Electromicrobiology of Dissimilatory Sulfur Reducing Bacterium *Desulfuromonas acetexigens*

Thesis by

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ABSTRACT

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Khaled Bin Bandar

Bioelectrochemical systems (BES) are engineered electrochemical devices that harness hidden chemical energy of the wastewater into the form of electricity or hydrogen. Unique microbial communities enrich in these systems for oxidation of organic matter as well as transfer of resulted electron to anode, known them as “electricigen” communities. Exploring novel electricigenesis microbial communities in the nature and understanding their electromicrobiology is one the important aspect for BES systems scale up. Herein, we report first time the electricigenesis property of an anaerobic, fresh water sediment, sulfur reducing bacterium Desulfuromona acetexigens. The electrochemical behavior of D. acetexigens biofilms grown on graphite-rod electrodes in batch-fed mode under an applied potential was investigated with traditional electroanalytical tools, and correlate the electron transfer from biofilms to electrode with a model electricigen Geobacter sulfurreducens electrochemical behavior. Research findings suggest that D. acetexigens has the ability to use electrode as electron acceptor in BES systems through establishing the direct contact with anode by expressing the membrane bound redox proteins, but not due to the secretion of soluble redox mediators. Preliminary results revealed that D. acetexigens express three distinct redox proteins in their membranes for turnover of the electrons from biofilm to electrode, and the
whole electricigenesis process observed to be unique in the *D. acetexigens* compared to that of well-studied model organism *G. sulfurreducens*. 
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INTRODUCTION

Chapter I.  

I.I.  Bioelectrochemical systems

Bioelectrochemical systems are systems in which a biological organism is responsible for electron provision (M. Rimbound). The basics of a bioelectrochemical system are very similar to that of electrochemical systems. Electrochemical systems generally are systems that may induce a chemical reaction from electricity or produce electricity from a chemical reaction. Fuel cells can be considered part of the latter subclass of electrochemical systems.

The first modern fuel cell has been dated back to the 19th century where Sir William Grove first invented a cell for electrical energy conservation proposes (Appleby). Before that the Bagdad battery is said to be one of the oldest if not the oldest fuel cell system known this day (L. Carrette). A fuel cell is said to be an enclosed system in which energy from chemical...
reactions are converted to electrical energy. A general scheme of a fuel cell consists of an anode, a cathode, a source of energy i.e. fuel, and an energy acceptor. The anode and cathode are connected in order to facilitate electron movement from the anode to the cathode. The fuel is broken down chemically at the anode donating its electron, which moves to the cathode and is up-taken by an acceptor that undergoes a chemical reaction. For example hydrogen gas could be used as fuel where it is chemically broken to H\(^+\) ions at the anode then join the oxygen that was broken down after the cathode after accepting the hydrogen’s electron to form water. (L. Carrette)

\[
\begin{align*}
\text{H}_2 & \rightarrow 2\text{H}^+ + 2e^- , \\
\text{O}_2 + 4e^- & \rightarrow 2\text{O}^-^2 \\
2\text{H}^+ + \text{O}^-^2 & \rightarrow \text{H}_2\text{O}
\end{align*}
\]

Fuel cells are generally divided based on electrolytes used, the temperature at which reaction takes place, type of fuel used and so on. For example alkaline cells are under low
temperature cells and molten carbonate cells are placed under high temperature cells.

Table 1 Fuel Cell Comparison. (US Department of Energy Office of Energy Efficiency & Renewable Energy)

<table>
<thead>
<tr>
<th>Fuel Cell Type</th>
<th>Common Electrolyte</th>
<th>Operating Temperature</th>
<th>Typical Stack Size</th>
<th>Efficiency</th>
<th>Applications</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymer Electrolyte Membrane (PEM)</td>
<td>Perfluoro sulfonic acid</td>
<td>50-150°C (122-212°F) typically 80°C</td>
<td>&lt;1kW-100kW</td>
<td>60%</td>
<td>• Backup power • Portable power • Distributed generation • Transportation • Specialty vehicles</td>
<td>• Solid electrolyte reduces corrosion &amp; electrolyte management problems • Low temperature • Quick start-up</td>
<td>• Expensive catalysts • Sensitive to fuel impurities • Low temperature waste heat</td>
</tr>
<tr>
<td>Alkaline (AFC)</td>
<td>Aqueous solution of potassium hydroxide soaked in a matrix</td>
<td>90-100°C (194-212°F)</td>
<td>10-100 kW</td>
<td>60%</td>
<td>• Military • Space</td>
<td>• Cathode reaction faster in alkaline electrolyte, leads to high performance • Low cost components</td>
<td>• Sensitive to CO₂ in fuel and air • Electrolyte management</td>
</tr>
<tr>
<td>Phosphoric Acid (PAFC)</td>
<td>Phosphoric acid soaked in a matrix</td>
<td>150-200°C (302-392°F)</td>
<td>400 kW 100 kW module</td>
<td>40%</td>
<td>• Distributed generation</td>
<td>• Higher temperature enables CHP • Increased tolerance to fuel impurities</td>
<td>• Pt catalyst • Long start up time • Low current and power</td>
</tr>
<tr>
<td>Molten Carbonate (MCFC)</td>
<td>Solution of lithium, sodium, and/or potassium carbonates, soaked in a matrix</td>
<td>600-700°C (1112-1292°F)</td>
<td>300 kW-5 MW 500 kW module</td>
<td>45-50%</td>
<td>• Electric utility • Distributed generation</td>
<td>• High efficiency • Fuel flexibility • Can use a variety of catalysts • Suitable for CHP</td>
<td>• High temperature corrosion and breakdown of cell components • Long start up time • Low power density</td>
</tr>
<tr>
<td>Solid Oxide (SOFC)</td>
<td>Yttria-stabilized zirconia</td>
<td>700-1000°C (1202-1832°F)</td>
<td>1 kW-2 MW</td>
<td>60%</td>
<td>• Auxiliary power • Electric utility • Distributed generation</td>
<td>• High efficiency • Fuel flexibility • Can use a variety of catalysts • Solid electrolyte • Suitable for CHP &amp; CCHP • Hybrid/HT cycle</td>
<td>• High temperature corrosion and breakdown of cell components • High temperature operation requires long start up time and limits</td>
</tr>
</tbody>
</table>

Unlike fuel cells, electrochemical cells are more versatile as they can create or use electricity. What determines the mode of action is the potential and whether it is added from an external power source or from the reaction of the cell itself. The direction of the reaction within the cell is determined by the difference in cell potential of the two electrodes. This potential is measured compared to a set standard hydrogen electrode and has the value of 0 volts. This measurement occurs by taking two half cells—a half cell is a container containing the electrolyte and an electrode- and connecting them with a salt bridge to facilitate ion contact in each half cell without mixing, and also connecting the electrodes together and measuring the cell potential between the electrodes. As many elements have been studied against the
standard hydrogen electrodes many set and accepted tables are provided in which the accepted cell half-cell potential is stated. In order to calculate the full cell potential both half potentials are added and from them the direction and magnitude of the potential can be computed.

**Equation 2** cell potential equation

\[ E_{\text{cell}} = E_{\text{reduction}}^o - E_{\text{oxidation}}^o \]

Microbial Fuel cells and bioelectrochemical cells are similar to electrochemical cells and fuel cells respectively, however a biological element is added. In microbial fuel cells and bioelectrochemical cells, microorganisms are used in order to produce energy, or energy is used to induce current generation. A microbial fuel cell has the same elements of a normal fuel cell except that fuel is generally a complex polysaccharides or organic compound that is broken down by specific bacteria. During the breakdown energy is transferred to the anodic electrode. Likewise in bioelectrochemical cells, however a potential difference is induced between the anode and cathode thus allowing and facilitating elec. to the anode from bacteria. (B. Logan)

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### Appendix: Synopsis of Standard Potentials

Selected half-reaction potentials are ranked in order of increasingly positive (anodic) assignments, providing a convenient guide to order reactions in a given range of potential values. Potential is represented on a redox scale, that is, the more negative is the more positive. The potential of the reference electrode is taken as zero for all reactions. Where no additional reference is given it is the standard hydrogen electrode.

<table>
<thead>
<tr>
<th>Coupler</th>
<th>Potential (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Li^+ + e^- → Li</td>
<td>0.000</td>
</tr>
<tr>
<td>H₂ + e^- → H</td>
<td>0.000</td>
</tr>
<tr>
<td>Na⁺ + e^- → Na</td>
<td>-2.711</td>
</tr>
<tr>
<td>Co²⁺ + e^- → Co</td>
<td>-1.452</td>
</tr>
<tr>
<td>Fe³⁺ + e^- → Fe²⁺</td>
<td>-0.779</td>
</tr>
<tr>
<td>Ce⁴⁺ + e^- → Ce³⁺</td>
<td>-0.133</td>
</tr>
<tr>
<td>Sn²⁺ + 2e^- → Sn</td>
<td>0.151</td>
</tr>
<tr>
<td>Pb⁺⁺ + 2e^- → Pb</td>
<td>0.126</td>
</tr>
<tr>
<td>Bi³⁺ + e^- → Bi²⁺</td>
<td>0.163</td>
</tr>
<tr>
<td>Al³⁺ + e^- → Al³⁺</td>
<td>0.335</td>
</tr>
<tr>
<td>Zn²⁺ + 2e^- → Zn</td>
<td>0.763</td>
</tr>
<tr>
<td>Cu²⁺ + e^- → Cu⁺</td>
<td>0.572</td>
</tr>
<tr>
<td>Fe³⁺ + e^- → Fe²⁺</td>
<td>0.374</td>
</tr>
<tr>
<td>H⁺ + e^- → ½ H₂</td>
<td>0.000</td>
</tr>
<tr>
<td>Cu²⁺ + e^- → Cu⁺</td>
<td>0.151</td>
</tr>
</tbody>
</table>

**Figure 4** an excerpt from Standard Potentials in Aqueous Solutions Book by Bard, Allan et al. 1985
Microbial fuel cells are divided into mediator microbial fuel cells, mediator free microbial fuel cells, and microbial electrolysis cells. The difference between a mediator free microbial fuel cell and a microbial fuel cell is in the mechanism of electron transfer, which will be explained shortly. On the other hand a microbial electrolysis cell requires the addition of voltage to the cell that leads to the electrolysis of water and/or the production of methane gas. Sometimes a complete reversal may be possible where carbon dioxide gas is reduced to form polycarbon organic compounds.

The mechanism in which the electron is transferred has been attributed to three mechanisms. All mechanisms are related to the breakdown of organic substances during cellular metabolism in order to produce ATP –Adenosine TriPhosphate, the molecule that stores energy needed for cellular functions-. In ATP production, electrons are transferred from the organic matter degraded metabolically through various different mechanisms in the cell depending on the type of bacterium and environment to terminal electron acceptor. This facilitates H⁺ flow back in the

![Image](image_url)

**Figure 5** Electron transfer mechanisms in bioelectrochemical cells.
cell generating electrical and mechanical energy needed for ATP synthesis. In both microbial fuel cells and bioelectrochemical cells the terminal acceptor is the anode. Mediators excreted by the cell or shuttles that transport electrons from the inside of the cell outward to the anode may mediate the actual transfer of the electron to the anode. A second pathway is by nanowires that extend from the bacterium and are in contact with the anode thus allowing transfer of electrons directly. Bacteria may also do this by direct contact with the anode. A fourth type of transfer which is still ambiguous, is the transfer of electrons by through help of different types of bacteria, such as by the help of mediators excreted by a different bacterium, or by contact with a different bacterium that is itself able to transfer electrons to the anode.

Microorganisms that are able to participate in electron transfer may be categorized by the mode of electron transfer. *Geobacter* and *Shewanella* are two genera that are most notable for their conductive pili that were adequately named nanowires. Grown cultures of *Synechocytis* and a co-culture of *Pelotomaculum thermopropionicum* and *Methanothermobacter thermoautotrophicus* also show nanowires formation (Y. Gorby). Other types of excoelectrogenic microorganisms that are able to transfer electrons through direct membrane contact include *Pichia anomala* and *Klebsiella pneumoniae* by way of...
enzyme on its outer membrane (Logan). *Pseudomonona aeruginosa* was also shown to participate in direct electron transfer through shuttles synthesizes within the cell.

Table 2 Different exoelectrogenic microorganisms studied as presented by Dr. Bruce Logan. (Logan)

<table>
<thead>
<tr>
<th>Year</th>
<th>Microorganism</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1999</td>
<td><em>Shewanella putrefaciens</em> IR-1 (REF. 30)</td>
<td>Direct proof of electrical current generation in an MFC by a dissimilatory metal-reducing bacterium (Gammaproteobacteria)</td>
</tr>
<tr>
<td>2001</td>
<td>Clostridium butyricum EG3</td>
<td>First Gram-positive bacterium shown to produce electrical current in an MFC (phylum Firmicutes)</td>
</tr>
<tr>
<td>2002</td>
<td><em>Desulfomonas acetoxidans</em>&lt;sup&gt;51&lt;/sup&gt;</td>
<td>Identified in a sediment MFC community and shown to produce power (Deltaproteobacteria)</td>
</tr>
<tr>
<td></td>
<td><em>Geobacter metallireducens</em>&lt;sup&gt;21&lt;/sup&gt;</td>
<td>Shown to generate electricity in a poised potential system (Deltaproteobacteria)</td>
</tr>
<tr>
<td>2003</td>
<td><em>Geobacter sulfurreducens</em>&lt;sup&gt;46&lt;/sup&gt;</td>
<td>Generated current without poised electrode (Deltaproteobacteria)</td>
</tr>
<tr>
<td></td>
<td><em>Rhodoferax ferrireducens</em>&lt;sup&gt;54&lt;/sup&gt;</td>
<td>Used glucose (Betaproteobacteria)</td>
</tr>
<tr>
<td></td>
<td>A3 (Aeromonas hydrophila)&lt;sup&gt;12&lt;/sup&gt;</td>
<td>Deltaproteobacteria</td>
</tr>
<tr>
<td>2004</td>
<td><em>Pseudomonas aeruginosa</em>&lt;sup&gt;10&lt;/sup&gt;</td>
<td>Produced low amounts of power through mediators such as pyocyanin (Gammaproteobacteria)</td>
</tr>
<tr>
<td></td>
<td><em>Desulfovibrio propionicus</em>&lt;sup&gt;35&lt;/sup&gt;</td>
<td>Deltaproteobacteria</td>
</tr>
<tr>
<td>2005</td>
<td>Geospirillum electrophilus&lt;sup&gt;48&lt;/sup&gt;</td>
<td>Psychrotolerant (Deltaproteobacteria)</td>
</tr>
<tr>
<td></td>
<td>Geotrichum fermentans</td>
<td>Produced an unidentified mediator (phylum Acidobacteria)</td>
</tr>
<tr>
<td>2006</td>
<td><em>Shewanella oneidensis</em> D5P10 (REF. 55)</td>
<td>Achieved a high power density (2 W per m&lt;sup&gt;2&lt;/sup&gt; or 500 W per m&lt;sup&gt;3&lt;/sup&gt;) by pumping cells grown in a flask into a small (1.2 mL) MFC (Gammaproteobacteria)</td>
</tr>
<tr>
<td></td>
<td>S. oneidensis MR-1 (REF. 54)</td>
<td>Various mutants identified that increase current or lose the ability for current generation (Gammaproteobacteria)</td>
</tr>
<tr>
<td></td>
<td>Escherichia coli&lt;sup&gt;59&lt;/sup&gt;</td>
<td>Found to produce current after long acclimation times (Gammaproteobacteria)</td>
</tr>
<tr>
<td>2008</td>
<td><em>Rhodopseudomonas palustris</em> DX-1 (REF. 37)</td>
<td>Produced high power densities of 2.72 W per m&lt;sup&gt;2&lt;/sup&gt; compared with an acclimated waste-water inoculum (1.74 W per m&lt;sup&gt;2&lt;/sup&gt;) (Alphaproteobacteria)</td>
</tr>
<tr>
<td></td>
<td><em>Ochrobactrum anthropi</em> YZ-1 (REF. 11)</td>
<td>An opportunistic pathogen, such as <em>P. aeruginosa</em> (Alphaproteobacteria)</td>
</tr>
<tr>
<td></td>
<td><em>Desulfovibrio desulfuricans</em>&lt;sup&gt;38&lt;/sup&gt;</td>
<td>Reduced sulphate when growing on lactate; resazurin in the medium was not thought to be a factor in power production (Deltaproteobacteria)</td>
</tr>
<tr>
<td></td>
<td>Acidiphilium sp. 3.2'Sup5 (REF. 57)</td>
<td>Current at low pH and in the presence of oxygen in a poised potential system (Alphaproteobacteria)</td>
</tr>
<tr>
<td></td>
<td>Klebsiella pneumoniae&lt;sup&gt;17&lt;/sup&gt; (REF. 58)</td>
<td>The first time this species produced current without a mediator (Gammaproteobacteria)</td>
</tr>
<tr>
<td></td>
<td>Thermus sp. strain JK&lt;sup&gt;48&lt;/sup&gt;</td>
<td>Phylum Firmicutes</td>
</tr>
<tr>
<td></td>
<td>Pichia anomala&lt;sup&gt;*&lt;/sup&gt; (REF. 5)</td>
<td>Current generation by a yeast (Kingdom Fungi).</td>
</tr>
</tbody>
</table>

*Air cathode microbial fuel cells (MFCs), except where noted. *Ferricyanide cathode.
I.II. Objectives of the study

In a previous work we observed a high abundance of *Desulfuromonas acetexigens* in anodic biofilms enriched from a mixed anaerobic sludge inoculum using surface functionalized electrodes as anode material (unpublished data). However, the electro-microbiology behavior of *D. acetexigens* was not investigated before. Therefore, the objective of this study was to investigate the electro-microbiology behavior of anaerobic, Gram-negative, sulfur reducing bacterium *D. acetexigens* biofilms induced on graphite carbon rod electrodes under set potential.

In this report, I evaluated the:

(i) Electricigenesis property of the novel bacterium *D. acetexigens*,

(ii) Interaction of *D. acetexigens* with electrode during electricigenesis,

(iii) Electron transfer mechanisms of *D. acetexigens* using traditional electroanalytical tools, and

(iv) Electro-microbiology property of *D. acetexigens* in comparison with a well-known electricgen, *Geobacter sulfurreducens*. 
Chapter II. MATERIALS AND METHOD

II.I. Reactor set up

The reactor is set up as a single chambered electrochemical cell container with a set volume of 100 mL. The container is bottle shaped with a main top neck (A); two necks at the top shoulder about 45° relative to the ground. One of which (B) is opposite to a protruding neck (E) used to connect two bottle reactors and one neck (C) between the latter neck and the protrusion E. A final neck (D) is place near the bottom under neck B. In neck A, a graphite anodic rod of a 0.5cm radius is placed through the lid along with a gas relief pipe attached to a collection bag. Through neck B the counter electrode of the same material is placed and is neck C the reference electrode is placed. Neck D is used to drain reactor when media change is taking place, while neck E is sealed.
Figure 7 Reactor set up with components that are annotated, A, main lid where working electrode and gas collection ports are connected. B, port for anodic electrode, C for working electrode, D, is the draining port, and E is used to convert two single chambers to one two-celled reactor with membrane separation, however for experimental proposes it is sealed by glue and a stopper.
II.II. Microbial culture development

Microbial pure strains *Geobacter sulfurreducens* (DSMZ 12127) and *Desulfuromonas acetexigens* (DSMZ 1397) were purchased from German Collection of Microorganisms and Cell Culture center, and were used as a source of electro-active bacteria. The strain was sub-cultured in 150 mL air tight, rubber septa-sealed, anaerobic syringe bottles containing 1250 mL of culture medium. The culture medium was prepared according to the protocol supplied by the culture center (http://www.dsmz.de, medium No. 826 for *G. sulfurreducens* and 148 for *D. acetexigens*). The bacteria, prior to inoculation in the electrochemical cell, were cultured separately in fumarate-containing respective culture medium for ~2 weeks (3 sub-cultures) and subsequently grown in the presence of growth medium and 10 mM acetate initial concentration under applied potential.
II.III. Growth medium preparation for electroanalytical studies

The growth medium was prepared by dissolving the required concentration of acetate (10 mM) and inorganic salts [NH₄Cl – 1.5 g/L; Na₂HPO₄ – 0.6 g/L; KCl - 0.1 g/L; NaHCO₃, trace element solution – 10 ml/L and vitamin solution - 10 ml/L] in 980 mL of distilled water. Before autoclaving, the medium was purged with N₂:CO₂ (80:20) gas mix vigorously for 60 min at 10 mL/min gas-flow rate to establish anaerobic conditions. After autoclaving (121 °C, 10 min) the medium was transferred into anaerobic glove box to maintain the anaerobic environment in the medium.
II.IV. Reactor operation and biofilm formation

*Desulfuromonas acetexigens* and *Geobacter sulfurreducens* biofilms were formed on graphite rod working electrodes (6 cm² geometric area) individually in a separate single chambered electrochemical cells (120 ml), under constant applied potential (-0.1 V vs Ag/AgCl) using a multi-channel potentiostat (CHI-1030a, CH Instruments, USA). Graphite rod (6 cm² geometric area) and Ag/AgCl electrode (BioAnalytical Systems, USA) were used as counter and reference electrodes, respectively. Biofilms were formed using anaerobic synthetic growth medium with 10 mM acetate added (final concentration) as a source of fuel. Fresh medium having 10 mM acetate was periodically replenished from the reactors when the amperometric current decreased to half of the peak currents attained during the individual batch cycle operations. All inoculations were carried out in an anaerobic glove box (Coy Laboratory, USA) and a temperature of 30°C and pH range of 7.2-7.5. (P. Jana)
II.V. Flow cytometry

In order to make a fair comparison in electrochemical activity between the cultures tested, the initial inoculum concentration (~2.6*10^7 cells/ml) for startup of both biofilms formation was maintained same. Thus, bacterial cell counts for the *D. acetexigen* and *G. sulfurreducens* inoculum were measured individually by flow cytometry (BD Accuri® C6 flow cytometer, BD Biosciences, USA) prior to inoculation into electrochemical cells. A volume of 700 μL of pre-diluted (200 times) samples was transferred to a sterile Eppendorf tube and incubated at 35 °C for 10 minutes prior to staining. The samples were stained with SYBR Green I (7 μL of 100× stock solution in 700 μL sample), vortexed and then incubated again at 35 °C for 10 minutes. A sample (200 μL) was then transferred to a 96-well plate for cell counting using the flow cytometer.
II.VI. Electroanalysis

II.VI.I. Cyclic Voltammetry

In situ CV was performed for the developed anodic biofilms at different time intervals to determine the redox behavior and electron transfer behavior between biofilm and electrode using a potentiostat (Biologic, VMP3, France). The CV was performed with a standard 3 electrode arrangement consisting of biofilm covered anode as working electrode, graphite rod as counter and Ag/AgCl electrode as reference electrode. Growth medium containing acetate was used as an electrolyte.

Prior to nonturnover CV analysis, acetate was gradually removed from the electrode-attached biofilms by washing the electrodes, under anaerobic conditions, in acetate-free culture medium. The washed electrodes were subsequently transferred into a separate electrochemical cell containing 100 mL of acetate-free culture medium and polarized continuously at -0.1 V (vs Ag/AgCl) until the anodic current approached zero. Polarization was then stopped to undertake nonturnover CV analysis in the same cell.
II.VII. SEM analysis

II.VII.I. Fixation

Sample fixation carried out prior to SEM imaging to preserve the morphology of the biofilms. At the end of experiment, electrodes were removed from the electrochemical cell and sectioned into two pieces for subsequent SEM imaging followed by fixation step. Sample fixation was undertaken by placing the electrode in the following solutions: (a) 1% glutaraldehyde, for 1 day, and (b) 1% osmium tetroxide overnight, with washing using 10 mM phosphate buffer (pH 7.0) between steps. Then the samples were dehydrated in a graded series of aqueous ethanol solutions (10–100%) and oven-dried (1 h at 40 °C) to remove residual moisture.

II.VII.II. Coating and SEM imaging

The dried samples were mounted over SEM stubs with double-sided conductivity tape and a thin layer of platinum metal applied (45s at 20 mA current in an argon atmosphere) using an automated sputter coater (Emitech, K550) for 1 min and imaged using a model Quanta 200D, FEI instrument (The Netherlands). The imaging was performed using an accelerating voltage of 15 kV and working distance of 10 mm.
Chapter III. **RESULTS AND DISCUSSION**

III.I. **Amperometric Response**

Figure 8 Amperometric response of *D. acetexigens* biofilms on GC at -0.1 V vs. Ag/AgCl applied potentials, where small arrows (black) indicate a change in feed (10 mM acetate in cell culture medium) and gray filled arrow and black unfilled arrow represent the time where in-vivo CV analysis...
Amperometric analysis was done in order to observe and study potential generation and current measurements to indicate whether *D. acetexigens* is capable of generating electrical potential and current individually in an acetate medium as a biofilm. In this study we induced current generation in a single chamber electrochemical cell at an applied potential of -0.1 V vs Ag/AgCl for graphite anodes using from *D. acetexigens* and acetate as inoculum and fuel. A wide range of applied potentials ranging from -0.4 V to +0.2 V vs Ag/AgCl tested (Rimbound et al., 2014) in the literature to enrich the electricigen biofilms on anodes from different inoculum sources. In this study, anode applied potential of -0.1 V (vs Ag/AgCl) was selected randomly to just act as an electron acceptor by providing a sufficient driving force.
for acetate oxidation and for electron transfer from the *D. acetexigens* to the electrode surface.

The evolution of amperometric current density as a function of time (Fig. 8) demonstrates an increase in current over time, and with feeding phases, from the *D. acetexigens*. During fed-batch operation over this period, *D. acetexigens* biofilms performance followed a trend of rapid rise in current density, a period of constant operation and then a relatively sharp fall, presumably related to *D. acetexigens* biofilms exoelectrogenesis activity/growth with time, followed by substrate depletion. The magnitudes of current generation from *D. acetexigens* increased with increase in number of fed-batch cycles operation, this trend is related to biofilm electrode attachment and acclimatization during an initial phase, followed by growth as a function of time. Once attachment and acclimatization occurs, current density rises at a higher rate for the next fed-batch cycle (from 4\(^{\text{th}}\) batch, i.e. ~410 h). The fed-batch amperometric response profiles in Figure 8 are similar to those observed for *G. sulfurreducens* biofilm growth on electrodes, although the pattern of growth can vary depending on a range of factors. The profile of current generation with time from *D. acetexigens* biofilms was observed to be similar to that of *G. sulfurreducens* biofilms activity (Fig. 9) under imposed potential. However, no lag phase in current generation was observed for *D. acetexigens* biofilms during the initial phase of biofilm colonization (Inset image of Fig. 8) as typically observed for *G. sulfurreducens* biofilms (Fig. 9). During batch operation over the startup period (0 – 75 h growth time), *D. acetexigens* performance followed a trend of rapid rise in current density, presumably related to its exoelectrogenesis activity. However, in the case of *G. sulfurreducens* biofilms, the profile of current generation followed a typical
bacterial growth curve behavior. Though, both *D. acetexigens* and *G. sulfurreducens* biofilms induced on graphite rod under same operational conditions with uniform initial inoculum concentration (~2.6 \( \times \) 10^7 cells/ml, based on flowcytometry analysis), noticeable change in terms of the magnitudes of current generation and the substantial time required to complete the individual fed-batch cycle operation was observed between the biofilms. This behavior perhaps attributed to the individual bacterial culture physiology to use electrode as an electron acceptor and associated biofilm growth on the electrode surface. The profile of current generation from *D. acetexigens* biofilms increased with increase in fed batch cycle operation, and substantial current generation reached to a maximum of ~6000 mA/m^2 over the period of ~480 h growth. Whereas *G. sulfurreducens* biofilms yielded a maximum current density of ~10000 mA/m^2 in ~400 h growth time at similar operational conditions. The difference in lag period, the profile of the growth in current density as a function of time and magnitudes of current density generation between the *D. acetexigens* and *G. sulfurreducens* biofilms perhaps attributed to there’s differences in anode interaction mechanisms and efficacy of individual bacterium electron exchange kinetics. The longer growth period followed by either several cycles of fed-batch or continuous operation presumably increases the *D. acetexigens* biofilm density on electrode surface to maximize the current density yield.
III.II. Cyclic voltammetry

Figure 10 Cyclic voltammetric (1 mV/s scan rate) response of *D. acetexigens* at different time intervals of batch cycle operation. (A) CV measured at peak amperometric current (unfilled black arrow of Figure 8) and (B) at the end of batch operation (gray filled arrow of Figure 8). Stars in Figure 3B represent the formal potential of the expressed redox moieties from *D. acetexigens* biofilm.
Figure 11 Cyclic voltammetric (1 mV/s scan rate) response of *G. sulfurreducens* at different time intervals of batch cycle operation. (A) CV measured at peak amperometric current (unfilled black arrow of Figure 9) and (B) at the end of batch operation (gray filled arrow of Figure 9). Stars in Figure 5B represent the formal potential of the expressed redox moieties from *G. sulfurreducens* biofilm.
Initial characterization of current generation at the electrodes was undertaken using slow-scan cyclic voltammetry (CV), to evaluate current-potential behavior of the bioanodes developed with the *D. acetexigens* inoculum. *In-situ* CV of the *D. acetexigens* biofilm electrodes (Fig. 10), performed at two time intervals over the growth period (4th batch cycle of Fig. 8) following initial inoculation, provides a sigmoidal shaped current-potential response (Fig. 10A), and the current increase observed in amperometry (represented with black unfilled arrow in Fig. 8) over the period is mirrored by a current increase in the steady-state region in the CV response. Figure 10A, retain the characteristic sigmoidal shape, indicative of electrocatalytic oxidation of substrate by a *D. acetexigens* biofilm, observed by others for substrate oxidation by biofilms induced to grow from mixed cultures (Liu et al., 2008; Katuri et al., 2012; Patil et al 2012) or single cultures of electroactive bacteria (Katuri et al., 2010 & 2012). Based on the CV results for turnover (in the presence acetate, Fig. 10A) electron transfer, the peak currents for the dominant redox couple centered around −0.37 V vs Ag/AgCl (pH 7.5) assumed to be that responsible for bioelectrocatalytic electron transfer in *D. acetexigens*.

In order to confirm the effect of substrate limited conditions on current generation by electrode-attached biofilms, the voltammetric behavior of biofilm was evaluated when the current generation started to decline during the batch cycle operation (represented with gray filled arrow in Fig. 8). At substrate limited concentration the catalytic activity of *D. acetexigens* biofilms ceased thus the voltammogram behavior (Fig. 10B) different from the voltammogram recorded at substrate illimitable condition. Moreover, the CV analysis of *D. acetexigens* biofilms at low substrate concentration reveals the possible redox signals that are
confounded within the catalytic wave recorded (Fig. 8A) in the presence of high substrate concentration. The expression of possible multiple redox moieties (-0.58V, -0.37V, -0.2V vs Ag/AgCl) and their redox reactions, and exact mid-point potentials of their respective redox moieties are cleared visualized from voltammogram recorded under low substrate concentration. However, the expressed redox moieties from _D. acetexigens_ biofilms were seems to be different from _G. sulfurreducens_ biofilms (Fig. 9A), as due to quantitative differences in redox moieties expression as well as differences in their respective mix point potentials.

In order to better understand the electron transfer mechanism of _D. acetexigens_ through expression of membrane expressed redox moieties, the CV analysis performed at acetate limiting (non-turnover) conditions. The non-turnover CV analysis helps to (i) observe the redox moieties confined inside the biofilms, and (ii) better identify the redox reactions that can occur at close potentials.
III.III. Cyclic Voltametric Analysis of Filtered Spent Wash

The capability of extracellular redox molecules secretion and its involvement in current generation by *D. acetexigens* was also investigated with cyclic voltammetry. At the end of batch operation, the CV analysis was performed for the filtrate (0.2 µm) of reactor’s spent wash solution to show whether election shuttles affects the electron transfer observed in earlier analyses. CV analysis occurred in a separate electrochemical cell by placing fresh working, counter and reference electrodes. The voltammogram did not detected any redox molecule in the spent wash solution (Fig. 12), which confirmed that substantial current generation through electrocatalytic oxidation of substrate by the *D. acetexigens* biofilms but not due to secretion of extracellular electron shutting compounds in the reactors.

![Cyclic voltammogram (1 mV/s) of the reactor's spent-washed medium (filtered, 0.2 µm) in a separate electrochemical cell, solution collected at the end of batch operation of *D. acetexigens* biofilms growth](image-url)
III.IV. Amperometric Response in Acetate-less Feed

Figure 13 Amperometric response of ~480 hours grown *D. acetexigens* biofilms at -0.1 V vs. Ag/AgCl applied potentials in acetate limited culture medium (A). Cyclic voltammogram (1 mV/s) under non turnover conditions (in the absence of acetate) for *D. acetexigens* biofilms, grown (~480 hours) under an applied potential of -0.1 V vs Ag/AgCl in culture medium.
Figure 14 Amperometric response of ~400 hours grown *G. sulfurreducens* biofilms at -0.1 V vs. Ag/AgCl applied potentials in acetate limited culture medium (A). Cyclic voltamogram (1 mV/s) under non turnover conditions (in the absence of acetate) for *G. sulfurreducens* biofilms, grown (~400 hours) under an applied potential of -0.1 V vs Ag/AgCl in culture medium.
The CV analysis of both *D. acetexigens* (Fig. 10) and *G. sulfurreducens* (Fig. 11) revealed that the behavior of electron transfer mechanism qualitatively similar as they both are formed as biofilm on electrode surface for turnover of the electrons generated during bacterial oxidation. Non-turnover (in the absence of electron donor) CV analysis was performed for the individual biofilms (Fig. 13 and 14) to understand the biofilm-electrode surface phenomenon involved in the current generation. The CV response of the biofilm recorded under substrate depleted (Fig. 10) as well as acetate limited (Fig. 13) conditions in culture medium reveals at least 3 distinct membrane bound redox couples from the *D. acetexigens* biofilms are responsible for the current generation. The estimated mid-point redox potentials (at pH 7.5) of the redox centers, from the voltammogram of substrate depleted condition, is −0.58, -0.37 and -0.2 V vs Ag/AgCl, and these values are comparable to that observed for acetate oxidation by biofilms of *G. sulfurreducens* (Fig. 11 and 13) and biofilms developed from wastewaters (Liu et al 2008; Katuri et al., 2012; Patil et al 2012). However, *G. sulfurreducens* expressed 4 distinct redox moieties in the biofilms, and their redox potentials centered at -0.52, -0.4, -0.33 and -0.13 V vs Ag/AgCl (pH 7.5). The observed high current density from *G. sulfurreducens* biofilms over the *D. acetexigens* perhaps due to (i) over expression of multiple redox protein in the biofilm, (ii) physiological distinct redox protein expression, (iii) biocompatibility of considering anode as electron acceptor, (iv) variations in electron transferring kinetics, etc.
III.V. pH Influence on Amperometric analysis

Figure 15 Influence of culture pH on *D. acetexigens* biofilm voltammogram. Experiments conducted with 480 h aged biofilm grown under potential control (-0.1 V vs Ag/AgCl) in acetate limited conditions (A). Figure B, shows potential versus pH for the major peaks over this range.
Figure 16 Influence of culture pH on G. sulfurreducens biofilm voltammogram. Experiments conducted with 400 h aged biofilm grown under potential control (~0.1 V vs Ag/AgCl) in acetate limited conditions (A). Figure B, shows potential versus pH for the major peaks over this range.
The role of growth medium pH on electron turnover and the interaction between the *D. acetexigens* and electrode was evaluated under substrate limited conditions (non-turnover CV, Fig. 15). It is well known that pH strongly influences the physico-chemical properties of the redox proteins, which in turn, influences the thermodynamics of the electron exchange in the prosthetic groups of redox proteins in general (Lehninger et al., 2004). In the pH range of 6.5 - 8.5 (Fig. 15A), the relationship between midpoint potential of the dominant membrane expressed redox proteins versus pH was not found to be linear (Fig. 15B) with a slope values of 23 mV and 31 mV for the dominant redox protein observed at -0.37 V and -0.2 V vs Ag/AgCl respectively. If the relationship between pH and redox proteins close to the theoretical value i.e. 59 mV/pH (Bard and Faulkner, 2001), it is expected that the *D. acetexigens* has reversible proton-coupled electron transfer mechanism as observed for *G. sulfurreducens* (Fig. 16). In addition, the growth medium pH not influenced the peak current (both oxidation and reduction) of the redox proteins (Fig. 15A) involved in *D. acetexigens* electron transfer, which confirms that, the biological activity of the *D. acetexigens* for current generation not effected by the growth medium pH in the range of 6.5 to 8.5.

In the case of *G. sulfurreducens* biofilms, the electron transfer mechanism observed to be proton coupled as the relationship between pH versus redox potential of the dominant redox protein involved in the electron turnover between biofilm and electrode was ~60 mV/pH (Fig. 16B), which is close to the theoretical value expected for proton-coupled electron transfer mechanism (Bard and Faulkner, 2001). Also, the peak oxidation and reduction current of the redox proteins involved in electron transfer from *G. sulfurreducens* biofilms to electrode was influenced by the growth medium pH (Fig 16A), thus optimization of the
growth medium pH for maximizing electricigen communities activity is one the considering parameter for scale up.

Based on the in-sites observed from above voltammetric investigation, it is clear that redox proteins expression and their catalytic activity during electricigenesis in *D. acetexigens* is different from the *G. sulfurreducens* physiology. Thus we are concluding that the physiology of *D. acetexigens* interaction with anode electrode is unique, and this study needs further confirmations through integration of molecular biology tools for identification and characterization of the genes and membrane proteins involved in this process.
III.VI. SEM Imaging

Figure 17 Scanning electron microscope images of 480 hours aged *D. acetexigens* biofilms on graphite rod electrode, grown under -0.1 V vs Ag/AgCl in acetate fed conditions. SEM images recorded at different magnification.
Scanning electron microscopy imaging of the both *D. acetexigens* and *G. sulfurreducens* biofilms electrodes sampled at the end of experiment provides additional evidence that current generation is provided by electrode-attached biofilms. It is important to note that cracks observed in SEM images are perhaps due to sample drying followed by fixation procedure, it is expected in thick biofilm structures. The spatial structure in both biofilms SEM consists of irregularly distributed rod shaped bacterial cells, along with some

Figure 18 Scanning electron microscope images of 400 hours aged *G. sulfurreducens* biofilm on graphite rod electrode, grown under -0.1 V vs Ag/AgCl in acetate fed conditions. SEM images recorded at different magnification.
aggregates presumably of exo-polysaccharide matrix with incorporated bacterial cells, and the overall the biofilm fully covered the surface of the electrodes (Fig. 17 & 18). Also SEM imaging indicates that the both biofilm consists of multiple layers of cells, and the overall topography is heterogeneous. From SEM imaging, it is important to note that, the ~400 h aged *G. sulfurreducens* biofilm forms a thicker and more densely-packed biofilm (Fig. 18), compared to that for the ~480 hr aged *D. acetexigens* biofilm. This clearly revealing that, the kinetics of *D. acetexigens* respiration considering anode as electron acceptor is slower than *G. sulfurreducens*, perhaps this slows down the *D. acetexigens* growth on electrode surface, and it leads to the poor electricigenesis performance as observed from the amperometric analysis (Fig. 8)
Chapter IV. **CONCLUSION**

Based on the observations from the work carried out here, following conclusions were drawn:

- Current generation by *Desulfuromonas acetexigens* biofilms induced to grow on graphite-rod electrodes under a potential of -0.1 V (vs Ag/AgCl) confirms that particular sulfur reducing bacterium has the capability to use anode as electron acceptor.

- Based on electroanalysis, the observed electricigenesis in *D. acetexigens* is due to direct contact of the bacterial cells to the electrode, but not due to the involvement of any extracellular secreted mediators.

- In situ cyclic voltammetry lends support to an electron transfer from the biofilm to the electrode through a dominant redox species. Typical slow scan CV analysis of a *D. acetexigens* biofilm under nonturnover conditions can provide distinct redox couples responsible for the electron transfer. It reveals that at least three different redox transitions (centered at -0.2V, -0.37V and -0.58V vs Ag/AgCl) occur between the biofilm and the electrode.

- The expression of membrane bound redox proteins in *D. acetexigens* (3 redox moieties centered at -0.2V, -0.37V and -0.58V vs Ag/AgCl at pH 7.5) were different from *G. sulfurreducens* biofilms (4 redox moieties centered at -0.13V, -0.33V, -0.4V and -0.52V vs Ag/AgCl at pH 7.5) for turnover of electrons to electrode.
• The electron transfer from *D. acetexigens* biofilm to electrode is not proton coupled, this behavior contradicting the observations noted in the case of *G. sulfurreducens* biofilms. This confirms that the whole electron transfer mechanism is unusual (a non proton-coupled electron transfer) in *D. acetexigens* biofilm, perhaps specific proteins expressed in their function. These kind of differences in electron transfer between the electricigens communities memorizing some of the biochemical functions which can occur in the nature microbes, where a protein that reduces ‘NO’ but pumps no protons is similar to a protein that reduces O₂ and pumps protons.

• The variation in physiological distinct membrane proteins expression between *D. acetexigens* and *G. sulfurreducens* biofilms perhaps responsible for differences in the electricigenesis properties as well for variations in the magnitudes of current generation. Further investigations coupled with microbial molecular tools necessary for better understanding of these variations at genetic level.
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