MICROBIAL COMMUNITY AND ELECTROCHEMISTRY OF BIOELECTROCHEMICAL SYSTEMS

A Dissertation in

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by

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Abstract

Microbial fuel cells (MFCs) are an emerging technology that converts organic matter to electricity using an exoelectrogenic biofilm on the electrode as the biocatalyst. The complexity of anode biofilms makes it hard to elucidate electrochemical mechanisms at the bioanode, but a precise understanding of exoelectrogenesis and competition in anode biofilms will aid in improving performance of these systems. Various factors such as substrate, external resistance, pH, or exoelectrogenic mode were tested to investigate the bioanode process and its influence on system performance.

The external resistance in MFCs regulates both the anode availability as an electron acceptor and the electron flux through the circuit. I evaluated the effects of external resistance ($R_{ext}$) on MFCs using acetate or glucose. I showed that $R_{ext}$ affects not only anode potential ($E_{an}$) and current generation but also anode biofilm community and methanogenesis. Acetate-fed MFCs produced higher Coulombic efficiencies and energy efficiencies than glucose-fed MFCs because of electron and potential losses from glucose fermentation. However, total methane production in acetate-fed reactors increased as $R_{ext}$ increased, suggesting that $E_{an}$ might influence the competition for substrate between exoelectrogens and methanogens with this substrate. Increasing $R_{ext}$ to 9800 Ω significantly changed the anode bacterial communities for both acetate- and glucose-fed reactors relative to communities in systems operating at 970 Ω, but decreasing $R_{ext}$ to 150 Ω had little effect.

pH influences both anode and cathode performance in MFCs. The polarization behavior and impedance of the bioanode in an MFC was analyzed in medium pH of 6.0 to 8.0, at 0.5 pH-unit intervals. In this research, I characterized the performance of the
anode, cathode, and whole cell using polarization behavior. I also developed a new equivalent circuit model for fitting electrochemical impedance spectroscopy (EIS) data, and it was used to explain electrochemical phenomena of the bioanode. The electrode potentials changed with pH (ca. -60 mV/pH for anode and ca. -68 mV/pH for cathode), coincident with thermodynamic estimations. Open circuit voltage reached a maximum (-517 mV) at pH 7 and maximum power density was highest (741 mW/m²) at pH 6.5 due to the effect of cathode performance. Maximum current density increased, and apparent half-saturation potential ($E_{KA}$) decreased with increasing medium pH due to improved bioanode performance. Impedance analysis showed that intracellular processes were the rate-limiting step for acetate-oxidizing exoelectrogenesis based on charge transfer resistance estimates, and a majority of charges were stored in intracellular processes based on capacitance values. Intracellular resistance was lowest near $E_{KA}$ and increased by several orders of magnitude above $E_{KA}$, while extracellular resistance decreased by only a few ohms as potential increased. However, EIS fittings deviated slightly at potentials above $E_{KA}$ due to an emerging impedance presumably associated with diffusive impedance and excessive potential.

The two model exoelectrogens *Geobacter sulfurreducens* PCA and *Shewanella oneidensis* MR-1 have very different electrochemical physiologies. To understand exoelectrogenic processes better, the electrochemistry of these distinct exoelectrogens was characterized. Duplicate anode electrodes were enriched with *G. sulfurreducens*, *S. oneidensis*, or their coculture. In both the PCA anodes and the MR-1 anodes, extracellular resistance was a few orders of magnitude larger than intracellular resistance, showing that the extracellular resistance process was rate limiting. Total capacitance values of both
cases were dominated by $C_2$ ranging from 3 to 10 mF, whereas $C_1$ ranged from $10^{-3}$ to $10^{-2}$ mF. Different morphologies of HF arcs were created among the three defined cultures. The coculture reactors were expected to increase current production due to exoelectrogenic contribution from both community members, but these anodes produced lower current and higher impedance at the bioanode, probably due to substrate limitation as the more exoelectrogenically productive community member (i.e., *G. sulfurreducens*) was constrained by the conversion of lactate to acetate by *S. oneidensis* MR-1.

The impedance attributes and thermodynamics of a mixed-culture bioanode and an abiotic anode were comparatively analyzed, along with the effects of riboflavin on EIS-determined charge transfer processes. Our results successfully identified and quantified their extracellular electron transfer properties. Activation overpotential was insignificant in the bioanode compared with the abiotic anode. The bioanode had ~30-fold lower charge transfer resistance and 2-fold higher capacitance for the low frequency reaction than the abiotic anode.
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Chapter 1

Introduction

Microbial fuel cells (MFCs) are different from electrochemical fuel cells in that they use microbes to catalyze reactions on the anode (and on the cathode in biocathode designs) instead of a chemical catalyst. The bioanode, a crucial part in bioelectrochemical systems (BESs), is composed of an anode biofilm and a conductive electrode. The main catalytic components of anode biofilms are *exoelectrogens*, microorganisms that are capable of exocellular electron transfer (57). A precise understanding of bioanode processes would aid in improving performance of BESs. However, the complexity of anode biofilms makes it hard to decipher the electrochemistry of the bioanode.

The first part of my dissertation research evaluated how external resistance affects the anode microbial community, electrogenesis, and methanogenesis. Regulating external resistance can be a promising operational tool for MFC-based wastewater treatment processes, as it is a system element that can be variable after design. It is a simple method for studying bioelectrochemistry and exoelectrogenic ecology in MFCs. Potentiostats polarize the anode by varying electrical energy input, while external resistances plainly control anode potential without energy input. In mixed-culture systems, exoelectrogens compete for electron donor with other functional groups such as fermenters, acetogens, and methanogens. It was hypothesized that their competition could be controlled by anode potential. I showed that external resistance affected anode microbial community evolution when anode potential substantially decreased. I also showed for the first time
that acetoclastic *Methanosacetaceae* was the main methanogenic group in acetate-fed MFCs, and their methanogenesis rate was affected by anode potential. This paper was published in *Applied and Environmental Microbiology*, and it is entitled “Influence of External Resistance Electrogenesis, Methanogenesis, and Anode Prokaryotic Communities in MFCs” (38).

The second part of my doctoral research investigated effects of pH on impedance characteristics and polarization behavior in an MFC. A viable biocatalyst on an anode electrode requires physiologically permissive medium conditions for their growth. Because medium condition is important for anodic catalytic activity, an improved understanding of the bioanode process as a function of solution conditions would contribute to enhanced MFC operation and performance. pH is a key medium condition. An acidic medium provides increased cathode performance due to increasing availability of protons, but low pH is inhibitory to microbial growth. In varying pH conditions, impedance elements of the bioanode in a single-chamber MFC were characterized using a novel equivalent circuit model. Furthermore, I characterized performance of the anode, cathode, and whole cell using polarization and electrochemical impedance spectroscopy (EIS) behavior. In this work, pH effects on the bioanode process, resistance, capacitance, and nonideality constants in each reaction were successfully differentiated and quantified. This chapter is in preparation for peer review submission.

Impedance and thermodynamics of a mixed-culture bioanode were compared with an abiotic anode. I found that anode biofilm greatly reduced activation overpotential and decreased charge transfer resistance significantly relative to the abiotic anode. In addition, effects of riboflavin on charge transfer processes were tested to investigate the influences
of redox mediator addition on exoelectrogenesis. Riboflavin affected charge transfer to
the anode surface and decreased total charge transfer resistance in the bioanode process.
Finally, EIS was performed on anode biofilms enriched with *Geobacter sulfurreducens*,
*Shewanella oneidensis*, or their coculture to test their charge transfer characteristics with
different exoelectrogenic modes. Each culture condition produced unique Nyquist
morphology in high frequency arcs. This work is also in preparation for submission.

With the EIS analyses tools developed in this work, and the demonstrated ability
to influence biofilm composition and performance through the external resistance as a
control parameter, this work collectively contributes to understanding bioanode
processes and their response to changing conditions (applied load and solution pH).
These findings offer advancements in analytical electrochemistry applied to MFC
systems as well as insights into enhancing bioanode and MFC performance.
Chapter 2

Literature Review

2.1 Effects of anode potential on BESs

The anode potential \( (E_{an}) \) is defined as the potential difference between the anode and the surrounding electrolyte, and as the electromotive force that drives electrons to flow into an anode; it is regarded as a measure of electron affinity (29). There are conflicting results about the influence of \( E_{an} \) on start-up and sustained performance in BESs. Application of a high \( E_{an} \) (+200 mV vs. Ag/AgCl) shortened the start-up of electrogenesis compared with an \( E_{an} \) of -360 mV in two-chamber MFCs (96). However, another study showed that \( E_{an} \) did not affect start-up time, but this was attributed to the use of exoelectrogenic inocula from an active MFC (1). In this latter study, an MFC with the anode poised at -200 mV vs. Ag/AgCl had the highest maximum power density among MFC systems poised at 0, -200, and -400 mV in the beginning, but the performance of these systems converged by the end of the one-month study.

\( E_{an} \) was demonstrated to influence anode biofilm communities in microbial electrolysis cells (MECs) (90). Both performance and bacterial community were analyzed at different anode potentials. A high \( E_{an} \) (170 mV vs. Ag/AgCl) created a thin anode biofilm with high bacterial diversity (91). This anode electrode harbored a low proportion of \textit{Geobacteraceae}-like cells and produced low current density. In lower \( E_{an} \) (-350, -290, and -180 mV vs. Ag/AgCl), \textit{Geobacteraceae}-like cells were preferentially selected on the
anode electrodes in thick anode biofilms (91). Theses anode electrodes produced high current densities and thick biofilms (91). One the other hand, it was recently suggested that changing $E_{an}$ by controlling the external resistance ($R_{ext}$) was not a valid tool for the improvement of anode biofilm performance (60), showing that $E_{an}$ does not affect anode biofilm performance. In this research, similar levels of maximum power were produced from potentially different anode biofilm communities enriched at different $R_{ext}$ (60). However, this study did not show if the system was anode limited. If cathode performance limits overall MFC performance, similar maximum power density could be produced from dissimilar anode biofilms. A more recent study showed that $R_{ext}$ affected power densities in cellulose-fed MFCs (83). The highest maximum power density was observed for reactors with the lowest $R_{ext}$ (83). This study characterized differences in 16S rRNA genes from anode-biofilm and planktonic communities, but the effect of $R_{ext}$ on microbial community evolution was not determined. These previous studies indicate that $E_{an}$ could affect anode microbial community and anode biofilm performance.

### 2.2 Methanogenesis in BESs

Electrogenesis or electrohydrogenesis are the main processes of energy generation in BESs. Methanogenesis can decrease the Coulombic efficiency (CE) as it diverts a fraction of the electrons from electogenesis into methane. Due to similar growth conditions of exoelectrogens and methanogens, methane is often produced and results in coulombic loss in BESs. Hence, minimizing methanogenesis is very important to increase electrical energy recovery from renewable energy resources. Suppression of
methanogenesis in MFCs was investigated by applying various approaches. Inhibitor (2-bromoethanesulfonate) addition and deliberate oxygen intrusion increased CE by suppressing methane production (10, 12), but changing the temperature or pH in MFCs had little effect (12).

Another system factor that can affect bioanode performance and community and may also influence methanogenic losses is $E_{an}$. In a glucose-fed MFC incorporating a ferricyanide catholyte, $E_{an}$ did not affect methane production in the range of -220 to 200 mV vs. SHE (26). In this system with glucose-acclimated anode communities, acetate was injected as the sole substrate for one batch-fed cycle and it showed negligible production of methane when the circuit was connected through 20 Ω of $R_{ext}$ and $E_{an}$ was above 100 mV vs. SHE (26). The authors concluded that acetoclastic methanogenesis might not be significant in MFCs (26). However, an alternative explanation is that the high current density at this low $R_{ext}$/high $E_{an}$ condition might not allow acetate utilization by acetoclastic methanogens because the exoelectrogenic community consumes acetate at a high rate. Methanogenesis was also tested in a flow-through system fed with acetate at a high loading rate (5.4 kg-COD/m$^3$ day) (93). This system had increasing methane production as $E_{an}$ decreased between -100 and -300 mV vs. SHE (93), showing methanogenesis might be affected by $E_{an}$.

Only hydrogenotrophic Methanomicrobiales was detected in an ethanol-fed MFC (73). Hydrogenotrophic methanogenesis has been also suggested to be the main methanogenic pathway in MEC systems, due to cathode-derived hydrogen flux toward the anode (17, 48). On the other hand, acetoclastic methanogenesis was the main
methanogenic process rather than syntrophic acetate oxidation in MFCs, where *Methanosarcinaceae* was the primary methanogenic population (12). In this work, lowering $R_{ext}$ reduced the methanogen production by 24%, which might be also due to shortened batch time (12).

### 2.3 pH effects on BESs

pH is also an important factor in BES operation. pH affects thermodynamics in BESs according to the Nernst equation. An acidic medium provides increased cathodic performance due to increasing availability of protons when protons are involved in the cathodic reaction. In contrast, anodic performance decreases in low pH because low pH is inhibitory to exoelectrogenic microbial growth. The maximum achievable potential of an anode and a cathode in a specific medium condition was calculated according to Eq. 1 - 4 at various pH values. $E^o$ is the standard potential (at 298K, 1 bar, 1M, reported vs. SHE), $E_{an/cat,M}$ is the maximum achievable electrode potential in a specific medium condition, $R$ is the ideal gas constant (8.314 J/Kmol), $T$ is temperature (K), and $F$ is the Faraday constant (96,485 C/mol).

\[
2HCO_3^- + 9H^+ + 8e^- \leftrightarrow CH_3COO^- + 4H_2O \quad (1)
\]

\[
E_{an,M} = E_{an}^o - \frac{RT}{8F} \ln \left( \frac{[CH_3COO^-]}{[HCO_3^-]^2[H^+]^8} \right), \quad E^o = 0.187 \quad (2)
\]

\[
O_2 + 2H^+ + 2e^- \leftrightarrow H_2O_2 \quad (3)
\]

\[
E_{cat,M} = E_{cat}^o - \frac{RT}{2F} \ln \left( \frac{[H_2O_2]}{[O_2][H^+]^2} \right), \quad E^o = 0.695 \quad (4)
\]
\[
\text{CH}_3\text{COO}^- (\text{acetate}) + 4\text{H}_2\text{O} \leftrightarrow 2\text{HCO}_3^- + 9\text{H}^+ + 8\text{e}^- \quad (5)
\]
\[
\text{CH}_3\text{CH(OH)COO}^- (\text{lactate}) + 2\text{H}_2\text{O} \leftrightarrow \text{Acetate} + \text{HCO}_3^- + 5\text{H}^+ + 4\text{e}^- \quad (6)
\]

Exoelectrogenic substrate oxidation increases the proton concentration in the anode chamber. For example, *G. sulfurreducens* PCA completely oxidizes acetate and produces 9-mole proton/1-mole acetate (Eq. 5). Anaerobic lactate oxidation by *S. oneidensis* MR-1 produces 5-mole proton/1-mole lactate. Proper proton diffusion out of the anode biofilm can relieve acidification of the anode biofilm. *G. sulfurreducens* grown using fumarate as an electron acceptor had lower growth rates at pH 6 (0.04 h\(^{-1}\)) than pH 7 (0.21 h\(^{-1}\)) (24). Synthesis of redox mediators by *S. oneidensis* DSP10 decreased in low pH. The concentration of riboflavin reduced considerably at pH 5, coupled with a substantial decrease in current generation without an appreciable change of cell density (4). This indicates that mediator concentration rather than just cell concentration are important in current generation from *S. oneidensis* DSP10 (4).

It was found that acidification of the anode biofilm affects current generation because microbial activity is inhibited in low pH (24, 27, 88). Maximum current density at pH 6 was only 25% of that at pH 8 (88). Alkaline medium and high buffer concentration enhanced bioanode performance due to the increasing flux of proton shuttles out of the anode biofilm (23, 88). In a cloth-electrode assembly (CEA) system buffered with 200 mM bicarbonate, increasing the pH from 7 to 9 resulted in a 42% improvement in maximum power density (\(P_{\text{max}}\)) (23). However, \(P_{\text{max}}\) decreased at pH 9.5, showing pH 9 was the optimum pH for attaining highest power density in this system.
Increasing performance in alkaline medium is mainly attributed to the high availability of proton shuttles such as less-protonated phosphate species ($\text{PO}_4^{3-}$ or $\text{HPO}_4^{2-}$) or carbonate species ($\text{CO}_3^{2-}$). To confirm if alkaline medium relieves anode biofilm acidification, the anode biofilm pH was visualized using fluorescent dye staining in different medium pH (24). This work demonstrated the formation of a pH gradient in the anode biofilm and acidification of the anode biofilm in low pH medium (24).

The cathodic performance increases with decreasing pH and increasing oxygen concentration for cathodic oxygen reduction in an acidic electrolyte system. One study observed lower pH created better performance in terms of current and power density in a carbon-granular cathode (25). Another study showed a 2.5-fold increase in maximum power density with a catholyte of pH 1 compared to that of pH 7.5 in two-chamber MFCs (21). Cathodic oxygen reduction was not limited by proton concentration in catholyte pH below 3 (21). Even in a biocathode, neutralizing the catholyte using strong acid was beneficial in current generation (51). Lowering the pH of the catholyte benefited cathodic oxygen reduction, resulting in increasing power production in an upflow MFC (101). One exception was a study that showed pH 9 was the optimal condition in air-cathode MFCs because cathodic impedance was minimized at pH 9 according to measurement of open-circuit EIS (30).

### 2.4 Impedance, Nyquist plot, and Bode plot

A recent investigation demonstrated the evaluation of ohmic resistance in three different MFC configurations using the current interruption method (52). However, this method
precludes the differentiation of the charge transfer resistance, the double layer capacitance at the electrodes, and the mass transfer resistance. A complete understanding of MFC constraints requires a detailed investigation of ohmic, kinetic, and mass transport properties. Internal resistance ($R_{in}$) mainly consists of three parts: charge transfer/polarization/activation resistance ($R_p$), ohmic/solution resistance ($R_{ohm}$), and concentration/diffusion resistance ($R_d$). The internal resistance in MFCs originates from electrode materials, catalyst coatings on electrodes, poor biofilm development, and electrochemical reactions on the anodes and the cathodes. EIS is a powerful nondestructive technique that has been used for analyzing the distinct contributions to internal resistance in MFCs.

Ohm’s law defines resistance ($R$) in terms of voltage ($E$) and current ($I$). While Ohm’s law is limited to an ideal resistance, circuit elements in the real world are complex. To predict complex circuit behavior, impedance ($Z$) is used in place of resistance. Impedance is a measure of the ability of a circuit to resist the flow of electrical current. Impedance is measured in EIS by applying AC (alternating current) potential to an electrochemical system and reading the AC response. Linear or pseudo-linear systems do not have phase difference between voltage and current.

Impedance ($Z$) is expressed analogously to Ohm’s law as follows:

$$Z = \frac{E_t}{I_t} = \frac{E_0 \sin(\omega t)}{I_0 \sin(\omega t + \varphi)} = Z_0 \frac{\sin(\omega t)}{\sin(\omega t + \varphi)} \quad (7)$$
where $E_t$ is the potential at time $t$, $E_0$ is the amplitude of the signal, $\omega$ is the radial frequency, $\phi$ is the phase shift, $I_t$ is the response signal at time $t$, and $I_0$ the amplitude of the response. With Euler’s relationship, it becomes simplified to

$$Z = Z_0 \left( \cos \phi + j \sin \phi \right) = ReZ + ImZ \quad (8)$$

Impedance is composed of a real part ($ReZ = Z_0 \cos \phi$) and an imaginary part ($ImZ = Z_0 j \sin \phi$), where $j$ is the imaginary unit. Each point on a Nyquist plot is the imaginary impedance versus the real impedance at each frequency. Higher frequencies are on the left side and low frequencies are on the right side in the Nyquist plot. However, a Nyquist plot does not present the respective frequencies. Another representation of impedance that explicitly shows the frequency is the Bode plot, in which either the absolute value of impedance ($|Z| = Z_0$) or the phase shift is plotted versus log frequency.

To calculate impedance components, impedance data are analyzed by equivalent circuit (EC) modeling. Common impedance components are resistance ($R$), capacitance ($C$), and inductance ($L$) (Table 2.1). Resistance of an object is the property that resists a steady current flow. Capacitance is the ability of electrical bodies to hold electrical charges. Inductance is the property of electrical bodies by which a change of current induces an electromotive force that opposes the change of current.

The impedance of capacitance ($Z_C$) decreases as the frequency increases and has only an imaginary component. The current is phase-shifted $90^\circ$ with respect to the voltage. The impedance of inductance ($Z_L$) increases as frequency increases and also has
only an imaginary component. The current is phase-shifted -90° with respect to the voltage. The impedance of resistance \(Z_R\) is independent of frequency and has no imaginary component because the current is in phase with the voltage.

Infinite Warburg (W), finite Warburg (O), and CPE (constant phase element, Q) are other components of ECs. Diffusion resistance creates the Warburg impedance. The Warburg impedance is small at high frequencies and big at low frequencies because the reactants have to diffuse further at lower frequency. If the diffusion layer has infinite thickness, the infinite Warburg impedance is applied. When a capacitor does not behave ideally, a CPE is used to explain the capacitor. The nonideality constant \(\alpha\) is usually treated as an empirical constant.
Table 2-1. Common impedance components, equations for their current vs. voltage relationship, and equations for their impedance relationship

<table>
<thead>
<tr>
<th>Component</th>
<th>I vs. E</th>
<th>Impedance (Z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resistance</td>
<td>$E = IR$</td>
<td>$R$</td>
</tr>
<tr>
<td>Capacitance</td>
<td>$i = C \frac{dE}{dt}$</td>
<td>$\frac{1}{jC\omega}$</td>
</tr>
<tr>
<td>Inductance</td>
<td>$E = L \frac{di}{dt}$</td>
<td>$jL\omega$</td>
</tr>
</tbody>
</table>

Table 2-2. Impedance components and equations for their impedance relationship

<table>
<thead>
<tr>
<th>Component</th>
<th>Impedance (Z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infinite Warburg (W)</td>
<td>$\frac{1}{Y_0 \sqrt(j\omega)}$</td>
</tr>
<tr>
<td>Finite Warburg (O)</td>
<td>$\frac{\tanh(B\sqrt(j\omega))}{Y_0\sqrt(j\omega)}$</td>
</tr>
<tr>
<td>CPE (Q)</td>
<td>$\frac{1}{Y_0 (j\omega)^{\alpha}}$</td>
</tr>
</tbody>
</table>

$Y_0 = C = \text{capacitance}$, $\alpha = \text{exponent that equals 1 for an ideal capacitor}$, $B = \delta/D^{1/2}$ ($\delta = \text{Nernst diffusion layer thickness, cm}$; $D = \text{Average diffusion coefficient, cm}^2/\text{s}$)
2.5 EIS research in BESs

EIS has been demonstrated to elucidate anodic electrochemical reactions in BESs. EIS was used to show that development of a mixed-culture anode biofilm significantly decreased charge transfer resistance. (7, 64, 67, 80). These results indicated that anode biofilm facilitated electron transfer to the anode. For example, in a ferricyanide-cathode MFC, initial development of anode biofilm during the first five days decreased anodic charge transfer resistance by ~40% (from 2.6 to 1.5 kΩ cm$^2$ at 0.27 A/m$^2$) and it also increased power density by ca. 120% (80). In an air-cathode MFC, anodic charge transfer resistance reduced by ~75% (from 0.073 to 0.017 kΩ cm$^2$ at 2.63 A/m$^2$) with increasing anodic capacitance during 70 days of long-term operation (7). However, this research did not provide the accuracy of EC model fitting, which is critical to evaluate the validity of impedance component values.

For more constrained impedance analysis on extracellular electron transfer, pure cultures were tested by EIS. Potentiostatic EIS was performed on a bioanode covered with *G. sulfurreducens*. It showed that charge transfer resistance was the lowest at the potential near the midpoint potential (67). In their impedance analysis, a second time constant through an additional circuit element did not improve data fitting (67). In a later work using *Shewanella oneidensis* DSP10, a second time constant was added (two time constant model) to differentiate two charge transfer reactions (79). They hypothesized that the first reaction was associated with electron shuttles and the second reaction was associated with microbial substrate oxidation (79). However, they provided neither EC fitting nor goodness of fit.
Chapter 3

External Resistance Influences Electrogenesis, Methanogenesis, and Anode Prokaryotic Communities in MFCs

Abstract

The external resistance ($R_{\text{ext}}$) of microbial fuel cells (MFCs) regulates both the anode availability as an electron acceptor and the electron flux through the circuit. I evaluated the effects of $R_{\text{ext}}$ on MFCs using acetate or glucose. The average current densities ($I$) ranged from 40.5 mA/m$^2$ (9800 Ω) to 284.5 mA/m$^2$ (150 Ω) for acetate-fed MFCs (ARs), with a corresponding anode potential ($E_{\text{an}}$) range of -188 to -4 mV (vs. SHE). For glucose-fed MFCs (GRs), $I$ ranged from 40.0 mA/m$^2$ (9800 Ω) to 273.0 mA/m$^2$ (150 Ω) with a corresponding $E_{\text{an}}$ range of -189 to -7 mV. ARs produced higher Coulombic efficiencies and energy efficiencies than GRs over all tested $R_{\text{ext}}$ because of electron and potential losses from glucose fermentation. Biogas production accounted for 14-18 % of electron flux in GRs, but only 0-6 % for ARs. GRs produced similar levels of methane regardless of $R_{\text{ext}}$. However, total methane production in ARs increased as $R_{\text{ext}}$ increased, suggesting that $E_{\text{an}}$ might influence the competition for substrate between exoelectrogens and methanogens in ARs. Increasing $R_{\text{ext}}$ to 9800 Ω significantly changed the anode

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1 This is a modification of the paper published as the following: Jung and Regan (2011). "Influence of External Resistance on Electrogenesis, Methanogenesis, and Anode Prokaryotic Communities in Microbial Fuel Cells." Appl. Environ. Microbiol. 77(2): 564-571.
bacterial communities for both ARs and GRs, while operating at 970 Ω and 150 Ω had little effect. *Deltaproteobacteria* and *Bacteroidetes* were the major groups in anode communities in ARs and GRs. *Betaproteobacteria* and *Gammaproteobacteria* were found only in ARs. *Bacilli* were abundant only in GRs. The anode methanogenic communities were dominated by *Methanosacetaceae*, with significantly lower numbers of *Methanomicrobiales*. These show that $R_{ext}$ affects not only $E_{an}$ and current generation but also anode biofilm community and methanogenesis.
3.1 Introduction

The bioanode, a crucial component in bioelectrochemical systems (BESs), is composed of an anode biofilm and a conductive electrode. The main catalytic components of interest in anode biofilms are exoelectrogens, microorganisms that are capable of exocellular electron transfer (57). In mixed-culture systems, exoelectrogens compete for electron donor with other functional groups such as fermenters, acetogens, and methanogens. The complexity of anode biofilms makes it hard to elucidate electrochemical mechanisms at the bioanode, but a precise understanding of exoelectrogenesis and competition in anode biofilms will aid in improving performance of BESs. Several reviews provide insightful summaries and perspectives regarding bioanodes (57, 65, 75, 78, 89).

The anode potential ($E_{an}$) is defined as the potential difference between the anode and the surrounding electrolyte (29). $E_{an}$ also refers to the electromotive force that drives electrons to flow into an anode and it is regarded as a measure of electron affinity (29). $E_{an}$ is affected by intrinsic factors such as electrode material, electrolyte composition, electrochemical reactions, and catalysts (anode biofilm in BESs) (29). $E_{an}$ also can be controlled extrinsically using a potentiostat or external resistances ($R_{ext}$). Potentiostats polarize the anode by varying electrical energy input, while external resistances simply regulate $E_{an}$ and current without energy input. For example, the application of a low external resistance allows a relatively high $E_{an}$ and current when the anode is connected to a highly oxidizing cathode.
There are conflicting results about the influence of $E_{an}$ on start-up and sustained performance in BESs. Application of a high $E_{an}$ (+200 mV vs. Ag/AgCl) shortened the start-up of electrogensis compared with an $E_{an}$ of -360 mV (96). In another study, $E_{an}$ did not affect start-up time due to the use of an exoelectrogenic inoculum from another BES (1). At the start of this study, an anode poised at -200 mV vs. Ag/AgCl had the highest maximum power density among systems poised at 0, -200, and -400 mV, but the performance of these systems converged by the end of the one-month study. $E_{an}$ was demonstrated to influence anode biofilm communities in microbial electrolysis cells (MECs) (90). A high $E_{an}$ (370 mV vs. SHE) created highly diverse and thin anode biofilms with low current density and a low proportion of *Geobacteraceae*-like cells, while lower $E_{an}$ (-150, -90, and 20 mV) preferentially selected *Geobacteraceae*-like cells and produced high current density and thick biofilms (91). It was recently suggested that $R_{ext}$ cannot be a valid tool for the improvement of anode biofilm performance because similar levels of maximum power were produced from potentially different anode biofilm communities enriched at different $R_{ext}$ (60). However, this study did not provide electrochemical characterization to demonstrate whether the system was anode limited, nor did it address whether changes in $R_{ext}$ influenced electron losses to competing metabolisms. Another recent study of cellulose-fed MFCs showed that $R_{ext}$ affected power densities, with the highest maximum power density observed for reactors with the lowest $R_{ext}$ (83). While this study showed screening-level differences in 16S rRNA genes from anode biofilm and planktonic communities, the effect of $R_{ext}$ on specific microbial populations was not determined.
Methanogenesis in BESs has also been investigated in terms of $E_{an}$. Methane production in a glucose-fed microbial fuel cell (MFC) incorporating a ferricyanide catholyte was not affected by $E_{an}$ between -220 to 200 mV vs. SHE (26). An acetate-fed batch test with these glucose-acclimated communities showed negligible production of methane at an $R_{ext}$ of 20 $\Omega$ ($E_{an} > 100$ mV vs. SHE), suggesting acetoclastic methanogenesis was not significant in these MFCs. That may have been influenced by the high current not allowing acetate utilization by the slow-growing acetoclastic methanogens. Another study showed that a flow-through system fed with acetate (5.4 kg-COD/m$^3$/day) had increasing methane production as anode potential decreased in the anode potential range of -300 < $E_{an}$ < -100 mV vs. SHE (93). In an ethanol-fed MFC, hydrogenotrophic *Methanomicrobiales* was the only detected methanogen (73). In MEC systems, hydrogenotrophic methanogenesis has been proposed as the main methanogenic pathway due to hydrogen flux to the anode (17, 48).

Controlling $R_{ext}$ is a simple method for studying bioelectrochemistry and exoelectrogenic ecology in MFCs. Furthermore, it can be a promising operational tool for MFC-incorporated wastewater treatment processes, as it is a system element that can be variable after design. The purpose of this study was to evaluate how $R_{ext}$ affects the anode microbial community, electricity production, methane production, and electron flow without energy input from potentiostats. To achieve this goal, triplicate anode biofilms were developed at the same $R_{ext}$ in H-shape MFCs using glucose or acetate. Variations among anode microbial communities in triplicate MFCs were small and their compositions were stable after four batch cycles (36). Then they were operated and
monitored at different $R_{ext}$. H-shape MFCs provided comparatively stable $E_{an}$ during most of the batch cycles, which maintained nearly constant pressure for each anode microbial community. Our results showed that $R_{ext}$ affected anode microbial community evolution when $E_{an}$ substantially decreased. It also showed for the first time that acetoclastic *Methanosetaetaeae* was the main methanogenic group in acetate-fed MFCs, and their methanogenesis rate was affected by $E_{an}$. $R_{ext}$ was demonstrated as a potential tool for controlling electrogenss, methanogenesis, and anode microbial community.

### 3.2 Materials and Methods

**MFC construction and operation.** H-shape MFCs (two chambers separated by a Nafion membrane) were constructed as previously described with 25.2 cm$^2$ of anode (carbon paper, E-TEK) and 13.0 cm$^2$ of cathode (carbon paper coated with 0.35 mg/cm$^2$-Pt, E-TEK) (36). Anode chambers were inoculated with anaerobic sludge from a secondary digester of the Pennsylvania State University Wastewater Treatment Plant. Two sets of triplicate reactors, acetate-fed reactors (ARs) fed sodium acetate (Sigma-Aldrich, MO) and glucose-fed reactors (GRs) fed D-glucose (EM Science, NJ), were operated with an $R_{ext}$ of 970 Ω for four batches (stages 0 to 3, ca. 65 days). Afterwards, an $R_{ext}$ of 9800, 970, or 150 Ω was correspondingly applied to AR1/GR1, AR2/GR2, and AR3/GR3 for an additional three batch cycles (stages 4 to 6). Acetate- and glucose-fed control reactors (AC and GC) were operated in open circuit for stages 0 to 3, and then in closed circuit at 970 Ω for stages 4 to 6 (Table 3-1). Anode chambers were filled with 210 ml medium (50 mM phosphate-buffered GM medium with 200 mg COD/L or 25 meq e$^-$/L) in an
anaerobic glove box. At the start of stages 1 to 3, the anode bottles were cleaned during the medium change, but this was discontinued at the end of stage 3. Cathode chambers were refilled with phosphate buffer (50 mM, pH 7.0) in each cycle and continuously aerated. Reactors were operated at 30°C.

**Electrochemical measurements.** Cell voltage was monitored using a multiple data acquisition system (PICO, UK). Cathode potential was intermittently measured with a Ag/AgCl reference electrode (MF-2079, BASi) located about 5 mm from the cathode electrode, and anode potential was calculated by subtracting cathode potential from cell voltage. Electrode potentials were converted into SHE values by adding 195 mV, and are reported throughout vs. SHE. The Coulombic efficiency (CE) and the energy efficiency (EE) were calculated as previously described (53). Ohmic resistances of abiotic MFCs filled with the anolytes and catholyte mentioned above were measured using a potentiostat (PC 4/750, Gamry Instrument Inc., PA), and internal resistance was calculated from the slopes of polarization curves.

**Chemical analyses.** Liquid samples were filtered with a syringe filter (0.2 µm Supor Membrane, PALL Life Science, NY), and soluble COD was measured using the colorimetric method (Cat 21258-15; HACH, CO). Duplicate headspace gas samples (100 µl) were collected using a gas-tight syringe for gas composition analyses at the end of each batch (44). Methane, carbon dioxide, hydrogen, and nitrogen concentrations in the headspace were analyzed by GC (Model 8610, SRI Instruments, CA) equipped with a thermal conductivity detector and a stainless steel column (1.8 m × 1/8”) packed with Porapak Q (Alltech, Deerfield, IL).
**DNA extraction and PCR.** Anode electrode samples (2 cm²) were sliced with sterilized scissors at the end of each batch in an anaerobic chamber for isolation of genomic DNA. DNA was extracted with the PowerSoil™ DNA Isolation Kit (MO BIO, CA) according to manufacturer instructions. The V3 region of 16S rRNA genes were amplified with 534r (5′-ATTAC CGCGG CTGCT GG-3′) and 341f (5′-CCTAC GGGAG GCAGC AG-3′) with a GC-clamp (5′-CGCCC GGGGC GCGCC CCGGG CGGGG CGGGG GCACG GGGGG-3′) attached to the 5′-terminus (71). PCR amplifications were performed in 10-µl volume containing 1.75 µl of DNA, 0.25 µM of each primer (IDT, Inc, IA), 0.4 mg/ml of bovine serum albumin (Promega, WI), and Taq PCR Master Mix (QIAGEN, CA), giving final concentrations as follows: 0.5 U of Taq polymerase, 200 µM of each deoxynucleoside triphosphate, 3.0 mM of MgCl₂, and KCl, Tris Cl, and (NH₄)₂SO₄. An iCycler IQ™ thermocycler (BioRad, CA) was used for the PCR with the following program: initial denaturation at 95°C for 10 min; 10 touchdown cycles of denaturation at 94°C for 1 min, annealing at 65-55°C for 1 min (decreasing 1°C each cycle), and extension at 72°C for 2 min; 25 standard cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min; and a final extension at 72°C for 30 min. Archaeal 16S rRNA genes were amplified with primers Arch958r (5′-YCCGGCGTTGAMTCCAATT-3′) and A20f (5′-TTCCGGTTGATCCYGCCRG-3′) in the above PCR composition using a PCR program described elsewhere (20).

**Community analysis.** Denaturing gradient gel electrophoresis (DGGE) was performed using the DCODE™ Universal Mutation Detection System (BioRad, CA) for bacterial community analysis. PCR amplicons (10 µl) mixed with a loading dye were
loaded onto 8% (wt/vol) polyacrylamide gels containing a gradient of denaturant ranging from 30-60% (100% denaturant was 7 M urea and 40% formamide). DGGE was run in 0.5 × TAE (Tris-acetate-EDTA) buffer at 25 V for 15 min and subsequently at 200 V for 6 hr (60°C). DGGE gels were silver stained (81). DGGE bands were excised and transferred to 30 µl elution buffer (QIAGEN, CA), and incubated overnight at 4°C. Eluted DNAs were purified by PCR-DGGE until a single band was obtained in the target region to remove commigrated DNA with other melting domains. Operational taxonomic units (OTUs) were defined based on melting domain. Single bands were eluted as above and purified using DNA Clean & Concentrator™-5 (Zymo, CA), followed by sequencing. Archaeal clone libraries were constructed as previously described (36, 72). DNAs were sequenced at the Penn State University Nucleic Acid Facility. Nucleotide sequences were deposited in the GenBank database (Accession Numbers HM193296 - HM193368). The DGGE profiles were converted into a matrix based on the locations and intensities of DGGE bands, and Minitab15® (Minitab Inc., PA) was used for Principal Component Analysis (PCA) to convert the matrix into a two-dimensional PCA plot.

**Quantitative PCR (qPCR).** Quantification of *Geobacteraceae* was performed with the primer set *Geobacteraceae*-494f (5′-AGGAAGCACCGGCTAACTCC-3′) and Geo825r (5′-TACCCGCRACACCTAGT-3′) (32). *Methanosetaceae* and *Methanomicrobiales* were quantified using primer sets Mst702f (5′-TAATCCTYGARGGACCACCA-3′) and Mst862r (5′-CCTACGGCACCRACMAC-3′), and MMB282f (5′-ATCGRTACGGGTTGTGGG-3′) and MMB832r (5′-CACCTAACGCRACATHGTTTTAC-3′) (99), respectively. qPCR was done in 20-µl
volumes containing 1 µl DNA, 0.25 µM of each primer (IDT, Inc, IA), 0.4 mg/ml bovine serum albumin (Promega, WI), and *Taq* PCR Master Mix (QIAGEN, CA). qPCR was done in an iCycler IQ™ thermocycler (Bio-Rad, CA) with the program used Yu el al (99). Standard curves were made using plasmids containing an insert sequence of each targeted group. Plasmid concentrations were determined with a NanoDrop 2000 (Thermo Scientific). To estimate total cell numbers, 16S rRNA genomic operon numbers were 2 for *Geobacteraceae* (70), 2 for *Methanosetaceae*, and 2.67 for *Methanomicrobiales* (49).

### 3.3 Results

**Electrochemistry.** Exoelectrogenic community evolution, system performance, and replicate reactor reproducibility at 970 Ω for batches 0 to 3 was reported elsewhere (36). Following the stable and reproducible performance that was observed after two months at identical $R_{ext}$, the $R_{ext}$ was increased in one of the triplicate reactors and decreased in another for acetate- and glucose-fed systems. Low $R_{ext}$ allowed high current flow and elevated $E_{an}$ (Table 3-2). Average currents in the voltage plateau ranged from 40.5 mA/m² (9800 Ω) to 284.5 mA/m² (150 Ω) for ARs, with a corresponding $E_{an}$ range of -188 to -4 mV. For the GRs, currents ranged from 40.0 mA/m² (9800 Ω) to 273.0 mA/m² (150 Ω) with a corresponding $E_{an}$ range of -189 to -7 mV. Cathode potential was also affected by $R_{ext}$, ranging from ~50 to ~330 mV. Lower average internal resistances of ARs (1322 ± 107 Ω) than GRs (1520 ± 126 Ω) contributed to higher power from ARs.

**Electron flow and energy recovery.** In lower $R_{ext}$ (higher $E_{an}$) systems, the total substrate consumption rate was higher due to increased rates of electrogensis (Table 3-3).
COD loss not accounted for in measured current, hydrogen, and methane production and estimated aerobic respiration increased with $R_{ext}$, reaching 74 % (AC) and 64 % (GC) in open circuit ($R_{ext} = \infty$). Acetate consistently resulted in higher CEs and EEs than glucose because of potential losses from glucose fermentation (26, 36). Biogas production accounted for 14-18 % of electron loss in GRs, but only 0-6 % for ARs. The high availability of the anode at low $R_{ext}$ resulted in high CE for both substrates. EEs were highest at 970 $\Omega$ (14 % for AR2 and 9 % for GR2), which was closest to the internal resistances of tested MFCs. Higher power density is earned when $R_{ext} = R_{int}$ according to the equation $P = OCV^2 \{R_{ext}/[A(R_{ext} + R_{int})^2]\}$ (15).

**Biogas production.** Headspace biogas mainly consisted of methane and carbon dioxide, with negligible hydrogen detected. For acetate-fed reactors, total methane production increased as $R_{ext}$ increased and current decreased (Table 3-3). Non-washing of anode chambers between batches did not affect methane production in ARs. Regardless of current generation, AC produced very little methane. GRs produced 43 to 54 $\mu$mol methane during stages 1 to 3 and 84 to 92 $\mu$mol in stages 5 and 6, during which the anode bottles were not washed with each medium change as they had been during stages 1 to 3 (Table 3-3). Regardless of $R_{ext}$, GRs produced similar levels of methane, but cycle length-averaged methanogenesis rates varied significantly due to longer operation in higher $R_{ext}$. The non-electrogenic reactor GC produced a similar amount of methane as the closed-circuit GRs. The amount of produced carbon dioxide increased with decreasing $R_{ext}$ and increasing current generation, and no carbon dioxide was detected in non-electrogenic AC and GC.
**$R_{ext}$ effect on anode prokaryotic communities.** Bacterial 16S rRNA genes were screened by DGGE and identified by band sequence analysis to determine the $R_{ext}$ effect on the anode community composition and evolution (Fig. 3-1 and Table 3-4). Anode bacterial communities enriched at 970 Ω for four batches were compared with those that developed after the $R_{ext}$ changes. Densitometry-based PCA showed that increasing $R_{ext}$ to 9800 Ω for both acetate- and glucose-fed MFCs changed the anode bacterial communities significantly, while continued operation at 970 Ω and a reduction to 150 Ω had little effect on them. The principal components for the community evolution observed in the 9800-Ω systems were OTUs Ac, Ad, and Ai for AR1 and OTUs Ga, Gg, and GJ for GR1. OTUs G1, G2, G3, G4, and G5 of glucose-fed anode communities had nearly identical sequences and shared the same melting domains in DGGE correspondingly with OTUs A1, A2, A3, A4, and A5 of acetate-fed communities. *Deltaproteobacteria* and *Bacteroidetes* were the major taxonomic groups commonly found in the anode communities of ARs and GRs. *Clostridia* was also a common group in ARs and GRs. *Betaproteobacteria* (*Acidovorax* sp.) and *Gammaproteobacteria* (*Pseudomonas* sp.) were found only in ARs, and *Bacilli* (*Enterococcus* sp.) was abundantly detected only in GRs. In order to identify targets for quantifying methanogenic communities, clone libraries of archaeal 16S rRNA genes were constructed. The methanogenic communities in our anode biofilms were dominated by *Methanosaetaceae*. Most of the retrieved clones (40 out of 42) had the closest match to *Methanosaeta concilii* Opfikon (NR028242) with >99 % similarity. The other two clones were affiliated with uncultured hydrogenotrophic *Methanomicrobiales* (39).
**Quantification of three functional groups in anode biofilms.** qPCR was used to quantify the cell number changes of three major functional groups (*Geobacteraceae, Methanosetaecae, and Methanomicrobiales*) in the anode biofilms. In general, the $R_{ext}$ change did not consistently affect abundance of these functional groups (Fig. 3-2), though these data do not reveal their relative abundance in the total anode communities. The average cell numbers of *Geobacteraceae* were $1.7 \times 10^6 \pm 5.8 \times 10^5$ cells/cm$^2$ in ARs and $2.5 \times 10^6 \pm 1.6 \times 10^6$ cells/cm$^2$ in GRs. When AC and GC produced electricity, *Geobacteraceae* cell numbers increased drastically. Average *Methanosetaecae* density was $2.0 \times 10^4 \pm 2.2 \times 10^4$ cells/cm$^2$ in ARs and $3.9 \times 10^4 \pm 1.9 \times 10^4$ cells/cm$^2$ in GRs. *Methanomicrobiales* was $1.1 \times 10^3 \pm 2.1 \times 10^2$ cells/cm$^2$ in ARs. However, average *Methanomicrobiales* in GRs increased ca. 5 times from $2.0 \times 10^3$ in stage 3 to $1.2 \times 10^4$ cells/cm$^2$ in stage 6, with a doubling of methane production (Table 3-3). This coincided with the discontinuation of anode chamber washing between feedings, but non-washing did not affect methane production or methanogen cell number in ARs. Regardless of current generation, AC produced very little methane and had only *Methanomicrobiales* cells. The non-electrogenic reactor GC had less methanogenic cells in the anode biofilm, but produced a similar amount of methane as the closed-circuit GRs.

**Electricity production of open circuit-acclimated anode biofilms.** The open-circuit $E_{an}$ of AC and GC were ca. 50 mV initially, gradually decreasing to ca. -300 mV at the end of stage 1. In stage 1, acetate was barely consumed in AC, while nearly all of the glucose added to GC was converted to and accumulated as acetate. In successive batches, $E_{an}$ remained ca. -300 mV and metabolite concentrations fluctuated in both
reactors. When circuits were connected with 970 Ω at stage 4, current was generated immediately in both systems and their $E_{an}$ rapidly increased to similar levels as AR2 and GR2. DGGE profiles of anode communities during stages 1 to 3 were similar and showed that Geobacteraceae sequences existed in the anode biofilms during open-circuit operation. Despite the unavailability of the anode for exoelectrogenic activity in open circuit condition, substantial substrate was consumed in these reactors during stage 2-3 (Table 3-3). Bands representing Geobacteraceae increased their intensities after circuit connection, which is consistent with the qPCR quantification showing that Geobacteraceae cell number increased ca. $10^3$ times (Fig. 3-3). Anode biofilm profiles of electrogenic AC and GC had high similarities to AR1 and GR2, respectively.

3.4 Discussion

External resistance effects on anode community evolution. Our results show that high $R_{ext}$ affected the exoelectrogenic communities that had established at 970 Ω. $R_{ext}$ regulates $E_{an}$, which is equivalent to the anode availability as an electron acceptor. $E_{an}$ influenced the competition between exoelectrogenic and non-exoelectrogenic community members, as supported by changes in the MFC performance with respect to electron distribution and biogas production (Table 3-3). $E_{an}$ might also influence competition among exoelectrogens, indirectly through microenvironmental conditions or directly through anode utilization, though we do not have direct evidence of this. For example, $R_{ext}$ influences substrate consumption rate (Table 3-3) and the associated proton production rate in the anode biofilm, so it can affect pH in the anode biofilm (24, 88).
The anode biofilm community has been shown previously to be affected by pH in biohydrogenesis processes (81).

Regarding direct effects of $E_{an}$ on competition among exoelectrogenic populations, the lowest $E_{an}$ (where the community changes were most pronounced) would select for exoelectrogens that can still meet their metabolic energy needs with such a small potential gradient between the redox potential of their electron donor and the anode. Most known *Geobacter* strains become significantly limited below potentials of about -0.15 V (82). Bacteria might adapt themselves to a specific potential by regulating intracellular reducing equivalents such as the NADH/NAD$^+$ ratio depending on the potential of the electron acceptor (56). For *Escherichia coli*, NADH/NAD$^+$ was 0.094 in aerobic growth and 0.22 in anaerobic growth (50), and increased progressively with decreasing midpoint potential of the electron acceptor (19). More fundamental bioelectrochemical studies are needed to address this redox issue.

**Anode bacterial ecology.** *Geobacter* strains appear to need a small amount of bicarbonate in the medium for crucial reactions, such as carbon fixation mediated by pyruvate-ferredoxin oxidoreductase (62). Accordingly, a low density of *Geobacter* cells in MFCs was thought to be associated with their poor growth in phosphate-buffered medium because high numbers of *Geobacter* 16S rRNA gene sequences were detected in carbonate-buffered anode chambers with scarce oxygen (11, 33). However, our result showing *Geobacter*-like cell dominance in phosphate-buffered MFCs having headspace filled with nitrogen is coincident with previous results with the same condition (36, 47, 98). It shows that initial buffer species might not be an important factor in terms of
*Geobacter* dominance in mixed-culture BESs, in which bicarbonate can be produced by other community members.

*Desulfuromonas*-like sequences were found in our GRs and were also detected in the anode community of an ethanol-fed MFC (43). *D. acetoxidans* is an acetate-utilizing electricity-producing species (5) and also oxidizes ethanol coupled with iron reduction (85). *Pelobacter*-like sequences detected in our systems were observed in sediment MFCs (31) and were predominant in ethanol-fed MECs (74). *P. propionicus*-like sequences were also abundant in an acetate-fed MEC system (13). *Acidovorax* sp. Ic3 is a hydrogenotrophic denitrifying bacterium (92), and similar sequences were detected in acetate-fed anode biofilms (8, 98) and a cathode biofilm (77). These molecular evidences imply more research opportunities on potential electrogenesis of these genera. *Pseudomonas aeruginosa* produces electricity using electron shuttles (pyocyanin) (76), and related sequences were found in the anode biofilm of an acetate-fed MFC (98), consistent with our results. *Enterococcus* is a genus of fermentative lactic acid bacteria, and an isolate of *E. gallinarum* exhibited oxidation and reduction peaks in cyclic voltammetry (41).

*Bacteroidetes* and *Clostridia* were common taxonomic groups in the anode biofilms of ARs and GRs. *Bacteroidetes* have been found in anode microbial communities of BESs fed starch-processing wastewater (40), cellulose (84), and acetate or glucose (98), but whether they contribute an exoelectrogenic phenotype in these communities is not known due to the absence of isolates from BESs. *C. sticklandii* performs sticklandii reactions, oxidizing an amino acid by reducing another amino acid
31

(86). *C. aminobutyricum* (X76161, 99%) ferments 4-aminobutyrate to ammonia, acetate, and butyrate. Both species were also found in acetate- or glucose-fed single chamber MFCs (98).

**Methanogenic archaeal ecology.** Methanogenesis was not affected by $E_{an}$ in GRs. Non-washing of anode chambers might have resulted in higher methanogenesis during stages 5 and 6 in GRs due to adhered microbial communities on the reactor walls that were not reflected in the qPCR data from anode electrodes. In ARs, non-washing of the anode chambers did not increase methanogenesis. Methanogenesis occurred only in MFCs harboring *Methanosetaeae*-like sequences in their anode biofilm, suggesting that acetoclastic *Methanosetaeae* can be the main methanogenic source in acetate-fed MFCs. As $E_{an}$ became higher, current generation increased and methanogenesis diminished (Table 3-3). $E_{an}$ might affect competition between exoelectrogens and acetoclastic methanogens in acetate-fed MFCs, with *Methanosetaeae* being out-competed in higher-current MFCs due to their lower affinity for and specific substrate utilization rate with acetate. For instance, pure culture *G. sulfurreducens* has a half-saturation concentration ($K_s$) for acetate of 6.4 mg COD/l (0.10 mM) and a specific substrate utilization rate ($q_{max}$) of 22.7 mg COD/mg VSS-d in ferric citrate-respiring conditions, while *Methanosaeta* sp. in methanogenic reactors has a $K_s$ of 49 mg COD/l and a $q_{max}$ of 10.1 mg COD/mg VSS-d (18, 22). Thus, if anode availability is not limited, exoelectrogens could have a kinetic advantage over methanogens for acetate.

Not only acetoclastic *Methanosetaeae* but also hydrogenotrophic *Methanomicrobiales* were detected in our systems. Hydrogenotrophic methanogenesis
was suggested to be a main methanogenic pathway in MECs (17, 48). In an ethanol-fed MFC, Methanomicrobiales was the only methanogen (73); Methanosaetaceae might have been suppressed by the high concentration of substrate (initial COD 2500 mg/L of ethanol) in this system due to their inhibition by high organic carbon concentrations (39). No Methanosaetaceae in the anode biofilm of AC could be due to the initial oxic condition. During anode biofilm acclimation, the higher biomass of the exoelectrogenic communities in closed-circuit ARs could create an ecological niche for Methanosaetaceae by consuming oxygen.

The differences between internal resistances and ohmic resistances were 282 ± 84 Ω for ARs and 355 ± 104 Ω for GRs. Assuming that the overpotentials of abiotic cathodes were similar due to the high reproducibility of commercial cathode electrodes used in this experiment, the larger difference for GRs could be due to current-dependent anode overpotential such as microbial activation or metabolic loss, such as indirect glucose oxidation by mixed cultures (26, 36, 98) and higher diversity of anode biofilms in GRs. However, the high ohmic resistance of H-shape MFCs precludes further electrochemical analysis on anode biofilm kinetics because power densities and current densities are limited by ohmic resistances in H-shape MFCs as shown previously (36). This ohmic limitation should be resolved by using BESs with low ohmic resistances.
3.5 Acknowledgements

This research was supported by National Science Foundation Grant CBET-0834033. The authors thank Jungrae Kim (Sustainable Environment Research Center, UK) and Kion Kim (Department of Statistics, Penn State University, USA) for their useful suggestions.
**Figure 3-1.** Bacterial 16S rRNA gene-derived DGGE profiles (A & C) and their corresponding PCA plots (B & D) showing community evolution of anode biofilms in ARs (A & B) and GRs (C & D). AR: Acetate-fed MFC, GR: Glucose-fed MFC, S3 to S6: Stage 3 to 6. Different external resistances were applied after stage 3. (Anode communities at stage 4 were not characterized).
Figure 3-2. Density of Geobacteraceae (Geo), Methanosaetaceae (Mst), and Methanomicrobiales (Mmb) in anode biofilms at stage 3 (white) and stage 6 (gray) measured by quantitative PCR (n=2).
Figure 3-3. Evolution of bacterial 16S rRNA gene-derived DGGE profiles from control reactor anode biofilms before and after electromogenesis. A: Acetate-fed MFCs, B: Glucose-fed MFCs. S1 to S6: Stage 1 to 6. Control reactors were changed from open to closed circuit after stage 3.
Table 3-1. MFC operation scheme depicting applied external resistance ($R_{\text{ext}}$).

<table>
<thead>
<tr>
<th></th>
<th>Stage 1-3</th>
<th>Stage 4-6</th>
<th>Stage 1-3</th>
<th>Stage 4-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC</td>
<td>Open Circuit</td>
<td>970 $\Omega$</td>
<td>GC</td>
<td>Open Circuit</td>
</tr>
<tr>
<td></td>
<td>970 $\Omega$</td>
<td>9800 $\Omega$</td>
<td>GR1</td>
<td>970 $\Omega$</td>
</tr>
<tr>
<td>AR1</td>
<td>970 $\Omega$</td>
<td>970 $\Omega$</td>
<td>GR2</td>
<td>970 $\Omega$</td>
</tr>
<tr>
<td>AR2</td>
<td>970 $\Omega$</td>
<td>970 $\Omega$</td>
<td>GR3</td>
<td>970 $\Omega$</td>
</tr>
<tr>
<td>AR3</td>
<td>970 $\Omega$</td>
<td>150 $\Omega$</td>
<td></td>
<td>150 $\Omega$</td>
</tr>
</tbody>
</table>
Table 3-2. Electrochemical properties of MFCs and performances in different $R_{ext}$ conditions.

<table>
<thead>
<tr>
<th>MFC</th>
<th>$R_{ext}$ (Ω)</th>
<th>$R_{ohm}$ (Ω)$^a$</th>
<th>$R_{int}$ (Ω)$^b$</th>
<th>$E_{an}$ (mV)$^c$</th>
<th>$E_{cat}$ (mV)$^c$</th>
<th>$I$ (mA/m$^2$)$^d$</th>
<th>$P$ (mW/m$^2$)$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC$^e$</td>
<td>$\infty$</td>
<td>-</td>
<td>-</td>
<td>-274±10</td>
<td>-</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>AR1</td>
<td>9800</td>
<td>1003</td>
<td>1200±28</td>
<td>-188±5</td>
<td>328±5</td>
<td>40.5±0.6</td>
<td>20.9±0.6</td>
</tr>
<tr>
<td>AR2</td>
<td>970</td>
<td>1003</td>
<td>1368±16</td>
<td>-76±3</td>
<td>160±4</td>
<td>187.5±0.9</td>
<td>44.3±0.4</td>
</tr>
<tr>
<td>AR3</td>
<td>150</td>
<td>1115</td>
<td>1398±43</td>
<td>-4±3</td>
<td>52±3</td>
<td>284.5±5.6</td>
<td>15.8±0.6</td>
</tr>
<tr>
<td>GC$^e$</td>
<td>$\infty$</td>
<td>-</td>
<td>-</td>
<td>-269±9</td>
<td>-</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>GR1</td>
<td>9800</td>
<td>1179</td>
<td>1449±18</td>
<td>-189±4</td>
<td>320±4</td>
<td>40.0±0.4</td>
<td>20.4±0.4</td>
</tr>
<tr>
<td>GR2</td>
<td>970</td>
<td>1195</td>
<td>1666±41</td>
<td>-70±3</td>
<td>142±4</td>
<td>168.1±1.7</td>
<td>35.6±0.7</td>
</tr>
<tr>
<td>GR3</td>
<td>150</td>
<td>1122</td>
<td>1445±33</td>
<td>-7±3</td>
<td>46±3</td>
<td>273.0±3.5</td>
<td>14.5±0.4</td>
</tr>
</tbody>
</table>

a. Ohmic resistance of abiotic MFCs measured by a potentiostat at open circuit

b. Internal resistance of MFCs measured from polarization curves

c. Anode potential ($E_{an}$) and cathode potential ($E_{cat}$) in the voltage plateau.

$E_x$ (vs. SHE) = 195 mV + $E_x$ (vs. Ag/AgCl), n = 8

d. Current/power density based on cathode (13 cm$^2$) because the anode area was variable due to anode sampling after each batch.

e. $R_{ext}$ is $\infty$ under open circuit during stages 2-3
Table 3-3. Average headspace gas compositions and electron flux distributions before and after changing external resistances.

<table>
<thead>
<tr>
<th>MFC</th>
<th>Headspace Gas Composition (µmol)</th>
<th>Electron flux (µeq/day) in stages 5-6</th>
<th>Electron distribution and energy efficiency (%) in stages 5-6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stage 2-3</td>
<td>Total</td>
<td>CE&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Stage 5-6</td>
<td>Electricity</td>
<td>Methane</td>
</tr>
<tr>
<td>AC&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1 ±2</td>
<td>143±17</td>
<td>0±0</td>
</tr>
<tr>
<td>AR1</td>
<td>28 ±6</td>
<td>193±58</td>
<td>43±2</td>
</tr>
<tr>
<td>AR2</td>
<td>30 ±8</td>
<td>347±20</td>
<td>207±3</td>
</tr>
<tr>
<td>AR3</td>
<td>13 ±3</td>
<td>481±4</td>
<td>327±6</td>
</tr>
<tr>
<td>GC&lt;sup&gt;f&lt;/sup&gt;</td>
<td>51 ±6</td>
<td>170±5</td>
<td>0±0</td>
</tr>
<tr>
<td>GR1</td>
<td>46 ±28</td>
<td>224±105</td>
<td>45±2</td>
</tr>
<tr>
<td>GR2</td>
<td>43 ±67</td>
<td>343±31</td>
<td>191±2</td>
</tr>
<tr>
<td>GR3</td>
<td>54 ±42</td>
<td>543±23</td>
<td>297±0</td>
</tr>
</tbody>
</table>

a. Coulombic efficiency

b. Biogas = methane and hydrogen.

c. Aerobic oxidation calculated based on oxygen transfer coefficient (1.45×10<sup>-4</sup> cm/s) through PEM (42)

d. The undefined electron sink = 1 – (CE + Biogas + Aerobic)

e. Energy efficiency

f. Control reactors were changed from open to closed circuit after stage 3

g. For AC and GC, values were taken from stages 2-3 when they were operated in open circuit (R<sub>ext</sub> = ∞)
Table 3-4. Phylogenetic identification of prominent DGGE bands.

<table>
<thead>
<tr>
<th>Band</th>
<th>GenBank closest isolate or enrichment-derived sequence</th>
<th>Class</th>
<th>Identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1/G1</td>
<td><em>Alistipes putredinis</em> (AJ518876)</td>
<td>Bacteroidetes</td>
<td>92</td>
</tr>
<tr>
<td>A2/G2</td>
<td><em>Geobacter sulfurreducens</em> (AE017180)</td>
<td>Deltaproteobacteria</td>
<td>98</td>
</tr>
<tr>
<td>A4/G4</td>
<td><em>Clostridium sticklandii</em> (M26494)</td>
<td>Clostridia</td>
<td>100</td>
</tr>
<tr>
<td>A5/G5</td>
<td><em>C. aminobutyricum</em> (X76161)</td>
<td>Clostridia</td>
<td>99</td>
</tr>
<tr>
<td>Aa</td>
<td><em>Pseudomonas aeruginosa</em> (EF650089)</td>
<td>Gammaproteobacteria</td>
<td>100</td>
</tr>
<tr>
<td>Ac</td>
<td>Bacterial enrichment (FJ802369)</td>
<td>n.a.</td>
<td>93</td>
</tr>
<tr>
<td>Ad</td>
<td><em>Acidovorax</em> sp. Ic3 (DQ421392)</td>
<td>Betaproteobacteria</td>
<td>100</td>
</tr>
<tr>
<td>Ae</td>
<td>Bacterial enrichment (EU082059)</td>
<td>n.a.</td>
<td>93</td>
</tr>
<tr>
<td>Af</td>
<td><em>G. sulfurreducens</em> (AE017180)</td>
<td>Deltaproteobacteria</td>
<td>96</td>
</tr>
<tr>
<td>Ag</td>
<td><em>Sphingobacterium faecium</em> (NR025537)</td>
<td>Sphingobacteria</td>
<td>88</td>
</tr>
<tr>
<td>Ah</td>
<td><em>G. sulfurreducens</em> (AE017180)</td>
<td>Deltaproteobacteria</td>
<td>96</td>
</tr>
<tr>
<td>Ai</td>
<td><em>Pelobacter propionicus</em> (CP000482)</td>
<td>Deltaproteobacteria</td>
<td>96</td>
</tr>
<tr>
<td>AJ</td>
<td><em>G. sulfurreducens</em> (AE017180)</td>
<td>Deltaproteobacteria</td>
<td>98</td>
</tr>
<tr>
<td>Ak</td>
<td><em>G. sulfurreducens</em> (AE017180)</td>
<td>Deltaproteobacteria</td>
<td>95</td>
</tr>
<tr>
<td>Ga</td>
<td><em>Enterococcus raffinosus</em> (FN600541)</td>
<td>Bacilli</td>
<td>99</td>
</tr>
<tr>
<td>Gb</td>
<td><em>Enterococcus raffinosus</em> (FN600541)</td>
<td>Bacilli</td>
<td>100</td>
</tr>
<tr>
<td>Gd</td>
<td><em>Enterococcus raffinosus</em> (FN600541)</td>
<td>Bacilli</td>
<td>99</td>
</tr>
<tr>
<td>Ge</td>
<td><em>Enterococcus raffinosus</em> (FN600541)</td>
<td>Bacilli</td>
<td>99</td>
</tr>
<tr>
<td>Gg</td>
<td><em>Alistipes putredinis</em> (AJ518876)</td>
<td>Bacteroidetes</td>
<td>93</td>
</tr>
<tr>
<td>Gh</td>
<td><em>Desulfovibrio desulfuricans</em> (FJ655841)</td>
<td>Deltaproteobacteria</td>
<td>97</td>
</tr>
<tr>
<td>Gi</td>
<td><em>Pelobacter propionicus</em> (CP000482)</td>
<td>Deltaproteobacteria</td>
<td>96</td>
</tr>
<tr>
<td>GJ</td>
<td><em>Desulfitomonas chloroethenica</em> (NR026012)</td>
<td>Deltaproteobacteria</td>
<td>91</td>
</tr>
</tbody>
</table>
Chapter 4

Impedance Characteristics and Polarization Behavior of an MFC in Response to Medium pH

Abstract

pH oppositely influences anode and cathode performance in microbial fuel cells. The differential electrochemical effects at each electrode and the resultant full-cell performance were analyzed in medium pH from 6.0 to 8.0. Potentials changed -60 mV/pH for the anode and -68 mV/pH for the cathode, coincident with thermodynamic estimations. Open circuit voltage reached a maximum (-517 mV) at pH 7, and maximum power density was highest (741 mW/m²) at pH 6.5 as the cathode performance improved at lower pH. Maximum current density increased and apparent half-saturation potential \(E_{KA}\) decreased with increasing medium pH due to improved anode performance. An equivalent circuit model composed of intracellular and extracellular processes accurately fit bioanode impedance data. Intracellular processes were the rate-limiting step for acetate-oxidizing exoelectrogenesis, with the pH-varying intracellular charge transfer resistance \(R_2\) ranging from 2 to 321 fold higher than the pH-independent extracellular charge transfer resistance \(R_I\). Intracellular capacitance \(C_2\) was two to three orders of magnitude larger than \(C_I\). \(R_2\) was lowest near \(E_{KA}\) and increased by several orders of magnitude at anode potentials above \(E_{KA}\), while \(R_I\) was nearly stable. However, fits
deviated slightly at potentials above $E_{KA}$ due to an emerging impedance possibly associated with diffusion and excessive potential.
4.1 Introduction

Microbial fuel cells (MFCs) differ from electrochemical fuel cells in that they use microbes to catalyze reactions on the anode (and on the cathode in biocathode designs) instead of an inorganic catalyst. This requirement for maintaining a viable biocatalyst necessitates physiologically permissive medium conditions. An improved understanding of the catalytic activity of the bioanode as a function of solution conditions would contribute to enhanced MFC operation and performance. However, the electrochemistry of the bioanode is complex due to changing microbial communities and interactions within these communities in the anode biofilm (38).

Electrochemical impedance spectroscopy (EIS) has been used to elucidate anodic electrochemical reactions in MFCs. Development of anode biofilms significantly decreased anodic polarization resistance (interchangeable with charge transfer resistance), indicating their catalytic role in electron transfer to the anode (7, 64, 67, 80). In a ferricyanide-cathode MFC, initial development of anode biofilm during the first five days decreased anodic polarization resistance by ~40% (from 2.6 to 1.5 kΩ cm² at 0.27 A/m²) with a simultaneous increase in power density by ca. 120% (80). In an air-cathode MFC, anodic charge transfer resistance decreased by ~75% (from 0.073 to 0.017 kΩ cm² at 2.63 A/m²) with increasing anodic capacitance during 70 days of long-term operation (7). To enable precise impedance analysis on extracellular electron transfer (exoelectrogenesis), pure cultures were also studied. Potentiostatic EIS on a *Geobacter sulfurreducens*-covered anode showed that charge transfer resistance was the lowest at the imposed
potential near the midpoint potential in cyclic voltammetry (67). In their impedance analysis, adding a second time constant through an additional circuit element did not improve data fitting (67). In a subsequent study using *Shewanella oneidensis* DSP10, three RC time constants were used to differentiate three charge transfer reactions (79).

One important controlling parameter for MFC performance is the medium pH. It was found that acidification of the anode biofilm affects current generation because microbial activity is inhibited in low pH (24, 27, 88). For example, *G. sulfurreducens* grown using fumarate as an electron acceptor had a considerably lower growth rate at pH 6 (0.04 h\(^{-1}\)) than pH 7 (0.21 h\(^{-1}\)) (24), and riboflavin production by *S. oneidensis* DSP10 was reduced significantly at pH 5 with appreciably decreasing power compared with neutral pH (4). It was demonstrated that alkaline medium and high buffer concentration enhances bioanode performance due to the increasing flux of proton shuttles out of the anode biofilm (23, 88). Increasing the pH from 7 to 9 resulted in a 42% improvement in maximum power density in a cloth-electrode assembly system (23). Maximum current density at pH 8 was 9.9 A/m\(^2\)-anode, and only ca. 25% of this current was attained at pH 6 in a single-chamber microbial electrolysis cell (MEC) (88). Anode biofilm acidification was experimentally substantiated by fluorescent detection of the pH gradient in a pure *G. sulfurreducens* biofilm (24). On the other hand, low pH enhances cathodic oxygen reduction, presumably due to increased availability of protons to participate in this reaction. It was observed that a lower pH created better performance in terms of current and power density in a granular-carbon cathode (25), and it was also observed in an upflow MFC that lower catholyte pH benefitted cathodic oxygen reduction (101). A 2.5-
fold increase of maximum power density was observed with a catholyte of pH 1 compared to that of pH 7.5 in a two-chamber MFC (21). Even in a biocathode, neutralizing the catholyte using strong acid was beneficial for current generation (51).

We focused on characterizing the tradeoff associated with differential performance effects of pH on each electrode, with acidic medium enhancing cathode performance but inhibiting bioanode performance. In varying pH conditions, impedance elements of the bioanode in a single-chamber MFC were characterized using a new equivalent circuit model. Furthermore, we characterized performance of each electrode and the whole cell using polarization and EIS behavior.

4.2 Materials and Methods

**MFC construction and operation.** A single-chamber bottle MFC (350 ml) with a carbon paper anode (3.14 cm$^2$ projected area, E-TEK) and an air cathode (7 cm$^2$ of Pt-coated area, 0.5 mg Pt cm$^{-2}$ Pt and four PTFE diffusion layers) was constructed as previously described (14, 54). A Nylon membrane filter with 0.22 µm pores (Magna nylon, N02SP04700) was used to cover the catalytic side of the cathode to prevent the formation of biofilm directly on the cathode. The small anode size and large medium volume allowed monitoring over long time periods without appreciable changes in the medium condition. A Ag/AgCl reference electrode was located 5 mm from the anode electrode. Anode biofilm enrichment was conducted in 50 mM phosphate-buffered GM medium (pH 7) (36). The MFC was inoculated with suspension from an electricity-producing MFC and operated with 10 mM sodium acetate (Sigma-Aldrich, MO) as an
electron donor under 600 Ω of external resistance ($R_{ext}$) at 30°C for 60 days for anode biofilm enrichment. Electrochemical tests were conducted upon observation of reproducible polarization behavior at a medium pH of 7. To characterize pH effects, GM media with pHs ranging from 6.0 to 8.0 at 0.5 pH unit increments were created with 5 M solutions of HCl or NaOH. The conductivities of these media were kept uniform at 8.9 mS/cm by adjusting with a 5 M solution of NaCl as needed.

**Electrochemical analysis.** Before each electrochemical measurement, the MFC was operated for 4 hours in the fresh medium condition, starting with a medium pH of 8.0 and decreasing incrementally to pH 6.0. Polarization tests, linear sweep voltammetry (LSV), and EIS were performed in separate batches for each pH condition. Between each pH change, the MFC was operated at pH 7 for 12 hours. Polarization curves were created by decreasing $R_{ext}$ from open circuit to 2 Ω and recording voltage after 30 min at each $R_{ext}$ using a multimeter (Keithley Instruments, OH). This test was done twice for each pH condition. LSV and EIS were performed using a potentiostat Reference 600 (Gamry Instrument Inc.). For LSV, the MFC circuit was disconnected for 2 hours to create open circuit potential (OCP) for the anode. Then, a scan rate of 0.1 mV/s with a 1-mV step size was applied to the anode over the potential range from OCP to -0.2 V. For potentiostatic EIS, the bioanode was first either poised at each test potential until it produced stable current or disconnected for 2 hr to attain OCP. Then EIS was performed at each DC potential with the following conditions: AC potential of 10 mV rms, initial frequency $10^6$ Hz, final frequency 10 mHz, 10 points/decade of data acquisition.
**Equivalent circuit model.** Our BIOANODE-1 model (Fig. 1) was composed of RC time constants representing two successive reactions of exoelectrogenic acetate oxidation based on the following hypotheses: (1) a low frequency arc (LFA) is associated with the intracellular processes (e.g., substrate oxidation and ATP/NADH generation), (2) a high frequency arc (HFA) is associated with extracellular electron transfer from a reduced intermediate to the solid anode surface, and (3) exoelectrogenic respiration is not limited by substrate diffusion due to the low current density of this MFC configuration. A constant phase element (CPE) was incorporated to model a non-ideal capacitor, defined as $1/Z = T(j\omega)^\alpha$, where $T (S^{\alpha})$ is a numerical value of the admittance ($1/|Z|$) at $\omega = 1$, $\alpha$ is an empirical nonideality constant, $j^2 = -1$, and $\omega (s^{-1})$ is the radial frequency ($\omega = 2\pi f$) (61). Capacitance ($C$) is calculated using $C = (TR_p)^{1/\alpha}/R_p$, where $R_p (\Omega)$ is the charge transfer resistance (61). Impedance spectra were fitted with the BIOANODE-1 model by $\chi^2$-minimization using Echem Analyst (Gamry Instrument Inc.).

**Calculations.** The theoretical potentials of the anode and cathode in a specific medium condition were calculated using the Nernst equation according to Eqs. 1 - 4 for the tested pH range at 303 K (30 °C).

$$2\text{HC}O_3^{-} + 9\text{H}^+ + 8\text{e}^- \leftrightarrow \text{CH}_3\text{COO}^- + 4\text{H}_2\text{O} \quad \text{(Eq. 1)}$$

$$E_{an,M} = E_{an}^0 - \frac{RT}{8F} \ln \left( \frac{[\text{HC}O_3^-]}{[\text{H}^+]^2} \right), \quad E^0 = 0.187 \quad \text{(Eq. 2)}$$

$$\text{O}_2 + 2\text{H}^+ + 2\text{e}^- \leftrightarrow \text{H}_2\text{O}_2 \quad \text{(Eq. 3)}$$

$$E_{cat,M} = E_{cat}^0 - \frac{RT}{2F} \ln \left( \frac{[\text{H}_2\text{O}_2]}{[\text{O}_2][\text{H}^+]^2} \right), \quad E^0 = 0.695 \quad \text{(Eq. 4)}$$
$E^\circ$ is the standard potential (at 298 K, 1 bar, 1 M, reported vs. standard hydrogen electrode (SHE)), $R$ is the ideal gas constant (8.314 J/Kmol), $T$ is temperature (K), and $F$ is the Faraday constant (96,485C/mol-eq). Potential values were reported with respect to Ag/AgCl using Ag/AgCl = SHE – 197 mV (2). Power density and current density were calculated using the apparent surface area of biofilm-covered anode (3.14 cm$^2$).

4.3 Results

**Polarization behavior.** Anode and cathode potentials were affected by the pH over the entire tested range of 6.0 – 8.0. As pH was lowered, both the anode potential ($E_{an}$) and the cathode potential ($E_{cat}$) increased (Fig. 4-2 and Table 4-1), indicating that anode performance deteriorated and cathode performance was enhanced as bulk pH decreased. The OCP was described with high accuracy using the Nernst equation (Fig. 4-2), assuming concentrations of 0.01 mM H$_2$O$_2$ at the cathode and 5 mM HCO$_3^-$ and 10 mM acetate in the solution at the anode. Potential changes per pH unit increase were -60 mV/pH for the anode and -68 mV/pH for the cathode. Open circuit voltage (OCV) reached a maximum at pH 7, but maximum power density ($P_{max}$) was highest at pH 6.5 (Table 4-1). Maximum current density increased and the midpoint potential (apparent half-saturation potential $E_{KA}$ derived from LSV curves) decreased as pH increased (Table 4-1, Fig. 4-3). At each medium pH, maximum current density was attained when $E_{an}$ was approximately 100 mV greater than $E_{KA}$. Anode polarization curves superimposed on LSV curves illustrated that both methods yielded comparable results (Fig. 4-3), except
that an $E_{an}$ range with a maximum current plateau was not observed using $R_{ext}$ alterations (i.e., the polarization curve data) in high pH conditions.

**Impedance parameters of the bioanode.** The BIOANODE-1 model generated excellent fits to the impedance data (Fig. 4-4). Goodness of fit ($87$) ranged from approximately $10^{-4}$ to $10^{-3}$. Fits deviated slightly from experimental data above $E_{KA}$ due to the emergence of a third arc at the lowest frequencies (Fig. 4-4). The low frequency arc (LFA) and this third arc enlarged as $E_{an}$ further increased above $E_{KA}$ and they finally combined into a single arc. However, goodness of fit was still in the same order. Because the third impedance arc was not considered in the BIOANODE-1 model, impedance spectra at potentials below $E_{KA}$ should be well-characterized using our model.

Medium pH did not affect $R_1$, but reducing medium pH increased $R_2$ (Fig. 4-5). At potentials below $E_{KA}$, $R_1$ averaged $11.3 \pm 0.8 \, \Omega$, and $R_2$ ranged from 2- to 321-fold higher than $R_1$. $C_2$ ($1.20 \times 10^{-2} \pm 0.47 \times 10^{-2} \, \text{F}$) was two or three orders of magnitude larger than $C_1$ ($5.89 \times 10^{-5} \pm 0.77 \times 10^{-5} \, \text{F}$). $\alpha_1$ and $\alpha_2$ were $0.80 \pm 0.03$ and $0.78 \pm 0.08$, respectively. Below $E_{KA}$, $C_2$ increased and $C_1$ decreased with increasing $E_{an}$. pH and $E_{an}$ did not affect ohmic resistance ($R_\Omega$, $12.4 \pm 0.8 \, \Omega$) because it is related to electrode resistance, medium conductivity, and proximity of the reference electrode to the working electrode, all of which were constant throughout these experiments.

The presence of the third arc significantly affected fitting as indicated by the $C_2$ surge at potentials above $E_{KA}$ (Fig. 4-5). The nonideality constant $\alpha_2$ fluctuated with $E_{an}$ variations, but $\alpha_1$ did not. Over the entire potential range, $\alpha_1$ averaged $0.82 \pm 0.04$ and $\alpha_2$ was $0.73 \pm 0.12$. In the overall potential range, $R_2$ ($1201.8 \pm 2552.4 \, \Omega$) was two orders of
magnitude greater than \( R_1 \) (10.8 ± 1.1 \( \Omega \)) (Fig. 4-5). \( R_2 \) was lowest near the \( E_{KA} \) and increased two or three orders of magnitude above \( E_{KA} \), while \( R_1 \) decreased by only a few ohms as \( E_{aa} \) increased (Fig. 4-5). Though impedance parameters generated from current-producing potentials had a consistent trend, those at OCP showed neither any consistency with the medium pH changes nor any correlation with the values attained from current-generating potentials (Table 4-1).

An abiotic control anode had an OCP of 510 mV at pH 7. The Nyquist plot of the abiotic anode (not shown) consisted of two arcs. Impedance parameters of the abiotic anode at open circuit and pH 7 were 13.5 \( \Omega \) for \( R_1 \), 4.3 \( \times \) 10\(^{-5} \) F for \( C_1 \), 0.88 for \( \alpha_1 \), 8.0 \( \times \) 10\(^7 \) \( \Omega \) for \( R_2 \), 6.0 \( \times \) 10\(^{-4} \) F for \( C_2 \), and 0.99 for \( \alpha_2 \). \( R_1 \) and \( C_1 \) in the abiotic anode were slightly higher than the bioanode, but \( R_2 \) was four orders of magnitude larger and \( C_2 \) was two orders of magnitude larger.

**4.4 Discussion**

Sequential reactions of microbial substrate oxidation and electron transfer via riboflavin were modeled in a previous equivalent circuit (EC) with three time constants (79). However, this EC did not generate accurate fitting to our data. An EC for hydrogen oxidation in the anode of a PEM fuel cell (16) was used in this experiment. It consists of two processes, the electrosorption of hydrogen (\( H_2 \leftrightarrow 2H_a \)) and the electron transfer from hydrogen onto the anode interface (\( H_a \leftrightarrow H^+ + e^- \)) (16). The hydrogen electrosorption was replaced conceptually by intracellular processes (substrate oxidation and reducing equivalent generation) in the BIOANODE-1 model because these reactions precede
charge transfer on the electrode surface (Fig. 4-1). Assuming this attribution of EC components is correct, the larger resistance of $R_2$ than $R_1$ demonstrated that the intracellular processes are rate limiting, which is consistent with the previous suggestion that the rate of electron delivery to the outer membrane proteins remains rate limiting compared to the rate of electron transfer to electrodes (68). Increasing $E_{an}$ lowered both HF and LF impedance in this study, showing that increasing $E_{an}$ facilitated the overall kinetics of exoelectrogenic electron transfer to the solid anode surface at potentials below $E_{KA}$. However, current did not proportionally increase with increasing $E_{an}$ above $E_{KA}$.

The pH gradient between the anode biofilm and the medium controls the diffusion of protonated buffer species out of the biofilm, so that low medium pH limits current density (88). Our results showed that $R_2$ was greatly affected by pH, while $R_1$ was insensitive to pH change (Fig. 4-5). This is consistent with the hypothesis that the LFA characterized by $R_2$ is related to intracellular processes because microbial processes are sensitive to pH, even without the need for extracellular electron transfer. If the conceptual description of the EC model is correct, this would suggest insensitivity to pH change of the prevailing charge transfer reaction(s) in this system.

Diffusion of protonated species from the anode biofilm may be related to the third arc detected in the low frequency domain (Fig. 4-4). Protons are produced during microbial substrate oxidation, which combine with buffer species such as carbonate or phosphate. Proton diffusion can be modeled by a Warburg element in equivalent circuits, usually shown as a straight line in the low frequency domain of a Nyquist plot. Pure diffusion creates a 45° line, but other factors such as capacitance influence the angle and
curvature of the Warburg element. For example, mass transport processes for a porous gas diffusion electrode usually give a semicircle rather than a straight line in a Nyquist arc due to finite diffusion (97, 100). In PEM fuel cell systems, the main contributor to the overall impedance in low overpotential is normally charge transfer resistance ($R_p$), which is dependent on potential according to the Tafel equation $V \sim \log R_p^{-1}$ (100), but diffusion resistance (mass transport resistance) becomes dominant in the overall impedance in high overpotential (100). In our data, the third arc became a significant portion of total impedance at high current density in the saturating $E_{an}$ region, appearing at potentials above $E_{KA}$ and finally combining with the LFA, creating a semicircle (Fig. 4-4).

Considering the evidences demonstrating that the LFA indeed represents the intracellular processes, increasing LFA impedance possibly indicates damage of the anode biofilm due to slow diffusion of protonated species at the higher current densities associated with these $E_{an}$ or possibly due to additional energy from the potentiostat. A two-chamber MFC did not have such an increase of impedance because of its low maximum current density (ca. 0.3 A/m²-anode) (36, 80). In a previous study, the lowest charge transfer resistance (0.408 kΩ cm²) was measured at an applied potential near the $E_{KA}$ of -355 mV (-158 mV vs. SHE) for $G. sulfurreducens$ (67). Our results also demonstrate charge transfer resistance was lowest near $E_{KA}$ at each pH condition. We showed that medium pH affected $E_{KA}$, which has been regarded as an intrinsic constant of an anode biofilm (65). These results imply that proton diffusion affect $E_{KA}$.

Extracellular impedance ($R_I$, average ~10 Ω) was minor compared with intracellular impedance ($R_2$, average ~400 Ω). At the maximum current density of our
anode biofilm (6.8 A/m²), extracellular and intracellular processes created ~20 mV and ~260 mV of potential loss, respectively (3.14 cm² of anode area × 6.8 A/m² × impedance Ω at 6.8 A/m²). Future research should focus on engineering the exoelectrogenic microbial processes to minimize this significant intracellular overpotential. In addition, extracellular processes in the bioanode could be further minimized by a novel anode material which allows enhanced contact between conductive microbial pili and anode surface, though our analyses suggest that this is not the main contributor to potential loss at the anode.

$C_2$ was several orders of magnitude greater than $C_1$, demonstrating higher charge storage in the LFA process than the HFA process, again supporting the interpretation that the LFA represents intracellular processes that might store more charge than extracellular charge transfer processes. The CPE is attributed to heterogeneous electrode properties or reactions (e.g., surface roughness, nonhomogeneous reaction rates on a surface, or variation of catalyst thickness) (3). The LFA had a higher $\alpha$ (0.82) than that of the HFA (0.73), but it is unknown whether this difference in nonideality is significant.

Anode performance was enhanced as pH became higher. This result was not consistent with a previous study using open circuit EIS, which showed the bioanode process preferred a neutral pH (30). Perhaps open circuit EIS might not be a representative way to evaluate electrode impedance in MFCs (Table 4-2). Because anode catalysts are living microorganisms, the unavailability of an electron acceptor at open circuit can create unrepresentative impedance values. Current production (microbial respiration) might be allowed to characterize living microbial catalysts. Even in hydrogen
fuel cells, the impedance data at current-generating potentials were suggested to be more representative of overall electrode processes than that at OCV with an exception that EIS of a symmetrical gas-feeding can be measured only at OCV (100).

Alkaline electrolyte fuel cells has less cathodic activation overpotential than PEM fuel cells with acidic electrolyte system (69). In an alkaline electrolyte system, hydroxide ($O_2 + 4e^- + 2H_2O \leftrightarrow 4OH^-$) generated at the cathode is utilized for the anodic hydrogen oxidation ($2H_2 + 4OH^- \leftrightarrow 4H_2O + 4e^-$), where protons are not involved in the electrochemical reaction (69). In previous MFC study, charge transfer resistance of the cathode decreased in high pH condition (30), suggesting that their system might be alkaline electrolyte system. On the other hand, in our result, high proton availability in low electrolyte pH lead to increasing cathodic potential, showing cathodic performance increased. Our thermodynamic estimations suggested that protons instead of hydroxide are involved in electrochemical reactions in our MFC system. This result also indicates that our MFC system is based on an acidic electrolyte system.

Although an acidic medium is beneficial for cathodic oxygen reduction, it decreases anodic substrate oxidation. High anode surface area, buffer concentration, or medium pH can prevent the acidification of anode biofilm because diffusion of protonated species is proportional to diffusive surface area, concentration of diffusive species, or pH gradient. Acidotolerant exoelectrogens might be another solution to pH inhibition. A strain of *Acidiphilium* sp. was able to produce up to $3 \text{ A/m}^2$-anode at ca. 390 mV in aerobic low-pH medium, which leads to a low ohmic resistance configuration due
to shorter distance between anode and air-cathode and high cathode performance due to high proton concentration (63).

The BIOANODE-1 model created a nice fit to the experimental data as indicated by goodness of fit, even in the presence of a third arc. Addition of a CPE component allows the arc to elongate and match data, even though the real physical process may not be understood (61). In our results, the third arc was not discretely modeled, but rather was fitted with the LFA charge transfer resistance. A Finite Warburg element was added to our model, but it did not generate a well-matched fitting (data not shown). For future study, the BIOANODE-1 model can be improved by considering diffusion resistance in a high-flux system or excessive electrical energy in EC modeling.

4.5 Acknowledgements

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**Figure 4-1.** A schematic depicting exoelectrogenic macroprocesses (A). Extracellular electron transfer (the red dotted line) can occur via conductive wire, redox mediator, or redox enzyme contacting the anode. The pH gradient between the bulk medium and the anode biofilm (shown here without a pH gradient in the biofilm) affects the driving force of diffusive ionic movement, as indicated by arrows. The proposed equivalent circuit, BIOANODE-1, models exoelectrogenic acetate oxidation occurring in the bioanode (B).
Figure 4-2. Averages and standard deviations of anode potential ($E_{an}$, circles) and cathode potential ($E_{cat}$, diamonds) measured at different external resistances in medium pHs ranging from 6.0 to 8.0 (n=2). Thick solid lines are theoretical potentials of the anode (bottom) and cathode (top) at approximate experimental conditions (cathode: $[\text{H}_2\text{O}_2] = 0.01 \text{ mM and } [\text{O}_2] = 0.2 \text{ atm}$, anode: $[\text{CH}_3\text{COO}^-] = 10 \text{ mM and } [\text{HCO}_3^-] = 5 \text{ mM, 303 K (30 °C)}$), and thick broken lines are standard potentials at 1 M or 1 atm of chemical species except for varying pH.
Figure 4-3. Linear sweep voltammetry (LSV, scan rate 0.1 mV/s) curves superimposed with anode polarization curves. Vertical lines are the theoretical open circuit potentials of the bioanode at a given pH calculated using the Nernst equation. Yellow circles designate midpoint potential ($E_{KA}$) values.
Figure 4-4. Bioanode impedance plots at $E_{\text{an}}$ of -250, -300, -350, -425, and -450 mV in medium of pH 7. Points are experimental data, lines are fitted curves using the BIOANODE-1 equivalent circuit. A - Nyquist plot, B - bode plot. High frequency arc (HFA) models terminal electron transfer from cells to the anode surface and low frequency arc (LFA) models intracellular catabolic processes.
Figure 4-5. Parameter values from fittings of impedance data with the equivalent circuit BIOANODE-1. The lowest $E_{an}$ for EIS was the OCP.
Table 4-1. Electrochemical data in each medium pH from polarization curves. $E_{KA}$ and $j_{max}$ were calculated from LSV curves.

<table>
<thead>
<tr>
<th>pH</th>
<th>6.0</th>
<th>6.5</th>
<th>7.0</th>
<th>7.5</th>
<th>8.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anode OCP (mV)</td>
<td>-438</td>
<td>-482</td>
<td>-517</td>
<td>-539</td>
<td>-555</td>
</tr>
<tr>
<td>Cathode OCP (mV)</td>
<td>288</td>
<td>251</td>
<td>224</td>
<td>170</td>
<td>145</td>
</tr>
<tr>
<td>OCV (mV)</td>
<td>725</td>
<td>733</td>
<td>741</td>
<td>709</td>
<td>700</td>
</tr>
<tr>
<td>$P_{max}$ (mW/m$^2$)</td>
<td>495</td>
<td>712</td>
<td>687</td>
<td>654</td>
<td>636</td>
</tr>
<tr>
<td>$j_{max}$ (A/m$^2$)</td>
<td>1.85</td>
<td>4.69</td>
<td>5.01</td>
<td>5.05</td>
<td>6.15</td>
</tr>
<tr>
<td>$E_{KA}$ (mV)</td>
<td>-352</td>
<td>-375</td>
<td>-416</td>
<td>-440</td>
<td>-431</td>
</tr>
</tbody>
</table>
Table 4-2. EIS parameter analysis on bioanode impedance at the open circuit potential (top half of table) and an $E_{an}$ of -400 mV.

<table>
<thead>
<tr>
<th>$E_{an}$ (mV)</th>
<th>pH 6.0</th>
<th>pH 6.5</th>
<th>pH 7.0</th>
<th>pH 7.5</th>
<th>pH 8.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>$I$ (A/m$^2$)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>$R_{\Omega}$ (Ω)</td>
<td>10.88</td>
<td>13.16</td>
<td>11.84</td>
<td>12.88</td>
<td>13.3</td>
</tr>
<tr>
<td>$R_1$ (Ω)</td>
<td>11.61</td>
<td>12.19</td>
<td>11.76</td>
<td>12.45</td>
<td>12.54</td>
</tr>
<tr>
<td>$C_1$ (F)</td>
<td>7.8×10$^{-5}$</td>
<td>6.3×10$^{-5}$</td>
<td>6.6×10$^{-5}$</td>
<td>6.6×10$^{-5}$</td>
<td>6.7×10$^{-5}$</td>
</tr>
<tr>
<td>$\alpha_1$</td>
<td>0.74</td>
<td>0.78</td>
<td>0.75</td>
<td>0.80</td>
<td>0.77</td>
</tr>
<tr>
<td>$R_2$ (Ω)</td>
<td>1550</td>
<td>856</td>
<td>4037</td>
<td>1121</td>
<td>2575</td>
</tr>
<tr>
<td>$C_2$ (F)</td>
<td>1.5×10$^{-2}$</td>
<td>1.6×10$^{-2}$</td>
<td>1.6×10$^{-2}$</td>
<td>1.5×10$^{-2}$</td>
<td>1.3×10$^{-2}$</td>
</tr>
<tr>
<td>$\alpha_2$</td>
<td>0.90</td>
<td>0.81</td>
<td>0.82</td>
<td>0.78</td>
<td>0.82</td>
</tr>
<tr>
<td>Goodness of fit</td>
<td>1.6×10$^{-3}$</td>
<td>5.3×10$^{-4}$</td>
<td>4.5×10$^{-4}$</td>
<td>4.3×10$^{-4}$</td>
<td>3.3×10$^{-4}$</td>
</tr>
</tbody>
</table>

| $E_{an}$ (mV) | -400   | -400   | -400   | -400   | -400   |
| $I$ (A/m$^2$) | 0.43   | 1.94   | 2.8    | 3.75   | 4.34   |
| $R_{\Omega}$ (Ω) | 10.86 | 13.08  | 12.26  | 12.91  | 12.39  |
| $R_1$ (Ω)    | 10.64  | 10.66  | 10.95  | 10.82  | 10.75  |
| $C_1$ (F)    | 6.8×10$^{-5}$ | 5.1×10$^{-5}$ | 5.2×10$^{-5}$ | 5.2×10$^{-5}$ | 5.7×10$^{-5}$ |
| $\alpha_1$  | 0.77   | 0.84   | 0.79   | 0.85   | 0.82   |
| $R_2$ (Ω)    | 156.1  | 32.22  | 35.8   | 20.63  | 19.2   |
| $C_2$ (F)    | 1.3×10$^{-2}$ | 1.1×10$^{-2}$ | 1.0×10$^{-2}$ | 1.1×10$^{-2}$ | 1.0×10$^{-2}$ |
| $\alpha_2$  | 0.87   | 0.74   | 0.81   | 0.64   | 0.68   |
| Goodness of fit | 4.8×10$^{-4}$ | 5.1×10$^{-4}$ | 2.7×10$^{-4}$ | 6.0×10$^{-4}$ | 5.3×10$^{-4}$ |
Chapter 5

Impedance Analysis on *Geobacter sulfurreducens* PCA, *Shewanella oneidensis* MR-1, and Their Coculture in Bioelectrochemical Systems

Abstract

The impedance signatures of duplicate defined-culture bioanodes enriched with *Geobacter sulfurreducens* PCA, *Shewanella oneidensis* MR-1, or their coculture were analyzed using an equivalent circuit model BIOANODE-1 composed of a low-frequency reaction ($R_2$ and $C_2$) and a high-frequency reaction ($R_1$ and $C_1$). The PCA anodes had lower impedance, higher capacitance, and higher current production than the MR-1 anodes. In both the PCA anodes and the MR-1 anodes, $R_2$ was a few orders of magnitude larger than $R_1$, showing that the $R_2$ process was rate limiting. Total capacitance values of both cases were dominated by $C_2$ ranging from 3 to 10 mF, whereas $C_1$ ranged from $10^{-3}$ to $10^2$ mF. Binary extracellular electron transfers could be expected to increase current production due to electron transfers contributions from both populations, but the coculture anodes produced lower current and higher bioanode impedance, probably due to substrate limitation associated with MR-1 converting lactate into acetate. Different morphologies of HF arcs were created among the three defined cultures. This finding might be extended to allow electrochemical identification of exoelectrogenic pathways.
5.1 Introduction

The anode biofilm is the key electrochemical catalyst in microbial fuel cells (MFCs) (55, 57). Electrochemical impedance spectroscopy (EIS) is increasingly used to analyze exoelectrogenic processes of the anode biofilm because EIS is a nondestructive method and generates a quantitative characterization of reactions (34). EIS characterization of anode biofilm properties has included both pure-culture experiments (6, 66, 67) and mixed-culture experiments (5, 31, 36, 37, 57).

Early EIS studies of bioanodes used one time constant equivalent circuit models with one charge transfer resistance and capacitance (7, 64, 67, 80). With a *Geobacter sulfurreducens*-colonized anode, a two time constant model was tested but did not improve EIS fitting (67). Subsequent efforts have focused on improved identification and characterization of each impedance component in detail. A three time constant model consisting of three sets of charge transfer and capacitance in series was used to identify reactions with a *Shewanella oneidensis* DSP10 anode (79). This model was developed to describe redox mediator reactions, microbial substrate oxidation, and other redox reactions. However, only resistance components were reported without the values of capacitance and nonideality constants (79). In a recent study of pH effects on the bioanode process, resistance, capacitance, and nonideality constants in two reactions were successfully differentiated and quantified in the equivalent circuit model BIOANODE-1 (34). This model has two time constants to differentiate intracellular processes (for example, substrate oxidation and reducing equivalent production) and
extracellular electron transfer processes, and it generated highly accurate EIS fitting for a mixed-culture bioanode in different medium pHs (34).

The two model exoelectrogens *G. sulfurreducens* and *S. oneidensis* have very different electrochemical physiologies. Conductive pili are the dominant extracellular electron transfer pathway for *G. sulfurreducens*, whereas endogenous electron shuttles are predominant for *S. oneidensis* (66). While *G. sulfurreducens* prefers to use acetate as an electron donor in anaerobic conditions, lactate is a favored electron donor for *S. oneidensis*. In addition, *G. sulfurreducens* lives mainly on the anode electrode in MFCs, but *S. oneidensis* favors growth in suspension. To understand exoelectrogenic processes better, the electrochemistry of these two different exoelectrogens was characterized. Duplicate anode electrodes were enriched with *G. sulfurreducens*, *S. oneidensis*, or their coculture. We successfully tested impedance parameters for each culture condition.

5.2 Materials and Methods

**Bacterial strains and culture media.** *G. sulfurreducens* strain PCA (ATCC 51573) was purchased from ATCC and *S. oneidensis* strain MR-1 was donated from Dr. Daniel Bond’s group (University of Minnesota). Growth medium for suspended cultures contained 0.6 g/L of Na$_2$HPO$_4$, 1.5 g/L of NH$_4$Cl, 0.1 g/L of KCl, 10 ml/L of trace elements, 8 g/L (50 mM) of sodium fumarate as the electron acceptor (45, 58, 59), and 10 ml/L amino acids (22 mg/L of L-arginine, 22 mg/L of L-glutamine, and 44 mg/L of DL-serine). Acetic acid (10 mM) was added as the electron donor for PCA growth, and lactic
acid (10 mM) was added for MR-1 growth. Because MR-1 does not grow without amino acids or yeast extract (59), amino acids were added in growth medium.

**Reactor construction.** Duplicate two-chamber fuel cells were constructed by joining chambers (borosilicate glass, 250 ml capacity, Penn State University) with glass tubing (7 cm$^2$ of exposed area to the medium) as previously described (36). The ends of each tube were glued with silicon (Loctite Superflex Cat. 59530, CA), attached with an anion exchange membrane (AMI-7001, Membrane International Inc., NJ), and assembled with a pinch clamp. Anode bottles, including a sidearm sampling port to facilitate suspension sampling, were sealed with a butyl rubber stopper clamped with an aluminum cap to prevent gas exchange. Anode electrodes (2.5 cm×6 cm) were made of carbon cloth (BASF Fuel Cell Inc., Somerset, NJ; CC-A) and cathode electrodes (2.5×6 cm) were made by applying platinum (0.5 mg/cm$^2$ Pt) and four diffusion layers on a 30 wt % wet-proofed carbon cloth (type B-1B, E-TEK) as previously described (14).

**MFC operation.** Acetic acid (10 mM) was added as the electron donor for PCA, and lactic acid (10 mM) was added for MR-1 and for the coculture. For the anode medium, the suspended-growth medium was used except the 50 mM of fumarate was replaced with NaCl (2.9 g/L, 50 mM) to maintain osmolarity and conductivity. Medium was adjusted to pH 6.8 using 1 N HCl or 1N NaOH. Then, 2.5 g of NaHCO$_3$ was added, and medium was flushed with N$_2$-CO$_2$ (80:20) to remove oxygen before autoclaving in sealed bottles. Filter-sterilized vitamin solution (10 ml/L) was added after medium had cooled (58). Final medium pH was 6.8. Catholyte contained 0.6 g Na$_2$HPO$_4$, 1.5 g of NH$_4$Cl, 0.1 g of KCl, 2.9 g/L of NaCl, and 2.5 g of NaHCO$_3$. All cultivation was done at
30 °C. The anode chamber contained 120 mL of medium and the cathode chamber was filled with 220 ml of catholyte. Cells (50 ml) grown in fumarate as the electron acceptor were centrifuged (× 9000 cfg) for 15 min at 5 °C, resuspended in 10 ml of the medium, and inoculated into the anode chamber. For the coculture reactors, 50-ml cells for each strain was centrifuged and resuspended in 5-ml medium. Circuits were connected through 460 Ω of external resistance between anode and cathode. The cathode chamber was provided with air passed through a 0.45-µm-pore-size filter for cathodic oxygen reduction. The reactors were operated for ~ 150 hours for bacterial acclimation in the first batch and electrochemical measurement was performed in the second batch as described below.

**Electrochemical measurement.** Cyclic voltammetry (CV) and EIS were performed using a potentiostat Reference 600 (Gamry Instrument Inc.). Before CV and EIS measurements, the reactors were operated for 2 hours to stabilize the bioanode in the fresh medium condition at -0.2 V vs. Ag/AgCl of poised anode potential. The circuit was disconnected for 30 min to create open circuit potential, and CV was performed with the following conditions: scan rate 1 mV/sec, step size 1 mV, Auto I/E Range, Scan limits -0.6 and 0.1 V. For potentiostatic EIS, the anode electrodes were poised at -0.4 V for 30 min and EIS was performed with following conditions: AC voltage 10 mV rms, initial frequency $10^3$ kHz, final frequency 50 mHz, 10 points/decade, and -0.4 V of DC voltage.

**Equivalent circuit (EC) model.** The BIOANODE-1 model was composed of RC time constants representing two successive reactions of exoelectrogenic electron donor oxidation based on the following hypotheses (35): (1) a low frequency arc (LFA) is
associated with substrate oxidation and reduced intermediate generation, (2) a high frequency arc (HFA) is associated with electron transfer from a reduced intermediate to the solid anode surface. Impedance spectra were fitted into the BIOANODE-1 model by $\chi^2$-minimization using Echem Analyst (Gamry Instrument Inc.). A constant phase element (CPE) was incorporated to model a non-ideal capacitor, defined as $1/Z = T(j\omega)^\alpha$, where $T (\text{s}^{\alpha})$ is a numerical value of the admittance ($1/|Z|$) at $\omega = 1$, $\alpha$ is an empirical nonideality constant, and $\omega (\text{s}^{-1})$ is the radial frequency ($\omega = 2\pi f$) (61). Capacitance ($C$) was calculated using $C = (TR_p)^{1/\alpha}/R_p$, where $R_p (\Omega)$ is the charge transfer resistance (or polarization resistance) (61). Potential values were reported with respect to the Ag/AgCl reference electrode.

5.3 Results and Discussion

All reactors produced current immediately as circuits were connected through 460-$\Omega$ resistance, but their times to reach stabilized voltage generation were different. Current developed rapidly in the PCA reactors, followed by the coculture reactors and the MR-1 reactors. The duplicate PCA MFCs reached a stabilized cell voltage (290 ± 10 mV) and anode potential (-338 ± 8 mV) in 24 hours. The coculture MFCs attained a stabilized voltage (200 ± 29 mV) and anode potential (-277 ± 62 mV) in 120 hours. The MR-1 reactors reached a stabilized voltage (85 ± 4 mV) and anode potential (-40 ± 5 mV) in 160 hours.

CVs showed that the PCA anodes generated 2.72 ± 0.54 mA of maximum current at -299 ± 7 mV of anode potential and maximum current for MR-1 in the tested potential
range was $0.12 \pm 0.07$ mA (Fig. 5-1B). Binary extracellular electron transfer mechanisms in the coculture reactors was expected to increase current production, but the coculture anodes produced lower current, probably due to substrate limitation. Because acetate supply to PCA can be limited by lactate oxidation of MR-1 in these experiments, the lower activity of MR-1 might be a bottle neck in the overall kinetics of the coculture systems (Fig. 5-1 C). It was reported that PCA cannot utilize lactate (9), but recent results in our lab showed that PCA can utilize lactate after long acclimation time (unpublished result).

Impedance of the bioanodes was measured at -400 mV of potential. This anode potential was used because impedance parameters derived from data collected at open circuit potential can produce misleading results in bioanode studies (34), and the small current associated with this potential allows identical measurement conditions for the exoelectrogens that vary in their current production capacities. In our equivalent circuit model, the LFA is associated with bacterial generation of reduced intermediates, and the HFA is associated with electron transfer from the reduced intermediates to the anode. In both the PCA anodes and the MR-1 anodes, $R_2$ was a few orders of magnitude larger than $R_1$, showing that the $R_2$ process was rate limiting. Total capacitance values were dominated by $C_2$ (from 3 to 10 mF), whereas $C_1$ ranged from $10^{-3}$ to $10^{-2}$ mF. The HF reaction showed more ideal capacitance behavior than the LF reaction, as indicated by $\alpha_2$ and $\alpha_1$. Higher $R_2$, $C_2$, and $\alpha_2$ in LF in PCA and MR-1 anodes was also observed in mixed-culture bioanodes (34), suggesting that this might be a general impedance feature of the bioanode.
Total charge transfer resistance of the PCA anodes was from 26- to 44-fold lower than for the MR-1 anodes, indicating that PCA relieves electron transfer resistance better than MR-1. Exoelectrogenic transfer processes of PCA conclusively lowered $R_2$ and $R_2$ than MR-1. There are many steps involved in exoelectrogenesis of MR-1, such as riboflavin diffusion through the cell membrane, their oxidation and reduction, and their diffusion through the medium. This complex process might induce energetic loss and create much higher resistance of MR-1 than PCA. Total capacitance of the PCA anodes was ~2.5 fold higher than for the MR-1 anodes, indicating that the PCA anodes had higher capability of charge storage. Our results indicate that PCA has higher electrogenic activity and harbor efficient exoelectrogenic machinery than MR-1. Previous investigation also showed that PCA had 10-folder higher current density (3 A/m$^2$) than MR-1 (0.3 A/m$^2$) at ~0 mV of anode potential (66, 67).

Different morphologies of HF arcs were created among the three culture conditions. In particular, Nyquist plots of the coculture anode had a unique HFA consisting of two semi-arcs (Fig. 5-2 C). The MR-1 anodes had larger HF arcs than those of the PCA anodes, and HF arcs of the coculture anodes consisted of two small arcs. However, HF arcs in the coculture anodes were smaller than the MR-1 anodes. Considering different exoelectrogenic electron transfers in three bioanodes, this finding suggests that EIS measurement can be used as a method for electrochemical identification of exoelectrogenic pathways.
5.4 Acknowledgements

This research was supported by National Science Foundation Grant CBET-0834033. The authors thank Dr. Daniel Bond (Associate Professor, Department of Microbiology and Biotechnology Institute, University of Minnesota) for sharing his experience on bacterial physiology and donating strain MR-1.
Figure 5-1. CVs of PCA (A), MR-1 (B), and the coculture (PCA+MR-1) (C). Open and closed symbols represent duplicate reactors.
Figure 5-2. Nyquist plots of anode impedance spectra from PCA (A), MR-1 (B), the coculture (PCA+MR-1) (C), and their composite (D). Impedance spectra were measured at -0.40 V of DC anode potential. Duplicate reactors were operated and representative spectra were shown here. Open and closed symbols represent duplicate reactors. Impedance parameters are reported in Table 5-1.
Figure 5-3. Bode plots of PCA (A), MR-1 (B), the coculture (PCA+MR-1) (C), and their composite (D). Impedance spectra were measured at -0.40 V of DC anode potential. Open and closed symbols represent duplicate reactors. Symbols with the black border indicate phase angle shifts (Y2 – Zphz °).
Table 5-1. Impedance parameters of bioanodes inoculated with *G. sulfurreducens* PCA, *S. oneidensis* MR-1, or their coculture. Impedance spectra were measured at -400 mV of DC anode potential (n=2). Impedance (*R*), capacitance (*C*), nonideality constant (*α*), $\sum R = R_1 + R_2$, $\sum C = C_1 + C_2$.

<table>
<thead>
<tr>
<th></th>
<th>PCA-1</th>
<th>PCA-2</th>
<th>MR-1-1</th>
<th>MR-1-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R_1$ (Ω)</td>
<td>36.1 ± 1.7</td>
<td>35.0 ± 5.8</td>
<td>319.6 ± 4.7</td>
<td>323.9 ± 4.9</td>
</tr>
<tr>
<td>$R_2$ (Ω)</td>
<td>120.0 ± 15.2</td>
<td>192.4 ± 50.1</td>
<td>6,555 ± 4,045</td>
<td>5,997 ± 4,443</td>
</tr>
<tr>
<td>$\sum R$ (Ω)</td>
<td>156.0 ± 16.9</td>
<td>227.3 ± 44.4</td>
<td>6,875 ± 4,040</td>
<td>6,321 ± 4,439</td>
</tr>
<tr>
<td>$C_1$ (mF)</td>
<td>$1.3 \times 10^{-3} ± 0.01 \times 10^{-3}$</td>
<td>$5.0 \times 10^{-3} ± 0.4 \times 10^{-3}$</td>
<td>$2.3 \times 10^{-2} ± 0.3 \times 10^{-2}$</td>
<td>$2.7 \times 10^{-2} ± 0.4 \times 10^{-2}$</td>
</tr>
<tr>
<td>$C_2$ (mF)</td>
<td>9.08 ± 1.51</td>
<td>9.30 ± 1.76</td>
<td>3.42 ± 0.02</td>
<td>3.25 ± 0.01</td>
</tr>
<tr>
<td>$\sum C$ (mF)</td>
<td>9.08 ± 1.51</td>
<td>9.31 ± 1.76</td>
<td>3.44 ± 0.01</td>
<td>3.28 ± 0.01</td>
</tr>
<tr>
<td>$\alpha_1$</td>
<td>0.57 ± 0.01</td>
<td>0.75 ± 0.01</td>
<td>0.73 ± 0.01</td>
<td>0.80 ± 0.00</td>
</tr>
<tr>
<td>$\alpha_2$</td>
<td>0.91 ± 0.03</td>
<td>0.85 ± 0.01</td>
<td>0.96 ± 0.01</td>
<td>0.94 ± 0.01</td>
</tr>
<tr>
<td>Goodness of fit</td>
<td>$2.6 \times 10^{-5} ± 0.6 \times 10^{-5}$</td>
<td>$2.0 \times 10^{-3} ± 0.4 \times 10^{-3}$</td>
<td>$4.3 \times 10^{-4} ± 0.4 \times 10^{-4}$</td>
<td>$4.3 \times 10^{-4} ± 0.3 \times 10^{-4}$</td>
</tr>
</tbody>
</table>
Chapter 6

Impedance and Thermodynamic Analysis of Biotic, Abiotic, and Mediator-Amended Anode Electrodes in MFCs

Abstract

Impedance and thermodynamics were evaluated on biotic, abiotic, and riboflavin-amended anode electrodes for characterization of exoelectrogenic processes. Activation overpotential was insignificant in the bioanode compared with the abiotic anode. Anodic polarization behaviors and impedance spectra were almost identical in the absence and the presence of acetate. The bioanode had lower charge transfer resistance and higher capacitance for the low-frequency reaction than the abiotic anode. The impedance characteristics for the high-frequency reaction were relatively similar in the abiotic anode and the bioanode. As anode potential increased, total resistance in the bioanode rapidly increased without any increase of current production, whereas it reduced as current increased in the abiotic anode. At open circuit, the bioanode had a high-frequency arc in Nyquist plot, and the abiotic anode did not have one.
6.1 Introduction

Electrochemical impedance spectroscopy (EIS) is an attractive technique to study the bioanode process. EIS doesn’t sacrifice the bioanode and produces a quantitative values of the bioanode process (34). Early EIS studies of the bioanode process used simple circuit models (7, 64, 67, 80). However, in a recent study of pH effects on the bioanode process, resistance, capacitance, and nonideality constants in two reactions were successfully differentiated and quantified (34). For a detailed electrochemical characterization of anode biofilm properties, impedance and thermodynamics of a mixed-culture bioanode were tested relative to an abiotic anode. In addition, effects of riboflavin on charge transfer processes were tested to investigate its influence on fitted EIS parameters. Our results successfully identified and quantified their extracellular electron transfer properties.

6.2 Materials and Methods

MFC construction and operation. Single-chambered MFCs (250 ml) (54) were used with a carbon cloth anode (2.5 cm × 6 cm, BASF Fuel Cell Inc., Somerset, NJ; CC-A) and an air cathode (2.5 cm × 6 cm, four PTFE diffusion layers on a 30 wt % wet-proofed carbon cloth type B-1B, E-TEK) by applying platinum (0.5 mg Pt /cm²) on one side. Anode bottles were sealed with a butyl rubber stopper clamped with an aluminum cap to prevent gas exchange. Phosphate-buffer medium (50 mM, pH 7) containing trace
elements (10 ml/L) and vitamins (10 ml/L) was used with amendment of acetic acid (10 mM) for electron donor (36). Suspension (100 ml) from an electricity-producing acetate-fed MFC was used to inoculate 100 ml of reactor medium in a single-chamber MFC. The circuit was connected with 460-Ω resistance in the acclimation step (10 days) and 100-Ω resistance thereafter (an additional 20 days). Riboflavin (MW 376.36, 98%, CAS# 63-88-5, Alfa Aesar) was added at a final concentration of 1 mM to test the effect of exogenous mediator addition. Ag/AgCl reference electrodes (BASi MF-2079, IN) were inserted though a rubber stopper in a sidearm sampling port and sealed using silicon glue.

**EIS analysis.** EIS was performed using a potentiostat Reference 600 (Gamry Instrument Inc.) in a fresh medium condition. For mixed-culture bioanode and abiotic experiments, anodes were poised at each test potential for 30 minutes, and EIS was performed at each potential with the following conditions: AC potential 10 mV rms, initial frequency $10^6$ or $10^5$ Hz, final frequency 50 or 10 mHz, and 10 points/decade of data acquisition frequency. Impedance parameters were calculated using an equivalent circuit model BIOANODE-1, which is composed of a low-frequency reaction ($R_2$ and $C_2$) and a high-frequency reaction ($R_1$ and $C_1$) (34). Impedance spectra were fitted with BIOANODE-1 by $\chi^2$-minimization using Echem Analyst (Gamry Instrument Inc.). A constant phase element (CPE) was incorporated in each reaction to model a non-ideal capacitor, defined as $1/Z = T(j \omega)^{\alpha}$, where $T$ ($S\cdot s^\alpha$) is a numerical value of the admittance ($1/|Z|$) at $\omega = 1$, $\alpha$ is an empirical nonideality constant, and $\omega$ ($s^{-1}$) is the radial frequency ($\omega = 2\pi f$) (61). Capacitance ($C$) was calculated using $C = (TR_p)^{1/\alpha}/R_p$, where $R_p$ (Ω) is the charge transfer resistance (61).
**Calculations.** Electrode potential at pH 7 and 303 K (30 °C) was calculated using the Nernst equation (Eq. 1 – 4).

\[ 2HCO_3^- + 9H^+ + 8e^- \leftrightarrow CH_3COO^- + 4H_2O \quad \text{(Eq. 1)} \]

\[ E = E^o - \frac{RT}{8F} \ln \left( \frac{[CH_3COO^-]}{[HCO_3^-]^2[H^+]^9} \right), \quad E^o = 0.187 \, V \quad \text{(Eq. 2)} \]

\[ O_2 + 4H^+ + 4e^- \leftrightarrow 2H_2O \quad \text{(Eq. 3)} \]

\[ E_{\text{cat,M}} = E_{\text{cat}}^o - \frac{RT}{4F} \ln \left( \frac{1}{[O_2][H^+]^4} \right), \quad E^o = 1.229 \, V \quad \text{(Eq. 4)} \]

where \( E^o \) is the standard electrode potential (at 298 K, 1 bar, 1 M, V vs. standard hydrogen electrode (SHE)), \( R \) is the ideal gas constant (8.314 J/Kmol), \( T \) is temperature (K), and \( F \) is the Faraday constant (96,485.339 C/mol). By applying the obtained \( R_p \) from EIS, the exchange current (\( i^0 \)) was calculated using the Tafel equation \( R_p = RT/nFi^0 \), where \( n \) is the number of electrons involved in the reaction (8 for complete acetate oxidation) (46, 95). Potential values were reported with respect to Ag/AgCl using Ag/AgCl = SHE – 197 mV (2).

### 6.3 Results

**Impact of anode biofilm on activation overpotential and impedance.** Activation overpotential was insignificant in the bioanode compared with the abiotic anode. The open circuit potential (OCP) difference between the bioanode and the abiotic anode was 1049 mV, and increased to ~1700 mV during high current generation due to the ~600 mV of activation overpotential in the abiotic anode (Fig. 6-1). OCPs were 533 mV for the abiotic anode and -516 mV for the bioanode (Fig. 6-1). Anodic polarization behaviors
and impedance spectra were almost identical for both systems in the absence and the presence of 10 mM acetate (data not shown).

The bioanode had lower charge transfer resistance and higher capacitance for the low frequency (LF) reaction than the abiotic anode (Fig. 6-2). In the abiotic anode, $R_2$ ranged from 32 to 10680 Ω, $C_2$ ranged from 2.7 to 3.1 mF, and $\alpha_2$ ranged from 0.95 to 0.96 within current range of 5 and 1915 µA. In the bioanode, $R_2$ ranged from 36 to 93 Ω, $C_2$ ranged from 7.6 to 14.9 mF, and $\alpha_2$ ranged from 0.70 to 0.87 within current range of 160 and 1424 µA. The impedance characteristics for the high frequency (HF) reaction were relatively similar in the abiotic anode and the bioanode. Charge transfer resistances for the bioanode were ~10% lower than the abiotic anode at the higher current values.

The bioanode had higher capacitance and showed more non-ideal capacitance behavior. In the abiotic anode, $R_1$ ranged from 11.6 to 12.2 Ω, $C_1$ ranged from 0.025 to 0.026 mF, and $\alpha_1$ ranged from 0.86 to 0.87 within current range of 5 and 1915 µA. In the bioanode, $R_1$ ranged from 10.3 to 11.6 Ω, $C_1$ ranged from 0.047 to 0.063 mF, and $\alpha_1$ ranged from 0.75 to 0.82 within current range of 160 and 1424 µA. Except for the linear decrease of $R_2$ with increasing current, other impedance parameters of the abiotic anode were relatively constant over the tested current range (Fig. 6-2). On the other hand, impedance parameters changed during current development in the bioanode. Total resistance in the bioanode rapidly increased without any increase of current production, whereas it reduced as current increased in the abiotic anode (Fig. 6-3).

**Effect of riboflavin on anode impedance.** In open-circuit EIS tests, the bioanode had a high frequency arc in the Nyquist plot that was absent with the abiotic anode,
regardless of riboflavin addition (Fig. 6-4). Riboflavin (1 mM) mainly affected the HF impedance in the abiotic anode and the LF impedance in the bioanode. In the abiotic anode, riboflavin induced a phase angle shift and created curvature in the HF impedance (Fig. 6-4 A and B). Riboflavin addition in the open-circuit bioanode system (Fig. 6-4 C and D) affected a drastic reduction in $R_2$ from 531 to 162 $\Omega$, $C_2$ increased from 7.2 to 10.0 mF, and $\alpha_2$ decreased slightly from 0.97 to 0.92. The effects of riboflavin addition on the bioanode HF reaction were less pronounced; $R_1$ increased 14.0 to 15.1 $\Omega$, $C_1$ decreased from 0.53 to 0.25 mF, $\alpha_1$ decreased from 0.79 to 0.72, and $i^0$ increased from $0.6 \times 10^{-2}$ to $1.8 \times 10^{-2}$ mA.

6.4 Discussion

Electrochemistry of bioanode and abiotic anode. Measured OCPs of the bioanode (-516 mV) and the abiotic anode (533 mV) were close to the thermodynamic estimations of acetate oxidation or water hydrolysis, respectively. At pH 7 and 303 K, acetate oxidation has a potential of -503 mV at the initial batch conditions of 5 mM of HCO$_3^-$ and 10 mM of CH$_3$COO$^-$ used in these experiments, and water hydrolysis has a potential of 585 mV assuming 10% of O$_2$ saturation (0.02 mM) at 30 C°. Initiation of water hydrolysis ($E^\circ = 611$ mV) needed 1.095 V more potential than acetate oxidation ($E^\circ = -484$ mV). Acetate addition did not change anode polarization. Our results demonstrate that acetate is not oxidized in the absence of anode biofilm, but rather only water hydrolysis occurs and at a high anode potential (water hydrolysis need ~1.1 V more than biological acetate
oxidation). This indicates that the EIS-derived bioanode impedance parameters are not influenced by abiotic redox reactions.

In the abiotic anode, a huge activation potential (~600 mV) was detected. Activation overpotential is caused by the slowness of the reaction taking place on the surface of electrode (46). It can be minimized by more effective catalyst, increasing surface area, or high temperature (46). Exoelectrogenic biofilm on the anode electrode created an OCP close to the acetate oxidation potential, suggesting that it had only minimal potential loss for acetate oxidation. The presence of the anode biofilm increased capacitance due to its properties as a charge storage body or conductive matrix (28, 65). Charge double layer is a build-up of charges between an electrode and its surrounding electrolyte (46). The feasibility of electrochemical reactions depends on the charge densities in the charge double layer; the more charge, the greater is the current (46). Hence, effective catalysts increase both the accumulation of charges (capacitance) and the probability of a reaction. In our experiment, total average capacitances (~30 mF) of the bioanode was 10-fold larger than the abiotic anode (~3 mF) due to the presence of \textit{Geobacter sulfurreducens} PCA, a dominant exoelectrogenic strain in many bioanodes (5, 36, 37, 47, 98).

**Effects of riboflavin on bioanode electrochemistry.** \textit{Shewanella} species secrete flavins such as riboflavin and FMN (flavin mononucleotide or riboflavin-5’-phosphate) to perform exoelectrogenesis (94). The ratio of FMN and riboflavin in solution was reported to be 50:50 initially, but riboflavin became dominant (>90%) as the biofilm matured in a lactate-fed MEC (66). The flavin concentration accumulated to 250 - 500 nM in BESs
(66) and 100 - 600 nM in non-BES suspended growth (94). In a BES experiment with *S. oneidensis* MR-1 and MR-4, more than 70% of electron transfer was accounted for by flavin mediation. To induce an observable effect, a high riboflavin concentration (1 mM) was applied in our research. The phase angle shift and curvature occurrence in HF occurred when riboflavin was added to the abiotic anode. Considering the electron mediator role of riboflavin, riboflavin might activate the HF reaction. In the bioanode, riboflavin addition increased charge storage and charge transfer in the LF reaction, which was demonstrated by the changes of capacitance and charge transfer resistance. Exchange current calculated from the Tafel equation increased three times, possibly suggesting that activation overpotential decreased by several orders of magnitude (46, 69). These results also show riboflavin might increase exoelectrogenic microbial activity because the LF reaction was suggested to be intracellular microbial activity (34). However, riboflavin addition decreased energy storage (capacitance) with a minimal change on charge transfer resistance in the HF reaction of the bioanode.

### 6.5 Acknowledgements

This research was supported by National Science Foundation Grant CBET-0834033.
Figure 6-1. Current values after 30 minutes at each poised potential were used for anode polarization curves (A). Representative Nyquist plots (B) and Bode plots (C) measured when the bioanode (Bio) was poised at 0.37 mA (-0.45 V) and the abiotic anode (Abio) was poised at 0.42 mA (1.2 V). Blue symbols in Bode plots show shifts of phase angle ($Y_2 - Z_{phz}$).
Figure 6-2. Transitions of charge transfer resistance ($R$), capacitance ($C$), and nonideality constant ($\alpha$) with respect to current development in the bionanode (Bio) and the abiotic anode (Abio).
Figure 6-3. Total charge transfer and diffusion resistance in the bioanode (Bio) and the abiotic anode (Abio) with respect to current development.
Figure 6-4. Impedance spectra from the abiotic anode (A and B) and the bioanode (C and D) measured at open circuit. Red symbols represent impedance spectra when 1 mM riboflavin was added. Blue symbols in Bode plots (B and D) indicate phase angle (\(Y_2 - Z_{phz}\)).
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