV</i> encodes Nm phosphoribosyltransferase. The module is expressed in the Nm-utilization pathway	481,600	(Li et al., 2018b)
<i>nadD*-nadE*</i>	<i>nadE</i> encodes NAD ⁺ - synthetase and <i>nadD</i> encodes nicotinate mononucleotide adenylyltransferase. Both genes originated from <i>E. coli</i>	528,640	(Li et al., 2018b)
<i>nadM</i>	<i>nadM</i> encodes NMN adenylyltransferase from <i>F. tularensis</i>	488,320	(Li et al., 2018b)
<i>nadM-nadD*-nadE*</i>	Genes expressed as a module	570,560	(Li et al., 2018b)
<i>ycel-pncB-nadM-nadD*-nadE*</i>	Genes expressed as a module	781,440	(Li et al., 2018b)

2.5.4 Biofilms formation and metabolic reducing pathway (*mtr*) of *S. oneidensis*

Biofilms are referred to as a complex of embedded microorganisms that stick with each other that could be a pure or a mixed culture within an extracellular matrix composed of self-produced extracellular polymeric substances (Snider et al., 2012; Parameswaran et al., 2013). Electroactive biofilms are imperative for exoelectrogens to efficiently carry out EET for bioelectricity generation. Current generated are directly proportional to biomass of the biofilm and type of electrode surface where negatively charged and hydrophobic anode surface are less favourable for electroactive biofilm formation and will also consequently affects the performance of the fuel cell. Optimum thickness is preferable to achieve substantial current densities as very thick deposition of biofilm are shown to limit electron flow (Wrighton et al., 2011; Parameswaran et al., 2013). Electrons generated during respiration of substrate by the exoelectrogens will go through their respective processes, depending on the type of organisms, and will finally be transported to the terminal electron acceptor – the surface of the electrode through the biofilm. Additionally, biofilms are also needed for the reduce of soluble of insoluble electron acceptors. These process are mediated by the c-type cytochromes (*c*-Cyts), or also known as the redox-active proteins (Brutinel and Gralnick, 2012; Kotloski and Gralnick, 2013). Microbial proteins such as type-IV pili, and electron shuttles like flavins, pyocyanin, *et cetera*, also enhances the conductivity of the biofilm, increasing electron transfer (Malvankar and Lovley, 2012; Orellana et al., 2013; Parameswaran et al., 2013).

Electron transfer of *S. oneidensis* biofilm are based on both direct electron transfer (DET) and mediated electron transfer (MET) where removal of electron shuttle flavin resulted in more than 70% loss in electron transfer in the biofilm (Marsili et al., 2008). Three factors, including electrode material (Dolch et al., 2014; Kipf et al., 2013), environmental parameters (Kipf et al., 2013; Rosenbaum et al., 2010), and biofilm thickness (Thormann et al., 2005) will affect the characteristics of biofilms formed by *S. oneidensis*. The genes *msh* (encodes Mannose-sensitive hemagglutinin, Msh)/*pilT* and *mxl* (encodes a putative carbohydrate containing cell associated component) are involved in surface attachment and biofilm formation (Saville et al., 2010; Thormann et al., 2004). A clusters of chemotaxis genes allow the strain to sense the electron acceptors. Genes within the clusters which are essential for chemotactic response to electron acceptor during anaerobic respiration is *cheA-3* and *cheA-1* where CheA-3 histidine protein kinase will be expressed when there are presence of electron acceptors (Li et al., 2007; Tai et al., 2010). The downregulation of motility protein flagellin and proteins associated with oxidative stress, along with the upregulation of *quorum sensing* proteins, riboflavin synthesizing

proteins, heme and transporter components ATP-Binding cassette (ABC), causes metabolic shifts in *S. oneidensis* biofilms (De Vriendt et al., 2005).

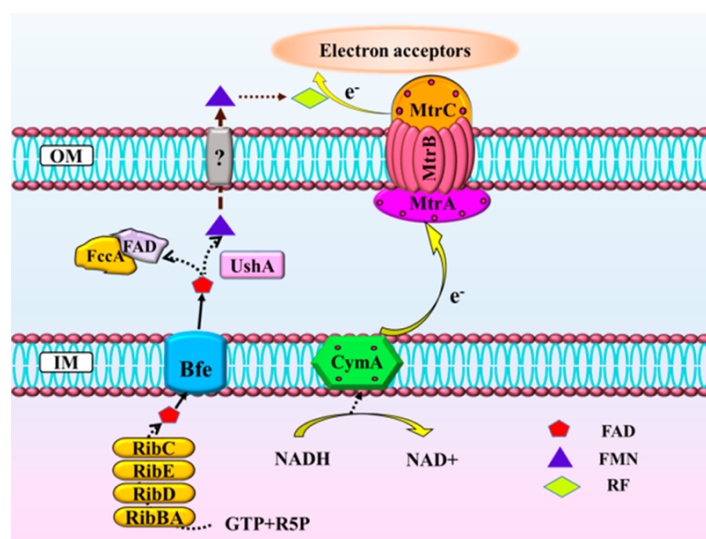


Figure 5: Schematic illustrates the flavin and mtr pathway in *S. oneidensis* (Min et al., 2017).

For *S. oneidensis*, a metal reducing conduit (Mtr pathway) is responsible for the flow of current from interior of cells to the outer membrane (OM) and subsequently to the extracellular anodes as a terminal electron acceptor, very often minerals that contains Fe(III), Mn(III), or Mn(IV) (Myers and Nealson, 1988), via the outer membrane *c*-type cytochromes (OMCs) (Lovley, 2012). A six multihaem *c*-Cyts, consists of (i) CymA (Inner membrane tetraheme *c*-Cyts), (ii) Fcc₃, (iii) MtrA (periplasmic decaheme *c*-Cyts), (iv) MtrC, (v) OmcA and a small tetrahaem cytochrome (STC), and lastly (vi) porin-like OM protein MtrB (Beliaev and Saffarini, 1998; Myers and Myers, 2000, 2002; Sturm et al., 2015; Beliaev et al., 2001; Coursolle and Gralnick, 2010). Electrons from organic matters and the electron donor, typically lactate in the case of *S. oneidensis*, go through an intracellular electron transfer pathway, from NADH through the quinol pool in the inner membrane, then subsequent through CymA where quinol will be oxidised and the electron transferred to STC and Fcc₃ (Firer-Sherwood et al., 2011; Marritt et al., 2012; McMillan et al., 2012, 2013), then to a terminal reductase complex consist of MtrA, MtrB, and MtrC. While it is unsure on the exact pathway in how the electrons are being transferred from STC and Fcc₃, a study where mutant with STC and Fcc₃ inactive shows reduced efficiency in Fe(III) oxides or oxyhydroxides reducing activities has proposed that they may play a role in the transportation of electrons from CymA to MtrA (Sturm et al., 2015; Leys et al., 1999, 2002). This complex will then transfer electron from the periplasmic proteins to the surface of bacteria (Hartshorne et al., 2009; Ross et al., 2007; White et al., 2013; Richardson et al., 2012), where physical interactions

of MtrC and OmcA can transfer electrons directly to Fe(III) containing minerals (Figure 5). To transport electrons to the exogenously to the surface of electrodes, *S. oneidensis* secretes co-factors to OMC, riboflavins (RF) for OmcA, and flavin mononucleotide (FMN) for MtrC, illustrated by the exhibition of complementary binding sites for these molecules on the cytochromes (Okamoto et al., 2014; Kotloski and Gralnick, 2013; Brutinel and Gralnick, 2012). These flavins also act as an “electron shuttle” that mediate the transfer of electron between the cells and the electrode. Recent studies shown that adding these electron shuttles exogenously or through amplifying their expression in *S. oneidensis* shows promising results in increasing power generated (Kotloski and Gralnick, 2013; Brutinel and Gralnick, 2012; Kumar et al., 2016).

2.5.5 Enhancing MFC performance through engineering biofilms formation

One of the strategies in enhancing MFC performance is to promote biofilm formation in exoelectrogens through biotechnology. Bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) were reported in multiple literatures that they play a central role in bacterial biofilm formation by promoting expression of adhesive matrix components that will in turn facilitates formation of bacterial biofilm. Liu et al. (2015) had demonstrated where expression of heterologous YdeH in *S. oneidensis*, which catalyse the biosynthesis of c-di-GMP, are able to enhance power generation of MFC. It was reported that the YdeH engineered strain with a constitutive promoter are able to produce maximum power density 2.8 times higher than of controlled strain. Lin et al. (2017) demonstrated that the expression of porin gene, *oprF* from *Pseudomonas aeruginosa* in *S. oneidensis*, coupled with addition of recombinant *Saccharomyces cerevisiae* that produce lactate with ethanol synthesis pathway knocked-out, are able to enhance power generation of MFC by up to 70% compared with the controlled. These studies shown that heterologous expression of genes related to biofilm formation could be one potential strategies in enhancing performance of an MFC. One of the major bottlenecks in MFC technology remains to be the long-term robust and efficient biofilms. Ding et al. ((2014) had demonstrated that where the disruption of the putrescine biosynthesis gene, *speF* stated previously, can allow *S. oneidensis* to form highly cohesive biofilms. Studies on detachment of *S. oneidensis* had found out that in-frame deletions of the global tranacriptional regulators ArcA, and CRP, which the mutants in *arcA* and *crp* have their detachment severely impact. This suggest a role for the genes to play in the formation of biofilm (Thormann et al., 2005). These mutants were not tested on an MFC and could be of a potential further thing for consideration in future experimental design.

2.5.6 Enhancing MFC through engineering EET mediators – Flavins

Several studies had shown that expression of the mediator that plays a role in EET, flavin, are able to increase performance of MFC. In one study where flavin synthetic pathway from *Bacillus subtilis*, *ribADEHC*, are expressed heterologously in *S. oneidensis* where an increase in power generation at 13.2 times compared with WT were reported (Yang et al., 2015). As previously stated, flavins acts as an electron shuttle for EET where *S. oneidensis* will secretes electrons as a product of anaerobic respiration, where these flavins will facilitate the transportation of these electrons to the electrode. Flavin synthetic pathway of *S. oneidensis* were also identified and expression amplified by Min et al. (2017) where both the flavin synthetic gene cluster, *ribD-ribC-ribBA-ribE*, and the Mtr pathway gene cluster, *mtrC-mtrA-mtrB* were co-expressed in *S. oneidensis*, with an increased in power generated of approximately 1.1 times were reported. In the first study, it was reported that 25.7 times of increased flavin concentration produced by *S. oneidensis* as compared with the controlled strain, whereas in the second study there is only an increase of 2 times flavin secreted. Detachment of *S. onedensis* cells from biofilms were also studied, it was reported that rapid decrease in concentration of oxygen will stimulate cell detachment. In-frame deletion of individual genes *arcA*, *crp*, and *etrA* was reported to be able to allow the transformed *S. oneidensis* to be defective in the detachment response, where most profound effect was observed in the transformed cell with *crp* deleted.

2.5.7 Enhancing MFC through engineering respiratory mechanisms of *S. oneidensis* – NAD^+

Sources for electrons that are transported through the Mtr pathway are derived from dehydrogenases such as lactate dehydrogenase, or $NAD(H^+)$ dehydrogenases (Madsen and TerAvest, 2019). In the past, the role of NADH in EET and under anoxic conditions are not well characterised. Recent research found out that *S. oneidensis* utilises Nuo or Nqr1, which are NADH dehydrogenases, for growth and EET in anaerobic respiration of lactate in high potential electron acceptor, which are also the similar dehydrogenases that is needed under aerobic conditions (Hirose et al., 2018; Madsen and TerAvest, 2019).

Li et al. (2018) had recently demonstrated that modifications in expression of NADH can enhance power generation capabilities of MFC. This is due to the role of NADH in respiration and also as an intracellular electron carrier and source for EET in *S. oneidensis*. NADH is regenerated by essential redox enzymes, such as GapA2, to serve as an electron acceptor in glycolysis. In his studies, several genes are identified from carbon metabolism pathways and are overexpressed together, resulting in

overall enhancement in power generated from MFC and also increased regeneration of NADH in the mutant *S. oneidensis*. These genes are namely *gapA2* and *pflB* from glycolysis, and *mdh* from TCA cycle. *pflB* encodes enzyme pyruvate-formate lyase which catalyse the conversion of acetyl-CoA and formate from pyruvate. As formate is a reduced metabolite, it can be utilized as an electron donor to *S. oneidensis* for power generation purposes. To enhance the release of stored electrons from formate to anode, *fdh** from *Candida boidinii*, which encodes a NAD⁺-dependent formate dehydrogenase which facilitates the conversion of NADH and CO₂ from formate, was also expressed exogenously, both had shown to have an effect in enhancing power generation in MFC. TCA cycle, also known as the Krebs cycle, is an essential for ATP production in most organism. The overexpression of the native gene *mdh* in *S. oneidensis* that encodes a NAD⁺-dependent malate dehydrogenase that converts oxaloacetate from malate shown promising results in enhancing power generating ability of an MFC. Overexpressing each of these genes discussed previously individually in *S. oneidensis* are able to increase voltage output significantly when compared with a wild-type strain by 26%, 28%, 9%, and 29% for *gapA2*, *pflB*, *fdh**, and *mdh*. Li et al. (2018a) had then generated a mutant *S. oneidensis* with all these genes identified that could enhance MFC performance. This mutant strain is reported to be able to generate higher power which is 3 times higher than the controlled wt *S. oneidensis*.

In a separate study, Li et al. (2018b) had identified genes in the NAD⁺ biosynthetic pathway and subsequently overexpressed to increase power generated of MFC through increasing NADH synthesized. Engineered genes are mainly identified from the salvage pathway through assimilating Nm and also utilizing nicotinic acid (Na) as a precursor, and the universal biosynthesis pathway which synthesizing NAD⁺. *S. oneidensis* are unable to utilize Na as a precursor due to the absence of Na transporter and nicotinate phosphoribosyltransferase that convert nicotinic acid mononucleotide (NaMN) from Na. Hence, Na assimilation pathway were reconstructed through *ycel* from *B. subtilis* that encodes a bifunctional niaP transporter for Na and Nm, and endogenous Nm metabolic pathways through the gene *pncB* which encodes the nicotinate phosphoribosyltransferase from *S. typhimurium* for the conversion of Na to NaMN and is introduced to create a mutant *S. oneidensis*. It is reported these mutants harbouring both *ycel* and *pncB* are able to generate voltage higher than of WT strain) and also showing increased intracellular NADH, NAD⁺, and NAD(H⁺) level at 78%, 44% and 54% times, respectively. Li et al. (2018c) then subsequently overexpressed endogeneous gene *nadV* that encodes a Nm phosphoribosyltransferase and *pnc* which encodes the Nm mononucleotide deaminase in the Nm-utilization pathway. It is reported that both mutants harbouring *ycel*, and with the genes *ycel*, *nadV*, and *pncC*, shows superior voltage output of 493 and 482 V/m² respectively. For the universal biosynthesis pathway, *nadE** and *nadD** from *E. coli* are expressed heterogeneously along with native

nadM in *S. oneidensis*, all genes identified from both pathways are assembled, resulting in mutant *S. oneidensis* strains that were able to produce maximum power density which is 4.4 times more as compared with the control strain. Additionally, intracellular NAD(H⁺) pool size of the mutant *S. oneidensis* increased by 2.1 times when compared with the WT strain.

2.5.8 Engineering *S. oneidensis* for enhanced utilities

In the sense of wastewater treating capabilities, studies also attempted to engineer *S. oneidensis* in hope to enhance their potential in treating pollutant which include incorporating foreign metabolic pathway that allow the organism to metabolise substrates that they normally will not utilise. An example of such cases would be the incorporation of xylose metabolic pathway into *S. oneidensis* which allow xylose to be used as a substrate in an MFC, resulting in an improved voltage generation up to 117 mV/m² (Li et al., 2017b). This demonstrated the potential in reprogramming *S. oneidensis* with foreign metabolic pathways for utilisation of different kinds of substrates commonly found in wastewater.

2.5.9 Conclusions

Manipulations can be categorized into gene functions which include biofilm formation, respiration, mediators, substrate utilization, and EET itself. The power generation results shown in Table 1 were found to be comparable to those studies utilizing different organisms and optimizing the MFC design configuration (Gude, 2016). Developing more engineering approaches on *S. oneidensis* is very important for the development of MFC as *S. oneidensis* serves as a model organism for EET, and more studies on EET should be focused at the molecular level. From the studies of gene functions, no attempts were found on the overexpression of biofilm-formation related genes. While it was proven that biofilm formation plays an important role for optimal MFC performance, manipulations had been attempted from the electrochemical aspects in which treatments were directed at carbonaceous electrode to study the surface attachment of the organisms (Cornejo et al., 2015). Coupling electrode modification with genetic manipulations can be one potential aspect where future research can venture into.

The advancement in the field of molecular biology has enabled rapid development of strategies in manipulating *S. oneidensis* for optimal and enhanced capabilities when applied in an MFC. Even with these findings, there are several obstacles that need to be addressed from the biological,

electrochemical, and physical aspects of the MFC technology. Namely, novel respiratory or metabolic pathway could be discovered from bacteria that utilize different types/ranges of substrates, and changes in the formation of biofilms that allow fine-tuning of electroactive bacteria for optimal performance in EET. Dynamics of different combination of electroactive or non-electroactive organisms can also be further investigated, as shown in the complementary studies of the combination of both *S. cerevisiae* and *S. oneidensis*. Effects of different promoters on both native and heterologous expression of genes in *S. oneidensis* can also be explored, as demonstrated by several studies showing increased utilization and more precise control of EET performed by the organisms.

2.6 Trends in actual wastewater treatment using MFC technology

In the past few years, MFC had been tested by various researcher to generate electricity by treating various type of real wastewater. These experiments were all conducted within laboratory scale, and various results were obtained. Several examples will be discussed. A study of utilising real electroplating wastewater as a fuel in dual-chambered MFC was demonstrated, power density of 1200 mW/m² and 99.5% removal of Cr⁶⁺ were being reported (Li et al., 2008). In a separate study, potential of utilising wastewater from food processing in MFC were investigated as well with power produced at 230 mW/m² and 86% of COD removed after treatment period (Mansoorian et al., 2013). Other potential sources of wastewater that MFC can utilise from is palm oil mill effluent (POME) and domestic sewage with power density obtained at 107.35 mW/m² and 2000 mW/m² respectively, with 32% and 80% of COD removed respectively (Zhang et al., 2015; Baranitharan et al., 2015). Rice bran, a by-product produced during the rice milling process had been reported to generate electricity in a laboratory-scale single chamber MFC at 520 mW/m² (Takahashi et al., 2016). These examples only demonstrated how can MFC technology be integrated into a conventional wastewater treating system and these individual values cannot be compared directly as different parameters in MFC, which include MFC design, separator, cathode and anode material, and the organism responsible for EET, were being used while these experiments were conducted.

Chapter 3: Materials and Methods

3.1 Introduction

This chapter describes the experimental details involved in the thesis, which include all chemicals, reagents, and culture media used. The work described below were carried out in a laminar air hood using sterile techniques to prevent microbiological contamination. All chemicals used were of reagent grade. All solutions and components of MFC used were autoclaved (except for the Nafion Membrane) prior to the experiment using a vertical autoclave at 121°C and 100 kPa for 40 minutes.

3.2 Chemicals, reagents, strains, and culture media used

Table 3: Chemicals and reagents used in this thesis

Chemicals	Product number	Source
D-Glucose, $C_6H_{12}O_6$	G8270	Sigma-Aldrich
Sodium D-L Lactate, $C_3H_5NaO_3$	31605-85	NACALAI TESQUE, INC.
Sodium Acetate, $C_2H_3NaO_2$	3470-01	avantor
Sodium Fumarate, $C_4H_2Na_2O_4$	F1506	Sigma-Aldrich
Sodium Phosphate Monobasic, NaH_2PO_4	A1047	ITW Reagents
Sodium Phosphate Dibasic, Na_2HPO_4	A1046	ITW Reagents
Potassium Ferricyanide, $K_3[Fe(CN)_6]$		R&M Chemicals
Hydrogen Peroxide, H_2O_2 (30%)	H-0155	KATAYAMA CHEMICAL
Sulphuric Acid, H_2SO_4 (98%)	339741	Sigma-Aldrich
Glutaric Dialdehyde, $C_5H_8O_2$ (50%)	W512303	Sigma-Aldrich
Plate Count Agar	1054630500	MERCK
MacConkey Agar		MERCK
Nutrient Broth	1054430500	MERCK

Table 4: Chemical composition for preparation phosphate buffer

Chemicals	Amount added/L
Na_2HPO_4	8.92g
NaH_2PO_4	2.30g

Phosphate buffer solution, pH = 7.2, containing (per liter) was prepared by dissolving the chemical listed (Table 4) in deionized water. pH was then adjusted with NaOH and HCl to desired value and sterilized for 40 minutes at 121°C.

3.3 Preparation of anolyte and catholyte

Synthetic wastewater (the anolyte) was prepared based on previous studies (Santoro et al., 2017; Stager et al., 2017), with pH = 7.2, containing (per liter) of their respective substrates at 50 mM (Table 5), were dissolved or topped up to 1L with 50mL Nutrient broth (5%), 50 mM phosphate buffer (Table 4), and sterilized for 40 minutes at 121°C with the exception of glucose, for which a 0.45µm filter syringe is used to filter sterilize the solution. Three types of synthetic wastewater, glucose, lactate, and acetate were prepared accordingly. These substrates were chosen due to their position in the respiratory pathway that is present in most common metabolic pathways in prokaryotes, as well as their extensive use in *in-vitro* studies of MFC (Pant et al., 2010). Catholyte solution of 50mM K₃[Fe(CN)₆] in 50mM phosphate buffer, pH = 7.2, containing (per liter) was prepared similarly and the chemical used are listed in Table 6. Ferricyanide was chosen as the catholyte due to the higher power output being recorded in MFC and its efficiency in basic laboratory studies that is widely reported (Ucar et al., 2017). All solutions were wrapped in aluminium foil as it is prone to degradation in the presence of light.

Table 5: Amount of substrate used for the preparation of individual 50mM synthetic wastewater

Substrates	Amount added/L
Glucose, C ₆ H ₁₂ O ₆	18.50g
Sodium lactate, C ₃ H ₅ NaO ₃	11.21mL
Sodium acetate, C ₂ H ₃ NaO ₂	3.69g
Sodium fumarate C ₄ H ₂ Na ₂ O ₄	8.00g

Table 6: Chemical composition for preparation of catholyte solution

Na ₂ HPO ₄	8.92g
NaH ₂ PO ₄	2.30g
K ₃ [Fe(CN) ₆]	16.47g

3.4 Bacteria strain

Two water samples were obtained, source A which is nearby a small pond in the National Taipei University of Technology (NTUT), and source B which is from a local household aquarium, to obtain bacterial strain needed. 10 μL of each sample was inoculated into 10mL of nutrient broth respectively and incubated at 30°C overnight. From the broth culture, a streak plate, and 50mL of Nutrient broth were added with 10 μL of the bacteria culture along with 2 graphite electrodes and incubated at 30°C overnight. A streak plate was also prepared and incubated at 30°C overnight. Colonies were then viewed under a digital microscope (VHX-5000, Keyence).

3.5 MFC set-up

3.5.1 Membrane pre-treatment

Chemours Nafion® N115 membrane was utilized to separate both anodic and cathodic chamber. Prior to the experiment, the membrane was activated by lightly boiling in 5% H_2O_2 at 80°C for 1h, followed by rinsing and lightly boiled again in deionized water at 80°C for 2h, then rinsed and boiled again in 2M H_2SO_4 at 80°C for 1h, and lastly rinsed and stored in deionized water.

3.5.2 Reactor set-up

Three custom glass-made dual-chambered MFC set up were used in this experiment (Figure 1 and 2B). The graphite plate (Shenzhen Hongwang Molding, China) was obtained by cutting with a vertical drilling machine; and the carbon cloth was cut using scissors. Titanium wire were used to connect the reactor. Reactors and dimensions of graphite plates and carbon cloth electrodes used for both cathodic and anodic chamber of the reactors are shown in Table 7.

Table 7: List of dimensions for the electrodes used in the experiments

Reactor	Water Source	Cathode type	Anode type	Surface area of cathode (cm)	Surface area of anode (cm)	Total surface area of anode (m^2)
A	A	Graphite Plate	Graphite Plate	2.9 x 7.4	2.9 x 7.3	0.0042
B	A	Graphite Plate	Carbon Cloth	2.9 x 7.3	2.4 x 9.9	0.0048
C	B	Graphite plate	Graphite Plate	2.9 x 7.4	3.7 x 5.2	0.0048

In the anodic chamber, 450 mL of nutrient broth and anode inoculated with bacteria culture were utilized for inoculation and the chamber was sealed to prevent oxygen from entering and the outlet gas port was connected to a gas bag for CO₂ emission. In the cathodic chamber, 50mM K₃[Fe(CN)₆] in 50mM phosphate buffer solution was used along with a graphite plate electrode, an aquarium pump was also utilized to supply oxygen to the chamber. When stable voltage was achieved, the substrate in the anodic chamber was then changed to different synthetic wastewaters as shown in Table 5 to evaluate the respective power generation and wastewater treating capabilities over an external resistance of 1k Ω .

3.6 Evaluation of MFC performance

3.6.1 Polarization curve

Variable resistors were constructed into a board to be used to generate a polarization curve (Figure 6). Initially, 1000 Ω resistor was connected to the set-up and the voltage was measured using a 580-battery test system (Scribner). A voltage over time graph was generated and the maximum current generated was determined. When voltage reached a plateau, the resistor was then replaced with a range of different resistors in a variable resistor box to obtain voltage readings on different values of resistances. The list of individual resistant value for the corresponding switch are listed in Table 8. The data were then recorded in terms of mV/m², mA/m², and mW/m².

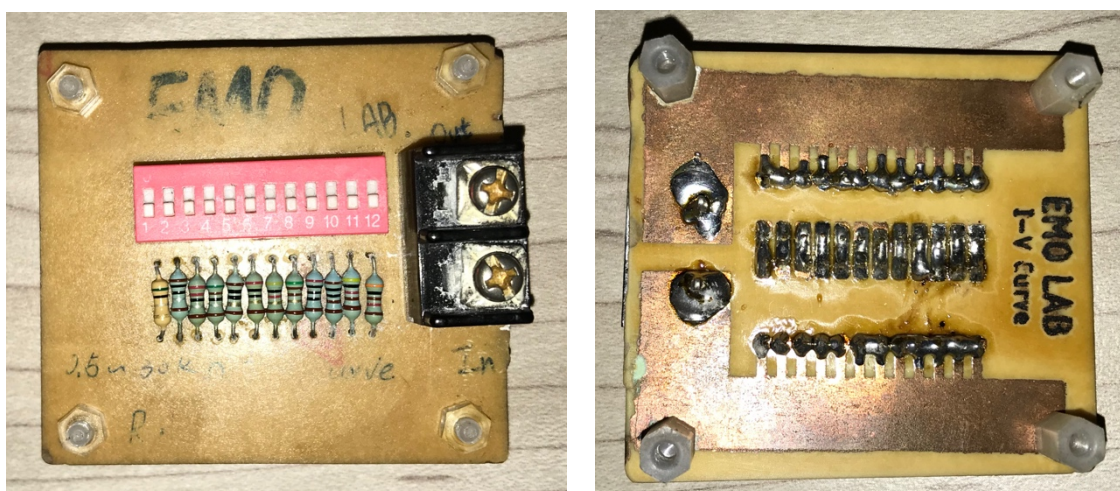


Figure 6: Variable resistor box used in the experiment to measure polarization curve

Table 8: Individual ohmic values for the variable resistor box

Switch Number	Ohmic value	Switch Number	Ohmic value
12	0.6	6	20.1
1	0.8	78	22.5
23	0.9	7	40.1
2	1.3	89	40.6
34	1.8	8	40.9
3	2.5	910	165.8
45	3.6	9	199.0
4	5.4	1011	796
56	6.8	10	994
5	10.1	1112	3.527K
67	13.5	11	3.997K
		12	30.01K

3.6.2 Chemical oxygen demand (COD) test

COD Digestion vials (High range plus) (HACH, USA) were utilized to measure the COD content in all synthetic wastewater (except glucose) before and after MFC treatment. 2 mL of the samples were injected into vials and the COD values were obtained by DRB200 Reactor (HACH, USA) after 2 hours of digestion. The COD test was duplicated for each sample to obtain the average value.

3.6.3 Determination of concentration of glucose

Glucose GO Assay kit (Sigma-Aldrich, United States) was used to measure concentration of glucose before and after inoculating into the reactor. The assay was firstly activated according to manufacturer's instruction by dissolving a capsule of kit reagent in an amber bottle with 39.2mL deionized water. One mL of deionized water was added into o-Dianisidine reagent preweighted vial and 0.8mL of the reagent added into the amber bottle with glucose reagent. Glucose sample was then diluted with deionized water to approximately 20-80 $\mu\text{g/mL}$. Several dilutions from the standard glucose solution provided were then prepared to generate a calibration curve. These samples were then transferred to a cuvette and the absorbance value measured by using a UV-Vis spectrophotometer

(Agilent, United States). Concentration of the glucose sample was then obtained by comparing with the calibration curve.

3.6.4 Coulombic efficiency

CE was calculated through formula listed in Equation 1 for glucose samples and Equation 2 for the others, area under the curve for the respective substrate was obtained by using the integrate function in OriginPro software.

3.6.5 Scanning electron microscopy

A scanning electron microscope (JEOL, Japan) was used to capture microorganism in the culture. Culture underwent fixation in 3% glutaric dialdehyde buffered with 0.1% phosphate buffer for 4 hours at 40°C, then washed in 0.1% phosphate buffer for 10 minutes then dehydrated by successive immersion in graded ethanol (30, 50, 70, 80, 90, and 100%) prior to visualization for 15 minutes each. Specimen was then air-dried and mounted onto specimen tubs using carbon tape, then coated with platinum.

Chapter 4: Characterisation and biochemical identification of water-borne bacteria

4.1 Introduction

The overall objectives of the thesis are to use common waterborne pathogens in hope to replace the dilemma of microbial population control in an MFC. Hence, this chapter aims to obtain the bacteria required for MFC inoculation. Two water sources were used, one from a lake and another from a local household aquarium. The identity of the bacteria was determined through a series of biochemical and carbohydrate fermentation tests. Identification of these bacteria are mandatory to determine the type of microorganism present and to ease optimisation of conditions that could promote their growth in MFC. Different trends will be obtained from the power generation in MFC as different organism have different patterns in substrate metabolism and different communities of organisms will also affect the overall stability in the biofilm formed and ultimately the power generated. The identified organism was then inoculated into MFC in subsequent chapters and their potential in operating an MFC cell will be evaluated.

4.2 Methodology

4.2.1 Water samples

Current chapter investigated the microbial population of two water sources used in this study: Source A- sediment of a lake in the National Taipei University of Technology (NTUT); and Source B- a local household aquarium. These water sources were chosen due to their difference in climate, hoping to isolate a more diverse bacterial population from two different sources. For each water source, sample was obtained from 3 different points of the source and mixed together for isolation of bacteria. Weather conditions was clear upon sample collection from source A, and this is not applicable for source B. Water condition of source A was murky, while it is clear from source B.

4.2.2 Microscopic characterisation of isolates from water sources

A total of 10 μL water sample was added into 10mL of Nutrient Broth respectively and incubated at 37°C for 24h. After incubation, 10 μL of the culture suspension obtained was inoculated into 50mL of Nutrient broth along with the graphite and carbon cloth electrodes in a petri-dish and incubated at 37°C for 24h to allow biofilm formation on electrode surface. After incubation, the culture was plated on Plate Count Agar (PCA) and incubated at 37°C overnight. A total of 10 bacteria colonies were isolated

from Source A, and four colonies from Source B, were streaked on new PCA plates to obtain for pure culture. Cell morphology was then determined by Gram-staining through gram-staining kit (Sigma).

4.2.3 Biochemical identification of isolates from water sources

Fourteen bacterial isolates were tested for fermentation of lactose by using MacConkey Agar, followed by production of oxidase using Oxidase test reagent. Colour changes in the oxidase reagent to blue indicates that the organism is oxidase positive. Red colonies formed indicates that organism is lactose fermenter (coliforms) and yellow colonies indicates that organism are non-lactose fermenter (Refer to appendix). Next, the bacterial isolates were then identified using API® 20E test (Biomérieux, USA). Isolate that obtained identification similarity percentage (% I.D) of less than 75% will be further confirmed using GN-ID A+B Panel test (Microgen). These test kits allowed determination of the carbohydrate fermentative profiles of the organisms in the *Enterobacteriaceae* family. The abbreviations for the various carbohydrate tests are listed in Table 6.

Table 9: List of abbreviations for biochemical and carbohydrates tests contained in both test strips

Abbreviations	Reactions / Enzymes
ONPG	Ortho-Nitrophenyl-β-galactoside
ADH	Arginine Dihydrolase
LDC	Lysine Decarboxylase
ODC	Ornithine Decarboxylase
CIT	Citrate utilization
H ₂ S	Sulfuric Acid production
URE	Urease
TDA	Tryptophan Deaminase
IND	Indole production
VP	Acetoin production (Voges Proskauer)
GEL	Gelatinase
GLU	Oxidation of Glucose
MAN	Oxidation of Mannitol
INO	Oxidation of Inositol
SOR	Oxidation of Sorbitol
RHA	Oxidation of Rhamnose
SAC	Oxidation of Saccharose
MEL	Oxidation of Melibiose
AMY	Oxidation of Amygdalin
ARA	Oxidation of Arabinose

4.3 Results

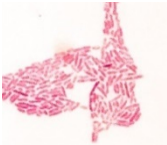
4.3.1 Gram-staining and biochemical tests of unknown isolates

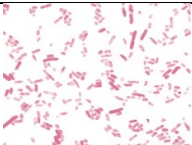
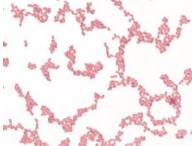
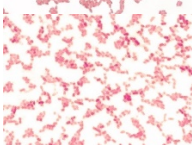
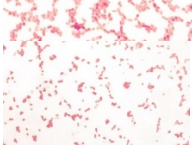
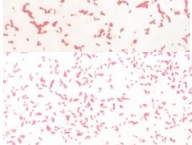
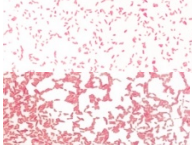
All 14 isolates were identified to be Gram-negative, rod shaped organism (Table 10), Isolates 1, 2, 3, 11, 12, 13, 14, exhibiting white colony morphologies with the medium turned yellow while the others exhibited red, circular colony on MCA (See appendix). From source A, namely isolates organism 4, 5, 6, 7, 8, and 9 are coliforms, while the remaining isolates 1, 2, 3, and 10 are non-coliforms (Table 10). From source B, all four isolates are non-coliforms (Table 10). All isolates exhibited negative result in Oxidase test, except for Isolate 1 (Table 10; see Appendix. While general macroscopic colony morphologies on PCA are not significant in the identification of bacterium, hence, further biochemical assays were adopted.

4.3.2 Carbohydrate fermentation profiles

Table 11 and 13 show the carbohydrate fermentation patterns of the 14 isolates by using API® 20E and Microgen GN-ID A+B Panel test kits. Results from API20® 20E shows that all isolates were able to ferment glucose (except for isolate 5), and mannitol (except for isolate 7). All isolates also do not contain enzyme tryptophan deaminase as indicated in the negative TDA test. From the API® 20E test, bacteria of *Aeromonas spp.*, *Serratia spp.*, *Kluyvera spp.*, *Chromobacterium spp.*, *Citrobacter spp.*, and *Klebsiella spp.*, were identified. Most organism from water source A were of *Enterobacteriaceae* family (Table 12), while all organism in water source B was identified to be *Serratia marcescens* (Table 15). Identity of isolates 2 and 3 from water source A were reconfirmed to be *Serratia marcescens* and *Enteric Gp68* respectively through Microgen GN-ID A+B Panel test due to the low %ID obtained from the previous API® 20E test.

Table 10: Macroscopic and microscopic observations, and oxidase tests of unknown isolates, unknown 1 to 10 were isolated from source A while unknown 11 – 14 was isolated from source B

Unknown	Source	Gram stain	Lactose fermenting	Oxidase
1	A		-	+
2	A		-	-
3	A		-	-
4	A		+	-
5	A		+	-
6	A		+	-
7	A		N/A	-

8	A		+	-
9	A		+	-
10	A		-	-
11	B		-	-
12	B		-	-
13	B		-	-
14	B		-	-

4.3.3 API® 20E test

The bacterial isolates were respectively streaked on PCA plates and incubated for 18h at 37°C. Single colony was selected and emulsified into 5mL 0.85% (w/v) sterile saline water. Then, 3mL of sterile water was distributed evenly into the bottom tray provided for holding the test strip (biomerieux, USA). The bacterial suspension was then pipetted into all wells on the test strip to the tube level except for CIT, VP, and GEL tests, which both tube and cupule was filled. Cupules of ADH, LDC, ODC, H₂S, and URE was filled with sterile liquid paraffin and test strip was covered with lid provided and incubated at 37°C for 18 hours. The strip was then removed from incubation and all spontaneous results indicated by colour changes was recorded. Additional test reagents were added in including TDA, IND, VP, and NO₂ and all results was tabulated based on the standard provided on the manufacturer's website (Figure 7). All results were then computed into the API software provided from the manufacturer for confirmation of bacteria identity.

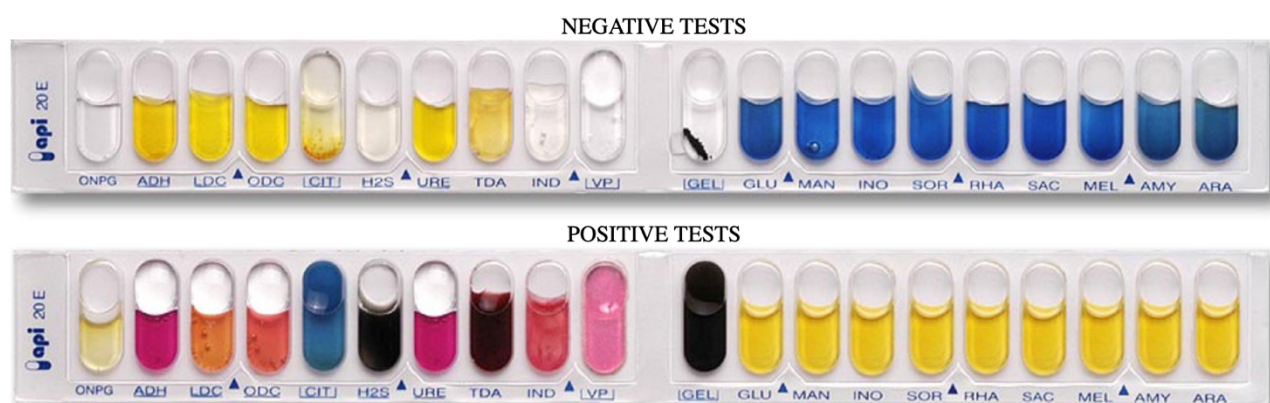


Figure 7: Colour standards for API® test provided on manufacturer's website (BIOMERIEUX, 2019)

4.3.4 Microgen GN-ID A+B Panel test

The bacterial isolates were respectively streaked on PCA plates and incubated for 18h at 37°C. Single colony was selected and emulsified into 5mL 0.85 (w/v) sterile saline water. 100µL of the bacterial suspension was pipetted into microwells on both GN-ID A and B test strips (Microgen, UK). Microwells with black indicators was further filled with 100µL sterile paraffin oil. The test strip was then sealed and incubated at 37°C for 18 hours. Results are then recorded by comparing to the colour chart provided (Figure 8). Additional test reagent for IND, VP, and TDA was then added into their respective wells indicated by a green circle and the results are recorded. All results were then computed into the software provided from the manufacturer for the final confirmation of bacteria identity.

Microgen™ GN A ID

WELL/NAPFCHEN /GODET	1	2	3	4	5	6	7	8	9	10	11	12	7
Reaction	Lysine	Ornithine	H ₂ S	Glucose	Mannitol	Xylose	O.N.P.G.	Indole	Urease	V.P.	Citrate	T.D.A.	Nitrate
Negative													
Positive													

Microgen™ GN B ID

WELL/NAPFCHEN /GODET 13

									Adonitol	Raffinose	Salicin	Arginine 24hrs	Arginine 48hrs
Negative													
Positive													

Figure 8: Colour chart provided for Microgen GN-ID A+B Panel test

Table 11: Carbohydrate fermentation profiles of 14 bacterial isolates using API® 20E test unknown 1 to 10 were isolated from source A while unknown 11 – 14 was isolated from source B

Unknown	1	2	3	4	5	6	7	8	9	10	11	12	13	14
ONPG	+	+	-	-	+	-	-	+	-	+	+	+	+	+
ADH	+	-	-	-	-	-	+	+	-	-	-	-	-	-
LDC	+	+	-	-	+	-	-	-	-	-	+	+	+	+
ODC	-	+	-	+	-	+	-	-	-	-	+	+	+	+
CIT	-	+	-	+	+	+	+	+	+	+	+	+	+	+
H ₂ S	-	-	-	-	-	-	-	+	+	-	-	-	-	-
URE	-	-	-	-	+	-	-	-	-	-	-	+	+	-
TDA	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IND	+	-	-	+	-	+	-	-	-	-	-	-	-	-
VP	+	+	-	-	+	-	-	-	-	-	+	+	+	+
GEL	+	+	-	-	-	-	+	-	-	-	+	+	+	+
GLU	+	+	+	+	-	+	+	+	+	+	+	+	+	+
MAN	+	+	+	+	+	+	-	+	+	+	+	+	+	+
INO	-	+	-	-	+	-	-	+	+	+	+	+	+	+
SOR	-	+	-	-	+	-	-	+	+	+	+	+	+	+
RHA	-	+	+	+	+	+	-	+	+	-	-	-	-	-
SAC	+	+	+	-	+	-	-	+	+	-	+	+	+	+
MEL	-	+	-	+	+	+	-	+	+	+	-	-	-	-
AMY	+	+	+	+	+	+	-	-	-	+	+	+	+	+
ARA	-	+	+	+	+	+	-	+	+	+	-	-	-	-

*+ indicates a positive reaction where – indicates a negative reaction for that particular substrate

Table 12: Results obtained through API® 20E test on identification of unknown isolates unknown 1 to 10 were isolated from source A while unknown 11 – 14 was isolated from source B

Unknown	Organism	% ID
1	<i>Aeromonas hydrophila/caviae/sobria 2</i>	99.0%
2	* <i>Serratia odorifera 1</i>	69.7%
3	Invalid identification (<i>Pantoea spp 4</i>)	57.1%
4	<i>Kluyvera spp.</i>	98.6%
5	<i>Klebsiella pneumoniae ssp pneumoniae 1</i>	97.3%
6	<i>Kluyvera spp.</i>	98.6%
7	* <i>Chromobacterium violaceum</i>	76.8%
8	<i>Citrobacter freundii</i>	99.9%
9	<i>Citrobacter freundii</i>	99.7%
10	<i>Klebsiella pneumoniae ssp ozaenae</i>	87.0%
11	<i>Serratia marcescens</i>	97.7%
12	<i>Serratia marcescens</i>	99.8%
13	<i>Serratia marcescens</i>	99.8%
14	<i>Serratia marcescens</i>	97.7%

*Indicates low discrimination of organism identity

Table 13: Carbohydrate fermentation profiles of unknown isolates obtained from Microgen GN-ID A+B Panel test. Unknown 2, 3, 5, and 7 were isolated from source A while unknown 11 was isolated from source B.

Unknown	2	3	5	7	11
Lysine	+	-	+	-	+
Ornithine	-	-	-	-	+
H ₂ S	-	-	-	-	-
Glucose	-	+	+	+	-
Mannitol	-	+	+	+	-
Xylose	-	-	+	-	+
ONPG	+	-	+	-	+
Indole	-	-	-	-	-
Urease	-	-	+	-	-
VP	+	-	-	-	+
Citrate	+	-	+	-	+
TDA	-	-	-	-	-
Gelatine	+	-	-	-	+
Malonate	-	-	+	-	-
Inositol	+	-	+	-	+
Sorbitol	+	-	+	-	+
Rhamnose	-	+	+	-	-
Sucrose	+	+	+	+	+
Lactose	-	-	-	-	-
Arabinose	-	-	+	-	-
Adonitol	-	-	-	-	+
Raffinose	-	-	+	-	-
Salicin	-	-	+	+	-
Arginine	+	-	-	-	-

Table 14: Organism identified through Microgen GN-ID A+B Test strip. Unknown 2, 3, 5, and 7 were isolated from source A while unknown 11 was isolated from source B.

Unknown	Organism	% ID
2	<i>Serratia marcescens</i>	99.29%
3	<i>Enteric Gp68</i>	87.50%
5	<i>Klebsiella pneumoniae</i>	99.25%
7	<i>Enteric Gp68</i>	99.88%
11	<i>Serratia marcescens</i>	99.93%

4.4 Discussion

Where bacteria are concerned this thesis aim to evaluate the potential of bacteria to be utilised in an MFC, hence waterborne bacteria were considered. As the purpose of the MFC is to treat wastewater, hence the isolation of bacteria from water sources were considered. This thesis would like to evaluate waterborne bacteria in a diverse environment and open system with changes in climate, and those in a closed and controlled system. The hypothesis is that bacteria isolated from these water sources would be of majority *Enterobacteriaceae* consist of mostly *Escherichia coli*, *Salmonella* spp., and *Shigella* spp. This would be based on the fact from previous identifications being reported similarly from lakewater (Kumar et al., 2013; Panneerselvam, 2012). The results obtained from Source A partly supports the hypothesis, where *Enterobacteriaceae*, including *Kluyvera* spp., *Klebsiella* spp., and *Citrobacter* spp., were identified. Bacteria from other species were also present. Shockingly, commonly found coliforms such as *E. coli* or *Salmonella* spp. were not detected in both water sources. For source B, only *S. marcescens* was isolated. *S. marcescens* is a water-borne pathogen commonly found in aquarium tanks (Musa et al., 2008). This supports our identification of this bacteria from Source B.

Hence, for the subsequent chapter, growth of these bacteria on electrode will be determined, and to generate power subsequently. *Serratia marcescens* was not attempted in MFC so their capacities for EET were not determined. Hence, our experiment also uses a monoculture of *S. marcescens* inoculated in MFC. To compare the identification protocol used, the justification for using API® 20E for most of the identification is attributed to the low cost and high accuracy of it being able to identify bacteria from a specific family (Awong-Taylor et al., 2008). While Microgen was reported to be of higher accurate than of API® 20E, thus it was utilised for re-identification in the case where API® 20E failed to identify a certain isolate. For both commercialised products, there are still limited to the identification of certain groups of bacteria. For complete and the most accurate profiling, 16s rRNA sequencing remains the most accurate method for complete identification. However, the time needed for identification is longer when compared with commercial API kits due to the steps required such as DNA extraction, primers synthesis, PCR, and lastly sequencing.

4.5 Conclusion

This chapter seeks to obtain and isolate the required bacterial strain for MFC inoculation. Source A which is an open and diverse system, and source B, which is a closed and controlled system. Single isolates were obtained from these sources and were subjected to identification tests. Identification were done based on the respective biochemical fermentation patterns of the isolates. For source A of the microorganisms, most were identified from a diverse group, with majority from the family *Enterobacteriaceae* and for source B, all bacteria were identified to be *S. marcescens*. Identification was confirmed with API test strip initially for strains with %ID of over 85%. Strains with %ID of lower than 75% are identified again with GN-ID A+B test strip.

Chapter 5: Effect of electrode material in dual-chambered MFC inoculated with mixed consortium of waterborne bacteria isolated from lake water

5.1 Introduction

This chapter focuses on the how both electrode material, graphite plate and carbon cloth affect the growth of common bacteria found in lake water in the anodic chamber of the MFC in treating three common substrates in wastewater – glucose, lactate, and acetate, subsequently affecting MFC efficiency. After synthetic wastewater treatment, the ability of these organism to utilize fumarate and subsequently fumarate coupled with lactate were also determined. Two MFC reactors which differ in electrode are set-up, with electrode materials being graphite plate (Reactor A) and carbon cloth (Reactor B). This is to evaluate if different electrode surface will have an effect on the formation of biofilms by these bacteria. As previously reviewed, the internal structure of these electrodes affects growth of bacteria and also electroconductivity. To recap, *G. sulfurreducens* will form thick homogenous cell layer on carbon cloth and heterogenous pillar structure in graphite plate, while *S. oneidensis* will undergo surface attachment, consequently forming biofilms, with both bacteria undergoing either direct electron transfer or mediated electron transfer mechanisms for electron transfer. Common waterborne bacteria were isolated from lake sediment are used instead for specified exoelectrogenic strains. As previously mentioned, previously undiscovered microorganism could potentially carry out EET, as demonstrated by *Listeria monocytogenes*. This experiment demonstrates the potential of a mixed consortium of bacteria isolated from lakewater identified previously in Chapter 4 in forming biofilms on two different types of electrodes, graphite plate and carbon cloth, and subsequently generating bioelectricity through metabolising various substrates provided in a fed-batch operation. Both reactors were firstly set-up as explained in chapter 3, then glucose synthetic wastewater was then added. Voltages for both reactors were then measured, when stable voltage was achieved, polarization curve was then obtained by varying external resistance load to obtain a set of voltage value. Substrate was then replaced with lactate, and lastly acetate. The feed was measured for change in COD before and after MFC treatment. After the experiment, FE-SEM was used to the visualise growth of microbes on their respective electrodes. Finally, fumarate was added into the reactor with graphite plate anode to be used as the sole electron acceptor, and then coupling fumarate with lactate as a combination to determine the effect in both power generation and wastewater treating in MFC.

5.2 Results and discussion

5.2.1 Power generation

Reactors are set-up as described in chapter 3. For Reactor A treated with glucose, peak voltage of 217 mV was achieved when $R = 60.4\text{k}\Omega$ (Figure 9), and a stable voltage of 7mV was achieved over a period of 28 hours (Figure 10A). Substrate was replaced with lactate and voltage fluctuations between 5mV to 13mV were observed at time, $t = 46\text{h}$, with voltage peaked at approximately 25mV at $t = 77\text{h}$. Peak voltage of 220mV was achieved when $R = 90.4\text{k}\Omega$ (Figure 9). At $t = 80\text{h}$, the lactate feed was replaced with acetate. Voltage increased steadily from 7mV to 15mV at $t = 80\text{h}$ until $t = 100\text{h}$, where a stable voltage of approximately 11mV was achieved. Peak voltage of 198mV was achieved when $R = 60.4\text{k}\Omega$ (Figure 4). At $t = 122\text{h}$ voltage started decline to 5mV (Figure 10A). All voltage values obtained are available in Appendix. Voltage fluctuations were observed in Reactor A and one plausible explanations could be due to the structure of the less porous graphite plate than hindered flow of electrons from the anode across the circuit.

For Reactor B, a stable voltage of 5mV was achieved over a period of 40 hours (Figure 10B). Peak voltage of 64mV was achieved when $R = 30.1\text{k}\Omega$ (Figure 9). Substrate was then replaced with lactate. From $t = 40\text{h}$ to $t = 86\text{h}$, voltage steadily increased from 5mV to approximately 12mV. Peak voltage of 165mV was achieved when $R = 150\text{k}\Omega$ (Figure 9). At $t = 87\text{h}$, the lactate feed was replaced with acetate. A stable voltage of approximately 27mV was achieved over a period of 20 hours, after which it started to decline (Figure 10B). Peak voltage of 242mV was recorded when $R = 100\text{k}\Omega$ (Figure 9).

All reactors showed a direct relation for cell voltage and external resistance until a point where the cell voltage plateaus. Several research shows similar trend (Ghangrekar and Shinde, 2007; Veluchamy, 2006) and this can be explained by the limitations imposed on the charge-transfer processes at the current-limiting electrode, on mass transfer, and on electrode reaction kinetics.

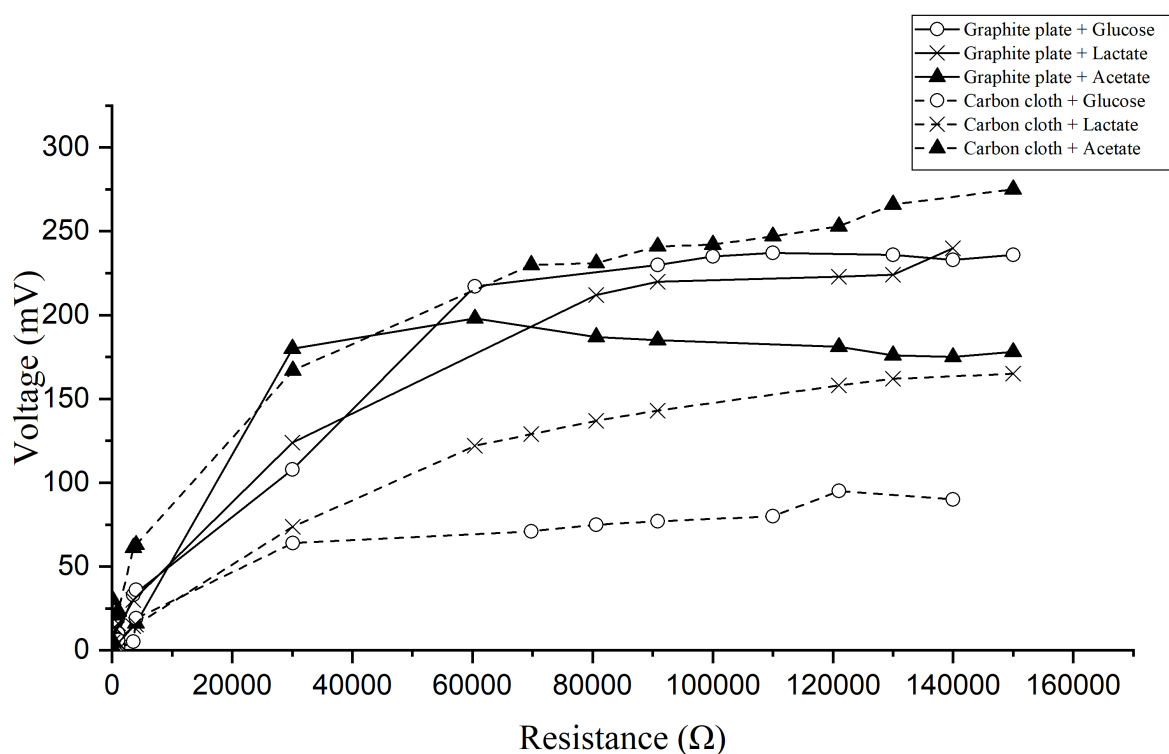


Figure 9: Voltage versus resistance graph for both reactors with different electrode materials and substrates

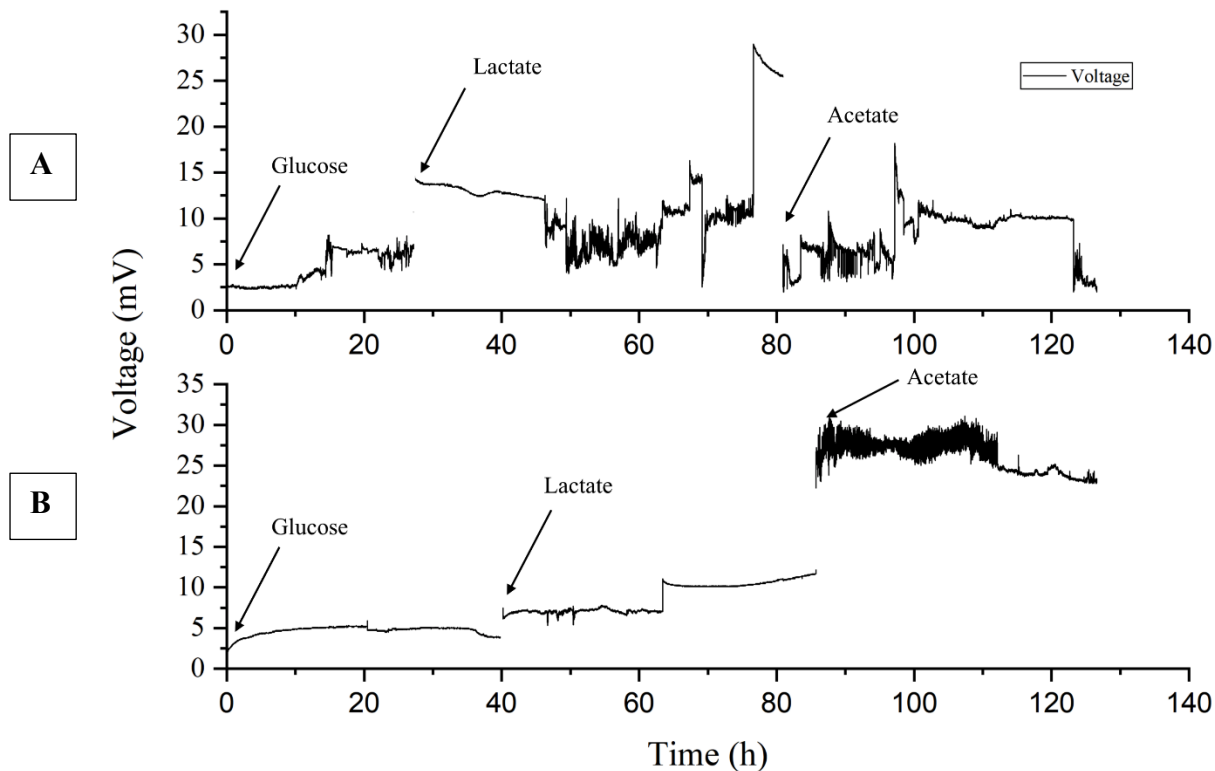


Figure 10: Voltage over time graphs for reactors with different electrodes consisting of graphite plate (A) and carbon cloth (B). Arrow indicates the time where substrate are being replaced.

5.2.2 Power density and polarization curve

Power density curve (Figure 11A) were plotted based on the list of voltage values obtained divided by their respective external load used (Data available in Appendix), the short circuit densities were 8.54, 4.27, and 2.85mA/m² on graphite plate, and 8.02, 1.04, and 1.69mA/m² on carbon cloth for acetate, lactate, and glucose, respectively. For maximum power density, 0.26, 0.23, and 0.18mW/m² was recorded from reactor A, while 0.22, 0.07, and 0.03mW/m² were achieved on reactor B for acetate, lactate, and glucose, respectively. Reactor A has higher maximum power density at 115.3, 232.5, and 254.5%, for the substrates acetate, lactate, and glucose, when compared with reactor B. Comparing these data obtained with fuel cells inoculated with *S. oneidensis* shows that power produced by these organisms is inferior to these specialised EAB as up to 632 mW/m³ of power density produced were reported (Gude, 2016). There might be several explanations for this, as the exact EET mechanism for these bacteria were not established yet at the moment, it could be due to the absence of suitable mediators, inhibitory effect due to different species of bacteria present within the biofilm, or growth condition not optimum for EET for these organism. However, there are trends shown in both voltage and when different substrate are supplied, this indicates certain multitudes in which these organisms can be used to generate power, albeit more studies should be done on the exact mechanisms in which how these organisms utilizes the substrated being supplied.

Comparing these results of power generated with those using *S. oneidensis* as the EAB as reported from literature, it is clearly shown that the EET capabilities of common waterborne bacteria are inferior as compared to *S. oneidensis*. Reactor inoculated with these specialised EAB were reported to be able to generate power density up to 156 mW/m² from glucose substrate in a similarly set-up double chambered MFC (Kim et al., 2011). However, one important thing to note that is in studies of transformed *S. oneidensis* which generated high amount of power densities have several limitations such as the difficulty to maintain transformed population for a long amount of time, typically which is required if this were to be used in an actual wastewater treating plant.

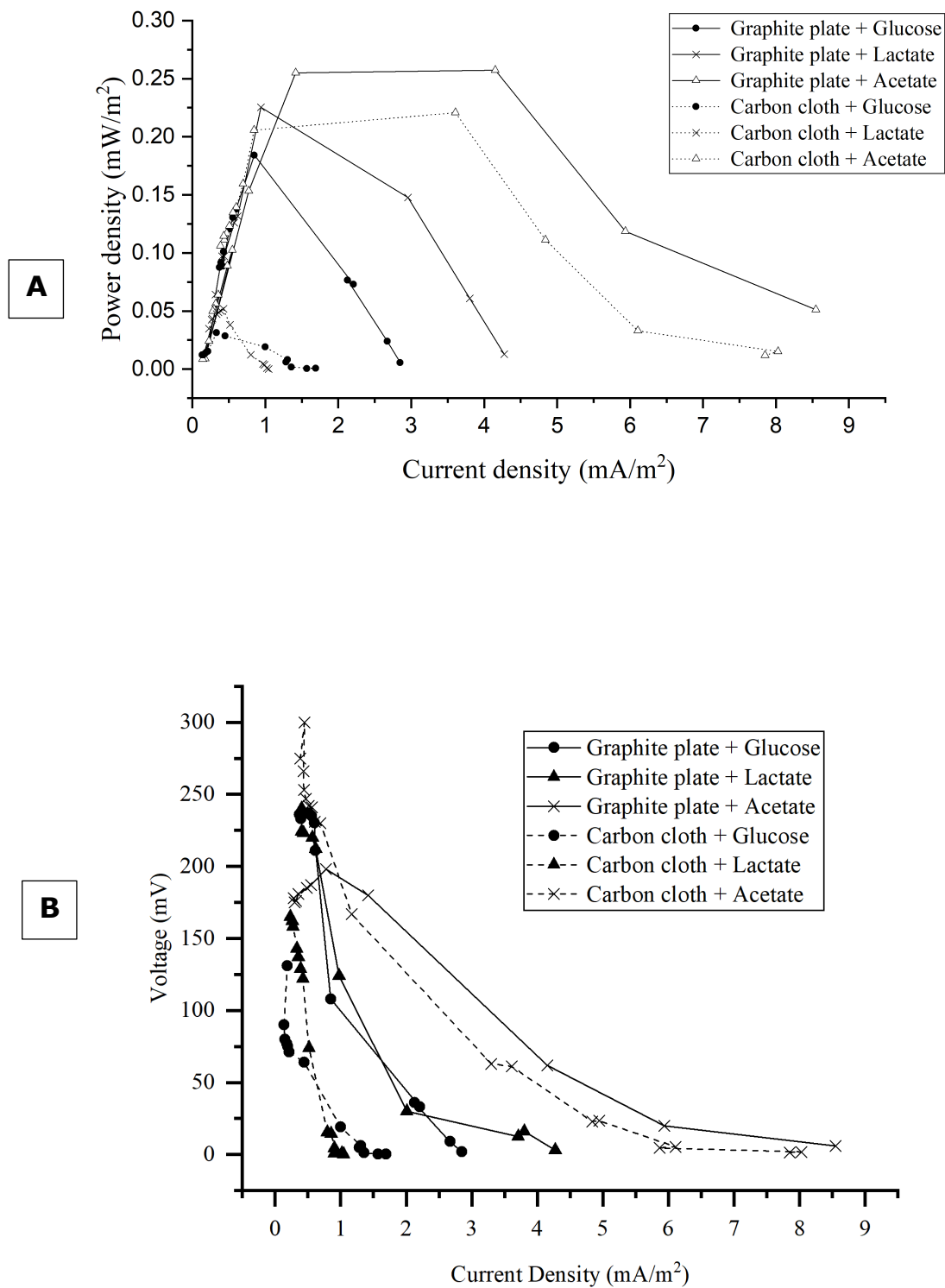


Figure 11: Power density (A) and polarization curves (B) for both reactors using different anode materials

In both reactors with different electrodes, the highest power density (shown by higher voltage) are achieved from acetate substrate, followed by lactate, and lastly glucose. These results are supported by literatures as glucose is a unrefined substrate and is in an earlier position within the metabolic pathway as compared to acetate and is usually the end product of bacterial fermentation for many prokaryotic metabolic pathways, and is a better substrate for electricity generation (Chae et al., 2009). Overall, reactor using graphite plate has higher power density output as compared with reactor using carbon cloth for all three substrates used. While this results contradicts with literatures being reported, with carbon cloth being superior than graphite plate (Dewan et al., 2008; ter Heijne et al., 2008) due to their porosity nature which ultimately increases total surface area. We hypothesized this could be due to the bacteria that have higher affinity for surface attachment to less porous surfaces, having more grown on the graphite plate electrode as compared to the carbon cloth electrode, ultimately affecting power generation. This hypothesis can be further tested through FE-SEM imaging which will be covered subsequently.

While it is also worthwhile to mention that a change in substrate will affect a shift in microbial community which will ultimately affects these results indirectly. Separate studies shown that MFC supplied with a single substrate have different capacity to acclimatize to change in substrate (Chae et al., 2010). These studies showed that change in substrate had caused a shift in microbial community which will affect the initial start-up period when different substrate are accumulated (Sun et al., 2015; Zhang et al., 2011).

Polarization curves (Figure 11B) (Data available in Appendix) at the peak output voltages were obtained to characterize the performance of the reactor. Slope for the reactor using graphite plate are less steep than of carbon cloth regardless of the substrate used. As for the substrate, glucose exhibits the steepest slope, followed by lactate and acetate. This signifies that reactors using graphite plates have lower internal resistance. While there is also less internal resistance when acetate is being used as the substrate, the highest internal resistance is recorded when glucose is used. The reason for the differences in resistance, which subsequently had caused the difference in power density when different substrates were used can be attributed to the change in pH of electrolyte by oxidation of the substrates provided. Similar trends were reported that electrolyte resistance decreases when sodium acetate was used due to the addition of sodium ions that increases electrolyte conductivity in the anodic chamber (Jung and Regan, 2011). The high internal resistance observed in glucose-fed reactor was proposed to be due to the production of fatty acids from fermentation of glucose which cause electrolyte acidification, resulting in the increase in resistance.

Polarization curve suggests that different substrates on both reactors gives a different value of internal resistance. With acetate fed reactors exhibiting the lowest internal resistance. One plausible reason might be due to the aquarium pump in the cathodic chamber that could potentially interfere with the cathode and consequently the circuit itself. In contrast, voltage for reactor using carbon cloth increase in a steadier state without much fluctuations when compared against the graphite plate reactor. As carbon atoms in carbon fibres are less densely packed compared to the graphite plate counterpart, this allow organisms to interact with the electrode more freely.

SEM imaging of the electrode showed aggregation of foreign material at the PEM that could obstruct proton transport from anodic chamber to cathodic chamber. Images of PEM after the experiments (Figure 12) shows foreign organic matter and bacteria that clustered across the membrane surface. Growth of unwanted organism (such as fungi) can potentially causes some problem as they can hinder EET to the electrode. Hence in subsequent experiments it is imperative to check for the growth or accumulation of unwanted material on the membrane surface in order to ensure efficient migration of protons across the membrane.

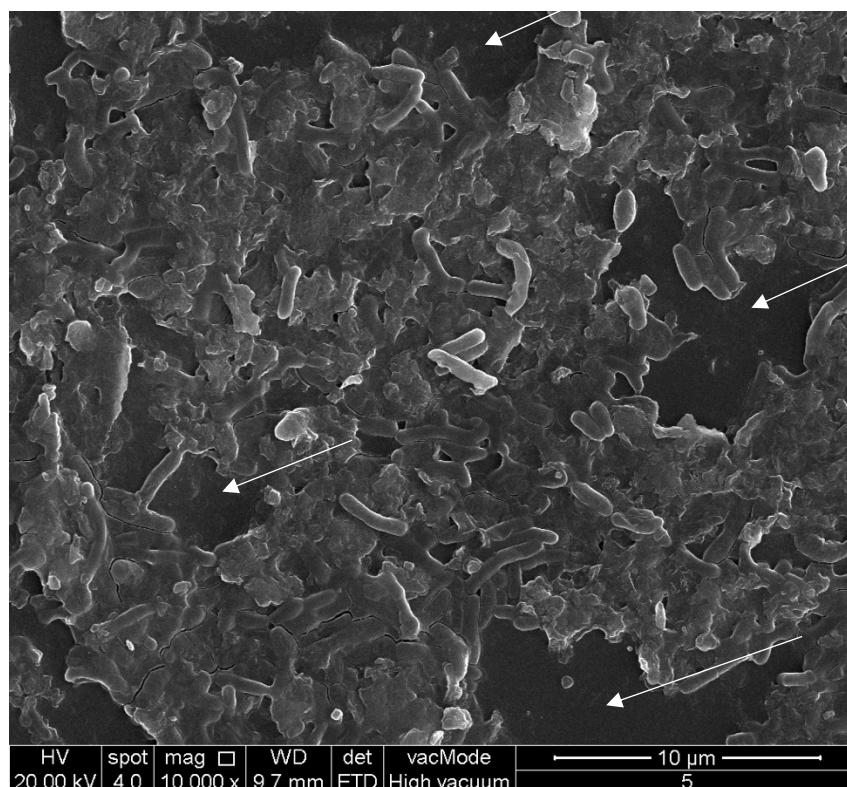


Figure 12: FE-SEM of a PEM after MFC treatment. Arrow indicated the area that had not been contaminated by foreign matter

5.2.3 COD removal, CE, and FE-SEM visualisation

Initial and final chemical oxygen demand (COD) concentration, and coulombic efficiency (CE) were shown in Table 15. COD removal is lowest for glucose with 16.49% and 36.51% being recorded for graphite plate and carbon cloth, while highest is 78.36% when acetate substrate is being used along with graphite plate electrode. The differences shown in percentage of COD removed from glucose and acetate in both reactors is attributed to the surface structure of the electrodes that selectively enhances the growth of certain groups of bacteria within the mixed consortium. This caused a shift of microbial community within both reactors which produced different patterns in glucose fermentation and acetate metabolism, subsequently affecting COD removal. Graphical output from OriginPro software for the acquisition of area under the curve is included in Appendix section. Contradictorily, highest CE value of 0.0024% and 0.0028% are also being observed for glucose substrate, with acetate being the lowest at 0.0006% and 0.0011% for both graphite plate and carbon cloth electrodes.

Results indicated the capabilities of both reactors in generating a small amount of power. Comparing the values for the power generated and removed COD with results being reported that use *S. oneidensis*

as the EAB with CE being achieved at up to 96% (Bhargavi et al., 2018), we suspected the microorganism utilizes an indirect or mediator-required electron transfer mechanism to the electrode, with this theory being further strengthened by looking at the CE values. As CE are defined as the number of coulombs being recovered from the oxidation of substrates, with the high percentage of COD removal and low CE infers several possible explanations. The low level indicates that the electron generated from the biological processes of the bacteria were being used for other processes other than current generation. Such examples such as methanogenesis, which is a major cause for electron loss carried out by methanogens. Study showed that controlling the methanogenesis processes had resulted in an increase in CE (Chae et al., 2010). Some bacteria in the chamber might be using a metabolic pathway that does not lead to electron being donated to the electrode. Examples such as the electrons produced during substrate metabolism is being used to reduce intermediate compounds to alcohol, ammonia, carbon dioxide, or hydrogen. While there is still power being generated, this can infer possibilities of either presence of exoelectrogenic strains or strains that produce electron shuttles that serve as mediators for electron transfer (such as phenazines in *Pseudomonas aeruginosa*) such as *Lactobacillus*, *Pseudomonas*, or *Enterococcus*.

Table 15: Initial and final COD, COD removal, and Coulombic efficiencies for both reactors in different substrates

	Glucose		Lactate		Acetate	
Reactor	A	B	A	B	A	B
Anode	GP	CC	GP	CC	GP	CC
Cathode	GP	GP	GP	GP	GP	GP
Initial COD (g/L)	19.41	11.44	33.80	33.80	96.10	96.10
Final COD (g/L)	16.21	7.26	9.20	9.50	20.80	33.10
COD Removal (%)	16.49	36.51	72.78	71.89	78.36	65.56
CE (%)	0.0024	0.0028	0.0017	0.0011	0.0006	0.0011

*GP = Graphite Plate, CC = Carbon Cloth

As observed from the SEM images of the electrodes prior to MFC treatment (Figure 13A and 13C), there is growth of rod-oval shaped organisms on electrodes of both reactors that attach to surface of both electrodes (Figure 13B and 13D). On carbon cloth electrode, it is very imminent seen that the organism attaches to the carbon cloth fibre, along with the proteins associated with surface attachment, the organism also does not grow “between” two carbon cloth fibres. On graphite plate electrode, organisms are tightly packed with each other on the surface of the graphite crystals, while the exact

mechanism in which this arrangement is possible cannot be concluded at this moment. From the SEM it is shown that there was more bacterial growth on the graphite plate electrode as compared with the carbon cloth electrode. This further strengthens our hypothesis previously that the bacteria showing higher affinity for surface attachment for this electrode.

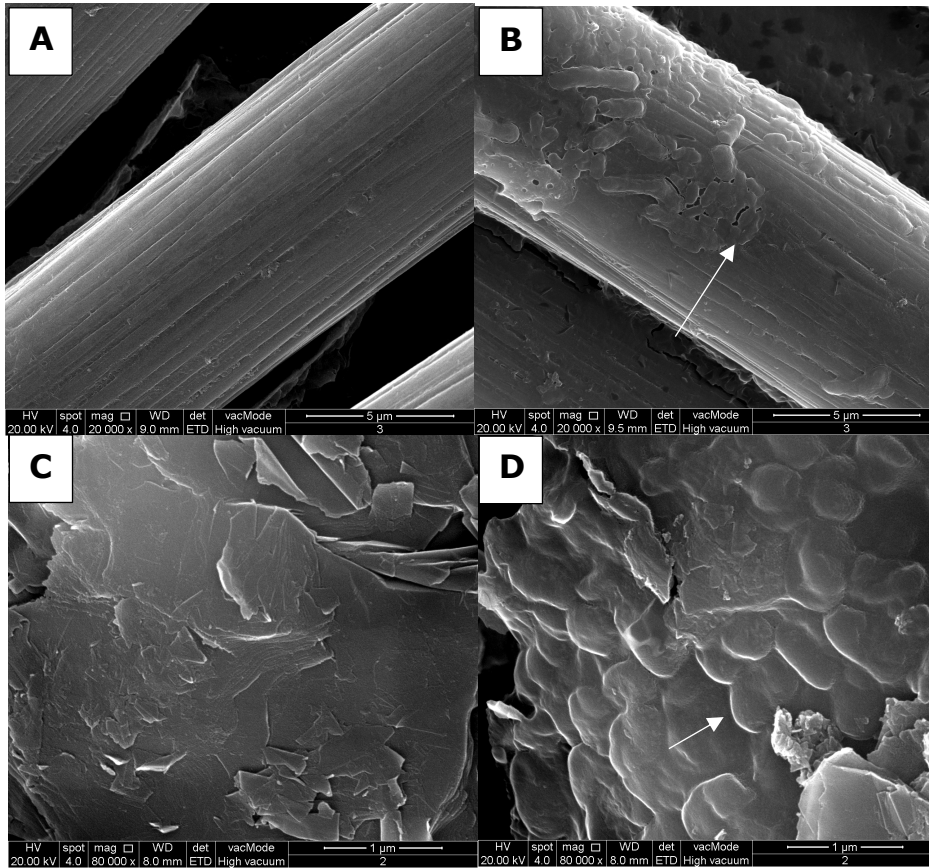


Figure 13: FE-SEM visualization of carbon cloth electrodes (AB) at 20 000x magnification and graphite plate (CD) at 80 000x magnification. B and D illustrates how microorganisms are grown on the surface of both electrodes while A and C are the electrode surface prior to MFC treatment

5.2.5 Fumarate and fumarate coupled with lactate as electron donor

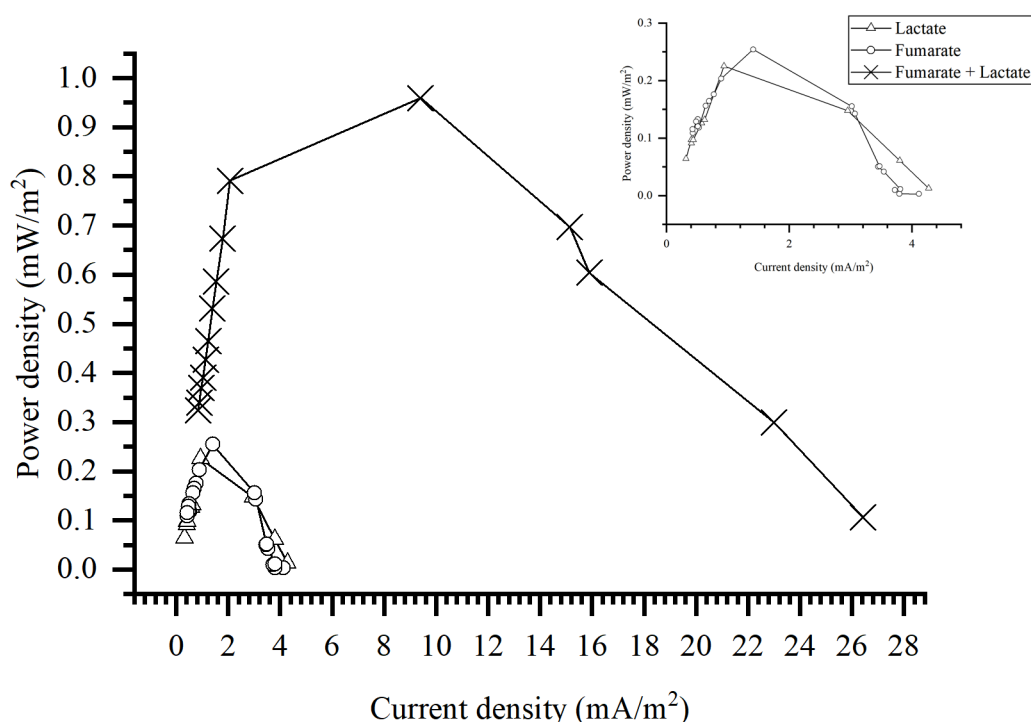


Figure 14: Power density curve when lactate, fumarate, and fumarate coupled with lactate were supplied into Reactor A

Reactor A, with SEM imaging carried out which confirmed the earlier hypothesis that this mixed consortium of organisms showing enhanced growth on graphite plate, was then subsequently inoculated with fumarate and subsequently fumarate coupled with lactate. Results shown that these organisms can use fumarate as the electron donor as well, albeit with low power density produced at 0.27 mW/m^2 , which power density value was similar when compared with lactate supplied previously (0.23 mW/m^2) (Figure 14). However, when fumarate was coupled together with lactate, power density increased by approximately 3-fold to 0.90 mW/m^2 . Exact reasons for this are unclear but it is speculated that the addition of fumarate could potentially enhance growth of bacterial biofilms on the surface of anode, thus increasing the substrate utilization of lactate to generate power. A study by Zhang et al. (2017) had demonstrated that the addition of fumarate can enhance bacterial growth on electrode surface, increasing maximum power densities produced by 2.41 times. However, they also stated that the addition can impair secretion of riboflavin which is imperative to mediate EET among bacteria and the anodic surface. As there are collectively a diverse species of organisms present in the bacterial biofilm grown on the anodic surface, this can explain the ability for the reactor to be able to use only fumarate, which *S. oneidensis* were not able to do so. Comparing the power densities produced through coupled fumarate and lactate with that of solely lactate, the former able to achieve 3.91 times

more in terms of power densities. In terms of COD removal, results indicated that the organisms were able to treat both fumarate and fumarate coupled with lactate with the latter showing high percentage in COD removal at 95.18% (data not shown). Our results had demonstrated that the coupling of fumarate with lactate were able to increase not only power generation, but also wastewater treating potential. While studies on the cellular level are required to answer this discovery, we speculated that it is due to the role of fumarate in formate regeneration that plays a role in the enhanced capabilities in MFC.

5.3 Conclusion

In this chapter, it was found that both reactors using either graphite plate or carbon cloth are able to generate electricity, albeit with low power obtained. With graphite plate electrode generates higher power than of reactor using carbon cloth electrode despite being the other way around if according to literatures reported. This could be explained by the difference surface attachment mechanism of bacterial community found in our experiment, which require further investigation and characterisation in the future. Highest power was generated from acetate substrate, in-regardless of electrode used, followed by lactate, and lastly glucose. The bacteria also show promising results in eliminating the substrate from the synthetic wastewater, with high percentage of COD removal, albeit with low CE. This can be explained through the electron loss during the oxidation of substrate due to non-exoelectrogenic activities such as methanogenesis, carried out by the microbial community that leads to less electrons being recovered from the substrate supplied. Finally, the reactor was also able to utilize fumarate as the sole electron donor, with both the power generation and wastewater treating effect being enhanced while addition of fumarate is coupled with lactate.

Chapter 6: Using *Serratia marcescens* in dual-chambered MFC

6.1 Introduction

In previous chapter, it was demonstrated that a mixed culture of common waterborne bacteria which was isolated from sediment of lake (Source A) was able to both produce electricity by treating wastewater. This chapter focuses on the effect of utilizing *Serratia marcescens*, which also is a common waterborne bacterium found in aquariums, in a typical dual-chambered MFC as described in the previous experiments. The usage of exoelectrogens such as *S. oneidensis* and *G. sulfurreducens* was widely accepted as the common bacteria used in the fuel cell due to their exoelectrogenic capabilities. However, in an actual wastewater treating environment it will be not feasible to control the population of these specific strains due to the highly contaminated nature of wastewater which will introduce in unwanted contaminants. Moreover, even if presence of such exoelectrogens are maintained in the system, a shift in bacterial community could still affect the MFC performance drastically. In previous experiments, it was concluded that (i) graphite plate showing higher power generated in mixed culture of common waterborne pathogens, and (ii) These organisms were able to generate power through utilizing synthetic wastewater supplied, albeit a low power density and coulombic efficiency were obtained. The current experiment will investigate the usage of a single isolate of *S. marcescens*, which is also a common waterborne pathogen found in aquatic environment. *S. marcescens* was isolated from a closed and controlled system in a local household aquarium (Source B) as described previously in Chapter 4. To further explore how different organisms utilises substrate for EET will affect MFC operation, the strain is sub-cultured and plated and will then be inoculated into the anode chamber of a dual-chambered MFC (Reactor C) as described in Chapter 3, using graphite plate as anode and cathode as found in Chapter 4 for promoted growth. Different substrates were then inoculated into the fuel cell for power generation consecutively. Changes in voltage over time was monitored and concentration of substrate before and after MFC operation was determined through COD tests. FE-SEM was then used to visualize growth of bacteria on the electrode surface. Finally, fumarate and fumarate coupled with lactate are added in to determine if the organism shows enhanced effect as previously observed in the diverse biofilm.

6.2 Results and discussion

6.2.1 Power generation

Peak voltage of 106, 157, 170, and 218 mV, was achieved when $R = 60.4\text{k}\Omega$, $69.8\text{k}\Omega$, $30.1\text{k}\Omega$ for glucose, lactate, and acetate, respectively (Figure 15). When resistance was fixed at 1000Ω , stable voltage of 6.8mV was achieved over a period of 36 hours by using glucose as substrate. Substrate was then replaced with lactate and voltage dropped to 3.10 mV and rised steadily again, plateaud at approximately 11.50 mV. Finally, lactate was replaced with acetate and voltage drops to 3.7mV and increased steadily up to 9.30 mV where it dropped starts to fall (Figure 16). Based on the Figure 16 , lactate substrate generated the highest amount of energy compared with the other substrates by *S. marcescens*, at 169% higher than of glucose, and 123% higher than acetate. Similar results were also reported by Ghanapriya et al. (2010), which their study had shown that *S. marcescens* using lactose as sole substrate achieved 126% higher energy than of glucose. Based on these results, it is speculated that *S. marcescens* were able to utilize lactate as sole carbon source for respiration, hinting at the presence of gene(s) encoding lactate dehydrogenase for the conversion of lactate to pyruvate for energy production. Ability to utilize acetate as carbon source were also as expected as previously mentioned, acetate are located at a further position down the glucose metabolic pathway, which explains the higher voltage generated by acetate as compared to glucose.

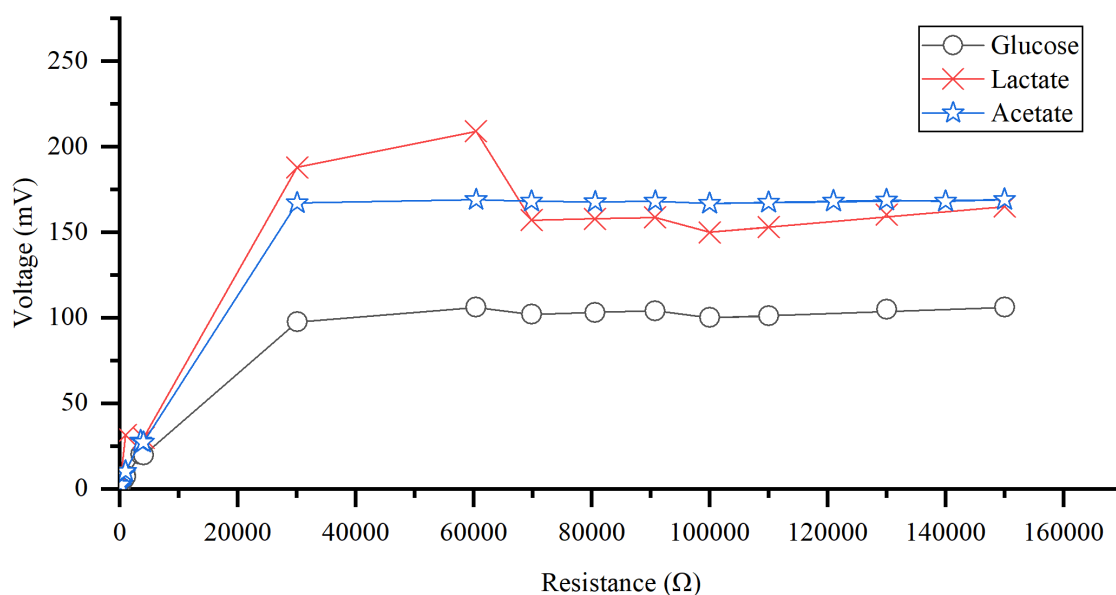


Figure 15: Voltage versus resistance graph of reactor C where the maximum external resistance required for peak voltage production were illustrated

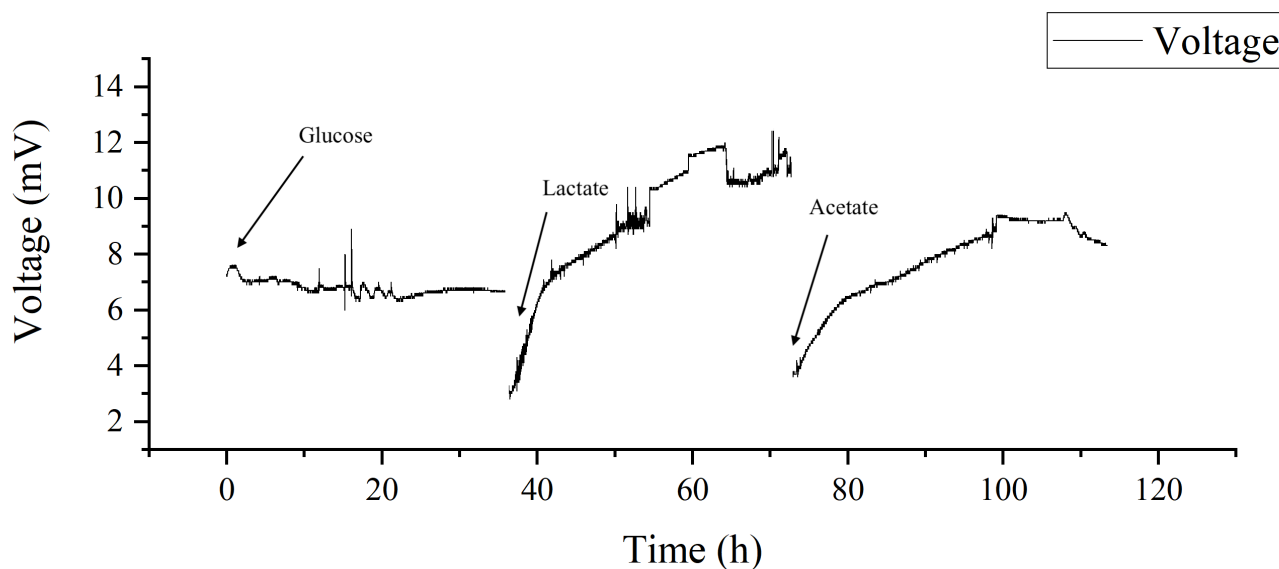


Figure 16: Growth of voltage when different substrates are supplied into Reactor C

6.2.2 Power density and polarization curve

Lactate exhibited the smallest dropping slope of polarization curve, which is then followed by acetate, and finally, glucose (Figure 17). This indicates the differences in internal resistances across different substrate used. By multiplying the current with its corresponding voltage, power density curves were then calculated. From power density curve (Figure 18), the short circuit densities and maximum power densities generated were 2.42, 5.45, and 3.31 mA/m² and 0.07, 0.26, and 0.20 mW/m² respectively for

glucose, lactate, and acetate. Highest power density were obtained from lactate substrate as expected, at 371% higher than glucose and 130% higher than acetate. Highest power density was observed from utilising lactate, which leads to the deduction that *S. marcescens* contained a metabolic or utilisation pathway which allowed direct oxidation of lactic acid where unlike *S. oneidensis*, lactate is firstly oxidised to pyruvic acid, which is then subsequently oxidised to acetate through the more common bacterial metabolic pathway and for electricity generation. This is something that could be investigated and explored further in subsequent work. Voltage results from glucose and acetate supports the results as explained previously, with the low amount of power generated from glucose due to the higher molecular weight of glucose when compared to acetate, and also the possibility that glucose were used in the fermentation pathway instead of EET pathways. Comparing with literature, power densities generated from *S. marcescens* were still inferior when compared to specialised *S. oneidensis* where power density can reach up to 1970 mW/m² using a single chamber MFC system (Kim et al., 2011) and clearly efforts are required for further optimisation for this organism to be able to be used in MFC. However, there are distinct benefits in using these non-optimised bacteria as they are more easily controlled in a system due to their abundance in water samples. *S. marcescens* are common water-borne pathogen and can be found easily in water sample with generally acquired resistances to be able to be readily used as organism in a highly contaminated environment, where *S. oneidensis* will be at a disadvantage in this sense and tight control are required to ensure growth of *S. oneidensis* in a typical contaminated environment in a wastewater treating system.

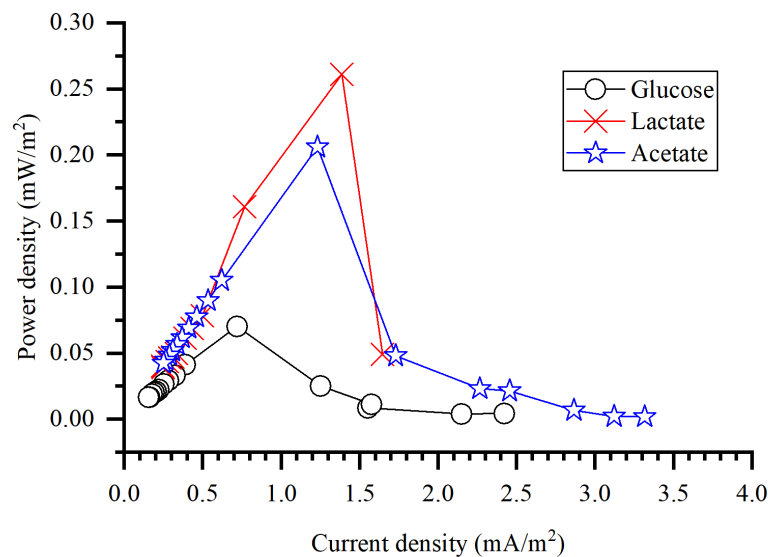


Figure 17: Power density curve of reactor C when different substrates were supplied

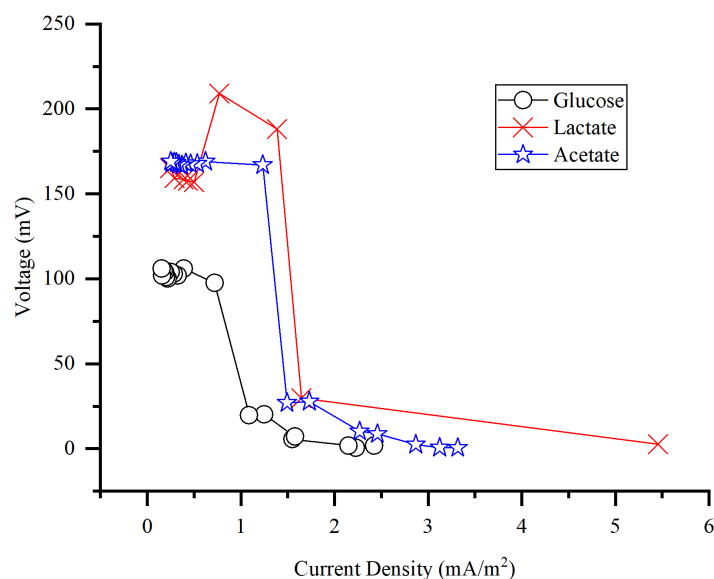


Figure 18: Polarization curves of reactor C when different substrates were supplied

6.2.3 COD, CE, and FE-SEM visualization

S. marcescens exhibited potential in treating wastewater with high highest COD removal rate recorded when acetate was being used as substrate, which is then followed by lactate, and lastly glucose (Table 16). It was reported previously that lactate utilisation had achieved higher power density generated than acetate, but this experiment had observed higher COD being removed from acetate. This hinted that the lactate metabolic pathway that *S. marcescens* had utilised as previously mentioned undergoes incomplete oxidation of lactic acid, which formed intermediary compound that serves as a by-products that accumulates in the anode chamber which had affected COD removed, while acetate is a common substrates that is positioned at a very late stage in the glucose metabolic pathway that is broken down for ATP production. As for the overall trend for COD removed, similar results were widely reported to the fact that acetate is a substrate that is more readily degradable substrate as compared to glucose due to its position in the respiratory pathway (Pant et al., 2010; Kim et al., 2011). Low CE obtained indicated that electron was not fully recovered for EET by *S. marcescens*. Several explanations could be attributed to this results, including both the penetration of oxygen to the anodic chamber and also aerobic activity of the organisms (Wang et al., 2013). To overcome these, Capodaglio et al. (2015) stated that the optimization of external load can generate a selective pressure within the anodic chamber, leading to the enhancement of exoelectrogenic bacteria with respect to methanogens, increasing CE. Kim (2011) on the other hand, had built a dual anode MFC which could reduce the metabolic electron losses caused by dissimilar bacterial communities on the anode. Wang et al. (2013)

had also found out that low CE can be caused by cathode characteristics in which area of cathode being inversely proportional to CE. They also reported that the cathode coated with Pt has increased the efficiency of MFC in terms of current generation. While this organism had shown potential in treating wastewater, further studies to characterize the metabolic role of this organism in MFC is required in order to facilitate efficient EET from *S. marcescens* for their maximum utilization. Gene knockouts or deletions could be performed to enhance this organism by eliminating genes responsible for fermentation, then introducing genes responsible for efficient EET (such as *mtr* pathway from *S. oneidensis*), facilitating both efficient wastewater treating and current generating abilities while at the same time requiring less facilitation in the inoculation process as compared to *S. oneidensis*. Cells were visualised under SEM (Figure 20) and was observed that *S. marcescens* can grow very well on graphite plate.

Table 16: COD remove efficiency of reactor C on different substrates supplied

	Glucose	Lactate	Acetate	Fumarate + Lactate
Initial COD (g/L)	11.44	33.80	96.10	50.2
Final COD (g/L)	7.26	25.00	33.10	2.5
COD Removal (%)	36.51	26.03	65.57	95.02
CE (%)	0.0036	0.0030	0.00042	NA

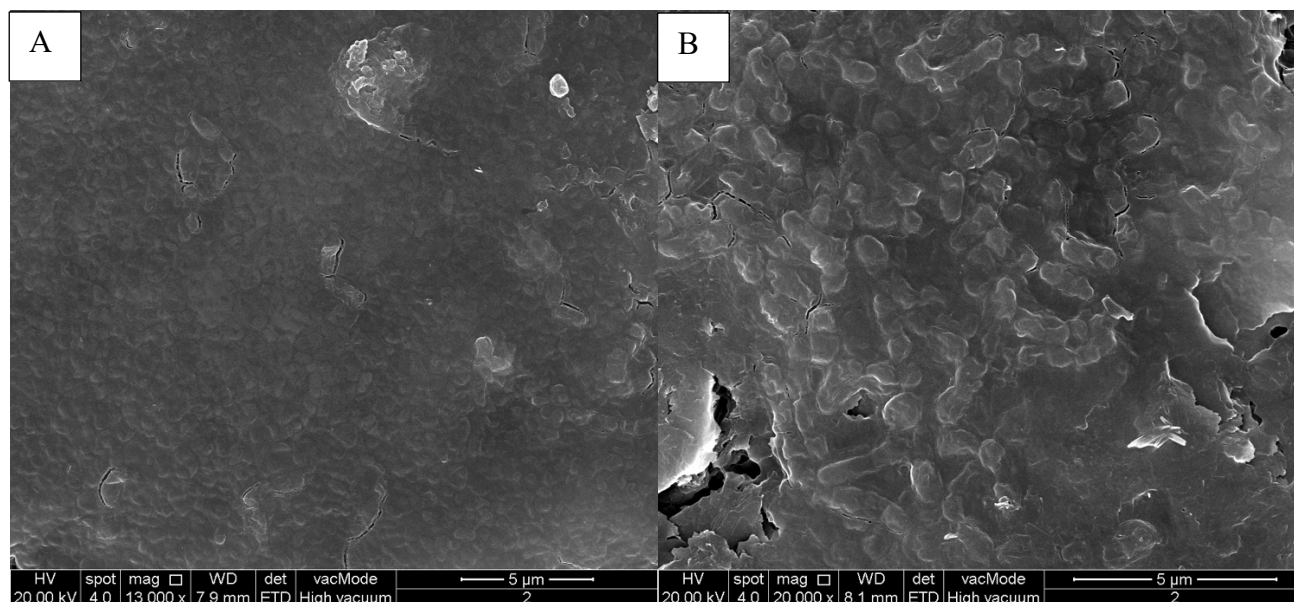


Figure 19: FE-SEM of anode used in reactor C in 13000x (A) and 20000x (B) magnification. Figure shows that the organism were grown packed with each other, showing formation of biofilms

6.2.4 Fumarate and fumarate coupled with lactate as electron donor

The reactor was then subsequently inoculated with fumarate as substrates. The reactor had shown maximum power density produced at around 0.2 mW/m^2 when fumarate was utilized as the sole electron acceptor. This value is increased by around 2.3 times when lactate was added in, increasing power generated to 0.43 mW/m^2 . Power generated were superior compared when another substrate was being used. Again, as previously mentioned in chapter 5, the exact reason for this was not determined but we speculate that this is due to the increased growth of biofilms from fumarate that can potentially allow the organism to utilize lactate more readily. Fumarate was described as a common terminal electron acceptor that can be utilised by bacteria to form biofilms in anaerobic condition. It was also described that it could be a limiting factor on growth of biofilms that utilises fumarate (Atci et al., 2017). However, electron transfer mechanisms in this context must be characterized in order to have more in-depth understandings of the biochemical effect and the role that fumarate plays in EET to allow this enhanced effect. For COD tests, COD removed when fumarate was utilised were negligible, while when coupled with lactate, COD removal increased to 95% (Table 16).

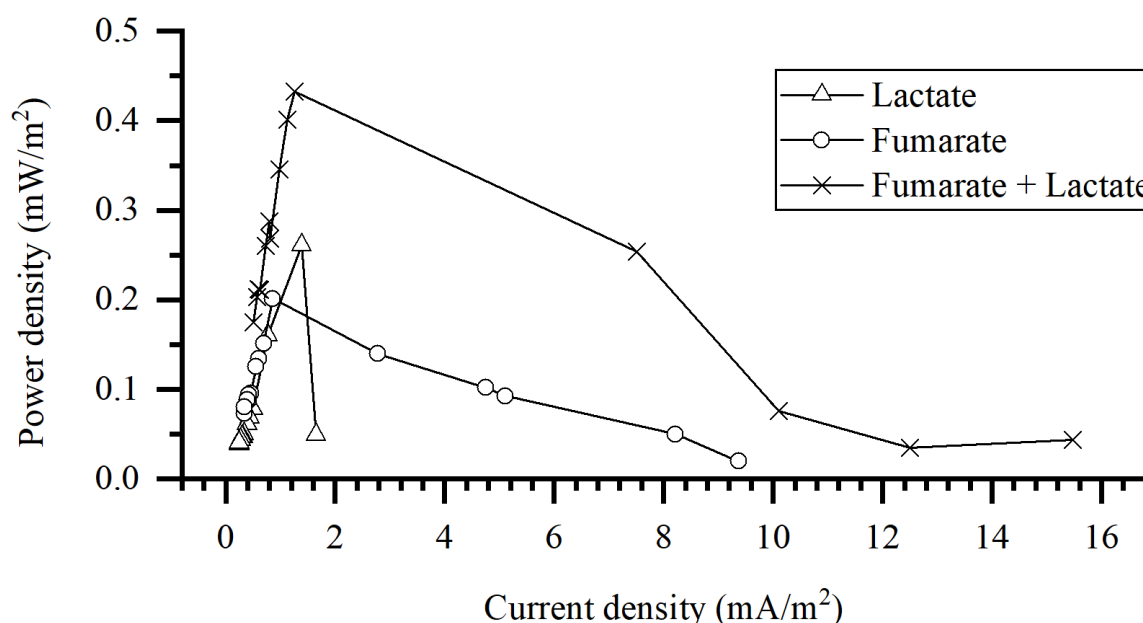


Figure 20: Power density curve when fumarate and fumarate coupled with lactate were supplied into Reactor A

6.3 Conclusions

In this chapter, we report that using *S. marcescens* as a monoculture inoculated into the anodic chamber of MFC were able to generate power by treating glucose, acetate, lactate, fumarate. Highest power density was observed with substrate being fumarate coupled with lactate, with 0.43 mW/m² recorded, similar as the previous chapter. COD removal potential were also demonstrated with high COD removal rate observed at up to 95%. Both power density generated, and CE were still inferior when compared to *S. oneidensis*, which is a generally well established EAB. However, there are benefits associating using common water-borne pathogens such as *S. oneidensis* such as the more flexible microbial control when the MFC technology were to be employed in an actual wastewater treating plant. At this stage, in-depth studies are required to explore the substrate metabolic pathways for this organism, along with further optimization and troubleshooting either by transformation or gene knockouts to allow efficient power generation and improved oxidation of substrate for effective conversion of wastewater to energy.

Chapter 7: General discussion

This study had managed to isolate and subsequently identify waterborne bacteria from two sources of different system, namely one with an open and diverse environment and another which the system is closed and controlled. After identification, identities of the bacteria from the first source were as expected to be of the major common waterborne bacteria with majority of the group belonging to the family *Enterobacteriaceae*. Out of all these bacteria identified, only *Klebsiella pneumoniae* had been reported in literature for MFC utilisation. In recent studies, a mutant *Klebsiella pneumoniae* with modified glycerol to lactate metabolic pathway were reported to be utilised in an MFC, coupled with mutant *S. oneidensis*. This coupling allows glycerol to be fed to *K. pneumoniae*, with enhanced effect where lactate is produced which will be subsequently used by the transformed *S. oneidensis* for EET, resulting in maximum power density produced of 19.9 mW/m² (Li, Yin, et al., 2018). On a separate study, *K. pneumoniae* was utilised to use glucose as substrate for power generation, with addition of electron mediator 2-hydroxy-1,4-naphthoquinone, allow power generation of up to a staggering amount at 409.71 mW/m² (Zhang et al., 2009). In these studies, *K. pneumoniae* was either coupled with mutant *S. oneidensis*, or added with expensive electron mediators which achieve high level of power density generated. While in theory these allow high power generation, it would be not feasible to apply this into a wastewater treating plant due to the complex monitoring required and the highly contaminated nature of the environment.

The thesis subsequently focuses on to evaluate whether *K. pneumoniae* coupled with the other bacteria mostly from the family *Enterobacteriaceae* found together in their natural habitat can perform EET related mechanisms in an MFC to generate power through treating synthetic wastewater supplied. Results demonstrated that these organisms were able to generate power, albeit at a low amount, at 0.18 mW/m², 0.23 mW/m² and 0.26 mW/m² from reactor with graphite plate anode, and 0.03 mW/m², 0.07 mW/m², and 0.22 mW/m² from reactor with carbon cloth anode for the substrates glucose, lactate, and acetate. The results demonstrated a trend by these organisms in which different substrates were able to generate different values of power density. The higher amount of power produced from reactor with graphite plate anode is due to the affinity of the organisms to form biofilm on the less porous carbonaceous surface instead of the porous carbon cloth. Fumarate and lastly fumarate coupled with lactate were then supplied and the highest power densities were recorded when fumarate was coupled with lactate at 0.90 mW/m².

Similarly, these organisms also demonstrated their potential in treating synthetic wastewater supplied, with higher being recorded also from fumarate coupled with lactate. These data demonstrated that the power density produced is proportionate to the COD removing efficiency. However, CE value remains inferior which infers that electrons were not fully recovered by the organisms from the substrate supplied for electricity generation. Additionally, results demonstrated that these organisms were able to grow better on graphite plate as compared to carbon cloth, demonstrated through the increased power production of approximately 4~8 times for all substrate used. While microbial population studies were required to study the interactions of these community of bacteria on the biofilm. *K. pneumoniae* will be used as an example to discuss the biofilm formation on the electrode surface. It was reported that *K. pneumoniae* can form biofilms and is attributed to the development of invasive infections especially in immunocompromised individuals through the colonization of the respiratory, urinary, and gastrointestinal tract. Stages of biofilm formation of *K. pneumoniae* starts from cell adherence, followed by formation of microcolonies maturation and finally dispersal of free cells from the matrix (Piperaki et al., 2017). Structure involved in biofilm formation process includes type 3 fimbriae and the capsular polysaccharides which the former will mediate stable adherence while the latter will ultimately affect the architecture of biofilm and the communication between cells. Having said this model alone does not constitute to the explanation of the entire polybacterial biofilm formed as it is difficult to characterise the biofilm formed and studies of the interactions amongst the bacterial populations were needed.

In chapter 6, power density were generated at 0.07 mW/m², 0.26 mW/m², and 0.20 mW/m² respectively for glucose, lactate, and acetate, this demonstrated similar results in terms of substrate utilization except for glucose. Results demonstrated that higher power density were generated when mixed community of bacteria were utilised in Chapter 5, this could be due to the wide range of bacteria present that presumably allow a wider range of substrate to be utilised for EET. However, using a mixed consortium pose their own disadvantages which include the difficulty to control microbial populations that could potentially fluctuates if different substrates were being supplied that could potentially enhance the growth of certain species in the biofilm. For *S. marcescens*, SEM imaging were able to show the surface of the graphite plate electrode were fully colonised by these bacteria. For *S. marcescens*, OxyR contributes to their formation of biofilm at the early stges by influencing the gene expression of fimbrial and plays a role in the early primary attachment of cells to the surface (Shanks et al., 2007). It was also reported that reduced carbon or nitrogen conditions will affect the biofilm morphotype of *S. marcescens* in which high nutrients will stimulate filamentous biofilm formation where low nutrients will signals classic microcolony biofilm formation (Rice et al., 2005). The exact

mechanisms or effect of either biofilms on performance on MFC were not evaluated and could be of future study subject. Even with the observed high growth, CE were still inferior which indicates that the loss of electrons during the utilisation of the substrate provided. These can be investigated through studies of gene expression in which the metabolic pathway of the organism could be further established.

For both set-ups with different sources of organisms being inoculated, the highest power densities generated were observed when fumarate coupled with lactate were supplied (0.90 mW/m² for mixed culture and 0.43 mW/m² for *S. marcescens*), with high COD removal being recorded as well at 91% and 95% respectively. The fact that reactor B having lower power densities being produced albeit having a more superior COD removing capabilities could be attributed to the loss of electrons in part of the respiratory processes in the bacteria cell and this indicated the need to study the mechanisms in which the organisms utilises the substrate provided. These results collectively demonstrated that both set-ups were able to carry out the functions of an MFC, each having their own advantages. The idea of having utilising mixed consortia has been proposed in the last decade, this is to overcome the limitations of using pure cultures such as strict operating conditions and the poor range of substrate that could be utilised. Having being said, pure culture were still needed for the clarifications of EET. For more complex substrates such as wastewater or activated sludge, it was reported that mixed consortia always achieve the best performance as compared with pure cultures (Cao et al., 2019).

Chapter 8: Conclusion

8.1 Conclusion

This study had firstly isolated and identified organism from two water sources to be subsequently inoculated into MFC. Two sources of bacteria were successfully isolated from lake sediment and local household aquarium respectively. Microbiological identification of these organisms through commercial biochemical test kits (API® 20E and Microgen GN-ID A+B Panel) were carried out. From lake sediment, *Aeromonas spp.*, *Serratia spp.*, *Kluyvera spp.*, *Chromobacterium spp.*, *Citrobacter spp.*, and *Klebsiella spp.*, were identified. Most organism isolater from this source were of *Enterobacteriaceae* family. From household aquarium, all bacteria was identified to be *Serratia marcescens*. Identity of isolates 2 and 3 from water source A were reconfirmed to be *Serratia marcescens* and *Enteric Gp68* respectively through Microgen GN-ID A+B Panel test due to the low % ID obtained from the previous API® 20E test.

Effects of carbon and graphite, on the performance of MFC, in terms of the growth of biofilms, and how will this subsequently affect power generation, and removal of organic matters was then determined. Common waterborne bacteria inoculated into MFC reactor were able to form biofilm on electrode surface and had shown higher affinity for biofilm formation on graphite plate as compared to carbon cloth, demonstrated through the increased power densities produced. Next, potential of MFC inoculated with common waterborne bacteria obtained from lake and water samples in generating electricity and treating synthetic wastewater was determined. Common waterborne bacteria inoculated into MFC reactor were able to generate power density while simultaneously treating synthetic wastewater supplied. Highest power density obtained of 0.90 mW/m² was recorded when fumarate coupled with lactate was supplied as synthetic wastewater. These organisms also exhibited a trend in substrate utilisation, with fumarate and lactate managed to achieve the highest power density generated, followed by fumarate, acetate, lactate, and finally glucose. The reactor were able to remove high percentage of COD at up to 95.18% recorded from fumarate coupled with lactate. Low CE had been recorded across the reactors, inferring electron loss during EET.

Lastly, the potential of MFC inoculated with a single isolate of *S. marcescens* obtained from household aquarium as compared with MFC inoculated with common waterborne bacteria were determined. *S. marcescens* were able to form biofilm on graphite plate, and also able to generate power density while simultaneously treating synthetic wastewater supplied. Highest power density obtained of 0.43 mW/m²

was recorded when fumarate coupled with lactate was supplied as synthetic wastewater. *S. marcescens* exhibited trends in substrate utilisation similar with mixed culture of common waterborne bacteria. The reactor were able to remove high percentage of COD, at 95%, also when fumarate coupled with lactate were supplied as synthetic wastewater. Low CE obtained from the reactor, this was attributed to non-exoelectrogenic processes carried out by the bacteria.

8.2 Contribution of the Thesis

This thesis has contributed to the literature in the following aspects:

1. Determine the feasibility of using common waterborne bacteria in operating an MFC reactor and the surface of anode which favours their biofilm formation. These bacteria were isolated from lake sediments which is easily accessible obtained and do not require stringent population control when operated in an MFC reactor.
2. Explored if a pure culture of *S. marcescens* was able to generate bioelectricity comparable to a mixed culture of waterborne bacteria in terms of power density, and COD removal efficiency.

8.3 Future work

There are several questions that can be investigated further, namely the mechanisms in which these organisms' forms biofilms on different electrode surface, the metabolic pathways in which these organisms utilise the substrates supplied, and the exact reason in the increased MFC performance through coupling of fumarate with lactate. Further studies could answer these questions by either gene deletions to study gene functions. For further optimisation for these organisms to be feasible when used in MFC, development are imperative in both biological and electrochemical aspects, including the introduction of substrate utilisation genes to allow an ever-wider range of substrates to be utilised, introduction and overexpression of heterologous EET genes from either *S. oneidensis* or *G. sulfurreducens*, and modifications on MFC set-up including electrode studies, electrolyte studies, and reactor set-up.

Microbial population studies can be also a potential further study topic to study the interactions of different microbial communities within the biofilm formed on the anode surface. This can be done through 16s rRNA sequencing and subsequently identifying the 16s rRNA sequence via BLAST software on NCBI. Different species of organisms should also be explored to study for more different mechanics in terms of EET. Studies can focus on the exploitation of both biofilm formation and substrate utilisation genes either through overexpression of heterologous gene or gene amplification for identified genes responsible for EET, as demonstrated by studies related to genetic engineering in *S. oneidensis*. More efforts are still required for this technology to be applicable in a real-world wastewater treatment system.

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Appendix

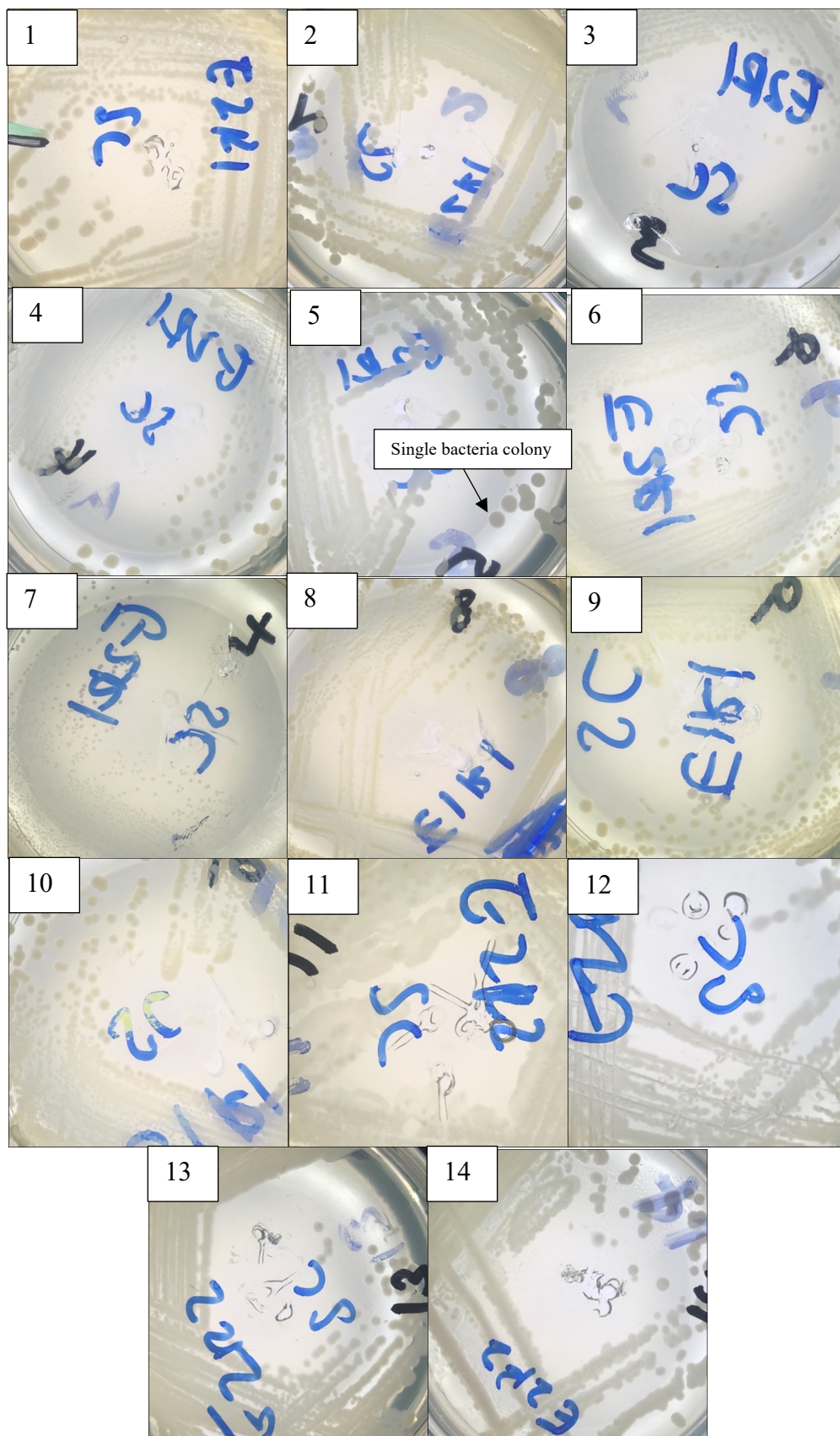


Figure 21: Colony morphology of unknown isolates on plate count agar after incubation at 37°C

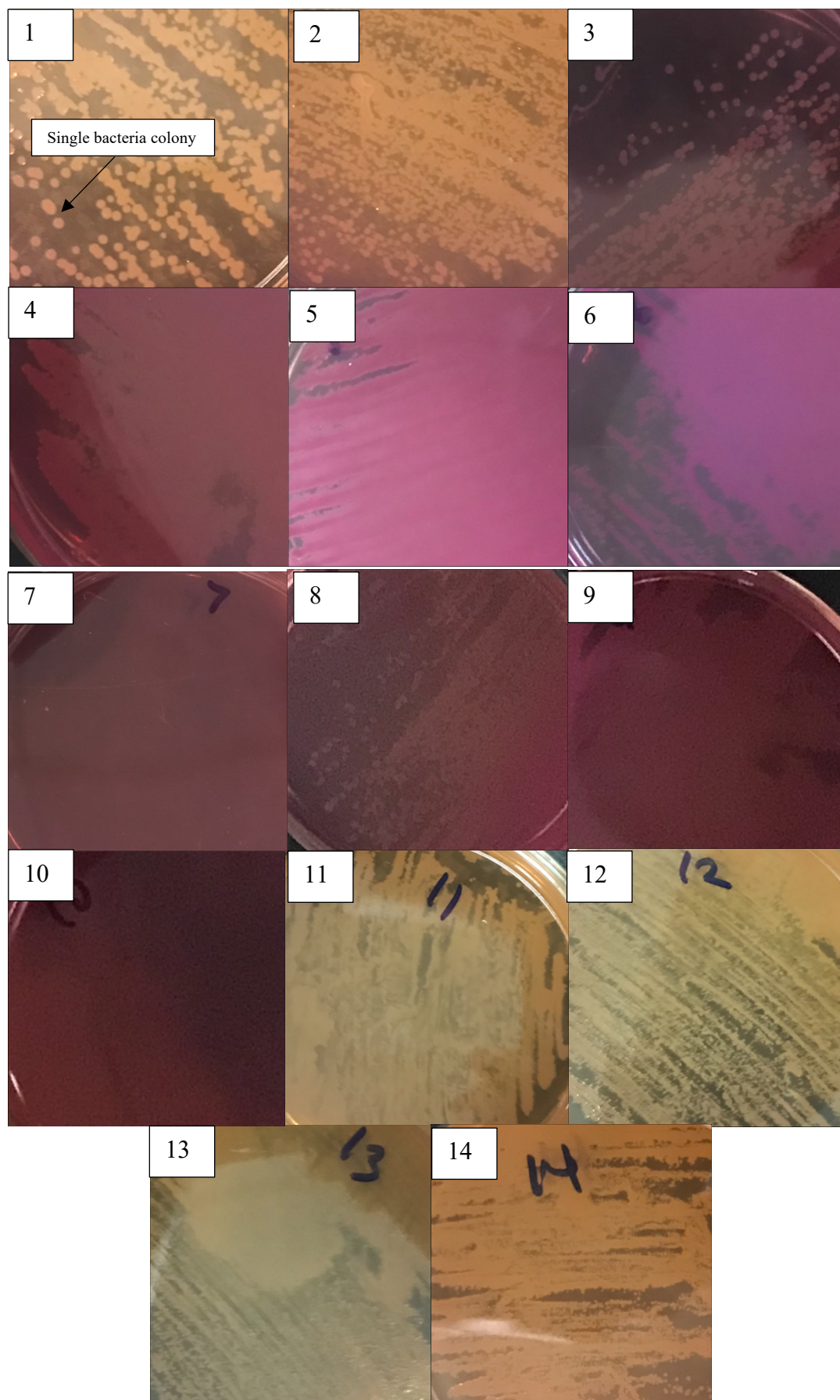
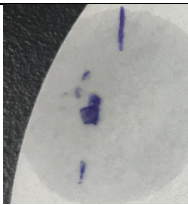
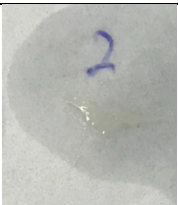
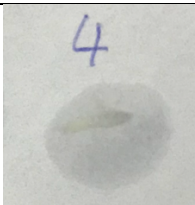
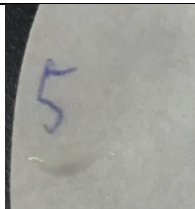
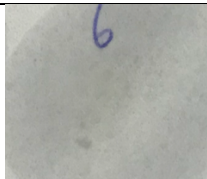
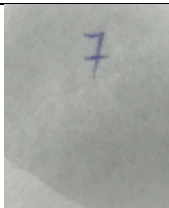


Figure 22: Colony morphology of unknown isolates on MacConkey agar after incubation at 37°C overnight

Table 17: Oxidase tests for unknown isolates

Unknown	1	2	3	4	5	6	7
Oxidase test							

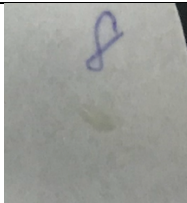
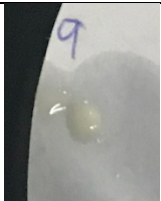
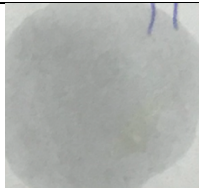
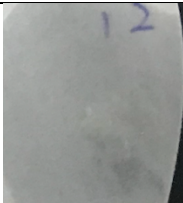
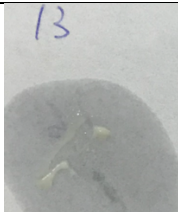
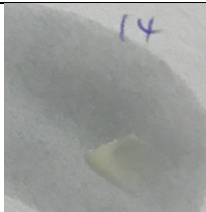
Unknown	8	9	10	11	12	13	14
Oxidase test							

Table 18: API® 20E Test strip for unknown isolates after 18 hours incubation at 37°C

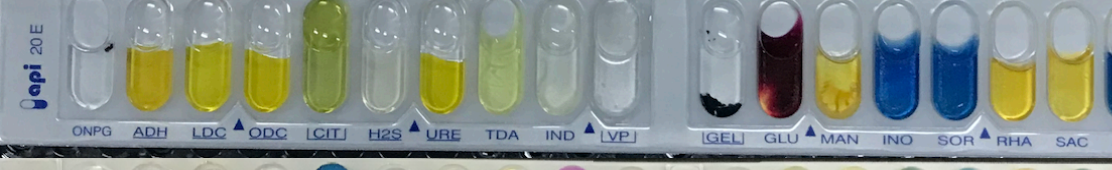
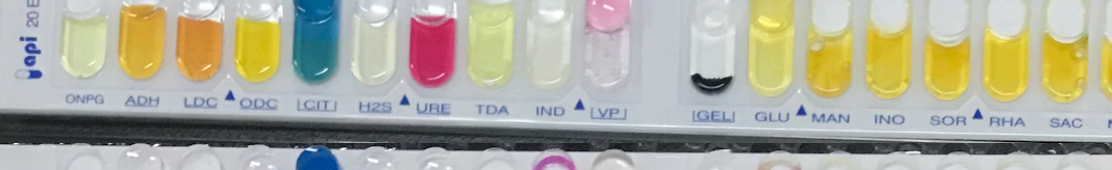
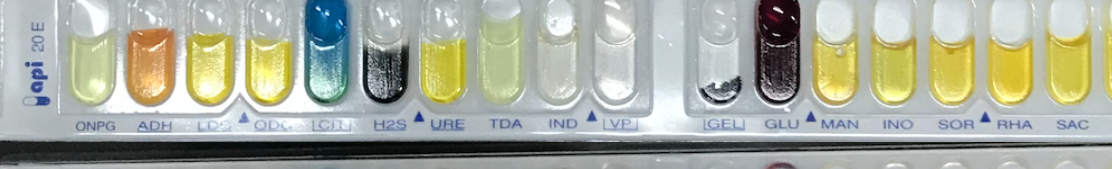
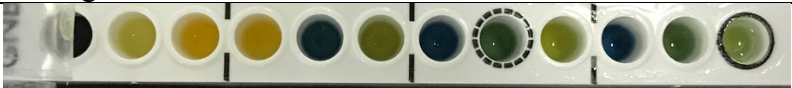
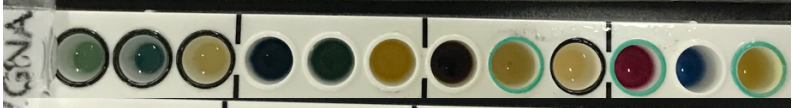
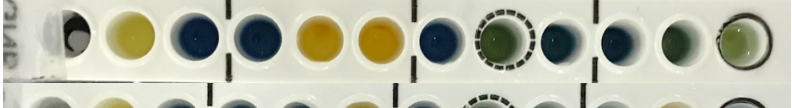
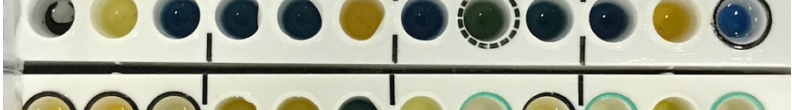
Unknown	API® 20E Test strip
1	
2	
3	
4	
5	
6	
7	
8	
9	



Table 19: Microgen GN-ID A+B Test strip after 18 hours incubation at 37°C

Unknown	Microgen GN-ID A+B Panel test
2	
3	
5	
7	
11	

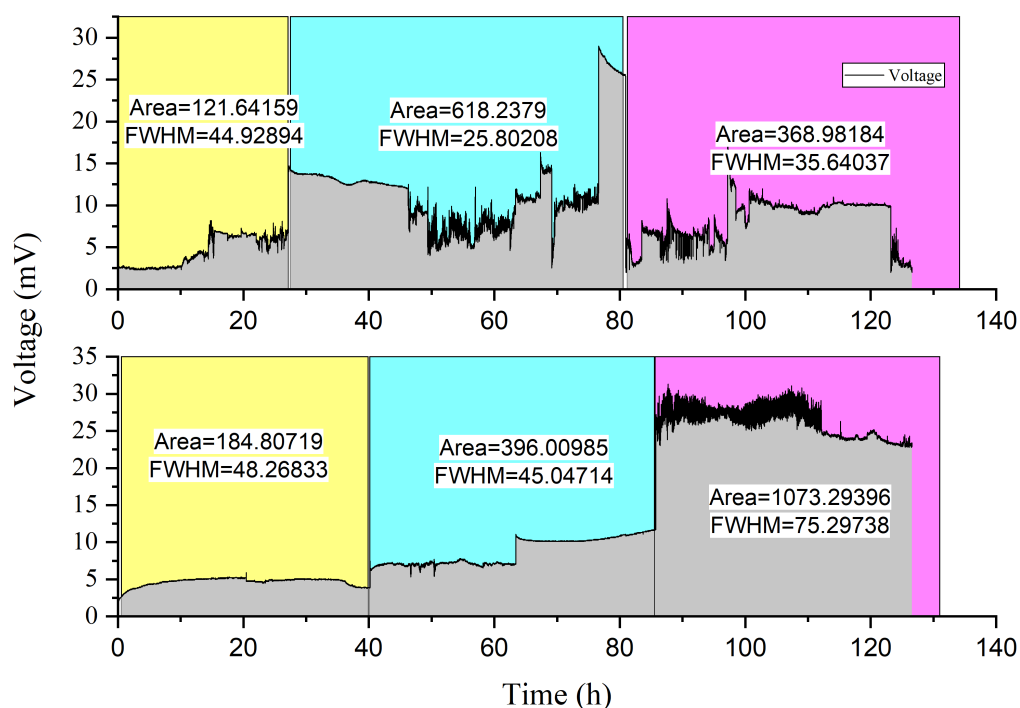


Figure 23: OriginPro output for the calculation of area under curve for voltage versus time graphs for the calculation of coulombic efficiency for reactor A and B using graphite plate and carbon cloth as electrode inoculated with glucose, lactate, and acetate

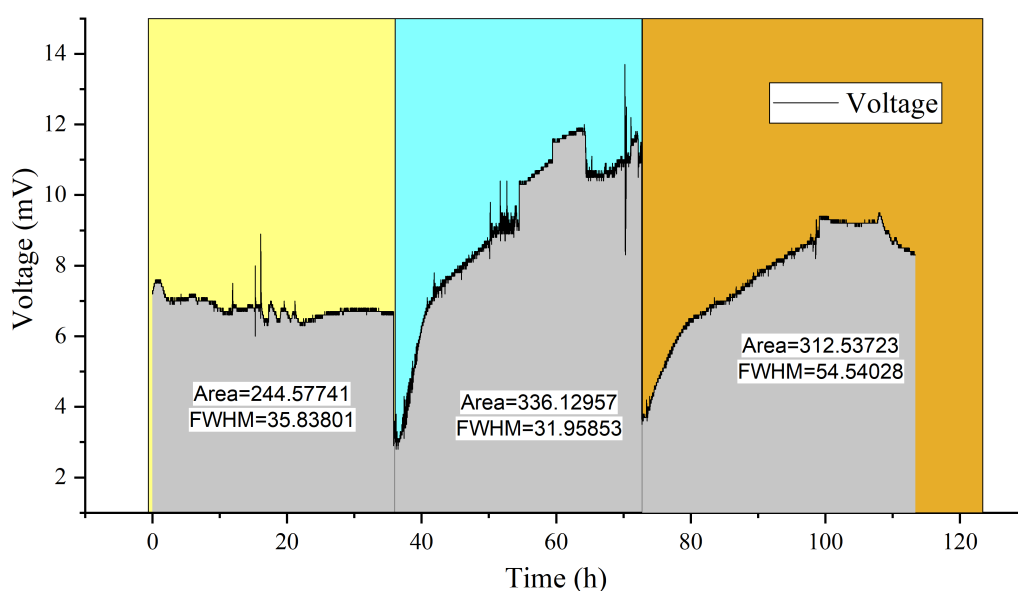


Figure 24: OriginPro output for the calculation of area under curve for voltage versus time graphs for the calculation of coulombic efficiency for reactor C using graphite plate electrode inoculated with glucose, lactate, and acetate