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ABSTRACT

Bioelectrochemical systems (BESs) combine the fields of microbiology and electrochemistry for the production of electricity, gaseous fuels, or chemicals from biodegradable material. Advancing these systems towards large-scale applications requires improvements in both reactor designs and our understanding of the microbial ecology of exoelectrogenic biofilms. The work described in this dissertation addresses these areas through the development of a scalable microbial electrolysis cell (MEC), characterization of exoelectrogenic biofilms using the molecular technique fluorescent in-situ hybridization (FISH), and investigation into the current-producing and substrate-utilizing capabilities of the exoelectrogenic bacterium *Geobacter sulfurreducens*.

**Stainless Steel Brush Cathodes.** MECs are a promising alternative method for producing hydrogen (H$_2$) from biomass, yet they have been limited to the lab scale due in part to the inherent non-scalability and costs of electrodes. To address this problem, I investigated alternatives to the traditionally used platinized cathode. Large electrode surface area can be used as an alternative to precious metal catalysts to improve the H$_2$ evolution kinetics, yet electrode material properties are also important, and the nickel content of stainless steel (SS) is known to be catalytically active for H$_2$ evolution in water electrolysis under alkaline conditions. Combining these two factors, I examined high surface area SS brushes as cathodes in MECs. Using a SS brush cathode with a specific surface area of 810 m$^2$/m$^3$, a H$_2$ production rate of 1.7 ± 0.1 m$^3$-H$_2$/m$^3$-d (current density of 188 ± 10 A/m$^3$) was produced at an applied voltage of 0.6 V (mixed culture inoculum, acetate as electron donor). The energy efficiency relative to the electrical energy input was 221 ± 8%, and the overall energy efficiency was 78 ± 5% based on both
electrical energy and substrate utilization. These values compare well to previous results obtained using platinum on flat carbon cathodes in a similar system. Reducing the cathode surface area by 75\% decreased performance from 91 ± 3 A/m$^3$ to 78 ± 4 A/m$^3$. A brush cathode with graphite instead of SS and a specific surface area of 4600 m$^2$/m$^3$ generated substantially less current (1.7 ± 0.0 A/m$^3$), and a flat SS cathode (25 m$^2$/m$^3$) produced 64 ± 1 A/m$^3$, demonstrating that both the SS and the large surface area contributed to high current densities. These results demonstrated for the first time that H$_2$ production can be achieved at rates comparable to those with precious metal catalysts in MECs.

**Small-Scale MECs.** Membrane-free MECs employing SS cathodes provide a model for building larger scale reactors, but I also investigated if this simplified design could be scaled down to allow for an improved method of conducting lab scale research. I developed a new method based on using two electrodes placed ca. 0.5-cm apart in small (5 mL) serum bottles, with multiple reactors operated in parallel using a single power supply. Several anode and cathode materials were examined, with the highest current production and most robust design consisting of graphite plate anodes and SS mesh cathodes. This configuration, built entirely from commercially available materials, cost $1.50 per reactor, which is substantially less than the cost of traditional reactor designs. Operating the reactors in parallel did not lead to differences in current production or electrode potentials, which can allow for operating more than 1,000 reactors using a single power supply. This method will not only benefit established researchers with a high throughput method for conducting BES research, but it will also provide new researchers with an affordable way to become established in this field.
Microbial Ecology of Exoelectrogenic Biofilms. The bacteria that compose anodic biofilms are critical components of BESs, functioning as converters of chemical to electrical energy. To characterize exoelectrogenic biofilms, I used the molecular technique fluorescent in-situ hybridization (FISH), which involves fluorescently labeling bacteria for visual identification and quantification. In both microbial fuel cells (MFCs) and MECs, a strong correlation between the presence of the exoelectrogen G. sulfurreducens and reactor performance was apparent from FISH analysis targeting this bacterium. However, the presence and/or high abundance of a bacterium on an anode did not necessarily translate into exoelectrogenic activity, as a mixed community consisting of roughly 30% of the Paracoccus genus yielded an isolate, Paracoccus denitrificans, which was incapable of generating electrical current as a pure culture in an MEC. FISH was also used as a tool to corroborate or challenge results from 16S rDNA clone libraries. An acetate-fed MFC anode contained an abundant population of cloned sequences matching the bacterium Pelobacter propionicus, yet a species-specific FISH probe targeting this bacterium indicated the lack of this species in the mixed community. Conversely the community was found to be dominated by G. sulfurreducens when a FISH probe specific for this bacterium was tested. These results highlight the importance of using multiple molecular methods for studying mixed communities and the important role G. sulfurreducens plays in current generation.

Geobacter vs. Mixed-Culture MECs. Since G. sulfurreducens was found to be a predominant exoelectrogen based on FISH analysis of mixed communities, I examined if this bacterium could perform comparably to a mixed community in an MEC supplied acetate. Reactors with G. metallireducens, a non-H₂ oxidizing exoelectrogen, were also
included to better understand the role of H₂ recycling in membrane-free MECs. At an applied voltage of 0.7 V, *G. sulfurreducens* initially produced higher current densities than a mixed consortium, but both cultures converged to roughly 160 A/m³ after several fed-batch cycles, while *G. metallireducens* produced substantially less current. When the reactors were supplied H₂ as the sole electron donor, current was generated by *G. sulfurreducens* and the mixed consortium, while no current was produced by *G. metallireducens*, indicating that internal H₂ recycling by some exoelectrogens can increase estimates of coulombic efficiency. Cyclic voltammetry analysis of *G. sulfurreducens* and the mixed culture revealed similar oxidative current profiles, with a slightly higher current observed for the mixed culture. This larger current may have been due to a more tightly packed catalyst or biofilm on the electrode surface, and scanning electron microscopy revealed that more compact biofilms were formed by the mixed culture compared to the looser and less dense biofilms of *G. sulfurreducens*. Community analysis of the mixed culture using 16S rDNA clone libraries indicated a predominance (72% of clones) of *G. sulfurreducens* despite its relative absence (2% of clones) in the inoculum, again highlighting this bacterium as a key exoelectrogen in BESs.

**Lactate Oxidation by Geobacter sulfurreducens.** Although *G. sulfurreducens* frequently predominates in mixed-culture acetate-fed BESs, it is often found in communities supplied substrates that have been suggested unusable by this bacterium. In particular, *G. sulfurreducens* is commonly found in MFCs fed with lactate. Earlier work indicated that *G. sulfurreducens* could not reduce Fe(III) when supplied lactate, however, the genome of this bacterium encodes genes for both a putative lactate permease and lactate dehydrogenase, two genes critical for lactate utilization by bacteria of the
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Chapter 1

General Introduction

The fossil-based energy resources that the world has become dependent on are unsustainable, detrimental to human and environmental health, and causes of political and economic tensions around the world. As a result, scientists and engineers are being called upon to re-think and re-invent the way we generate energy. Solar energy is the most ideal renewable energy source because of its abundance, yet because of its other characteristic—intermittence—developing methods for extracting stored solar energy from biomass are needed. Technologies currently exist for converting concentrated biomass streams, such as plants and agricultural crops, into usable energy, but a substantial portion of biomass ends up diluted in waste streams, such as industrial, agricultural, and domestic wastewaters. Converting these unconventional energy sources using biological methods into biogas (methane and hydrogen) are two promising approaches because they allow for the separation of the desired product (biogas) from the input (wastewater). This introduction will therefore focus on established and developing technologies for harvesting energy from dilute biomass streams in order to provide a basis for comparison with the emerging bioelectrochemical methods discussed in the next chapter.
1.1 The Solar-Based Economy

In one hour, more energy hits the earth in the form of solar radiation than the world currently consumes in one year [1]. In 2001, world energy consumption reached 13.5 TW, whereas total solar income in one year reaches an estimated 170,000 TW [1, 2]. Clearly there is an abundant supply of solar energy reaching the earth, yet due to several technological and economic hurdles it is unlikely that an economy run entirely on daily solar energy income will be feasible. Solar radiation also contributes to other forms of renewable energy that with the appropriate technologies can be used to extract additional energy from the sun. Wind, produced from the flux of solar radiation, has the potential to generate 2,000 TW [2]. Biomass, which is produced through photosynthesis, is estimated to contain 20 TW, and roughly 6 TW is available in the form of hydropower from the sun-driven water cycle [2]. Most of the technologies for harnessing these available energy sources are currently being developed, with some larger scale commercialized projects already well established.

Although the argument for a solar-based economy is quite strong, one major limitation is the storage and/or transport of excess energy [1]. Solar energy is only present during the day and wind is highly unpredictable, therefore a dilemma exists of how to provide energy when neither of these sources is available. Energy production is also limited to regions in which solar income and wind production are economically viable. Transport of energy produced in the most suitable locations will be necessary for regions that lack a strong solar economy, such as the northeastern United States.
1.2 Storing the Sun as Biomass

When solar radiation is harnessed by photovoltaic cells or wind turbines, the energy captured is ultimately converted into electricity. These technologies, therefore, act as converters, transforming energy in the form of photons (photovoltaics) or kinetic energy (wind turbines) into electrical energy. Nature also developed a method for storing solar radiation, but in another form: chemical energy. Plants and other autotrophs convert carbon dioxide into biomass (i.e., carbohydrates) using solar energy through photosynthesis. Currently, many processes for converting this stored chemical energy into usable energy carriers, such as ethanol or biodiesel, are being developed [3-6]. For more dilute sources, such as domestic, industrial, and agricultural wastewaters, methods for converting concentrated biomass streams, such as gasification and pyrolysis, into energy are not used. Instead, the biomass in these streams is viewed as a liability and substantial amounts of energy are invested in order to remove and treat it. Energy recovery, if performed, for these types of waste streams is typically done through an anaerobic biological process, producing either methane or H₂, as this process allows for the separation of the desired product (gas) from the liquid phase. The technologies for producing these gases will be discussed in the next sections, but first an analysis of the energy content of wastewaters will be reviewed.
1.3 The Water Energy Nexus

The development of modern sanitation has dramatically improved the health of humans and the environment. Providing clean drinking water and adequate sanitation in developed countries has led to the construction of complex and massive water and wastewater infrastructures. To keep these systems operating, a substantial amount of energy has to be invested. In the US, roughly 5% of electricity generated is used to operate the water/wastewater infrastructure, which includes pumping, processing, filtering, and aeration [7]. Many developing countries lack access and the infrastructure to the fossil energy reserves which developed countries have used to develop their sanitation infrastructure. With close to three billion people lacking adequate sanitation, and most of them located in undeveloped countries, accessibility to energy is a critical step towards improving global sanitation [8].

The energy intensity of conventional wastewater treatment can be primarily attributed to the constant aeration of large basins to encourage organic matter removal through bacterial respiration. Removal of biomass, measured as chemical oxygen demand (COD) or biochemical oxygen demand (BOD), is a primary goal of the treatment process, as the release of this material to a receiving waterway can contribute to oxygen depletion. Engineers rely on the natural aerobic respiration of bacteria to remove this biomass by providing oxygen as a terminal electron acceptor. Aerating large basins in a process called activated sludge provides oxygen, yet this process is energy intensive. Aeration alone can account for up to 50% of a wastewater treatment plant’s (WWTP) electrical energy demand [9]. Depending on the method of aeration, the specific energy demand
can range from 0.7 – 2 kWh/kg-COD (kWh invested per kg-COD removed) [9]. Other processes within the plant, such as clarifier sludge pumps and dewatering equipment, consume considerably less than aeration, yet are still contributors to the overall energy demand. Newer alternatives to the activated sludge process include membrane bioreactors (MBRs), which aid in decreasing the footprint of a WWTP yet consume more energy than traditional aeration processes in part due to the need to reduce membrane biofouling [10, 11]. Continuing to develop more effective treatment technologies such as MBRs is part of the solution, yet reducing their energy demand is critical for implementing a sustainable infrastructure for both developed and undeveloped countries.

There is potential to convert wastewater treatment into an energy-producing process. The reason bacteria grow during the activated sludge process is because the organic matter they consume provides energy. Biomass in wastewater consists of the remains of plants, animals, grease, and other organic sources, all of which contain energy in the form of chemical bonds. Plant material, for example, consists of carbohydrates, such as glucose, and this chemical is often used as a model substrate when discussing the breakdown of organics in wastewater. One kilogram of glucose (1.06 kg-COD) contains ca. 4.4 kWh (based on complete combustion), which could yield about 1 kWh of useful energy [12]. Although using glucose provides a straightforward means for estimating energy potential, the complexity of wastewaters requires an alternative approach for more accurate energy content estimates. Examining dried wastewater using bomb calorimeters, Shizas and Bagley concluded that domestic wastewater in Canada contained more than nine times the energy that is invested to process and treat it [13]. Using improved methods to minimize losses of volatile compounds, Heidrich et al. re-visited this issue
and provided an estimate of \( \text{ca. } 7.5 \text{ kJ/L} \) for domestic wastewater and \( \text{ca. } 17 \text{ kJ/L} \) for mixed wastewater [14]. If we apply these values to the total amount of municipal wastewater treated per day in the US, which is estimated at \( 1.3 \times 10^{11} \text{ L/d} \) [15], we reach a range of approximately \( 9.8 \times 10^{11} \text{ to } 2.2 \times 10^{12} \text{ kJ/d} \) or \( \text{ca. } 100 \text{ to } 225 \text{ TWh per year} \) (0.01 TW to 0.03 TW). This is roughly 2.5% to 5.7% of total electricity production in the US in 2009 [16], although actual energy recovery would be lower due to inefficiencies in generation and utilization. Energy recovery from waste streams could provide a part of the solution to renewable energy; however, the question of which technologies are best suited to harness this energy is still an area of intense research, with methane production using anaerobic digesters already a commercially viable option.

1.4 Biological Methane Production

If we use glucose as a model substrate and we want to convert it to methane, we can see that based on the Gibbs free energy the overall reaction is highly favorable [17].

\[
\text{Glucose} \rightarrow 3\text{CO}_2 + 3\text{CH}_4 \quad \Delta G^{\circ} = -418 \text{ kJ/mol}
\]

It is important to note, however, that there is no single microbe that can perform this reaction alone. Rather, a suite of microbes working together are needed in a multi-step process [18]. In the first stage, cellulolytic and hydrolytic bacteria break down long chained substrates, such as cellulose, into simpler units, such as glucose. Next a group of primary fermentative bacteria convert these monomers into a mix of short chain fatty
acids, such as acetate and butyrate, alcohols, H₂, and other fermentation end products. In order for the final conversion to methane by a group of microorganisms called methanogens to occur, the fermentation end products must be converted to acetate, H₂, carbon dioxide, or other C₁ compounds such as formate or methanol [17, 18]. Based on thermodynamics, we can see that in order for this to occur, H₂ partial pressures must remain low. These syntrophic bacteria rely on methanogens to consume the H₂ to low enough levels for the reactions to proceed. If H₂ accumulates then H₂ production from NADH is not possible due to thermodynamic limitations. Instead alternative routes for NADH oxidation occurs, leading to the accumulation of short chain fatty acids. This buildup of acids, leads to a drop in pH, which can inhibit methanogenic growth. Careful maintenance of this syntrophic relationship is one of the challenges facing digester operators. The methane produced in digesters is typically combusted on site for the production of heat or electricity, and in some cases the heat is used to keep the digester at a constant temperature.

1.5 Biological H₂ Production

Although methane production from waste is already commercially viable, on a COD basis methane is worth about $0.11/kg CH₄-COD, whereas H₂ is worth about $0.75/kg H₂-COD [19]. This means that if one is only considering the finished product, producing H₂ from wastewater is a much better economical decision (assuming equal production costs). Furthermore, methane is primarily used as an energy source for heating or electricity production, while H₂ has an array of uses in the industrial world [20].
Upgrading fossil fuels, saturating foods, producing acid (i.e., HCl), and serving as a reductant for metal production are all applications where H₂ is needed. Unfortunately, since most renewable production methods are not commercially viable, over 95% of the H₂ used for these applications are generated from fossil fuel reformation [20]. This approach is especially problematic when H₂ is described as a clean fuel for powering H₂ fuel cell vehicles. Although the end products in these fuel cells are water and electricity, substantial greenhouse emissions still occur due to the carbon dioxide released during reformation. Alternative H₂ production methods include water electrolysis, but if fossil fuel derived electricity is used for this process, then fossil based carbon emissions must be taken into account. Biological production is another approach, yet as described in the next section, this method suffers from the endothermic nature of typical fermentation end products.

If instead we look to convert glucose to H₂ gas, we find that the reaction is not favorable under standard conditions. First, the Gibbs free energy is positive, indicating that this reaction is not spontaneous under standard biological conditions (pH 7, \( T = 25^\circ C, P = 1 \text{ bar} \)).

\[
C_6H_{12}O_6 + 12H_2O \rightarrow 12H_2 + 6HCO_3^- + 6H^+ \quad \Delta G^\circ = +3 \text{ kJ/mol}
\]

Under conditions of very low H₂ partial pressures this reaction is favorable, but in most natural and engineered environments where hydrogen accumulates, this reaction will not yield enough energy for ATP generation (ca. 40 – 50 kJ) [17]. Sufficient amounts of ATP are available if glucose is converted to other fermentation end products in addition to
hydrogen. Acetate production from glucose for example yields both a spontaneous reaction and the production of 4 mol-H₂/mol glucose. This yield can only be obtained if H₂ partial pressures are kept low because under these conditions the reducing equivalent NADH formed during conversion of glucose to pyruvate will be able to contribute electrons for the production of H₂. However, if the partial pressure of H₂ remains high, then alternative metabolic routes occur resulting in the production of various fermentation end products, such as butyrate, lactate, and ethanol. These accumulated substrates contain additional H₂ that is not recovered, reducing actual yields to a maximum of two mol-H₂/mol-glucose. Additional H₂ could be recovered from these substrates (maximum of six mol-H₂/mol-ethanol), but due to thermodynamic limitations imposed by accumulating H₂ concentrations, the H₂ remains locked within these substrates. [18].

Attempts to improve H₂ yields have included both operational and biological manipulations [21]. Strategies to reducing H₂ partial pressures include gas stripping, continuous gas collection, and H₂ selective membranes [22-24]. Genetic modification of bacteria, such as the dark fermentative clostridia, by blocking pathways towards fermentation end products other than acetate have also improved H₂ yields; however, these approaches can only gain at most an additional 2 mol-H₂/mol glucose [25]. In acetate, another 8 mol-H₂ could potentially be recovered, and finding strategies to do this is important to meeting the US Department of Energy’s goal of 10-12 mol-H₂/mol glucose [26].

Spontaneous acetate conversion to H₂ is not possible because the reaction does not yield enough energy available for ATP generation.
\[
\text{CH}_3\text{COO}^- + 4\text{H}_2\text{O} \rightarrow 2\text{HCO}_3^- + \text{H}^+ + 4\text{H}_2 \quad \Delta G^0 = +105 \text{ kJ/mol}
\]

If the partial pressure of H\(_2\) remains below 1 Pa (reaction conditions), then sufficient energy is available, yet it is difficult to create such conditions in practice. In natural environments, where bacteria and methanogens are either in close proximity or in tight aggregates, this conversion can occur [27-29]. Through the mechanism of interspecies H\(_2\) transfer, methanogens rapidly consume H\(_2\) produced by acetate oxidizers, although resulting in methane as the final end product instead of H\(_2\). Since engineered systems will likely not obtain low enough H\(_2\) partial pressures for acetate conversion to H\(_2\), additional approaches involving an input of energy are needed to improve yields.

Photofermentation is an alternative strategy for converting acetate to H\(_2\) that relies on an energy input in the form of sunlight [30-32]. Photoheterotrophic bacteria, such as \textit{Rhodopseudomonas} and \textit{Rhodobacter} use sunlight to convert fermentation end products such as acetate and butyrate into H\(_2\) [33]. Limitations to this approach include the large reactor surface area required for capturing solar energy, the production of H\(_2\) only during daylight, the need to maintain pure culture conditions, and the impact of inhibitors such as ammonia and oxygen on the H\(_2\) generating enzymes [21, 34].

Due to the limitations of both dark and photo fermentative H\(_2\) production methods and the lack of any commercial scale reactors, alternative and novel approaches are needed to improve H\(_2\) yields from waste streams. The research described in this dissertation advances forward a recently discovered approach for generating H\(_2\) gas called electrohydrogenesis, which lies at the interface of microbiology and
electrochemistry [19]. In the next chapter, I will provide a detailed overview of this technology, the reactors and materials used, and the microorganisms involved.

1.6 Dissertation Scope and Outline

The objectives of this dissertation was threefold: 1) to develop a scalable and affordable microbial electrolysis cell (MEC) design, 2) characterize the communities of exoelectrogenic biofilms, and 3) examine the current producing and substrate utilizing capabilities of the exoelectrogenic bacterium Geobacter sulfurreducens.

In Chapter 3, I showed that costly platinized cathodes can be replaced by high surface area cathodes made of stainless steel (SS) for producing H₂ in MECs. Both the material properties of the SS and the increased surface area were shown to be critical for high current densities, as large surface area graphite surfaces did not produce measurable current. Reactors with close electrode spacing and half hemispherically shaped SS brush cathodes placed directly above brush anodes obtained similar current densities as ones using a platinized cathode.

In Chapter 4, I combined the results of my Master’s thesis (membrane-free MEC design) with the SS cathodes of Chapter 3 to develop a small scale design that allows for high throughput BES research. Parallel operation using a single power supply allows for high throughput operation of these mini-MECs and improve the ability to conduct pure culture investigations.

In Chapter 5 I applied the molecular technique fluorescent in situ hybridization (FISH) to analyze the presence and abundance of both exoelectrogenic and non-
exoelectrogenic bacteria in BESs. This technique was also used to corroborate other molecular methods, such as 16S rDNA clone libraries. In a formic acid fed MFC, both clone libraries and FISH revealed an abundance of bacteria matching the genus *Paracoccus*, but when an isolate of this genus was tested in an MEC, no current was produced, suggesting growth on oxygen or syntrophic interactions with other bacteria. Conversely, the abundance of the known exoelectrogen, *G. sulfurreducens*, was shown to be correlated with reactor performance. The importance of including a second molecular technique for characterizing mixed communities, such as FISH, was shown when a culture dominated by *Pelobacter* species according to clone library results was found to lack this bacterium when examined by FISH. Instead, the closely related bacterium, *G. sulfurreducens*, was found throughout the biofilm, again highlighting this bacterium as a key exoelectrogen.

Since Chapter 5 revealed that *G. sulfurreducens* was the predominate exoelectrogen in mixed BES communities, in Chapter 6 I examined its ability to produce current relative to a mixed culture and another exoelectrogenic bacterium, *G. metallireducens*, in an MEC. *G. sulfurreducens* generated similar current to the mixed culture, while *G. metallireducens* produced substantially less. Cyclic voltammetry revealed similar catalytic activity for both the mixed community and *G. sulfurreducens*, with a slightly higher current production from the mixed culture. This higher current production may have been due to tighter and more compact biofilms for the mixed community, which were observed in scanning electron microscopy imaging. The similarity in performance of these two cultures could also be attributed to the presence of
G. sulfurreducens in the mixed culture community, which predominated (72% of clones) the clone library.

Chapter 7 showed that G. sulfurreducens can oxidize lactate as the sole electron donor. Previous results suggested that this was not possible, but this bacterium contains genes encoding both a lactate permease and lactate dehydrogenase. When supplied lactate, G. sulfurreducens was capable of generating current in mini-MECs or reducing iron (Fe(III)). Complete lactate oxidation occurred, with the production and consumption of both pyruvate and acetate as intermediates. This finding allows for better comparative studies with the lactate utilizing and exoelectrogenic bacteria of the Shewanella genus.

1.7 Additional Research Publications

In addition to the Chapters listed in this dissertation for which I was the primary investigator, the following list includes publications on which I participated as a co-investigator.

Publications in which I performed FISH experiments (results summarized in Chapter 5):


Other publications (not presented in this dissertation):


1.8 Literature Cited


Chapter 2

Introduction to Bioelectrochemical Systems

2.1 Historical Perspective

Research in bioelectrochemical systems (BESs) dates back as far as 1911, when evidence for electricity production from microorganisms was first discovered [1]. Until 1999, advancement of the field was hindered by the belief that electron mediators or shuttles needed to be provided in order for bacteria to generate current [2-5]. These first generation microbial fuel cells (MFCs) were not viewed as being practical for real world applications, such as wastewater treatment due to the need to continuously supply mediators. In the 1990’s, mounting evidence suggested that metal reducing bacteria, such as members of the *Geobacter* and *Shewanella* genera, could directly reduce insoluble metals, such as ferrihydrite and manganese dioxide, without the need for mediators [6-12]. In 1999, Kim and co-workers hypothesized that since electrodes are similar to insoluble metals that direct electrode reduction could be possible [13]. By controlling the potential of an electrode, they showed that electrical current could be generated by *Shewanella oneidensis* cells supplied lactate without the addition of electron mediators. At about the same time, other researchers were exploring current production in sediment microbial fuel cells (SMFCs) buried within marine sediments [14-16]. They noted that a graphite anode buried in marine sediments and connected to a cathode suspended above it in oxygen rich waters generated current. Previously it was shown that abiotic reactions can lead to current production in similar systems [14], but when anodes harvested from marine sediment MFCs were examined, they were found enriched with bacteria of the
family *Geobacteraceae* [15, 17]. Control electrodes operated in open circuit were not highly enriched with these bacteria, suggesting a biological role for current production [15, 17]. Subsequent pure culture studies with members of the *Geobacteraceae*, including *Geobacter sulfurreducens*, *Geobacter metallireducens*, and *Desulfuromonas acetoxidans*, clearly established the ability of bacteria to generate current without the need for mediators [15, 18]. Elucidating the mechanisms of electron transfer is ongoing, yet early evidence suggests the role of membrane bound cytochromes [19-21], bacterial nanowires [22-24], and/or the production of mediators [25-28], such as flavins as routes for transferring electrons to an anode.

After early studies established the ability of direct electron transfer, investigations into using mixed cultures found in wastewater treatment plants were conducted as a potential method for generating electricity from these waste streams [29-35]. Continuous power generation could be generated from domestic and industrial wastewaters without the need for additional mediators. Once this was established, changes in reactor designs, materials, and catalysts were investigated in order to improve performance while also reducing capital costs for larger scale designs. Initially MFCs had large electrode spacing and membrane separators [18, 36, 37]. These designs had large internal resistances due to the distance between electrodes and the diffusion resistance of ions through the membranes. Removing membrane separators and increasing the solution conductivity reduce internal resistance, allowing for higher power densities [29, 31, 36, 38]. This led to power densities as high as 1300 mW/m² [36]. In order to further increase volumetric power densities, large surface area electrodes, such as graphite brushes and granules were used to provide a massive area on which bacteria could grow [35, 39].
Typically platinum was used as cathodic catalyst in MFCs, but non-precious alternatives have been investigated, such as activated carbon and biological catalysts [40-45]. For air based cathodes, maximum power densities of 2800 mW/m² or 1550 W/m³ were obtained in 2007 [46], and since then only incremental improvements in power production have been made. Although MFCs may have reached a plateau in power production, their development has also led to new reactor types and applications that have continued to push the field of BESs forward.

In 2005, two research groups independently discovered a new technology that was a derivative of MFCs that allowed for the production of hydrogen gas instead of electricity [47-49]. Initially called the bioelectrochemically assisted microbial reactor (BEAMR) process [47] or biocatalyzed electrolysis [48], both rely on the principle of applying a small voltage to a completely anaerobic MFC to drive the formation of hydrogen at a cathode. These reactors were renamed to microbial electrolysis cells (MECs) to better match the naming convention of MFCs (51). To understand why voltage must be added, a review of the thermodynamics of the involved reactions will be presented.

2.2 Thermodynamics of MECs

Reaction spontaneity or thermodynamic favorability is determined using the Gibbs free energy of reaction under standard biological conditions (\(\Delta G^o_r; T = 25^\circ C, \text{pH} = 7, \text{P} = 1 \text{ bar}\)), which is an estimate of the total amount of work that can be obtained from a reaction. Negative \(\Delta G^o_r\) values indicate spontaneous reactions, while positive values
indicate reactions that will not occur unless additional energy is added. The conversion of acetate to hydrogen gas, for example, is not thermodynamically favorable as indicated by the positive $\Delta G_r^{\circ}$ [50].

$$\text{CH}_3\text{COO}^- + 4\text{H}_2\text{O} \rightarrow 4\text{H}_2 + 2\text{HCO}_3^- + \text{H}^+ \quad \Delta G_r^{\circ} = +104.6 \frac{\text{kJ}}{\text{mol}}$$

In order for hydrogen production to occur, additional energy larger than $\Delta G_r^{\circ}/nF$ must be invested, where $F$ is faraday’s constant (96,500 C/mol e⁻) and $n$ is the number of electrons ($n = 8$ for acetate) [51]. This value is referred to as the equilibrium voltage, $E_{eq}$, and for acetate is:

$$E_{eq} = -\frac{\Delta G_r^{\circ}}{nF} = -\frac{104.6 \times 10^3 \text{ J/mol-acetate}}{(8 \text{e}^- / \text{mol-acetate})(96500 \text{ C/mol e}^-)} = -0.14 \text{ V}$$

This theoretical value translates into an energy demand of 0.29 kWh/m³-H₂ (assuming 100% cathodic hydrogen recovery), which is substantially less than the 5.6 kWh/m³-H₂ or 1.6 V required for water electrolysisis [51]. In order to supply the necessary voltage to make the reaction occur, a power supply or battery can be used. Although in theory only $-0.14 \text{ V}$ is required, in practice $>-0.2 \text{ V}$ (more negative than 0.2 V as invested voltage is represented with a negative sign) is required to measure appreciable hydrogen production rates [47, 48, 52]. This applied voltage ($E_{AP}$) will always be greater than $E_{eq}$ due to internal losses in the system.. Once current begins to flow, there are losses at both electrodes leading to overpotential, or the difference between the theoretical value and
the actual recorded value. At the anode, bacteria consume some energy for cell maintenance and growth, and at the cathode losses occur due to the large activation losses needed for the hydrogen evolution reaction. Typically anodic overpotentials are much lower than at the cathode, which highlights the effectiveness of bacteria as biocatalysts [48]. Interestingly, in traditional water electrolysis, cathodic overpotentials are quite low even at current densities in the range of 10,000 A/m² [48]. It is still unclear why cathodic overpotential in MECs are much higher at substantially lower current densities, but this may relate to slower kinetics of the hydrogen evolution reaction in neutral conditions (pH 7) compared to highly alkaline conditions in traditional water electrolysis. Ohmic losses include the resistance of electron flow through connections and electrodes and ions through solution and membrane separators (if included). The sum of the electrode overpotential and the ohmic losses add up to consume a part of the applied voltage. The remainder is stored as hydrogen gas. When the voltage is increased, current increases, and as a result electrical energy losses also increase, which translates into a higher energy demand per unit (m³) hydrogen produced. Therefore a goal in MEC research is to reduce the internal losses through improvements in reactor designs, electrode materials, and exoelectrogens so that energy recoveries remain high.

2.3 MEC proof of concept

MECs were discovered in 2005 by two independent research groups, one at Penn State University and the second at Wageningen University in the Netherlands [47-49]. These first two studies were designed to prove the concept of microbial electrolysis and
were not optimized for maximum performance. Liu et al. used large electrode spacing (15 cm), small electrode specific surface areas (6 m²/m³), and small membrane surface areas (3.5 cm²) in their first MEC design, which are factors known to contribute to high internal resistance and low current densities [36, 47]. In this system, maximum anodic current densities (Iₐ) reached ca. 0.4 A/m² (Eₘₐₓ = 0.5 V), but when the authors used closer electrode spacing (0.5 cm) and equal electrode and membrane surface areas (7 cm²), current densities improved to ca. 2 A/m² at the same applied voltage [47]. However, hydrogen recoveries dropped by 20% in this more optimized reactor likely due to diffusion of hydrogen through the membrane and into the anode chamber. Current densities of ca. 0.5 A/m² (Eₘₐₓ = 0.5 V), similar to the first reactor described in Liu et al., were obtained in the second proof of concept study by Rozendal et al [48]. A detailed analysis of potential losses by these authors revealed that both the cathode and membrane played substantial roles in decreasing reactor performance. In particular, of the 0.5 V applied to their reactor, 0.28 V was lost by cathodic overpotentials, while diffusion of hydrogen gas through the membrane prevented the recovery of 15 of the predicted 35 mL of hydrogen gas (based on current production). Both of these studies provide the critical foundation for MECs, and subsequent studies have focused on optimizing reactor performance through improvements in i) electrode materials and catalysts, ii) reactor configurations and operation, and iii) understanding the microbial ecology of anodic biofilms.
2.4 Electrode materials and catalysts

2.4.1 Anodes

Materials that are commonly employed as anodes in MFCs are also used in MECs as the same anodic reaction occurs within both types of reactors. Typically a carbon based electrode such as carbon cloth and paper, graphite granules, or carbon fiber brushes are used [39, 52-56]. Initially anodes with low specific surface areas were used, but incorporating high surface area electrodes with specific surface areas of $10^3$ m$^2$/m$^3$ or larger provided a massive surface area for the biocatalysts to grow and in turn improve volumetric current production [53, 57, 58]. For example, Logan et al. showed that replacing a carbon paper anode with a graphite brush anode increased current density from ca. 3 A/m$^2$ to ca. 8 A/m$^2$ (normalized to cathode surface area) in MFCs [39]. To improve bacterial adhesion to the anode, the surface can be positively charged through a heated ammonia gas treatment [59]. This approach reduces startup time and allows for higher maximum currents in MFCs, and it has been applied for several anodes in MECs [52, 53, 60]. Similarly, heat treatment of carbon based materials can improve performance, likely due to a cleaning effect of the surface [61].

2.4.2 Cathodes

Cathodic materials applied in MFCs are also used in MECs, yet because the reaction phases involved at the MEC cathode are different than MFCs, cathode design
can be simplified. In MFCs, the cathodic reaction occurs at the junction of three phases: water, solid, and gas. MFC cathodes, therefore, must be designed to allow enough oxygen in to the reactor (through the use of diffusion layers for example) in order for the reduction of oxygen to water to occur [62]. In MECs, the cathodic reaction occurs at the solid and liquid phases. The cathode is typically fully immersed in liquid, yet gas diffusion electrodes that release hydrogen to empty cathode chambers have also been tested [63, 64]. The hydrogen evolution reaction under neutral pH conditions is very slow, which translates into large overpotentials to drive this reaction on small surface area carbon electrodes. In order to reduce this overpotential, a catalyst such as platinum can be added, yet there are many disadvantages to using platinum, including the high cost, detrimental environmental impacts during extraction, and the inhibition due to wastewater constituents such as sulfide. Most lab-scale MECs use platinum, yet intensive research into alternatives is ongoing, as it is expected that full scale MECs treating wastewater will incorporate non-platinum based cathodes [57, 65-70].

One such alternative is the use of microorganisms to catalyze the hydrogen evolution reaction [71, 72]. Hydrogenases are enzymes that reduce protons for the production of hydrogen gas and these enzymes are common in many bacteria. After a mixed culture bioelectrode was enriched for current production using acetate and hydrogen, the electrode potential was reversed, the electrons donors removed, and current was consumed by the bioelectrode [71]. This procedure resulted in the startup of the first biocathode established on a graphite electrode capable of producing higher hydrogen production rates than a platinized control electrode. The advantage of using microorganisms on the cathode is their ability to self regenerate and their essentially free
cost for construction. However, the long term viability of biocathodes is unknown, and the potential for methanogenic invasion and conversion of hydrogen into methane needs further examination. Alternative non-precious metal based cathodes include stainless steel (Chapter 3), nickel, nickel alloys, and molybdenum disulfide [57, 65, 69, 73-77]. Many of these alternative cathodes are obtaining similar performances as platinized cathodes, which is promising result in regards to reducing costs of larger scale reactors.

2.4.3 Membranes

Ion selective membranes are often placed between the anode and cathode of MECs in order to reduce crossover and consumption of hydrogen by bacteria on the anode. Several types of membranes, including cationic, anionic, and bipolar, have been tested in MECs, yet they can substantially reduce reactor performance [48, 63, 78-80]. In both synthetic and real wastewaters, cations such as Na\(^+\), K\(^+\), Mg\(^{2+}\), and Ca\(^{2+}\), are in much higher concentration than free protons (10\(^{-7}\) M at pH 7). As a result, these species contribute to charge balancing of the electrons that travel to the cathode. This leads to proton accumulation at the anode and hydroxide accumulation at the cathode. For every unit change in pH between the anode and cathode, an additional 60 mV of the applied voltage is irreversibly lost. Rozendal et al. estimated that close to 0.4 V of the 1.0 V applied to an MEC using an anionic exchange membrane was lost due to the development of a pH gradient [48]. In principle, membranes should allow for the collection of high purity hydrogen gas from the cathode. However, hydrogen crossover through membranes is also common, which leads to reduced hydrogen recoveries and the development of
hydrogen consuming microbes within the reactor. Some commercially available membranes show poor ion transport specificity also, with anionic exchange membranes capable of transferring positively charge species such as Na\(^+\) [79, 80].

### 2.5 MEC Designs and Operation

Traditional MEC designs incorporated membranes as discussed above, but it was recently shown that membrane-free designs can lead to both high hydrogen recoveries and production rates [52]. Since then multiple studies have developed reactors that operate without membranes [55, 58, 81-83]. Membrane-free operation removes the associated pH gradient and potential losses and reduces the internal resistance of the system, which in turn allows for more of the applied voltage to be invested in driving the electrode reactions. However, the tradeoff is that both carbon dioxide and methane (as low as 1\%) will be present in the collected gas if a mixed culture is used. This occurs due to the presence of microorganisms within mixed cultures that consume hydrogen and reduce carbon dioxide for the production of methane. These methanogens are typically slow growing organisms that are sensitive to oxygen, and intermittent air exposure and short retention times can help reduce methane generation, but not completely eliminate it [52, 58, 83, 84].

In order to improve reactor performance, MECs are often operated using high strength buffers. These buffers serve two purposes: improving solution conductivity and stabilizing pH. Phosphate buffers are commonly used and can range from 10 to 300 mM [48, 52, 55]. A 50 mM phosphate buffer, for example, has a conductivity of 7.5 mS/cm,
which is substantially larger than most domestic wastewaters (ca. 1 mS/cm). Because of
this low conductivity of typical wastewater, every cm of distance between the anode and
cathode electrodes leads to an ohmic loss of ca. 1 V, assuming the reactor was producs 10
A/m² [85]. Supplementing large scale reactors treating wastewater with buffer salts is
neither an economical nor environmentally (eutrophication potential of phosphate) sound
approach. As an alternative, advective flow directed through the anode toward the
cathode can help improve mass and charge transfer in MECs and MFCs.

In order to operate an MEC, an additional energy input must be added, and this is
typically provided in the form of electrical energy. Power supplies are used to apply a
negative whole cell voltage by connecting the positive terminal to the anode and the
negative terminal to the cathode [52, 86]. Alternatively, potentiostats can also be used to
either control the whole cell voltage or fix either electrode potential to set value [48, 71].
Potentiostats also provide an array of electrochemical techniques for analyzing MEC
performance, including voltammetric sweep methods for quick assessment for current
production at different potentials. This equipment is well suited for the laboratory, but
will likely not be used to control large scale reactors due to the high cost (up to $80,000).
Ideally, MECs will be powered using renewable produced electricity, such as through
photovoltaic panels, wind turbines, or salinity gradients (Kim and Logan, PNAS, in
press). The concept of using photovoltaics has already been demonstrated [87]. It is also
possible to power an MEC using an MFC, however, the overall energy efficiency is very
low and the combined costs for both reactor types will likely be prohibitive for larger
scale operation [88, 89].
2.6 Microorganisms

The bacteria capable of current production in BESs are typically Proteobacteria, yet others from the Firmicutes and Acidobacteria have been shown to be exoelectrogenic [90]. Within mixed anodic biofilm communities, however, a wide diversity of bacteria have been detected [91, 92]. The presence of these microbes does not mean that they are exoelectrogenic and only a few microbes are actually capable of current generation.

There are three generally agreed upon mechanisms of how bacteria transfer electrons from inside the cell, across the membrane, and to an electrode [90]. Direct electron transfer occurs when a membrane bound protein makes physical contact with the electrode. Electrons are then transferred from that protein, such as a cytochrome, to the electrode. Since direct contact is required, this mechanism would lead to thin biofilms made up of a mono-layer of cells. Mediated electron transfer occurs when a soluble electron shuttle that is either added to the medium or is produced in-situ by the microbe is reduce internally by the cell, diffuses to the electrode, transfers electrons, and returns to the cell in an oxidized state [28]. Examples of shuttles include flavins, humic acids, and methyl viologen. Bacteria, such as *Shewanella* species are known to produce flavins that assist in current production [28]. The third mechanism is through pilin directed electron transfer. Also called nanowires, these appendages that extend from the cell have been suggested to be electrically conductive [22, 23]. The exact mechanism of how electrons are transferred along or within these nanowires has yet to be determined, yet they open the door for potential long range electron transfer. Both *Geobacter* and *Shewanella* species are known to produce these appendages, and when genes coding these nanowires
are knocked out in *G. sulfurreducens*, current production in MFCs is substantially hindered [24].

Compared to MFCs, very little is known about the mixed culture communities that develop in MECs. In MFCs, oxygen diffusion through the cathode can lead to substrate loss to oxygen by aerobic or facultative microbes. The environment within MECs can be different from MFCs in that it is completely anaerobic, so it would be expected that anodic communities would develop differently. When an acetate fed MFC anode was transferred and operated in an MEC, the resulting community showed a decrease in diversity with a more pronounced enrichment of bacteria from the Delta-Proteobacteria [56]. Another acetate fed MFC anode also showed a decrease in diversity when switched to an MEC, with a significant increase in the percentage of clones represented by *Geobacter sulfurreducens* [93] (Chapter 5).

In addition to bacteria, a group of organisms from the Archaea called the methanogens are common in MECs operated with and without membranes. The two main types of methanogens are acetoclastic and hydrogenotrophic. Acetoclastic methanogens split acetate into methane and carbon dioxide, which results in a loss of electron donor for exoelectrogenic bacteria [94]. Hydrogenotrophic methanogens convert hydrogen and carbon dioxide into methane, resulting in decreased hydrogen recoveries in MECs. In MECs fed acetate, exoelectrogenic bacteria are capable of outcompeting acetoclastic methanogens for acetate, thus the contribution from this group of methanogens is little to none. In MECs using membranes, hydrogen can diffuse from the cathode chamber into the anode chamber where it is converted by hydrogenotrophs into methane. This crossover has been observed in several studies, and in some cases, methane generated in
the anode compartment can diffuse into the cathode chamber [48, 53, 72]. In MECs lacking membranes, hydrogenotrophic methanogens can significantly reduce hydrogen recoveries especially at long retention times or low applied voltages. This decrease in performance is the primary tradeoff of membrane-less designs. Methanogens have been discovered on both anode and cathode electrodes, and have even been suggested to directly accept electrons from cathodes for the production of methane [95]. Methods to reduce methanogen development include intermittent air exposure, pH reduction, heat shocking, addition of methanogen specific inhibitors, and decreased retention times [55, 58, 84]. All of these methods result at best in temporary reduction of methane production, but in all cases, methane concentrations gradually increase over time. This suggests that methanogens are either deeply embedded within anode/cathode biofilms where they are not affected by inhibition methods or they develop syntrophic strategies with exoelectrogens that enable them to survive harsh conditions.

2.7 Substrates

In full scale applications, MECs are expected to treat mixtures of carbohydrates, fatty acids, proteins, and other biodegradable substrates, yet in the lab most studies are conducted using a single tractable substrate. Acetate is the most widely used substrate because it is non-fermentable and it is a common fermentation end product from dark fermentation. The best MEC performances have been obtained using acetate as the substrate, with the highest hydrogen production rates of 50 m$^3$/H$_2$/m$^3$/d obtained in a two chamber MEC at an applied voltage of 1 V [68]. Hydrogen recoveries are also high using
this substrate and have approached close to the theoretical of 4 mol-H₂/mol-acetate [52]. Other substrates yield lower production rates and recoveries because bacteria with alternative anaerobic metabolic routes often compete with the exoelectrogens for the substrate [53]. Ethanol for example can be fermented by bacteria such as those in the *Pelobacter* genus rather than converted directly into current by exoelectrogens [96]. Although the endproducts of this fermentation (acetate and/or hydrogen) can subsequently be converted into current, the initial substrate conversion step reduces the overall production rates and leads to inefficient substrate conversion. More complex substrates, such as cellulose and straw hydrolysate have also been tested, yet due to the slow hydrolysis steps required to produce usable electron donors for exoelectrogenic bacteria, performance is reduced compared to acetate fed MECs [53, 97]. Despite one of the intended applications of MECs, domestic wastewater has only been examined once yielding low hydrogen production rates and recoveries [54]. However, this analysis was an early study and improvements in reactor designs and operation suggest that better performance from wastewater might be possible.

### 2.8 MEC Performance

MECs are analyzed and compared in terms of current production, hydrogen production rates, hydrogen recoveries, and energy recoveries [51]. Current is typically normalized to either an electrode surface area (m²) or the reactor volume (m³), which allows for better comparison among different reactors than simply reporting the current (mA). However, in some cases electrodes with large surface areas, such as graphite felt or
cloth, are normalized to projected surface areas, which have a much larger actual surface area. This normalization can lead to values that appear larger than current densities obtained by solid graphite block electrodes that have almost identical actual and projected surface areas (except when surface roughness is included). Current directly relates to the hydrogen production rate as the electrons that travel to the cathode are eventually converted into hydrogen gas. The applied voltage has a significant impact on the hydrogen production rate because more energy to drive the reactions is invested at higher applied voltages. Hydrogen production rates with reactors fed acetate have varied from ca. 0.01 to 50 m$^3$ H$_2$/m$^3$ d, with the largest value of 50 m$^3$ H$_2$/m$^3$ d obtained at an applied voltage of ca. 1.0 V [51, 68]. This high applied voltage reduces the overall energy efficiency of the process and is comparable to the energy typically invested (−1.5 to −2.0 V) for traditional water electrolysis. Hydrogen production rates can also be overestimated due to the re-oxidation of hydrogen on the anode. When recycled current was removed from a membrane-less MEC generating an estimated 18 m$^3$ H$_2$/m$^3$ d, the actually hydrogen production rate was lowered to ca. 4 m$^3$ H$_2$/m$^3$ d [83].

The recovery of hydrogen from the MEC is another metric for comparing reactors and this can be calculated based on both substrate and current. Energy recoveries relative to only the electrical energy added to the system has reached as high as 680% at an applied voltage of 0.2 V for an acetate fed MFC [53]. However, when the energy of the substrate is factored in to calculate the overall energy recovery, efficiencies are below 100%, but can remain as high as ca. 80% [51].
2.9 Literature Cited


Chapter 3

High Surface Area Stainless Steel Brushes as Cathodes in Microbial Electrolysis Cells (MECs)

Abstract

Microbial electrolysis cells (MECs) are an efficient technology for generating hydrogen gas from organic matter, but alternatives to precious metals are needed for cathode catalysts. We show here that high surface area stainless steel brush cathodes produce hydrogen at rates and efficiencies similar to those achieved with platinum-catalyzed carbon cloth cathodes in single-chamber MECs. Using a stainless steel brush cathode with a specific surface area of 810 m$^2$/m$^3$, a hydrogen production rate of 1.7 ± 0.1 m$^3$.H$_2$/m$^3$.d (current density of 188 ± 10 A/m$^3$) was produced at an applied voltage of 0.6 V. The energy efficiency relative to the electrical energy input was 221 ± 8%, and the overall energy efficiency was 78 ± 5% based on both electrical energy and substrate utilization. These values compare well to previous results obtained using platinum on flat carbon cathodes in a similar system. Reducing the cathode surface area by 75% decreased performance from 91 ± 3 A/m$^3$ to 78 ± 4 A/m$^3$. A brush cathode with graphite instead of stainless steel and a specific surface area of 4600 m$^2$/m$^3$ generated substantially less current (1.7 ± 0.0 A/m$^3$), and a flat stainless steel cathode (25 m$^2$/m$^3$) produced 64 ± 1 A/m$^3$, demonstrating that both the stainless steel and the large surface area contributed to high current densities. Linear sweep voltammetry showed that the stainless steel brush cathodes both reduced the overpotential needed for hydrogen evolution and exhibited a
decrease in overpotential over time as a result of activation. These results demonstrate for the first time that hydrogen production can be achieved at rates comparable to those with precious metal catalysts in MECs without the need for expensive cathodes.

This chapter was published as:

### 3.1 Introduction

The performance of microbial electrolysis cells (MECs) has improved considerably in only a few years since their discovery [1-5]. The use of high surface area anodes, close electrode spacing, different membrane materials, and improved reactor designs has rapidly increased both current densities and hydrogen recoveries [6-8]. However, high efficiency of the MEC process has required the use of a precious metal such as platinum (Pt) on the cathode to efficiently catalyze hydrogen evolution. One alternative to a precious metal catalyst is to use a biocathode, but current densities using microorganisms are substantially lower than those with a platinized electrode at a given applied voltage. For example, Rozendal et al. obtained a current density of 1.2 A/m² with a biocathode at a cathode potential of −0.7 V [9]; however, current densities in the range of 4 – 10 A/m² are typically achieved when Pt is used on the cathode [1, 6, 10, 11].

Precious metal catalysts reduce cathodic overpotential by lowering the activation energy and by affecting the overall rate of reaction [12]. Thermodynamically, reactions
proceed at some finite rate, and so even on non-catalyzed surfaces there is some rate of reaction. In some cases this rate is sufficient to obtain an overall increased rate of reaction just by using a large enough surface area. For example, Freguia et al. used graphite granules in a microbial fuel cell (MFC) with a specific surface area of 3150 m²/m³ to increase the rate of oxygen reduction to 63 A/m³ compared to 0.6 A/m³ obtained using a non-catalyzed flat graphite cathode with a specific surface area of 2.3 m²/m³ [12, 13]. Large surface area cathodes of graphite fiber brushes and stainless steel (SS) wool have also been used in sediment MFCs to increase oxygen reduction rates in seawater [14, 15]. Tests in water electrolyzers using SS have shown that hydrogen evolution rates are increased using SS with an increased Ni content [16, 17]. Olivares-Ramirez et al. tested three different grades of SS in water electrolyzers under alkaline conditions, and determined there was a positive correlation with improved hydrogen evolution rates and the nickel content of the SS [17]. However, the performance of SS has not previously been examined under neutral pH conditions for hydrogen evolution.

In this study, we examine the use of SS in an MEC using high surface area brush cathodes, and compare performance of these brush electrodes with those obtained using Pt-catalyzed carbon cloth (Pt/C) cathodes. We selected a SS with a high Ni content (SS304) and examined performance in terms of current densities, hydrogen production rates, and hydrogen recoveries. We also determined the effect of SS brush surface area on current generation and cathode overpotential. To examine the effect of material composition on current production, we compared the SS brushes to a graphite brush cathode. Linear sweep voltammetry (LSV) scans were used to examine cathodic overpotentials of Pt/C cathodes relative to those obtained using SS brush cathodes.
3.2 Materials and Methods

3.2.1 Cathodes

The SS brush cathodes (Gordon Brush Mfg Co., Inc., Commerce, California) were made of grade 304 SS, which has the composition: 0.08% C, 2% Mn, 0.045% P, 0.03% S, 1% Si, 18 – 20% Cr, and 8 – 11% Ni (balance Fe) [18]. The bristles (0.008 cm diameter) were wound into a twisted SS core (0.20 cm diameter) using an industrial brush manufacturing machine. The brushes were 2.5 cm long and 2.5 cm in diameter. Based on the mass and estimated surface area of the bristles, each brush (100% loading case) had 310 cm$^2$ of surface area, producing 2500 m$^2$/m$^3$-brush volume (95% porosity), for a specific surface area of $A_s = 650$ m$^2$/m$^3$ of reactor volume. In some tests, brushes with reduced bristle loadings of 50%, 25%, and 10% were used, with surface areas of 160 cm$^2$ ($A_s = 340$ m$^2$/m$^3$), 110 cm$^2$ ($A_s = 240$ m$^2$/m$^3$), and 79 cm$^2$ ($A_s = 170$ m$^2$/m$^3$), respectively. These areas include the surface area of the SS core, which is estimated at 2.4 cm$^2$ (5.1 m$^2$/m$^3$) based on the projected area of a cylinder. A flat piece of grade 304 SS (McMaster-Carr, Cleveland, Ohio) was used in some tests (surface area of 7 cm$^2$). SS cathodes were cleaned before use by sonication in an ultrasonic cleaner (model 1510, Branson, Danbury, Connecticut) for 10 minutes in 70% ethanol, followed by rinsing with DI water, and sonication again for 5 minutes in fresh DI water. In one test a graphite fiber brush electrode containing a titanium wire core (surface area of 0.22 m$^2$; $A_s = 4600$ m$^2$/m$^3$) [19] was used as the cathode.
3.2.2 Reactor Construction

Three different MEC architectures were examined to determine the effect of cathode brush architecture, with all reactors containing an ammonia treated, graphite fiber brush anode prepared as previously described (Figure 3.1) [19, 20]. The first reactor (V = 28 mL) contained a 100% loaded SS brush oriented vertically above and parallel to the core of the anode (Reactor VB). In order to reduce the spacing to 0.5 cm between the electrodes, both brushes were cut in half using scissors, each one forming a half cylinder. The half SS brush had a reduced surface area of $A = 230 \, \text{cm}^2$, but an increased specific surface area of $A_S = 810 \, \text{m}^2/\text{m}^3$. An anaerobic gas collection tube was installed above the brush cathode. A second reactor (V = 48 mL) was made by combining a cube-shaped MFC [19] to a second cube-shaped reactor that was 2.5 cm in length and had a gas collection tube attached on top (Reactor HB). Reactor HB was used to examine SS brush cathodes with different surface areas and the graphite brush cathode, with each cathode brush inserted perpendicular to the core of the anode. A third reactor contained either a Pt/C cathode (0.5 mg-Pt/cm$^2$) as described previously or a flat SS cathode (Reactor FC) [6, 19, 20]. Both flat cathodes had specific surface areas of $A_S = 25 \, \text{m}^2/\text{m}^3$. Prior to starting a batch cycle the gas collection tubes were crimped shut.

3.2.3 Startup and Operation

The brush anodes were first enriched in an MFC using the effluent from an active MFC as previously described [6]. The anodes were transferred to MECs and fed sodium
acetate (1 g/L; J.T. Baker) in a 50 mM phosphate buffer medium (PBS; Na₂HPO₄, 4.58 g/L; and NaH₂PO₄•H₂O, 2.45 g/L, pH = 7.0) and nutrient solution (NH₄Cl, 0.31 g/L; KCl, 0.13 g/L; trace vitamins and minerals [21]) having a final solution conductivity of 7.5 mS/cm. At the end of each batch cycle, the crimp tops were removed, the contents drained, and the reactors were left exposed to air for 20 minutes to help inhibit the growth of methanogens [6, 22]. After adding the medium and re-crimping the collection tubes, the reactors were sparged for 15 minutes with ultra high purity nitrogen (UHP) (99.998%), covered with aluminum foil to prevent the growth of phototrophic microorganisms, and placed in a constant temperature room (30°C). Performance of the reactors was evaluated in terms of current density and continuous gas production rate using a respirometer [6]. Gas analysis as previously described was performed for the optimized reactor (Reactor VB) [6]. Complete substrate removal was assumed for each batch cycle, equivalent to a chemical oxygen demand (COD) of 0.022 g-COD.

A fixed voltage (E_{ap}) of 0.6 V was applied to the reactor circuit using a power source (model 3645A; Circuit Specialists, Inc., Mesa, Arizona), and the current determined by measuring the voltage across a 10 Ω resistor [6]. An Ag/AgCl reference electrode (RE-5B; BASi, West Lafayette, Indiana) was placed in each reactor, with the cathode potential recorded using a multimeter (Model 2700; Keithley Instruments, Inc., Cleveland, Ohio). All hydrogen production, energy recovery, and hydrogen recovery results were calculated as previously described [3, 6].
3.2.4 Voltammetry

Linear sweep voltammetry (LSV) was performed on a potentiostat (model PC4/750, Gamry Instruments, Warminster, Pennsylvania) with 1 mV/s rates on the cathodes (100% loaded SS brush, flat SS, and Pt/C) at 30°C in a 28 mL reactor. The LSV reactor also included an Ag/AgCl reference electrode (Princeton Applied Research, Oak Ridge, Tennessee) and a 2 cm² pure platinum foil counter electrode. The reactor was filled with 50 mM PBS (pH = 7.0) without trace nutrients and sparged with UHP nitrogen. Chronopotentiometry at 50 mA for 24 hours in 50 mM PBS was performed to simulate accelerated use of a 100% loaded SS brush. Stripping was performed with cyclic voltammetry in 0.5 M H₂SO₄ from -0.5 to +1.5 V vs. Ag/AgCl at 250 mV/s.

3.3 Results

3.3.1 Effect of Cathode Surface Area

The impact of cathode surface area was evaluated using MECs with horizontally-placed brush cathodes (Reactor HB). Varying the SS brush bristle loadings did not substantially impact current generation (Figure 3.2). For brush bristle loadings of 50 – 100%, the current density remained around 90 A/m³. Lowering the bristle loading below 50% resulted in a slight decrease in current density to 85 ± 3 A/m³ for the 25% loaded brush and 78 ± 4 A/m³ for the 10% loaded brush. With no brush bristles (base core only),
the MEC generated 24 ± 0 A/m³, indicating there was a significant level of activity due to the SS core on current density.

The cathodic overpotential decreased with the increasing bristle loadings from no bristles up to 25% bristle loading (Figure 3.3). The 50% loaded brush exhibited the lowest cathodic overpotential of −0.968 ± 0.007 V, while the 100% loaded brush reached −0.990 ± 0.002 V. The brush core with no bristles had the highest cathodic overpotential of −1.082 ± 0.005 V (vs. Ag/AgCl)

### 3.3.2 Current Densities using other Cathodes

To examine the impact of material composition on current generation, a graphite fiber brush cathode containing a titanium wire core was tested in Reactor HB. Although the specific surface area of the graphite brush was 7 times larger than the 100% SS brush tested, current production was substantially lower. Only 1.7 ± 0.0 A/m³ was achieved after three days (Figure 3.4). The SS brush core with no bristles and identical electrode spacing generated a current density 14 times larger than the graphite brush. Thus, large surface area alone could not account for the performance of the SS brushes.

The importance of the SS as a catalyst was further verified by using a flat SS cathode in Reactor FC. Although the specific surface area of the flat SS cathode was more than a hundred fold smaller than the graphite brush cathode, current generation was greater (64 ± 2 A/m³). The current density produced by the flat SS cathode (2.6 cm electrode spacing) was also 2.7 times greater than the SS brush core (24 ± 0 A/m³; 3.5 cm electrode spacing). Although, the flat SS cathode had a slightly larger surface area (A = 7
cm$^2$) than the SS brush core ($A = 2.4$ cm$^2$), the higher current density of the flat SS cathode suggests that the orientation and distance of the cathode was more important for increased current density than surface area.

### 3.3.3 Comparison to a Platinized Cathode

Since the brush bristle loadings did not have an appreciable impact on current production, it was believed that the main factor limiting power generation was electrode distance. Therefore, a fully loaded SS brush was trimmed in half and placed as close as possible above a similarly trimmed graphite brush anode (Reactor VB, $A_s = 810$ m$^2$/m$^3$) in order to create a configuration capable of generating current densities similar to Pt/C cathodes [6]. During the first few cycles, the current density was greater in the MEC using the Pt/C cathode (Reactor FC) than the MEC with the vertically-aligned SS brush cathode (Figure 3.5). Within four cycles, however, Reactor VB was producing the highest current density of 194 ± 1 A/m$^3$, compared to 182 ± 2 A/m$^3$ for Reactor FC. For the final three batch cycles, both reactors generated a similar average current density, with Reactor FC reaching 188 ± 10 A/m$^3$ and Reactor VB obtaining 186 ± 2 A/m$^3$.

The higher current density of Reactor VB with the SS brush was a result of a lower cathodic overpotential than Reactor FC with the Pt/C cathode (Figure 3.6). During the first batch cycle, the Pt/C cathode had a higher overpotential than the SS brush, likely due to the higher current density. By the second cycle, both the SS brush and Pt/C cathode exhibited roughly the same overpotential, but several later cycles the Pt/C cathode showed an increase in overpotential (cycles 3 – 4). This trend may have been due
to minor Pt catalyst inactivation in combination with an activation of the SS for the hydrogen evolution reaction. After the first two cycles of reactor acclimation, the SS cathode in Reactor VB produced a cathode potential of $-0.910 \pm 0.002$ V, whereas the Pt/C cathode exhibited a higher overpotential with a value of $-0.924 \pm 0.003$ V. These potentials correspond to cathodic losses of about 0.29 V for the SS brush and 0.30 V for the Pt/C cathode relative to the equilibrium potential of hydrogen formation ($-0.62$ V vs Ag/AgCl).

3.3.4 Energy Recoveries and Production Rates

The recoveries and production rates for the SS brush in Reactor VB were averaged over the last three cycles in Figure 3.2. Relative to only the electrical energy input, the energy recovery reached $\eta_E = 220 \pm 8\%$. When the substrate energy was also included, the overall energy recovery was $\eta_{E+S} = 78 \pm 5\%$. The cathodic hydrogen recovery was $r_{\text{CAT}} = 83 \pm 8\%$, and the average hydrogen production rate was $Q = 1.7 \pm 0.1$ m$^3$-H$_2$/m$^3$-d.

3.3.5 Linear Sweep Voltammetry

LSV scans performed only on the cathodes and not the assembled MECs indicated that the Pt/C cathode could initially operate at 0.1 – 0.2 V lower cathodic overpotentials than the 100% loaded SS brush (Figure 3.7). The activity of the SS brush for hydrogen evolution improved after simulating accelerated use, resulting in catalytic
activity similar to the Pt/C cathode. In the initial LSV, the SS brush had a resting potential of +0.06 V vs. NHE (where the current was zero) and small positive currents for more positive potentials. After accelerated use the resting potential shifted to -0.08 V vs. NHE. To remove any possible SS surface corrosion products that may have accumulated during the accelerated use, the SS brush was stripped using cyclic voltammetry until the currents corresponding to hydrogen and oxygen evolution became constant (~5 cycles). A third LSV performed on the SS brush after cyclic voltammetry produced results very similar those obtained after the initial use LSV (data not shown), suggesting that corrosion products on the surface of the SS that occur with use cause the SS to become more active towards hydrogen evolution. Compared to a flat SS cathode, the SS brush exhibited a lower overpotential, particularly at lower currents, thus confirming the effectiveness of the high surface area. Current generation observed below the standard state theoretical potential for hydrogen production (−0.42 V vs. NHE; $P_{H2} = 1$ atm) in Figure 3.7 occurred because the LSV was performed under atmospheric conditions where the partial pressure of hydrogen ($P_{H2} = 5 \times 10^{-5}$ atm) reduces the theoretical potential to be −0.29 V.

3.4 Discussion

High current densities were achieved in MECs without a precious metal catalyst by using high surface area SS cathodes. The highest current density using a SS cathode MEC of 194 ± 1 A/m$^3$ (applied voltage of 0.6 V) was obtained by placing a high surface area SS cathode in close proximity to the graphite fiber brush anode (Reactor VB). Close
electrode spacing was achieved by cutting each brush into a half-hemisphere and orienting the flat portions of the brushes next to each other (0.5 cm between the flat surfaces). When two full brushes were used in the same reactor (1.2 cm between the brush edges; 3.5 cm between the electrode midpoints), the current density was lower (91 ± 3 A/m³), despite the greater surface areas of the two electrodes in Reactor HB than in Reactor VB. Reducing the surface area of the cathode in Reactor HB had little impact on the current density (over a limited range), demonstrating that the better performance of Reactor VB than Reactor HB was mainly due to the electrode spacing. Close electrode spacing is important to avoid large ohmic losses [23], particularly for solutions with low conductivities, such as domestic wastewater.

The use of SS brush cathode shows great promise for the development of low cost reactors not only due to high current densities, but also as a result of high energy recoveries. Hydrogen production rates and overall energy recoveries obtained here for Reactor VB of $Q = 1.7 \pm 0.1$ m³-H₂/m³-d and $\eta_{E+S} = 78 \pm 5\%$ are comparable to previous studies using Pt/C cathodes ($Q = 0.5 – 2.0$ m³-H₂/m³-d and $\eta_{E+S} = 58 – 82\%$) (Table 3.1).

One aspect of using SS brush cathodes in an MEC that must be further addressed is potential hydrogen gas bubble holdup in the brushes. When using highly loaded brushes (> 50%), we visually observed that gas bubbles became trapped within the brush bristles. Bubble capture is a problem for two reasons. First, the accumulation of hydrogen gas on the surface decreases the active area available for hydrogen evolution [24]. This could increase cathodic overpotential, resulting in reduced reactor performance. We observed here that cathodic overpotential with a full brush was greater than that obtained with a 50% loaded brush, although it cannot be conclusively determined that this was
directly a result of the entrapment of hydrogen gas bubbles due to the different geometries of the brushes. Second, the retention of gas bubbles in the brush (or anywhere in the reactor) can result in increased microbiological losses of hydrogen. When bubble residence times are increased in the reactor, the total hydrogen gas diffusion into the liquid can increase and can result in greater losses of hydrogen gas to hydrogenotrophic bacteria and methanogens. The lower cathodic hydrogen recovery and higher methane concentrations of the SS brush ($r_{\text{CAT}} = 83 \pm 8\%$ and $C_{\text{CH}_4} = 2.3 \pm 2.3\%$) compared to the Pt/C cathode ($r_{\text{CAT}} = 95 \pm 2\%$ and $C_{\text{CH}_4} = 0.9 \pm 0.1\%$) in a previous study [6] are likely evidence of these processes.

Further investigation into SS corrosion and its impacts on current generation is needed. Of the tests performed using the SS brush in reactor VB, the current density was lowest for the first batch cycle and increased with subsequent batch cycles (Figures 3.5 – 3.6). LSV of the SS before and after accelerated use indicated that hydrogen evolution overpotentials can decrease by 0.15 – 0.2 V. The large increase in hydrogen catalytic activity, and the shift in resting potentials in combination with the reversion of activity after stripping, indicated that SS corrosion products occur with use that are beneficial for HER (28). Conversely, the Pt/C cathode showed a slight decrease in current generation along with a decrease in overpotential (cycles 2 – 4), which may have been due to minor catalyst poisoning. Thus, it appears that over time corrosion or other changes associated with the SS improve its performance as a cathode in an MEC. However these changes may be dependent on the current density or electrolyte composition, and therefore they require further exploration.
Using high surface area SS brushes as cathodes in MECs is an important step towards building larger scale reactors that are economical because it avoids the use of expensive catalysts. Platinum currently costs ~$43,000/kg compared to $4.82/kg for 304 grade SS [25, 26], which translates into a cost of $0.03 for the half SS brush cathode and $0.15 for the Pt on the Pt/C cathode used in this study (excluding the cost for the carbon cloth, Nafion binder, and current collector). Rozendal et al. [23, 27] determined that the cathode (including catalyst) accounts for the greatest percentage (47%) of the total capital costs for both MFCs and MECs. Their model predicts that cathodes will play a smaller role in the total reactor cost by identifying materials that have substantially lower costs than those currently used. Clearly, the use of SS instead of Pt moves cathode designs closer to that goal.

3.5 Literature Cited


### 3.6 Tables

Table 3.1. Overall Energy Recoveries, Electrical Efficiencies, Cathodic Hydrogen Recoveries, Volumetric Current Densities, and Hydrogen Production Rates at $E_{ap} = 0.6$ V.

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<th>$\eta_E$ (%)</th>
<th>$r_{\text{CAT}}$ (%)</th>
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3.7 Figures

Figure 3.1. Reactor schematics. Reactor VB (vertical brush): half brush anode, HBA; half brush cathode, HBC. Reactor HB (horizontal brush): full brush anode, FBA; full brush cathode, FBC. Reactor FC (flat cathode): platinized carbon cloth cathode, Pt; stainless steel cathode, SS. Power supply, PS. 10 Ω resistor, R.

Figure 3.2. Current densities versus time for SS brush cathodes with different bristle loadings of 100%, 50%, 25%, 10%, or 0% (brush base core only) at $E_{ap} = 0.6$ V.
Figure 3.3. Cathode potentials (versus Ag/AgCl) versus time for consecutive batch cycles using SS brush cathodes with different bristle loadings at $E_{ap} = 0.6$ V.

Figure 3.4. Current densities versus time for a 100% loaded SS brush cathode (SSB 100%), a flat SS cathode (SS flat), a SS brush core (SS core), and a graphite brush cathode (GB) at $E_{ap} = 0.6$ V.
Figure 3.5. Current density versus time for both the platinized carbon cloth cathode (Pt) and the SS brush cathode cut in half (Half SS) at $E_{ap} = 0.6$ V.

Figure 3.6. Cathode potentials (versus Ag/AgCl) versus time for both the Pt/C cathode and the SS brush cathode cut in half (Half SS) at $E_{ap} = 0.6$ V.
Figure 3.7. LSV curves for the platinized cathode (Pt), 100% loaded SS brush cathode before (pre) and after (post) accelerated use, and flat SS cathode (SS Flat).
Chapter 4

A Method for High Throughput Bioelectrochemical Research Based on Small Scale Microbial Electrolysis Cells

Abstract

There is great interest in studying exoelectrogenic microorganisms, but existing methods can require expensive electrochemical equipment and specialized reactors. We developed a simple system for conducting high throughput bioelectrochemical research using multiple inexpensive microbial electrolysis cells (MECs) built with commercially available materials and operated using a single power source. MECs were small crimp top serum bottles (5 mL) with a graphite plate anode (92 m²/m³) and a cathode of stainless steel (SS) mesh (86 m²/m³), graphite plate, SS wire, or platinum wire. The highest volumetric current density (240 A/m³, applied potential of 0.7 V) was obtained using a SS mesh cathode and a wastewater inoculum (acetate electron donor). Parallel operated MECs (single power source) did not lead to differences in performance compared to non-parallel operated MECs, which can allow for high throughput reactor operation (> 1,000 reactors) using a single power supply. The utility of this method for cultivating exoelectrogenic microorganisms was demonstrated through comparison of buffer effects on pure (Geobacter sulfurreducens and Geobacter metallireducens) and mixed cultures. Mixed cultures produced current densities equal to or higher than pure cultures in the different media, and current densities for all cultures were higher using a 50 mM phosphate buffer than a 30 mM bicarbonate buffer. Only the mixed culture was
capable of sustained current generation with a 200 mM phosphate buffer. These results demonstrate the usefulness of this inexpensive method for conducting in-depth examinations of pure and mixed exoelectrogenic cultures.

This chapter was published as:

### 4.1 Introduction

Applications of bioelectrochemical systems (BESs) have advanced in the past decade to include producing electricity, biogases, high value chemicals, and desalinated water [1-4]. Although the end products of BESs differ, they share several fundamental aspects that must be improved to advance these systems, such as electrode materials and reactor design and a better understanding of microbial community interactions. New methods of conducting BES research are needed that reduce both the time required for experiments and reactor cost, while allowing for high throughput operation that can be used to screen for new strains of exoelectrogenic bacteria and maintain pure culture conditions.

One approach for performing high throughput research is to use miniaturized BESs, such as multiple-well electrode arrays. Several small-scale (mL and µL) BESs have been designed, but most have been based on microbial fuel cell (MFCs) with the primary focus on screening bacteria for exoelectrogenic activity [5]. Some of these designs require expensive materials (Nafion membranes, platinum catalysts, gold electrodes), access to state of the art materials technology (photolithography, sputtering,
polymer molding), and custom machining [6-11]. These designs are excellent tools for researchers with access to the appropriate resources, yet the slow adoption of these systems by other researchers suggests that simpler designs are needed to enable more widespread experimentation with exoelectrogenic microorganisms. For example, the simple and robust methods developed by Hungate and Balch led to a paradigm shift in conducting anaerobic microbiology, in large part to the incorporation of a simple tube design that is now ubiquitous in microbiology labs across the world [12, 13]. However, in the field of BES research, there is currently no equivalent platform or procedure that has been as widely adopted.

In order to make BES research more accessible for microbiological study, we developed a new procedure that relies on a simple and affordable reactor that can be assembled using commercially available materials and a single power source capable of powering thousands of reactors. This reactor design is based on a single-chamber (membrane-free) microbial electrolysis cell (MEC) [14-16]. A commonly used electrode material for both the anode and cathode in most MECs is carbon cloth, but this material cannot be effectively used in smaller systems as the cloth can unravel and lead to short circuiting when close electrode spacing is used. Therefore, we examined the use of several alternative materials that could be used at a small scale and at close proximity, with the electrodes held apart by rigid connecting wires to avoid short circuiting. The electrodes were built to fit through the top of small reactors (serum bottles) so that the complete reactor system could be easily assembled, autoclaved, and sealed gas-tight with a crimp top allowing for pure culture tests and gas build up in the system. The use of a single power source for the operation of multiple reactors was validated by comparing the
performance of many reactors operated in parallel to those attached to individual power supplies. We used this new system to demonstrate that high phosphate buffer concentrations can adversely affect current generation by pure strains of exoelectrogens but not mixed cultures.

4.2 Materials and methods

4.2.1 Reactor Construction and Setup

Single chambered MECs were constructed using 5 mL clear glass serum bottles (Wheaton). Anodes were isomolded graphite plates with a thickness of 0.32-cm (Grade GM-10; GraphiteStore.com, Inc.), cut to dimensions of 1.5-cm (L) × 1-cm (W), providing a specific surface area of $A_{\text{AN}} = 92 \, \text{m}^2/\text{m}^3$. All anodes were polished using sandpaper (grit type 400), sonicated to remove debris, cleaned by soaking in 1 N HCl overnight and rinsed three times in Milli-Q water. Titanium wire (0.08 cm diameter; McMaster-Carr), cleaned with sandpaper, was cut to 5-cm lengths and bent at one end into a J-shape. After inserting the non-bent portion of the wire through a hole drilled near the top center of the graphite plate (0.08-cm diameter drill bit), the bent end was inserted into a second hole and crimped to provide a tight connection of the wire to the plate. Other materials, including carbon cloth and carbon paper, did not yield secure connections when crimped with wire and therefore were not used due to unacceptably large contact resistances (> 2 $\Omega$). Any anode or cathode with a contact resistance > 0.5 $\Omega$ was discarded.
The cathode can limit current generation in an MEC, and therefore many MEC designs use carbon cloth with platinum (Pt). Cloth was difficult to use in these systems due to poor electrical connections, and it was found in preliminary tests that it unraveled over time. Stainless steel (SS) mesh has also been recently used in MECs [17] and therefore we tested cathodes constructed from SS mesh (Type 304, Mesh size 90 × 90; McMaster-Carr) cut to produce the same projected area as the graphite plates, providing a mesh wire specific surface area (based on the area of the individual wires) of $A_{\text{CAT}} = 86 \text{ m}^2/\text{m}^3$ [17]. SS mesh electrodes were connected to a 5-cm length of SS wire (Type 302/304, 0.08-cm diameter; McMaster-Carr) by crimping the wire around the mesh using a drilled hole. All SS was roughened and cleaned using sandpaper, and rinsed with Milli-Q water. Several alternate cathode materials were also examined to see if simpler materials could provide comparable results. These included a single wire of SS (Type 302/304, 0.08-cm diameter; McMaster-Carr) or platinum (Pt) (0.25-mm diameter; Hauser & Miller Co.), and a graphite plate prepared as described above for the anode. Replicate reactors were included for each cathode type, including uninoculated control reactors containing SS mesh cathodes.

Electrodes were installed by pushing each respective wire through a thick butyl rubber stopper (20-mm diameter; Chemglass). The stopper and electrode assembly was inserted into the glass serum bottles by lubricating the sides of the stopper with water, while maintaining an electrode spacing of $ca. 0.5$-cm through bending the rigid wires (Figure 4.1). After crimp sealing the vials shut using aluminum caps, reactors were vacuumed empty and filled three times with anaerobic gas ($\text{CO}_2/\text{N}_2$, [20/80] or $\text{N}_2$ [100%] as noted), and autoclaved. Reference electrodes (Ag/AgCl; $+200 \text{ mV}$ vs standard
hydrogen electrode (SHE); BASi) were used in some mixed culture experiments to record anode and cathode potentials by inserting the electrode (0.57-cm diameter) through a hole cut in the butyl stopper using a 0.79-cm diameter cork punch and positioning the tip between the anode and cathode. All electrode potential values are reported versus SHE.

MECs were operated using a programmable power supply (model 3645A; Circuit Specialists, Inc.). For reactor operation in parallel, two separate sets of 20 test leads were soldered together at one end, with one set connected to the positive terminal of the power supply and the second set connected to the negative terminal. Each test lead attached to the positive terminal had a 10 Ω resistor connected in series for recording the voltage produced by each reactor. A multimeter (model 2700; Keithley Instruments, Inc.) was used to record the voltage at 20 minute intervals, with the current calculated using Ohm’s law \( I = V/R \). Volumetric current density \( I_V; \text{A/m}^3 \) was found by dividing the current by the liquid volume and current density per area \( I_A; \text{A/m}^2 \) was normalized by the anode surface area. To operate the reactors, the test leads attached to the positive terminal were connected to the anodes and the test leads attached to the negative terminal were connected to the cathodes (Figure 4.1). To ensure there were no effects of operating the reactors in parallel, identical reactors were also operated using individual power supplies.

4.2.2 Microorganisms and Media

Domestic wastewater from the primary clarifier at the Pennsylvania State University wastewater treatment plant served as the inoculum for the mixed culture MECs. *Geobacter sulfurreducens* and *Geobacter metallireducens* were obtained from frozen
laboratory stocks. Both *Geobacter* sp. were pre-cultured as described previously [18] in a bicarbonate buffered medium (BCM-30) with sodium acetate (1 g/L) as the electron donor and ferric citrate (20 mM) as the electron acceptor. MEC tests were conducted using 1 g/L sodium acetate and either 50 mM phosphate buffer (PBM-50), 200 mM phosphate buffer (PBM-200), or BCM-30 lacking ferric citrate. Prior to MEC inoculation, 5 mL of sterile, anaerobic medium was added to each reactor. Both *Geobacter* sp. were added during late stationary phase growth by directly injecting 0.5 mL of cells plus growth medium into the MECs. For the mixed culture, wastewater (10%) was added. All cultures were started in MECs using BCM-30 and operated in duplicate.

MEC media were prepared in advance and stored in 125 mL sealed serum bottles as sterile, anaerobic stocks. Each medium contained a base composition of (per liter): 0.25 g NH₄Cl, 0.1 g KCl, 10 mL vitamins, and 10 mL minerals [19]. The media differed in the buffer used in conjunction with the base composition, with BCM-30 containing 0.6 g NaH₂PO₄, 2.5 g/L NaHCO₃ (pH = 7; conductivity \( \gamma = 4.5 \) mS/cm), PBM-50 containing 2.5 g NaH₂PO₄, 4.6 g Na₂HPO₄ (pH = 7; \( \gamma = 7.5 \) mS/cm), and PBM-200 containing 10 g NaH₂PO₄, 18.4 g Na₂HPO₄ (pH = 7; \( \gamma = 20 \) mS/cm). The BCM-30 medium was sparged and maintained under a CO₂/N₂ [20/80] atmosphere, whereas 100% N₂ gas was used for both PBM-50 and PBM-200 media.
4.2.3 Reactor Operation

During startup, all reactors were operated at $E_{AP} = 0.7$ V as this has been shown to be a suitable voltage for producing high current densities, while avoiding the potential for water electrolysis [18, 20]. Reactors were operated in fed-batch mode, with medium replacement after current dropped below 0.01 mA. Medium was replaced by piercing the butyl stopper with a needle and syringe, withdrawing the liquid contents, and refilling with sterile, anaerobic medium. This procedure avoids the need to remove the crimp top, and if performed under sterile conditions in a laminar flow hood eliminates possible reactor contamination. Between each cycle, reactor headspace was vacuumed and filled three times with either CO$_2$/N$_2$, [20/80] (BCM-30) or 100% N$_2$ (PBM-50 and PBM-200) in order to remove residual gases produced during the previous cycle. For the varied applied whole cell voltage scans, reactors were supplied fresh medium, operated for one hour at $E_{AP} = 0.7$ V, and then the applied voltage was changed from 0.2 V to 1.2 V at 0.2 V intervals every 10 minutes.

After multiple cycles, the mixed and pure culture reactors were analyzed for headspace composition. Headspace analysis was conducted by taking 200 µL samples using an airtight syringe (Hamilton) and injecting into two separate gas chromatographs (SRI Instruments) for measuring H$_2$, O$_2$, N$_2$, CH$_4$, and CO$_2$ as described previously [18]. Soluble chemical oxygen demand (COD) of the mixed culture reactors was performed at the beginning and end of the cycle by pre-filtering samples using a 0.2 µm filter and following a standard method (TNTplus COD Reagent; HACH Company). COD was used
for calculating the coulombic efficiency ($C_E$) by dividing the coulombs recorded as current by the coulombs of substrate consumed during a cycle [2].

4.3 Results

4.3.1 Cathode Material Evaluation

After several batch cycles at $E_{AP} = 0.7$ V (BCM-30), current densities of mixed culture MECs using SS mesh and graphite plates were considerably higher (ca. 120 A/m$^3$) than MECs using either SS wire (ca. 55 A/m$^3$) or Pt wire (ca. 35 A/m$^3$) (Figure 4.2). The current density of reactors using Pt wire exhibited the most variation, and a black precipitate formed on the Pt surface. Further examination of cathodes was performed by using a range of applied whole cell voltages (Figure 4.3), which indicated better current production for SS mesh; therefore this material was used in all further experiments.

4.3.2 Method Validation

Mixed culture MECs using SS mesh cathodes were compared in parallel (all reactors attached to the same power supply) and non-parallel operation (reactors attached to individual power supplies). Similar current densities were observed under both operation modes, with current densities of $I_V = 116 \pm 2$ A/m$^3$ recorded for parallel operation and values of $I_V = 107 \pm 2$ A/m$^3$ observed for non-parallel operation (Figure 4.4A). There
were minor differences in recorded anode potentials (parallel, −0.23 V; non-parallel, −0.22 V) and cathode potentials (parallel, −0.92 V; non-parallel, −0.91 V), with < 0.01 V standard deviation for all measurements (Figure 4.4B).

Coulombic efficiencies were similar for reactors operated in parallel ($C_E = 92 \pm 1\%$) and individually ($C_E = 90 \pm 0\%$). Gas pressure accumulated to ca. +0.22 ± 0.05 bar at the end of each cycle. The headspace contained methane, carbon dioxide, and nitrogen, and no hydrogen. Low hydrogen gas recovery is common in some membrane-free MECs, as methanogens can readily convert hydrogen into methane [14, 21, 22]. Typically hydrogen produced in MECs is collected in gas bags [14, 17] reducing the partial pressure present in the headspace and improving hydrogen recoveries. Since hydrogen was not collected in this study and it was allowed to accumulate inside the MEC, complete conversion of hydrogen to methane by methanogens occurred.

4.3.3 Culture Comparison

The utility of this method for comparative analysis of reactor conditions such as solution chemistry was explored by examining the effect of two different buffers on two known exoelectrogens (G. sulfurreducens and G. metallireducens) compared to a wastewater derived mixed culture. After recording stable current production for five fed-batch cycles ($E_{AP} = 0.7$ V, BCM-30), the solutions in the MECs were changed. Improvements in current production were noted for all cultures with the higher conductivity 50 mM phosphate buffer (PBM-50) with the mixed culture producing the highest current of $I_V = 238 \pm 15$ A/m$^3$ [averaged over two cycles] (Figure 4.5). Increased
current production was expected due to the increased solution conductivity of 7.5 mS/cm for the PBM-50 compared 4.5 mS/cm for the BCM-30.

After operating two cycles under these conditions, the buffer concentration was further increased to 200 mM (PBM-200), which resulted in appreciable drops in current for both pure cultures, but with steady current production of $I_V = 238 \pm 20$ A/m$^3$ for the mixed culture. *G. metallireducens* typically is cultivated in freshwater media [23]. The high increase in ionic strength using PBM-200 likely was responsible for the sharp drop in current for this bacterium despite the expected increase in performance due to the increased solution conductivity. *G. sulfurreducens* is capable of growth in medium containing up to one half the salt concentration of seawater [24], but here it showed poor current production with the high concentration of phosphate buffer for reasons not well understood. Following two cycles of operation with PBM-200, the medium was switched back to BCM-30 for two additional cycles. These final two cycles were consistent with the initial cycles using this medium, indicating good reproducibility and a lack of impact on culture viability despite changes in the buffer.

At the end of each cycle, the gas pressure accumulated in the headspace of *G. metallireducens* (a non-H$_2$ oxidizing exoelectrogen) reactors reached *ca.* 0.5 bar, and the reactors withstood this pressure without damage or apparent leaking, indicating the robustness of this design. The headspace of the pure culture reactors contained primarily hydrogen, along with smaller amounts of nitrogen and carbon dioxide and no methane. Although the maximum allowable pressure for these reactors has not been tested, similarly designed pressurized tubes used for culturing methanogens were shown to hold up *ca.* 4 bar of pressure even with multiple needle penetrations [13].
4.4 Discussion

A method for conducting high throughput BES research was developed by using a simple MEC reactor design with two electrodes immersed in serum bottles, and operating all reactors in parallel on a single power source. This technique was shown to be useful for examining materials (electrodes and catalysts) and the electromicrobiology (exoelectrogen performance) of BESs. Cathode screening indicated that the highest current densities could be obtained using SS mesh cathodes, with maximum volumetric current densities of 240 A/m$^3$ (2.6 A/m$^2$-anode) obtained with a mixed culture in PBM-50. This current density is comparable or better than previous results obtained using other single-chamber systems (Table 4.1) [15, 18, 22, 25], but the design used here is simpler, more affordable and did not require the use of precious metals. The reactors in this study are similar to a previously described design, but that MEC included a separator between the carbon cloth electrodes and separate power supplies were used for each reactor, producing ca. 43 A/m$^3$ in a highly conductive medium (~320 mM phosphate buffer) at $E_{AP} = 0.6$ V [15] (Table 4.1). The design of our mini-reactors has several improvements compared to that system, including a reduced electrode spacing (0.5 cm here versus 2 cm) [26], the lack of a separator which can improve proton diffusion from the anode to the cathode [27], and the use of a non-precious metal cathode (SS mesh here versus Pt on a carbon cathode) [17, 28]. These changes produced a five-fold increase in volumetric current density in a medium with lower conductivity (50 mM PBM), but at a slightly larger applied voltage (0.7 V here versus 0.6 V). Operating the reactors in parallel here
rather than using individual power supplies for each reactor further reduces operating costs and allows for easy inclusion of replicate reactors.

The effect of solution chemistry on current production of *G. sulfurreducens* and *G. metallireducens* was examined here using this new method. Consistent with previous work comparing these two cultures with a mixed culture in MECs, *G. sulfurreducens* generated the highest current density using identical medium (BCM-30, 1 g/L sodium acetate) [18]. Changing the BCM-30 medium in this study to a phosphate buffer with a higher conductivity (PBM-50) led to an increase in current production for all cultures, but the use of a very high concentration of phosphate buffer (PBM-200) produced an appreciable decrease in current for both pure cultures. The mixed culture produced the same current density in both phosphate buffer solutions. Most BES comparative culture studies have been conducted with a single medium [18, 29-31], yet these results suggest that the choice of medium and ionic strength may adversely affect current generation by some microorganisms. The method developed here allows for a more thorough analysis of culture performance with different media and microorganisms.

When this method is used for the examination of mixed or pure cultures, it is important to consider hydrogen utilization by exoelectrogens and other microbes. When exoelectrogens oxidize a substrate at the anode, such as acetate, the current results in hydrogen evolution at the cathode. This produced hydrogen can be consumed by exoelectrogens for the generation of current (hydrogen recycling) [18, 32] or used for the reduction of carbon dioxide to produce methane (methanogenesis) or acetate (acetogenesis) [33]. It is possible to sparge the reactors with other gases (such as N_2 or a N_2:CO_2 mixture) to remove any gases produced in the system. Providing gas lines to a
large number small reactors would be difficult, although a multiple line gas manifold placed next to a series of bottles could be used to minimize space. These different metabolisms possible should be taken into account when considering the use of any single-chamber MEC as electron recycling through hydrogen oxidation or acetogenesis can result in elevated coulombic efficiencies. Alternatively, single-chamber methods provide a unique approach for the enrichment of biocathodes [4] or the investigation of interspecies interactions among competing metabolisms.

The results of this work show that many of the challenges of developing miniaturized high throughput methods can be overcome using small-scale membrane-free MECs. First, small electrode spacing and the lack of separators used in this design reduced factors such as pH gradients and proton diffusion resistance that contribute to high internal resistance [26, 27]. Several previous miniaturized designs have noted limitations in current production due to high internal resistance, which is often the primary factor limiting current generation, not the exoelectrogenic microorganisms [11, 34]. Second, the design is based on inexpensive and commercially available materials, with each MEC described here costing ca. $1.50. The cost and time required for manufacturer of some other miniaturized designs, in particular multiple-well array architectures, can be prohibitive for researchers entering the field of BESs or for those lacking access to the appropriate resources. Third, this method allows for high throughput research on a scale greater than previously described. Based on the maximum current limitation of the power supply used in this study (3 A), roughly 6,000 reactors could be operated in parallel using a single power supply assuming current production comparable to that observed at $E_{AP} = 0.7$ V (ca. 0.0005 A per reactor). Previous small scale designs have ranged from a single reactor
to 24 individual wells in a multiple electrode array [5]. Therefore, this simplified and robust method will provide both new and established researchers with a powerful tool for conducting small-scale based BES research at a large scale.

4.5 Literature Cited


Table 4.1. Summary of current densities from previous membrane-free MECs at similar applied voltages.

<table>
<thead>
<tr>
<th>Anode</th>
<th>$E_{AP}$ (V)</th>
<th>Buffer</th>
<th>$V$ (mL)</th>
<th>$I_V$ (A/m$^3$)</th>
<th>$I_A$ (A/m$^3$)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>carbon cloth</td>
<td>0.6</td>
<td>PBM-320</td>
<td>300</td>
<td>43</td>
<td>9.3</td>
<td>[15]</td>
</tr>
<tr>
<td>graphite plate</td>
<td>0.7</td>
<td>PBM-50</td>
<td>5</td>
<td>240</td>
<td>2.6</td>
<td>this study</td>
</tr>
<tr>
<td>graphite brush</td>
<td>0.7</td>
<td>PBM-50</td>
<td>28</td>
<td>250</td>
<td>--</td>
<td>[14]</td>
</tr>
<tr>
<td>carbon cloth</td>
<td>0.7</td>
<td>BCM-30</td>
<td>18</td>
<td>160</td>
<td>6.7</td>
<td>[18]</td>
</tr>
<tr>
<td>carbon cloth</td>
<td>0.7</td>
<td>PBM-50</td>
<td>120</td>
<td>98</td>
<td>3.9</td>
<td>[25]</td>
</tr>
<tr>
<td>graphite granules</td>
<td>0.8</td>
<td>PBM-50</td>
<td>256</td>
<td>223</td>
<td>--</td>
<td>[22]</td>
</tr>
</tbody>
</table>

*a* na – data not available from source
4.7 FIGURES

Figure 4.1. Schematic (left) and photos of MEC design (middle) and parallel operation setup (right) using single power supply located behind reactors. Reference electrodes were omitted from the MECs shown in the photos.

Figure 4.2. Current densities recorded for different cathodes over the course of multiple fed-batch cycles using a mixed culture. All reactors were operated in duplicate at $E_{AP} = 0.7 \text{ V (BCM-30)}$ [SS – stainless steel, Pt – platinum, Graphite – graphite plate].
Figure 4.3. Current densities at different applied whole cell voltages (Mixed cultures in BCM-30, with reactors tested in duplicate. Ctrl indicates uninoculated reactor with a SS mesh cathode).
Figure 4.4. (A) Current densities recorded for mixed culture reactors using SS mesh cathodes operated in parallel (∥) or non-parallel (∥) mode. (B) Anode and cathode potentials recorded during the same cycles from (A). Vertical dotted lines indicate start of second cycle. All reactors were operated in duplicate at $E_{AP} = 0.7$ V (BCM-30).
Figure 4.5. Current densities recorded for pure and mixed cultures using varied buffers. All reactors were operated sequentially as shown in the figure [BCM-30 – 30 mM bicarbonate buffer, PBM-50 – 50 mM phosphate buffer, PBM-200 – 200 mM phosphate buffer, MC – mixed culture, GS –*G. sulfurreducens*, GM –*G. metallireducens*]. All reactors were operated in duplicate at $E_{AP} = 0.7$ V.
Chapter 5

Applying Fluorescent In-Situ Hybridization to Study Exoelectrogenic Communities

Abstract

The bacteria that compose anodic biofilms are critical components of bioelectrochemical systems (BESs), functioning as converters of chemical to electrical energy. Deciphering which bacteria are present and their roles is therefore important for advancing our understanding of the capabilities of BESs and how they can be improved. Molecular techniques are required for identifying bacteria in mixed communities, and this chapter focuses on the use of fluorescent in situ hybridization (FISH) as a tool to corroborate other molecular techniques, analyze species abundance, and visualize anodic biofilm structure in BESs.

5.1 Introduction

Molecular methods have significantly improved our understanding of microbial communities in natural and engineered environments. Before these techniques were created, identifying bacteria required isolation and classification according to characteristics such as morphology, physiology, and response to simple staining procedures. Molecular methods have taken us a step further by identifying bacteria based on highly conserved regions of their DNA [1]. In particular, the region that encodes the
16S rRNA is most commonly used as a bacterial fingerprint because it is subject to less variation than other regions that encode, for example, proteins. When these sequences are recovered from a mixed community, they can be aligned against other sequences associated with known and unknown bacteria in order to aid in identification. The laboratory methods for obtaining 16S rRNA sequences have advanced rapidly in the past decades, with the ability now available to obtain thousands of sequences within a fraction of the time of the earliest methods. Although these techniques can provide an abundance of information regarding the microbial diversity of a sample, precise quantification of microbial communities remains a challenge.

One method that allows for the in-situ visualization and quantification of bacteria is fluorescent in situ hybridization (FISH). First developed back in 1989, FISH relies on the evolutionary conservation of 16S rRNA sequences to characterize communities from the phylum to the species level using fluorescently labeled probes [2]. These probes are short oligonucleotide sequences, typically 18 – 20 nucleotides long, with a fluorescent dye attached at one end. Rather than directly targeting the DNA as described above, FISH targets the complementary sequence on the RNA molecule that is located on the ribosome. Typically the 16S rRNA (small subunit) is targeted because the sequences of this molecule for almost all described bacteria and archaea are known, but the large subunit, or 23S and 5S rRNA, can also be targeted. Each 16S rRNA consists of ca. 1,600 nucleotides and each bacterium can contain several ribosomes, which means that the target molecule can range from 100’s to 100’s of thousands per cell. Multiple probes each bearing different fluorescent dyes and targeting different phylogenetic levels can be added to a mixed community to distinguish different organisms. The ease with which
oligonucleotide probes can be synthesized combined with the availability of 16S rRNA sequences has therefore allowed FISH to become a powerful tool for studying microbial communities [3].

5.2 FISH Methodology

FISH involves three main steps: fixation, hybridization, and visualization. During fixation, bacterial cell membranes are permeabilized using a fixative such as paraformaldehyde or ethanol. This step also stabilizes the cell shape and prevents loss of cell integrity through lysis. After fixation, the cells are dehydrated in a series of ethanol rinses to remove water while maintaining cell structure. Next, the fluorescently labeled probes are added in conjunction with a hybridization buffer and the samples are allowed to sit for several hours to allow for the probe to hybridize to the target rRNA.

Figure 5.1. Steps involved in FISH. Samples are first fixed, then the probe is hybridized to the rRNA of the target cells, and finally the cells are visualized using microscopy or cytometry. From Amann and Fuchs, 2008 [3].
Instead of using temperature to control the specificity of probe binding, formamide can be added during hybridization to reduce non-specific binding of the probe to closely related organisms. Non-labeled helper probes that flank either one or both sides of the FISH probe target site can be added during the hybridization procedure to improve target site accessibility [4]. Afterwards, the cells are washed to remove excess probe and the samples are prepared for observation or identification using either epifluorescent microscopy or flow cytometry. Using different fluorescent filters, multiple images can be captured using microscopy to visualize the localization of target organisms or cells can be sorted based on the emission wavelength of the attached fluorescent dye using cytometry. A more in-depth description of the methodology used for the analysis of BES communities in this work can be found in the published and submitted papers in listed at the end of Chapter 1 (section 1.7).

5.3 BES Applications of FISH

The microbial communities of BESs are typically investigated using 16S rDNA clone libraries, with only a few studies incorporating FISH to quantify or confirm clone library results. FISH has also been used to observe the distribution of pure and defined culture biofilms. Using a co-culture of *Geobacter sulfurreducens* and *Pelobacter carbinolicus*, Richter et al. used species specific probes for these two bacteria to study the anode biofilm structure in a microbial fuel cell (MFC) [5]. The MFC was supplied a fermentable substrate (ethanol) which cannot be utilized by *G. sulfurreducens*, therefore *P. carbinolicus* fermented ethanol to acetate and hydrogen, which then provided suitable
substrates for *G. sulfurreducens* to generate electrical current. *P. carbinolicus* is incapable of current generation, yet FISH revealed an equal distribution of *P. carbinolicus* and *G. sulfurreducens* throughout the anode biofilm. These results also confirmed those obtained by 16S clone libraries, which estimated an even distribution of the two bacteria on the anode. In a separate defined culture study, several gram negative and gram positive bacteria were tested together for current production in an MFC, with the resulting biofilms examined using FISH [6]. Fluorescence microscopy revealed that some of the tested species developed into tall towering biofilms that extended above the surface of other cultures, suggesting that some bacteria have developed strategies for outcompeting other anode colonizing bacteria. However, no other species competition studies have been conducted using FISH despite its usefulness in visualizing biofilm structure.

In non-defined mixed culture biofilms, FISH has also been used to examine the community structure on both anodes and cathodes. In a mixed culture MFC fed propionate, subclass probes for the Proteobacteria were used, and it was discovered that both Delta- and Betaproteobacteria colonized the outer edges of the biofilm [7]. However, no quantification or comparison with clone libraries was performed. In a cellulose fed MFC, sub phylum level FISH probes were used to confirm the presence and morphology of particular bacteria involved in cellulose degradation [8]. Based on 16S clone library results, the community contained sugar fermenting bacteria from the *Chloroflexi* sub phylum, and FISH probes targeting this group confirmed both their presence and filamentous morphology. Mixed biocathode communities performing simultaneous nitrification and denitrification were also examined using FISH and revealed
that the outer layer closest to oxygen consisted of nitrifying organisms while denitrifying organisms were located closer to the electrode surface [9].

5.4 Objective

The objective of the work described in this chapter is to apply FISH for the characterization of mixed microbial anodic communities in different BESs. In particular, the aims are to use FISH to corroborate 16S clone library results, reveal the distribution and abundance of exoelectrogenic and non-exoelectrogenic bacteria, and examine the abundance of the high power producing exoelectrogen, *G. sulfurreducens*, in relation to reactor performance. These studies will be written as brief summaries of the manuscripts, with the entire manuscripts from which they are derived listed in Chapter 1 (section 1.7).

5.5 Study 1 – Abundant yet Non-Exoelectrogenic Bacteria in MFCs

One envisioned application of BESs is the conversion of cellulosic fermentation end products into useable energy such as electricity or hydrogen gas [10-12]. Most BESs are supplied acetate as this is both a common fermentation end product and is non-fermentable under anaerobic conditions [13]. There are various other end products that have not been fully examined in BESs, including the acids lactic, formic, and succinic, and the alcohol, ethanol [10]. Under anaerobic conditions, a variety of metabolic pathways are possible from these substrates, including conversion to acetic acid, hydrogen, and eventually methane. To test the feasibility of converting typical fermentation end products into electricity in an MFC, these typical substrates produced
during hydrogen production from cellulosic fermentation were supplied to separate MFCs [12]. Reactor performance for each substrate will not be reported here, as the primary focus of this section is on the mixed culture community within the formic acid fed MFC.

5.5.1 Community Analysis

After operating duplicate formic acid fed MFCs for one year, anode biofilm samples were obtained for both DNA extraction and FISH analysis. Up to this point, the MFCs exhibited poor performance relative to the other fermentation end products tested. Maximum power reached \(ca.\ 60 \text{ mW/m}^2\) compared to \(ca.\ 830 \text{ mW/m}^2\) for acetic acid and \(ca.\ 740 \text{ mW/m}^2\) for lactic acid. Extracted DNA was used to construct clone libraries for the determination of community diversity. Clone libraries revealed that \(ca.\ 30\%\) of the community was similar to \textit{Paracoccus denitrificans}, a facultative denitrifying bacterium, and another \(15 – 30\%\) of the community was represented by clones most similar to \textit{Geobacter sulfurreducens} (Figure 5.2).
Figure 5.2. Clone library results of formic acid fed MFC anode community using primer pairs (a) 338F – 518R and (b) 530F – 1490R. Figure from [14].

FISH analysis using Paracoccus genus specific probes confirmed the presence of Paracoccus within this mixed culture biofilm based both on positive hybridization and the coccoidal morphology of detected cells (Figure 5.3). Images showed that this genus was well distributed throughout the biofilm, and cell counts revealed that ca. 30% of the community consisted of this genus.
5.5.2 Performance of an Isolate in an MEC

A *Paracoccus* strain was isolated after the mixed culture was serially diluted and plated on agar plates containing Luria-Bertani (LB) medium. The strain recovered most closely matched *Paracoccus denitrificans* PD 122, but due to a more diverse carbon source utilization, the isolate was considered a novel strain and named *Paracoccus denitrificans* PS-1. After growing the isolate overnight in LB to a cell OD₆₀₀ of ca. 0.5, the cells were centrifuged, resuspended in sterile medium, and inoculated into sterile, anaerobic mini-MECs [15] containing a diversity of carbon sources in a based medium.
containing (per L): 0.31 g NH4Cl, 0.13 g KCl, 2.45 g NaH2PO4•H2O, 4.57 g Na2HPO4.

Substrates examined included formate, acetate, lactate, glucose, ethanol (all at 1 g/L), and LB (at 10 g/L). Reactors were operated in duplicate at an applied voltage of 0.7 V and a wastewater inoculum was used as a positive control. None of the substrates tested resulted in the production of current from the isolate *P. denitrificans* PS-1, while current was generated for all substrates except LB by the wastewater inoculum (Figure 5.4).

![Graphs showing current densities for various substrates](image)

Figure 5.4. Current densities recorded for both the wastewater derived mixed culture (MC) and replicates of *P. denitrificans* (PD A, PD B) in mini-MECs at an applied voltage of 0.7 V.
5.5.3 Implications

A bacterium shown to be highly abundant and well distributed throughout the biofilm of an anode was found to be non-exoelectrogenic. These results suggest that the predominance of a bacterium on an anode does not necessarily translate into a functional role as a current producer. Instead, this bacterium may have served as a converter of formic acid into a substrate that could be used by the known exoelectrogen \textit{G. sulfurreducens}. Hydrogen production from formate may have been a possible route, as \textit{P. denitrificans} is known to use the enzyme formate dehydrogenase to convert formate into hydrogen and carbon dioxide [16]. This metabolism would provide hydrogen as a suitable electron donor for current generation by \textit{G. sulfurreducens}, but a suitable carbon source for this bacterium would not be available as neither formate nor carbon dioxide can be used for biomass production [17, 18]. The relatively low percentage of clones matching to \textit{G. sulfurreducens} compared to formate fed MFCs operated under different conditions (discussed in Study 3) where acetate was produced by acetogenic bacteria, suggests that carbon source limitation may have been a factor in the poor reactor performance. \textit{P. denitrificans} may also have served a role as an oxygen scavenger for bacteria that prefer anaerobic conditions, such as \textit{G. sulfurreducens}. \textit{G. sulfurreducens} can grow with up to 10% oxygen as the terminal electron acceptor, however biofilms of \textit{G. sulfurreducens} have been shown to be compromised in MFCs using air based cathodes [19, 20]. These results also suggest that operating MFCs without air based cathodes (ie – using a ferricyanide catholyte) or using microbial electrolysis cells (MECs) may lead to more effective recovery of exoelectrogenic bacteria.
5.6 Study 2 – *Geobacter* or *Pelobacter*: The Importance of Using Multiple Molecular Methods

As with all scientific methods, molecular methods for characterizing communities are subject to biases and limitations. Typically only one technique is used to decipher the members of a mixed culture BES community, with conclusions drawn based on the single technique. However, biases inherent in the technique may not provide an accurate picture of the community members present and the use of multiple techniques may therefore be needed. In this study, FISH was used as a second method to corroborate the results of a 16S rDNA clone library derived from a mixed culture MFC anode.

5.6.1 *Pelobacter* Dominates Clone Library

After operation of an acetate fed MFC for over one year in fed-batch operation, a portion of the anode was obtained for both DNA extraction and FISH analysis. Up to this point, the reactor was producing high power densities (*ca.* 830 mW/m²), comparable to previously described acetate fed MFCs. Extracted DNA was used to construct clone libraries for the determination of community diversity. Clone libraries revealed that more than 60% of the community was most similar to *Pelobacter propionicus*, with no clones representing *G. sulfurreducens* detected (Figure 5.5).
The genus *Pelobacter* contain secondary fermenters (i.e., they cannot ferment sugars) with some species (*P. carbinolicus*) capable of dissimilatory iron reduction [21, 22]. All previously described *Pelobacter* species are incapable of acetate oxidation, likely due to a lack of acetate transporters throughout this genus [21, 23]. Cytochromes are believed to be critical enzymes needed for enabling current production, yet *Pelobacter* sp. lack the c-type cytochromes necessary for extracellular electron transfer to electrodes [23], which explains why the bacterium *P. carbinolicus* is incapable of current generation as a pure culture in an MFC [5]. Therefore, it is surprising that the MFC anode in this study was dominated by this genus.

5.6.2 *Geobacter* Dominates FISH Results

During peak current production in a fed-batch cycle, the anode biofilm was sampled for fluorescent in situ hybridization (FISH) analysis. Several fibers were cut from the mixed culture anode, carefully transferred to sterile tubes (2 mL capacity) to
minimize disruption of the biofilms, and covered with 0.5 mL of a phosphate-buffered saline (FISH-PBS) solution (10 mM Na\textsubscript{2}PO\textsubscript{4}, 130 mM NaCl, pH 7.2–7.4). Cells were fixed by adding 1.5 mL of 4% paraformaldehyde and stored for 3 h at 4°C. Following fixation, the samples were rinsed using FISH-PBS and stored in 50% ethanol, 50% FISH-PBS at \( -20^\circ C \). Before hybridization, several fibers were transferred to sterile 200 \( \mu \)L PCR tubes and allowed to dry at 46°C for 15 min. The fibers were then successively dehydrated in 50%, 80%, and 100% (vol/vol) ethanol for 3 min each. After air drying, fibers were separated and placed in different tubes for each probe condition. Hybridization buffer containing 0.9 M NaCl, 20 mM Tris-HCl (pH 8.0), 0.02% SDS, and no formamide was added (100 \( \mu \)L) to each tube. A probe targeting the type strain of \textit{Pelobacter propionicus} was designed using the probe design tools in the ARB software (Table 5.1). A formamide series was not performed in order to optimize the specificity of this probe; however, the most closely matching species as shown in Table 5.1 contained several mismatches with the target sequence, suggesting a low probability of non-specific probe binding. Two helper probes designed to flank either side of the FISH probe were also included. The probe named PBP184 (targets location 184 on the \textit{E. coli}. 16S rRNA) was labeled with Alexa Fluor 488 (Invitrogen) and added (10 \( \mu \)L) for a final concentration of 4.5 ng/\( \mu \)L. No probe and nonsense probe controls were included to examine for autofluorescence and nonspecific binding of the dye, respectively. Probe GEO2, which targets \textit{G. sulfurreducens}, was 5'-labeled with Alexa Fluor 594 (Invitrogen) and added for a final concentration of 4.5 ng/\( \mu \)L. Two unlabeled helper probes HGEO2-1 and HGEO2-2 were added at the same concentration as the labeled probe. Hybridization was performed in the dark using a hybridization oven (Model 5430, VWR) at 46°C for
1.5 h. After hybridization, the buffer was removed and 100 μL of a pre-warmed (48°C) wash buffer containing 70 mM NaCl, 20 mM Tris-HCl, 5 mM EDTA, and 0.01% SDS was added, and the samples were incubated at 48°C for 15 min in the dark. After removing the wash buffer, the fibers were gently rinsed twice with 100 μL of cold (4°C) milli-Q water. Fibers from each tube were spread onto respective wells of a multi-well Teflon-coated slide (Electron Microscopy Sciences) and mounted and counterstained simultaneously using an antifade reagent containing 4’,6-diamidino-2-phenylindole (DAPI) (SlowFade Gold with DAPI, Invitrogen). Samples were examined on an Olympus BX61 epifluorescent microscope equipped with DAPI and TexasRed filter sets. Images were recorded using a DP72 digital camera and analyzed using the DP2-BSW software.

Table 5.1. FISH probe sequences used in this study compared to 16S sequences of closely related bacteria. Red font indicates mismatches with probe sequence.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Probes</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. propionicus</td>
<td>G. sulfurreducens</td>
<td>GACCAAAGCCGTCGTGGG</td>
</tr>
<tr>
<td>16S</td>
<td>G. metallireducens</td>
<td>ATCCGTAGACCCCGTGGG</td>
</tr>
<tr>
<td>P. carbinolicus</td>
<td>G. sulfurreducens</td>
<td>GAAGACAGGAGGCCCGAAA</td>
</tr>
<tr>
<td>hPBP165</td>
<td>P. propionicus</td>
<td>GATGACAGAAGGCCAAATG</td>
</tr>
<tr>
<td>hPBP194</td>
<td>G. metallireducens</td>
<td>AGTGACAAAAAGGCCGAAG</td>
</tr>
<tr>
<td>G. metallireducens</td>
<td>P. carbinolicus</td>
<td>GATGACGGTAGGCCGAAG</td>
</tr>
<tr>
<td>GBS194</td>
<td>G. sulfurreducens</td>
<td>GTCCCCCCTTTTCCCGCAAC</td>
</tr>
<tr>
<td>GBS226</td>
<td>hGBS194</td>
<td>GTCCCCCCTTTTCCCGCAAG</td>
</tr>
<tr>
<td>hGBS226</td>
<td>hPBP194</td>
<td>CTAATGGTAGCGGACTCATCC</td>
</tr>
</tbody>
</table>
Positive controls for probes PBP184 and GEO2 were carried out on pure cultures of both *P. propionicus* and *G. sulfurreducens*, respectively (Figure 5.6). Probe PBP184 did not hybridize to a pure culture of *G. sulfurreducens* (type strain) and probe GEO2 did not hybridize to a pure culture of *P. propionicus* (type strain) (data not shown).

![Image](image_url)

Figure 5.6. Validation of FISH probes targeting (A) *P. propionicus* (green) and (B) *G. sulfurreducens* (red). (C) Mixture of the two cultures showed no overlap of FISH signals and differences in cultures based on expected size differences. White bars represent 10 µm.

After confirmation of the probes specificity, FISH was performed on the mixed culture sample. Based on fluorescence imaging, no *P. propionicus* cells were detected, while the
biofilm was dominated by cells that positively hybridized to the *G. sulfurreducens* probe (Figure 5.7). Cell counts of *G. sulfurreducens* were not performed, but qualitative results revealed that a majority of cells were represented by this bacterium.

![FISH image of mixed culture anode biofilm](image)

Figure 5.7. FISH image of mixed culture anode biofilm spread onto a glass slide showing *G. sulfurreducens* (red) and all cells (blue; DAPI stain). White bar represents 10 µm.

### 5.6.3 Implications

Clone library analysis of an acetate fed MFC revealed a high percentage of clones matching to *P. propionicus*, yet FISH results indicated a lack of this bacterium and instead a predominance of *G. sulfurreducens*. These contradictory results highlight the importance of incorporating at least two molecular methods when characterizing mixed culture communities in natural and engineered systems. The reasons for the discrepancy may have been due to biases associated with these method and/or evolutionary changes in the cultures over time.
Prior to clone library construction, PCR is conducted to amplify a portion of the 16S rDNA. During this step bias towards particular species can occur because primers selected may favor one organism over the other [24, 25]. Typically multiple primers are used to reduce this bias, but in this work, only one primer set was incorporated. Among bacteria, the number of rRNA operons can vary anywhere from one to fifteen, with *P. propionicus* containing four and *G. sulfurreducens* with two [23, 26]. A higher number of rRNA encoding genes may therefore translate into an overrepresentation during PCR amplification, which in turn could overestimate the number of clones representing that organism. After clone libraries are constructed and sequences obtained, ribosomal databases to which the sequences are compared may also yield discrepancy depending on phylogenetic structure inherent in their design. For example, the nucleotide collection at the basic local alignment search tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) website from which the clone library in this work is derived reported a dominance of *P. propionicus*. However, when the ribosomal database project (RDP) (http://rdp.cme.msu.edu/) is used instead, these same sequences are classified within the *Geobacter* genus; however, this contradicts the results from BLAST as *P. propionicus* is classified within the *Pelobacter* genus. Further examination of the differences of these two databases is warranted in order to clarify this discrepancy.

The limitations of FISH include non-specific binding of the probe to non-target cells, cell membranes, and non-living material. To reduce these limitations, both positive (probe testing on type strains) and negative (nonsense and no probe) controls were performed in this work. The sequence of the *P. propionicus* probe designed in this study was located outside the sequences used in the construction of the clone library so it was
impossible to confirm the probe specificity against the *P. propionicus* sequences obtained. The type strain sequence of *P. propionicus* was used to design the FISH probe, yet a more accurate approach may have been to use the *P. propionicus* sequences obtained from the clone libraries. However, the fact that the *G. sulfurreducens* probe, which was designed using the type strain, exhibited positive responses for this sample and other mixed culture BES anodes (see Study 3), suggests that using the type strain sequence is acceptable for FISH analysis.

The second explanation for the discrepancy may be evolutionary changes or horizontal gene transfer that occurred within the biofilm. Adaptive evolution of a pure culture of *G. sulfurreducens* occurred when an anode was fixed at a potential and operated for over six months [27]. After this time period, a new variant from this biofilm was obtained that was 100% identical to the wild type based on 16S rDNA sequencing, yet it exhibited substantial phenotypic differences along the cell periphery. This new variant was also capable of increased current production and reduced startup times compared to the wild type. In this study the reactors were operated for a period of one year, which may have allowed for a similar adaptive evolution of the mixed community. Adaptation of *P. propionicus* for electron transfer to an electrode could therefore be an explanation or the transfer of genes involved in extracellular electron transfer from *G. sulfurreducens* to *P. propionicus* may have also occurred. Further investigation into the reasons causing this discrepancy and the potential for horizontal gene transfer in exoelectrogenic biofilms is therefore warranted.
5.7 Study 3 – Predominance of \textit{G. sulfurreducens} in BES Communities Revealed Using FISH

\textit{G. sulfurreducens} is commonly detected in clone libraries of wastewater derived mixed community MFCs fed acetate [28-30]. Its presence and importance when other substrates are supplied, different innocula are used, or different types of BES reactors are operated is not as well understood. The objective of this study was therefore to examine the abundance of \textit{G. sulfurreducens} throughout multiple BESs, each operated under different conditions and to examine if the abundance of this bacterium could be correlated to reactor performance.

5.7.1 Microbial Desalination Cells

Current desalination technology consumes a substantial amount of energy to produce fresh drinking water [31]. Finding ways to minimize the energy input is important for developing sustainable drinking water supplies for both developed and developing countries. Recently a new type of BES was discovered that is capable of removing a substantial portion of salt from water without continuous energy input [32]. In these microbial desalination cells (MDCs), two membranes are inserted between an anode and cathode of an MFC. An anionic exchange membrane placed against the anode compartment allows negatively charged $\text{Cl}^-$ ions to pass through, balancing the charge of the free protons ($\text{H}^+$) produced during bacterial oxidation of organic matter. Positively charge sodium ions ($\text{Na}^+$) pass through a cationic exchange membrane that is placed against the cathode compartment, balancing the charge of the electrons transported to the
cathode. When oxygen is present at the cathode, the MDC operates as a modified MFC, and the cell voltage decreases over time [33]. This operation leads to a decrease in the efficiency of the desalination chamber as the voltage between the two electrodes is not constant. As an alternative approach, a voltage can be applied between the anode and cathode, leading to the operation of a microbial electrodialysis cell (MEDC) [34]. In either modes of operation, Cl\(^-\) ions enter the anode chamber, potentially affecting the exoelectrogenic community. The effect of two different salt solutions (5 and 20 g/L NaCl) on MEDC reactor performance and abundance of *G. sulfurreducens* was therefore investigated. MEDCs desalinating the lower concentration salt solution showed improved performance in terms of desalination (68 vs. 37%), more negative anode potentials, and coulombic efficiency (48 vs. 16%). Similar total cell numbers were observed on the anodes under both salt concentrations, but a species specific FISH probe targeting *G. sulfurreducens* indicated a higher abundance of this bacterium in the low salt solution MEDC. Roughly 80% of cells were identified as *G. sulfurreducens* in the 5 g/L MEDC (Figure 5.8 A) compared to ca. 60% for the 20 g/L (Figure 5.8 B). These results confirm the predominance of this bacterium in BESs supplied acetate, and show a correlation between reactor performance and the quantity of *G. sulfurreducens* cells present.
5.7.2 Complex Wastewater fed BESs

In the laboratory, BESs are typically supplied an easily tractable substrate, such as acetate, but large scale BESs are envisioned for the treatment of complex waste streams, such as domestic and industrial wastewaters. Understanding how reactors perform when supplied complex wastewaters is therefore important for estimating large scale performance. Two wastewaters were examined for their ability to generate power in MFCs and subsequently converted into MECs to investigate the potential for hydrogen recovery [35]. Potato wastewater was obtained from a potato processing plant, and dairy manure wastewater from a dairy farm. These wastewaters were supplied in fed-batch operation to MFCs, with a slightly higher power density recorded for the potato wastewater fed MFC. The reactors were then operated as MECs by making the cathode anaerobic and applying a voltage of 0.9 V. Substantially higher current was obtained using the potato wastewater (ca. 4.5 mA) compared to the dairy wastewater (> 1 mA).

Figure 5.8. FISH images of anode biofilm suspensions from an MEDC operated with (A) 5 g/L or (B) 20 g/L NaCl. Red/pink – *G. sulfurreducens*; Blue – All Bacteria and Archaea (DAPI). White bar represents 10 µm.
FISH analysis of both anodes indicated a higher abundance of *G. sulfurreducens* in the community supplied potato wastewater.

Figure 5.9. FISH images of anode biofilm suspensions from (A) potato wastewater and (B) dairy wastewater fed MFCs. Red – *G. sulfurreducens*; Blue – All Bacteria and Archaea (DAPI). White bar represents 10 µm.

This bacterium was well distributed throughout the biofilm in the potato wastewater MFC (Figure 5.9 A), compared to small clumps of this bacterium scattered throughout the dairy wastewater anode (Figure 5.9 B). Although the wastewaters were not identical in composition (potato wastewater had more than double the conductivity of the dairy wastewater), these results suggest that wastewaters that can support the growth and development of *G. sulfurreducens* can lead to improved reactor performance.
5.7.3 Varied Innoculum Source

Domestic wastewater is the most common inoculum for BESs [13, 36, 37], yet little is known about the effect of inoculum source on BES performance and the resulting anodic community. The objective of this study was to examine the impact of three different innocula (two wastewaters and a bog) on MFC power production and community development [38]. All MFCs were operated under identical conditions with acetate as the carbon source. Although the bog inoculated MFC produced current faster than the wastewater inoculated MFCs, power production eventually converged for all reactors. Similarly, the anodic communities as analyzed by both 16S rDNA clone libraries and pyrosequencing initially were different but converged in composition by the end of multiple batch cycles. The abundance of *G. sulfurreducens* was confirmed using multiple molecular methods, with ca. 58% recorded for 16S rDNA clone libraries, ca. 63% for FISH, and ca. 81% for pyrosequencing. Despite the use of different inoculum sources, these results show good reactor performance is dependent on the presence of *G. sulfurreducens*.

5.7.4 Formic Acid Fed MFCs

As discussed in Study 1, cellulosic fermentation end products in addition to acetate are potential substrates for energy production in BESs. Study 1 showed that formic acid MFCs exhibit low power production compared to other fermentation end products, such as acetate, lactate, and ethanol when operated over a fixed external
resistance of 1000 ohms despite thermodynamic predictions of high cell voltages [12]. Those MFCs were operated under non-anode controlled conditions which allow the bacteria to control the potential of the anode presumably using respiratory enzymes. In an attempt to improve performance of MFCs supplied formic acid, this study looks at the effect of fixing the potential of the anode using a three electrode setup and an electrochemical device called a potentiostat. Two fixed anode potentials of −0.15 V and +0.15 V (vs standard hydrogen electrode) and a control reactor (operated using a 1000 Ω resistor) were selected and all reactors were supplied 1 g/L formic acid as the electron donor [39]. The −0.15 V fixed anode produced the highest current and power, while the other fixed anode performed slightly better than the control MFC. The largest percentage (58%) of clones matching to *G. sulfurreducens* was detected on the −0.15 V fixed anode, compared to 36% for the +0.15 V anode and 21% for the control anode. In the MFC with the largest number of *G. sulfurreducens* clones, 17% of clones also matched to the genus *Acetobacterium*, which consist of bacteria capable of generating acetate from formic acid. Acetate production was detected in the set anode potential reactors during the course of a batch cycle, suggesting that *G. sulfurreducens* used this substrate as a carbon source and electron donor for anode respiration, although direct formate oxidation by *G. sulfurreducens* may have been another route for current production with acetate present as a carbon source [18]. FISH was conducted to verify the presence of *G. sulfurreducens* and to quantify this bacterium in each reactor. At the −0.15 V fixed potential reactor, ca. 90% of cells were *G. sulfurreducens*, while lower percentages (ca. 45%) were noted for the +0.15 V fixed reactor and control MFC (Figure 5.9).
Figure 5.10. FISH images of anode biofilm suspension from MFCs operated with fixed anode potentials of (A) $-0.15$ V and (B) $+0.15$ V. Red/pink – *G. sulfurreducens*; Blue – All Bacteria and Archaea (DAPI). White bar represents 10 µm.

It is believed that the cytochromes responsible for current production by *G. sulfurreducens* have a midpoint potential of *ca.* -0.15 V, therefore by setting the anode potential to this value likely favored electron transfer through these respiratory enzymes [40, 41]. This operation led to the highest abundance of *G. sulfurreducens* and the best performance of all reactors, suggesting that operating conditions can have a significant impact on reactor performance.

## 5.8 Conclusions

The huge microbial diversity in natural and engineered environments requires close examination using molecular methods to understand which bacteria are present and how to better improve engineered systems. Due to biases in all molecular techniques, incorporating multiple methods is needed to obtain a more accurate picture of the
communities present. The results of this work show that FISH in conjunction with other methods, such as 16S rDNA clone libraries or pyrosequencing, can provide a more in-depth insight into the distribution and quantity of particular bacteria or groups of bacteria. In particular, FISH was used to confirm the presence and determine the abundance of the key exoelectrogen \( G. \text{sulfurreducens} \) in multiple BESs. Reactors with higher percentages of this bacterium were shown to perform better than those with lower quantities, and the way in which reactors were operated could determine whether biofilms were dominated by non-exoelectrogenic bacteria or \( G. \text{sulfurreducens} \). Future work examining electrode colonization and competition over time using FISH may help us understand how \( G. \text{sulfurreducens} \) can predominate when other bacteria are present and what mechanisms might be used to outcompete other bacteria. Further improvements of FISH methods to study intact biofilms, rather than biofilm suspensions are also needed to study community distribution relative to location on an electrode. Continuing to apply and advance FISH to study BES communities is therefore a critical tool for advancing our understanding of the microbial ecology and potential applications of these systems.

5.9 Literature Cited


18. Coppi, M. V.; O'Neil, R. A.; Leang, C.; Kaufmann, F.; Methe, B. A.; Nevin, K. P.; Woodard, T. L.; Liu, A.; Lovley, D. R., Involvement of *Geobacter*


Chapter 6

Hydrogen Production by Geobacter Species and a Mixed Consortium in a Microbial Electrolysis Cell

Abstract

A hydrogen utilizing exoelectrogenic bacterium (*Geobacter sulfurreducens*) was compared to both a non-hydrogen oxidizer (*Geobacter metallireducens*) and a mixed consortium in order to compare the hydrogen production rates and hydrogen recoveries of pure and mixed cultures. At an applied voltage of 0.7 V, both *G. sulfurreducens* and the mixed culture generated similar current densities (ca. 160 A/m$^3$) resulting in hydrogen production rates of ca. 1.9 m$^3$-H$_2$/m$^3$-d, whereas *G. metallireducens* exhibited lower current densities and production rates of 110 ± 7 A/m$^3$ and 1.3 ± 0.1 m$^3$-H$_2$/m$^3$-d, respectively. Before methane was detected in the mixed culture MEC, the mixed consortium achieved the highest overall energy recovery (relative to both electricity and substrate energy inputs) of 82 ± 8% compared to *G. sulfurreducens* (77 ± 2%) and *G. metallireducens* (78 ± 5%), due to the higher Coulombic efficiency of the mixed consortium. At an applied voltage of 0.4 V, methane production increased in the mixed culture MEC, and as a result, the hydrogen recovery decreased and the overall energy recovery dropped to 38 ± 16% compared to 80 ± 5% for *G. sulfurreducens* and 76 ± 0% for *G. metallireducens*. Internal hydrogen recycling was confirmed as the mixed culture generated a stable current density of 31 ± 0 A/m$^3$ when fed hydrogen gas, while *G. sulfurreducens* exhibited a steady decrease in current production. Community analysis
suggested that *G. sulfurreducens* was predominant in the mixed culture MEC (72% of clones) despite its relative absence in the mixed culture inoculum obtained from an MFC reactor (2% of clones). These results demonstrate that *Geobacter* species are capable of obtaining similar hydrogen production rates and energy recoveries as mixed cultures in an MEC and that high coulombic efficiencies in mixed culture MECs can be attributed in part to the recycling of hydrogen into current.

This chapter was published as:


### 6.1 Introduction

Electrohydrogenesis is an efficient method for generating hydrogen gas from organic matter in reactors known as microbial electrolysis cells (MECs) [1-3]. MECs differ from air-cathode microbial fuel cells (MFCs) in that the cathode remains anaerobic and voltage is added in order to generate hydrogen at the cathode. Under the biological conditions in MECs, hydrogen evolution is not a thermodynamically favorable reaction. However, combining the hydrogen formation reaction potential of $E_{\text{CAT}} = -0.41$ V at the cathode with the anode potential typically obtained in MFCs of $E_{\text{AN}} = -0.30$ V (1 g/L acetate) results in a minimum required voltage of only 0.14 V. Applied voltages ($E_{\text{AP}}$) of 0.2 V (0.45 kWh/m³-H₂) or larger are needed in practice to produce measurable quantities of hydrogen, but this input is substantially less than the average of 2.3 V (5.1 kWh/m³-H₂) required for water electrolysis [4].
Recent improvements in designs and materials have substantially improved hydrogen yields, production rates, and energy recoveries [1, 5-9]. Hydrogen recoveries using typical dead-end fermentation end products such as acetate and butyrate have reached 80 – 100%, while other complex substrates such as glucose and cellulose have yielded recoveries of around 70% [10]. Production rates larger than 6 m$^3$-H$_2$/m$^3$-d have been obtained using MECs [11], which are similar to an average rate of 2.5 m$^3$-H$_2$/m$^3$-d obtained for hydrogen production by biological fermentation [12]. Energy recoveries relative to the electrical energy input as high as 680% have already been shown [10], and overall energy recoveries that include the energy of the substrate have reached 85% [10, 13].

Hydrogen losses can occur using a mixed culture in an MEC, reducing hydrogen yields, production rates, and recoveries [5, 11, 14, 15]. Hydrogen recoveries can drop significantly at lower applied voltages in membraneless MECs because of methanogenic consumption of hydrogen [13, 15-17]. Using a membraneless MEC, Call and Logan [13] found that the overall hydrogen recovery of 90% at $E_{AP} = 0.6$ V was reduced to 18% at $E_{AP} = 0.2$ V, and that methane concentrations increased from 0.9% to 28% in the product gas. Reducing solution pH can help inhibit methanogens, but a methane concentration of 22% was observed in a membrane free MEC at pH 5.8 [15]. When hydrogen is the intended product of an MEC, methane production is detrimental to the process. However, biologically produced methane is a renewable energy source and membrane-less MECs can be used to generate methane instead of hydrogen, although energy recoveries are lower [16]. Hydrogen can also be consumed by chemolithotrophic bacteria in a mixed culture MEC. These bacteria may transfer the associated electrons to a suitable electron
acceptor, such as carbon dioxide, and in some cases, the anode. In the latter scenario, the electrons from hydrogen would be recycled internally, causing an increase in Coulombic efficiency [14]. Hydrogen losses reduce hydrogen and energy recoveries, and alternative methods for generating methane-free and high hydrogen content gas are needed.

Pure culture MECs are one method to avoid losses to methanogens, but production rates and efficiencies with pure cultures can be low compared to those with mixed cultures. Using a pure culture of *Shewanella oneidensis* MR-1 and lactate, Hu et al. obtained a hydrogen production rate of 0.025 m³-H₂/m³-d at an applied voltage of $E_{AP} = 0.6$ V [15]. However, production rates at this same applied voltage using mixed cultures have reached 1 – 2 m³-H₂/m³-d [10, 13]. In MFCs, *S. oneidensis* has produced low Coulombic efficiencies (< 10%) [18, 19] and maximum current densities of ca. 50 mA/m² [20] with lactate, compared to ca. 9900 mA/m² [21] for mixed cultures.

Several *Geobacter* species are commonly found in mixed culture MFCs, and tests with pure cultures of *Geobacter sulfurreducens* have demonstrated power and current densities close to or equal to those achieved with mixed cultures. In an air cathode MFC, *G. sulfurreducens* produced a lower power density (461 mW/m², 1.5 A/m²) than a mixed culture (576 mW/m², 1.3 A/m²) [22]. The reduced performance of *G. sulfurreducens* in the air cathode MFC may have been due to oxygen intrusion across the cathode. Using an MFC with a ferricyanide cathode, Nevin et al. [23] reported a power density of 1.9 W/m² (4.6 A/m²) for *G. sulfurreducens* compared to 1.6 W/m² (3.2 A/m²) for a mixed consortium. When the authors placed the *G. sulfurreducens* MFC in an anaerobic chamber, the Coulombic efficiency improved from 55% to ca. 100%, confirming the importance of strictly anaerobic conditions for *G. sulfurreducens*. This suggests that the
anaerobic environment of MECs may provide excellent conditions for obtaining current densities comparable to those of mixed cultures with pure cultures of *Geobacter* species, while at the same time eliminating methane gas production.

In order to investigate the performance of *Geobacter* species in MECs, we selected two *Geobacter* species based on their differences in hydrogen utilization. *G. sulfurreducens* was selected because it is capable of producing high current densities in MFCs and it can utilize hydrogen. *G. metallireducens*, which does not oxidize hydrogen, was examined to see if higher hydrogen recoveries were possible with a bacterium that cannot oxidize hydrogen. Both of these cultures were compared to a mixed culture under identical conditions in order to further examine the role of internal hydrogen recycling in MECs, and to show that methane-free gas can be produced in MECs at rates comparable to those obtained with mixed cultures.

### 6.2 Materials and Methods

#### 6.2.1 Microorganisms and culture media

*Geobacter sulfurreducens* PCA (ATCC 51573) and *Geobacter metallireducens* GS-15 (ATCC 53774) were obtained from the American Type Culture Collection (ATCC) and cultured using the ATCC media #1957 and #1768, respectively. Sodium acetate (6.8 g/L) was provided as an electron donor, and ferric citrate (13.7 g/L) or sodium fumarate (8.0 g/L) was supplied as an electron acceptor for *G. metallireducens* and *G. sulfurreducens*, respectively. Growth medium (75 mL) was added to serum bottles
(125 mL capacity), sparged with anaerobic N₂/CO₂ (80/20 [v/v]) gas, sealed with thick stoppers and aluminum crimp tops, and sterilized by autoclaving. To inoculate the *Geobacter* MECs, 40 mL of growth medium containing cells were centrifuged, the supernatant was removed, and a pellet of cells was resuspended in sterile MEC medium. The medium used in the MEC (MEC medium) was identical to the growth medium except that it contained 1 g/L sodium acetate and lacked ferric citrate or fumarate. The conductivity of the MEC medium was 4.0 mS/cm and the initial pH was 6.8. The mixed consortium was obtained from a previously acclimated MFC operating for several months with the same MEC medium except that a phosphate buffer (50mM PBS; Na₂HPO₄, 4.58 g/L and NaH₂PO₄•H₂O 2.45 g/L; pH = 7.0) was used instead of bicarbonate. After three batches of reproducible voltage generation, the mixed culture MFC anode was placed in sterile MEC medium containing glass beads, vortexed to suspend the cells, and used to inoculate the MECs (10% v/v).

### 6.2.2 MEC construction and operation

Identical single chamber MECs (no membrane) made from a solid polycarbonate block were drilled to form a cylindrical interior with a length of 2.5 cm and a diameter of 3 cm (empty bed volume of 18 mL) (Figure 6.1). A glass gas collection tube (5 mL) was attached to the top of the cubic reactor as previously described [13]. The anodes were ammonia treated carbon cloth (Type A, E-TEK), and the cathodes were carbon cloth (Type B, E-TEK) containing 0.5 mg-Pt/cm² catalyst on the side facing the anode [13, 24]. Both electrodes had projected surface areas of 7 cm². After crimping the collection tubes
shut with thick rubber stoppers, and sterilizing the fully assembled MECs by autoclaving, an ethanol sterilized Ag/AgCl reference electrode (+200 mV vs. standard hydrogen electrode (SHE); RE-5B, BASi) was installed in the middle of the reactor to record the anode potential ($E_{AN}$). The MECs were filled with anaerobic MEC medium and inoculated in an anaerobic glove box (N$_2$/H$_2$ ratio of 95/5 [v/v]). A control lacking an inoculum was also prepared using the same procedures. Each MEC was operated in fed-batch mode by draining the contents of the reactor at the end of each batch, refilling with sterile MEC medium containing substrate, and sparging the headspace with sterile, anoxic gas (N$_2$/CO$_2$ ratio of 80/20 [v/v]). Operating the MECs in this manner produced a rise in current upon feeding and then a drop in current after the substrate was depleted. Before and after each batch, the pH of the MEC medium for each culture was near neutral (ca. 7.0 – 7.5).

Continuous gas production was recorded using a respirometer (AER-200; Challenge Environmental) and the produced gas was collected using gas bags (0.1 L; Calibrated Instruments) as described previously [13]. A fixed voltage ($E_{AP}$) was applied to the MECs using a power supply (model 3645A; Circuit Specialists) and the voltage across a resistor (10 $\Omega$) was recorded using a multimeter (model 2700; Keithley Instruments, Inc.) in order to calculate the current [13]. The anode potential relative to the Ag/AgCl electrode was also recorded. All anode potentials are reported relative to the Ag/AgCl reference unless otherwise stated. For the H$_2$ utilization test, the MECs were filled with MEC medium lacking acetate and continuously sparged with sterile H$_2$/CO$_2$ (80/20 [vol/vol]) gas.
6.2.3 Acetate analysis

Acetate (Ac) was analyzed using a gas chromatograph (Agilent, 6890N) equipped with a flame ionization detector and a 30 m × 0.32 mm × 0.5 µm CA-FFAP fused-silica capillary column. Samples were filtered through a 0.2 µm pore diameter membrane and were acidified using formic acid (0.63 M; final concentration) before analysis. The temperature of the GC column was started at 60 °C, increased at 20 °C/min to 120 °C, and then at 30 °C/min to a final temperature of 240 °C for another 3 min. The temperatures of injector and detector were both 250 °C. Helium was used as the carrier gas at a constant column flow rate of 3.30 mL/min.

6.2.4 Gas analysis

At the end of each batch, the gas composition in the gas bag was analyzed by gas chromatography using a gastight syringe (250 µL; Hamilton Samplelock Syringe). The concentrations of H₂, N₂, and CH₄ were analyzed with one gas chromatograph (GC) (argon carrier gas; model 2610B; SRI Instruments), and the concentration of CO₂ was analyzed with a separate GC (helium carrier gas; model 310, SRI Instruments).

6.2.5 Calculations

MEC performance was characterized using calculations as described previously [1, 13]. Briefly, the current was normalized to either the liquid volume of reactor $I_V$
(A/m³), or the anode surface area $I_A$ (A/m³). Coulombic efficiency, $C_E$ (%), was calculated as $C_E = C_T/C_C$, where $C_T$ is the total coulombs calculated by integrating the current over time, and $C_C$ is the total charge consumed based on acetate removal (eight electrons per acetate). To convert the acetate concentration (g/L) to coulombs, a chemical oxygen demand (COD) of 1.07 g-COD/L was used along with Faraday’s constant ($F = 96,500$ C/mol). Cathodic hydrogen recovery, $r_{\text{CAT}}$ (%), was calculated as $r_{\text{CAT}} = n_R/n_E$, where $n_R$ (mol) is the amount of H₂ produced, and $n_E$ is the amount of H₂ expected based on the current. Overall hydrogen recovery, $r_{\text{H₂}}$ (%), was calculated as $r_{\text{H₂}} = C_E r_{\text{CAT}}$. The H₂ production rate, $Q_V$ (m³-H₂/m³-d) was calculated as $Q_V = 43.2 I_V r_{\text{CAT}} / F c_g(T)$, where $c_g$ is the concentration of a gas at a temperature $T$ calculated using the ideal gas law, and 43.2 is for unit conversion.

Energy recovery relative to only the electrical energy input, $\eta_E$ (%), was calculated as $\eta_E = W_{\text{H₂}}/W_E$ where $W_{\text{H₂}}$ is the energy of the produced hydrogen (for $\Delta H$ calculations, the higher heating value of 285.8 kJ/mol-H₂ was used; for $\Delta G$ calculations, the Gibb’s free energy of 237.2 kJ/mol-H₂ was used), and $W_E$ is the electricity input determined as $W_E = C_T E_{\text{AP}}$, where $E_{\text{AP}}$ is the applied voltage corrected for the power loss across the resistor used to measure the current. The overall energy recovery, $\eta_{E+S}$ (%), was calculated as $\eta_{E+S} = W_{\text{H₂}}/(W_E + W_S)$, where $W_S$ is the energy of the removed acetate (for $\Delta H$ calculations, 874.3 kJ/mol-acetate was used; for $\Delta G$ calculations, 844.1 kJ/mol-acetate was used). When noted, methane was included in the energy recovery for the mixed culture MEC ($\Delta H$=74.6 kJ/mol-CH₄; $\Delta G$ = 50.5 kJ/mol-CH₄). Calculated parameters are based on data collected over a fed-batch cycle after performance (based on current generation) had been stable for at least two successive batch cycles at each
applied voltage. The average values for each successive cycle (three or more separate cycles) was then used to calculate the reported averages and standard deviations.

### 6.2.6 Cyclic voltammetry

In order to further compare electrode reducing rates between *G. sulfurreducens* and the mixed culture MEC, cyclic voltammetry (CV) was performed using a potentiostat (PC 4/750, Gamry Instruments, Inc.) at a scan rate of 10 mV/s, over a potential range of −0.8 to 0.2 V relative to the Ag/AgCl reference electrode. Prior to the CV scans, the MECs were filled with fresh MEC medium and operated for at least one hour at $E_{AP} = 0.7$ V. The peak current was defined as the maximum current produced averaged over a period of three seconds.

### 6.2.7 Scanning electron microscopy

At the end of the experiments, the anodes of the *G. sulfurreducens* and mixed culture MECs were examined to compare biofilm morphology differences using a scanning electron microscope (SEM). The electrode samples were fixed with 1.5% glutaraldehyde for 1 h and then 1% osmium tetroxide for 30 min. After each fixation step, the samples were washed in 0.1 M cacodylate buffer three times. The fixed samples were dehydrated with a graded series of ethanol and dried using a critical point drying process in liquid CO₂ (BAL-TEC CPD030; Bal-Tec). Samples were then sputter coated
with 10 nm Au/Pd (BAL-TEC SCD050; Bal-Tec) and examined using a scanning electron microscope (JSM 5400; JEOL Ltd.) at an accelerating voltage of 20 kV.

6.2.7 Community analysis

Community and purity analysis was performed at the end of the experiments for the mixed culture and Geobacter species, respectively, and were compared to the cultures used for inoculation. The anodes were placed in sterile MEC medium lacking acetate and vortexed with glass beads to suspend the cells in solution. DNA was extracted using a PowerSoil DNA isolation kit (MO BIO Laboratories) according to the manufacturer’s instructions. For the Geobacter reactors, a 16S rRNA gene fragment of the extracted DNA was amplified by PCR using universal bacterial primers 338F (5’-ACTCCTACGGGAGGCAGCAG-3’) and 518R (5’- ATTACCGCGGCTGCTGG-3’), which flank the V3 region [25]. For the mixed community, a 16S rRNA gene fragment of the extracted DNA was amplified by PCR using universal bacterial primers 27F (5’-AGAGTTTGATCCTGGCTCAG-3’) and 1541R (5’-AAGGAGGTGATCCAGCC-3’) [26]. PCR products were purified using QIAquick PCR Purification Kit (Qiagen) and then ligated and cloned using a TOPO TA cloning kit (Invitrogen) according to the manufacturer’s instructions. Plasmids were extracted using the Ultraclean 6-minute Plasmid Prep kit (MoBio) according to manufacturer’s instructions. Eight plasmids from each Geobacter reactor and forty-six plasmids from each mixed reactor were sequenced with the T7 primer on the TOPO plasmid using an ABI 3730XL DNA sequencer (Applied Biosystems). The nucleotide collection (nr/nt) of the National Center for
Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) was searched using the BLASTn algorithm to analyze the sequences. Mega 4.0.2 [27] was used to align these sequences and generate a phylogenetic tree using the neighbor-joining method with a bootstrap test (500 replicates) of phylogeny. Sequences derived from the analysis of the mixed reactors were deposited in GenBank under accession numbers GQ152894–GQ152986.

6.3 Results

6.3.1 MEC performance at $E_{AP} = 0.7$ V

Each MEC was operated in batch mode at $E_{AP} = 0.7$ V until stable current generation was observed. During the first batch, *G. sulfurreducens* reached a peak in current production after 1.5 days, compared to 3 days for the mixed culture and about 8.5 days for *G. metallireducens* (Figure 6.2A). In the first batch, both *G. sulfurreducens* and the mixed culture generated similar peak current densities ($I_V$) of $80 \pm 1$ A/m$^3$ and $83 \pm 4$ A/m$^3$ respectively, whereas *G. metallireducens* produced $31 \pm 0$ A/m$^3$. After *G. metallireducens* reached a peak current density of $120 \pm 1$ A/m$^3$ (batch 3), current generation decreased with each subsequent batch, reaching $106 \pm 2$ A/m$^3$ by the final batch (data not shown). By the final batch at $E_{AP} = 0.7$ V, *G. sulfurreducens* generated $160 \pm 2$ A/m$^3$ compared to $166 \pm 0$ A/m$^3$ for the mixed culture.

The anode potentials ($E_{AN}$) for each reactor at $E_{AP} = 0.7$ V showed a sharp decrease in the overpotential for the initial batches (batches 1 – 3), and then were more
constant in subsequent batches (Figure 6.2B). *G. sulfurreducens* and the mixed culture had similar anode potentials for each batch, and both reached a potential of $-0.29 \pm 0.00$ V by the final batch. *G. metallireducens* operated at a higher anodic overpotential, with a recorded potential of $-0.23 \pm 0.00$ V by the final batch. For comparison, the theoretical anode potential under the experimental conditions ($pH = 6.8$, $NaH_2C_2O_2 = 12$ mM, $HCO_3^{-} = 30$ mM, $T = 30^\circ$C) was $-0.48$ V. Thus *G. sulfurreducens* and the mixed culture had $-0.19$ V anodic overpotentials, whereas *G. metallireducens* was around $-0.25$ V.

*G. sulfurreducens* and the mixed culture exhibited reproducible gas production ($V_G$) after the first few batches (Figure 6.2C), while *G. metallireducens* reached a peak in volume and then produced decreasing volumes with each subsequent batch (data not shown). Both *G. sulfurreducens* and the mixed culture produced similar volumes of gas after the first few batches, with an average of $24 \pm 1$ mL for *G. sulfurreducens* (batches 3 – 10) and $26 \pm 2$ mL (batches 4 – 12) for the mixed culture. *G. metallireducens* reached a peak volume of 22 mL at the end of batch 6, but between batches 7 – 10, *G. metallireducens* generated smaller quantities of gas with each subsequent batch. On average, *G. metallireducens* produced $21 \pm 2$ mL.

### 6.3.2 MEC performance at $E_{AP} = 0.4$ V

When the applied voltage was lowered to $E_{AP} = 0.4$ V, the current densities decreased as expected (Figure 6.3A). *G. sulfurreducens* generated an average of $57 \pm 2$ A/m$^3$, which was about 21% higher than the current density produced by the mixed culture ($47 \pm 1$ A/m$^3$) and roughly 84% larger than that of *G. metallireducens* ($31 \pm 5$ A/m$^3$).
Both *G. sulfurreducens* and the mixed culture exhibited consistent and reproducible batches of current generation, whereas *G. metallireducens* produced a slightly higher average maximum current density with each batch (batch 1, 22 ± 0 A/m\(^3\); batch 2, 27 ± 0 A/m\(^3\); batch 3, 35 ± 5 A/m\(^3\)).

The anode potentials followed a trend similar to that observed at \(E_{AP} = 0.7\) V, with a slight decrease in the overpotential with each consecutive batch (Figure 6.3B). By the fourth batch the anode potential for *G. sulfurreducens* was \(-0.40 ± 0.00\) V. The mixed culture had a more negative potential of \(-0.42 ± 0.00\) V, indicating that *G. sulfurreducens* had a slightly larger overpotential than the mixed culture. At this higher overpotential, however, *G. sulfurreducens* was capable of generating a higher current density (Figure 6.3A). *G. metallireducens* exhibited the lowest anode potential during the first batch (\(E_{AN} = –0.39 ± 0.00\) V), but showed a sharp reduction in the overpotential by the second batch. Combining this reduction in overpotential with the increase in current density suggests that the lower applied voltage was more favorable for current generation by *G. metallireducens* than the higher applied voltage.

Improvements in gas production with each batch at \(E_{AP} = 0.4\) V was also noted for *G. metallireducens* (Figure 6.3C). In the first batch, *G. metallireducens* produced 11 mL of gas, but by the end of the second and third batches, it produced 19 and 22 mL, respectively. In contrast, the mixed culture showed a decrease in the volume of gas collected with each batch, with 17 mL produced at the end of the first batch, and only 11 mL after the last. This decrease was likely due to methanogenic consumption of the produced hydrogen, as evidenced by an increase in methane concentration from 11% to
54% for the first and final batches, respectively. *G. sulfurreducens* generated reproducible volumes of gas for each batch, with an average of 23 ± 1 mL for all batches.

### 6.3.3 Hydrogen recoveries and production rates

The performance of each reactor in terms of recoveries, yields, and production rates, depended on the applied voltage and for the mixed culture, hydrogen losses to methanogens (Table 6.1). Before methane was detected in the mixed culture in experiments at $E_{AP} = 0.7$ V, Coulombic efficiencies averaged $C_E = 89 ± 4\%$. After methane appeared, the Coulombic efficiency ($C_E = 93 ± 2\%$) did not increase significantly (T-test, $p > 0.09$). The Coulombic efficiencies of both *G. sulfurreducens* (86 ± 2\%) and *G. metallireducens* (81 ± 5\%) were lower than those obtained for the mixed culture.

Cathodic hydrogen recoveries ($r_{CAT}$) in all the MECs were above 100\% at $E_{AP} = 0.7$ V, indicating that more hydrogen was recovered than expected based on the current. Values above 100\% have similarly been reported by others [2, 6]. At $E_{AP} = 0.4$ V, the cathodic hydrogen recovery of the mixed culture dropped to 43 ± 19\%, due to methanogenic conversion of hydrogen to methane. The longer reaction times at $E_{AP} = 0.4$ V of two to three days, compared to about one day at $E_{AP} = 0.7$ V, were presumably more favorable for methane production by slow growing methanogens. When a final batch was operated at $E_{AP} = 0.7$ V following the batches at $E_{AP} = 0.4$ V, the cathodic hydrogen recovery dropped to 74\%, compared to 105 ± 3\% originally obtained at this applied voltage, demonstrating the impact of methanogen growth on hydrogen recovery.
Overall hydrogen recoveries ($\eta_{E+S}$) were highest for the mixed culture (97 ± 1%) when methane concentrations remained below 1% at $E_{AP} = 0.7$ V. After methane concentrations increased, the hydrogen recovery ($r_{H2}$) decreased to 38 ± 16% at $E_{AP} = 0.4$ V. Both *G. sulfurreducens* and *G. metallireducens* exhibited lower hydrogen recoveries of 89 ± 4% and 88 ± 1% respectively at $E_{AP} = 0.7$ V because of their lower coulombic efficiencies. The similarity of these recoveries for the two strains indicates that the ability of *G. metallireducens* to consume hydrogen did not have a significant impact on hydrogen recovery.

*G. sulfurreducens* and the mixed culture produced hydrogen at similar production rates ($Q_V$) of ~1.9 m³-H₂/m³-d at $E_{AP} = 0.7$ V, as expected from comparable current densities and cathodic hydrogen recoveries. When the cathodic hydrogen recovery dropped for the mixed culture due to hydrogen losses to methanogens, however, the production rate subsequently dropped to 1.3 m³-H₂/m³-d, even though the current density was similar to that obtained at $E_{AP} = 0.7$ V in the initial batches. *G. metallireducens* obtained the lowest current density, and subsequently had the lowest production rate of 1.3 ± 0.1 m³-H₂/m³-d.

### 6.3.4 Internal hydrogen recycling

To examine the potential for hydrogen losses and coulombic efficiency increases due to internal hydrogen recycling, a hydrogen utilization test was performed using each MEC at $E_{AP} = 0.7$ V (Figure 6.4). During this test a gas mixture of 80% hydrogen and 20% carbon dioxide was constantly sparged through MECs containing medium lacking
acetate. After an initial drop in current density during the first two hours, *G. sulfurreducens* showed a slight increase in current production from 64 ± 2 A/m³ to 66 ± 1 A/m³, followed by a steady decrease. The mixed culture also showed an initial drop in current to 27 ± 1 A/m³ with a slight increase to 31 ± 0 A/m³, followed by steady current production for the remainder of the test. *G. metallireducens*, which does not utilize H₂, initially produced current, likely as a result of stored substrate. However, there was a sharp decrease in current production towards the zero current value of the control at the end of the test, indicating that hydrogen did not sustain current generation by this bacterium.

### 6.3.5 Energy recoveries

Relative to only the electrical energy input, all MECs at both applied voltages achieved efficiencies over 100% (Table 6.1). At $E_{AP} = 0.7$ V, each MEC obtained roughly the same energy efficiency ($\eta_E = 230\%$ vs. $\Delta H$; see Table 1 for efficiencies based on $\Delta G$), but the mixed culture MEC showed a sharp drop to $\eta_E = 163\%$ after methane concentrations increased to 9%. At $E_{AP} = 0.4$ V, *G. sulfurreducens* obtained the highest electrical energy recovery of $\eta_E = 344 \pm 12\%$ while *G. metallireducens* and the mixed culture reached $\eta_E = 338 \pm 15\%$ and $\eta_E = 164 \pm 72\%$, respectively. The electrical energy recoveries with the mixed culture were significantly different from those of the two *Geobacter* species (T-test, $p < 0.02$), but there was no significant difference between the two *Geobacter* species (T-test, $p > 0.58$). When the energy of the substrate was included as an energy input, the mixed culture obtained the highest value of $\eta_{E+S} = 82 \pm 1\%$ at $E_{AP}$.
0.7 V, but this was appreciably reduced to $\eta_{E+S} = 38 \pm 16\%$ at $E_{AP} = 0.4$ V. Both Geobacter species had slightly lower overall energy recoveries at $E_{AP} = 0.7$ V, with G. sulfurreducens and G. metallireducens reaching $\eta_{E+S} = 77 \pm 3\%$ and $\eta_{E+S} = 78 \pm 5\%$, respectively.

Including the energy of the methane produced in the mixed culture MEC did not substantially increase overall energy recoveries. At $E_{AP} = 0.4$ V, the overall energy recovery was 10% larger ($\eta_{E+S} = 42 \pm 14\%$) with methane and hydrogen than with just hydrogen ($\eta_{E+S} = 38 \pm 16\%$). In the final batch at $E_{AP} = 0.7$ V, the overall energy recovery increased by only 3% from $\eta_{E+S} = 58\%$ to $\eta_{E+S} = 60\%$ when methane was accounted for in the energy balance.

6.3.6 Cyclic voltammograms

Since no distinct differences were evident between the current densities of G. sulfurreducens and the mixed culture at both applied voltages, CVs were performed to examine the electrode reducing rates at a wider range of potentials (Figure 6.5). CVs were conducted after 35 days of operation for G. sulfurreducens and after 26 days for the mixed culture. CVs of both G. sulfurreducens and the mixed culture yielded a sigmoidal shape characteristic of catalytic activity and consistent with previous G. sulfurreducens CVs [28, 29] During the forward scan, G. sulfurreducens began producing positive current at $E = −0.55$ V, while the mixed culture initially operated at a higher overpotential at around $E = −0.47$ V. Between $E = −0.55$ V and $−0.16$ V, G. sulfurreducens operated at lower anodic overpotentials than the mixed culture, but above
\[ E = -0.16 \text{ V}, \] the mixed culture produced higher current. The mixed culture also produced a higher peak current of \( 5.9 \pm 0.0 \text{ mA} \) versus \( 4.6 \pm 0.0 \text{ mA} \) for \( G. \text{sulfurreducens} \).

6.3.7 \textit{G. sulfurreducens} and mixed culture current densities for \( 0.3 \text{ V} < E_{\text{AP}} < 1.0 \text{ V} \)

Since CV analysis indicated that the mixed consortium was capable of higher current densities at higher applied voltages, the mixed culture MEC and \( G. \text{sulfurreducens} \) were operated at applied voltages from 0.3 V to 1.0 V (Figure 6.6). For \( E_{\text{AP}} \geq 0.7 \text{ V}, G. \text{sulfurreducens} \) generated on average a 2% higher current density, but for \( E_{\text{AP}} \leq 0.6 \text{ V}, G. \text{sulfurreducens} \) produced on average a 30% larger current density. For all applied voltages, the mixed culture current density increased as a factor of \( 354 \pm 9 \text{ A/m}^3 \) per volt, while the current density of \( G. \text{sulfurreducens} \) was slightly lower at \( 343 \pm 2 \text{ A/m}^3 \) per volt. At the highest applied voltage \( (E_{\text{AP}} = 1.0 \text{ V}), G. \text{sulfurreducens} \) generated \( 263 \pm 3 \text{ A/m}^3 \) (6.8 ± 0.1 A/m²) and the mixed culture produced \( 260 \pm 24 \text{ A/m}^3 \) (6.7 ± 0.6 A/m²). At the lowest applied voltage \( (E_{\text{AP}} = 0.3 \text{ V}), G. \text{sulfurreducens} \) produced \( 25 \pm 0 \text{ A/m}^3 \) (0.6 ± 0.0 A/m²) and the mixed culture reached \( 15 \pm 0 \text{ A/m}^3 \) (0.4 ± 0.0 A/m²).

6.3.8 SEM images

SEM imaging revealed different biofilm morphologies of the mixed culture and \( G. \text{sulfurreducens} \) (Figure 6.7). \( G. \text{sulfurreducens} \) formed layers of cells stacked on top of each other and the graphite fibers, whereas the mixed culture formed tight structures that wrapped around individual graphite fibers. Higher magnification indicated that the mixed
culture covered a larger surface area of the graphite fibers compared to *G. sulfurreducens* which appeared to form looser biofilms around the fibers.

### 6.3.9 Community analysis and purity check

The mixed culture from the acclimated MFC used as the MEC inoculum contained primarily *Firmicutes* (26% of clones), *Proteobacteria* (30%) and *Bacteroides* (38%) (Table 6.2). *Actinobacteria* was also present, representing 4.3% of the community, while only one *G. sulfurreducens* clone (2.1% of the total) was detected. After the MEC experiments, the anode community was dominated by *G. sulfurreducens* (72%) (Table 6.3). *Proteobacteria* (including *G. sulfurreducens*) composed 87% of the overall population, with 6.5% represented by *Bacteriodes*, 4.3% by *Deferribacteres*, and 2.2% by *Firmicutes*. *Actinobacteria* was not present in the mixed culture MEC.

Sequence analysis from both the *G. sulfurreducens* and the *G. metallireducens* reactors confirmed (98-100% identity) that these reactors remained uncontaminated throughout the experiments (data not shown).

### 6.4 Discussion

These results show that hydrogen losses to methane generation can be avoided in MECs by using pure cultures, and that hydrogen production rates and yields similar to those of mixed cultures can be obtained with *G. sulfurreducens*. Although *G.*
metallireducens is exoelectrogenic, current densities produced using this bacterium were consistently lower than those of the mixed culture or G. sulfurreducens.

Evidence for internal hydrogen recycling was confirmed for both G. sulfurreducens and the mixed culture based on hydrogen utilization tests in the absence of acetate (Figure 6.4) and Coulombic efficiencies measured in the presence of acetate. Some current was always initially produced by enriched anodes (likely from endogenous respiration), but the magnitude and stability of these current varied in the tests. For example, ca. 24 A/m³ was initially produced by G. metallireducens when the reactor was sparged with hydrogen gas (no acetate). However, this current was only ca. 20% of that produced in MEC tests with acetate, and this strain cannot respire using hydrogen [30]. With G. sulfurreducens under the same conditions (hydrogen gas, no acetate), the current density was higher (ca. 66 A/m³), with values initially ca. 40% of that produced in MEC tests with acetate. This suggests that there was appreciable hydrogen utilization by G. sulfurreducens in MEC tests that resulted in current generation. In addition, the Coulombic efficiency for G. sulfurreducens (C_E = 86%) was higher than that of G. metallireducens (C_E = 81%) in acetate fed MEC tests, suggesting that the Coulombic efficiency may have increased slightly (from 81 to 86%) as a result of hydrogen utilization (assuming all other factors, such as cell yields, were constant between the strains). However, a comparison of Coulombic efficiencies in these MEC tests with those obtained in other MFC tests indicates that overall, the Coulombic efficiency values were not increased to unreasonable values by hydrogen utilization. In MFC tests with G. sulfurreducens using a ferricyanide catholytes (no oxygen), for example, Coulombic efficiencies were much higher than those obtained here, reaching 100 % [23]. Thus, while
hydrogen cycling was likely for *G. sulfurreducens*, the relatively low Coulombic efficiency values indicate that hydrogen recycling in MECs was a relatively small contributor to current production.

In the case of the mixed culture, the high Coulombic efficiency (*C*<sub>E</sub> = 93%) in MEC tests and sustained current production in hydrogen utilization tests suggested that hydrogen cycling contributed more substantially to current production. The mixed culture hydrogen utilization tests produced a current density of ca. 30 A/m<sup>3</sup> (no acetate). Although this is only ca. 19% of the maximum current (160 A/m<sup>3</sup>) generated with acetate, this current density was sustained for the mixed culture by hydrogen gas, whereas current density always decreased with the pure cultures in hydrogen gas tests. The observation of sustained current generation in hydrogen utilization tests, combined with a Coulombic efficiency value larger than those obtained for either *Geobacter* strain, suggests that hydrogen cycling increased the Coulombic efficiency of the mixed culture. This hydrogen cycling did not affect overall energy recoveries, however, as similar values were obtained for the mixed culture and *G. sulfurreducens* in MEC tests before the enrichment of methanogens.

Methanogenesis was observed in mixed culture tests and increased over time. However, the high Coulombic efficiency and the consumption of hydrogen by the mixed culture suggested that the main route for methane generation was hydrogenotrophic and not acetoclastic methanogenesis. This observation is consistent with most other mixed-culture MEC studies that have shown hydrogenotrophic methanogenesis predominates in MECs [14, 16, 17, 31]. It is also unlikely that methane generation occurred by electromethanogenesis [32], *i.e.* directly from electron release at the cathode. Hydrogen
production was catalyzed here using platinum on the cathode, and there was insufficient
time to acclimate a methanogenic biofilm on the cathode, and therefore
electromethanogenesis could not have substantially contributed to methane production
under these conditions.

The reduced performance of *G. metallireducens* compared to *G. sulfurreducens* in
the MEC was not expected based on iron reducing rates of these two bacteria. *G. metallireducens* can reduce iron at a rate of 887 fmol/cell-day with soluble iron, and 94
fmol/cell-day with insoluble iron, compared to 225 fmol/cell-day (soluble iron) and 23
fmol/cell-day (insoluble iron) for *G. sulfurreducens* [33, 34]. The magnitude of the
applied voltage may have also been a factor in the reduced performance of *G. metallireducens* compared to *G. sulfurreducens*. The current density, anode potential, and
volume of produced gas all improved with each successive batch at $E_{AP} = 0.4$ V
compared to a decrease in each of these metrics at $E_{AP} = 0.7$ V. Erratic performance of
MECs with mixed cultures has also been observed at higher applied voltages [13].

There was also a noticeable difference in biofilm morphology between *G. sulfurreducens* and the mixed culture despite the predominance of *G. sulfurreducens* in
the mixed culture MEC community. The mixed culture biofilms were tighter and
appeared more compact on the surface of the graphite fibers than those of *G. sulfurreducens*, which appeared less dense and comprised of more voids between the
cells and fibers. Combining this observation with the higher peak current generated by
the mixed culture during CV analysis suggests that the mixed culture achieved better
contact with the electrode surface than *G. sulfurreducens*. The higher current density of
the mixed culture is consistent with higher per-biomass electrode reducing rates of a mixed culture compared to *G. sulfurreducens* observed in MFCs [22].

There was a large change in the community structure between the bacteria detected in the inoculum taken from an MFC and that present in the MEC. To our knowledge, this is the first time that changes in bacterial communities have been studied for a culture transferred from an MFC to an MEC. While *G. sulfurreducens* was detected in only 1 of 46 clones from the MFC inoculum, in the MEC tests it accounted for 72% of the community based on 16S rRNA sequence analysis. This indicates that the conditions in the MEC provided a better competitive growth environment for *Geobacter* than the MFC. The reasons for this include both operational conditions and the medium. In an MFC oxygen can diffuse through the cathode, but the MECs were operated under completely anoxic conditions. Most importantly, a carbonate-rich medium was used for the MEC, but the original MFC reactor was operated with a medium containing a phosphate buffer. In MFC experiments by Ishii et al. [22] using an air-cathode MFC and a carbonate medium, *G. sulfurreducens* was also found to be predominant in the anode biofilm community. We have also found that *G. sulfurreducens* grows poorly or not at all in a phosphate buffer medium (unpublished results), and thus the use of a carbonate medium is critical for growth of this microorganism.

These results show that pure cultures can produce hydrogen gas in MECs at rates and recoveries comparable to mixed cultures. Depending on the species, the current densities may be higher than mixed cultures, but in all cases, methane-free gas is produced. Investigations of other pure cultures, in particular species that can derive energy and biomass from sources other than the substrate are needed in order to increase
coulombic efficiencies and hydrogen recoveries. Engineering and selection of strains adapted to electron transfer to anodes may also lead to an increase in efficiencies and production rates [35, 36]. Furthermore, other strains that can efficiently produce hydrogen in MECs using complex substrates will help create new applications such as localized hydrogen production from diverse and abundant waste streams.

### 6.5 Literature Cited


### 6.6 Tables

Table 6.1. Coulombic efficiencies, hydrogen recoveries, energy recoveries, acetate removal, and hydrogen production rates for each MEC at $E_{AP} = 0.7$ V and 0.4 V.

<table>
<thead>
<tr>
<th>Reactor</th>
<th>$C_E$ (%)</th>
<th>$r_{CAT}$ (%)</th>
<th>$r_H$ (%)</th>
<th>$\eta_E$ (%)</th>
<th>$\eta_{E+S}$ (%)</th>
<th>Ac removal (%)</th>
<th>$Q_V$ (m$^3\cdot$H$_2$/m$^3$-d)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>$E_{AP} = 0.7$ V</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>G. sulfurreducens</em></td>
<td>86</td>
<td>104</td>
<td>89</td>
<td>228</td>
<td>189</td>
<td>77</td>
<td>66</td>
</tr>
<tr>
<td><em>G. metallireducens</em></td>
<td>81</td>
<td>109</td>
<td>88</td>
<td>236</td>
<td>196</td>
<td>78</td>
<td>66</td>
</tr>
<tr>
<td>Mixed culture$^a$</td>
<td>89</td>
<td>108</td>
<td>96</td>
<td>236</td>
<td>195</td>
<td>82</td>
<td>70</td>
</tr>
<tr>
<td>Mixed culture$^b$</td>
<td>93</td>
<td>105</td>
<td>97</td>
<td>230</td>
<td>191</td>
<td>82</td>
<td>70</td>
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<tr>
<td>Mixed culture$^c$</td>
<td>92</td>
<td>74</td>
<td>68</td>
<td>163</td>
<td>135</td>
<td>58</td>
<td>49</td>
</tr>
<tr>
<td><strong>$E_{AP} = 0.4$ V</strong></td>
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<tr>
<td><em>G. sulfurreducens</em></td>
<td>89</td>
<td>91</td>
<td>80</td>
<td>344</td>
<td>285</td>
<td>80</td>
<td>61</td>
</tr>
<tr>
<td><em>G. metallireducens</em></td>
<td>82</td>
<td>90</td>
<td>74</td>
<td>338</td>
<td>235</td>
<td>76</td>
<td>53</td>
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<tr>
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<td>43</td>
<td>38</td>
<td>164</td>
<td>136</td>
<td>38</td>
<td>33</td>
</tr>
</tbody>
</table>

$^a$before the appearance of methane in the collected gas  
$^b$after methane appeared  
$^c$after operating several batches at $E_{AP} = 0.4$ V

$\Delta H$ (based on heat of combustion)  
$\Delta G$ (based on Gibbs free energy)
Table 6.2. Community profile of the MFC anode measured by 16S rRNA gene cloning.

<table>
<thead>
<tr>
<th>BLAST search results nearest match</th>
<th># clones with &gt;95% identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncultured bacterium clone R1B-15</td>
<td>6</td>
</tr>
<tr>
<td>Bacteroides sp. 22C</td>
<td>3</td>
</tr>
<tr>
<td>Uncultured bacterium BB23</td>
<td>3</td>
</tr>
<tr>
<td>Uncultured bacterium clone HDBW-WB48</td>
<td>3</td>
</tr>
<tr>
<td>Uncultured bacterium clone: YWB28</td>
<td>3</td>
</tr>
<tr>
<td>Bacterium B4C1-5</td>
<td>2</td>
</tr>
<tr>
<td>Ruminobacillus xylanolyticum</td>
<td>2</td>
</tr>
<tr>
<td>Thermomonas haemolytica strain A50-7-3</td>
<td>2</td>
</tr>
<tr>
<td>Uncultured bacterium clone R-1167 **</td>
<td>2</td>
</tr>
<tr>
<td>Uncultured Rhodocyclaceae clone MFC-B162-F11</td>
<td>2</td>
</tr>
</tbody>
</table>

* Represented by one clone:
  Acidovorax sp. R-24607, Azoarcus sp. BH72, Bacterium enrichment culture clone PA10, Comamonas sp., Geobacter sulfurreducens PCA, Hydrogenophaga sp. AH-24, Methylobacterium sp. F3.2, Niabella sp. GR10-1, Peptococcaceae bacterium Y5 *, Rhodococcus sp. T104, Uncultured bacterium clone 1As26, Uncultured bacterium clone 31, Uncultured bacterium clone 656048, Uncultured bacterium clone ATB-M1328, Uncultured bacterium clone B53, Uncultured bacterium clone PL-5B10, Uncultured bacterium clone PL-7B6, Uncultured bacterium clone Rhag4-33, Uncultured Candidatus Odyssella sp. clone 1-K
* 90% identity; ** 94% identity
Table 6.3. Community profile of the MEC anode measured by 16S rRNA gene cloning.

<table>
<thead>
<tr>
<th>BLAST search results nearest match</th>
<th># clones with &gt;97% identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geobacter sulfurreducens PCA</td>
<td>33</td>
</tr>
<tr>
<td>Uncultured bacterium clone:e03=d04</td>
<td>2</td>
</tr>
</tbody>
</table>

Represented by one clone:
Acidovorax sp. R-24607, Arcobacter butzleri RM4018, Pseudomonas sp. ZZ5, Uncultured Arcobacter sp. clone DS031, Uncultured bacterium clone HB99 **, Uncultured bacterium clone HDBW-WB48, Uncultured bacterium clone LTR-R12 **, Uncultured bacterium clone M3B06, Uncultured bacterium clone R1B-24, Uncultured Deferribacter sp. clone 25IIISN *, Wolinella succinogenes strain ATCC 29543

* 92% identity; ** 96% identity
Figure 6.1. MEC single chamber reactor design used in this work. Reference electrode is shown extending from the front of the reactor.
Figure 6.2. Current densities (A), anode potentials (B), and gas production (C) recorded at $E_{AP} = 0.7$ V for *G. sulfurreducens* (GS), mixed culture (MC), *G. metallireducens* (GM), and the control (CNTRL; no inoculum).
Figure 6.3. Current densities (A), anode potentials (B), and gas production (C) recorded at $E_{AP} = 0.4$ V for *G. sulfurreducens* (GS), mixed culture (MC), *G. metallireducens* (GM), and the control (CNTRL; no inoculum).
Figure 6.4. Current densities recorded during hydrogen utilization tests (no acetate provided) at an applied voltage of $E_{\text{AP}} = 0.7$ V for *G. sulfurreducens* (GS), mixed culture (MC), *G. metallireducens* (GM), and the control (CNTRL; no inoculum).

Figure 6.5. CV recorded at 10 mV/s from $-0.8$ V to $+0.2$ V for *G. sulfurreducens* (GS), mixed culture (MC), and the control (CNTRL; no inoculum).
Figure 6.6. Current densities recorded as a function of applied voltage for *G. sulfurreducens* (GS), and the mixed culture (MC).

![Graph showing current density vs. applied voltage](image)

Figure 6.7. SEM images of the control (A, D), *G. sulfurreducens* (B, E), and mixed culture (C, F) anodes at the end of all batches. Images were taken after day 63 for *G. sulfurreducens* and day 47 for the mixed culture. Bars in A-C are 100 µm. Bars in D-F are 10 µm.
Chapter 7

Lactate oxidation coupled to iron or electrode reduction by *Geobacter sulfurreducens*

Abstract

*Geobacter sulfurreducens* PCA completely oxidized lactate and reduced iron or an electrode, producing pyruvate and acetate intermediates. Compared to *Shewanella oneidensis* MR-1, *G. sulfurreducens* PCA produced ten times higher current in lactate-fed microbial electrolysis cells. The kinetic and comparative analyses reported here suggest a prominent role of *G. sulfurreducens* strains in metal and electrode reducing communities supplied lactate.

This chapter was published as:


7.1 Introduction

There is great interest in the study of bacteria capable of transferring electrons to solid electron acceptors, such as metal oxides or electrodes in different types of bioelectrochemical systems (BESs). The two most studied exoelectrogenic bacteria are *Geobacter sulfurreducens* PCA, and *Shewanella oneidensis* MR-1 [1-4], but these microorganisms have never been studied under exactly the same conditions in BESs. S.
*Oneidensis* MR-1 can grow anaerobically using lactate, but it produces acetate which it cannot further oxidize under these conditions. Sequences similar to *G. sulfurreducens* are commonly found in mixed culture microbial fuel cells (MFCs) and sediments supplied lactate, while those similar to the *Shewanella* genus are relatively rare in such studies [5-8]. It has been suggested that the presence of sequences matching *G. sulfurreducens* was primarily due to acetate consumption sustained by other bacteria that incompletely oxidized lactate to acetate [5-8]. This assumption was likely based on earlier work indicating that *G. sulfurreducens* PCA could not oxidize lactate, as the authors reported similar levels of Fe(II) in both lactate supplied cultures and negative controls after five days [9]. Although *G. sulfurreducens* PCA was thought not to respire with lactate [9], its genome includes genes for putative lactate permeases (GSU1622, GSU0226) and lactate dehydrogenases (GSU1620, GSU1621), which are highly conserved among lactate oxidizing bacteria, including those of the *Shewanella* genus [10].

It is now known that several *Geobacter* species can oxidize lactate [11-13], and it has recently been shown that *G. sulfurreducens* PCA can oxidize lactate with fumarate reduction [14, 15]. Gene regulation analysis of lactate versus acetate fed cultures of *G. sulfurreducens* PCA has been examined [14], but no data have yet been presented on lactate oxidation kinetics or intermediate product formation by strain PCA. There are indications of direct lactate oxidation in previous MFC studies with communities containing sequences matching *G. sulfurreducens* [6, 7]. For example, there was immediate current production by established mixed culture MFC biofilms at the onset of lactate fed-batch cycles [5, 7, 16-18]. Immediate current generation is unlikely a result of lactate conversion to acetate as indirect conversion results in a lag of current generation.
of several hours. Direct current production from lactate oxidation is more plausible in these MFCs, yet bacteria capable of this type of metabolism, such as *Shewanella* species, produce lower current densities than mixed cultures under identical conditions [5, 19, 20]. Furthermore, many *Shewanella* species use electron shuttles [21] that would be removed when the medium was replaced prior to fed-batch operation. This suggests that strains of *G. sulfurreducens* capable of high current production directly from lactate oxidation exist within mixed community biofilms fed lactate.

In order to better understand lactate utilization in BESs, we examined current generation by *G. sulfurreducens* PCA and *S. oneidensis* MR-1 in microbial electrolysis cells (MECs) under identical conditions (medium, electrode and temperature). Iron reduction by *G. sulfurreducens* PCA was also examined to relate lactate oxidation kinetics to previously reported rates of acetate oxidation.

### 7.2 Electrode reduction using lactate.

Biofilms of *G. sulfurreducens* PCA (strain obtained from −80°C frozen laboratory stocks, ATCC 51573) were initially established on graphite plate anodes ($A_A = 92 \text{ m}^2/\text{m}^3$) in serum bottle (5 mL) based microbial electrolysis cells (MECs) [22, 23] using stainless steel mesh cathodes ($A_C = 86 \text{ m}^2/\text{m}^3$) [24]. MECs (triplicate reactors) were supplied acetate (10 mM) in a freshwater (FW) medium (per liter: 0.1 g KCl, 0.3 g NH₄Cl, 0.6 g NaH₂PO₄, 2.5 g NaHCO₃, 10 mL vitamins, 10 mL minerals, headspace of 80:20 mixture of N₂:CO₂) [25], and operated in fed-batch mode at an applied voltage ($E_{AP}$) of 0.7 V and at 30°C (Fig. 1A). This applied voltage was chosen because it allowed for relatively fast
cycle times (< 2 days) compared to lower applied voltages, and this voltage was previously used for culturing *Geobacter* sp. in MECs [25].

After current production stabilized, the electron donor was switched to lactate (10 mM). Current density ($I_A$; normalized to total anode surface area) reached a maximum of 0.8 ± 0.2 A/m$^2$ over a period of four days, which was ca. 50% less than the maximum current produced when the cultures were supplied acetate. When MECs were emptied, sparged with sterile, anaerobic gas and refreshed with new medium, 0.3 ± 0.1 A/m$^2$ was immediately produced after applying the voltage, supporting direct conversion of lactate into current. Improvements in current production for the triplicate samples were noted for the second fed-batch cycle, possibly due to evolution of *G. sulfurreducens* PCA for more efficient lactate utilization. Electrons were recycled via hydrogen gas, as shown by estimated Coulombic efficiencies ($C_E$) above 100% for both acetate-fed (140 ± 15%) and lactate-fed (140 ± 33%) MECs. To eliminate the possibility that residual hydrogen produced in the MECs was the main contributor to current production, *G. sulfurreducens* PCA was also tested in a two-chamber reactor incorporating a membrane separator (Nafion) between the working and counter chambers (12 cm electrode spacing). The internal resistance of this two-chamber reactor is large, which results in a low current. Therefore, to increase current in this device, and to better define the anode conditions, the potential of the anode (graphite rod, 14.5 cm$^2$) was controlled at +0.4 V (vs standard hydrogen electrode). This potential was selected to allow comparison with previous reports of acetate-fed reactors at this potential [1, 26]. After 20 days, current reached a maximum of ca. 0.2 A/m$^2$ (data not shown) with an estimated $C_E$ of 12 ±1%, confirming that lactate could serve as the sole electron donor for current generation. Higher current
densities in the MEC tests (single-chamber) compared to the two-chamber tests may have been due to different operating conditions (fixed whole cell potential vs fixed anode potential), a decrease in internal resistance due to closer electrode spacing and no membrane, or the presence of hydrogen which could serve as an additional electron donor.

The use of lactate by *G. sulfurreducens* PCA allowed for the direct comparison of electrode reducing rates to *S. oneidensis* MR-1, under identical conditions. *S. oneidensis* MR-1 was grown overnight in Luria-Bertani medium at 30°C to an OD$_{600}$ of 1.1 ± 0.0, centrifuged, washed three times in sterile FW medium, and inoculated into sterile MECs (in triplicate) containing the same medium used for testing *G. sulfurreducens* PCA. *G. sulfurreducens* PCA was also inoculated into new, sterile reactors (in triplicate) for comparison. Current production reached a maximum of 0.06 A/m$^2$ for *S. oneidensis* MR-1 compared to 0.8 A/m$^2$ for *G. sulfurreducens* PCA (Fig. 1B). The low current densities observed here for *S. oneidensis* MR-1 are consistent with previous reports of low current generation by this bacterium in MFCs and MECs [5, 19, 20].

### 7.3 Iron reduction using lactate.

Cells of *G. sulfurreducens* PCA were thawed from frozen stocks, grown at 30°C in anaerobic culture tubes containing acetate (10 mM) ferric citrate (15 mM) in FW medium (10 mL) until complete Fe(III) reduction occurred and transferred (10%) three times in FW medium containing lactate (2 mM) and ferric citrate (15 mM) under Fe(III) limiting conditions. On the fourth transfer, lactate, acetate, and pyruvate concentrations
were recorded using high performance liquid chromatography (HPLC), Fe(II) production using a ferrozine assay [27], and cell counts using an acridine orange staining procedure [28]. After ten days, *G. sulfurreducens* PCA produced 12.8 ± 0.6 mM Fe(II) and removed 2.6 ± 0.0 mM lactate, indicating a stoichiometry of Fe(II) production to lactate consumption of 4.9 ± 0.2 (Fig. 2). These results are not consistent with incomplete lactate oxidation to acetate via eq. 1 as this would have required a stoichiometric ratio less than four when including substrate conversion to biomass.

\[
\text{lactate}^- + 2\text{H}_2\text{O} + 4\text{Fe(III)} \rightarrow \text{acetate}^- + \text{HCO}_3^- + 5\text{H}^+ + 4\text{Fe(II)} \quad (1)
\]
\[
\text{lactate}^- + 2\text{Fe(III)} \rightarrow \text{pyruvate}^- + 2\text{Fe(II)} + 2\text{H}^+ \quad (2)
\]
\[
\text{pyruvate}^- + 2\text{Fe(III)} + 2\text{H}_2\text{O} \rightarrow \text{acetate}^- + 2\text{Fe(II)} + \text{HCO}_3^- + 3\text{H}^+ \quad (3)
\]

Additional tests under lactate limited conditions (1 mM lactate, 30 mM Fe(III), conducted in triplicate) confirmed that complete oxidation of lactate occurred. Pyruvate was detected, suggesting lactate oxidation via eq. 2 with the transfer of two electrons, followed by pyruvate oxidation to acetate (eq. 3). This result is consistent with pyruvate oxidation by *G. sulfurreducens* PCA described previously [29].

All cultures were tested for purity by extracting DNA, conducting PCR of the 16S rDNA using forward primer 530F (5′-GTCCCAGCMGCCGCGG-3′) and reverse primer 1490R (5′-GGTTACCTTGTTACGACTT-3′), and sequencing purified PCR products. All sequences returned clean and were confirmed as pure using the BLASTn algorithm at the National Center for Biotechnology Information (NCBI) website.
7.4 Implications.

These results provide kinetics of lactate oxidation by *G. sulfurreducens* PCA coupled with either electrode or Fe(III) reduction along with the first direct comparison to a member of the *Shewanella* genus. Although sequences matching *G. sulfurreducens* in mixed communities supplied lactate may be associated with strains capable of lactate oxidation, researchers have previously concluded that these *Geobacter* species relied on acetate production from incomplete lactate utilization by other members of the community [5-7], likely due to the previous conclusion by Caccavo et al. that *G. sulfurreducens* PCA did not utilize lactate [9]. The results shown here expands upon more recent findings that strain PCA can grow using lactate, and show based on current generation in MECs that these rates are substantially greater than those of strain MR-1 under the same conditions. Elucidating the contribution of *G. sulfurreducens* to lactate oxidation within mixed communities warrants further investigation along with examining the functional role of the genes (lactate permease and dehydrogenase) associated with lactate utilization.

Expanding the substrate diversity of *G. sulfurreducens* PCA is promising for the further development of MFCs. Bacterial respiration rates can be a limiting factor in MFCs and other BESs, as it is well known that certain substrates (e.g. acetate) result in higher current and power densities than others (e.g. butyrate, and complex substrates such as wastewaters) [30, 31]. Therefore, methods to improve microbial kinetics in BESs can help to improve system performance. Adaptive evolution of *G. sulfurreducens* PCA for high-current producing strains has been shown with acetate [32], but wastewaters and
cellulosic fermentation effluents contain a diverse range of substrates. Although mixed communities can generate current from these waste streams [6], using strains of *G. sulfurreducens* that are highly adapted to acetate, lactate, pyruvate, and formate [33] may allow for increased current production compared to mixed cultures.

7.5 Literature Cited


Figure. 7.1. (A) Current densities of *G. sulfurreducens* in MECs fed acetate (Ac⁻) or lactate (Lac⁻) at $E_{AP} = 0.7$ V. Batch cycles were operated in triplicate in the order shown with dotted lines representing medium replacement. (B) Current densities of *G. sulfurreducens* and *S. oneidensis* in MECs fed lactate during an initial batch cycle at $E_{AP} = 0.7$ V. Both cultures were operated in triplicate.
Figure 7.2. (A) Fe(II) concentrations, cell numbers, (B) lactate, acetate, and pyruvate concentrations of \textit{G. sulfurreducens} oxidizing lactate as the sole electron donor under Fe(III) limited conditions. Experiments performed in triplicate with two uninoculated controls.
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