Thermophilic Microbial Electrochemical Cells

by

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Doctor of Philosophy

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Chapter 1: Introduction to Microbial Electrochemical Cells (MXCs)

Overview:

The increasing cost of fossil fuels, the impacts their consumption has on the environment, and climate change has encouraged the development of alternative energy sources (Rittmann 2008, Doney 2009, U.S. Global Change Research Program 2014, Sieminski 2015). Alternative energy sources often seek to mitigate carbon emissions and environmental impact while diversifying energy resources (Rittmann 2008). Over the past 65 years, there have been significant increases in consumption and production of alternative fuel technologies including wind, solar, electrochemical cells, and biofuels (US EIA 2015, Stocker 2014, Oliveira 2013). The focus of this research project is the development and maturation of microbial electrochemical cells (MXCs), a technology that focuses on biomass as a resource to produce electrical current and high-value chemicals (Rabaey 2005).

An MXC is an electrochemical cell that uses bacteria as a catalyst to convert the chemical energy stored in reduced organic compounds; often wastes measured using chemical oxygen demand (COD) or biochemical oxygen demand (BOD), into electrical energy, hydrogen, or a number of other useful chemical products (Kim 1999, Moon 2005, Cheng and Logan 2007, Ieropoulus 2005, Schröder 2007). There are two primary areas of focus when it comes to investigating microorganisms in MXC technology: one which looks at bacteria that are capable of passing electrons to an anode, or anode respiring bacteria (ARB), and the other which looks at bacteria or archaea that are capable of oxidizing a cathode, or electrode oxidizing microorganisms (Torres 2008, Lovely 2008). The ARB in MXCs are dissimilatory metal-reducing bacteria capable of performing extracellular electron transfer (EET) to insoluble metals

including sulfur compounds, iron oxides, humics, and AQDS. Due to the low conductivity of their membranes, ARB utilize a series of redox active proteins, or cytochromes, to transfer electrons from the cytoplasm to the cell surface (Carlson 2012, Bird 2011, Leang 2003, Lloyd 1999). The electrode oxidizing microorganisms in MXCs are capable of receiving electrons from insoluble metals and reducing terminal electron acceptors. This dissertation will focus on using ARB in the anode compartment of MXCs.

MXC technology operates with two primary modes of function: one which is called a microbial fuel cell (MFC), and the other which is called a microbial electrolysis cell (MEC) (Rittmann 2008). In an MFC, ARB are placed in an anaerobic anode chamber with reduced organic carbon as their food, or electron source, and an electrode, or anode, as their electron acceptor. The anode chamber is often separated from a cathode chamber using an ionically conductive membrane that is permeable to either anions, in the case of an anion exchange membrane (AEM), or cations, in the case of a cation exchange membrane (CEM). For all experiments in this dissertation, an AEM was employed to allow hydroxide ions (OH^-) to transfer from the cathode to the anode for the purpose of maintaining electroneutrality. The electrode in the cathode chamber, or cathode, is connected to the anode with a resistor or load, and the cathode compartment is kept under aerobic conditions. This establishes a potential gradient between the anode and the cathode. The electrons stored in the organic carbon are transferred to the ARB via bacterial metabolism, then transferred from the ARB extracellularly to the anode, and finally travel along the potential gradient to the cathode where they ultimately reduce an oxidative terminal electron acceptor (Torres 2014). The transfer of electrons, measured in amperes (A), along a potential gradient, measured in voltage

(V), produces power, measured in watts (W). Since the cell voltage in MFCs is not directly controlled, performance standards are often reported in power density, or the W per surface area of the anode or cathode measured in square meters (W m^{-2}). The transfer of electrons from ARB is coupled with the production of protons (H^+) in the biofilm anode. An overview is shown in Figure 1.1.

The benefit of operating MFCs for power production is determined by the net amount of energy that can be recovered from the process. For MFCs, there are two primary factors that determine the amount of power that can be recovered: the cell voltage (E_{cell}) and the current (I), measured in A, generated over this voltage. Given that: W=V*A, or W_{MFC}= E_{cell}* A m^{-2}, where W_{MFC} is the power density produced by the MFC, the ideal operation for an MFC is one that provides the highest W_{MFC}. In acetate-fed MFCs, the theoretical maximum cell potential is approximately 1.1 V (Logan 2006) vs SHE because O_2 has a potential of 0.75 V vs SHE and acetate has a potential of -0.35 V vs SHE at 60°C and pH=7. However, bacteria must be able to derive a net gain of energy from the oxidation of the reduced electron donor to generate ATP, NADH, or biomass (Schroder 2007). In order for bacteria to derive energy for cell growth, the working potential of the anode must be higher than the electron donor. This results in a loss of potential from $E°_{cell}$ and is referred to as overpotential. In the case of acetate, the anode potential must be higher than -0.35 V vs SHE, but ideally, must be as close to -0.35 V vs SHE as possible to simultaneously provide electrons for the biofilm anode to grow while optimizing power production. In MFCs, the potential of the anode is commonly established using resistors, which have the advantage of being relatively inexpensive and do not require costly machinery

(Franks 2009). However, resistors also have the disadvantage of making managing the anode potential difficult as the conditions of the MFC change.

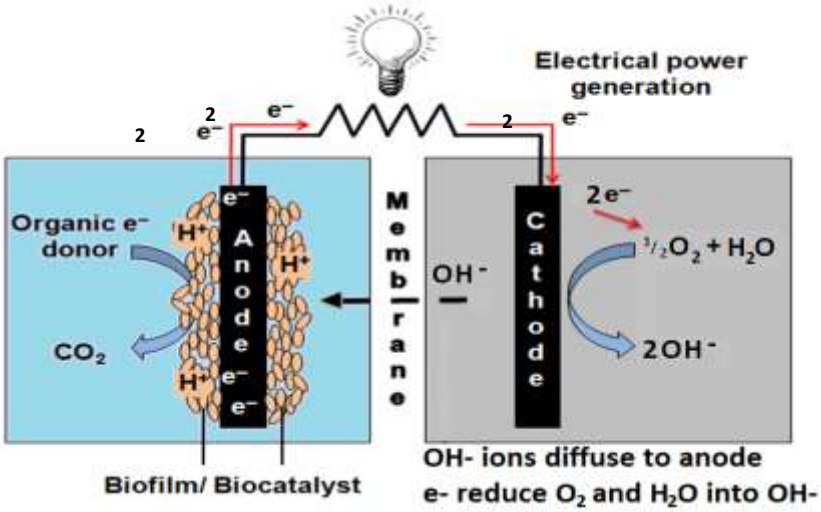

Figure 1.1: Electron flow in a typical microbial fuel cell (MFC). Adapted from Torres 2010 and equations adjusted per Popat 2012.

In an MEC, the conditions of the anode compartment are identical to the MFC, and the anode compartment is separated from the cathode compartment using an ionically conductive membrane; however, the cathode is kept under anaerobic conditions, and a power source is used to apply a specific voltage. As the electrons transfer from the anode to the cathode, an outside potential is supplied to enable the lysis of water (H_2O) into hydrogen gas (H_2) and OH^- at the cathode. The H_2 is then captured and stored for combustion or conversion to electricity in a conventional hydrogen fuel cell. Since the anode is poised at a specific potential in MEC technology and the cell voltage is specifically controlled, performance standards are often reported in current density, or the number of amperes per surface area of the anode or cathode (A m^{-2}). Since the ARB grow on the surface area of the anode, all current densities

shown in this dissertation will be displayed per surface area of the anode. An overview is shown in Figure 1.2.

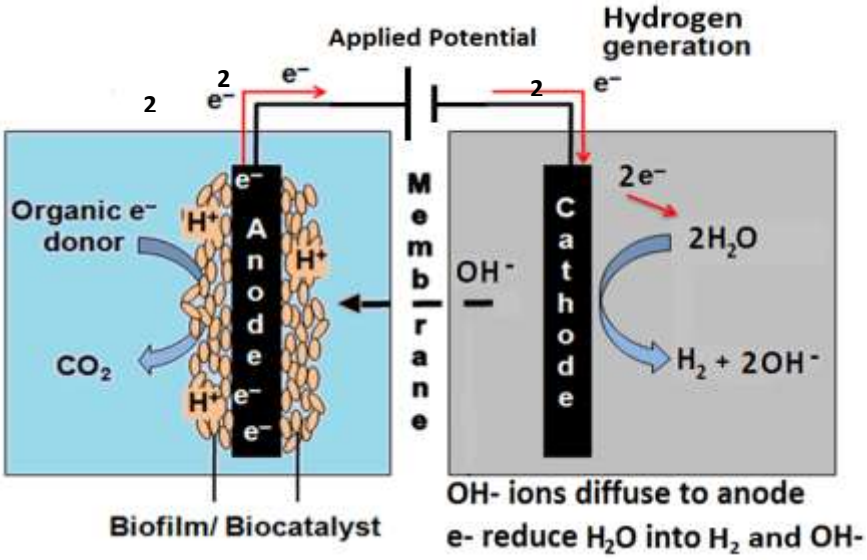

Figure 1.2: Electron flow in a typical MEC. Figure 2 adapted from Torres 2010 and equations adjusted per Popat 2012.

The benefit of operating MECs for H_2, or other useful end products, is determined by the total electrons captured in produced end products that were acquired from the substrate fed into the reactor (Lee 2008). The production of H_2 via the electrolysis of H_2O is an endergonic process, meaning that it is not spontaneous and requires the input of energy. For MECs at 60 °C and pH 7, a voltage of ~0.109 V vs SHE is applied, via a power source, to catalyze H_2O electrolysis at the cathode. The H_2 captured from the MEC cathode can be oxidized with O_2 and converted to electrical power and H_2O using a hydrogen fuel cell, yielding an $E°_{cell}$ net voltage of 1.12 V vs SHE. Similar to an MFC, the potential of the anode must be higher than that of the substrate being supplied; however, since MECs allow for the direct control of the anode

potential via a power source (an uncommon practice with MFCs), the voltage of the anode can be controlled to maximize E_{cell}. In MECs, the end products can be captured and stored as H_2 or other high value products. In addition, poising the potential of the anode enables controlled studies to determine physiological properties of ARB based on anode potential. For these reasons, MEC mode of operation with a poised anode potential established by a potentiostat is used in this dissertation. Figure 1.3 shows a typical 'H-type'MEC used in this research.

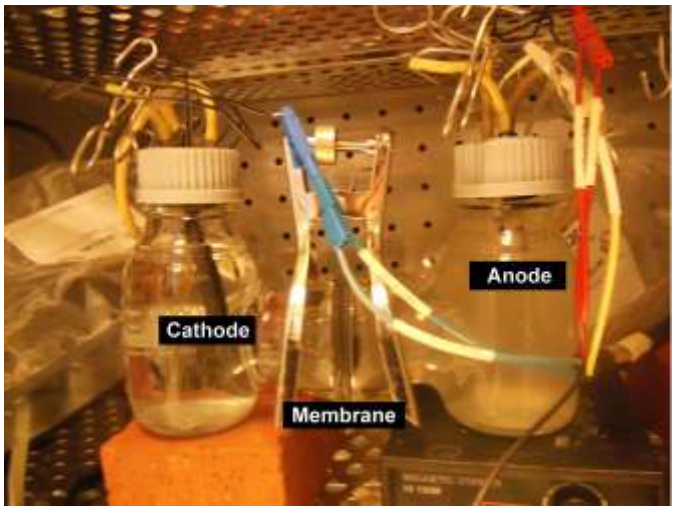

Figure 1.3: Typical lab scale H-type MEC set up.

Research looking at ARB in MXC technology has primarily focused on either modelling and assessing the effectiveness of microbial communities for the utilization of specific organic wastes (Marcus 2007, Du 2007, Pant 2010) or using electrochemistry to gain insights into the physiological and kinetic properties of ARB (Srikanth 2008, Marsili 2010, Yang 2012, Parameswaran 2013, Badalamenti 2013, Yoho 2014). The microbial communities under investigation may be a precise monoculture (a community consisting of only one species of bacteria) (Marshall 2009, Parameswaran 2013), a controlled mixed culture (a community

consisting of more than one bacterial species that are intelligently selected ahead of time for the conditions of the reactor) (Bourdakos 2014), an enriched culture (a community consisting of more than one bacterial species that has been naturally selected for the conditions of the experiment) (Jong 2006, Miceli 2012), or a random culture (a community consisting of several bacterial species that are not selected specifically for the reactor) (Torres 2009). Random culture communities can come from a diverse number of sources including soil samples or wastewater treatment sludge (Niessen 2006, Zhang 2006, Miceli 2012). In addition, the utilization of a diverse array of organic wastes has been assessed in MXC technologies including: organic acids, sugars, and highly reduced complex polymeric substances (Du 2007, Ren 2008, Pant 2010). Complex polymeric substances such of lignin and cellulose often, but not always, have limited bioavailability for ARB in mesophilic MXCs due to the thermodynamic and kinetic limitations of breaking them down into substrates that can be utilized for anode respiration (Lynd 2002, Rismani-Yazdi 2007, Ren 2008). However, thermophilic MXCs offer kinetic and thermodynamic benefits that make them great candidates for current generation from complex and cellulosic reduced organic polymers (Mathis 2008, Liu 2010, Parameswaran 2013).

The field of MXC technology has only limited information about the physiological features of Gram-positive thermophilic ARB, but research in the field is growing (Pham 2008, Ehrlich 2008, Wrighton 2012, Parameswaran 2013). Broadening understanding for thermophilic ARB will assist in elucidating novel metabolic pathways for substrate utilization and electron transport, reveal limitations encountered in thermophilic MXCs, and enhance the feasibility of using MXC technology to produce valuable products from diverse waste streams (Beveridge

and Murray 1980, Beveridge 1982, Erlich 2008, Mathis 2008, US Patent No. 20090017512). This research primarily focuses on four major thermophilic research projects:

1. A monoculture of *Thermincola ferriacetica* was used to construct a draft genome of *T. ferriacetica* to determine potential genetic markers associated with bacterial EET, including the presence of multiheme *c*-type cytochromes.
2. A monoculture of *Thermincola ferriacetica* was used to conduct experiments to establish the fundamental physiological properties and limitations of a model thermophilic microorganism in MECs.
3. A monoculture of *Thermonanaerobacter pseudethanolicus* was used to determine the viability of using a single thermophilic microorganism to ferment the complex substrates glucose, cellobiose, and xylose while simultaneously performing anode respiration.
4. A highly enriched mixed community of thermophilic cellulose degrading bacteria coupled with ARB was established to determine the feasibility of combining thermophilic microorganisms for the purpose of producing current from cellulosic wastes in a single anode chamber in an MEC.

Biological Principles of Gram-Positive Thermophilic Bacteria:

Thermophiles are microorganisms that belong to a specific class of extremophiles that survive and operate at temperatures that are anthropocentrically considered outside the realm of 'normal', or mesophilic, conditions. Thermophiles include two distinct classifications: thermophile- microorganisms that prefer a temperature range of 50-80°C- and

hyperthermophile- microorganisms that prefer a temperature range of 80-125°C (Kashefi and Lovley 2003, Seckbach 2004). Some of the first microorganisms to thrive on earth were chemoautotrophic thermophiles that lived underwater near hydrothermal vents and thus were in areas protected from UV radiation that contained plentiful amounts of dissolved minerals (Seckbach 2006). Positioned close to the root of the Bacteria kingdom on the tree of life (Ciccarelli 2006, Seckbach 2006), thermophiles may provide a glimpse into some of the earliest forms of dissimilatory metal reduction on Earth.

Thermophiles exist across several kingdoms and include everything from insects, to eukaryotic algae and fungi, to archaea and bacteria. Most thermophilic bacteria thrive at temperatures ranging from 55-<100°C and tend to persist in warm water, hot springs, hot geysers, and hydrothermal vents (Niu 2009, Onyenwoke 2007, Seckbach 2006, Slepova 2009, Slobodkin 2006, Sokolava 2005, Zavarzina 2007). Many thermophilic bacterial species thrive under anoxic or even anaerobic conditions and are chemotrophs: using oxidized minerals including SO_4^-, Fe(III) oxides, NO_3^-, and Mn(IV) as their terminal electron acceptors (Nealson and Conrad 1999, Knoll 2003). In addition, thermophiles produce thermostable enzymes (thermozymes), giving them higher metabolic rates and thus increasing the kinetics of organic waste consumption in MXCs (Mathis 2008, Liu 2008, Parameswaran 2013). (Perhaps the most famous example of a thermozyme is *Taq* polymerase, isolated from *Thermus aquaticus* and used universally in polymerase chain reaction (PCR) protocols (Brock 1969).) For the purpose of this dissertation, the focus will be on Gram-positive, chemoheterotrophic, thermophilic, anaerobic bacteria. These are ideal candidates for MXC research because they are very likely to

be physiologically capable of growing on an anode in high temperature conditions while consuming organic waste as their electron donor.

Within the kingdom Bacteria, there are two distinct classifications- Gram-positive and Gram-negative- that span across many bacterial phyla (Ventura 2007, Vesth 2013). Bacteria are classified as either Gram-positive or Gram-negative depending on whether they retain a stain with crystal violet dye after washing with water and alcohol- a protocol developed by Hans Christian Gram in 1884. This classification has profound implications on the physiology and structure of the bacterial cell- specifically the structure of the cell wall and membrane. This is significant because metal reducing bacteria are required to transport their electrons externally through their cell membranes and, thus, Gram-positive and Gram-negative ARB may have entirely distinct pathways for metal reduction, yielding different limitations in MXCs.

In Gram-negative bacteria, the cell wall consists of two membranes, an inner membrane and an outer membrane, that are separated by a periplasm. Within the periplasmic space is a thin layer of peptidoglycan (~5-10nm), or murein- a polymer of sugars, *N*-Acetylglucosamine (NAG), *N*-Acetylmuramic acid (NAM), and amino acids that accounts for approximately 10% of the dry weight of the cell. For external electron transport to occur, the electron must traverse a series of peripheral and integral proteins and cytochromes that are imbedded in the inner membrane, span across the periplasm, and are docked to the outer membrane (Birds 2011). In the field of MXC technology, most research and modelling has focused on understanding electron transport and biofilm limitations with Gram-negative bacteria (Marcus 2007, Marcus 2011, Liu 2011, Carlson 2012, Wrighton 2012, Vecchia 2014, Pirbadian 2014). In contrast, the

research in this dissertation focusses exclusively on understanding electron transport and anode respiration for Gram-positive ARB in MXCs.

Gram-positive bacteria have a cell wall composed of only one membrane and thus lack an outer membrane. In Gram-positive bacteria, the membrane is separated from the peptidoglycan by a periplasmic space. The peptidoglycan consists of many layers, is very thick (20-80nm), and can weigh as much as 90% of the cell's total dry weight (Pham 2008, Erlich 2008). In addition, Gram-positive bacteria have teichoic acids embedded in their cell walls that can extend from the cell membrane to the outer surface of the peptidoglycan layer, and have been implicated as the metal binding sites for the cells (Beveridge and Murray 1980, Beveridge 1982, Erlich 2008). The presence of a thick peptidoglycan layer in metal reducing bacteria makes it necessary for electron transfer to occur either via proteins and cytochromes that are packed into fissures within the cell wall, anchored to the peptidoglycan, or positioned along teichoic acids (Carlson 2012, Ehrlich 2008). Metal-like conductance along teichoic acids or electron hopping along cytochromes embedded in peptidoglycan may have profound influences on the limitations and performance of thermophilic MXCs compared to mesophilic MXCs including: changes in conductivity of the extracellular matrix (K_{bio}) and changes in the midpoint potential (E_{ka}) of electron channeling cytochromes (Marcus 2007).

The method through which ARB transport electrons to the anode in MXCs varies by bacterial species (Torres 2010, Mohan 2014). In ARB, there are three major theories for how EET occurs, including one method for mediated, or indirect, electron transfer (MET) and two methods for non-mediated direct electron transfer (DET) (Torres 2010, Mohan 2014). The MET

method involves redox mediators, or extracellular shuttles, that transfer electrons between the bacterium and the anode that are either produced by the bacteria or added by researchers to mitigate electron transfer (Schroder 2007, Mohan 2014). The two methods for DET to the anode include direct contact of a redox protein, or cytochrome, imbedded on a cell's outer membrane or peptidoglycan layer to the anode, and direct contact of an electrically conductive, or semiconductive, extracellular matrix made up of either pili/'nanowires' or membranous extensions embedded with cytochromes (Lovley 2008, Torres 2010, Carleson 2012, Parameswaran 2013, Pirbadian 2014). The two Gram-positive ARB used in this research, *T. ferriacetica* and *T. pseudethanolicus*, have been experimentally shown in this and prior research to use DET via long range electron transport through an extracellular matrix to the anode; therefore this paper will focus on the DET theory for electron transport that is based on long range electron transport (Parameswaran 2013).

The Gram-positive nature of these bacteria makes it probable that there may be alternative mechanism(s) for long range DET than those present in Gram-negative bacteria since Gram-positive bacteria have no secondary membrane; instead, they have a thick layer of peptidoglycan surrounded by an S-layer. As stated earlier, thermophiles and other extremophiles would have been the best adapted organisms for early Earth conditions; thus, by investigating metal reducing thermophilic bacteria from the Firmicutes phylum in the Clostridia class, we may be catching a glimpse into some of the earliest mechanisms for electron transfer to insoluble metals, and perhaps the process of respiration (Ciccarelli 2006, Puigo 2008). Figure 1.4 shows that the firmicutes phylum was one of the first major phyla to split from the last universal bacteria ancestor (represented by the black circle), indicating that EET mechanisms in

other bacteria, including many Gram-negatives, may not have the same evolutionary lineage. An increase in optimal growth temperature affects the amino acid sequence of almost every protein within the thermophilic proteome and has a large impact on the GC content of the bacterial RNA, suggesting that the proteins and protein structures involved in thermophilic EET may be different than those present in mesophilic ARB (Hurst 2001, Puigbo 2009). Although it is possible that all long range DET is the result of a single divergent evolutionary model or from horizontal gene transfer (HGT), it may be the case that long range DET in Gram-negative bacteria is the result of convergent evolution -an independent evolutionary process resulting in a similar outcome- leading to homoplasy. Considering that prokaryotes have been around for 3.5 billion years and survived for ~1 billion years before oxygen, it highly likely that we will observe convergent EET phenomena in prokaryotes.

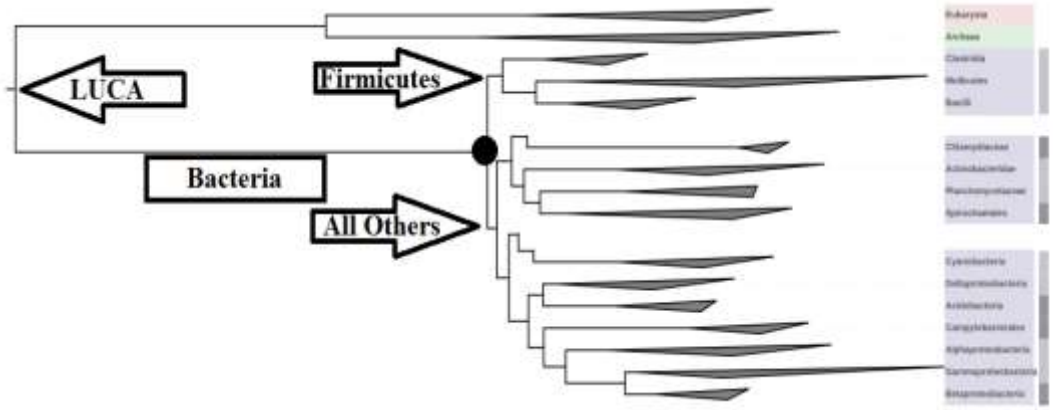

Figure 1.4: Phylogenetic tree created using iTOL 1.0. Tree shows phlogenetic lineage from the Last Universal Common Ancestor (LUCA) between Bacteria, Archaea, and Eukaryota. Black dot is last common ancestor between the Firmicutes phylum and all other bacteria phyla (Ciccarelli 2006, Letunic 2007). Figure reveals that Firmicutes are not the ancestors of the Gram-negtive

bacteria used to establish much of the literature regarding EET in the field of microbial electrochemical technology.

Biological Principles of *Thermincola ferriacetica*:

Thermincola ferriacetica strain Z-0001 (DSMZ 14005) is a metal reducing, thermophilic, obligate anaerobic, facultative chemolithoautotrophic, Gram-positive, straight or slightly curved rod-shaped bacterium that was isolated from an amorphous Fe(III) hydroxide [$Fe(OH)_3$] deposit taken from a terrestrial hydrothermal spring on Kunashir Island in Russia, near northern Japan. Based on 16SrDNA sequence analysis, *T. ferriacetica* is in the cluster of the Peptococcaceae family with 98% similarity to *Thermincola carboxydophila*. Low DNA-DNA hybridization (27 ± 1%) with *T. carboxydophila* has deemed *T. ferriacetica* a novel species. Morphological dimensions for *T. ferriacetica* are 0.4–0.5 µm in diameter and 1.0–3.0 µm in length, which can grow singularly, but often occur in chains of 4-50 cells. A scanning electron micrograph of *T. ferriacetica* is shown in Figure 1.5. Some cells within these chains form spores which are resistant to temperatures up to 121°C over 30 minutes and result in larger cell diameters: 2.0-3.0 µm. *T. ferriacetica* is motile by means of 1-4 peritrichous flagella (Zavarzina 2007).

T. ferriacetica grows within the temperature range of 45-70°C and has optimum growth between 57-60°C. In addition, *T. ferriacetica* grows in a pH range of 5.9-8.3 with optimum growth between 7.0-7.2. *T. ferriacetica* also has a salt tolerance of up to 35g/L, but maintains optimal growth under low salt conditions (Zavarzina 2007). *T. ferriacetica* has been shown to respire using Fe(III) Hydroxide [$Fe(OH)_3$], magnetite [Fe_3O_4], Mn(IV) [MnO_2], and

anthraquinone-2,6-disulfonate [AQDS] as electron acceptors. *T. ferriacetica* can grow using acetate [CH_3COOH], peptone, yeast and beef extracts, glycogen, glycolate [$C_2H_4O_3$], pyruvate [$C_3H_4O_3$], betaine, choline, N-acetyl-D glucosamine [$C_8H_{15}NO_6$], and casamino acids as electron sources. *T. ferriacetica* can also grow chemolithoautotrophicaly using H_2 as an electron source. Lastly, *T. ferriacetica* can also produce H_2 when CO is the electron source and acetate is the carbon source (Zavarzina 2007).

Previous experiments reveal that *T. ferriacetica* can produce current when used in MFCs and MECs with a doubling time of 1.2 ± 0.25 h (Parameswaran 2013, Marshall 2009). The ability to reduce metals in addition to the diverse range of electron sources makes *T. ferriacetica* an ideal candidate for current generation via incorporation into MXCs; however, little is known regarding the limitations and genetic framework of Gram-positive thermophilic ARB in MXCs (Wrighton 2012). This dissertation evaluates the limitations of using *T. ferriacetica* in MECs and also posts a draft genome to elucidate potential pathways for EET. Lastly, given the diverse range of electron donors and acceptors, it is likely that *T. ferriacetica* coexists naturally with other thermophilic bacteria in communities that are responsible for the mineralization of organic materials. It is therefore important to look into the possibilities of incorporating *T. ferriacetica* into syntrophic relationships to maximize its potential as a renewable energy provider. This research investigates incorporating *T. ferriacetica* with a thermophilic cellulolytic bacterial consortium for the purpose of converting cellulosic waste into current in the anode of an MEC.

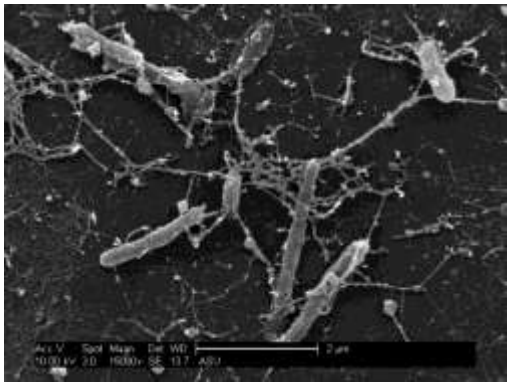

Figure 1.5: Scanning electron micrograph of *T. ferriacetica* cells on a glass slide. Image reveals rod shaped cells that have long extracellular appendages.

Biological Principles of *Thermonanaerobacter pseudethanolicus*:

Thermoanaerobacter pseudethanolicus 39E^T (ATCC 33223) was first isolated from the Octopus Spring algal mat in Yellowstone National Park, USA and is an anaerobic, thermophilic, Gram-positive, rod shaped bacterium that grows optimally at 65°C with a pH range of ~5.4-8.3 (Onyenwoke 2007, Lee 1993, Zeikus 1980, Hollaus and Sleytr 1972). Previous names, and now pseudonyms, for *T. pseudethanolicus* include *Clostridium thermohydrosulfuricum*, *Thermoanaerobacter thermohydrosulfuricus*, *Thermoanaerobacter ethanolicus* ATCC33223, and *T. ethanolicus* strain 39E (Hollaus and Sleytr 1972, Lee 1993, Onyenwoke 2007). Interestingly, the 'pseud' in *T. pseudethanolicus* comes from its previous nomenclature as *T. ethanolicus*- its name translates literally to 'false-ethanolicus'. *T. pseudethanolicus* was previously reported to be a novel subspecies of *Thermoanaerobacter brockii*, but DNA-DNA hybridization values are significantly below 70%, making *T. pseudethanolicus* a novel species in the genus *Thermoanaerobacter* (Onyenwoke 2007).

Literature reports cells from *T. pseudethanolicus* 39E^T form round mother-cells with distending drumstick shaped structures on their spores (Figure 1.6) (Zeikus 1980, Lee 1993). The cells are also motile and can reduce thiosulfate to H_2S (Onyenwoke 2007). *T. pseudethanolicus* can grow chemoheterotrophically with acetate in the presence of insoluble iron (III) oxides with a doubling time of 1.25 h (Onyenwoke 2007, Roh 2002). It also grows fermentatively, producing H_2 from xylose and glucose, and ethanol from glucose (He 2009, Hniman 2011). Use of pure culture *T. pseudethanolicus* (ATCC 33223) has not previously been reported in MXCs, but is an ideal candidate for use as an ARB due to its ability to respire insoluble metals and efficiently ferment complex reduced organics including xylose, cellobiose, starch, glucose, maltose, and sucrose into acetate (Roh 2002, Onyenwoke 2007).

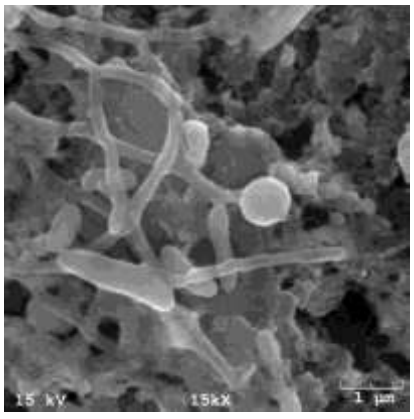

Figure 1.6: Scanning electron micrograph of *T. pseudethanolicus* grown in biofilm on an electrode. Image shows circular structure on some elongated cells while cells without circular structure appear thicker in size.

Biological Principles of Cellulose Fermentation:

Plant biomass is the most abundant form of biomass on Earth and consists of 3-30% lignin, 30-56% cellulose, and 10-27% hemicellulose (Carere 2008, Niessen 2006, Emtiazi 1999). Harnessing energy from plant biomass is difficult since the glycan polymers of which it is composed are difficult to biodegrade (Olsen 2012, Basen 2014). Since cellulose is a recalcitrant polymer, it is only susceptible to degradation from organisms containing cellulolytic enzymes, or cellulases. Bacterial organisms that have been shown to conduct cellulolytic activities are predominately Gram-positive thermophiles from the Firmicutes phylum and include members of the *Brevibacillus* genus (Liang 2009, Kato 2005), the *Clostridium* genus (Viljoen 1925, Akinosho 2014), and the *Caldicellulosiruptor* genus (Brunecky 2013). Bacterial cellulases may be associated with bacterial cell walls, exist in complex organelles called cellulosomes, or be excreted into the environment (Lynd 2002).

No bacterium is reported that is capable of cellulose fermentation and dissimilatory metal reduction; therefore, it is necessary to employ a microbial consortium consisting of both cellulolytic microorganisms and ARB that are capable of consuming cellulose derived fermentation products. Previously, thermophilic bacteria have been studied extensively for their high cellulolytic growth rate and ability to produce CO_2, H_2, ethanol, lactate, and acetate as final products of cellulose fermentation (Florenzano 1984, Freier 1988, Lynd 2002, Rydzak

2011, Niessen 2005). Although cellulolytic bacteria have been implicated to ferment cellulose in pure culture, many studies report high cellulolytic growth rates in mixed culture communities (Kato 2005, Olsen 2012, Zambare 2011). This study employs a highly enriched cellulolytic bacterial consortium including the bacteria mentioned above for the production of fermentation products that can be consumed by ARB for current production.

Thermodynamic Principles of Thermophilic Microbial Electrochemical Cells:

Figures 1.1 and 1.2 show the production of H^+ within the biofilm anode as the electron donor is oxidized. Accumulation of H^+ within the biofilm results in a decreased pH, which inhibits bacterial growth and thus lowers current production. Limitations, including H^+ diffusion, have held MECs to a maximum current production of 10-15A m^{-2} (Torres 2008). H^+ must diffuse out of the biofilm in order for the pH to remain neutral. To expedite the mass transfer of H^+ from the biofilm, a buffer is often added with a pKa within the ideal physiological range of the bacteria composing the biofilm anode. The pKa values for commonly used buffers at 30°C are: phosphate (pKa= 7.2), bicarbonate (pKa_1= 6.33) and HEPES (pKa= 7.6). Equation (1) shows how bicarbonate buffer acts to increase H^+ diffusion by working as a transporter:

(1) $HCO_3^- + H^+ \rightarrow H_2CO_3$

For this dissertation, all studies were conducted using sodium bicarbonate buffer at 60°C. Equation (2a and 2b), from Mook (1975), shows how the pKa_1 and pKa_2 of bicarbonate are affected by temperature:

(2a) $pKa_1 = \frac{3404.71}{T} + 0.032786*T - 14.8435$ (Mook 1975)

(2b) $pKa_2 = \frac{2902.39}{T} + 0.02379*T - 6.4980$ (Mook 1975)

where T = temperature (K).

From equation (2), a modest change in the pKa_1 and pKa_2 of bicarbonate occurs as temperature is increased, with increased temperature resulting in lower pKa values.

T (°C)	pKa_1	pKa_2
0	6.58	10.63
25	6.35	10.33
30	6.33	10.29
60	6.30	10.14

Table 1.1: Effect of temperature on pKa_1 and pKa_2 of bicarbonate buffered solutions.

The high temperature associated with thermophilic conditions is expected to work in concert with the buffer diffusion rate to increase the H^+ transport rate out of the biofilm, thus decreasing overpotentials. Bacteria that are in close proximity to the anode are least likely to receive electrons from an organic donor due to diffusion limitations associated with transporting electron donors through the biofilm (Marcus 2011). In addition, as electron donors are oxidized, H^+ ions are formed and must diffuse out of the biofilm (Marcus 2011). Thermophilic MXCs may be able to reduce the overpotentials associated with these diffusion limitations by increasing the rate of diffusion for ions and electron donors within the biofilm. The increased rates for diffusion at 60°C can be calculated using a simplified Einstein-Stokes equation (3):

(3) $$D_2 = D_1 * \frac{T_2 * Vis_{H_2O,2}}{T_1 * Vis_{H_2O,1}}$$

where D_2 = diffusion of ion at 60°C, D_1 = diffusion of ion at 25°C, T_2 = 333K, T_1 = 298K, $Vis_{H2O,\,2}$ = viscosity of H_2O at 60°C and $Vis_{H2O,\,1}$ = viscosity of H_2O at 25°C.

From this relationship, we can see that $D_2 = 2.153 * D_1$. This means that any ion in water at 60°C is theoretically expected to diffuse at greater than twice the rate than if it were at room temperature. Therefore, buffer and substrate diffusion in water is ~2.1x faster at 60°C than 25°C. Also important to consider is that the diffusion rate of O_2 will also be about 2x faster at 60°C which may introduce overpotentials associated with O_2 contamination.

In MXCs, the conditions of the anode should always be kept anaerobic, since O_2 contamination, measured in dissolved oxygen (DO), can have deleterious effects on cell performance, including increasing overpotentials and reducing coulumbic efficiencies (CE) (Jung 2007). Coulombic efficiency is the ratio of electrons delivered to the anode that are translated into captured electrical energy in an MFC or captured as electrical current in an MEC (Lee 2008). Aerobic conditions in the anode negatively affect the ARB in the biofilm. Some ARB are obligate anaerobes and cannot survive in the presence of O_2, which results in the loss of the biocatalyst and stops the anode reaction. Also, if the bacteria are not obligate anaerobes, they will likely favor using O_2 as their electron acceptor given its very high oxidative potential (0.75 V vs SHE at 60°C and pH 7), resulting in lower CE.

Thus, an added benefit of using thermophilic MXCs is that O_2 is approximately 35% less soluble at 60°C compared to 30°C which may decrease overpotentials caused by anodic DO

contamination. The solubility of O_2 in water can be calculated using Henry's Law constants and the Van't Hoff equation as shown in equation (4):

(4) $$K_{H,cp}(T) = K_{H,cp}(T^{\theta}) * e^{C*\frac{1}{T}-\frac{1}{T^{\theta}}}$$

where $K_{H,cp}(T)$ = Henry's Law constant of O_2 for a given concentration and pressure (mol/L*atm) at temperature (K), $K_{H,cp}(T)^{\theta}$ = Henry's Law constant of O_2 under standard concentration, pressure and temperature (K), and C = enthalpy of solution at standard temperature/ ideal gas constant.

Thermodynamic analysis reveals little change in cell potential when operating at higher temperatures. The half reaction potential (E_{an}^{0}) of acetate oxidation under standard conditions has been reported as 0.187 V vs SHE (Thauer 1977). The anode half reaction for MXCs consists of the complete oxidation of acetate in the anode to HCO_3^- and is represented in equation (5):

(5) $2HCO_3^- + 9H^+ + 8e^- \rightarrow CH_3COO^- + 3H_2O$ (Logan 2006)

However, given the operating conditions of an MFC ran at room temperature with 5 mM acetate, 5 mM bicarbonate, and a pH of 7, the E_{an}^{0} becomes -0.296 V vs SHE (Logan 2006). Temperature can be factored into this calculation via equation (6):

(6) $$E_{an} = E_{an^0} - \frac{RT}{nF} * \ln\frac{[CH_3COO^-]^a}{[HCO_3-]^b*[H^+]^c}$$

where E_{an} = theoretical operating potential of anode, R = ideal gas constant, T= temperature (K), *n* = moles of electrons (8), F = Faraday's constant, a= moles CH_3COO^- (1), b= moles HCO_3^- (2) and c= moles H^+ (9). From this equation, the actual operating potential for the oxidation of acetate

is about -0.305 V vs SHE at 30°C and about -0.353 V vs SHE at 60°C. This results in a negative shift of only 48 mV of operating potential in the acetate oxidation reaction of MFCs when comparing 60°C to 30°C.

The E_{cat}^0 of the cathodic O_2 reduction under standard conditions has been reported as 1.229 V vs SHE (Thauer 1977). The cathode half reaction for an MFC consists of the reduction of O_2 and H_2O in the anode to OH^- and is represented in equation (7):

(7) $½O_2 + H_2O + 2e^- \rightarrow 2OH^-$

However, given the operating conditions of an MFC ran at room temperature with an O_2 partial pressure of 0.2, and a pH of 7, the E_{cat}^0 becomes 0.805 V vs SHE (Logan 2006). Temperature can be factored into this calculation via equation (8):

(8) $\mathrm{E_{cat}} = \mathrm{E_{cat^0}} - \frac{\mathrm{RT}}{n\mathrm{F}} * \frac{\ln 1}{{}_{p}\mathrm{O_2} * [\mathrm{OH^-}]^{\mathrm{c}}}$ (Logan 2006)

Where E_{cat} = actual operating potential of cathode, *p* = partial pressure of O_2 (0.2 in air), O_2 = the concentration of O_2 (assumed to be 1 in an air cathode), *n* = moles of electrons (4) and c= moles OH^- (4). From this equation, the actual operating potential for the oxidation of acetate is about 0.797 V vs SHE at 30°C and about 0.754 V vs SHE at 60°C. This is a negative potential shift of only about 43 mV in the O_2 reduction reaction of the MFC when comparing 60°C to 30°C. Table 1.2 shows the thermodynamic properties for the half reaction under standard conditions, 25°C MFC, 30°C MFC and 60°C MFC conditions where E_{tot} = the total cell potential and is calculated by equation (9):

(9) $E_{tot} = E_{cat} - E_{an}$

	E_{cat} (V vs SHE)	E_{an} (v vs SHE)	E_{tot} (vs SHE)
Standard (E^0)[a]	1.229	0.187	1.042
25°C MFC[b]	0.805	-0.296	1.101
30°C MFC	0.797	-0.305	1.102
60°C MFC	0.754	-0.353	1.107

Table 1.2: Potential comparison for MFCs under standard, mesophilic and thermophilic conditions.

Anodic calculations assume 5 mM bicarbonate, 5 mM acetate, and pH = 7. Cathodic calculations assume O_2 has a partial pressure of 0.2 and a pH = 7. E_{tot} does not vary significantly with changing temperatures. [a]data from (Thauer 1977) and [b]data from (Logan 2006).

The E_{an} potential in an MEC is the same as the MFC since the same reaction occurs in the anode. Using equation (5), an E_{an} of -0.296 V vs SHE is calculated in an MEC at room temperature with 5mM bicarbonate and 5mM acetate, using a standard value for E_{an}^0 of 0.187 V vs SHE (Thauer 1977). Consult Table 1.2 for E_{an} at various temperature conditions.

The major thermodynamic distinction between and MEC and an MFC occurs in the cathode. In an MEC, the cathode is kept anaerobic in order to drive electrolysis of H_2O into H_2, as is shown in equation (10):

(10) $2H_2O + 2e^- \rightarrow H_2 + 2OH^-$

Taking the anode half reaction equation (4) and the cathode half reaction equation (9), the overall reaction for the production of H_2 in an MEC is equation (11):

(11) $CH_3COO^- + 3H_2O \rightarrow HCO_3^- + CO_2 + 4H_2$ (Logan 2008)

The Gibbs Free Energy of this reaction is ΔG_r^0 = 144.3kJ mol^{-1} with 5 mM bicarbonate and 5 mM acetate. This means that the conversion of acetate into H_2 is an endergonic process. Electrochemically, this means that a potential is applied to drive this reaction. The essential voltage that must be applied to drive the electrolysis of H_2O to H_2 can be calculated by converting the ΔG_r^0 into electrical potential via equation (12):

(12) $\mathrm{E_{tot}} = -\frac{\Delta \mathrm{G_r}}{n\mathrm{F}}$ (Logan 2008)

Where E_{tot} = the voltage which must be applied to drive hydrogen production in an MEC. Equation (12) informs us that the potential which must be applied to drive the production of hydrogen under standard conditions is equivalent to E_{tot} = -0.187 V vs SHE.

Since the E_{an} is the same as for an MFC, the E_{tot} determined in equation (9) can be used to deduce the E_{cat}. However, in a thermophilic MEC, temperature must be factored into the E_{cat} to measure its effect on E_{tot}. Under standard conditions, the E_{cat}^0 of equation (10) is 0.0 V vs SHE (Thauer 1977). For an MEC, the E_{cat} of the cathodic H_2O electrolysis under standard conditions can be determined by equation (13):

(13) $\mathrm{E_{cat}} = -\frac{\mathrm{RT}}{2\mathrm{F}} * \ln\frac{\mathrm{p_{H_2}}}{[\mathrm{H^+}]^2}$ (Logan 2008)

Where p_{H2} = partial pressure of H_2.

From equation (13), given the operating conditions of an MEC ran at 25°C with an H_2 partial pressure of 1.0 atm, and a pH of 7, the E_{cat} becomes -0.414 V vs SHE (Logan 2008). When temperature is adjusted, the E_{cat} at 30°C becomes -0.420 V vs SHE and the E_{cat} at 60°C becomes -0.462 vs SHE. Table 1.2 shows the thermodynamic properties for the half reaction under

standard, 25°C MEC, 30°C MEC, and 60°C MEC conditions where E_{tot} = the total cell potential and is calculated by equation (9).

	E_{cat} (V vs SHE)	E_{an} (V vs SHE)	E_{tot} (V vs SHE)
Standard (E^0)[a]	0.0	0.187	-0.187
25°C MEC[b]	-0.414	-0.296	-0.117
30°C MEC	-0.420	-0.305	-0.115
60°C MEC	-0.462	-0.353	-0.109

Table 1.3: Potential comparison for MECs under standard, mesophilic and thermophilic conditions.

Anodic calculations assume 5 mM bicarbonate, 5 mM acetate and pH = 7. Cathodic calculations assume H_2 has a partial pressure of 1.0 and a pH = 7. E_{tot} does not vary significantly with changing temperatures, but there may be a slightly advantageous reduction in overpotential when using thermophilic MECs. [a]data from (Thauer 1977) and [b]data from (Logan 2008).

E_{tot} is also dependent upon the pH of the anode and cathode compartments of MXCs. Through equation (6), the dependency of the E_{an} on pH can be calculated. In addition, the presence of temperature (T) in equation (6) reveals that anodic overpotential is also influenced by the temperature of the MXC. From this, a theoretical overpotential in the anode for each pH unit change can be calculated. Table 1.4 shows how many mV of overpotential are added per pH unit change at a given temperature.

T (°C)	ΔmV per pH unit
0	54.2
25	59.1
30	60.1
60	66.1

Table 1.4: Temperature dependency of mV shift in overpotential per pH unit change

A decrease in pH will contribute to overpotential in E_{an} by shifting its value more positive and thus decreasing E_{tot}. An increase in pH will make E_{an} shift negative, creating a more reductive anode reaction, and thus increasing E_{tot}. Table 1.4 shows that a change in pH in a thermophilic (60°C) MXC has a ~7.0 mV larger effect than does the same change in pH under mesophilic (25°C) conditions- about Δ1.0 mV per 5.0°C. Table 1.4 also reveals that changes in pH are one of the most significant factors in determining the energetics of E_{an} and E_{tot} in MXCs.

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