Thermophilic Microbial Electrochemical Cells

by

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ABSTRACT

Microbial Electrochemical Cell (MXC) technology harnesses the power stored in wastewater by using anode respiring bacteria (ARB) as a biofilm catalyst to convert the energy stored in waste into hydrogen or electricity. ARB, or exoelectrogens, are able to convert the chemical energy stored in wastes into electrical energy by transporting electrons extracellularly and then transferring them to an electrode. If MXC technology is to be feasible for 'real world' applications, it is essential that diverse ARB are discovered and their unique physiologies elucidated- ones which are capable of consuming a broad spectrum of wastes from different contaminated water sources.

This dissertation examines the use of Gram-positive thermophilic (60 ◦C) ARB in MXCs since very little is known regarding the behavior of these microorganisms in this setting. Here, we begin with the draft sequence of the *Thermincola ferriacetica* genome and reveal the presence of 35 multiheme c-type cytochromes. In addition, we employ electrochemical techniques including cyclic voltammetry (CV) and chronoamperometry (CA) to gain insight into the presence of multiple pathways for extracellular electron transport (EET) and current production (*j*) limitations in *T. ferriacetica* biofilms.

Next, *Thermoanaerobacter pseudethanolicus*, a fermentative ARB, is investigated for its ability to ferment pentose and hexose sugars prior to using its fermentation products, including acetate and lactate, for current production in an MXC. Using CA, current production is tracked over time with the generation and consumption of fermentation products. Using CV, the midpoint potential (E_{KA}) of the *T. pseudethanolicus* EET pathway is revealed.

Lastly, a cellulolytic microbial consortium was employed for the purpose ofassessing the feasibility of using thermophilic MXCs for the conversion of solid waste into current production. Here, a highly enriched consortium of bacteria, predominately from the Firmicutes phylum, is capable of generating current from solid cellulosic materials.

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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.

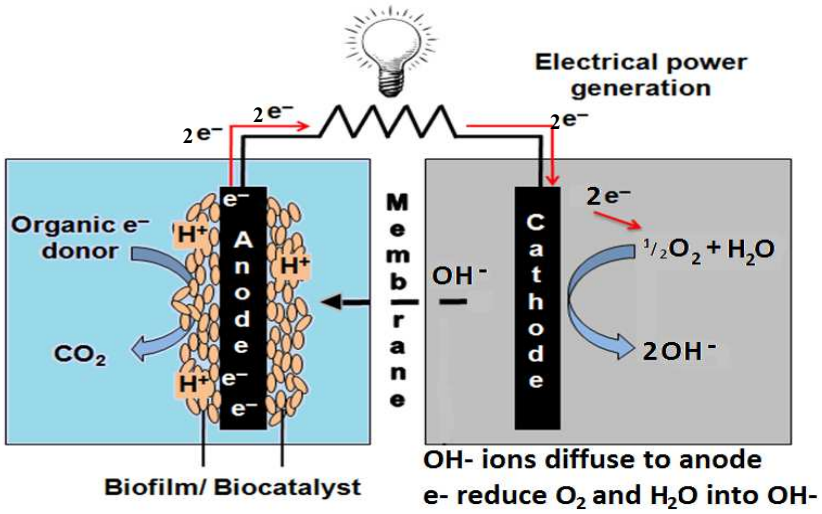

Figure 1.1: Electron flow in a typical microbial fuel cell (MFC). Adapted from Torres 2010 and equations adjusted per Popat 2012.

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.

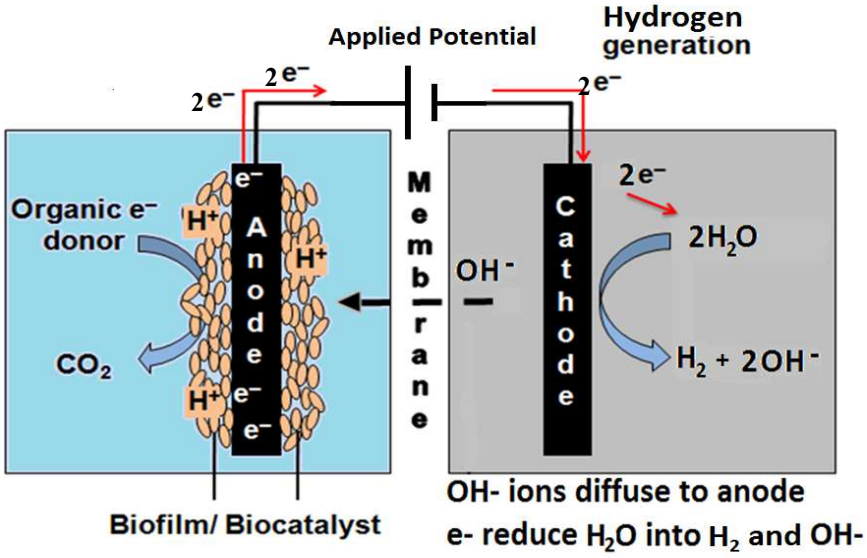

Figure 1.2: Electron flow in a typical MEC. Figure 2 adapted from Torres 2010 and equations adjusted per Popat 2012.

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.

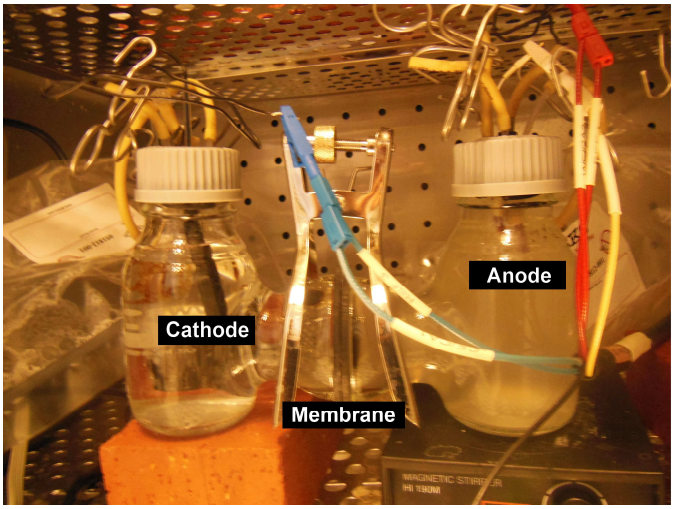

Figure 1.3: Typical lab scale H-type MEC set up.

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.

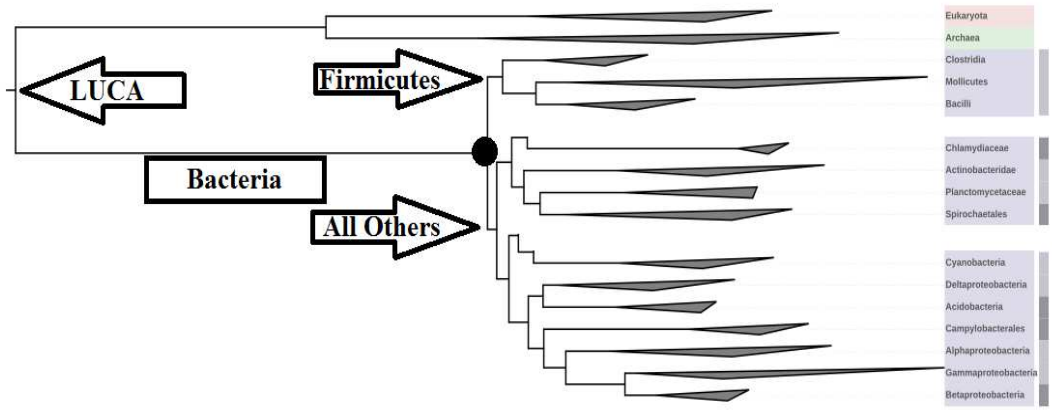

Figure 1.4: Phylogenetic tree created using iTOL 1.0. Tree shows phlogenetic lineage from the Last Universal Common Ancestor (LUCA) between Bacteria, Archaea, and Eukaryota. Black dot is last common ancestor between the Firmicutes phylum and all other bacteria phyla (Ciccarelli 2006, Letunic 2007). Figure 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 microgram 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.

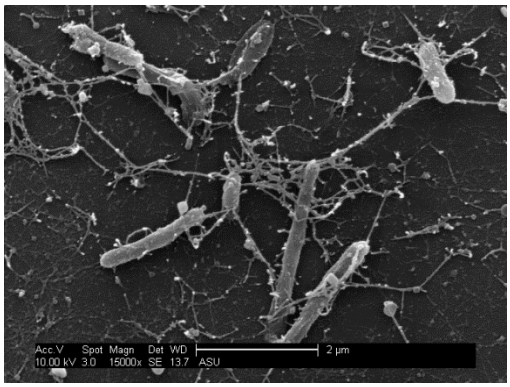

Figure 1.5: Scanning electron micrograph of *T. ferriacetica* cells on a glass slide. Image reveals rod shaped cells that have long extracellular appendages.

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).

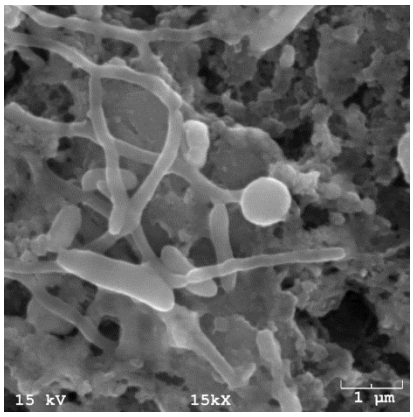

Figure 1.6: Scanning electron micrograph of *T. pseudethanolicus* grown in biofilm on an electrode. Image shows circular structure on some elongated cells while cells without circular structure appear thicker in size.

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) $\frac{1}{2}O_2 + H_2O + 2e^- \rightarrow 2OH^-$

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) $E_{cat} = E_{cat^0} - \frac{RT}{nF} * \frac{\ln 1}{p_{O_2} * [OH^-]^c}$ (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 $\Delta G_r^0 = 144.3 kJ\ mol^{-1}$ 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) $E_{tot} = -\frac{\Delta G_r}{nF}$ (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) $E_{cat} = -\frac{RT}{2F} * \ln\frac{p_{H_2}}{[H^+]^2}$ (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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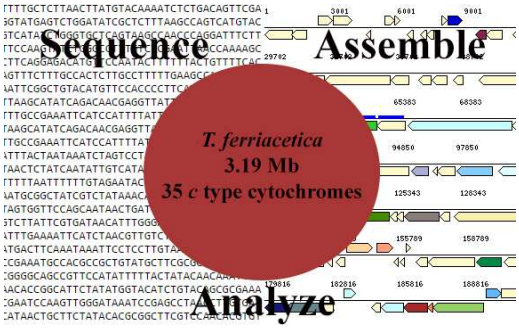
Chapter 2: Draft Genome of the Gram-Positive Thermophilic Iron Reducer *Thermincola ferriacetica* strain Z-0001[T1]

Overview

A 3.19-Mbp draft genome of the Gram-positive thermophilic iron-reducing *Firmicutes* isolate from the *Peptococcaceae* family, *Thermincola ferriacetica* Z-0001 was assembled at ~100x coverage from 100-bp paired-end Illumina reads. The draft genome contains 3,274 predicted genes (3,187 protein coding genes) and putative multiheme *c*-type cytochromes.



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Results

Thermincola ferriacetica strain Z-0001 (DSM 14005), first isolated from a terrestrial hydrothermal spring on Kunashir Island (Kurils) (Zavarzina 2007), is a Gram-positive, thermophilic (45-70 °C), spore-forming bacterium that is capable of dissimilatory metal reduction and anode respiration in a microbial electrochemical cell (MXC) (Marshall 2009, Mathis 2008, Parameswaran 2013), and it is one of only a limited number of sequenced Gram-positive thermophilic bacteria that has been documented to perform extracellular electron transfer (EET) to insoluble metal substrates (Byrne-Bailey 2010, Roh 2002, Wrighton 2008). Strain Z-0001 is capable of organotrophic growth with acetate and other organic compounds while reducing extracellular electron acceptors including amorphous Fe(III)-hydroxide, magnetite, Mn(IV), anthraquinone-2,6-disulfonate (AQDS), and anodes in MXCs (Marhsall 2009, Parameswaran 2013, Zavarzina 2007). Strain Z-0001 is also capable of chemolithoautotrophic growth using molecular hydrogen as the electron donor and Fe(III) as the electron acceptor (Zavarzina 2007). In addition, strain Z-0001 is able to produce H_2 and CO_2 while using CO as its electron donor and acquiring its carbon from acetate (Zavarzina 2007).

Among Gram-positive bacteria, little is known regarding the mechanism for EET or how the peptidoglycan layer impacts this pathway (Beveridge 1982, Beveridge and Murray 1980, Ehrlich 2008). Direct contact-dependent electron transfer has been suggested in *Thermincola potens* JR (Wrighton 2011) with genetic evidence for the presence of *c*-type cytochromes (Carlson 2012), proteins that are responsible for EET in other metal-reducing bacteria (Leang 2003). In contrast to *T. potens*, *T. ferriacetica* strain

Z-0001 has been suggested to transfer electrons long range via an extracellular matrix (Parameswaran 2013), suggesting it may encode additional electron transfer capabilities. T. *ferriacetica* has been reported to produce current densities up to 10 A m^{-2} despite having only half the cytochrome repertoire of *Geobacter sulfurreducens* (Methé 2003, Parameswaran 2013). Further genetic comparison of these strains could help elucidate the EET mechanism(s) of strain Z-0001.

The draft assembly presented here is from an axenic culture of electrode-grown *T. ferriacetica* strain Z-0001 cells in order to eliminate contamination by iron or AQDS. gDNA was collected and sequenced on an Illumina HiSeq 2000 lane, yielding > 45 million 2x100 bp reads. Raw reads were trimmed (sliding window 3 until quality > 28) and down-sampled to provide 100x coverage for assembly using the a5 pipeline (March 26, 2013 release, Tritt 2012). The 3,196,047-bp draft genome assembly yielded 53 contigs > 500 bp with an N_{50} of 112112 bp, an L_{50} of 8, and a G+C content of 45.69%.

The draft assembly was annotated using the JGI IMG/ER pipeline, yielding 51 tRNAs, 3,274 predicted genes (3,187 predicted protein coding genes), and 35 *c*-type cytochromes with three or more heme (CXXCH) binding motifs- including a 222.67 kDa *c*-type cytochrome containing 58 heme binding motifs. Table 2.1 displays all 35 putative *c*-type cytochromes. BLASTN sequence analysis of its 16S rRNA gene revealed 99.9% (1436/1438 nt) identity with *T. potens* JR and 99.7% (1399/1403 nt) identity with *Thermincola carboxydophila* (Byrne-Bailey 2010, Sokolova 2005). *T. ferriacetica* contains two multiheme *c*-type cytochromes and 429 genes that are not present in *T. potens*. However, based on an average nucleotide identity (ANI) of 98.3% between their

genomes, these two organisms may be members of the same species (Richter and Rosselló-Móra 2009).

Nucleotide Sequence Accession Number

This Whole Genome Shotgun project for *T. ferriacetica* strain Z-0001 was deposited at DDBJ/EMBL/GenBank under the accession LGTE00000000. The version described in this paper is version LGTE01000000. The raw and adaptor trimmed Illumina reads were submitted to SRA under accession SRX1100231.

Name	CDS (within Contig)	Heme motifs	Size (AA)	Size (kDa)
Tfer_0004_putative multiheme cytochrome c	5943→6977	10	344	39.19
Tfer_0070_putative multiheme cytochrome c	67185→68231	10	348	38.58
Tfer_0071_NapC/NirT cytochrome c domain protein	68315→69538	12	407	45.15
Tfer_0072_putative multiheme cytochrome c	69944→71470	17	508	57.33
Tfer_0075_putative multiheme cytochrome c	73143→74204	6	353	37.54
Tfer_0076_putative multiheme cytochrome c	74486→76330	15	614	66.86
Tfer_0077_putative multiheme cytochrome c	76721→83047	58	2108	222.67
Tfer_0082_putative multiheme cytochrome c	89349→91238	11	629	68.14
Tfer_0155_NapC/NirT cytochrome c domain-containing protein	157890→158333	4	147	16.70
Tfer_0156_ammonia-forming nitrite reductase	158416→159660	4	414	46.74
Tfer_0401_putative multiheme cytochrome c	63064→64845	10	593	65.21
Tfer_0712_putative multiheme cytochrome c	89029→93423	17	1464	158.72
Tfer_0719_putative multiheme cytochrome c	106821→110489	10	1222	131.33
Tfer_0721_putative multiheme cytochrome c	111354→113222	8	622	68.34
Tfer_0722_putative multiheme cytochrome c	113394→115028	6	544	58.60
Tfer_0725_NapC/NirT cytochrome c domain-containing protein	116864→118042	12	392	43.95
Tfer_0972_putative multiheme cytochrome c	119742→120392	3	216	24.34
Tfer_1028_putative multiheme cytochrome c	17876→19324	6	482	51.26

Tfer_1031_NapC/NirT cytochrome c domain-containing protein	21342→22505	12	387	43.48
Tfer_1797_methyl-accepting chemotaxis sensory transducer/ putative triheme cytochrome c	49319→50038	3	239	26.52
Tfer_1887_putative multiheme cytochrome c	49123→50214	10	363	40.63
Tfer_1988_putative multiheme cytochrome c	33118→34446	6	442	47.85
Tfer_1990_putative multiheme cytochrome c	35489→38845	6	1118	119.03
Tfer_1992_putative multiheme cytochrome c	39794→44566	15	1590	171.18
Tfer_2063_putative multiheme cytochrome c	14274→23423	45	3049	335.29
Tfer_2064_putative multiheme cytochrome c	23444→25891	6	815	89.97
Tfer_2066_putative multiheme cytochrome c	26970→28184	6	404	43.18
Tfer_2149_putative multiheme cytochrome c	30081→31427	15	448	51.09
Tfer_2153_putative multiheme cytochrome c	33759→34952	7	397	41.31
Tfer_2155_putative multiheme cytochrome c	35822→37108	7	428	45.36
Tfer_2210_NapC/NirT family cyctochrome c	13953→14465	5	170	18.84
Tfer_2544_chaperone protein DnaJ/putative triheme cytochrome c	17771→18904	3	377	41.23
Tfer_2880_putative multiheme cytochrome c	13229→14152	6	307	32.08
Tfer_3001_putative multiheme cytochrome c	32868→33416	5	182	20.97
Tfer_3193_putative multiheme cytochrome c	7756→9333	9	525	56.58

Table 2.1: Putative multiheme *c*-type cytochromes for *T. ferriacetica* are listed. Included

are: the protein name, the coding DNA sequence (CDS) location for each protein within its contig, and the number of heme motifs, amino acids, and kDa for each protein.

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Chapter 3: The Effect of pH and Buffer Concentration on Anode Biofilms of *Thermincola ferriacetica*[2]

Overview

We assessed the effects of pH and buffer concentration on growth and current production in biofilms of *Thermincola ferriacetica* – a thermophilic, Gram-positive, anode-respiring bacterium (ARB) – grown on anodes poised at a potential of -0.06 V vs. SHE in microbial electrolysis cells (MECs) at 60 °C. Maximum current density in biofilms grown with 10 mM bicarbonate buffer was 6.8±1.1 A m^{-2}. Increasing bicarbonate buffer concentrations from 10 mM to 100 mM resulted in an increase in the current density by 40±6% to 11.2±2.7 A m^{-2}. Adjusting pH between 5.2 and 8.3 resulted in an increase in j, although with a smaller increase in j from pH 7.0 to 8.3, suggesting inhibition in current production at low pH. Confocal laser scanning microscopy (CLSM) images indicated that higher bicarbonate buffer concentrations resulted in larger biofilm thicknesses (L_f), from 68±20 μm at 10 mM bicarbonate to >150 μm at 100 mM, suggesting that H^+ diffusion rates have a strong influence on L_f. Thus, *T. ferriacetica* follows a similar pattern to *G. sulfurreducens* biofilms, being inhibited by low pH and low buffer concentrations. However in comparison, the faster transport rates at higher temperatures and the ability to grow at lower pH values than *G. sulfurreducens* allows *T. ferriacetica* to produce higher current densities with lower buffer concentrations, making it an attractive alternative for low alkalinity wastewater applications.

[2] Chapter 3 unpublished with Bradley G. Lusk, Prathap Parameswaran, Sudeep Popat, Bruce E. Rittmann, Cesar I. Torres

Introduction

The limitation(s) to microbial respiration during growth on solid electron acceptors or electrodes in microbial electrochemical cells (MXCs) have been studied extensively with Gram-negative mesophilic anode-respiring bacteria (ARB) (Marcus 2007, Marcus 2011, Torres 2008a-b). However, such limitations for Gram-positive thermophilic ARB have not been studied in detail yet (Pham 2008**,** Ehrlich 2008, Wrighton 2012, Parameswaran 2013). Broadening this understanding for thermophilic ARB can reveal the difference in kinetic limitations encountered in thermophilic biofilms versus the mesophilic counterparts, possibly enhancing the feasibility of using MXC technology to produce valuable products from diverse waste streams (Beveridge and Murray 1980, Beveridge 1982, Ehrlich 2008, Mathis 2008, US Patent No. 20090017512). In this study, our aim was to determine proton (H^+) transport limitations present in biofilms of the Gram-positive thermophilic ARB *Thermincola ferriacetica*, a previously characterized (Zavarzina 2007) ARB that has been reported to perform anode respiration using a non-shuttling, direct long-range extracellular electron transfer (EET) mechanism (Marshall and May 2009, Parameswaran 2013).

Bacteria that perform direct, long-range EET in MXCs develop biofilms on the anode. As these biofilms grow and mature, bacteria form layers resulting in biofilms with thicknesses of >100 μm (Parameswaran 2013, Reguera 2006, Robuschi 2013). As bacteria in the biofilm consume the electron donor, they produce electrons, which result in current production, and protons (H^+), which need to diffuse out of the biofilm into the

bulk solution (Torres 2008b). In mature biofilms, multiple layers of active bacteria produce H^+, creating a pH gradient that becomes a major limiting factor for current production by ARB and biofilm growth (Marcus 2011, Torres 2008). This phenomenon has been well characterized, and to limit the inhibition caused by pH gradients within the biofilm, a buffer is be added to aid the transport of H^+ out of the anode biofilm (Marcus 2011, Torres 2008). The equation below shows how H^+ can be carried out of the biofilm by transporting bicarbonate in, which protonates to produce CO_2, which is then transported out of the system. The use of a buffer is advantageous over simple proton transport due to the low concentration of protons (and, thus, low fluxes) at neutral pH, which is required for growth of most known ARB that produce high current densities.

$HCO_3^- + H^+ \rightarrow H_2CO_3 \rightarrow CO_2 + H_2O$

All previous studies analyzing pH inhibition in anode biofilms have been performed with mesophilic Gram-negative bacteria. Based on the Einstein-Stokes equation, the rate of H^+ or buffer transport via diffusion will be >2x higher at 60 ºC when compared to mesophilic systems at 30 ºC. Correspondingly, previous studies have indicated that *T. ferriacetica* biofilms produce higher current densities at lower buffer concentrations when compared to mesophilic anode biofilms composed of mixed or pure cultures (Parameswaran 2013). Previous investigations have also implied that pH and biofilm growth play a major role in the energetics of anode respiration by ARB (Reguera 2006, Torres 2008b, Yoho 2014).

In this study, we used chronoamperometry experiments in microbial electrolysis cells (MECs) to assess the limitations in current production caused by pH inhibition resulting from H^+ and buffer diffusion limitations in thermophilic biofilms. First, we adjusted pH with the addition of either NaOH or HCl to evaluate its effect on current production. Second, we analyzed the effect of bicarbonate buffer concentration (10, 25, 50, and 100 mM) on current production and the corresponding biofilm thickness. This study is the first to analyze H^+ transport limitations for anode respiration within a thermophilic Gram-positive ARB. We determined that H^+ diffusion is a significant limitation in thermophilic ARM biofilms that affects current production as well as biofilm thickness, although not to the same levels as observed in mesophilic ARB biofilms.

Materials and Methods

Growth Media and Culture Conditions. We used a modified DSMZ Medium 962: Thermovenabulum medium to grow *T. ferriacetica* strain 14005 (DSMZ, Braunshweig, Germany). The modified medium contained: $NaAc*3H_2O$ (3.4 g l^{-1}), NH_4Cl (0.33 g l^{-1}), K_2HPO_4 (0.33 g l^{-1}), $MgCl_2*6H_2O$ (0.33 g l^{-1}), $CaCl_2*2H_2O$ (0.1 g l^{-1}), KCl (0.33 g l^{-1}), yeast extract (0.05 g l^{-1}), 1 mL selenite−tungstate stock solution (prepared by dissolving 3 mg $Na_2SeO_3.5H_2O$, 4 mg $Na_2WO_4*2H_2O$, and 0.5g NaOH in 1 L distilled water), ATCC vitamin solution (10 ml l^{-1}), and trace elements solution (10 ml l^{-1}). The trace elements solution was composed of the following ingredients in 1 L deionized water: 1.5 g nitrilotriacetic acid, 3 g $MgSO_4*7H_2O$, 0.5 g $MnSO_4*H2O$, 1 g NaCl, 0.1g $FeSO_4*7H_2O$, 0.18 g $COSO_4*7H_2O$, 0.1 g $CaCl_2*2H_2O$, 0.18 g $ZnSO_4*7H_2O$,

0.01 g $CuSO_4*5H_2O$, 0.02 g $KAl(SO_4)_2*12H_2O$, 0.01 g H_3BO_3, 0.01 g $Na_2MoO_4*2H_2O$, 0.03 g $NiCl_2*6H_2O$, and 0.3 mg Na_2SeO_3*5H2O. We prepared media in a condenser apparatus under N_2:CO_2 (80:20) gas conditions. Media was brought to boil and allowed to boil for 15 minutes per liter. We stored media in 100 ml serum bottles and autoclaved for 15 minutes at 121 °C. We added ATCC Vitamin Solution and Na_2CO_3 added after autoclaving; Na_2CO_3 concentrations were adjusted for each experimental condition. Initial *T. ferriacetica* stock cultures were grown in 100 ml serum bottles containing 10 mM $Fe(OH)_3$ on an Excella E24 Incubator Shaker (New Brunswick Scientific) at 60 ºC and 150 RPM. Subsequent cultures were inoculated using culture grown in the laboratory.

H-Type MEC Construction for Bicarbonate and pH Experiments. We used H-type MECs for all experiments. Each MEC used consisted of two 350 ml compartments separated by an anion exchange membrane (AMI 7001, Membranes International, Glen Rock, NJ). For all MECs, the operating temperature was 60 °C. All MECs contained two cylindrical graphite anodes with varying surface areas and an Ag/AgCl reference electrode (BASi MF-2052) unless otherwise noted (Table 3.1). Reference potential conversion to a standard hydrogen electrode (SHE) was conducted by constructing a two chambered cell with one chamber containing modified DSMZ Medium 962 and the other containing 1M KCl (Parameswaran 2013, Greeley 1960). Anode potential was poised at -0.06 V vs. SHE and the current continuously monitored every two minutes using a potentiostat (Bio-Logic, Model VMP3, Oak Ridge, TN). For all MECs, the anode chambers were mixed via agitation with a magnetic stir

bar. The cathode consisted of a single cylindrical graphite rod (0.3 cm diameter and a total area of 6.67 cm^2). Cathode pH was adjusted to 12 via addition of NaOH. Gas collection bags were placed on the anode compartments to collect volatile products and on the cathode to collect hydrogen.

Effect of pH on Current Density Experiments. To determine the effect of pH on *j*, we used two replicated MECs with 50 mM bicarbonate buffer and 25 mM acetate as the electron donor. Each MEC had an anode surface area of 3.89 cm^2. At each pH condition, after achieving a steady *j*, we altered the pH by the addition of HCl or NaOH. Results were also used to obtain a pH range for current production for *T. ferriacetica* when used during operation in MECs.

Effect of Bicarbonate Buffer on Current Density Experiments. To determine the effect of bicarbonate buffer on *j*, we used four replicate MECs with varying bicarbonate concentrations of 10, 25, 50, and 100 mM and 25 mM acetate as the electron donor. We added new medium into each MEC by continuously flowing at a rate of ~4.5 mL s^{-1} until the old medium was completely replaced (~1.3 min hydraulic retention time). Bicarbonate buffer concentration of 150 mM resulted in a decreased *j*. This is due to a major limitation caused by the development of CO_2 bubbles within the biofilm that resulted in shearing (Figure 3.5). This limitation made it not possible to assess the effect(s) or other limitation(s) resulting from increasing bicarbonate buffer beyond 100 mM bicarbonate. The anode surface areas for each MEC were: 6.78, 3.26, 3.02, and 3.64 cm^2 respectively. At each buffer concentration, after achieving a steady *j*, bicarbonate

buffer was added by continuously flowing in 1 L of new medium containing 10, 25, 50, or 100 mM bicarbonate. This experiment was conducted by either starting at 10 mM bicarbonate and increasing to 100 mM, or by starting at 100 mM bicarbonate and decreasing to 10 mM (Figure 3.4).

Thickness Measurements and Microscopic Analysis. Confocal laser scanning microscopy (CSLM) was used to measure L_f per the protocol from Parameswaran (2013). The protocal in this study differed in that all confocal images were acquired using an upright Leica SP5 CSLM after applying LIVE/DEAD (BacLight Cell vitality kit, Invitrogen, USA). For measuring L_f at various bicarbonate concentrations, we used an H-type MEC with six anodes for a total surface area of 23.52 cm^2. For growth MECs, media consisted of 10 mM bicarbonate and was inoculated with scrapped biofilm from a previous MEC. After a steady current was achieved, we replaced the medium with new media containing 25, 50, and 100 mM bicarbonate. After reaching constant j at each condition, cyclic voltammetry at 1 mV s^{-1} and 10 mV s^{-1} (data not shown) was performed and an anode was sacrificed for CSLM. For imaging purposes, we place anodes in open petri dishes containing fresh media and observed them using an HCX PL APO CS 20x/0.70NA immersion objective with a working distance of 260 µm. We also performed scanning electron microscopy (SEM) on mature intact biofilms from two MECs under 50 mM bicarbonate buffer and 25 mM acetate conditions. In addition, we prepared SEM samples using scrapped biofilm on glass slides (Figure 3.8 A-D). Preparation for SEM followed the protocol from Parameswaran (2013). We used an FEI

XL-30 environmental SEM (Philips) with an accelerating voltage of 5−20 kV and a working distance of 8−10 mm.

Effect of salt and acetate on current density experiments. To confirm that *j* was not influenced by ionic concentration, a mature biofilm was grown on an anode with medium containing 50 mM bicarbonate and 25 mM acetate. The anode surface area was 1.47 cm^2. Once a constant *j* was achieved, 10, 25, and 50 mM NaCl was added to the media (Figure 3.6). To confirm that *j* was not influenced by acetate concentration, a mature biofilm was grown on an anode with media containing 50 mM bicarbonate and 25 mM acetate. The anode surface area was 1.47 cm^2. To confirm that acetate was a non-limiting factor in all experiments, acetate was added to the reactor at 25, 50, 75, and 100 mM (Figure 3.7).

Results and Discussion

Effect of pH on Current Density Produced by *T. ferriacetica* Biofilms.

To determine the role that pH plays in limiting current density (*j*), *T. ferriacetica* biofilms were grown on anodes with medium containing 50 mM bicarbonate as buffer and 25 mM acetate as electron donor. Once the biofilms produced a constant *j*, bulk pH was adjusted using either NaOH or HCl. Results were normalized vs *j* at ~pH 8.0 ($j_{pH\,8}$) since this is the pH that indicated the highest current density (14.3 A m^{-2}). Data from all biofilms (n=2) is averaged in Figure 3.1 and indicates an appreciable *j* within pH range of ~pH 5.2 – 8.3. This range is larger than those reported for mesophilic *Geobacter*

sulfurreducens biofilms, especially showing that *T. ferriacetica* biofilms can produce appreciable *j* at pH <6 (Torres 2008, Franks 2009).

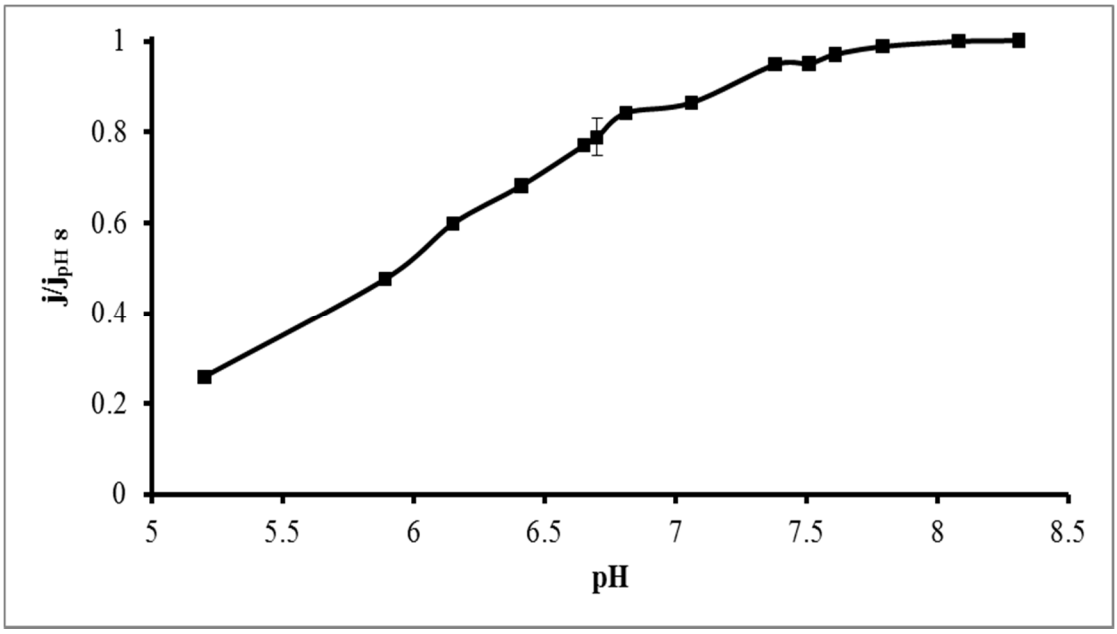

Figure 3.1: Effect of pH on current density normalized to that produced at pH 8.0 ($j_{pH\ 8}$ = 14.3 A m^{-2}).

Figure 1 also indicates that *j* increases with increasing pH – an observation similar to that for mesophilic Gram-negative biofilms (Torres 2008). *j* was the smallest at ~pH 5.2, where $j_{pH\ 5.2}$ was ~20% (2.8 A m^{-2}) of $j_{pH\ 8}$. The biofilms produced ½ $j_{pH\ 8}$ at pH ~6.0 ($j_{pH\ 6}$ = 6.5 A m^{-2}), and *j* increased consistently until ~pH 7.4, where it reached ~97% ($j_{pH\ 7.4}$ = 13.9 A m^{-2}) of the $j_{pH\ 8}$ value. The impact pH had on *j* was less significant above pH ~7.4. Given that the pKa_1 of bicarbonate is ~6.3 at 60 °C, the buffering capacity of bicarbonate in the biofilm may be less significant once bulk pH reaches pH 7.4. Higher than this pH, thermophilic ARB may have a limitation in *j* that is not directly related to

H^+ diffusion and may be the result of pH inhibition caused by basic conditions in the biofilm. pH inhibition may be observed in thermophilic ARB under high pH conditions because H^+ diffusion is significantly less limiting than in mesophilic biofilms due to the increased rate of diffusion caused by higher temperature. Losses in *j* are likely associated with pH inhibition of the biofilm caused by acidic conditions (Marcus 2011) in addition to H^+ diffusion limitations resulting from the buffering capacity of bicarbonate.

To monitor the impact of buffer concentrations on *j*, biofilms were grown on anodes with media containing 25 mM acetate and varying bicarbonate buffer concentrations. Biofilms were first grown with medium containing 10 mM bicarbonate buffer and then replaced with new media containing progressively 25 mM, 50 mM, or 100 mM bicarbonate buffer. Each data point was acquired when the biofilms produced a stable *j* (shown in Figure 3.2). *j* was normalized to $j_{100\ mM\ bicarbonate}$ – the current density of each biofilm at 100 mM bicarbonate (for *j* values, see Table 3.1). The *j* observed at 10 mM bicarbonate was ~61±6%, 25 mM was ~80±6%, and 50 mM was ~92±6% of $j_{100\ mM\ bicarbonate}$.

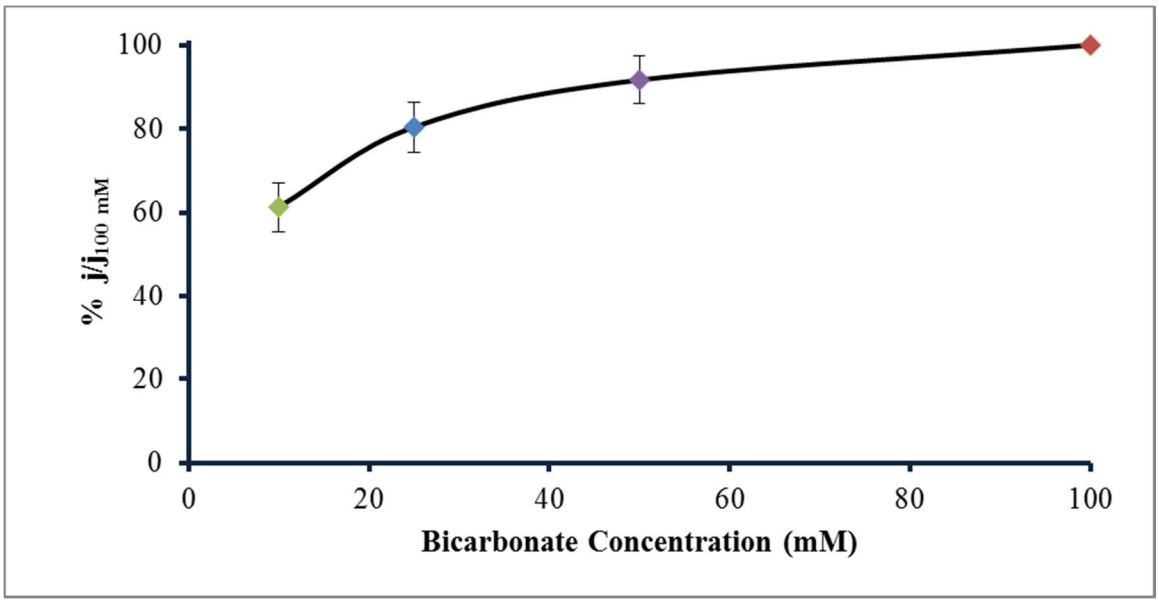

Figure 3.2: Effect of bicarbonate buffer concentration on current density as normalized to that produced with 100 mM bicarbonate buffer. Green box represents 10 mM bicarbonate, blue box represents 25 mM bicarbonate, purple box represents 50 mM bicarbonate, and red box indicates 100 mM bicarbonate buffer

To determine the statistical significance of each buffer condition, a one-way analysis of variance (ANOVA) was conducted with a post-hoc Tukey honest significant difference (HSD) test (Gleason 1999). ANOVA results indicate a p-value much lower than 0.01 which indicates that one or more condition is significantly different from the other. The Tukey HSD test results indicate that *j* increases with buffer ($p<0.01$) within the 10-25 mM bicarbonate concentration (pH = 6.9-7.1), but the increase becomes less significant ($p<0.05$) in the 25 mM-50mM bicarbonate range (pH = 7.1-7.3), and insignificant in the 50 mM- 100 mM bicarbonate range (pH = 7.3-7.8). For statistical tables for Figure 3.2, see Appendix A.

Name	Temp (°C)	Potential (V vs SHE)	Anode #	Surface Area (cm^2)	j (A m^{-2}) 10 mM	25 mM	50 mM	100 mM
RXR1	60	-0.06	2	6.78	8.8	10.9	13.2	15.5
RXR2	60	-0.06	2	3.26	5.0	7.8	9.29	9.4
RXR3	60	-0.06	2	3.02	6.1	7.2	8.31	8.7
RXR4	60	-0.06	2	3.64	6.4	8.2	8.81	10.4

Table 3.1: Results from reactors used for bicarbonate buffer experiments. Different colored boxes correspond to color scheme from Figure 3.2.

These data show that, as reported in previous studies (Parameswaran 2013), *T. ferriacetica* biofilms produce higher j at 10 mM bicarbonate buffer concentrations than mesophilic ARB. This is extremely advantageous for dilute wastewaters that have low alkalinities, as higher j is achievable with this ARB over *G. sulfurreducens*. Moreover, the data reported in this study indicates that although H^+ diffusion is a major limiting factor at low buffer concentrations, it does not show a limitation at higher buffer concentrations, as j in *T. ferriacetica* biofilms does not vary beyond 50 mM bicarbonate. In comparison, *G. sulfurreducens* showed a significant increase in current densities with increasing bicarbonate concentrations at all concentrations studied (0-100 mM, Marcus 2011).

Confocal laser scanning microscopy (CLSM) was employed to quantify the impact of bicarbonate buffer concentrations on biofilm thickness (L_f) with results shown in Figure 3.3. ANOVA results indicate a p-value much lower than 0.01 which indicates

that one or more condition is significantly different from the other. The Tukey-Kramer HSD (Gleason 1999) test results indicate that L_f does not change with buffer within the 10-25 mM bicarbonate concentration range- indicating that L_f is not a major limitation- with H^+ diffusion likely as the primary limitation for j as shown in Figure 3.2. In addition, increases ($p<0.05$) were observed in the 25 mM-50mM bicarbonate range and in the 50 mM- 100 mM bicarbonate range ($p<0.05$). Higher bicarbonate buffer concentrations resulted in more varied and dynamic peaks and valleys. L_f values for live portions of the biofilm are reported as 68±20 μm at 10 mM, 57±11 μm at 25 mM, 124±58 μm at 50 mM, and 180±47 μm at 100 mM. This phenomenon has been predicted for mesophilic biofilms and is attributed to the enhanced transport of H^+ from the biofilm (Hunter 2005, Marcus 2011). For raw images, see Appendix B. For statistical tables for Figure 3.3, see Appendix C.

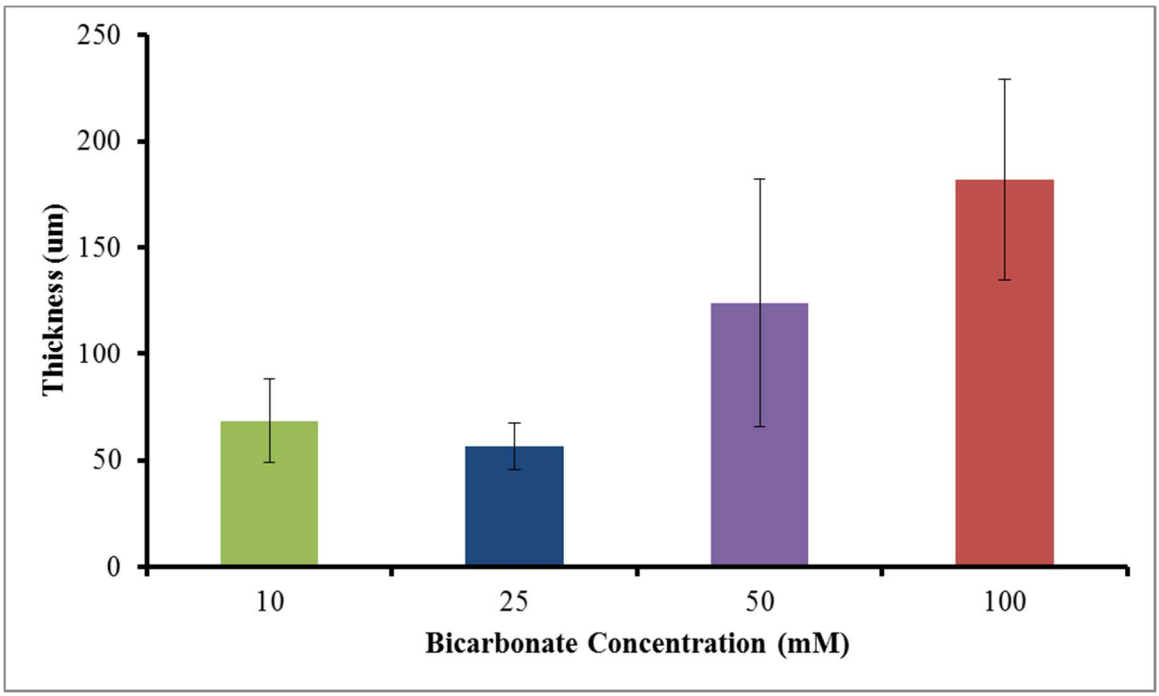

Figure 3.3: Effect of bicarbonate buffer concentration on biofilm thickness. Different colored boxes correspond to color scheme from Figure 3.2.

To determine if L_f, rather than bicarbonate buffer, was governing the j for the results shown in Figure 3.2, biofilms that had reached steady state at 100 mM bicarbonate were fed new media with gradually decreasing bicarbonate buffer concentrations (100 mM, then 50 mM, 25 mM, and 10 mM) (Figure 3.4). Results indicate that L_f had no significant impact on j at low or high buffer concentrations, but may have influenced j at 25 mM bicarbonate buffer. Similar to mesophilic *G. sulfurreducens* biofilms, *T. ferriacetica* biofilms are not limited by L_f after reaching a steady j value (Robuschi 2013). Results also indicate that H^+ diffusion is not a major limitation in mature biofilms until buffer concentration is as low as 10 mM bicarbonate (~pH 6.9) , compared to mesophilic

ARB biofilms, where 100 mM bicarbonate concentrations seem insufficient to alleviate H^+ transport limitations. This experiment also showed that limitations in *j* caused by H^+ diffusion do not appear at high buffer concentrations in mature *T. ferriacetica* biofilms in which pH > 7.3. This corroborates with the previous set of data that suggests that *j* in *T. ferriacetica* biofilms not become limited by H^+ diffusion until pH is lower than ~7.4.

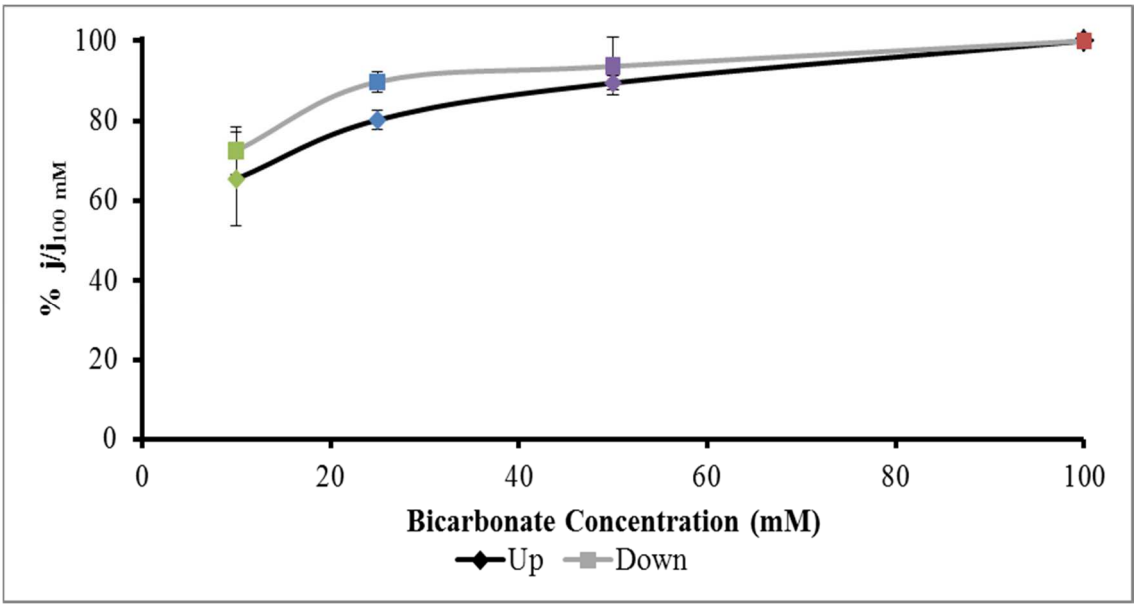

Figure 3.4: Effect of buffer concentration on *j* as normalized to 100 mM bicarbonate for both adding 10 mM bicarbonate to 100 mM (Up) and 100 mM bicarbonate to 10 mM bicarbonate buffer (Down). The graph above compares the difference in *j* for two reactors grown at 10 mM bicarbonate and 25 mM acetate. The two reactors were first grown at increasing bicarbonate concentrations: 10 mM, 25 mM, 50 mM, and then 100 mM (Figure 3.2) and then grown at decreasing bicarbonate concentrations: 100 mM, 50 mM, 25 mM, and 10 mM.

Bicarbonate buffer concentrations of 150 mM resulted in a decrease in *j* caused by the formation of CO_2 bubbles in sufficient quantities to stifle biofilm growth and cause significant sloughing. Results are shown in Figure 3.5. For this reason, it was not possible to measure the impact of bicarbonate buffer > 100 mM.

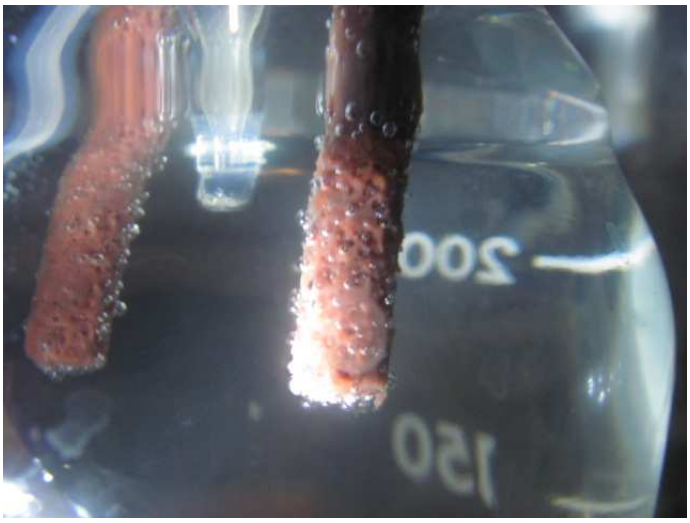

Figure 3.5: CO_2 bubbles resulting from 150 mM bicarbonate buffer.

Effect of NaCl and Acetate Concentration on Current Density in *T. ferriacetica* Biofilms.

In addition to pH and bicarbonate buffer concentration experiments, control studies were established to assess the importance of ionic strength in the biofilm via adding NaCl (Figure 3.6) and to determine if 25 mM acetate was limiting (Figure 3.7). Results indicate that as NaCl was added at various concentrations from 0 mM (9.6 A m^{-2}) to 50 mM (9.6 A m^{-2}) to biofilms grown with media containing 50 mM bicarbonate and 25 mM acetate, there was almost no change in *j*. Also, no significant change in *j* was observed as acetate concentrations were increased from 25 mM (7.9 A m^{-2}) to 100 mM

(7.5 A m^{-2}) in biofilms grown with 50 mM bicarbonate. These results reveal that the ionic strength of the media is not a major determining factor in *j* and that *T. ferriacetica* biofilms may be tolerant to slightly halophilic conditions. This was expected because we poised the anode potential and had placed the reference electrode adjacent to the anode. This resulted in negligible Ohmic losses even at the lower ionic strength. Finally, these results indicate 25 mM acetate is sufficient to be non-limiting in thermophilic biofilms of thicknesses in the range of 124±58 μm.

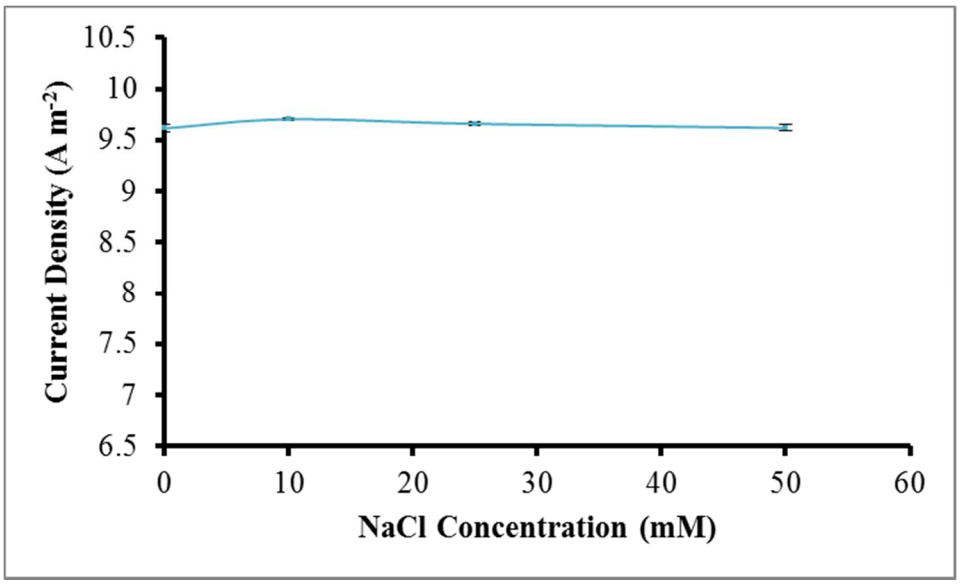

Figure 3.6: NaCl concentration shows little effect on *j*.

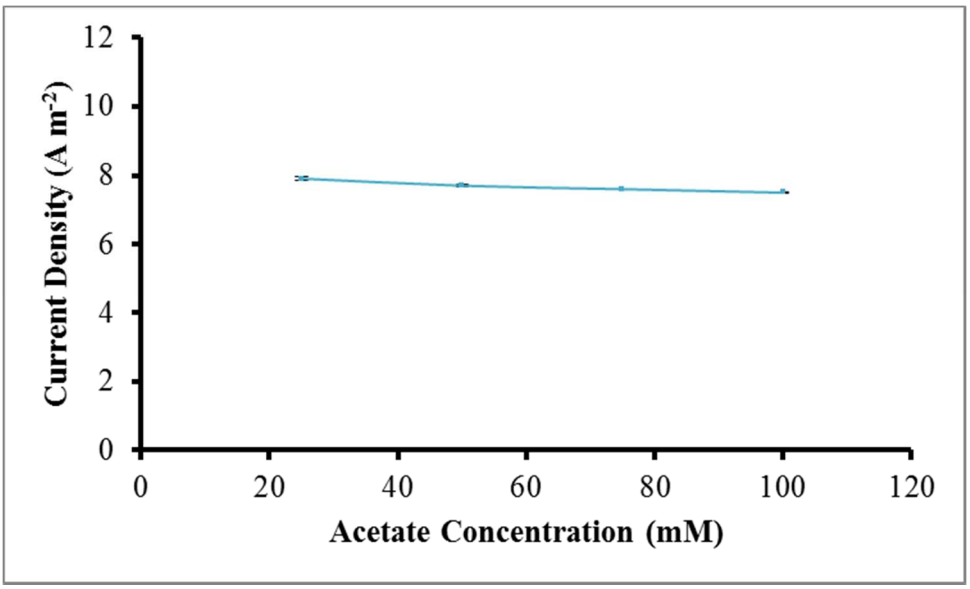

Figure 3.7: Acetate concentration shows little effect on *j*.

Scanning Electron Microscopy of *T. ferriacetica* Biofilms

SEM was conducted to evaluate the morphological characteristics of a *T. ferriacetica* biofilm grown under 50 mM bicarbonate conditions. (A) Shows a z-directional cross section of the anode at 1000X magnification with the anode highlighted with a white arrow- a thick biofilm can be observed. (B) Shows a biofilm sample that was transferred onto a glass slide and magnified at 25,000X. Appendages can be observed protruding from the cells. (C) Shows a stack of cells growing on an anode at 15,000X. (D) Shows a broad overview of the biofilm attached to the anode taken at 38X magnification.

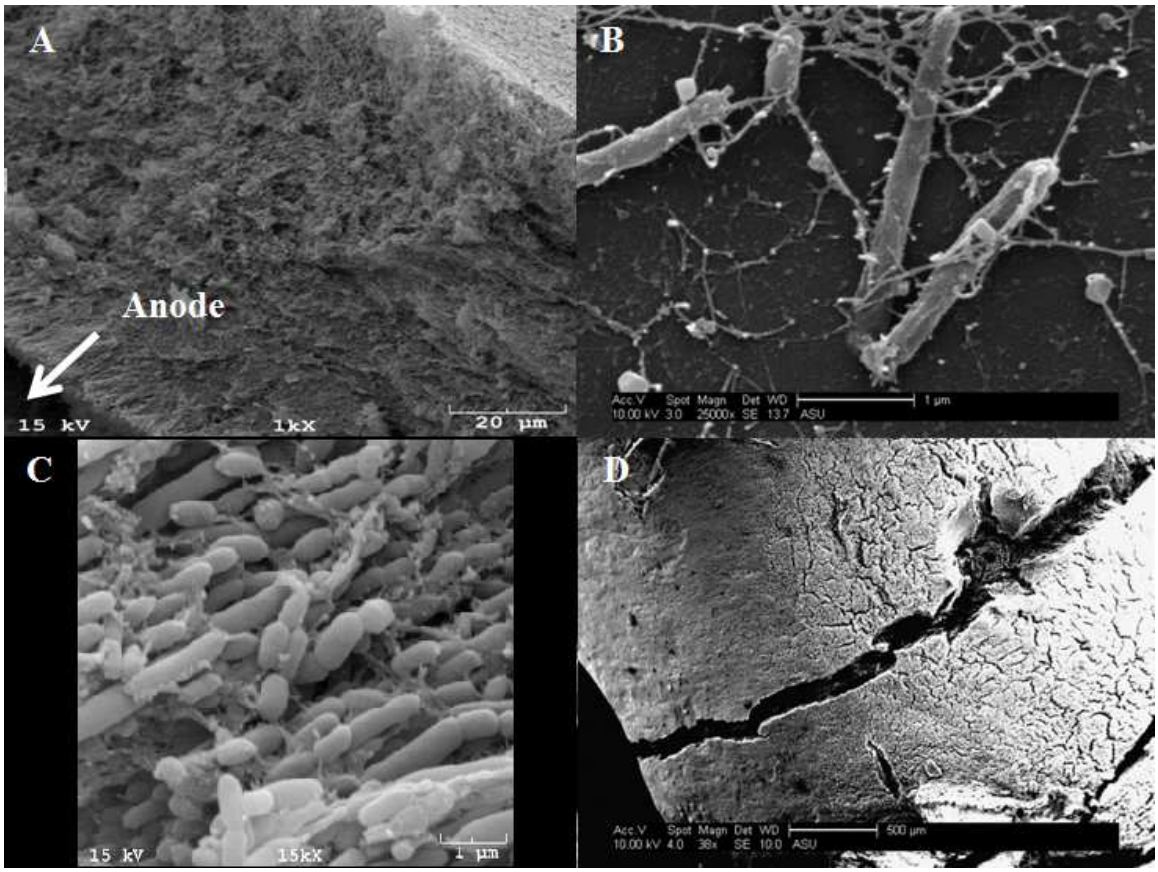

Figure 3.8A-D: Scanning Electron Micrograms for *T. ferriacetica* biofilm.

Conclusion

The impacts of bicarbonate buffer concentrations and pH on *j* in anode biofilms of *T. ferriacetica* indicate that, as with anode biofilms composed of mesophilic ARB, H^+ transport and pH inhibition are significant limiting factors up to a pH 7.4 and that L_f does not play a major role in *j* after steady state *j* is achieved. However, some important differences from *G. sulfurreducens* were identified. *T. ferriacetica* has a wider pH range, allowing current generation at lower bicarbonate buffer concentrations and pH values as

low as 5.2. This gives *T. ferriacetica* an advantage to produce higher *j* despite pH gradients in the biofilms.

Most importantly, we observe a maximum *j* that is constant at 50-100 mM buffer concentrations and pH values above ~7.4. This observation suggests that, while there is a H^+ transport limitation, this limitation is mitigated at certain conditions or does not completely govern *j*. As a consequence, a second limitation has been identified, at current densities of ~9.9 ± 2.2 A m^{-2}. It is possible that electron transport is the main limitation at higher *j*, given the extensive amount of studies that demonstrate potential gradients in *G. sulfurreducens*. Nonetheless, most conditions at which *G. sulfurreducens* is studied at fall under a H^+ transport limitation, as shown by (Marcus 2011). Thus, *T. ferriacetica* provides conditions that might be better suited for studying EET by ARB by elucidating the limitations experienced under high turnover conditions when H^+ diffusion is no longer the major limiting factor.

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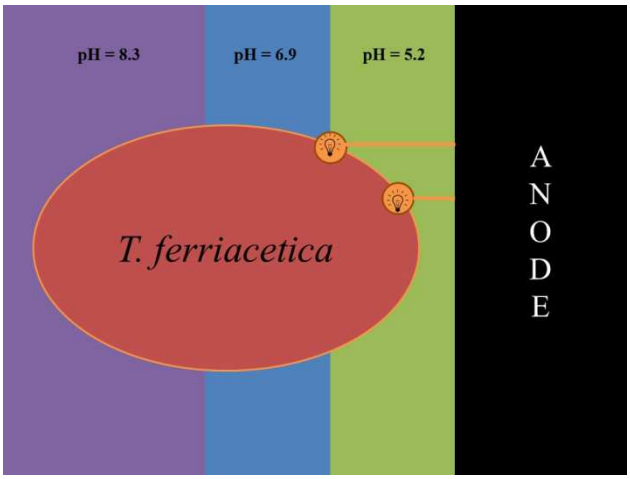
Chapter 4: pH Shifts in the Anode Potential Response from *Thermincola ferriacetica* Suggest the Presence of a Rate Limiting Proton-Coupled Electron Transfer Protein[3]

Overview

Thermincola ferriacetica, a thermophilic, Gram-positive, anode respiring bacterium (ARB) was grown in biofilms in microbial electrochemical cells (MXCs) to investigate its external electron-transport (EET) limitations. Electrochemical studies, including cyclic voltammetry (CV), are often used to elucidate the rate limiting step of electron transport in ARB. Previously reported CV analysis of *T. ferriacetica* indicated a sigmoidal Nernst-Monod response in electrical current (j) to changes in anode potential (V). This response suggests that a single proton (H^+) coupled electron (n = 1) transport reaction is responsible for the rate-limiting step in *T. ferriacetica* metabolism. The specific protein responsible for this response is thought to be a *c*-type cytochrome. Although *T. ferriacetica* has been shown to contain 35 *c*-type cytochromes, the one(s) responsible for EET has yet to be identified. Here, we show that *T. ferriacetica*'s response under certain growth conditions is composed of at least two separate n = 1 Nernst-Monod relationships; suggesting the presence of more than one pathway for anode respiration. By altering bulk pH, it is revealed that biofilms in neutral to high pH (6.9-8.3) show a very broad redox peak while biofilms in low pH (5.2) reveal multiple redox peaks. In addition, bicarbonate buffer concentrations show a similar trend, with lower bicarbonate leading to the presence of multiple redox peaks; consistent with pH gradients developing inside the *T. ferriacetica* biofilm. Finally, the redox respose is

[3]Chapter 4 unpublished with Bradley G. Lusk, Prathap Parameswaran, Sudeep Popat, Bruce E. Rittmann, Cesar I. Torres

analyzed as current density increases in *T. ferriacetica* biofilms- revealing the presence of multiple redox peaks as pH gradients form within the biofilm. This chapter reveals that *T. ferriacetica* contains more than one H^+ coupled EET pathway and that EET pathways within *T. ferriacetica* are sensitive to changes in bulk pH.



Introduction

Performance of electrode respiration by anode respiring bacteria (ARB) in microbial electrochemical cells (MXCs) is often monitored using a potentiostat equipped with the capability of applying an electrochemical technique known as low scan cyclic voltammetry (LSCV). LSCV analysis occurs when a potential (V) sweep is performed and the corresponding current (j) is measured in response to that V, referred to as a j-V response (Badalamenti 2013, Marsili 2010, Parameswaran 2013, Srikanth 2008, Yang 2012, Yoho 2014). With ARB, respiration occurs through electron transfer from the electron transport chain of the microorganism to the anode. A change in V at the anode alters the energy available to ARB via anode respiration, thus allowing us to monitor the metabolic energetics of redox active pathways in ARB (Fricke 2008, Yoho 2014). In this

chapter, LSCV at 1 mV s^{-1} was used to monitor the *j-V* response of *T. ferriacetica* biofilms to characterize the midpoint potentials (E_{ka}) of the redox processes occurring within anode biofilms under various pH conditions and bicarbonate buffer concentrations.

Many mechanisms for extracellular electron transfer (EET), including indirect shuttling, direct contact, and direct long-range transfer via a solid conductive matrix have been reported in Gram-negative mesophilic ARB, with little known in regard to Gram-positive ARB (Lovley 2008, Mohan 2014, Schroder 2007, Torres 2010, Parameswaran 2013). ARB that perform EET via direct contact often give a sigmoidal Nernst-Monod response with an n = 1 (Marcus 2007). This indicates that the mechanism through which EET occurs in these biofilms is a single H^+ coupled electron transport process (Torres 2010). *T. ferriacetica* has previously been indicated as an ARB capable of performing long-range direct EET via a conductive extracellular matrix with a Nernst-Monod fit of n = 1 (Marcus 2007).

It is well documented that H^+ transport within the biofilm anode is one of the primary limitations for anodic MXC performance (Torres 2008, Marcus 2011). Previous studies with *Geobacter sulfurreducens* indicate that current production in MXCs is enhanced by increasing either bicarbonate or phosphate buffer concentrations up to 100 mM- suggesting that pH gradients are the main kinetic limitation in these biofilms (Torres 2008). However, as shown in Chapter 3, *T. ferriacetica* biofilms are only limited at low bicarbonate buffer concentrations (< 50 mM) due to their lower pH limit for

growth and faster transport kinetics at high pH when compared to mesophilic *G. sulfurreducens*. The lack of H^+ transport limitations at high pH and buffer concentrations in *T. ferriacetica* biofilms is advantageous for studying electron transport kinetics in biofilm anodes.

Previous studies with mesophilic ARB have reported that redox active proteins, including multiheme *c*-type cytochromes, are primarily responsible for direct electron transfer to electrodes by ARB (Carlson 2012, Leang 2003). In addition, electrochemical analysis of many ARB, including *G. sulfurreducens* and *Geoalkalibacter ferrihydriticus*, is now revealing the presence of multiple EET pathways with Nernst-Monod responses working simultaneously to perform anode respiration (Yoho 2014, Yoho 2015). *T. ferriacetica* contains 35 multiheme *c*-type cytochromes (Lusk 2015) and thus may contain multiple pathways for EET. In this chapter, we investigate the electrochemical response of *T. ferriacetica* and its dependence on pH conditions, buffer concentrations, and current density. We observe a pH dependence of the electrochemical response of *T. ferriacetica* including the appearance of multiple redox peaks during LSCV analysis, suggesting H^+ coupled EET. We hypothesize that shifts in E_{ka} and the presence of multiple peaks during LSCV under certain conditions are the result of the expression of multiple multiheme *c*-type cytochromes in either the same pathway or multiple pathways.

Materials and Methods

Growth Media and culture conditions. Please refer to Chapter 3.

H-Type MEC Construction. Please refer to Chapter 3.

Effect of pH on Midpoint Potential Experiments. To determine the effect of pH E_{ka}, two MECs were constructed and operated using 50 mM bicarbonate buffer and 25 mM acetate as the electron donor. Each MEC had an anode surface area of 3.89 cm^2. At each pH condition, after achieving a steady j, pH was altered by either the addition of HCl or NaOH. LSCV was performed at 1.0 mV s^{-1} and 10 mV s^{-1} (Figure 4.1a-b) for various pH values to determine the effect of pH on E_{ka}.

Effect of Bicarbonate Buffer on Midpoint Potential Experiments. To determine the effect of bicarbonate buffer on E_{ka}, four MECs were constructed and operated using 10, 25, 50, and 100 mM bicarbonate buffer with 25 mM acetate as the electron donor. New medium was added by continuously flowing at a rate of ~4.5 mL s^{-1} until the old medium was completely replaced. The anode surface areas for each were: 6.78, 3.26, 3.02, and 3.64 cm^2 respectively. At each buffer condition, after achieving a steady j, LSCVs were performed at 1.0 mV s^{-1} (Figure 4.2a-b) and 10 mV s^{-1} (data not shown). After LSCV, bicarbonate buffer was added by continuously flowing in 1 L of new medium containing 10, 25, 50, or 100 mM bicarbonate. This experiment was conducted by either starting at 10 mM bicarbonate and increasing to 100 mM or by starting at 100 mM bicarbonate and decreasing to 10 mM.

Effect of Current Density on Midpoint Potential Experiments. To test whether L_f could be a determining factor in E_{ka}, an MEC was constructed using 25 mM acetate, 50 mM bicarbonate, and 3 mL inoculum. This MEC had an anode surface area of 3.89 cm^2 and was monitored over 36 hours as j increased from 1 A m^{-2} to 12.8 A m^{-2}.

LSCV was conducted at a scan rate of 1.0 mV s^{-1} (Figure 4.3a-b) and 10 mV s^{-1} (data not shown) at each gain in j of ~1 A m^{-2}. Samples were taken for pH before and after the experiment.

Non-Turnover low scan cyclic voltammetry. To analyze the presence of multiple redox peaks under non-turnover conditions, biofilms were established on anodes with media containing 50 mM bicarbonate buffer and 25 mM acetate. Anode surface areas were 3.02 and 3.64 cm^2. After a biofilm was established, the medium was replaced with new medium containing 50 mM bicarbonate buffer but no acetate. LSCVs were performed at 1.0 mV s^{-1} (Figure 4.5) and 10 mV s^{-1} (data not shown).

Results and Discussion

Effect of Direct pH Manipulation on Midpoint Potential in *T. ferriacetica* Biofilms.

To determine the effect of pH on midpoint potential (E_{KA}), low scan cyclic voltammetry (LSCV) (1.0 mV s^{-1} and 10 mV s^{-1}) was conducted under the 50 mM bicarbonate, 25 mM acetate condition as pH was adjusted. Results are standardized to the derivative value divided by the maximum derivative value (D/D_{max}). Results, shown in Figure 4.1a-b, indicate a shift in apparent E_{KA} towards negative values with increasing pH. Apparent E_{KA} values from Figure 4.1a (CV at 10 mV s^{-1}), show that an ~80 mV negative shift in E_{KA} was observed from pH 5.2 (-0.19 V vs SHE) to 8.3 (-0.27 V vs SHE). This indicates that the biofilm is receiving less energy from anode respiration as pH decreases and the theoretical potential of acetate increases (Katuri et al 2010, Logan 2006). Despite a similar pH change (~1.4 pH units) the ~60 mV E_{KA} shift between pH

5.2 and pH 6.9 (-0.25 V vs SHE) is much more pronounced than the 20 mV shift between pH 6.9 and pH 8.3.

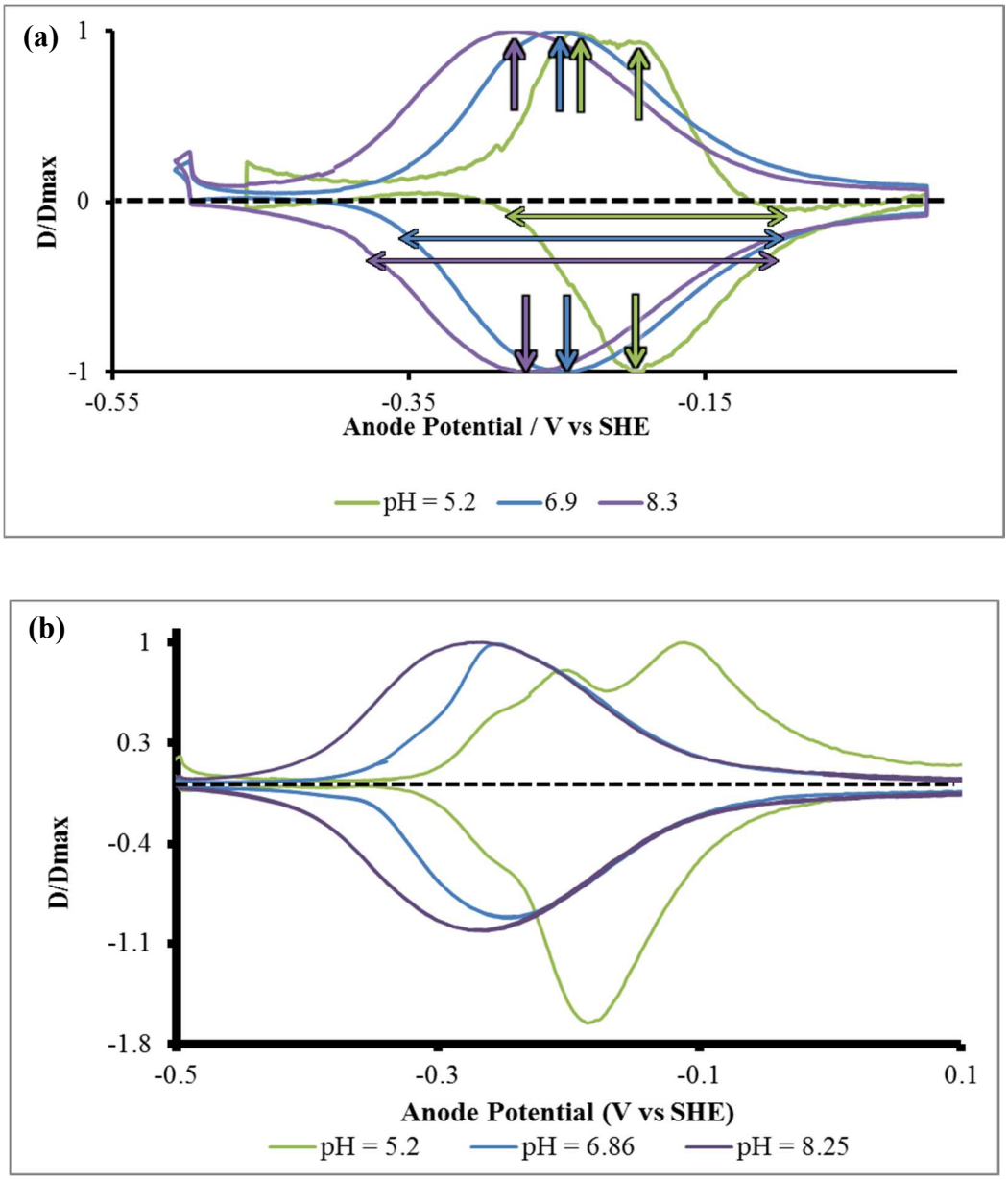


Figure 4.1a-b: (a) Derivative LSCV (10 mV s^{-1}) as a function of pH. Colored vertical arrows indicate midpoint potential(s) and colored horizontal arrows indicate range of

midpoint potential. (b) Derivative LSCV (1.0 mV s^{-1}) as a function of pH. The hashed black line in (a) and (b) differentiates the forward (top) and reverse (bottom) LSCV scans. Colors are based on pH values: green = pH 5.2, blue = pH 6.9, and purple = pH 8.3.

When comparing the E_{KA} values for the forward (above hashed black line) and reverse sweeps from Figure 4.1a (below hashed black line), the derivative plot reveals similar apparent E_{KA} values for pH 6.9 and 8.3. However, two separate peaks were observed at pH 5.2 - with E_{KA} values of -0.19 V vs SHE and -0.24 V vs SHE (E_{KA} values are indicated with colored vertical arrows). For the pH 6.9 and 8.3 conditions, a midpoint range of ~250 mV is observed. For the pH 5.2 condition, this range is closer to ~200 mV (midpoint ranges are represented with colored horizontal arrows). The observation of a larger potential range may indicate that the biofilm is using a larger sweet of cytochromes at pH values > pKa_1 of bicarbonate.

In addition, the observation of multiple peaks (including the previously reported E_{KA} for *T. ferriacetica* at -0.12 V vs SHE (Parameswaran 2013)) is emphasized in the 1.0 mV s^{-1} CV shown in Figure 4.1b, suggesting that *T. ferriacetica* biofilms may be using multiple cytochromes with non-equivalent heme groups (Katuri et al 2010) or multiple pathways for anode respiration (Yoho 2014). The presence of multiple redox peaks during various stages of biofilm development has been reported in mesophilic *G. sulfurreducens* and *Geoalkalibacter ferrihydriticus* biofilms as a result of differential expression of redox associated proteins including multiheme *c*-type cytochromes (Fricke

2008, Katuri 2010, Leang 2003, Yoho 2014, Yoho 2015). Under conditions in which pH is non-limiting, many of these pathways may be expressed and utilized simultaneously, leading to an apparent E_{KA} that is an average of several cytochromes or redox pathways. However, as pH drops, the conditions become limiting and the biofilm obtains less energy from anode respiration. This may catalyze *T. ferriacetica* biofilms to channel electrons only through pathways that are energetically favorable for these conditions. This selective pressure may have resulted in the utilization of fewer pathways by the biofilm for anode respiration - yielding a decreased midpoint range and the appearance of multiple redox peaks.

This hypothesis is supported in Figures 4.1a-b. By observing the forward CV sweep (above the black hashed line), the pH 5.2 condition reveals the presence of two distinct redox peaks. As the V is increased during the forward sweep j is driven more positive, resulting in the release of H^+ causing acidification within the biofilm and the development of a H^+ gradient. The reverse j-V response shown in Figure 4.1a-b reveals that one of the redox pathways is greatly inhibited by the acidic conditions created within the biofilm during the forward V sweep. It may also be the case that *T. ferriacetica* biofilms have only two pathways for electron transfer, and the shift in E_{KA} from altering pH makes these two pathways more distinguishable. Further studies, including the use of electrochemical impedance spectroscopy (EIS) (Yoho2014), are needed to verify this hypothesis.

Effect of Changing Bicarbonate Buffer Concentration on Midpoint Potential in *T. ferriacetica* Biofilms.

LSCVs were performed as the buffer concentration was increased to analyze if any shifts in E_{KA} were present (Figure 4.2a-b show derivative CVs for bicarbonate experiment reactors shown in Chapter 3, Figure 3.4). Looking at the reverse scan of Figure 4.2a shows that the E_{KA} exhibits a ~40 mV negative shift between 10 mM (-0.26 V vs SHE) to 100 mM bicarbonate (-0.30 V vs SHE). The 40 mV negative shift in E_{KA} produced by this experiment is the result of a pH shift from ~6.9-7.8. LSCVs were also conducted at 1 mV s^{-1} on each reactor as bicarbonate concentration was decreased (Figure 4.2b). During the course of this experiment, pH shifted from ~6.7-7.8. However, the E_{KA} shift from 100 mM bicarbonate (-0.30 V vs SHE) to 10 mM bicarbonate (-0.285 ± 0.005 V vs SHE) was ~15 ± 5 mV. These results give a clear indication that the change in E_{KA} from decreasing buffer concentration in a fully established biofilm is less pronounced than when changing from a low buffer to high buffer in a new biofilm. The decreased shift in E_{KA} that occurs while lowering bicarbonate buffer concentration is the result of decreased H^+ diffusion from the biofilm that may be due to increased L_f (> 150 μm) caused by first growing the biofilm at 100 mM bicarbonate (See Chapter 3, Figure 3.3). Under these conditions, the pH observed in the bulk is different from the pH experienced in the biofilm. For an overview of results, see Table 4.1.

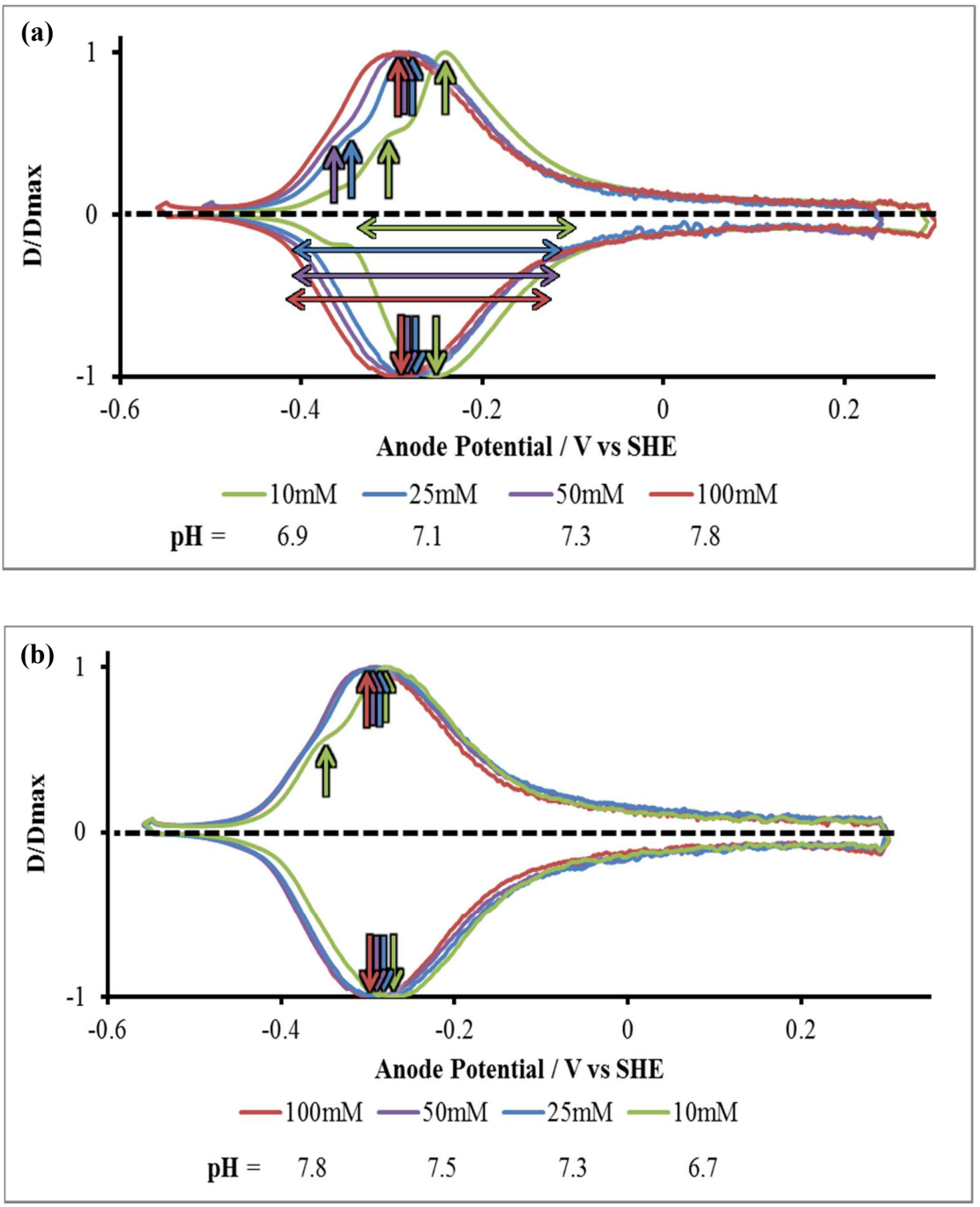


Figure 4.2a-b: Derivative LSCVs (1.0 mV s^{-1}) for increasing bicarbonate buffer conditions. (b) Derivative LSCVs (1.0 mV s^{-1}) for decreasing bicarbonate buffer conditions. Different colored lines correspond to the bicarbonate concentrations: red =

100 mM, purple = 50 mM, blue = 25 mM, and green = 10 mM. Hashed black line differentiates the forward and reverse LSCV scans. Colored vertical arrows indicate midpoint potential(s) and colored horizontal arrows indicate range of midpoint potential.

	10 mM Bicarbonate				**25 mM**			
RXR3	E_{ka}	pH	Age (d)	j (A m^{-2})	E_{ka}	pH	Age (d)	j (A m^{-2})
Up	-0.26	6.9	35	6.1	-0.28	7.2	43	7.2
Down	-0.28	7.2	64	7.1	-0.29	7.3	62	8.0
RXR4								
Up	-0.26	6.9	5	6.4	-0.28	7.1	13	8.2
Down	-0.27	6.7	31	6.8	-0.29	7.3	29	9.2

	50 mM				**100 mM**			
RXR3	E_{ka}	pH	Age (d)	j (A m^{-2})	E_{ka}	pH	Age (d)	j (A m^{-2})
Up	-0.29	7.4	46	8.3	-0.30	7.8	59	8.7
Down	-0.30	7.7	61	8.1	-0.30	7.8	59	8.7
RXR4								
Up	-0.28	7.3	19	8.8	-0.30	7.8	26	10.4
Down	-0.30	7.5	28	9.9	-0.30	7.8	26	10.4

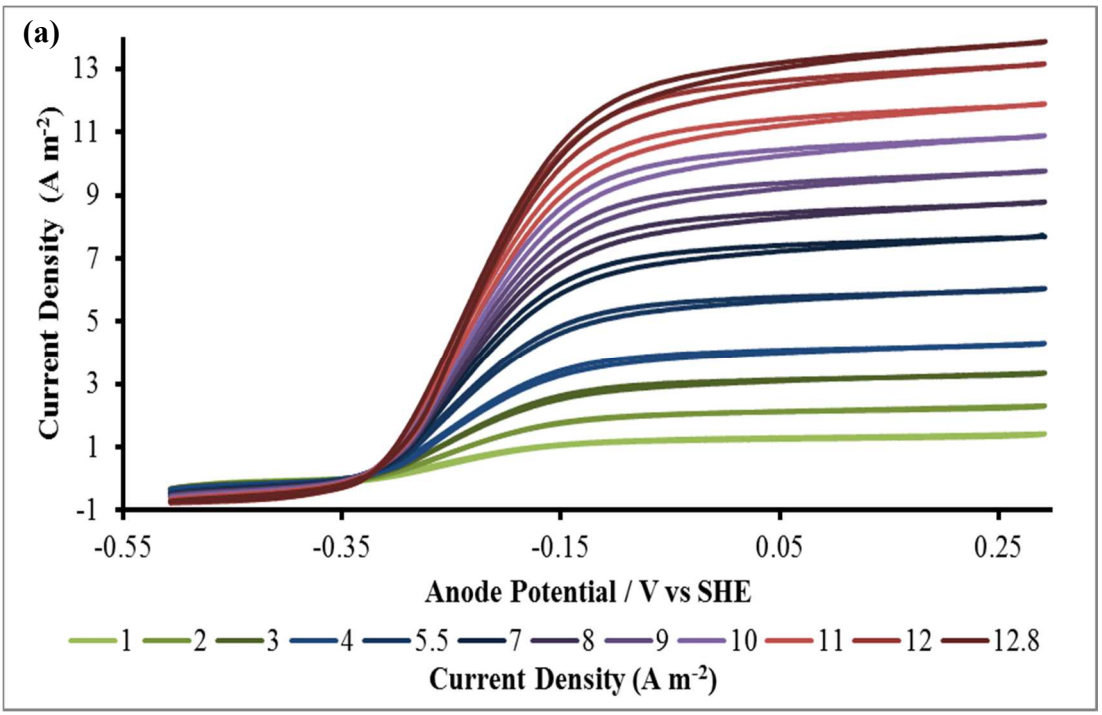
Table 4.1: List of values for reactors used in midpoint experiments and increasing (Up) vs decreasing (Down) bicarbonate buffer concentration experiments. Different colored boxes correspond to color scheme from Figure 4.2a-b.

Figure 4.2a shows that multiple peaks are present at 10, 25, and 50 mM bicarbonate buffer concentrations. These multiple peaks are present, but less pronounced when reducing buffer concentration from 100 mM to 10 mM bicarbonate (Figure 4.2b). The range of the E_{KA} at 100 mM bicarbonate buffer is ~250 mV, a similar range to that

observed for high pH (pH ≥ 6.9) conditions in the previous experiments. The E_{KA} range for the 10 mM bicarbonate buffer is ~225 mV, with lower pH again corresponding to a decreased midpoint range. The presence of multiple E_{KA} values at low buffer concentrations in *T. ferriacetica* biofilms is due to differences in pH as discussed previously. As in the pH experiments, lower buffer is associated with lower *j* as a result of pH inhibition of the biofilm and the increased potential of acetate which limits the pathways through which the biofilm can utilize acetate for cellular metabolism.

Effect of Current Density on Midpoint Potential in Growing *T. ferriacetica* Biofilms.

The effect of *j* on the E_{KA} during early development in *T. ferriacetica* biofilms was assessed at 25 mM acetate as electron donor and 50 mM bicarbonate buffer conditions since these concentrations were shown to be non-limiting in Chapter 3, Figures 3.6 and 3.7. For this experiment, *j* was monitored over ~36 hours as it increased from 0 A m^{-2} to 12.8 A m^{-2}. LSCV analysis was conducted at a scan rate of 1.0 mV s^{-1} (results shown in Figure 4.3a) at each data point. The derivative results are shown in Figure 4.3b to reveal shifts in E_{KA}. Over the course of this experiment, there was a modest 10 mV positive shift in E_{KA} as pH decreased from ~7.4 (-0.25 V vs SHE) to ~7.0 (-0.24 V vs SHE). This reveals that, similar to *G. sulfurreducens* biofilms (Yoho 2014), increased *j* results in changing E_{KA} values. However, contrary to mesophilic biofilms, the E_{KA} values *T. ferriacetica* biofilms shift more positive with increased *j*, with this shift attributed to changes in pH. Figure 4.3b shows that *T. ferriacetica* biofilms contain multiple redox peaks over a range of ~250 mV during early stages of development, with multiple peaks becoming more pronounced as pH decreases.



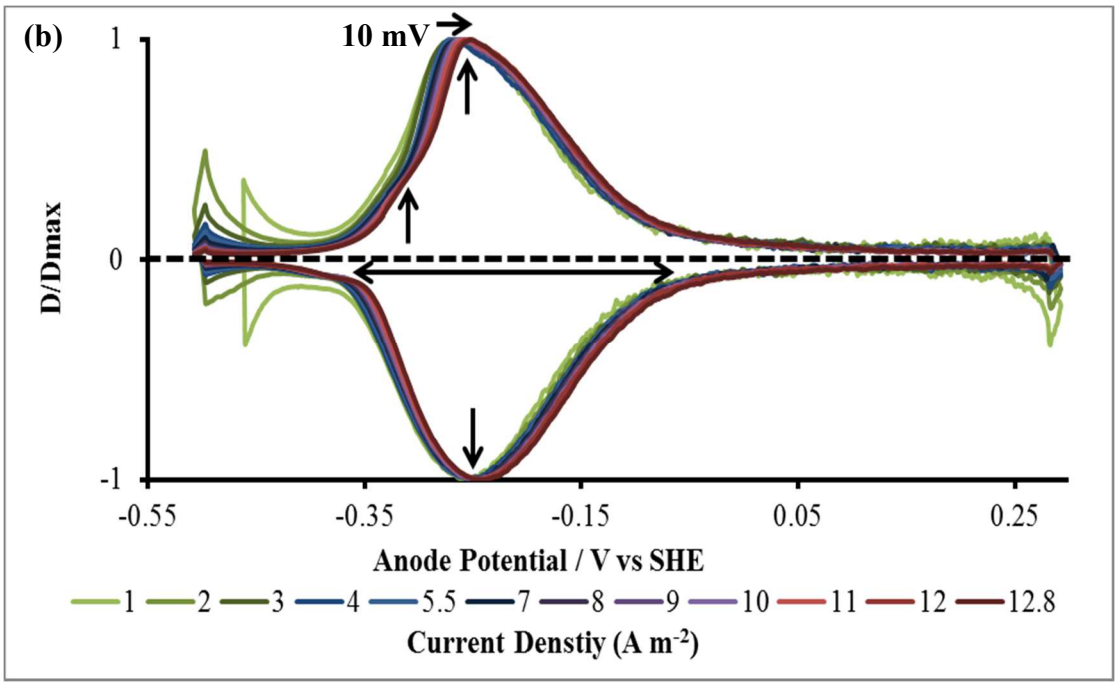


Figure 4.3a-b: (a) Effect of current density on E_{KA} in growing biofilm. (b) Derivative LSCV (1.0 mV s^{-1}) for effect of current density on E_{KA} in growing biofilm. Black vertical

arrows indicate midpoint potential(s) and black horizontal arrows indicate range of midpoint potential. Black arrow on top of graph indicates the 10 mV negative shift during the course of the experiment. Hashed black line differentiates the forward and reverse LSCV scans. Colors in (a) and (b) are consistent and are used to delineate the current density conditions of the LSCV scan.

Nernst-Monod Analysis of Multiple EET Pathway Hypothesis for *T. ferriacetica*.

In order to determine if *T. ferriacetica* is using two or more H^+ coupled EET pathways simultaneously, the Nernst-Monod model was eployed (Marcus 2007, Yoho 2015). Using the Nernst-Monod model, we determined that at least two EET pathways that conduct a single electron, single H^+ (n = 1) transfer are present in *T. ferriacetica* biofilms (Figure 4.4). Figure 4.4 shows the D/D_{max} value of LSCVs (1.0 mV s^{-1}) for *T. ferriacetica* biofilms that were producing 11 and 12 A m^{-2} at pH ~7 respectively.

Figure 4.4 shows that assuming only one EET pathway with a Nernst-Monod at n = 1 (either only P1 or only P2) does not have a very good fit to the data (j = 11 A m^{-2} or j = 12 A m^{-2}) as is shown by P1 and P2. However, under the assumption that P1 and P2 (taken from the multiple peaks present in figure 4.1a-b) are working simultaneously to perform EET (red hashed line), the Nernst-Monod model shows a good fit to the data. This analysis suggests that *T. ferriacetica* is performing anode respiration with at least two EET mechanisms simultaneously.

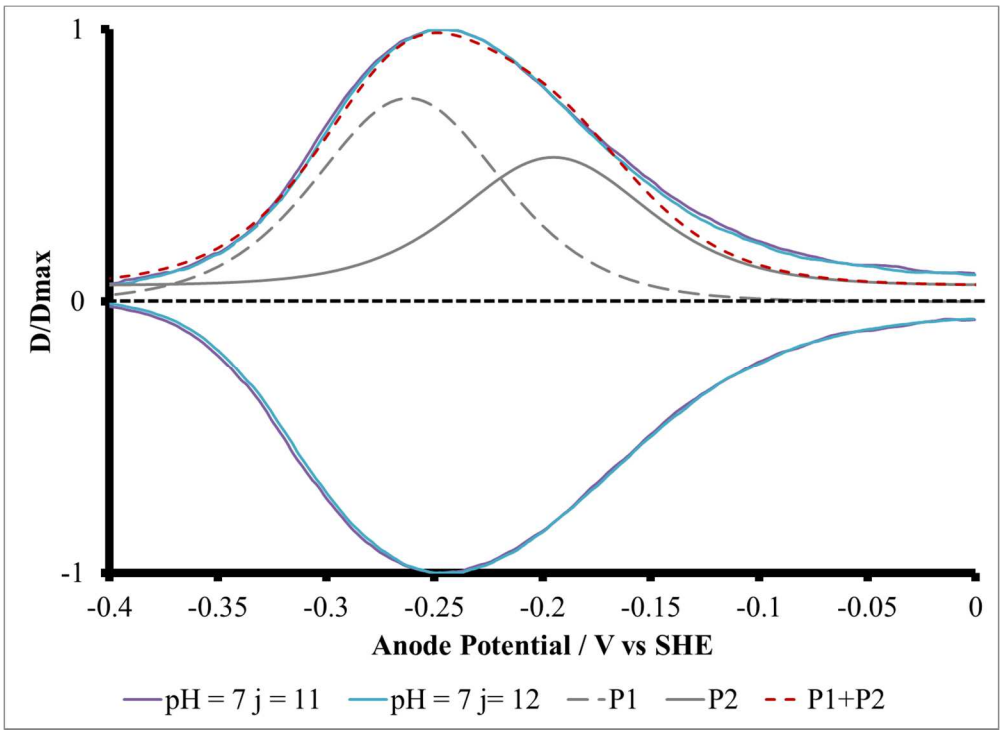


Figure 4.4: Nernst- Monod fit for D/D_{max} value of LSCVs (1.0 mV s^{-1}) for *T. ferriacetica* biofilms that were producing 11 and 12 A m^{-2} at pH ~7 respectively. Purple line = biofilm producing 11 A m^{-2}, blue line = biofilm producing 12 A m^{-2}, grey hashed line (P1) is Nernst-Monod fit for hypothesized EET pathway 1, grey solid line (P2) is Nernst-Monod fit for hypothesized EET pathway 2, and red hashed line (P1+ P2) is Nernst-Monod fit for simultaneous expression of hypothesized EET pathway 1 and 2. Hashed black line differentiates the forward and reverse LSCV scans.

Midpoint Potentials Under Non-Turnover Conditions in *T. ferriacetica* Biofilms.

LSCV under non-turnover conditions (Figure 4.5) allowed us to monitor the E_{KA} values of the redox processes occurring within a biofilm anode without the presence of acetate at pH ~7.0. For this experiment, the *j-V* response revealed the potentials at which

the electron transfer pathway(s) within the biofilm were oxidized and reduced by the electrode. Multiple redox peaks were observed over a ~250 mV potential range under non-turnover conditions. This range of redox peaks correlates with the ones observed under turnover conditions. These results indicate the possibility of either separate pathways for anode respiration or multiple redox proteins, including *c*-type cytochromes, within the same pathway (Fricke 2008, Yoho 2014). Further experiments observing potential shifts in apparent E_{KA} under non-turnover conditions will help to elucidate the importance of changing pH in the energetics of the redox processes associated with the proteins or cytochromes responsible for EET.

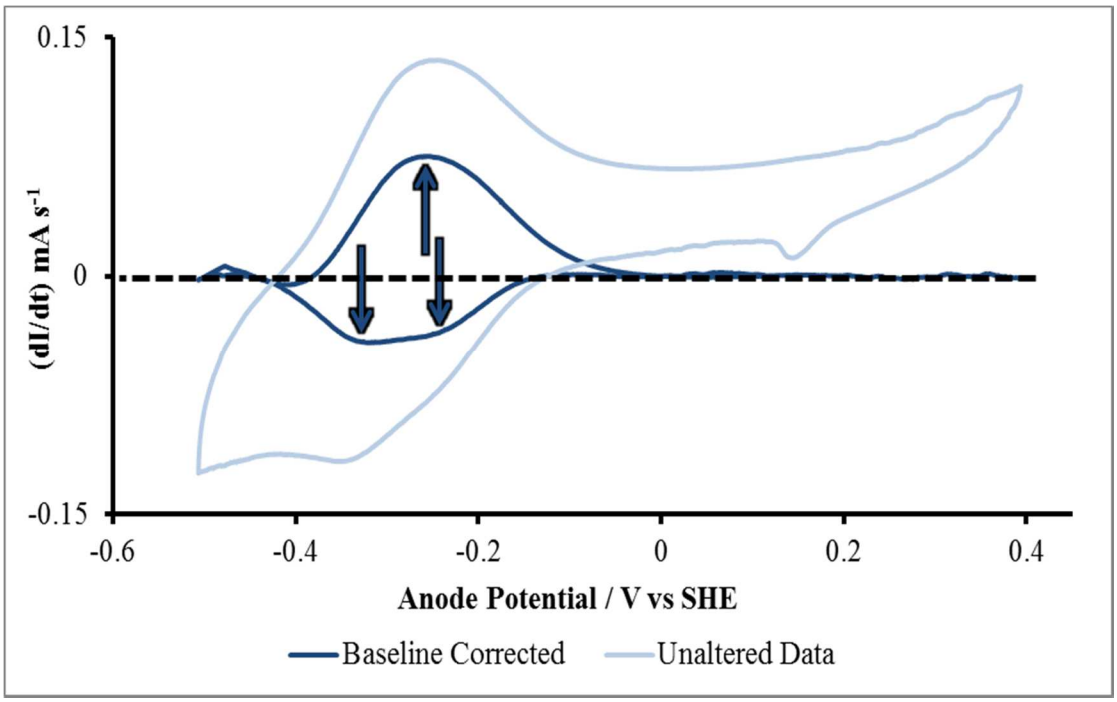


Figure 4.5: Non-Turnover LSCV (1.0 mV s^{-1}) at 50 mM bicarbonate and 0 mM acetate. Blue vertical arrows indicate midpoint potential(s). Hashed black line differentiates the forward and reverse LSCV scans.

Conclusion

For all conditions studied, pH was a major contributor to altering energetics within the biofilm anode, resulting in a shift in E_{ka} values. This suggests that, similar to mesophilic biofilms (Marcus 2011), the pH condition within *T. ferriacetica* biofilms plays a critical role in associated EET pathway(s). This phenomenon is observed due to the H^+ coupled reaction for current production in biofilms composed of ARB, in which the transfer of electrons is associated with the release of H^+. For example, increasing pH or bicarbonate buffer concentration indicated a negative shift in E_{ka}, a broadening of the

midpoint potential range, and an increase in *j*. These phenomena likely occur since these conditions enhance H^+ diffusion out of the biofilm and limit pH inhibition (Marcus 2011). However, at pH conditions <~7, the midpoint range shrinks and more distinct redox peaks appear in LSCV analysis. Although the positive shifts in E_{ka} as pH decreases may be accounted for by the theoretical ~66 mV shift in acetate potential for every pH unit, the emergence of multiple E_{ka} values and a shrinking midpoint range cannot.

Lower bicarbonate buffer concentrations (10-25 mM) and pH values (<~7) enhance the occurrence of multiple redox peaks in *T. ferriacetica* biofilms. In addition, non-turnover CV analysis indicates multiple redox peaks are present in *T. ferriacetica* that correlate with E_{ka} values observed in biofilms grown at low pH and low buffer concentrations under turnover conditions. This data suggests that *T. ferriacetica* uses multiple redox pathways or multiple redox steps for anode respiration that may be associated with *T. ferriacetica*'s repertoire of 35 *c*-type cytochromes. Little is known about the EET mechanism(s) in Gram-positive bacteria- the presence of a large peptidoglycan layer make these mechanisms even more elusive. Future studies should analyze the role peptidoglycan plays in EET and overpotential more closely to help elucidate the limitations experienced under high turnover conditions when H^+ diffusion is no longer the major limiting factor.

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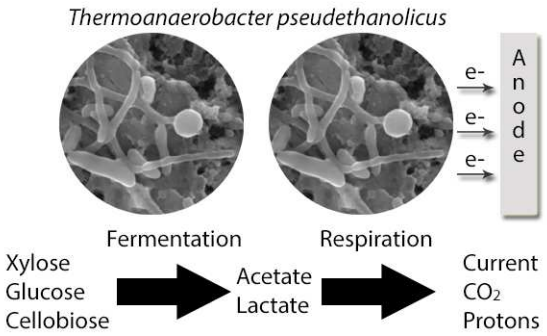
Chapter 5: Characterization of electrical current-generation capabilities from thermophilic bacterium *Thermoanaerobacter pseudethanolicus* using xylose, glucose, cellobiose, or acetate with fixed anode potentials[4]

Overview

Thermoanaerobacter pseudethanolicus 39E (ATCC 33223), a thermophilic, Fe(III)-reducing, and fermentative bacterium, was evaluated for its ability to produce current from four electron donors - xylose, glucose, cellobiose, and acetate -- with a fixed anode potential (+ 0.042 V vs. SHE) in a microbial electrochemical cell (MXC). Under thermophilic conditions (60 ºC), *T. pseudethanolicus* produced high current densities from xylose (5.8 ± 2.4 A m^{-2}), glucose (4.3 ± 1.9 A m^{-2}), and cellobiose (5.2 ± 1.6 A m^{-2}). It produced insignificant current when grown with acetate, but consumed the acetate produced from sugar fermentation to produce electrical current. Low-scan cyclic voltammetry (LSCV) revealed a sigmoidal response with a mid-point potential of -0.17 V vs SHE. Coulombic efficiency (CE) varied by electron donor, with xylose at 34.8% ± 0.7%, glucose at 65.3% ± 1.0%, and cellobiose at 27.7% ± 1.5%. Anode respiration was sustained over a pH range of 5.4-8.3, with higher current densities observed at higher pH values. Scanning electron microscopy showed a well-developed biofilm of *T. pseudethanolicus* on the anode, and confocal laser scanning microscopy demonstrated a maximum biofilm thickness (L_f) greater than ~150 μm for the glucose-fed biofilm.

[4] Chapter 5 published as: Lusk, BG, Khan, QF, Parameswaran, P, Hameed, A, Ali, N, Rittmann, BE, Torres, CI. 2015. Characterization of electrical current-generation capabilities from thermophilic bacterium Thermoanaerobacter pseudethanolicus using xylose, glucose, cellobiose, or acetate with fixed anode potentials**.** Environmental Science & Technology. Just Accepted Manuscript. DOI: 10.1021/acs.est.5b04036



Introduction

Anode-respiring bacteria (ARB) are capable of catalytically converting the chemical energy stored in organic compounds into electrical energy via extracellular respiration at an insoluble anode. In nature, these bacteria are known to reduce Fe(III) and Mn(IV) oxides (Badalamenti 2013) and potentially perform direct interspecies electron transfer (DIET) (Rotaru 2015) by using simple organic compounds (acetate, lactate) as electron donors; however, in a microbial electrochemical cell (MXC), these oxides are replaced with an anode having a set potential. Anode respiration has been achieved in over 30 metal-reducing bacterial isolates from various genera, including *Shewanella, Geobacter, Pseudomonas, Thermincola,* and *Rhodoferax*, but only a select few ARB, including *Geobacter sulfurreducens* (Bond and Lovley 2003), *Thermincola ferriacetica* (Parameswaran 2013) *Geoalkalibacter ferrihydriticus* (Badalamenti 2013)*, and Geoalkalibacter subterraneus* (Badalamenti 2013), are able to produce high current densities (*j*) through the formation of a biofilm that enables efficient extracellular electron transport (EET) (Lovley 2008) .

G. sulfurreducens and *S. oneidensis* are the two ARB most commonly studied over the past decade (Bond and Lovley 2003, Gorby 2006). Both microorganisms are mesophilic, Gram-negative, and from the Proteobacteria phylum. The identification of *Thermincola ferriacetica* and *Thermincola potens*, two ARB from the Firmicutes phylum, was an important physiological discovery, as these microorganisms are thermophilic and Gram-positive (Marshall 2009, Wrighton 2011). The discovery of new ARB is essential for the technological applications envisioned for microbial electrochemistry. For example, thermophilic ARB in the *Thermincola* family are known to consume only acetate and H_2 as electron donors (Parameswaran 2013, Marshall 2009, Carlson 2012), but conversion of organic waste streams into useful products using MXCs demand microbial communities (Mathis and May 2008) or ARB capable of producing *j* from complex organic materials, such as sugars (Bond and Lovley 2005, Luo 2013, Chaudhuri and Lovley 2003). Mesophilic Gram-negative ARB capable of converting sugars to current production in MXCs have been reported using *Geothrix fermentans* (Bond and Lovley 2005), *Tolumonas osonensis* (Luo 2013), and *Rhodoferax ferrireducens* (Chaudhuri and Lovley 2003). However, none of these studies produced more than 0.5 A m^{-2} from the sugars tested. *T. pseudethanolicus* is the first fermenter capable of producing high current densities from sugars and the most versatile in terms of the complexity of sugars utilized.

Thermophilic bacteria -- including *Thermoanaerobacter pseudethanolicus*, *Thermoanaerobacter ethanolicus, Clostridium thermocellum,* and *Clostridium thermohydrosulfuricum* -- contain thermozymes (enzymes that are stable at elevated

temperatures) capable of fermenting lignocellulosic materials or their hydrolysates into ethanol, lactate, or acetate (Mathis and May 2008, Cook 1993, Demain 2005, Roh 2002, Thomas 2014). Some of these fermentative bacteria also perform dissimilatory metal reduction (Roh 2002); thus, they may be able to convert xylose, glucose, and cellobiose into simple acids, including acetate, for consumption and current production in MXC technology. *T. pseudethanolicus* (ATCC 33223), a thermophilic, Gram-positive, rod-shaped bacterium, can grow with acetate in the presence of Fe(III) oxides and produces acetate from fermentation of xylose, glucose, and cellobiose (Roh 2002, He 2009, Hemme 2011, Hniman 2011, Onyenwoke 2007), making it an ideal candidate for use as an ARB on an anode.

In this study, we evaluated the ability of *T. pseudethanolicus* to respire to an anode while utilizing xylose, glucose, cellobiose, or acetate as an electron donor. We carried out, for the first time, detailed chemical, electrochemical, and microscopic evaluations on the *T. pseudethanolicus* biofilm anode to understand its simultaneous fermentation and anode-respiration capabilities. *Thermoanaerobacter* represents only the second thermophilic ARB family to be studied in monoculture in MXCs, and it is one of the first ARB shown to be capable of sugar fermentation (Bond and Lovley 2005, Luo 2013, Chaudhuri and Lovley 2003). This discovery opens the possibility of using fermentative bacteria that are also dissimilatory metal reducers as the primary ARB in microbial electrochemistry, especially to capture the energy in the carbohydrate fraction of biomass.

Materials and Methods

Growth media and culture conditions. A modified ATCC Medium 1118: Methanobacteria medium (ATCC medium 1045) was used to grow *T. pseudethanolicus* 39E (ATCC 33223): K_2HPO_4 (0.45 g l^{-1}), $(NH_4)_2SO_4$ (0.3 g l^{-1}), NaCl (0.6 g l^{-1}), $MgSO_4*7H_2O$ (0.12 g l^{-1}), $CaCl_2*2H_2O$ (0.08 g l^{-1}), yeast extract (0.2 g l^{-1}), and Wolfe's Mineral Solution (10 ml l^{-1}). Media was prepared in in a condenser apparatus under N_2:CO_2 (80:20) gas conditions. Medium was brought to boil and allowed to boil for 15 minutes l^{-1}. Medium was then stored in 100-ml serum bottle and autoclaved for 15 minutes at 121 °C. Wolfe's Vitamin Solution (10 ml l^{-1}) and Na_2CO_3 (4.2 g l^{-1}) were added after autoclaving. Substrate was added after autoclaving, either glucose (1.8 g l^{-1}), xylose (3.0 and 6.0 g l^{-1}), cellobiose (0.34 and 3.4 g l^{-1}), or acetate (0.82 g l^{-1}) (Cook 1993, Onyenwoke 2007). Reducing agent, including sodium sulfide and cysteine, was excluded from the media to minimize its background effect on electrochemical observations. *T. pseudethanolicus* stock cultures were grown under fermentative conditions with glucose as an electron donor in batch mode in 100 ml serum bottles on an Excella E24 Incubator Shaker (New Brunswick Scientific) at 60 ºC and 150 RPM.

H-Type MEC construction. Eight H-type reactors were constructed and operated in batch mode. Each reactor consisted of two 350-ml compartments separated by an anion exchange membrane (AMI 7001, Membranes International, Glen Rock, NJ). For all reactors, the operating temperature was 60 °C. Each reactor was fed with a different substrate at the concentrations mentioned above. All reactors contained two

cylindrical graphite anodes (anode surface area for Glucose and xylose MXCs = 5.2 cm^2; Cellobiose and acetate MXC = 5.0 cm^2) and an Ag/AgCl reference electrode (BASi MF-2052). Reference potential conversion to a standard hydrogen electrode (SHE) was conducted by constructing a two chambered cell with one chamber containing modified ATCC Medium 1118 and the other containing 1M KCl (Parameswaran 2013, Greeley 1960). The anode potential was poised at 0.042 V vs SHE, and the *j* was monitored continuously every two minutes using a potentiostat (Princeton Applied Research, Model VMP3, Oak Ridge, TN). The anode chambers were kept mixed via agitation from a magnetic stir bar at 200RPM. The cathode consisted of a single cylindrical graphite rod (0.3 cm diameter and a total area of 6.67 cm^2). Cathode pH was adjusted to 12 via addition of NaOH. Gas collection bags were placed on the anode compartments to collect volatile fermentation products and on the cathode to collect hydrogen.

MXC batch experimental setup. All MXC reactors were operated as microbial electrolysis cells (MECs), inoculated with 3 or 6 ml stock culture from serum bottles and grown under fermentative conditions in batch mode. During operation of the reactors, the pH was measured every 2-3 days, and 1 mL of medium was collected and stored in an amber high pressure liquid chromatography (HPLC) vial for tracking fermentation products including: ethanol, acetate, lactate, propionate, iso-propionate, butyrate, iso-butyrate, valerate, iso-valerate, xylose, glucose, cellobiose, and sucrose. All HPLC samples were filtered using a 0.2-μm membrane filter (Life Sciences Acrodisc 4450T) and stored at -20 °C until analysis was conducted using a Model LC-20AT HPLC (Shimadzu) equipped with an AMINEX HPX/87H column (Bio-Rad Laboratories,

Hercules, CA,1997) as described earlier (Parameswaran 2009). Coulombic efficiency (CE) was calculated based on initial and final TCOD and the current measurement on the potentiostat according to previous literature (Parameswaran 2009). Yeast extract TCOD was included in CE analysis since yeast extract utilization has been reported in previous literature (Roh 2002). Low Scan Cyclic voltammetry (LSCV) was conducted at scan rates of 1 mV s^{-1} and 10 mV s^{-1}, when the *j* of the xylose reactors reached ~7.5 A m^{-2} (pH = 7.58), to measure the midpoint potential (E_{KA}).

pH-effect experiments. To determine the effect of pH on *j*, two separate glucose-fed MECs were constructed and operated under the same conditions as the previous reactors. After achieving steady state conditions, pH was altered by either the addition of HCl or NaOH. Results are shown as a ratio of the highest *j* achieved vs. the *j* of a given pH. Results were used to obtain a pH range for operation in MECs and also to understand the role of pH in *j*.

Acetate spike experiment. A mature biofilm was grown on a xylose-fed MEC over ~70 days. The MEC was then stopped and media was replaced with media deplete of an electron donor. The MEC was then resumed and a stationary phase (~0.1 A m^{-2}) was achieved. Once the reactor reached a stationary phase, a 1mL injection containing 0.82 g l^{-1} acetate was added to the reactor.

Microscopic analysis. Scanning electron microscopy (SEM) and confocal laser scanning microscopy (CSLM) were accomplished by establishing a separate H-type batch reactor fed 20 mM xylose with the same operating conditions. After 60 days of

operation, the two anodes had developed mature biofilms that were sacrificed for imaging purposes. Preparation for SEM followed the protocol from (Parameswaran 2013). An FEI XL-30 environmental SEM (Philips) was used with an accelerating voltage of 5−20 kV and a working distance of 8−10 mm. CSLM was used to measure biofilm thickness (L_f) per the protocol from (Parameswaran 2013). Confocal images were acquired using an upright Leica SP5 CSLM after applying LIVE/DEAD (BacLight Cell vitality kit, Invitrogen, USA) staining of the biofilm anode.

Results and Discussion

Initial growth shows establishment of biofilm over five days. A representative xylose-fed electrochemical cell containing *T. pseudethanolicus*, with its anode potential set at + 0.042 V vs SHE, was allowed to ferment xylose into acetate and then establish a biofilm over five days, when it reached a stationary current density (j) of 6.5 A m^{-2}, as shown in Figure 5.1. The increase in current follows an exponential increase, similar to those observed by *G. sulfurreducens* and *T. ferriacetica* (Parameswaran 2013, Marsili 2010). The rate of current increase is faster than that of *G. sulfurreducens* (Marsili 2010), possibly due to the faster kinetic growth rates under thermophilic conditions and the fact that *T. pseudethanolicus* can also grow under fermentative conditions.

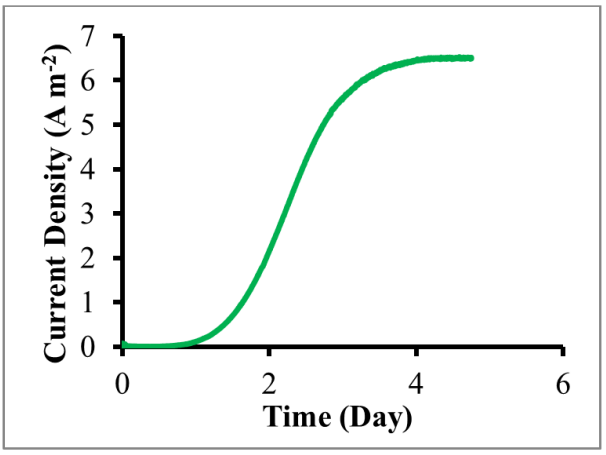
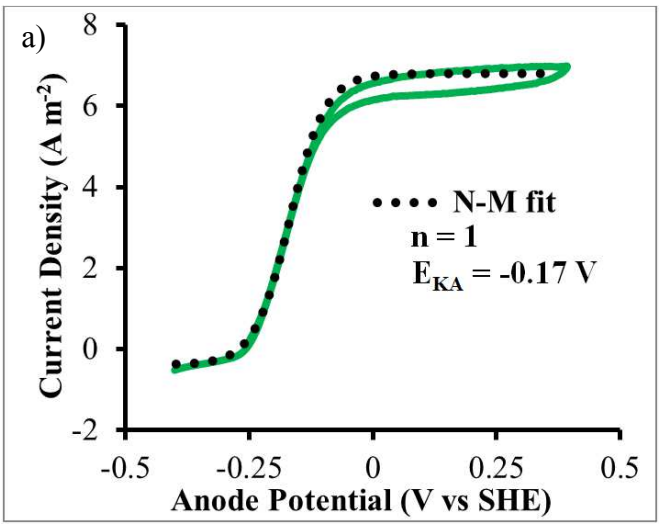


Figure 5.1: Initial growth phase in the xylose-fed MXC operated in batch for five days.

After the *j* reached stationary phase (day 4), CVs at a scan rate of 1.0 mVs^{-1} and 10 mVs^{-1} (data not shown) were performed. The midpoint potential (E_{KA}) was -0.17 V vs. SHE, and the maximum current density (j_{max}) was ~7 A m^{-2} at pH 7.6, as shown in Figure 5.2a, with the derivative shown in 5.2b. The data fit the Nernst-Monod equation with n = 1 with only slight deviation at the highest anode potential. The good fit suggests that, similar to *G. sulfurreducens*, the rate-limiting step for *T. pseudethanolicus* kinetics is an enzymatic step that is involved in an electron-transfer (Torres 2008). The E_{KA} for *T. pseudethanolicus*, however, is slightly lower than that usually observed for *G. sulfurreducens*, ~-0.15 V vs SHE (Marsili 2010, Srikanth 2008), and *T. ferriacetica*, another Gram-positive thermophilic ARB, at -0.128 V vs SHE (Parameswaran 2013).



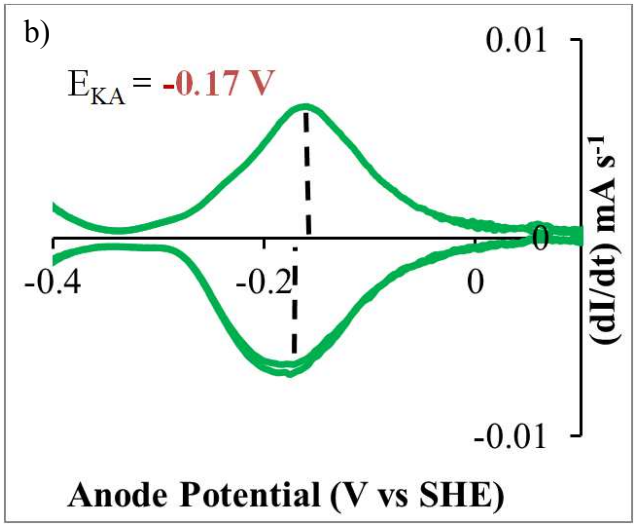


Figure 5.2a-b: (a) LSCV at 1 mV s^{-1} with observed data (green) for the xylose MXC and Nernst-Monod fit at n = 1 (black dashed line). (b) Shows derivative plot.

Figure 5.3 shows how *j* depended on the medium pH in the range of 5.4 to 8.27. At pH values lower than 5.40 or higher than 8.27 *j* dropped to ~0 A m^{-2}. As with other ARB, including *G. sulfurreducens* (Torres 2008), *T. pseudethanolicus* generated larger *j* at higher pH values, although the trend became less pronounced above pH 7. The strong

effect of pH at lower pH likely was due to a proton-transport limitation. Although the pH is that of the bulk, it is reasonable to assume that the pH of the outer layer of the biofilm is similar to that of the bulk and that the pH gradually decreased throughout the biofilm due to diffusion limitations (Marcus 2011). Proton diffusion out of the biofilm should have been enhanced by a higher diffusion coefficient of the transporting buffer with thermophilic conditions (Parameswaran 2013). *T. pseudethanolicus* generated current over a wider pH range (pH = 5.40 - 8.27) than that observed for *G. sulfurreducens* (pH = 5.8 - 8.0) (Torres 2008, Franks 2009).

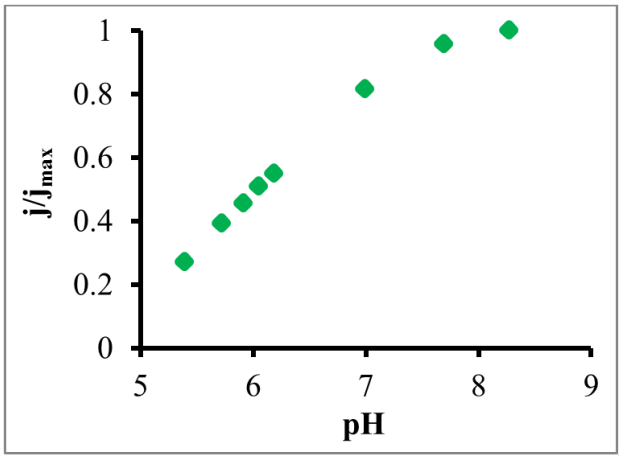


Figure 5.3: Effect of pH on current density normalized to the maximum value of 2.7 A m^{-2} (at pH 8.27).

Xylose fermentation. At the end of the low-scan cyclic voltammetry (LSCV) experiments, the reactor medium was replaced with new medium containing 20 mM xylose and left in batch mode for over 75 days. The production of fermentation products and *j* over time can be seen in Figure 4.5a, and the % electron equivalents for current and

substrate can be seen in Figure 4.5b. The mature *T. pseudethanolicus* biofilm did not produce current directly from xylose, but instead fermented xylose into acetate (almost exclusively), eventually producing *j* up to ~7.5 A m^{-2} from the fermentation products. The sharp decrease in *j* from day 0 shows the loss of current production, and acetate built up in parallel out to about day 8. Once the acetate concentration reached ~20 mM, the *j* sharply increased, after which it decreased again. Around day 29, the *j* recovered up to ~7.5 A m^{-2} and remained relatively stable until it began a slow decrease as acetate concentration declined to <10 mM. pH values remained relatively stable over the course of the experiment, although the pH was lowered initially as a result of acetate accumulation and increased towards the end of the experiment as a result of acetate depletion. The anion exchange membrane (AEM) (AMI 7001, Membranes International, Glen Rock, NJ) is thermostable up to 90 °C, and is rated for pH < 10. The pH increase observed after *j* reached 0 A m^{-2} may be the result of OH^- leakage through the membrane from the cathode to the anode. Coulombic efficiency (CE) was calculated to be 34.8% ± 0.7%.

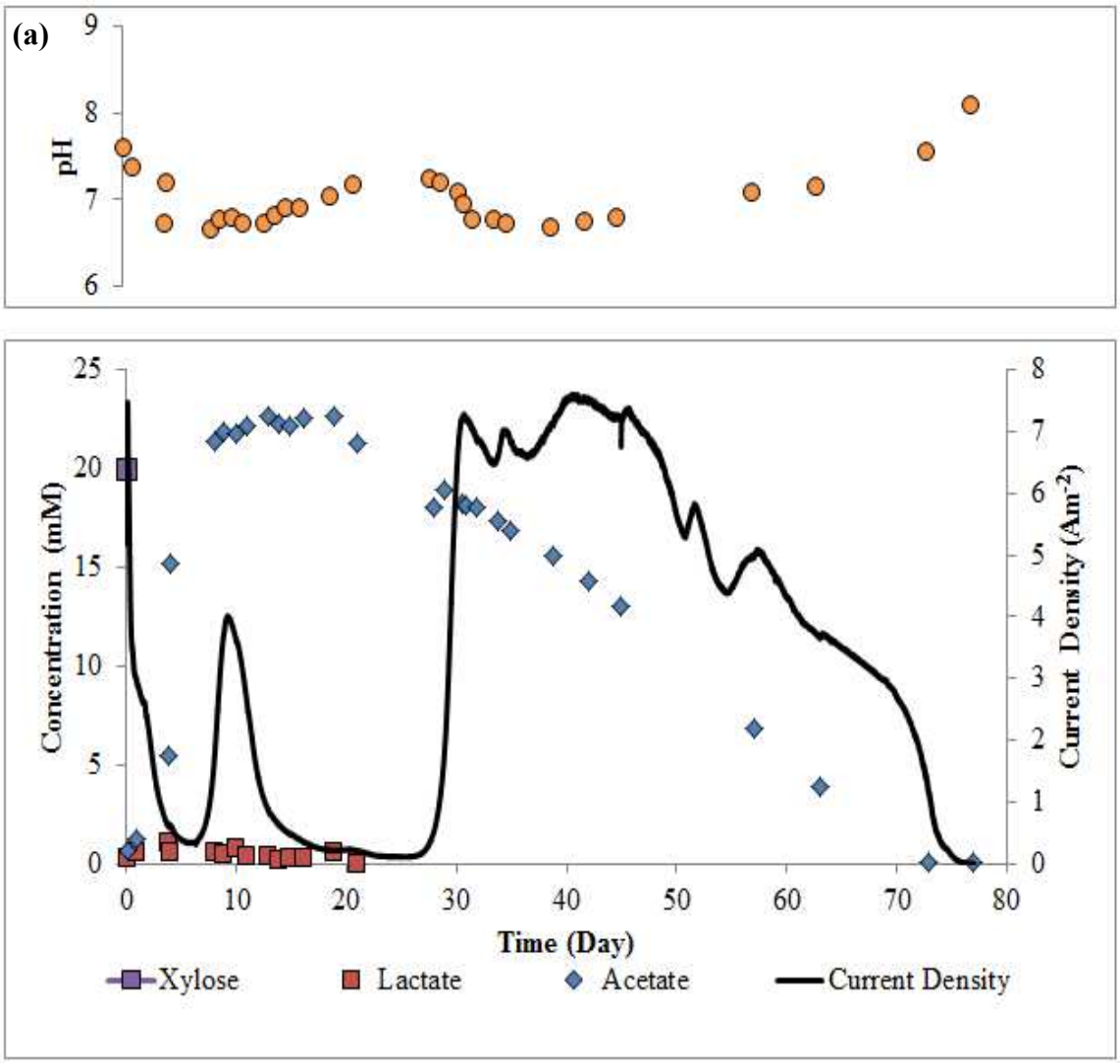


Figure 5.4a: Results from the xylose-fed MXC operated in batch for ~77 days. Orange circles indicate pH, black line indicates *j*, the purple square indicates the starting xylose (which declined to non-detectable by the next sampling point), red squares indicate lactate, and blue diamonds indicate acetate. An LSCV was conducted at time 0, just prior to replacing the medium.

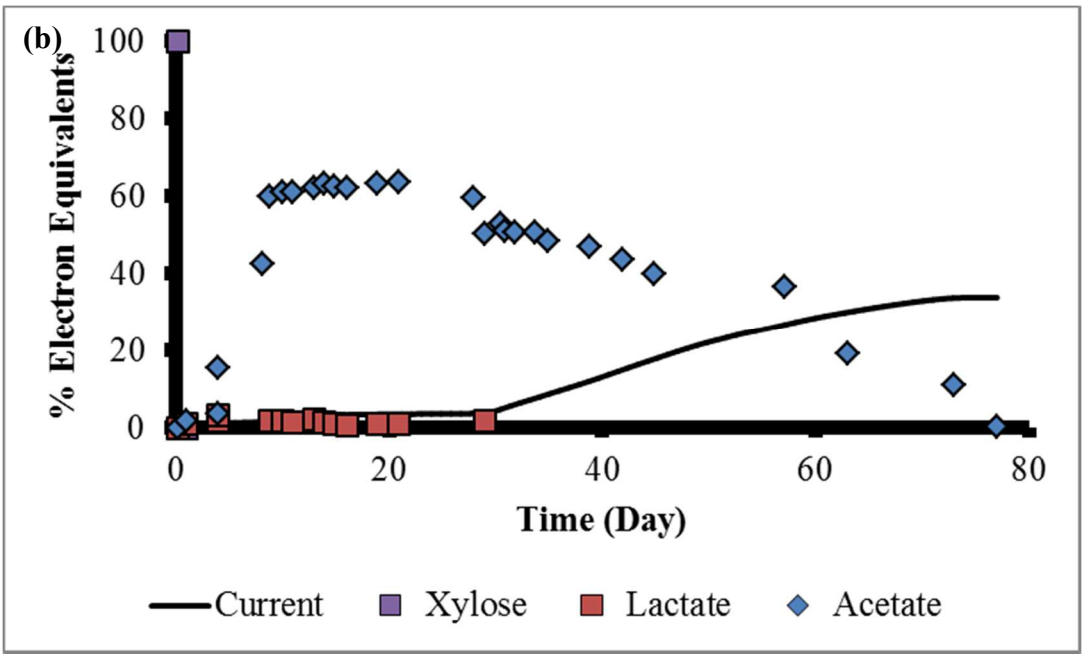


Figure 5.4b: Fraction of electrons captured as current, acetate, lactate and initial substrate are shown as a percentage of the total electrons present in the initial substrate for 20 mM xylose-fed MXC.

Glucose fermentation. A grown *T. pseudethanolicus* biofilm containing no electron donor and producing only a decay *j* (~0.5 A m^{-2}) was fed with new medium containing ~10 mM glucose and operated in batch mode for over 45 days. The production of fermentation products and *j* over time can be seen in Figure 5.5a and the % electron equivalents for current and substrate can be seen in Figure 5.5b. A mature *T. pseudethanolicus* biofilm did not produce current directly from glucose, but instead fermented glucose into lactate and acetate, producing *j* up to ~4.8 A m^{-2} from these fermentation products. Complete glucose fermentation occurred within 5 days, and lactate was fermented by day 12. Once the acetate concentrations reached ~25 mM, the *j*

sharply increased (up to ~4.8 A m^{-2}), after which the *j* gradually decreased in parallel with the acetate concentration for the remainder of the experiment. Lactate production was much more significant during glucose fermentation than it was with xylose fermentation, as was reported in a previous study (He 2009). The pH remained relatively stable over the course of the experiment, although it was initially lowered as a result of acetate and lactate production and increased at the end. The increase in pH at the end of the experiment is partly a result of acetate depletion; however the increase prior to complete acetate depletion may be the result of OH^- leakage from the cathode to the anode. The CE for glucose was 65.3% ± 1.0%, which is significantly higher than for xylose and similar to previous observations with *T. ethanolicus*, a closely related bacterium (Lacis and Lawford 1991).

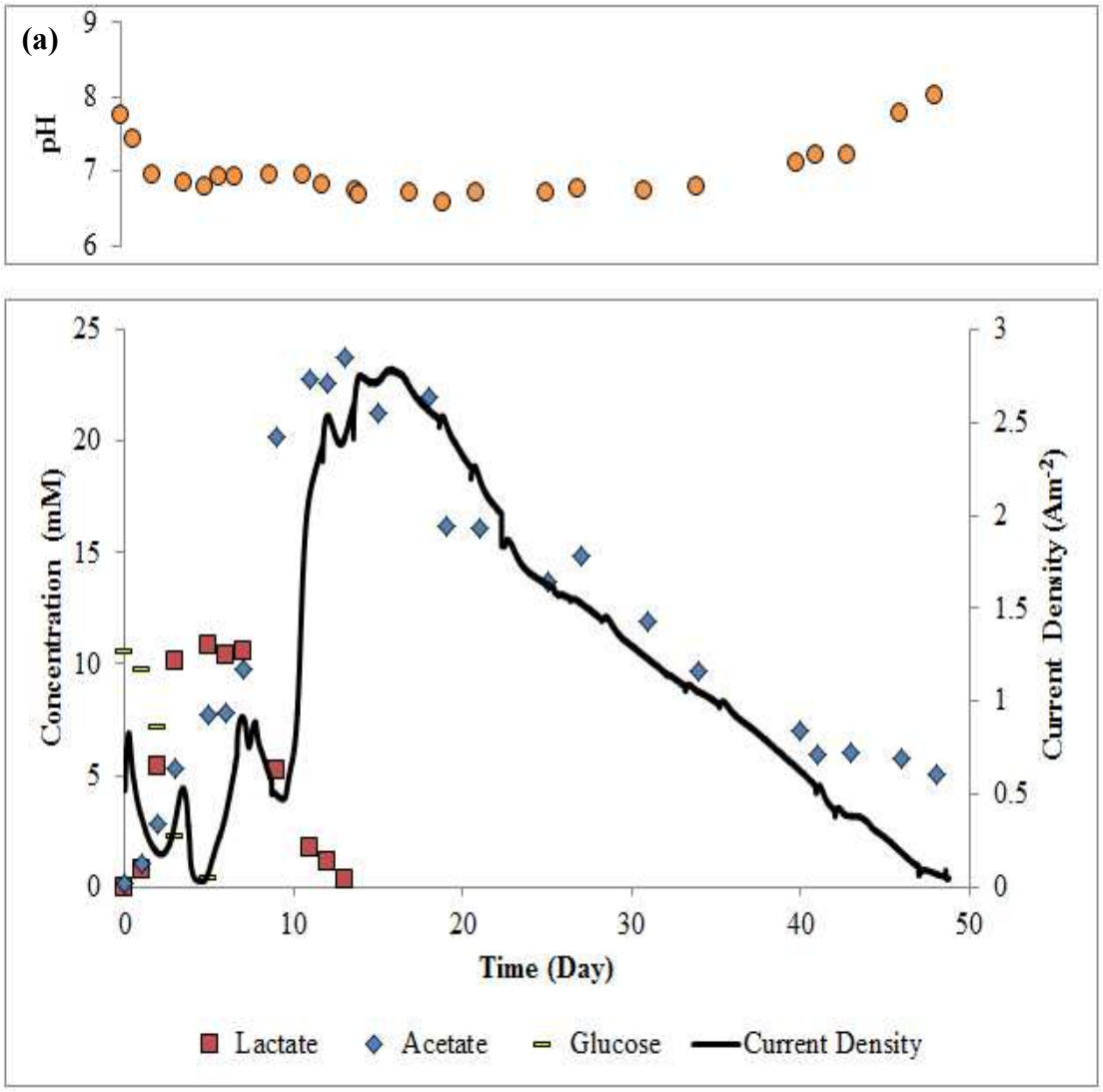


Figure 5.5a: Results for the glucose-fed MXC operated in batch for ~48 days. Orange circles indicate pH, black line indicates *j*, yellow dashes indicate glucose, red squares indicate lactate, and blue diamonds indicate acetate.

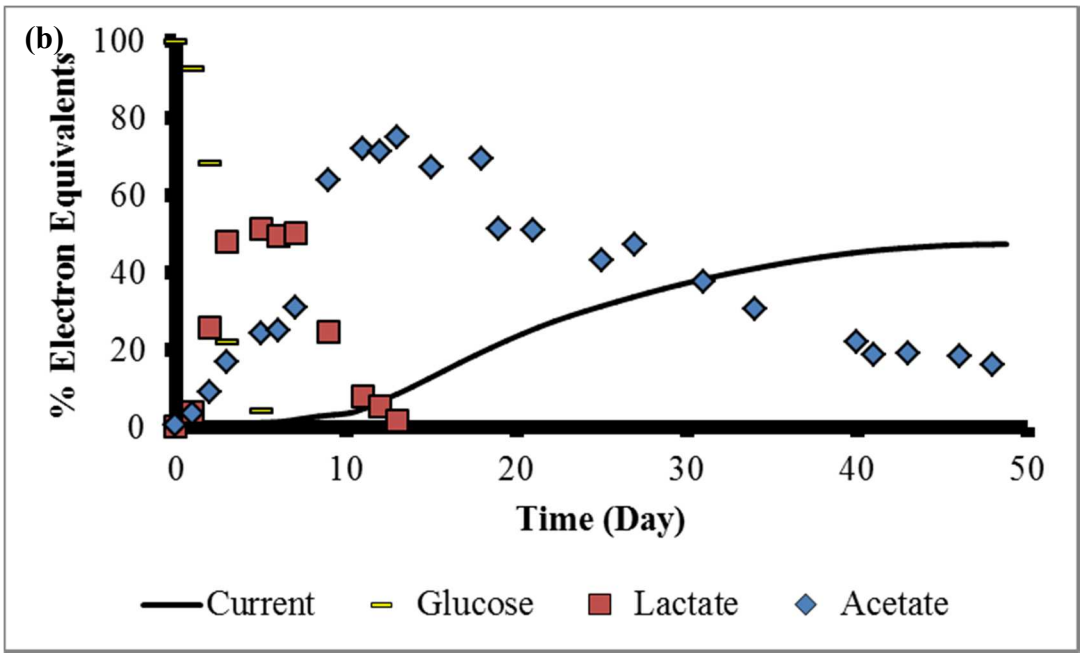


Figure 5.5b: Fraction of electrons captured as current, acetate, lactate and initial substrate are shown as a percentage of the total electrons present in the initial substrate for 10 mM glucose-fed MXC.

Cellobiose fermentation. A grown *T. pseudethanolicus* biofilm at stationary phase (~0.15 A m^{-2}) containing no electron donor, was fed with new medium containing ~7.5 mM cellobiose and left in batch mode for over 80 days. The production of fermentation products and *j* over time can be seen in Figure 5.6a, and the % electron equivalents for current and substrate can be seen in Figure 5.6b. As with the other substrates, *T. pseudethanolicus* did not produce current directly from cellobiose, but fermented it first into acetate and other volatile acids and subsequently produced *j* up to ~3.5 A m^{-2} from these fermentation products. Complete cellobiose fermentation occurred within 10 days of reactor operation. Once the acetate concentrations reached ~25 mM,

the *j* sharply increased, after which it gradually decreased with acetate concentration for the remainder of the experiment. Acetate and minimal lactate production was observed in both cellobiose-fed electrochemical cells. Consistent with the xylose- and glucose-fed reactors, pH remained relatively stable over the course of the experiment, with a pH drop during the initial phase resulting from the accumulation of acetate. The CE was calculated to be 27.7% ± 1.5%.

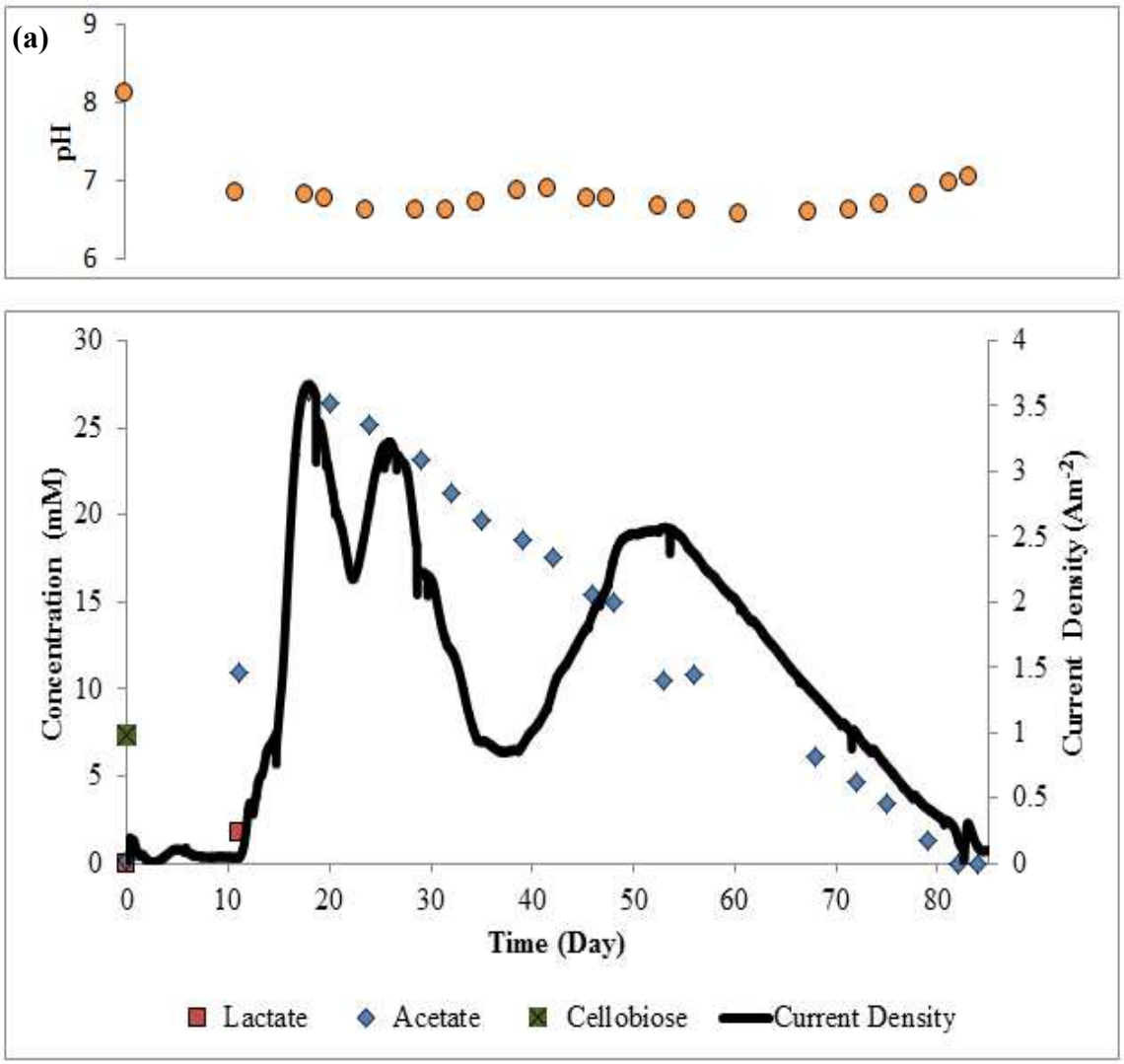


Figure 5.6a: Results for the cellobiose-fed MXC operated in batch for ~82 days. Orange circles indicate pH, black line indicates *j*, green boxes with Xs indicate cellobiose, red squares indicate lactate, and blue diamonds indicate acetate.

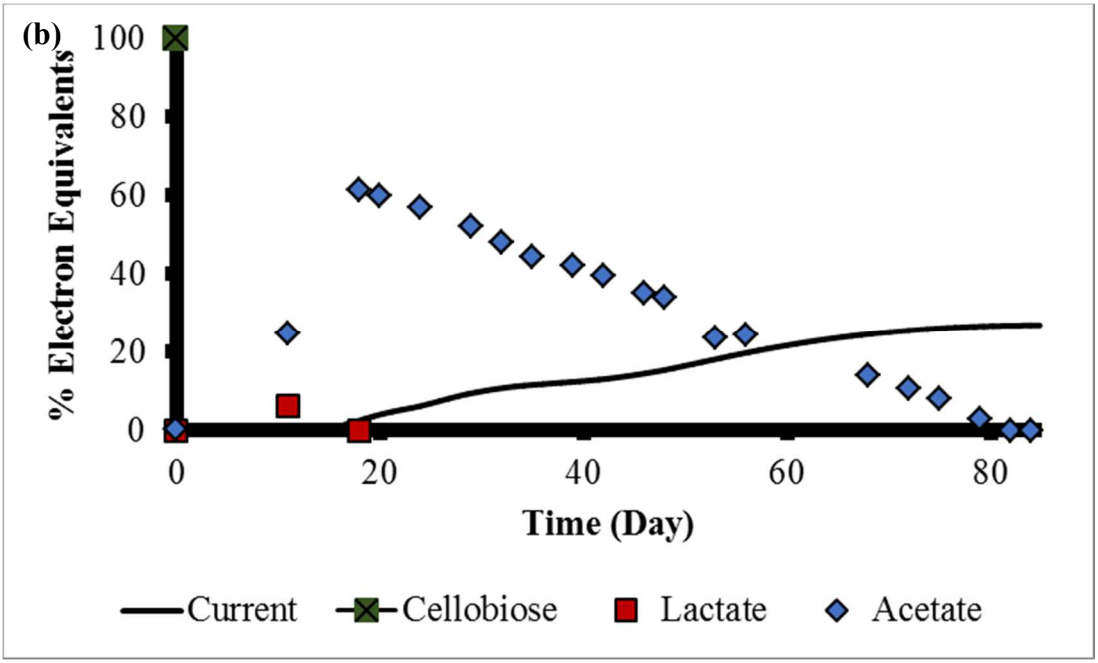


Figure 5.6b: Fraction of electrons captured as current, acetate, lactate and initial substrate are shown as a percentage of the total electrons present in the initial substrate for 7.5 mM cellobiose-fed MXC.

Coulombic Efficiency profiles. CE values varied among substrates: glucose at 65.3% ± 1.0% > xylose at 34.8% ± 0.7% > cellobiose at 27.7% ± 1.5%. No hydrogen or methane gas was observed in the gas phase of the anode chamber in any of the reactors. For comparison, previous reports for *T. ethanolicus*, a closely related thermophile, had 22-26% of electrons from xylose and 12-18% of electrons for glucose being utilized for cell yield (Lacis and Lawford 1991). It is likely that a significant fraction of electrons not counted in the CE were contained in biomass and/or EPS. Previous literature using fermentative MXCs (Lee 2008) reported the fraction of electrons used for growth by ARB in the biofilm anode were as much as 1.5- to 2-fold higher than those used for

fermentative growth. Substrate-specific differences in CE also may have been caused by differences in end-product formation as a result of the various metabolic pathways associated with transporting and metabolizing each substrate (Hemme 2011, Lacis and Lawford 1991, Stouthamer 1979).

Acetate consumption in *T. pseudethanolicus* biofilm anode. Results indicate that *T. pseudethanolicus* performs fermentation (mostly to acetate), not direct anode respiration from the fermentable substrates. This may be due to the thermodynamic advantage of ATP production from the fermentation of xylose, glucose, and cellobiose to acetate compared to the ATP-production capacity provided by the oxidation of acetate by anode respiration with a fixed potential of 0.042 V vs SHE. All reactors had accumulation of fermentation byproducts, primarily acetate, prior to consumption for anode respiration. This accumulation likely was due to the limited size of anode surface area (either 5.0 or 5.2 cm^2 per 350 mL). Future research can investigate the effects of anode surface area, and a larger specific surface area should minimize acetate accumulation by being a faster acetate sink.

While the *T. pseudoethanolicus* biofilms consumed acetate derived from fermentation, anode respiration was not established in an acetate-fed batch MXC. Replicate trials yielded almost no current generation, indicating that *T. pseudethanolicus* cannot develop mature biofilms in electrochemical cells without an initial fermentation stage. To determine whether or not a mature biofilm was able to produce current from acetate, 10 mM acetate was spiked into a xylose-fed electrochemical cell that had

developed a mature biofilm and was deplete of electron donors. The acetate spike generated *j* of around 1.2 A m^{-2}. This phenomenon is likely due to the limited inoculum size coupled with a minimal anode surface area. Without a fermentable substrate, *T. pseudethanolicus* may not have achieved the biomass necessary to attach to the anode.

Morphological characterization of biofilm. SEM images (Figure 5.7 a-d) reveal biofilm morphology consistent with previous literature reports on *T. pseudethanolicus*: a drumstick-shaped structure emanating from a terminal, round mother cell (see white squares in Figure 5.7 b-d) (Lee 1993, Zeikus 1980). The bacteria appear to be 2-3 μm in length, but some are branched or form chains of bacteria that are stacked in a network. In concert with previous observations, not all cells form spherical spores and cells without spores appear less elongated than those that do (Lee 1993). CLSM images (Figure 5.8a-c) reveal a biofilm at least 150 μm thick and with many peaks and valleys. CLSM images also show that live and dead bacteria are relatively evenly distributed throughout the biofilm, but with a higher number of dead cells closest to the anode. Characterization of the biofilm anode with light and electron microscopy, in concert with electrochemical observations, showed that high *j* was achieved from xylose, glucose, and cellobiose by a bacterial monoculture capable of forming thick biofilms and transferring electrons to an anode. These findings facilitate the exploration of new EET mechanism(s) used by an increasingly diverse set of thermophilic Gram-positive ARB.

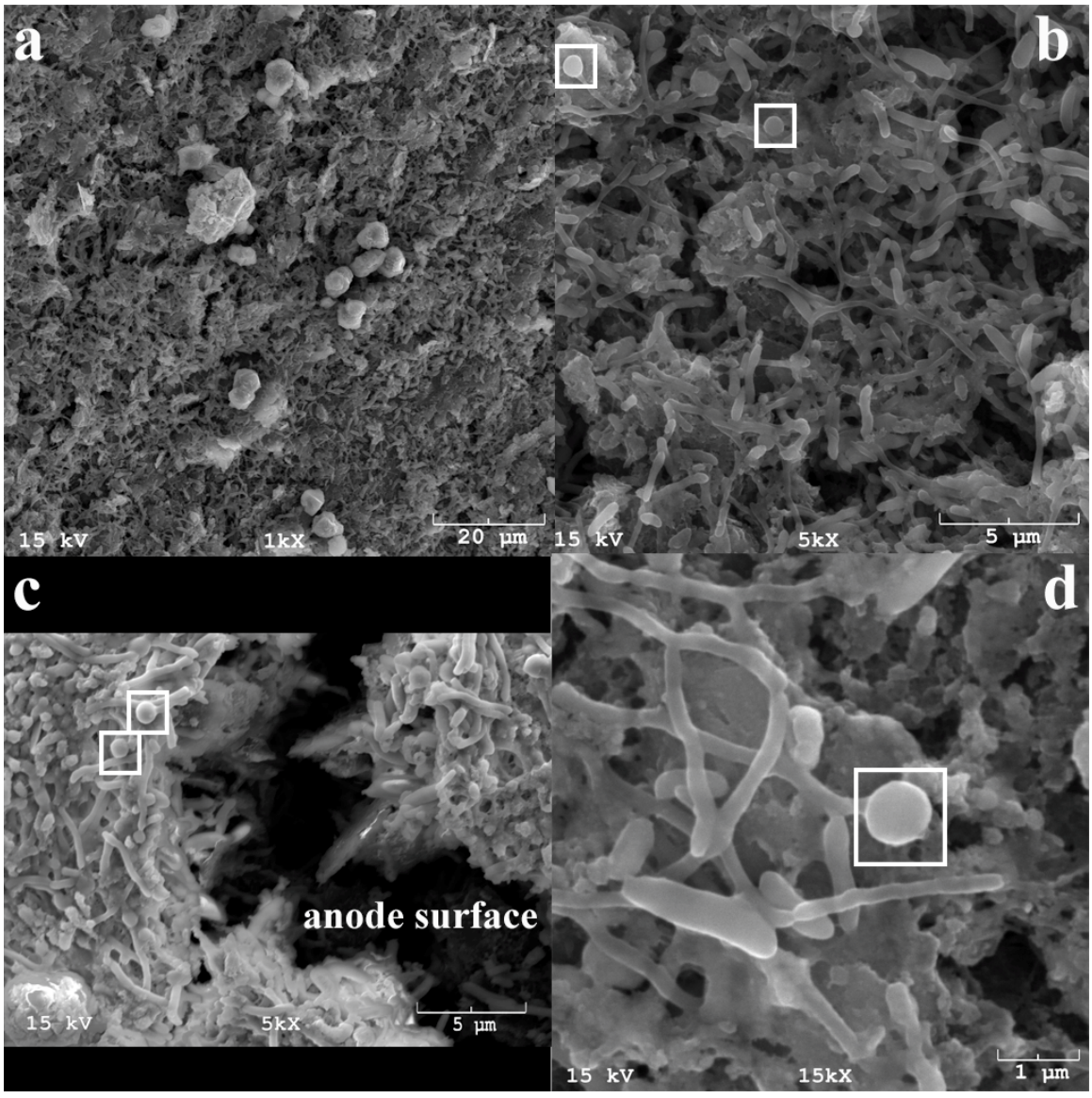


Figure 5.7 a-d: SEM images of an anode biofilm grown on 40-mM xylose. White squares indicate drumstick shaped structures which are indicative of *T. pseudethanolicus* morphology (Lee 1993, Zeikus 1980). (a) A broad overview of the biofilm at 1000X. (b) Complex biofilm morphology at 5000X magnification. (c) Multiple layers of cells extending from the anode of the MEC taken at 5000X magnification. (d) Drumstick structure extending from the cells at 15000X.

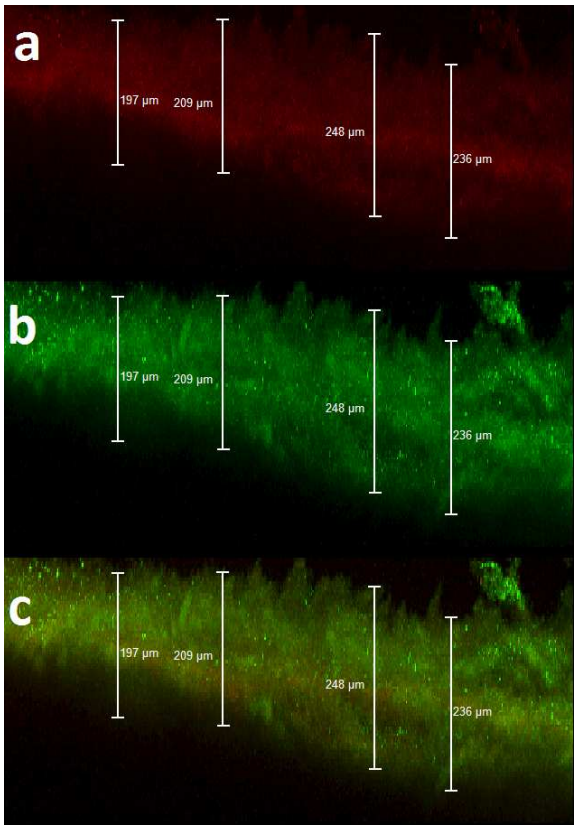


Figure 5.8 a-c: Representative CLSM images for a LIVE/DEAD assay for the anode biofilm grown on 40 mM xylose. Shown is a cross section of the z-axis with the cylindrical anode on the bottom black part of the image while the top part is the media. The white lines indicate where thickness values were captured. (a) Red shows the thickness (L_f) of DEAD cells within the biofilm. (b) Green shows the L_f of LIVE cells within the biofilm. (c) An overlay of red and green to show a holistic representation of LIVE/DEAD distribution within the biofilm.

Conclusion

Outlook of the physiological and practical implications of current production by *Thermoanaerobacter pseudethanolicus*. The lag phase (~10 days) observed in Figures 4.4a, 4.5a, and 4.6a are the result of a preference for *T. pseudethanolicus* to ferment all possible substrates prior to anode respiration. Fermentation of xylose, glucose, and cellobiose to acetate is more energetically favorable in terms of Gibbs free energy, than is oxidation of acetate for anode respiration. Thus, it is reasonable that *T. pseudethanolicus* would perform anode respiration when there are no fermentable sugars remaining in the MXC. This phenomenon is observed under all conditions investigated in this study. It is observed that, in all MXCs, *j* appears to rise and fall during operation. This may be caused by lower pH that develops in the biofilm towards the end of the fermentation process, which negatively affects the energetics of anode respiration. More investigation is needed to confirm the limitations in *T. pseudethanolicus* biofilms.

Our results show that *T. pseudethanolicus* 39E was able to produce *j* comparable to other ARB through the sequential fermentation of sugars and anode respiration of the fermentation products. *T. pseudethanolicus* joins a cohort of fewer than 10 ARB isolates, including *Geobacter sulfurreducens* (Band and Lovley 2003), *Thermincola ferriacetica* (Parameswaran 2013), *Geoalkalibacter ferrihydriticus* (Badalamenti 2013), *Geoalkalibacter subterraneus* (Badalamenti 2013), and *Tolumonas osonensis* (Luo 2013) capable of high *j* (> 2 A m^{-2}) Torres 2014). It is the third thermophilic ARB isolated and the only one capable of growing by fermentation (Parameswaran 2013, Wrighton 2011).

The capability to simultaneously ferment sugars and convert fermentation products to current opens up new MXC applications related to using bacterial monocultures for the thermophilic conversion of cellulosic and lignocellulosic byproducts into energy-rich products or electrical power. For this conversion, *T. pseudethanolicus* may be used alone or combined with other efficient cellulose degraders. *T. pseudethanolicus* biofilms are thick and are limited by proton diffusion; however, very little is known about the EET mechanisms used by Gram-positive bacteria (Carlson 2012), making future studies to elucidate these mechanisms of particular interest.

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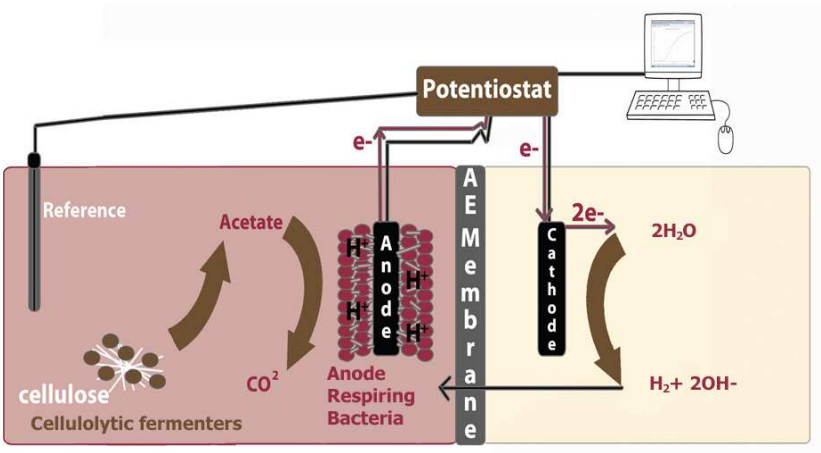
Chapter 6: Simultaneous fermentation of cellulose and current production with a highly enriched mixed culture of thermophilic bacteria in a microbial electrolysis cell[5]

Overview

A highly enriched mixed culture of thermophilic (60 °C) bacteria was assembled for the purpose of using cellulose to produce current in thermophilic microbial electrolysis cells (MECs). Current densities (j) were sustained at 6.54 ± 0.15 A m^{-2} in duplicate reactors with a coulombic efficiency (CE) of 84 ± 0.3% and a coulombic recovery (CR) of 54 ± 11.6%. Cellulose was fermented into sugars and acids before being consumed by anode respiring bacteria (ARB) for current production. Low scan cyclic voltammetry (LSCV) revealed a midpoint potential (E_{ka}) of -0.17 V vs SHE. LIVE/DEAD analysis using confocal laser scanning microscopy (CLSM) shows a heterologous biofilm with peaks and valleys ranging from 40-60 μm. Scanning electron microscopy (SEM) indicates the presence of diverse bacterial morphologies within the biofilm. Pyrosequencing analysis presents a diverse, although highly enriched thermophilic microbial community consisting mainly of the phylum Firmicutes with the Thermoanaerobacteracea and Peptococcacea families occupying the anode and *Brevibacillus* and *Tepidmicrobium* genera present in the bulk media. This study indicates that thermophilic consortia can be used to produce high j (>2 A m^{-2}) from cellulosic materials in MECs with > 80% CE and > 50% CR values.

[5] Chapter 6 unpublished with Bradley G. Lusk, Alexandra Colin, Prathap Parameswaran, Bruce E. Rittmann, and Cesar I. Torres



Introduction

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 2005, 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). For this reason, many conventional methods for extracting energy from plant biomass consist of combustion processes that produce large amounts of ash and are highly regulated to limit the release of volatile organic compounds (VOCs) (Badger 2002). Unlike the combustion process, biological technologies focus on discovering new ways to harness the energy stored in plant biomass to produce beneficial fermentation products including electricity, ethanol, acetate, or hydrogen (Saripan 2014, Hama 2014, Li 2012**,** Xia 2012, Wilson 2009, Demain 2005).

Cellulose is the most abundant polymer in plant biomass (Carere 2008, Niessen 2005). Since cellulose is a recalcitrant polymer, it is only susceptible to degradation from organisms containing cellulolytic enzymes, or cellulases. Consolidated bioprocessing, for

example, is a rapidly advancing field that often uses genetically modified bacteria to produce ethanol in high concentrations on an industrial scale from cellulosic biomass. This is accomplished in one step and without exogenous cellulase enzymes (Olsen 2012). Another employed biological technology is the utilization of isolated cellulolytic enzymes to degrade cellulose into glucose that is then fermented by yeasts to produce high concentrations of ethanol; however, the process of purifying these enzymes is costly. In the last five years, researchers have began to consider using thermophilic bacterial consortia, since they can degrade cellulose at higher activities than the isolated enzymes (Bryant 2011, Olsen 2012, Zambare 2011) and can operate at slightly acidic, neutral, or slightly basic pH conditions (Lynd 2002, Sizova 2011, Torres 2008).

Microbial electrolysis cells (MECs) utilize bacteria that are capable of consuming fermentation products from cellulose degradation for the production of electrical current (*j*) via anode respiration; they are referred to as anode respiring bacteria (ARB) (Oh 2005, Kim 1999, Mathis 2008, Parameswaran 2013). Previous studies have reported that cellulose fermentation products can be utilized in microbial electrochemical cells (MXCs) for the production of *j* hydrogen using mixed cultures (Ren 2008, Rismani-Yazdi 2007, Niessen 2005). Coupling ARB with cellulolytic bacteria provides the possibility of converting cellulose directly into *j* in an MEC without having to collect fermentation products. In addition, MECs may decrease inhibition caused by the accumulation of acids from cellulose fermentation (Demain 2005, Niessen 2005), since ARB consume these acids to produce *j*. Previous studies with mesophilic cellulolytic cultures coupled with ARB in microbial fuel cells (MFCs) have been shown to produce

low *j* and low or unreported coulombic efficiency (CE) values (Ren 2008, Rismani-Yazdi 2007). However, several thermophilic bacteria have been shown to exhibit cellulolytic activity both in pure and mixed culture studies (Sizova 2011, Demain 2005, Lynd 2002, Kato 2005) and using them in MECs may provide enhanced eletron recovery and capture efficiency.

Since, no bacterium is known to be capable of cellulose fermentation and dissimilatory metal reduction, we chose to employ a thermophilic microbial consortium for the efficient conversion of cellulosic material into *j*. Thermophilic MECs have the potential to convert cellulose into *j* at with high coulombic recoveries (CR) and high CE due to the increased growth kinetics, cellulase activity, stability, mixing rates, and diffusion rates of H^+ within the biofilm anode (McBee 1950, Mathis 2008, Parameswaran 2013, Sizova 2011, Taylor 2009, Torres 2008). For example, *Thermincola ferriacetica*, a thermophilic ARB capable of producing *j* from the consumption of acetate, has a doubling time five times faster than *Geobacter sulfurreducens* and can achieve high *j* (>2 A m^{-2}) and CE (93%) in MECs (Parameswaran 2013, Marshall 2009). In addition, as discussed in chapter 5, *Thermoanaerobacter pseudethanolicus* is capable fermenting cellulose degradation products, including xylose, glucose, and cellobiose, into acetate and ultimately producing *j*. This study employed thermophilic ARB with a cellulolytic microbial consortium for the pupose of producing *j* from cellulose in MECs.

Materials and Methods

Growth and Media Conditions for *Thermincola ferriacetica* (DSMZ 14005). The pure culture of *T. ferriacetica* strain 14005 was obtained from DSMZ, Braushweig, Germany. The strain was cultivated in serum bottles a modified DSMZ Medium 962: *Thermovenabulum* medium. The media consisted of the following in 1.0 L deionized water: 0.33 g each of NH_4Cl, KH_2PO_4, $MgCl_2*6H_2O$ and KCl; 0.1 g $CaCl_2*2H_2O$; 0.05 g yeast extract; 1 mL selenite-tungstate solution (prepared by dissolving 3 mg $Na_2SeO_3*5H_2O$, 4 mg $Na_2WO_4*2H_2O$ and 0.5 g NaOH in 1.0 L distilled water); 0.84 g $NaHCO_3$ (10 mM); 3.4 g of $NaCH_3COO*3H_2O$ (25 mM); 10 mM $Fe(OH)_3$ as electron acceptor; 10 mL ATCC vitamin solution; and 10 mL trace element solution. The trace elements solution consisted of the following ingredients in 1.0 L deionized water: 1.5 g nitrilotri-acetic acid, 3.0 g $MgSO_4*7H_2O$, 0.5 g $MnSO_4*H_2O$, 1.0 g NaCl, 0.1 g $FeSO_4*7H_2O$, 0.18 g $COSO_4*7H_2O$, 0.1 g $CaCl_2*2H_2O$, 0.18 g $ZnSO_4*7H_2O$, 0.01 g $CuSO_4*5H_2O$, 0.02 g $KAl(SO_4)_2*12H_2O$, 0.01 g H_3BO_3, 0.01 g $Na_2MoO_4*2H_2O$, 0.03 g $NiCl_2*6H_2O$, and 0.3 mg $Na_2SeO_3*5H_2O$. The pure cultures were grown in 160 mL batch serum bottles containing 100mL media and were incubated in an Excella E24 Incubator Shaker (New Brunswick Scientific) at 60 ºC and 150 RPM.

Growth Conditions and Media for Enrichment of Cellulolytic Bacterial Consortium. The strain was cultivated in serum bottles using the ATCC Medium 1190. The media consisted of the following in 1 L deionized water: 1.36 g KH_2PO_4 (10mM); 4.2 g $Na_2HPO_4.12H_2O$ (2.5mM); 0.5 g NH_4Cl; 0.18 g $MgCl_2.6H_2O$; 0.5 g yeast extract; 2 g glucose; 10 mL ATCC vitamin solution; 5 mL Wolfe's Modified Elixir; 40 mL

Reducing Solution (prepared by dissolving 1 g $Na_2S.H_2O$ and 2.5 g L^{-1} Cysteine-HCl in 200 mL 0.2 N NaOH). Cellulose was provided by adding either Whatman #0 filter paper (Qualitative Circles, Cat no. 1001 042) with a diameter of 42.5 mm and an average weight of 0.120g at ~2.4g l^{-1} or 2.40g l^{-1} of α-Cellulose powder (Sigma). The cultures were grown in 160 mL batch serum bottles containing 100mL media and were incubated in an Excella E24 Incubator Shaker (New Brunswick Scientific) at 60 ºC and 150 RPM. Degradation of cellulose was monitored visually and the products of cellulose fermentation were monitored via high pressure liquid chromatography. Colonies showing the highest affinity for cellulose degradation were transferred to new serum bottles containing cellulose. Due to increased rates of cellulose fermentation (Figure 5.2a-f), bottles containing filter paper (Figure 5.2d-e) were added to the MECs after glucose concentrations had diminished.

Construction, Operation, and Monitoring of Dual Chamber H-type Microbial Electrolysis Cells. Three MECs were constructed- each contained two 350 mL chambers for the anode and the cathode for a total reactor volume of 700 mL. An anion exchange membrane (AMI 7001, Membranes International, Glen Rock, NJ) was used to allow ion transfer between the anode and cathode. The anode electrode was comprised of two graphite rods with a total anode surface area of either 4.15 cm^2, 2.87 cm^2, or 2.40 cm^2. An Ag/AgCl reference electrode (BASi MF-2052) was placed in the anode chamber. The anode was poised at -0.06 V vs SHE using a potentiostat (Princeton Applied Research, Model VMP3, Oak Ridge, TN). The anode chambers were kept completely mixed via agitation from a magnetic stir bar. The cathode consisted of a

single cylindrical graphite rod (0.3 cm diameter and a total area of 6.67 cm^2). Cathode pH was adjusted to 12 via addition of NaOH. Gas collection bags were placed on the anode compartments to collect volatile products and on the cathode to collect hydrogen. Hydrogen concentration was not measured.

Reactors were inoculated with serum bottles containing an enriched cellulolytic culture, as well as with *T. ferriacetica*. For the enriched cellulolytic culture, after 10 days of growth (glucose concentration = 0 mM), 200ml of spent ATCC Medium 1190 media with visibly unfermented filter paper was transferred in a glove box under anaerobic conditions to the anode of an H-type MEC. In addition, under anaerobic conditions, 150ml of modified DSMZ Medium 962 media without acetate and 3ml of *T. ferriacetica* from stock serum bottles was added to the anode of the MEC. The MECs were operated in batch mode is a 60°C incubator.

EC-Lab software (version 10.31) was used to constantly monitor current in two minute intervals for chronoamperometry (CA) and to observe the j-V response of the biofilm anode during low-scan cyclic voltammetry (LSCV). LSCV scans were performed 1 mV s^{-1} and 10 mV s^{-1}.

Monitoring of Fermentation Product Formation and Consumption with High Pressure Liquid Chromatography, Total Chemical Oxygen Demand, and Gas Chromatography. To monitor the fermentation of cellulose, the consumption of fermentation products, and pH, 1 mL samples were taken from the serum bottles daily and from the reactors every ~four days. pH was monitored using an Orion 2 Star pH Benchtop apparatus (Thermoscientific). Liquid samples were filtered through a 0.2 µm

filter and stored at -20°C until they were analyzed using High Pressure Liquid Chromatography (HPLC) (Shimadzu, USA) equipped with an Aminex HPX-87H column. Fermentation products monitored included acetate, lactate, butyrate, ethanol, glucose, and cellobiose.

Initial and final concentrations of total chemical oxygen demand (TCOD) were measured using a Hach 20-1500 mg/L range TCOD kit. A biochemical methane potential (BMP) test was used on the yeast extract to determine its potential as an electron source since *T. ferriacetica* growth on yeast extract has been reported previously (Zavarzina 2007). TCOD measurements were used to calculate coulombic efficiency (CE) (Parameswaran 2013) and coulombic recovery (CR) (Ge 2013). CE is a measurement of the conversion efficiency of the electrons removed from the MEC utilized for current production while CR is a measure of the total electrons that entered the MEC that were recovered as current production.

Gas production in the headspace of serum bottles and MECs was periodically measured using a frictionless glass syringe (Perfektum, NY). H_2 and CO_2 production was quantified using a gas chromatograph (GC 2010, Shimadzu) equipped with a thermal conductivity detector. CH_4 was monitored, but not observed in any MECs or serum bottles. H_2 was not observed in the gas phase of the anode in any MECs.

Confocal Laser Scanning Microscopy (CLSM) and Scanning Electron Microscopy (SEM). Microscopy measurements were completed by sacrificing the live biofilms from an MEC after reaching a steady current. To ascertain the thickness of the active and inactive biomass on the anode, we employed the LIVE/DEAD technique

(BacLight Cell vitality kit, Invitrogen, USA) to an intact biofilm connected to an anode. Measurements were acquired using an upright Leica SP5 microscope. Images were taken every 5 mm with a 40X immersion objective.

SEM was performed on an intact biofilm connected to an anode. After removal from the reactor, the biofilm anode was fixed with 4% glutyraldehyde for 12 hours at 4°C and then washed and stored in 10 mM PBS solution. The sample was treated with 1% osmium tetroxide for 15 minutes, followed by graded-ethanol series dehydration (50%, 70%, 95%, and 100% for 5 minutes each). The sample was then dried by critical-point drying and then mounted on an aluminum stub before being sputter coated with a Au/Pd alloy with a Technics Hummer II sputter coater. Imaging was conducted using an FEI XL-30 environmental SEM (Philips) with an accelerating voltage of 10-20 kV and a working distance of 8-10 mm.

DNA Extraction and Pyrosequencing Community Analysis. For bacterial community analysis of the bacteria occupying the anode, a fraction of the biofilm was also collected in a sterilized 1.5 ml centrifuge vial. For bacterial community analysis of the bulk, 150 ml of liquid was removed and placed in three sterilized 50 mL Falcon tubes. The tubes were then centrifuged at 4000 RPM for 15 minutes using a centrifuge (5810 R, Eppendorf) and the pellets were preserved. All DNA collection was conducted at the end of the MEC batch runs. DNA extraction was performed using the Gram-positive bacteria method from the Qiagen DNEasy Blood and Tissue Extraction Kit (Qiagen Inc., Mississauga, ON) following the manufacturer's recommendations. DNA extraction was

confirmed and quantified using a Nanodrop ND-1000 spectrophotometer. Extracted DNA was stored at -20 °C until ready for processing.

Bacterial community analysis was performed via pyrosequencing following the protocol Mr. DNA Analysis Pipeline (Ontiveros-Valencia 2013). This analysis did not probe for the presence of Archaea. Amplicon pyrosequencing was conducted at the Research and Testing Laboratories LLC (Texas, USA), using a standard 454/GS-FLX Titanium (Sun et al., 2011). The V6 and V7 regions of the 16S rRNA gene were targeted with primers 939F (5′-TTGACGGGGGCCCGCAC-3′) and 1492R (5′-TACCTTGTTACGACTT-3′) to analyze the Bacterial domain (Zhao et al., 2011). Raw data was scrutinized using QIIME 1.4.0 suite (Caporaso et al., 2010a): sequences having < 200 bps, homopolymers > 6 bps, primer mismatches, or an average quality score < 25 were removed. The Greengenes 16S rRNA gene database with *uclust* (Edgar, 2010) was used to pick the operational taxonomic unit (OTU) based on ≥ 97% identity. OTUs that contain < two sequences (singletons) were removed. Remaining OTUs were aligned with the representative sequence in the Greengenes database using *PyNast* (DeSantis et al., 2006; Caporaso et al., 2010b). ChimeraSlayer was used to identify chimeric sequences (Haas et al., 2011) which were removed using a python script in QIIME. OTUs were assigned a taxonomy using a 50% confidence threshold with the ribosomal database project (RDP) (Wang et al., 2007).

Results

Establishing a highly enriched cellulolytic microbial consortium

In order to establish a culture of thermophilic cellulose degrading bacteria, serum bottles were established containing ~2.4g l^{-1} cellulose powder or ~2.4g l^{-1} cellulose paper. Inoculum for the serum bottles was sourced from other serum bottles in the lab that were exhibiting cellulolytic activity. The results for the batch bottle studies with cellulolytic cultures are shown in Figure 6.1a-f. Results indicate that the four primary products formed from cellulose fermentation were acetate, lactate, ethanol, and H_2. In all cases, the pH of the serum bottles containing cellulolytic bacteria (Figure 6.1a-b and d-e) dropped ~ 1 pH unit, while the pH in the non-cellulolytic control bottles (Figure 6.1c and f) remained roughly the same over the course of the experiment. In addition, fermentation rates show that cellulose paper (resctors d-e) was fermented in 3±1 days- a greater rate than cellulose powder (reactors a-b), which took ~11 days. For this reason, serum bottles d-e were used to inoculate additional cellulose-fed serum bottles containing ~2.4g l^{-1} cellulose paper.

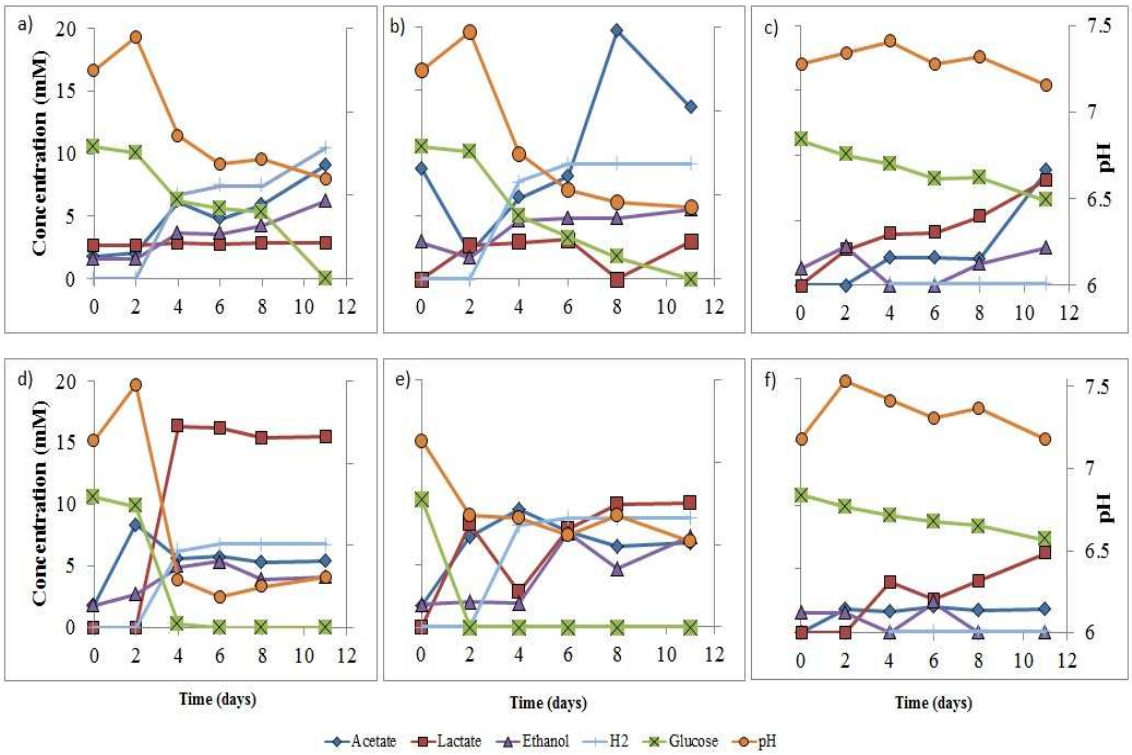


Figure 6.1a-f: Representative fermentation profiles tracked over ~11 days from six serum bottles. The primary y-axis (left) designates mM concentrations andthe secondary y-axis (right) represents pH. Concentrations of fermentation products are displayed on the x-axis. Acetate is shown with (dark blue diamonds), lactate (red squares), ethanol (purple triangles), H_2 (light blue lines), and glucose (green squares with x's). Corresponding pH is indicated by orange circles. (a-b) show cellulolytic cultures grown with ~2.4g l^{-1} cellulose powder that were capable of cellulose fermentation while (c) shows a non-cellulolytic culture that was grown in the presence of ~2.4g l^{-1} cellulose powder that was not capable of cellulose fermentation. (d-e) Show cellulolytic cultures grown with ~2.4g l^{-1} cellulose paper that were capable of cellulose fermentation while (f) shows a non-cellulolytic culture that was grown in the presence of ~2.4g l^{-1} cellulose paper that was

not capable of cellulose fermentation. Concurrent cellulolytic serum bottles were used to inoculate MECs with thermophilic ARB- results are shown in Figure 6.2a-b.

Initial growth and current generation from coupling cellulose fermentation with anode respiration

Results indicate that a consortium of cellulose fermenting bacteria and ARB were capable of producing a sustained current density (*j*) of ~6.54 ± 0.15 A m^{-2} in duplicate MECs using cellulose as the only substrate (Figure 6.2a-b). This is higher than previously reported cellulose-fed thermophilic MFCs (0.4 A m^{-2}) (Mathis 2008) and cellulose-fed mesophilic MFCs (0.05 A m^{-2}) (Ren 2008) and (< 0.18 A m^{-2}) (Rismani-Yazdi 2007). In addition to operating under thermophilic conditions, the higher *j* observed in this study may be the result of MEC mode of operation which limits O_2 contamination and allows for the potential of the anode to be poised at a specific voltage. For comparison, previous cellulose-fed mesophilic MECs produced current densities up to 1.8 A m^{-2} (Niessen 2005).

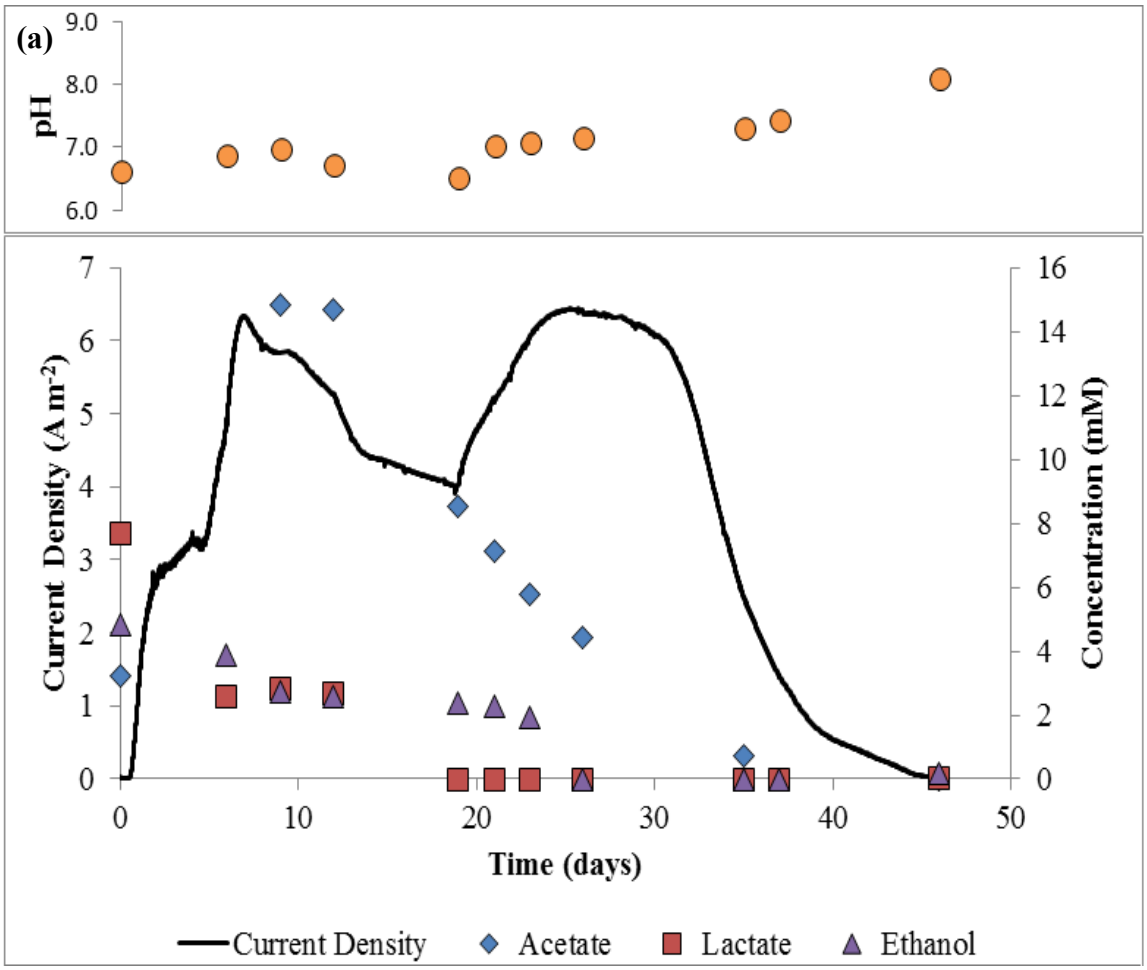


Figure 6.2a: Cellulose-fed MEC 1: current generation (black line) from cellulose-fed MEC along with concentrations of fermentation byproducts in mM are shown: acetate (blue diamonds), lactate (red squares), and ethanol (purple triangles). Corresponding pH is indicated by orange circles.

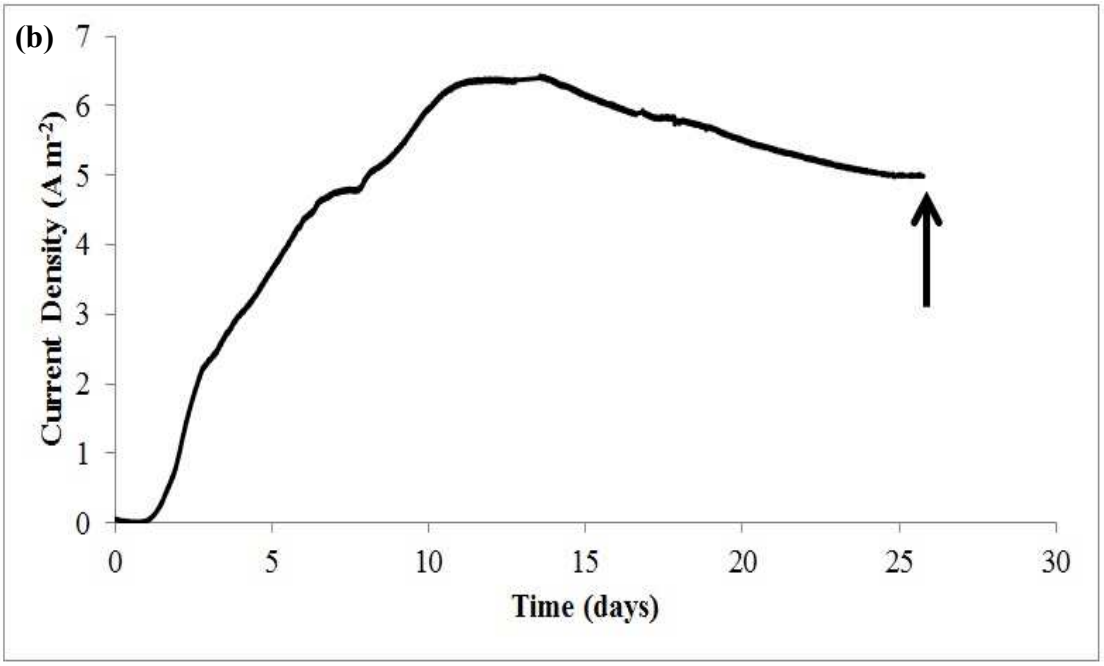


Figure 6.2b: Cellulose-fed MEC 2: current generation (black line) from duplicate cellulose-fed MEC. Black arrow indicates when biofilm was sacrificed for SEM and CLSM.

Figure 6.2a-b show MECs that were incoculated with 200 ml cellulolytic culture media containing bacteria and ~2.4g l^{-1} cellulose, and 150 ml ARB culture media containing thermophilic ARB. Figure 5.2a shows that acetate concentrations rose as cellulose fermentation occurred in MEC 1, and then fell as it was consumed by ARB for *j*. The initial acetate concentration (2 mM) is from fermentation of cellulose in the serum bottles prior to transfer to the MEC. However, since the cellulose paper was not completely degraded in the serum bottles before transferring to the MEC, an increasing acetate concentration (~15 mM) is the result of cellulose fermentation in the anode compartment. In addition, pH measurements indicate the acetate production correlated

with decreases in pH, while acetate and lactate depletion correlated with a rise in pH. A drop in pH between days 9-20 resulted in a decrease in *j*. This indicates that the MEC becomes pH inhibited as acids are produced from cellulose fermentation, but recovers as those acids are consumed. The limited anode surface area in comparison to the bulk volume created a scenario where fermentation products could accumulate faster than they were consumed resulting in acidic conditions within the biofilm anode.

During MEC operation, there was a gradual decrease in ethanol concentration; first coupled with an increase in acetate, then with a decrease in acetate concentration. Ethanol concentrations do not rise during the fermentation process within the MEC; therefore, it is unclear whether ethanol production occurs during batch MEC operation. The ethanol that came into MEC 1 from the cellulolytic media bottles may have been gradually fermented into acetate or consumed by other ARB in the reactor (Kim 2007, Parameswaran 2011). By day 37, all acetate, lactate, and ethanol concentrations had reached undetectable levels while TCOD analysis revealed that the media still contained reduced electron equivalents- suggesting that biomass decay sustained current production $< 1A\ m^{-2}$ for nine days.

There was no presence of H_2 or CH_4 gas in the anode of either MEC. Previous research indicates that thermophilic ARB are capable of H_2 consumption (Zavarzina 2007) and that homoacetogens can produce acetate from H_2 in MECs containing mixed cultures. Thus, it is likely that any H_2 produced during cellulose fermentation was quickly

consumed by homoacetogens or for anode respiration. The absence of CH_4 indicates that methanogenic Archaea were likely not present in the MECs.

TCOD analysis indicated a coulombic efficiency (CE) of 84 ± 0.3% and a coulombic recovery (CR) of 54 ± 11.6%. The lower CR values for MEC 2 (45.9%) compared to MEC 1 (62.3%) are the result of harvesting the anodes from MEC 2 prior to the MEC reaching ~0 A m^{-2}. Closing the carbon balance, and thus accounting for all electrons from cellulose fermentation, is a common challenge in cellulolytic research (Hogsett 2012). However, previous reports show that approximately 15-26% of electrons lost can be attributed to biomass (Lee 2008). Therefore, it is likely that missing (~14%) electrons in the CE calculation were contained in non-decayed biomass.

During runs for both MEC 1 (day 9) and MEC 2 (day 14), cyclic voltammetry (CV) scans at 1 mV s^{-1} and 10 mV s^{-1} were conducted to electrochemically characterize the anode biofilms of both MECs (Figure 6.3). At the end of the run for MEC 1, ~ day 37, biomass was collected from the anode biofilm and bulk media for the characterization of the microbial community using pyrosequencing (Figure 6.4). Lastly, for the cellulose-fed MEC 2, both anodes were sacrificed at day 25 (indicated by black arrow in Figure 6.2b) for the purpose of microscopic characterization of an active biofilm anode with scanning electron microscopy (SEM) (Figure 6.5a-d) and confocal laser scanning microscopy (CLSM) (Figure 6.6).

Characterization of the biofilm anode using cyclic voltammetry, pyrosequencing, scanning electron microscopy, and confocal laser scanning microscopy

Low-Scan Cyclic Voltammetry (LSCV) revealed a midpoint potential (E_{KA}) of -0.17 ± 0.003 V vs SHE for MECs 1 and 2 (Figure 6.3). This, coupled with the similar j (~6.64 A m^{-2} for MEC 1 and 6.43 A m^{-2} for MEC 2), indicates that the ARB present in these two MECs have similar electrochemical properties and thus the community composition may be similar to one another. In addition, the midpoint potentials of MECs 1 and 2 are identical to the midpoint potential reported for *Thermoanaerobacter pseudethanolicus* reported in Chapter 5- indicating the this ARB is likely present in both MECs.

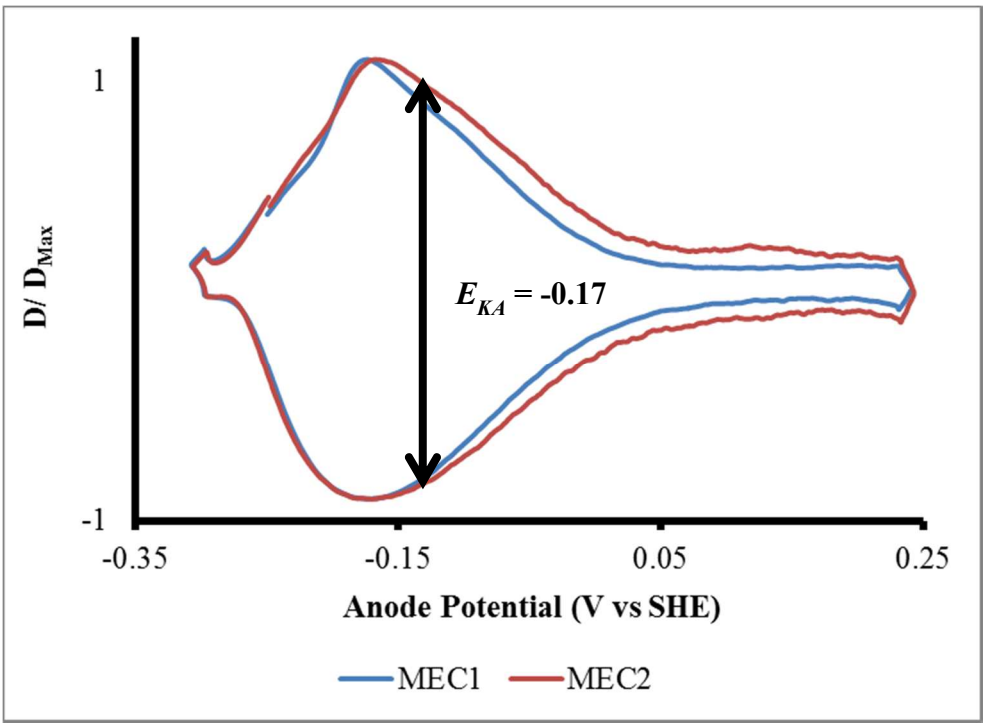


Figure 6.3: Derivative for LSCV at 1 mV s^{-1} for MECs 1 and 2 as normalized to D/D_{Max}. Black arrow indicates midpoint potential (E_{KA}).

Sequence analysis from MEC 1 showed that both the bulk media and biofilm anode were nearly completely inhabited by bacteria from the Firmicutes phylum (98.1%) with Proteobacteria (1%) and unassigned bacteria (0.9%) making up a small portion of the microbial population. The microbial community of the biofilm anode was more concentrated with Firmicutes (99.7%) than the anode bulk media (96.5%), while the anode bulk media contained Proteobacteria (1.9%) and the biofilm anode did not contain Proteobacteria. The anode biofilm is suspected to contain more ARB than the anode bulk media because ARB require the anode for respiration. Also, the bacteria responsible for

fermentation are expected to be ubiquitous throughout the MEC and occupy a higher percentage of the anode bulk media microbiome.

Results indicate that the biofilm anode contained a highly enriched culture of bacteria from the Peptococcaceae family (49.5%, light blue in Figure 6.4), which is likely *T. ferriacetica*, and from the Thermoanaerobacteraceae family. Interestingly, presence of the *Thermoanaerobacter* genus (brown in figure 6.4) is indicated in both the biofilm anode (47.4%) and the anode bulk media (1.5%). *Thermoanaerobacter* species, including *Thermoanaerobacter pseudethanolicus*, was implemented in Chapter 5 for its ability to ferment cellulose fermentation products while simultaneously performing dissimilatory metal reduction (Roh 2002). It is likely that this bacterium was selected for in the cellulolytic fermentation bottles and functioned as both a fermenter and ARB once it was used to inoculate the MECs.

Another major inhabitant of the biofilm anode was *Tepidmicrobium* (2.7%) from the Tissierellaceae family (pink in figure 6.4) which also made up a large portion of the anode bulk media (43.9%). Members of the *Tepidmicrobium* genus, including *Tepidmicrobium ferriphilum* and *Tepidmicrobium xylanilyticum*, have been reported as capable of oxidizing proteinaceous substrates or carbohydrates while simultaneously reducing either 9,10-anthraquinone 2,6-disulfonate (AQDS) or Fe(III) oxides (Niu 2009, Slobodkin 2006). The presence of these bacteria in both the anode bulk media and the biofilm anode is indicative of the cellulose fermentation products present and bacterial decay which was a major source of electrons during the final stages of MEC operation, when the biomass samples were collected.

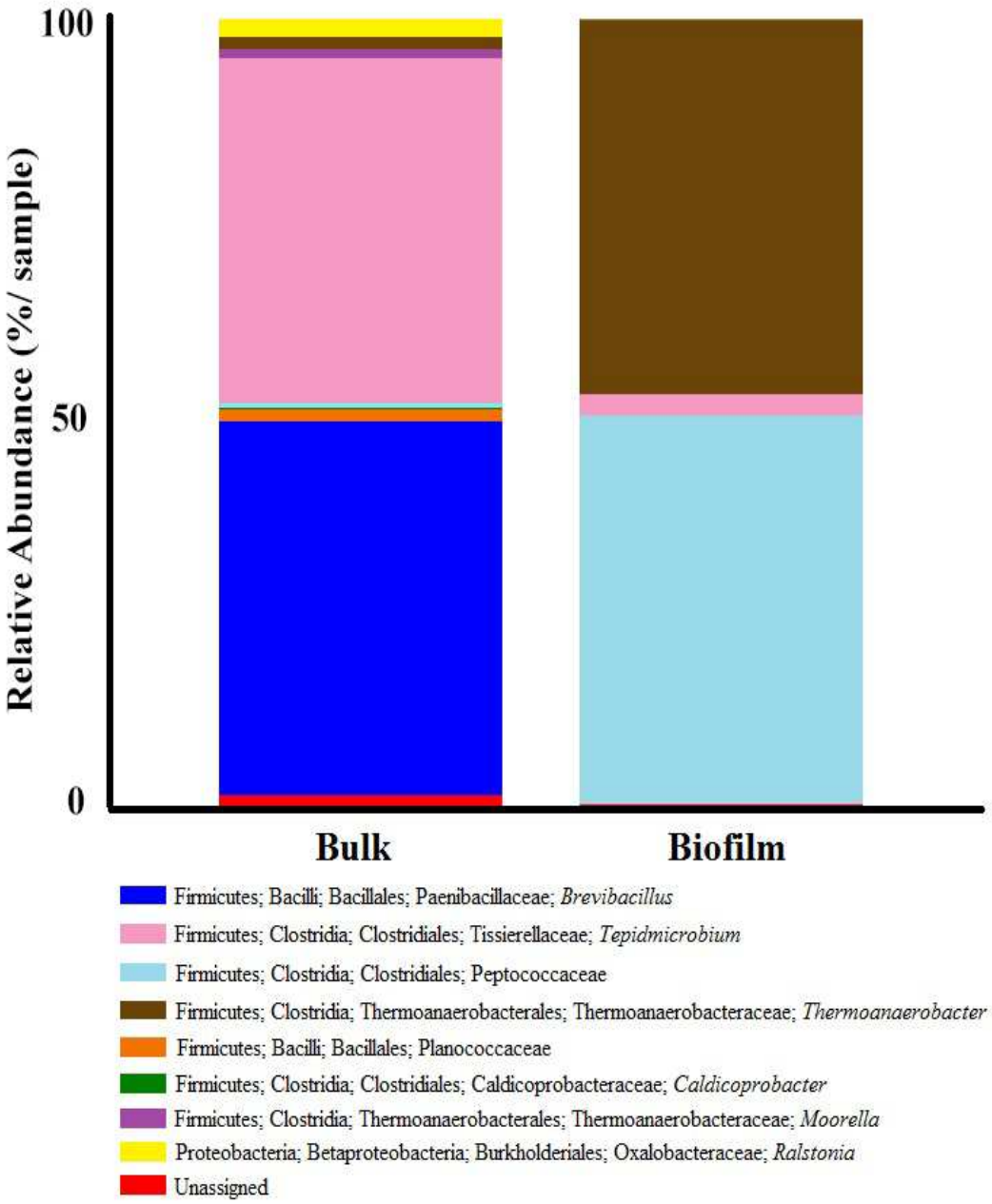


Figure 6.4: Overview of bacterial community for samples taken from either the anode bulk media ("Bulk") or the biofilm anode ("Biofilm").

For the anode bulk media, the *Brevibacillus* genus was the most abundant (47.5%, dark blue in figure 6.4). *Brevibacillus*, including *Brevibacillus* sp. strain JXL and *Brevibacillus laterosporus*, has been reported to produce cellulosomes (Liang 2009, Kato 2005) and is likely the key microbial player for cellulolytic activity in the MECs. *Ralstonia* (yellow in figure 6.4), a Gram-negative bacterial genus, was also present (1.9%) in the bulk anode; however its role remains unclear. Members of the *Ralstonia* genus, including *Ralstonia paucula*, have been identified in mixed thermophilic lipolytic cultures and may have played a role in fermentation of lipids from microbial decay (Hamid 2003). The Planococcaceae family (orange in figure 6.4) accounted for a small portion of the anode bulk media (1.7%). Members of this family vary in Gram stain, morphology, and fermentative ability (Shivaji 2013). *Caldicoprobacter* made up only a small portion (0.2%, green in figure 6.4) of the microbiome in the anode bulk media with members of this genus, including *Caldicoprobacter oshimai* and *Caldicoprobacter algeriensis*, reported as thermophilic, xylanolytic, fermentative bacteria (Bouanane-Darenfed 2011, Yokoyama 2010). Lastly, members of the *Clostridium* genus were found to be present in the anode bulk media; however, its concentration in the community was < 0.1%. Members of the *Clostridium* genus, including *Clostridium thermocellum*, produce cellulosomes and are well documented as cellulolytic thermophiles (Viljoen et al., 1926, Akinosho 2014).

Scanning Electron Microscopy (SEM) analysis reveals a biofilm which is composed of a diverse set of cell morphologies. Present are cocci (Figure 6.5a-b) and bacteria with medium, rod-shaped cells (Figure 6.5c)- a similar in morphology to *T.*

ferriacetica shown in Chapter 3 (Parameswaran 2013, Zavarzina 2007). Lastly, present are bacteria containing long, rod-shaped structures with spore like appendages (white boxes in figure 6.5d) which may indicate the presence of cellulosomes (Freier 1988).

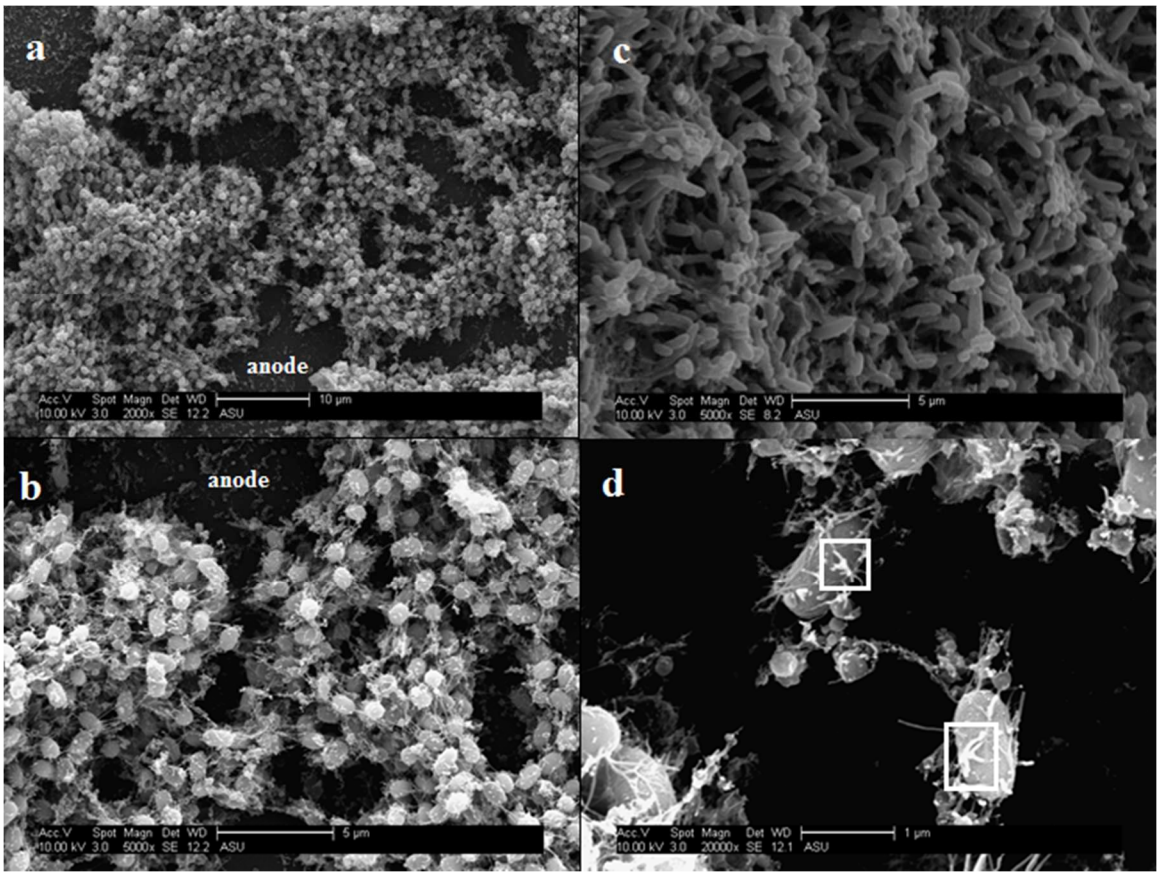


Figure 6.5a-d: SEM images reveal a biofilm anode with diverse bacterial morphologies. (a) 2k X magnification shows stacks of cocci occupying the anode surface. Anode surface is indicated by "anode". (b) 5k X magnification reveals coccus shaped bacteria are approximately 1-2 µm in diameter. Anode surface is indicated by "anode". (c) 5k X magnification reveals many bacilli occupying biofilm anode. (d) 20k X magnification

reveal two bacilli that have an appendage which appears to branch from one cell to another. White squares indicate shapes that may be cellulosomes.

Confocal Laser Scanning Microscopy (CSLM) LIVE/DEAD analysis (Figure 6.6) reveals heterogeneous biofilm morphology with peaks and valleys. Active biofilm thickness (L_f) ranges between 40 µm to 60 µm. Similar L_f has been observed in thermophilic biofilms under similar operating conditions in Chapter 3 and (Parameswaran 2013). This L_f is sufficient to cause a H^+ gradient within the biofilm that may limit j within the MEC (Marcus 2011) as is observed in MEC 1 when pH temporarily drops due to the accumulation of acids from cellulose fermentation.

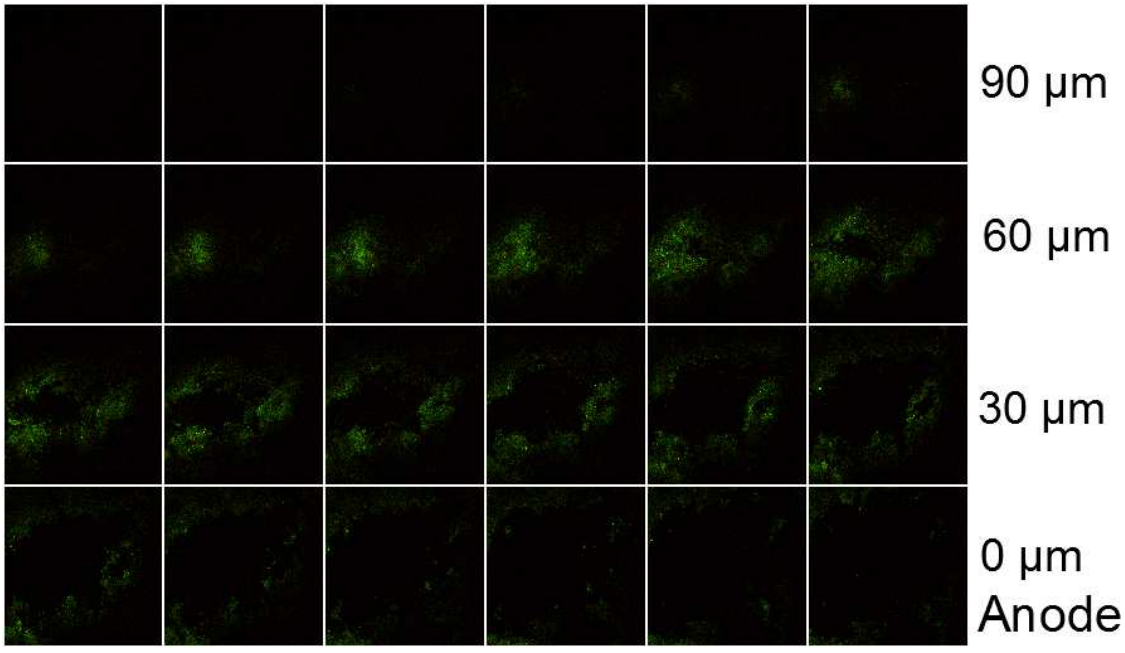


Figure 6.6: CLSM LIVE/DEAD analysis reveals an active biofilm layer approximately 40-60 µm thick.

Discussion

Many factors play a crucial role in the advancement of MEC technology as a possible solution for producing *j* or H_2 from cellulosic waste streams. These factors include: the operating temperature, bacterial consortia, and dimensional properties of thermophilic MECs. Although previously reported literature indicates that optimal activity for thermophilic ARB is at 60 °C (Parameswaran 2013, Marshall 2009, Mathis 2008, Zavarzina 2007), other reports indicate that cellulase activity may be optimal in other thermophilic and hyperthermophilic bacteria at higher temperatures (Johnson 1981, Curatolo 1983, Basen 2014, Blumer-Schuette 2012). Given that there are many potential thermophilic ARB, it may be possible to increase *j* or fermentation product utilization by employing a variety of dissimilatory metal reducing bacteria (Niu 2009, Roh 2002, Slepova 2009, Slobodkin 2006).

Many studies on cellulolytic microbial activity focus on production of fermentation products as their ends rather than *j* in MECs (Johnson 1981, Curatolo 1983, Florenzano 1984, Raman 2011, Li 2012). Future research should also focus on optimizing *j* from MECs by focusing on MEC geometry and design. For example, previous reports have indicated that anode surface area should be optimized to account for the rate of production of acids and alcohols from cellulose fermentation (Mathis 2008) compared to the amount of surface area needed for ARB to consume those fermentation products to produce *j*. Here, thermophilic cellulolytic MECs are shown to exhibit high *j*, CE, and CR. However, research into other potential bacterial consortia, temperatures, and MEC geometries is crucial to the optimization of *j* in cellulolytic

MECs. Thus, future studies should focus on analyzing the kinetics of: cellulase activity, fermentation product formation and consumption, anode respiration, bacterial growth, and nutrient balancing.

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Chapter 7: Conclusion and Future Outlook

Implications of MXC Research Using Thermophilic Bacteria

Throughout this dissertation, the use of microbial electrochemical cells (MXCs) has proven to be a powerful analytic tool for discovering fundamental properties about anode respiring bacteria (ARB). In addition, fundamental knowledge about ARB helps in the development of MXC technology for the purpose of enhancing energy recovery from diverse wastes. From this perspective, the practical importance of using MXC technology for fundamental and 'use-inspired' reseach is elucidated. MXCs are shown to be beneficial for a society with perpetually changing energy demands and needs.

Researching novel ARB may lead to new discoveries and catalyze the breakthrough needed to make MXC technologies energy neutral or positive, and thus increase their viability in the commercial world (Torres 2014). Researching Gram-positive bacteria, extremophiles, thermophiles, and other unique ARB is paramount to advancing the field. For example, by researching new organisms, including Gram-positives, new external electron transport (EET) pathways, beyond the three that are currently well documented within the field, may be discovered (Lovley 2008, Mohan 2014, Schroder 2007, Torres 2010, Parameswaran 2013). This research may also shine a light on the potential of ARB to use multiple redox pathways or processes to perform anode respiration (Badalamenti 2013, Fu 2013, Yoho 2014, Yoho 2015).

As was shown in Chapters 3 and 4 of this dissertation, using thermophilic ARB with larger pH ranges allows researchers to probe into the EET limitations of biofilm anodes. For example, since *T. ferriacetica* is not limited by buffer at high bicarbonate

concentrations, we are better able to probe other electron transport limitations occurring within the biofilm at non-H^+ limiting conditions. In addition, as was discussed in the introduction to this dissertation, thermophiles were likely the first inhabitants of Earth and existed ~1 billion years prior to the accumulation of oxygen in the Earth's atmosphere (Seckback 2004, 2006). Respiration in these organisms likely occurred via EET to a diverse set of metal oxides (Seckback 2004, 2006). New discoveries regarding the functionality of thermophilic ARB may provide insight into some of the earliest forms of respiration.

Discovering the properties and physiology of unique ARB also enables researchers and engineers to recover more energy from an increasingly diverse range of contaminants. As shown in Chapter 3, *T. ferriacetica* is more able to capture energy as current at a lower alkalinity than is *Geobacter sulfurreducens* due to the increased rate of H^+ diffusion experienced at high temperatures (Torres 2008). The studies in Chapter 5 reveal that a fermentative, thermophilic ARB called *Thermoanaerobacter pseudethanolicus* is capable of recovering energy as current from fermentable substrates, including sugars (Lusk 2015). And Chapter 6 shows how we can take the information gathered in Chapters 3-5 to develop an intelligently engineered thermophilic microbial consortium to recover energy from solid wastes (cellulose). This discovery opens up the possibility of using thermophilic MXCs to recover energy from a diverse array of 'real life' thermophilic wastewaters including agricultural and food manufacturing wastes (Juteau 2006, Linke 2006, Xiao 2009).

Future Research

The research methods in this dissertation set a good model for how to proceed with thermophilic research in the field of MXC technology. First, unique dissimilatory metal reducing bacteria should be discovered since they are potential candidates for ARB. This can happen either via bio-prospecting- where a research team isolates bacteria from samples- or by conducting *in silico* data searches through previously published literature. Once potential ARB are implicated, they should be characterized in MXCs using electrochemical techniques including cyclic voltammetry (CV), chronoamperometry (CA), and potentially electrochemical impedance spectroscopy (EIS) (Badalamenti 2013, Marsili 2010, Parameswaran 2013, Srikanth 2008, Yang 2012, Yoho 2014, Yoho 2015). If the bacteria are discovered to perform anode respiration, then their genome should be sequenced to enable genetic characterization, as was shown in Chapter 2 (Lusk 2015, Badalamenti 2015). Finally, using the newly discovered electrochemical and genetic data, these microoganisms should be used to develop 'use-inspired' technologies including MXCs which are capable of sustainably recovering energy from diverse wastes, such as in Chapters 4, 5, and 6.

In addition to the research presented in this dissertation, it is also important to acquire additional data in regards to ARB function and performance in MXCs. For example, the data provided by mapping the genome is limited in that it only gives us a glimpse into the potential genetic tools that ARB may be using when grown as biofilm anodes. In order to develop a more fundamental understanding of the processes occurring during anode respiration, it is important to recover RNA from biofilms and bulk media

for a transcriptomic analysis of the genes that are being transcribed by fermentative bacteria and ARB (Qiao 2009). In addition, it is also essential to probe into the proteomic data of these bacteria to gather a more complete understanding of which proteins are being translated and expressed during anodic fermentation and anode respiration (Costa 2015, Vecchia 2014).

Future research directions should have two primary goals:

1. Discover new ARB

2. Optimize energy recovery from diverse wastes using MXCs.

Here, a list of potential ARB is identified using an *in silico* probe of previously characterized dissimilatory metal reducing thermophilic bacteria. A comprehensive list of other dissimilatory metal reducing thermophilic bacteria can be observed in (Amend 2001, Slobodkin 2005).

Organism	Temp. (◦C)	Metabolism	Electron sources	Electron acceptors	pH range	Source
Tepidimicrobium ferriphilum	26-62	Anaerobic Respiration	tryptone, Casamino acids, yeast extract, beef extract, casein hydrolysate, proline, L-valine and n-propanol	AQDS, Fe(III) oxide, Fe (III) citrate, Fe (III) EDTA, Fe (III) nitrilotriacetate	5.5-9.5	Slobodkin 2006
Tepidimicrobium xylanilyticum	25-67	Fermentation, Anaerobic Respiration	xylan, xylose, glucose, cellobiose, peptone, tryptone, Casamino acids, yeast extract, beef extract, casein hydrolysate, L-cysteine, L-serine, L-lysine, L-glycine, L-threonine, L-methionine and pyruvate	AQDS, Fe(III), [acetate, ethanol, butyrate, hydrogen] [fermentation products]	5.8-9.3	Niu 2009

Organism	Temp. (◦C)	Metabolism	Electron sources	Electron acceptors	pH range	Source
Carboxydothermus siderophilus	52-70	Anaerobic Respiration	CO, yeast extract, glucose, Xylose, lactate	AQDS, Fe(III)	5.5-8.5	Slepova 2009
Thermolithobacter ferrireducens	50-75	Anaerobic Respiration	H_2, formate	AQDS, Fe(III), Thiosulfate, fumarate	6.5-8.5	Sokolova 2007
Bacillus thermoamylovorans SKC1	50	Fermentation, Anaerobic Respiration	Yeast extract, arabinose [glucose, cellobiose, xylose, fructose] [Fermentative growth only]	Fe (III) citrate, Fe (III)-EDTA, Cr(VI), Te(IV), Se(IV)	6-8	Slobodkina 2007

Table 7.1: List of potential thermophilic ARB for study in MXCs.

After the bacteria in Table 7.1 are characterized, then an intelligently selected microbial consortium can be assembled for the purpose of converting diverse wastes into electrical current or H_2 (Miceli 2014). This research should coincide with 'good' engineering techniques for the development of optimized MXCs. This includes the development of MXC designs that eliminate Ohmic resistance and ionic resistance by assuring that the anode and cathode are assembled in close proximity and that ion exchange membranes with proper temperature, pH, and salinity tolerance are employed. In addition, the anodic surface area needs to be optimized so that the metabolism of ARB is not inhibited by lack of surface area on the anode (Wei 2011, Zhang 2010, Zhou 2011). Also, cathodic conditions must be optimized so that the MXC is not limited by the cathodic section of the MXC as has been indicated as a primary source for overpotential in MXCs (Popat 2012, Popat 2014). Keeping these goals in mind, coupled with the discovery of new ARB, MXC technology is a valuable tool for discovering fundamental properties of dissimilatory metal reducing bacteria and can be a viable renewable energy option for a diverse set of waste water applications.

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APPENDIX A

ANOVA AND POST-HOC RESULTS FOR BICARBONATE BUFFER EXPERIMENTS SHOWN IN FIGURE 3.2 FROM CHAPTER 3

One-way ANOVA and post-hoc Tukey HSD Test statistical tables for Figure 3.2

Input Data	Bicarbonate Buffer Concentration (mM)			
	10	25	50	100
j/j_{max} value (%)	56.8	70.3	85.2	100
	53.3	82.8	98.6	100
	69.9	82.2	95.1	100
	61.7	78.6	84.8	100
	64.9	88.2	94.7	100

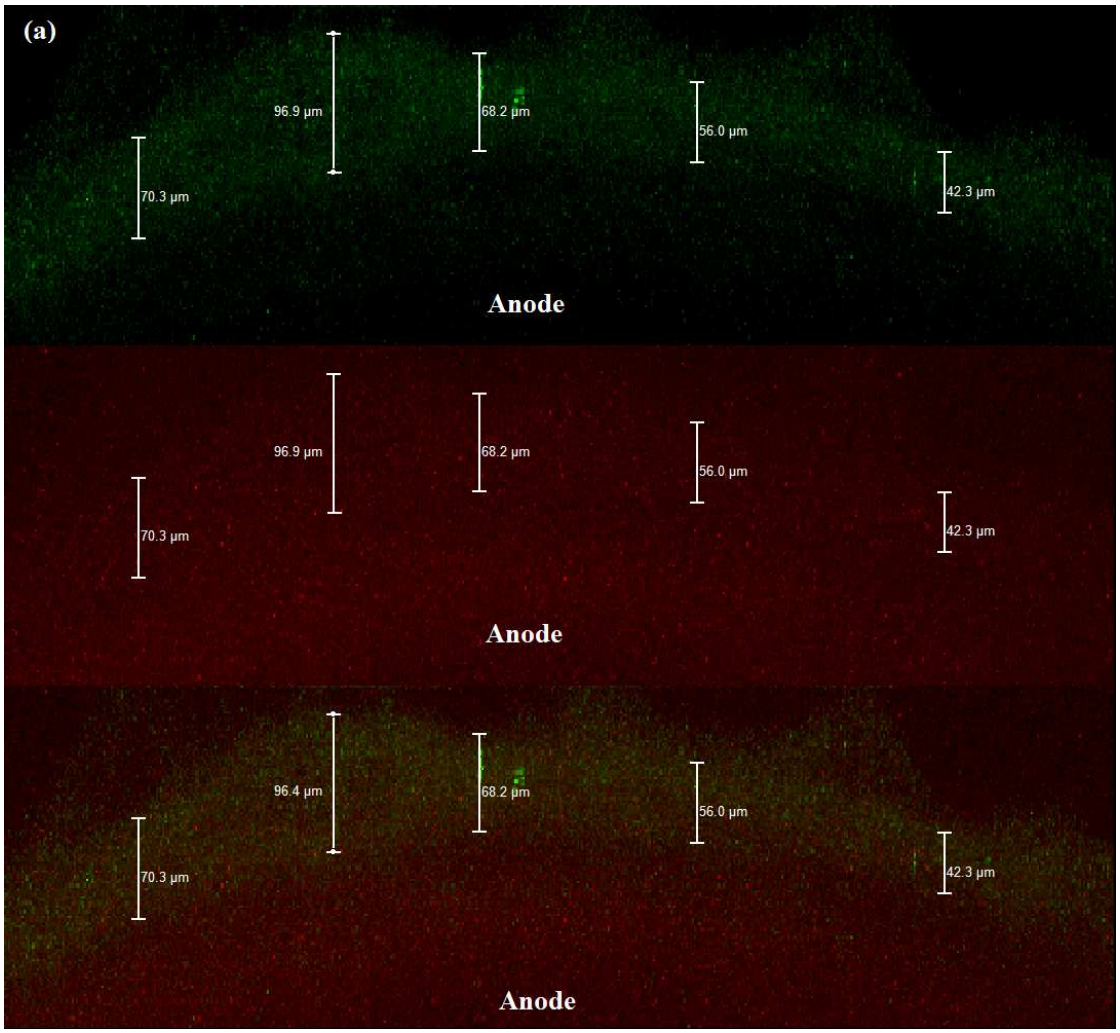
Descriptive Stats	Bicarbonate Buffer Concentration (mM)				
	10	25	50	100	Pooled Data
observations (N)	5	5	5	5	20
sum ($\sum\chi_i$)	306.6	402.1	458.4	500	1,667.10
mean (χ)	61.32	80.42	91.68	100	83.355
sum of squares ($\sum\chi_i^2$)	18,972.04	32,511.97	42,184.14	50,000.00	143,668.15
sample variance (s^2)	42.832	43.772	39.507	0	247.7384
Sample std.dev. (s)	6.5446	6.616	6.2855	0	15.7397
std. dev. of mean (SE_χ)	2.9268	2.9588	2.8109	0	3.5195

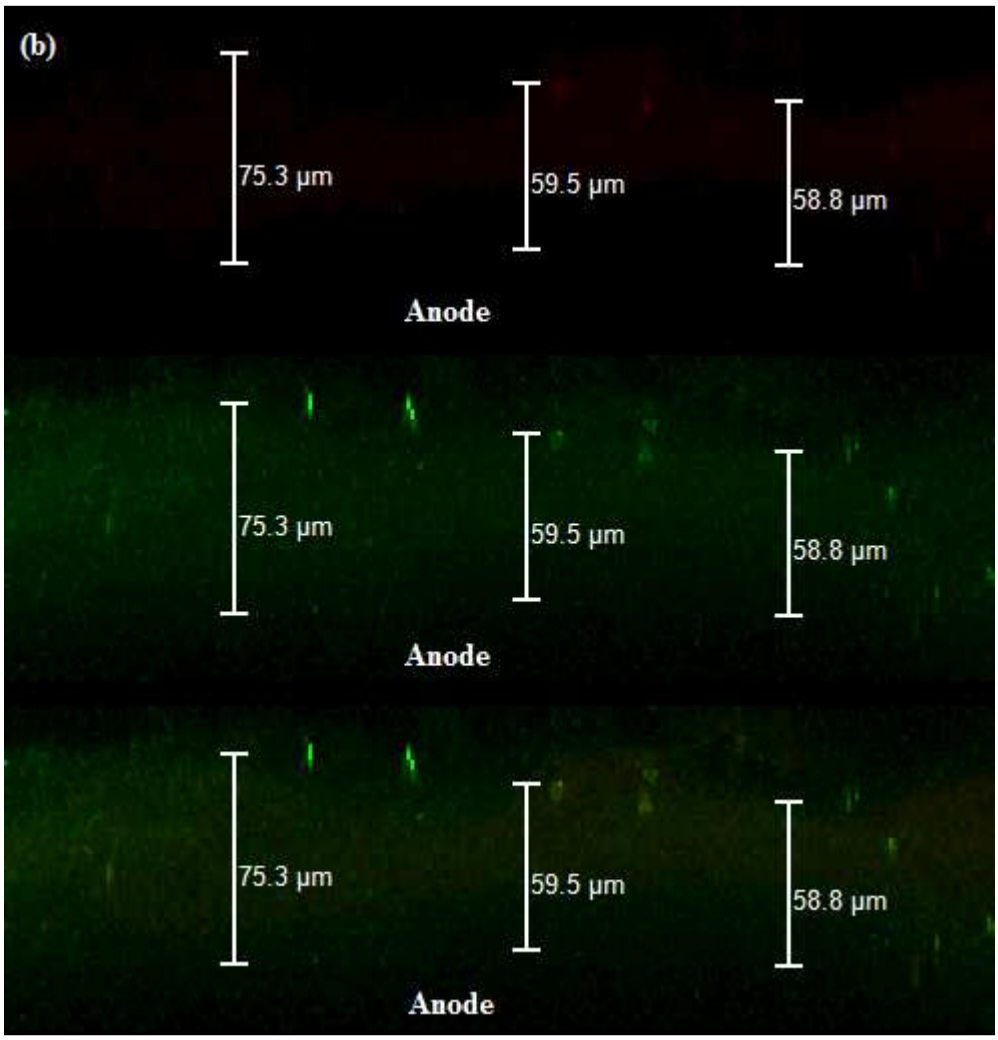
ANOVA Stats	sum of squares (SS)	degrees of freedom (v)	mean square (MS)	F statistic	p-value
Treatment	4,202.59	3	1,400.86	44.4327	5.53E-08
Error	504.444	16	31.5277		
Total	4,707.03	19			

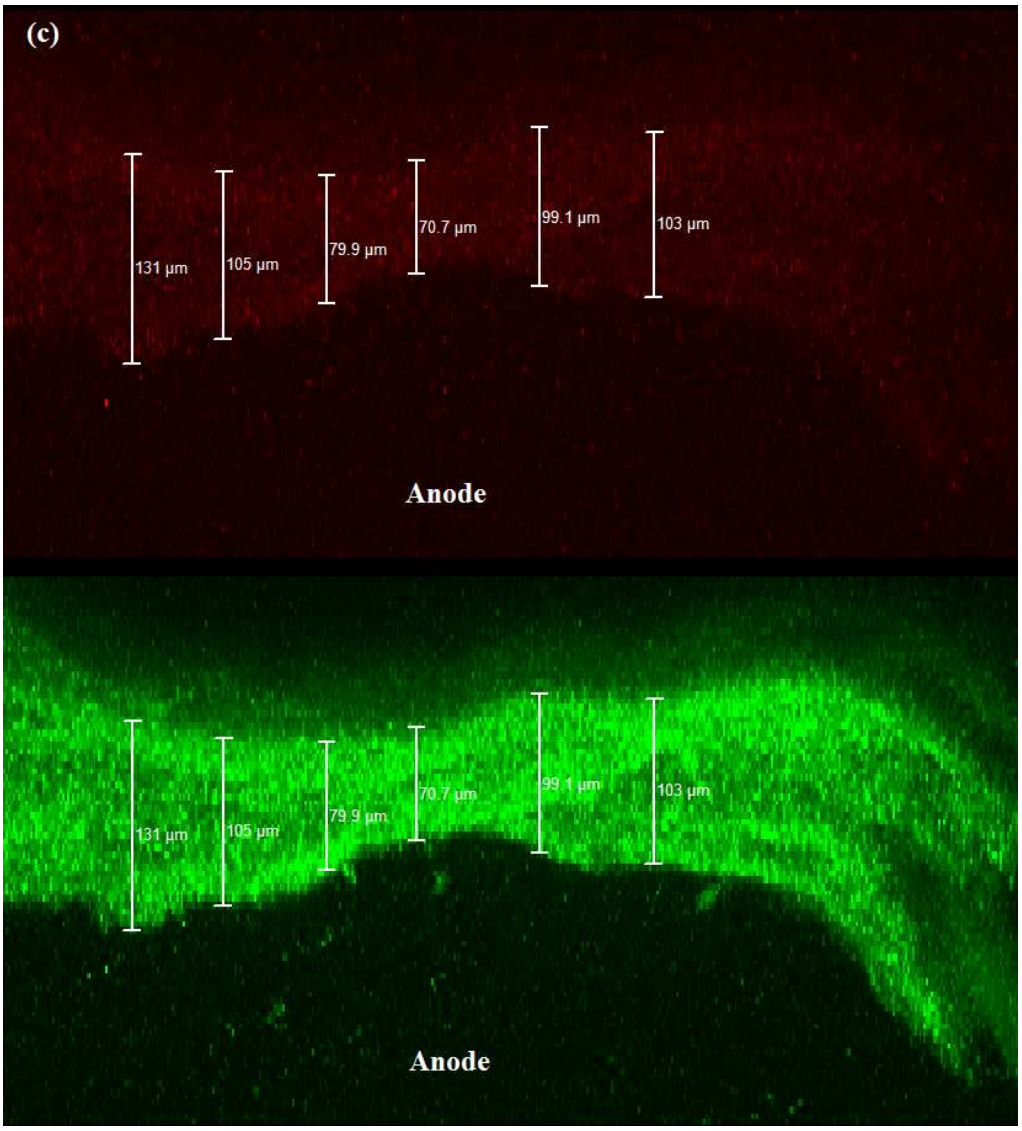
Bicarb. Conc. (mM)	Tukey HSD Q statistic	Tukey HSD p-value	Tukey HSD inferfence
10 vs 25	7.6063	0.001005	p<0.01
10 vs 50	12.0904	0.001005	p<0.01
10 vs 100	15.4037	0.001005	p<0.01
25 vs 50	4.4841	0.027347	p<0.05
25 vs 100	7.7974	0.001005	p<0.01
50 vs 100	3.3133	0.129495	insignificant

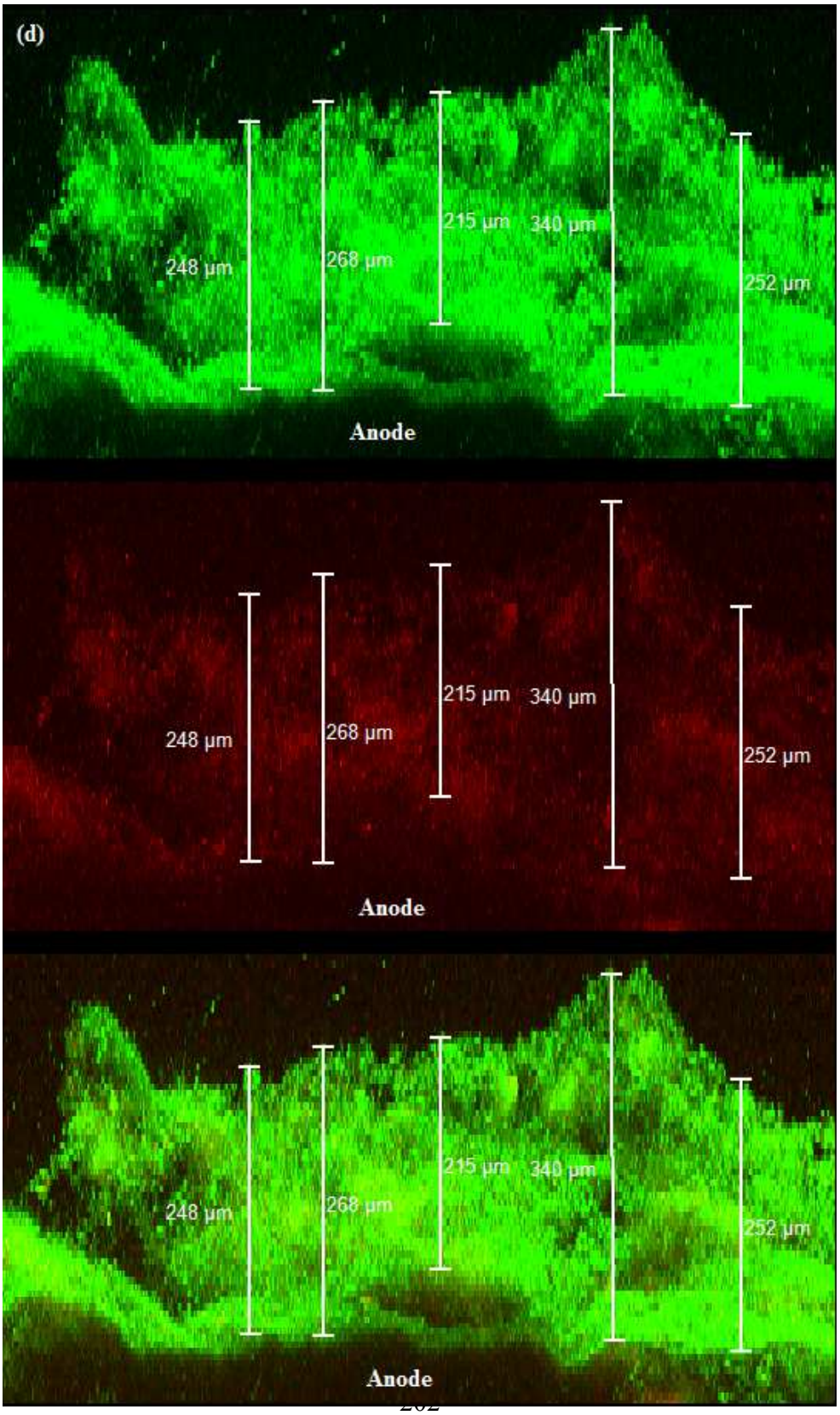
APPENDIX B

CONFOCAL LASER SCANNING MICROSCOPY RAW IMAGES FROM CHAPTER 3


(a)
96.9 µm
68.2 µm
56.0 µm
70.3 µm
42.3 µm
Anode
96.9 µm
68.2 µm
56.0 µm
70.3 µm
42.3 µm
Anode
96.4 µm
68.2 µm
56.0 µm
70.3 µm
42.3 µm
Anode


(b)
75.3 µm
59.5 µm
58.8 µm
Anode
75.3 µm
59.5 µm
58.8 µm
Anode
75.3 µm
59.5 µm
58.8 µm
Anode


(c)
131 µm
105 µm
79.9 µm
70.7 µm
99.1 µm
103 µm
Anode
131 µm
105 µm
79.9 µm
70.7 µm
99.1 µm
103 µm
Anode


(d)
248 µm
268 µm
215 µm
340 µm
252 µm
Anode
248 µm
268 µm
215 µm
340 µm
252 µm
Anode
248 µm
268 µm
215 µm
340 µm
252 µm
Anode

APPENDIX B: Confocal Laser Scanning Micrographs for *T. ferriacetica* biofilm at corresponding buffer concentrations. All pictures are shown as a cross section of the z-dimension with red = dead/ inactive biomass and green = live/ active biomass. The location of the anode is marked with "Anode". Displayed for all conditions are the dead segment, the live segment, and/or an overlay of the two. All measurements (in μm) displayed on graphs are relative to the thickness of the active layer of the biofilm. (a) at 10mM bicarbonate, (b) at 25mM bicarbonate, (c) at 50mM bicarbonate, and (d) at 100mM bicarbonate buffer.

APPENDIX C

ANOVA AND POST-HOC RESULTS FOR CONFOCAL LASER SCANNING MICROSCOPY RESULTS SHOWN IN FIGURE 3.3 FROM CHAPTER 3

One-way ANOVA and post-hoc Tukey-Kramer HSD Test statistical tables for Figure 3.3

Input Data	Bicarbonate Buffer Concentration (mM)			
	10	**25**	**50**	**100**
***Lf* value (µm)**	70	75	131	153
	97	60	105	138
	68	59	80	152
	56	50	99	173
	42	43	103	211
	95	53	226	276
	67		206	203
	51		188	235
			161	162
			53	137
			71	121
			64	224

Descriptive Stats	Bicarbonate Buffer Concentration (mM)				
	10	**25**	**50**	**100**	**Pooled Data**
observations (N)	8	6	12	12	38
sum ($\sum\chi_i$)	546	340	1,487.00	2,185.00	4,558.00
mean (χ)	68.25	56.6667	123.9167	182.0833	119.9474
sum of squares ($\sum\chi_i^2$)	39,948.00	19,864.00	221,719.00	422,447.00	703,978.00
sample variance (s^2)	383.3571	119.4667	3,404.99	2,235.90	4,250.21
Sample std.dev. (s)	19.5795	10.9301	58.3523	47.2853	65.1937
std. dev. of mean (SE_χ)	6.9224	4.4622	16.8449	13.6501	10.5758

ANOVA Stats	sum of squares (SS)	degrees of freedom (v)	mean square (MS)	F statistic	p-value
Treatment	91,927.23	3	30,642.41	15.9472	1.21E-06
Error	65,330.67	34	1,921.49		
Total	157,257.89	37			

Bicarb. Conc. (mM)	**Tukey HSD Q statistic**	**Tukey HSD p-value**	**Tukey HSD inferfence**
10 vs 25	0.692	0.8999947	insignificant
10 vs 50	3.9347	0.0414836	$p<0.05$
10 vs 100	8.0461	0.0010053	$p<0.01$
25 vs 50	4.3393	0.0208938	$p<0.05$
25 vs 100	8.0925	0.0010053	$p<0.01$
50 vs 100	4.5967	0.0132229	$p<0.05$

BIOGRAPHICAL SKETCH

Scientist, avid runner, businessman, and social entrepreneur, Brad Lusk spent 6.5 of his 10.5 years at Arizona State University (ASU) as a graduate student in the Swette Center for Environmental Biotechnology researching thermophilic bacteria in microbial electrochemical cells. During his time as a Ph.D. student, he has hosted and attended scientific conferences throughout the United States including the North American meeting for the International Society for Microbial Electrochemical Technology (ISMET) at Penn State University, the International meeting for ISMET at ASU, the Association of Environmental Engineering and Science Professors meeting at the Colorado School of Mines, and the general meeting for the American Society for Microbiology in Boston, Massachusetts. His experiences and friendships formed during his graduate studies have enabled him to travel to exotic locations in India and Pakistan, where he has given presentations about his research to a broad, global audience. He has also shared his love for science to live public audiences at local Phoenix events including IGNITE, Night of the Open Door, and COMICON. In his free time, he volunteers with a local non-profit called Camp Sparky -where he served as Chair from 2014-2015- to visit local Title 1 elementary schools through the Phoenix metropolitan area. This volunteer work allows him to spread a message of scientific awareness and emphasize the positive impacts of education to local ‘at-risk’ youth. In 2012, he turned his interest and longtime knack for vintage collectibles into a lucrative online business. He also uses his passion for nutrition and running to conquer 5Ks, 10Ks, and half marathons. With his degree, Brad is looking to travel the world to visit laboratories and industries that are interested in microbial electrochemical research in order to gain a global perspective on his field of interest and to share his experiences to spread cultural awareness.