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Microbial Electrochemistry and its Application to Energy and Environmental Issues

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Jason Thomas Hastings

Dr. Dev Chidambaram / Dissertation Advisor
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We recommend that the dissertation prepared under our supervision by

JASON THOMAS HASTINGS

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Dev Chidambaran, Ph.D., Advisor

Alan Fuchs, Ph.D., Committee Member

Vaidyanathan Subramanian, Ph.D., Committee Member

Qizhen Li, Ph.D., Committee Member

Maurice Fuerstenau, Ph.D., Graduate School Representative

Marsha H. Read, Ph. D., Dean, Graduate School

August, 2012
Abstract

Microbial electrochemistry forms the basis of a wide range of topics from microbial fuel cells to fermentation of carbon food sources. The ability to harness microbial electron transfer processes can lead to a greener and cleaner future. This study focuses on microbial electron transfer for liquid fuel production, novel electrode materials, subsurface environments and removal of unwanted byproducts. In the first chapter, exocellular electron transfer through direct contact utilizing passive electrodes for the enhancement of bio-fuel production was tested. Through the application of microbial growth in a 2-cell apparatus on an electrode surface ethanol production was enhanced by 22.7% over traditional fermentation. Ethanol production efficiencies of close to 95% were achieved in a fraction of the time required by traditional fermentation. Also, in this chapter, the effect of exogenous electron shuttles, electrode material selection and resistance was investigated. Power generation was observed using the 2-cell passive electrode system. An encapsulation method, which would also utilize exocellular transfer of electrons through direct contact, was hypothesized for the suspension of viable cells in a conductive polymer substrate. This conductive polymer substrate could have applications in bio-fuel production. Carbon black was added to a polymer solution to test electrospun polymer conductivity and cell viability. Polymer morphology and cell viability were imaged using electron and optical microscopy. Through proper encapsulation, higher fuel production efficiencies would be achievable. Electron transfer through endogenous exocellular protein shuttles was observed in this
study. Secretion of a soluble redox active exocellular protein by *Clostridium sp.* have been shown utilizing a 2-cell apparatus. Cyclic voltammetry and gel electrophoresis were used to show the presence of the protein. The exocellular protein is capable of reducing ferrous iron in a membrane separated chamber. In experiments where the redox active protein was allowed to pass through the permeable membrane, iron dissolution was 14-fold greater than experiments where the protein was held to one chamber by a non-permeable membrane. Confirmation of a redox active protein could reshape or understanding of subsurface redox processes. The final topic in this study discusses electron transfer within the cell for production of fermentation products. Glycerol, which is an unwanted side-product of biodiesel transesterification, is utilized as a carbon source for fermentation. Bacterial samples harvested from Galena Creek soil (NGC) are shown in this study to be efficient consumers of glycerol. NGC microbe was characterized through 16s rDNA genetic sequencing and determined to belong to genus *Clostridium*. *Clostridium* NGC was able to consume glycerol at 29.7gpl within 72hrs grown in a media containing 50gpl glycerol. All observed fermentation metabolites were characterized and quantified through an HPLC. Glycerol consumption rates and metabolite production rates were observed using varying media recipes. This study has found that NGC has higher selectivity for low weight acids at lower yeast extract concentration and higher selectivity for larger acids and alcohols at higher yeast extract concentrations.
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Chapter 1-Introduction

The World Energy Outlook states that if the governments around the world continue with current policies then the world’s energy demands will be 55% higher in 2030 than the usage in 2005 [1]. Approximately 84% of this increased energy demand will need to come from burning fossil fuels [2]. As more fossil fuels are combusted, the CO₂ emission levels will increase by 49% by 2030 when compared to 2005 values[2]. With these staggering values, it is apparent that there is a need for alternative and renewable energy sources [3]. Some avenues of energy production such as biodiesel, produce unwanted chemical by-products which will need to be considered before they are accepted as alternate fuel sources [4].

One possible method for addressing this problem is through the use of microorganisms. Multiple research laboratories are currently investigating the ability of microorganisms to produce fuel from standard carbon sources and convert unwanted by-products into useable chemicals. Microorganisms achieve this by means of electron transfer reactions. Electrons are transferred within the microorganisms for the production of fuel or from the microorganisms to external constituents where degradation will follow. Figure 1.1 illustrates the process by which a microbe will oxidize a food source to produce electrons, which will then be used for production of useful metabolite of reduction of external constituents. Traditionally the front end of Figure 1.1 used for fuel production and the back end is for the reduction of external
constituents.

Figure 1.1-A schematic of a microbial generation of electrons from the oxidation of a food source and its utilization in other processes.

Microbial electrochemistry and its application to energy and environment is introduced in **Chapter 1** and the experimental tools are described in **Chapter 2**. The focus of this study will be discussed as described below.

**Chapter 3** discusses the use of an electrochemical microbial reactor for the enhanced production of ethanol. The focus of this chapter is to enhance fuel production by focusing on the front end of the schematic shown above. Electrons will be removed to enhance microbial growth rate, which will translate in to enhanced kinetics of fuel production.

Electrodes that are utilized in the above electrochemical reactors require to be of high surface area to enhance electron transfer kinetics between the microbe and the electrode. It is hypothesized that the kinetics can be vastly improved if these electrodes can be made in a manner in which the microbes are actually encapsulated within the electrode matrix, thereby accelerating the transfer of electrons from the microbe to the electrode. The focus for this chapter is to engineer the electron removal end of
schematic shown in Figure 1.1 to oxidize glycerol. A process to engineer and fabricate such an electrode for above electrochemical reactor is presented in Chapter 4.

**Chapter 5** discusses the microbial conversion of glycerol, an unwanted alternative energy byproduct created during the production of biodiesel, into more useful compounds. The focus for this chapter is to utilize the front end of schematic shown in Figure 1.1 to oxidize glycerol. The glycerol oxidation will produce more value added constituents which is desired in the liquid transportation fuels industry. A member of the genus Clostridium was locally isolated and found to be vastly superior in terms of total glycerol consumption and conversion compared to strains reported in the literature.

**Chapter 6** discusses the natural biological processes that have evolved to address the electron utilization or back end of the schematic shown in Figure 1.1. It describes the isolation and characterization of an exocellular protein, for the first time, from a gram positive, obligate anaerobe that is used in redox reactions. The chapter will describe a novel experimental methodology that was developed to rapidly identify such proteins in future.

The dissertation concludes with a summary of results and a section on suggested future work.
Chapter 2 - Materials and Methods

All gases used were ultrahigh purity grade. All reagents were of analytical grade or better. Ultrapure water (18.2 MΩ) was used in throughout this study.

2.1 Analytical Techniques

The following analytical techniques were used for the characterization and analysis. Metabolites and reduction products were analyzed using multiple techniques: High Performance Liquid Chromatography (HPLC), Ultraviolet-Visible spectroscopy (UV-Vis), Cyclic Voltammetry and Dynamic Light Scattering (DLS).

2.1.1 Ultraviolet-Visible Spectroscopy

All UV-Vis experiments were conducted on the UV-1800 spectrophotometer from Shimadzu (Figure 2.1).

Figure 2.1- UV-1800 spectrophotometer from Shimadzu.

There are two existing light sources within a UV-VIS spectrophotometer: one for each (UV-deuterium lamp and visible light-halogen lamp) spectrum. While the
spectrophotometer is running, the wavelengths will change, warranting the use of multiple bulbs. The sample chamber is equipped with multiple slots to allow for continuous measurements of several sample a at a particular wavelength. The instrument is blanked after any change in wavelength. Quartz cuvettes were used to prevent any interference and allow measurements at lower wavelengths. The Beer-Lambert law states that there is a logarithmic dependence among transmittance of the light through a substance, the product of the absorption coefficient of the substance and the distance the light travels through the substance, as shown in equation 2.1.

\[ A = abc \]  

(Equation 2.1)

Where \(a\) is the absorbance coefficient, \(b\) is the light path length and \(c\) is the concentration. Equation 2.1 implies that the absorbance becomes linear with the concentration. Beer’s law must be obeyed; otherwise a non-linear relation will develop between concentration and absorbance.

0.3ml aliquots of growth were measured in the UV-Vis at predetermined time intervals. The growth was measured @ 600nm.

A standard curve for the color of the iron was generated prior to any experiments. A full spectrum, from 190nm to 900nm, was run on every sample; this allowed for visual confirmation of the presence of divalent iron formed from the reduction of goethite.
2.1.2 High Performance Liquid Chromatography

The production of ethanol from *Z. mobilis* and the formation of metabolites from NGC were measured using HPLC (Shimadzu LCsolution-20-AB) (Figure 2.2) with Refractive Index Detector (RID). Degradation products and spent media were separated using a Biorad® 87H column. The oven was set at 50°C. The eluent stream used was 0.05M sulfuric acid in water with a flow of 0.6 mL/min. All samples containing biomass were centrifuged at 10000 RPM for 5min. The filtrate was then aliquoted into HPLC vials and ready for use. Samples with high concentrations were diluted prior to placement in HPLC vials. Injection volume was set at 20µl.
2.1.3 Gas Chromatography Mass Spectrometry

Gas samples from NGC fermentation of glycerol were analyzed using gas chromatography with a Thermal Conductive Detector (GC-TCD); SRI 8610C GC (Figure 2.3). Haysep D and Molecular Sieve columns (6 ft) were used for the analysis. Ultra pure argon gas was used as the carrier gas. The temperature gradient was set from 40°C to 200°C during the course of 15 min, which showed good resolution between hydrogen and carbon dioxide peaks.

Figure 2.3- SRI 8610C GC-TCD.
2.1.4 Cyclic Voltammetry

Determination of the electron transfer abilities of the synthesized protein from *C. pastuerianum* was conducted using Cyclic voltammetry (CV). A platinum wire was used as the counter electrode. All potentials were measured with respect to a Ag/AgCl reference electrode. Electrochemical tests were performed using a PC4/FAS1 model potentiostat supplied by Gamry Instruments, Inc. Gamry Framework version 4.1 was used to control the potentiostat and Gamry’s ECHEM analyst version 1.1 was used to analyze the data.

Figure 2.4- Gamry PC4/FAS1 potentiostat.
2.1.5 Dynamic Light Scattering

Liquid spent media samples from *C. pastuerianum* were placed in the Dynamic Light Scattering (DLS) equipment for determination of endogenous electron shuttle size. All samples were separated into an air tight vial then transferred to the DLS. A background of plain media was run before the analysis of each sample. Samples were removed from the air tight vial and paced directly into the DLS as fast as possible. The measurement was conducted without hesitation as to not allow time for the protein to oxidize in an aerobic environment. Once finished, the DLS gave endogenous electron shuttle size.

Figure 2.5- Microtac nanotrac DLS.
2.1.6 Scanning Electron Microscopy

The Scanning Electron Microscope (SEM) (Hitachi 4700) was utilized for the imaging of bacterial growth on electrodes and bacterial growth in polymeric material. All biological samples were coated with platinum for 28s at 90mA. This coating time allowed for clear imaging without sacrificing surface features. All images were taken with a secondary electron detector set in mixed mode. The microscope electron beam energy was set at 15kV and 10mA. This electron beam energy was chosen as it did not damage the samples immediately and allowed for high resolution images. The microscope software remained in analysis mode as this focuses the electron beam energy into a narrower field thus allowing better resolution images.

Figure 2.6- Hitachi 4700 SEM.
2.1.7 Optical Microscopy

Viability as well as microbial entrapment verification was determined through optical microscopy. A Zeiss Axioskop 2 plus optical microscope with a Zeiss MRc imager was utilized. Microbial viability post-entrapment was determined through a fluorescent live/dead stain. A filter cube will be utilized to remove unwanted light from the imager. The fluorescent stain was used in tangent with a Mercury-vapor high pressure arc lamp (HBO). Images of bacteria entrapped in polymeric material will be conducted with a halogen lamp (HALO). All images will be taken using a 100x oil required objective. The images taken will be processed in ZEN, a Zeiss supplied photo-processing software.

Figure 2.7- Zeiss Axioskop 2 plus optical microscope.
Chapter 3 - Electrochemical Enhancement of Microbial Growth Kinetics

3.1 Introduction

The microbial conversion of carbohydrates to alcohol is known as fermentation. Fermentation is used to produce ethanol, one of the most important biofuels. Fermentation can be conducted with a plethora of starting carbohydrates; such as, xylose, fructose, sucrose, glucose and many more [5-15]. Fermentation from glucose is the main focus of this study. Fermentation from glucose yield two main products: (I) Ethanol and (II) Carbon Dioxide [16]. Globally, there is an increased need for alternative transportation energies. Ethanol can be used in tandem with gasoline to power motor vehicles. In the United States, ethanol forms 10% of all gasoline sold at the pump. By using ethanol as a fuel source, production of smog-forming compounds and accumulation of CO$_2$ are limited.

To achieve maximum cell growth two requirements need to be achieve: (I) adequate aeration and (II) optimal sugar content in the media [17]. Inability to fulfill these two requirements leads to anaerobic respiration and limited cellular growth [18]. Fermentation of sugars to alcohols can be achieved with various cell culture methods. Cell culture methods can be divided into three main categories: (I) Continuous, (II) Batch and (III) Fed-Batch [19]. Continuous processes show the best product yield. Continuous processes maintain optimum cell growth and product formation by controlling the flow rate of the nutrient broth. By utilizing an optimum nutrient feed, the cell-mass to
nutrient ratio remains in a regime that is inherently advantageous. When nutrient concentrations are above or below optimum values, there will be increased side product formation and a limited cellular growth rate. In addition to controlled nutrient flow, the product stream is removed. Fermentation products, such as ethanol, are harmful at high concentrations to the microbes that produce them. Removing the fermentation products before they reach toxic levels is an advantage to continuous flow. Batch processes are the simplest methods to implement. Batch processes do not have continual flows in or out of the system. Microbial growth proceeds in an environment of continually changing nutrient and product concentration. The fermentation products remain and can hinder cellular respiration. The advantages to batch processes are simple set-up, maintenance and operation. Due to these advantages most industrial processes are conducted using batch processes. Fed-batch processes control the nutrient flow into the fermentation vessel throughout fermentation and any products formed remain until the end of the process.

Yeast are facultative anaerobic eukaryotic fungi [20]. Yeasts are chemoorganotrophs and the most common carbon sources they use are hexose sugars. Studies have shown the ability of yeast to metabolize pentose sugars, and biomass sugars such as xylose [5, 6, 21, 22]. The fermentation of biomass sugars is an important source to meet current energy demands.

*Zymomonas mobilis* is a facultative anaerobic gram negative bacteria, which utilizes the Enter-Doudoroff glucose degradation pathway, which can be seen in Figure
1.1. Only a few know facultative anaerobic bacteria can capitalize on this pathway, which is found mostly in strictly aerobic bacteria [23]. In 1979, Rogers et. al. reported on the improved fermentation capabilities of *Zymomonas mobilis*. That study demonstrated ethanol productivity to be several times greater than that of yeast and ethanol yields up to 97% [24]. *Zymomonas mobilis* wild type can utilize three carbon sources: (I) glucose, (II) fructose and (III) sucrose [23]. Fermentation of glucose and fructose leads to the formation of primarily ethanol [25]. Sucrose fermentation tends to form larger quantities of byproducts, such as levan and sorbitol [26]. With higher byproduct formation, a lower ethanol yield is observed. *Zymomonas mobilis*, when grown on glucose produces minimal byproducts when compared to yeast [27]. Ethanol will ultimately reach toxic concentrations and become the growth inhibitor for *Zymomonas mobilis* in batch and fed-batch processes. Increased concentrations of ethanol will lead to increased plasma membrane permeability, which in turn will allow cofactors, coenzymes and intermediate metabolites to leak from the cell and inhibit the fermentation kinetics [28].

Industrial applications of fermentation can be viewed in multiple fields, other than fuel production, including pharmaceuticals, chemical production and agriculture. As mentioned earlier, industry utilizes the three methods for fermentation control. Continuous fermentation processes are used in food technology and biological purification of waste water [29, 30]. The large volume of water needed to be purified is the reason for the implementation of a continuous process. When products require stringent quality control, such as in pharmaceutical companies, a batch process will be
implemented[29]. Fed-batch processes have numerous operation strategies, regulating feed rate or by using feedback control [31]. Most fed-batch processes are maintained manually by a human operator, since fermentation processes are dependent on multiple variable, which can lead to non-optimized production yield [32]. As a result of the inability to accurately monitor the growth environment, control of the fermentation process is difficult [33].

Figure 3.1-Schematic of the Entner-Doudoroff pathway [34].
Typically, environmental microbes are evolved for utilizing low levels of carbon source as environments for growth are generally poor. However, these microbes are now be provided with carbon sources at concentrations 100X what might be found in the environment. In anaerobic processes, that means electron removal has to occur at a more rapid pace. It is our hypothesis that the electron removal process is the rate determining step and its enhancement would lead to faster kinetics.

It is our specific hypothesis that electron removal at initial stages will increase cellular growth and lead to increased ethanol production kinetics. Figure 3.2 provides an example of electron removal.

![Figure 3.2-A schematic showing the generation of electrons in a molecule from oxidation of a carbon source and the removal of electrons through generation of metabolites or reduction of dissolved substances.](image)

### 3.2 Material and Methods

*Zymomonas mobilis* strain ZM4 (ATCC 31821) was used in this study. Cultures were grown in an incubator at 28°C. The culture was grown with no active shaking or mixing in an effort to limit dissolved oxygen content. 2ml of one day old growth was transferred to a new flask and allowed to grow for 8hrs. After 8hrs, 14ml of growth was transferred to 250ml of media consisting of (g l⁻¹) glucose, 100; yeast, 10; KH₂PO₄, 1;
MgSO₄·7H₂O, 0.5; (NH₄)₂SO₄, 1. All chemicals used in this study were analytical grade or better. Dionized water was used throughout this study.

Table 3.1- Composition of the growth medium for Z. mobilis ZM4.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Maintained Recipe (g)</th>
<th>Experimental Recipe (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

1L of media was made for each experiment. The media was divided into four 500ml flask at 250ml each. The flasks were covered with rolled cotton and aluminum foil. Once covered, the flasks were sterilized in an autoclave held at 121°C for 20min. Two of the four media containers were inoculated with 14ml of growth. The 2-cell apparatus’ are custom glassware where one side on a 100ml bottle had an arm with a portal. The arm tip has a grooved section to accompany an O-ring. A complete cell is made by connecting two cells via the arm bridge with a stainless steel clamp. In between the cells, a 0.4μm polycarbonate membran is placed along with one O-ring. The O-ring is placed on the bacterial growth side of the 2-cell. The polycarbonate membrane is placed with the shiny side toward the bacterial growth side. After completing the 2-cell apparatus’, they were filled with DI, covered with rolled cotton and aluminum, and autoclaved with the same settings as the media. The un-inoculated
media was placed on the right side of the 2-cell apparatus, shown in Figure 3.3. Both inoculated and un-inoculated media was added to the each cell at a volume of 66ml.

Figure 3.3-a.) An image of the control apparatus used in the experiments. The left chamber is where bacterial growth occurs, hence the opaqueness of the chamber. The 0.4um membrane can be seen in between the cells. b.) An image of the electrochemical cell used in the experiments. The electrodes always face the arms. The electrodes are connected completing a circuit.

The experimental set-up used in this paper utilizes an electrochemical cell as shown in Figure 3.3. The electrochemical cell is created identical to the cell described above, except for the addition of electrodes to each chamber. The electrodes were created by cutting 1 by 2.25in sections of carbon paper (Fuel Cell Earth, Av Carb MGL200). A small strip of copper conductive tape (3M) was placed around the top of the carbon paper coupon. The copper tape is used to solder a wire as electrode lead. A 6in piece of 18-gauge wire was solder to the copper tape using a 60/40 tin to lead rosin core (Alpha Fry). After the solder had cooled, the resistance was measured. Only when the electrode resistance was lower than $2\,\Omega$, the electrode was used in the experiment. The electrodes were autoclaved along with the 2-cell apparatus: once autoclaved, the electrodes were placed in each side off the apparatus. The electrodes were placed as
close to the arm as possible and attached to one another. The process used in this paper is a semi fed-batch. The glucose from the media chamber will flow into the growth chamber, through diffusion, as respiration occurs.

Samples were taken at the following time intervals: 16, 20, 24, 26, 28, 30, 32, 36 and 40 hr. The growth of the bacteria was measured with a UV-Vis (Shimadzu, UV-1800) @ 600 nm. The UV-Vis has a 6-cell changer, so each cuvette retained its own cell-blank value for the entire fermentation reaction. This feature is important as it limits the variability in the process. The media chamber did not have its O.D. checked, as there was no growth. Approximately 1 ml of each chamber was placed in labeled microcentrifuge tubes for the determination of the ethanol concentration. The microcentrifuge tubes were centrifuged (RevSci, RevSpin 200T) @ 10000 RPM for 5 min. Samples were centrifuged to remove cellular biomass, as it will interfere with the High Performance Liquid Chromatography (HPLC). After centrifugation was completed, 0.5 ml from each sample was mixed with 9.5 ml of water in a glass vial to achieve a 1:20 sample: water dilution which was necessary to prevent detector saturation in the HPLC. 1 ml of diluted mixture was analyzed using an HPLC (Shimadzu) with a Refractive Index Detector (RID) (RID-10A). A Biorad Organic Acid Analysis Column (Aminex HPX-87H Ion Exclusion) was used with the following parameter: flow rate, 0.6 ml min⁻¹; oven temperature, 50°C; effluent stream, 0.01 N H₂SO₄; and rinse solution, water. Samples were run in batch format with a 106 auto sampler (Shimadzu, SIL-20A). An ethanol calibration curve was created to accurately determine the ethanol concentration.
Effect of various parameters on cell growth and fermentation were also studied. They include:

(1) Exogenous electron shuttle: AQDS was added to the inoculated compartment of the 2-cell apparatus.

(2) Various electrode materials were used: The electrodes used in the experiment were changed to carbon cloth and graphite.

(3) Resistors were added between the leads of the electrodes: Resistance of $10\text{M}\Omega$ was used in the experiment.

The exogenous electron shuttle will be added as a means to accelerate the electron transfer to the electrode. Addition of the electron shuttle was required in the inoculated chamber to ensure proper contact with viable, fermenting cells. Electrode material was varied to confirm high surface area and conductivity. Resistors mounted between the leads will generate power, which will be measured through monitoring the potential in the wires.

Upon completion of the experiment, the electrodes were imaged using a Scanning Electron Microscope (SEM) to verify and confirm the growth of bacteria on the electrodes.

3.3 Results

Electrochemically assisted fermentation shows many advantages over conventional methods. By electrochemically removing the electrons produced during respiration, the growth rate and ethanol production rate can be greatly enhanced. A high surface area electrode is ideal for increased efficiency, as it allows for microbial
growth to occur on the electrode. The higher the bacterial content on the electrode, the faster the electron transfer will be. Electrons are transferred from the growth chamber to the media chamber. Once in the media chamber, the electrons are taken up by dissolved oxygen. Dissolved oxygen has been shown to limit ethanol production by increasing biomass concentration\cite{35}. In an aerobic environment, the energy generated and utilized by the consumption of one molecule of glucose is 36-38 ATP. This large energy production enables higher production rates of microbial cells. During respiration, the carbon source is used to produce either new cells or growth products. Figure 3.2 illustrates the path of the electron from glucose to oxygen. The glucose is consumed to produce two main products, namely, ethanol and carbon dioxide as shown below.

\[ C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 \]

(Equation 3.1)

*(Zymomonas mobilis* utilizes the Entner-Doudoroff pathway. This pathway is extremely efficient at producing ethanol, thus giving near theoretical ethanol production rates. This pathway leads to the generation and utilization of 1 ATP of energy per molecule of glucose consumed in an anaerobic environment. When compared to the 2 ATP generated by yeast, the reason for a higher ethanol yield is clear. ATP is utilized by the bacteria as an energy storage molecule. Microbes use ATP for the production of new cells. Thus, a higher ATP production will lead to a higher production of cellular biomass and fewer ATP generation will lead to a lower cell growth rate.)
The enhanced fermentation in an electrochemical cell was observed with varying process control. The effect of exogenous electron shuttles, electrode material and the addition of a resistor will be discussed in the following section.

### 3.3.1 Electrochemical Fermentation Reactor

As stated earlier, all experiments were conducted with a control. The control is a 2-cell apparatus without the addition of electrodes. Growth will be permitted in the left chamber and prevented in the right. Figure 3.4 shows an illustration for the growth of bacteria in the control set-up.

![Figure 3.4-Schematic illustrating the experimental apparatus for the control set-up.](image)

The base case for this study is the addition of a carbon paper electrode in each compartment of the 2-cell apparatus. A schematic illustrating the base case can be seen in Figure 3.5.
Figure 3.5 - Schematic illustrating the experimental set-up used in the Electrochemical Fermentation Reactor.

Figure 3.6 - Growth rate of experimental set-up. The top curve represents the EFR and the bottom the control.
Figure 3.6 shows the growth rate of the microbial cells in the Electrochemical Fermentation Reactor (EFR) and control without electrodes experimental set-ups. The initial increase in growth rate represents an increased cell count, which correlates to an increase in glucose consumption. The absorbance at 600nm of the control culture is lower than the absorbance of the EFR culture. The cell count is 22.2% higher for the EFR than that of the control without electrodes. The difference between the absorbance at 600nm of the duplicates was 0.013 at 26hrs. Table 3.2 shows growth values normalized to growth of control after 30hrs for EFR and control cultures up to 30 hrs.

Table 3.2 - Shows growth values normalized to growth of control after 30hrs for EFR and control cultures up to 30 hrs.

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>Control</th>
<th>EFR</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>45.7</td>
<td>38.0</td>
</tr>
<tr>
<td>18</td>
<td>50.9</td>
<td>55.7</td>
</tr>
<tr>
<td>20</td>
<td>61.8</td>
<td>71.2</td>
</tr>
<tr>
<td>22</td>
<td>73.4</td>
<td>87.4</td>
</tr>
<tr>
<td>24</td>
<td>81.9</td>
<td>97.2</td>
</tr>
<tr>
<td>26</td>
<td>89.2</td>
<td>109.0</td>
</tr>
<tr>
<td>28</td>
<td>96.3</td>
<td>112.0</td>
</tr>
<tr>
<td>30</td>
<td>100.0</td>
<td>110.8</td>
</tr>
</tbody>
</table>

The EFR process utilizes the added passive electrodes for the removal of electrons from the microbes and the inoculate chamber. The increase represents the
produced electrons being used in biomass production instead of ethanol production. The removed electrons are transferred to the un-inoculated chamber where they are used in the following electrochemical reaction:

\[ O_2 + H_2O + 4e^- \rightarrow 4OH^- \]

\( (Equation \ 3.2) \)

Equation 3.2 illustrates the one mole of dissolved oxygen, O\(_2\), in an aqueous environment combining with 4 electrons to produce 4 hydroxide ions. Figure 3.7 is an image of the electron transfer process. The carbon source is oxidized, left of microbe, which generate electrons. The electrons are removed by the passive electrodes to the un-inoculated chamber where they interact with dissolved oxygen.

![Figure 3.7-Illustrates the electron transfer process from the oxidation of a carbon source to the removal of dissolved oxygen](image)

As the carbon source is oxidized by microbial respiration, electrons are generated which will need to be removed for the microbe to remain charge neutral. Equation 3.2 and Figure 3.7 demonstrate the pathway an electron takes during microbial respiration with the assistance of an electrode. With the assistance of the
electrodes in the 2-cells, the electron is transferred from the inoculated side to the un-
inoculated side where they are used by oxygen. Typically, after the consumption of
dissolved oxygen, the generated electrons are used to reduce intermediate metabolites
resulting in the production of ethanol, as shown in Figure 3.1, and slowing cellular
growth. In the electrochemical cell, the electrons are removed before metabolite
formation thus increasing the growth rate of the microbes. Once all the dissolved
oxygen is depleted, the electrons will then be used for generation of ethanol as seen in
Figure 3.8. At this depletion point, the electrodes could be removed and ethanol
generation is expected to be the same as in experiments where they are not removed.
The decrease in dissolved oxygen has a beneficial effect on the production of ethanol as
shown by Figure 3.9. If the un-inoculated compartment is provided with a continuous
supply of oxygen, electron removal can be maintained at high rates, further increasing
kinetics.

Figure 3.8- Illustrates the electron transfer process from the oxidation of a carbon source to
the production of ethanol.
At this depletion point, the electrodes could be pulled and ethanol generation would theoretically be the same as keeping them in the cells. The decrease in dissolved oxygen has a beneficial effect on the production of ethanol as shown by Figure 3.9.

![Normalized Electrochemical Process](image)

**Figure 3.9**- Ethanol Production of EFR experimental set-up. The top curve represents the EFR and the bottom the control.

Figure 3.9 provides evidence for the benefit of using electrodes as a means for the transfer of electrons to enhance the microbial growth rate and ethanol production kinetics. The percent enhancement was observed at 22.7% at 26hrs. The difference in the concentration of ethanol for the EFR process was 0.01gpl. The maximum observed production increase was at 26 hrs. Table 3.3 shows ethanol production values normalized to 32hrs of control ethanol production.
After the maximum production increase, the difference between electrochemically enhanced and traditional becomes smaller as we approach the theoretical yield for ethanol production. At 30hrs, the overall production of ethanol is the same for both process controls. This is because the total amount of ethanol production is determined stoichiometrically based on initial glucose amount as shown in equation (3.1).

\[ C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 \]

\[(Equation \ 3.1)\]

Once all of the dissolved oxygen is removed from the growth compartment, the cellular respiration switches from aerobic to anaerobic. At 30hrs, the ethanol production of the control will be greater than that of the electrochemically enhanced
process. The increased overall production of ethanol in the control is attributed to the utilization of carbon for ethanol production throughout the entire experiment. The electrochemically enhanced process utilizes carbon for ethanol production once all of the dissolved oxygen had been removed. This will limit the amount of total ethanol production. However the goal of this research was to increase kinetics not overall production.

Electrochemically assisted fermentation shows many advantages over conventional methods. By electrochemically removing the electrons produced during respiration, the cellular growth rate and ethanol production rate can be greatly enhanced. A high surface area electrode is ideal for increased efficiency as it allows for microbial growth to occur on the electrode as shown in Figure 3.10. The higher bacterial content on the electrode leads to faster electron transfer kinetics. However, it should be emphasized that the mechanism of electron transfer is not known at this time; it could be direct transfer at the cell surface or via a dissolved electron shuttle. In either case, a large area electrode will help. Electrons are transferred from the growth chamber to the media chamber. Once in the media chamber, the electrons are used by dissolved oxygen.
3.3.2 Addition of an Exogenous Electron Shuttle

The addition of an exogenous electron shuttle is discussed as this could further increase the electron transfer kinetics of the system. The observed process is assumed to be direct electron transfer by contact with the electrode. The addition of an electron shuttle could allow for a more rapid removal of electrons from the microbe as no contact is necessary, which the ultimate consequence would be a higher growth rate.

An electron shuttle is a compound that is capable of electron uptake and removal. Ideally electron shuttles can be cycled multiple times before they are of no further use to the system. An exogenous electron shuttle is an electron shuttle that originates...
outside of the cell. In the case of this experiment, an exogenous electron shuttle was added. The electron shuttle added was Anthriquinonedisulfide, AQDS. This study was conducted to evaluate whether AQDS would uptake the electrons from the microbes and transfer them to the electrode. Figure 3.11 shows an illustration for the growth of bacteria in the addition of exogenous electron set-up.

Figure 3.11-Schematic illustrating the experimental apparatus for the growth of bacteria in the addition of exogenous electron shuttle experimental set-up.
Figure 3.12 shows the growth rate of the EFR with the addition of an exogenous electron shuttle and microbes in control without electrodes experimental set-ups. The initial increase in growth rate represents an increased cell count, which correlates to an increase in glucose consumption. The absorbance of the control culture is lower than that of the EFR process. The cell count is 16.2% higher for the electrochemically assisted than that of the control without electrodes. The difference in absorbance at 600nm between the duplicates of EFR with the addition of an electron shuttle was 0.25 at 26hrs. Table 3.4 shows growth values normalized to growth of control after 30hrs for exogenous electron shuttle and control cultures up to 30 hrs.
Table 3.4- Shows growth values normalized to growth of control after 30hrs for exogenous electron shuttle and control cultures up to 30 hrs.

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>Control</th>
<th>Exogenous Electron Shuttle</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>45.7</td>
<td>35.7</td>
</tr>
<tr>
<td>18</td>
<td>50.9</td>
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</tr>
<tr>
<td>30</td>
<td>100.0</td>
<td>107.4</td>
</tr>
</tbody>
</table>

This experimental set-up does not give a growth increase that is equal to that of the electrochemically enhanced fermentation. The AQDS will uptake the electrons and since the AQDS is small enough to pass through the membrane the electrons could be transferred to the un-inoculated chamber. However, the AQDS movement is by diffusion and is not as fast as with electrodes in the electrochemical reactor. However, this slight increase over the control represents the produced electrons being used in biomass production instead of ethanol production. The removed electrons are transferred to the un-inoculated chamber where they are used in the electrochemical reaction in equation 3.2 shown earlier.
Figure 3.13 is an image of the electron transfer process. The carbon source is oxidized, left of microbe, which generate electrons. The electrons are removed by the passive electrodes and AQDS. The removed electrons transfer through the electrode to the un-inoculated chamber where they interact with dissolved oxygen and minor amounts of diffused AQDS. The AQDS will transfer electrons to the oxygen not adjacent to the electrode in the un-inoculated chamber. The process outlined in Figure 3.13 occurs only at the beginning of the experiment. After the dissolved oxygen has been depleted, the electrons will then be utilized for the production of ethanol.
Figure 3.14—Ethanol Production of exogenous electron shuttle experimental set-up. The top curve represents the exogenous electron shuttle and the bottom the control.

Figure 3.14 shows the contribution of an exogenous electron shuttle on the production of ethanol. The percent enhancement was observed at 20.2% at 26hrs. The difference in ethanol production between the duplicates was 0.98gpl. The maximum observed production increase was at 26 hrs. Table 3.5 shows ethanol production values normalized to 32hrs of control ethanol production.
Table 3.5 - Shows ethanol production values normalized to ethanol production of control after 32hrs for exogenous electron shuttle process and control cultures up to 30 hrs.

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>Control</th>
<th>Exogenous Electron Shuttle</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>16.9</td>
<td>19.0</td>
</tr>
<tr>
<td>18</td>
<td>27.1</td>
<td>26.7</td>
</tr>
<tr>
<td>20</td>
<td>34.3</td>
<td>35.7</td>
</tr>
<tr>
<td>22</td>
<td>43.3</td>
<td>51.2</td>
</tr>
<tr>
<td>24</td>
<td>60.1</td>
<td>67.1</td>
</tr>
<tr>
<td>26</td>
<td>73.8</td>
<td>88.8</td>
</tr>
<tr>
<td>28</td>
<td>90.0</td>
<td>102.5</td>
</tr>
<tr>
<td>30</td>
<td>97.7</td>
<td>100.2</td>
</tr>
</tbody>
</table>

At 30hrs, the ethanol production of the control was greater than that of the electron shuttle enhanced process. The addition of AQDS produces a smaller disparity between the control than that of the EFR process above. Since the growth rate is smaller, the expected overall yield would be higher. Comparing the EFR process and electron shuttle process between 30hrs and 40hrs, the curve for the electron shuttle process is much closer to the control than the EFR. As the production of ethanol reaches control levels after 36hrs, the amount of carbon utilized for production of ethanol is increased as compared to the EFR process. This increase in the overall yield is evidence that less carbon was used for biomass production at the beginning of the process. At 36hrs, the EFR process is at a value of 94.9% whereas the electron shuttle
process is at 98.2% of the ethanol yield observed in the control. These values prove that the electron shuttle is not an efficient addition to the experimental set-up for the enhancement of ethanol production kinetics.

The microbes grow on the surface of the electrode as shown in Figure 3.15. In this image a bio-film is noticed on the surface of the electrode. The formation of a bio-film could prove to be beneficial for the transfer of electron through the electrode system. A bio-film will allow for microbes not to be in direct contact of the electrode as observed Figure 3.15.

![Figure 3.15-SEM image of microbial growth on carbon paper with addition of an electron shuttle.](image)
3.3.3 Effect of Electrode Material

The electrode material is expected to have noticeable effect on the growth rate of the microbes. The surface area of the electrode will affect how much growth can occur on the surface of the electrode, which will in turn influence the growth rate of the microbes. Conductivity is another very important parameter for the increased growth kinetics. If the electrode chosen has a large surface area, but no ability to transfer electrons the electrode will then be only a growth substrate for the microbes. The material chosen in the electrochemically enhanced reactor was a carbon paper electrode. This electrode is easily machined, has great surface area and is very electrically conductive. The electrodes that will be used as possible replacements will be carbon cloth and graphite electrodes. Figure 3.16 shows an illustration for the growth of bacteria in the presence of various electrode material.

Figure 3.16-Schematic illustrating the experimental apparatus for the growth of bacteria in the presence of various electrode material.
Figure 3.17 - Growth rate of electrode material selection experimental set-ups. The top curve represents the graphite electrode, the middle represents the carbon cloth electrode and the bottom the control.

Figure 3.17 shows the growth rate of the control without electrodes and the effect of the electrode material. The initial increase in growth rate represents an increased cell count, which correlates to an increase in glucose consumption. The absorbance of the control culture is lower than that of both alternative electrode materials. The cell count is 15.1% and 19.3% higher for the cloth and graphite electrodes, respectively than that of the control without electrodes. The difference in the absorbance at 600nm of the electrode material duplicates was 0.02 and 0.005 for the cloth and graphite electrodes, respectively. Table 3.6 shows growth values
normalized to growth of control after 30hrs for various electrode material and control cultures up to 30 hrs.

*Table 3.6 - Shows growth values normalized to growth of control after 30hrs for various electrode material and control cultures up to 30 hrs.*

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>Control</th>
<th>Carbon Cloth</th>
<th>Graphite</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>45.7</td>
<td>36.9</td>
<td>38.3</td>
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<tr>
<td>18</td>
<td>50.9</td>
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<td>56.1</td>
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<tr>
<td>20</td>
<td>61.8</td>
<td>68.2</td>
<td>71.2</td>
</tr>
<tr>
<td>22</td>
<td>73.4</td>
<td>84.2</td>
<td>87.6</td>
</tr>
<tr>
<td>24</td>
<td>81.9</td>
<td>94.2</td>
<td>98.2</td>
</tr>
<tr>
<td>26</td>
<td>89.2</td>
<td>102.7</td>
<td>106.4</td>
</tr>
<tr>
<td>28</td>
<td>96.3</td>
<td>106.7</td>
<td>110.4</td>
</tr>
<tr>
<td>30</td>
<td>100.0</td>
<td>105.3</td>
<td>108.4</td>
</tr>
</tbody>
</table>

The cloth electrode is not as efficient at enhancing cell growth as both the carbon paper and graphite electrodes. This deficiency is most likely attributed to the non-rigid nature of the electrode. When placed in the 2-cell apparatus, the cloth electrode was easily bent and stuck to the side. With half of the electrode stuck to the wall of the apparatus, the electron transfer mechanism was likely hindered. The carbon cloth electrode showed limited electron transfer capabilities when compared to the carbon paper electrode. The graphite electrode however was extremely difficult to machine, but was stationary once placed in the media. The graphite electrode showed
extremely low resistance, 0.3Ω, when compared to the carbon paper and carbon cloth electrodes, 1.6Ω. This decrease in the resistance should theoretically increase the electron transfer process. The graphite electrode provided enhanced growth kinetics when compared to the control. The carbon cloth and graphite electrodes utilize the same electron transfer mechanism as the electrochemically enhanced process as seen in Figure 3.18.

![Normalized Effect of Electrode](image)

**Figure 3.18**- Ethanol Production of various electrode material experimental set-ups. The top curve represents the graphite electrode, the middle represents the carbon cloth electrode and the bottom the control.

Figure 3.18 illustrates the effect of electrode material on the production of ethanol. The maximum observed production increase of 22.2% was observed at 26 hrs for the carbon cloth electrode. The difference in the ethanol production of the carbon
cloth electrode was 0.6gpl at 26hrs. The maximum observed difference for the graphite electrode was 24.63±0.01% at 24hrs. The difference in the ethanol production of the graphite electrode was 0.2gpl at 24hrs. Table 3.7 shows ethanol production values normalized to 32hrs of control ethanol production.

Table 3.7- Shows ethanol production values normalized to ethanol production of control after 32hrs for various electrode material and control cultures up to 30 hrs.

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>Control</th>
<th>Carbon Cloth</th>
<th>Graphite</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>16.9</td>
<td>19.8</td>
<td>18.7</td>
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<tr>
<td>18</td>
<td>27.1</td>
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<td>73.8</td>
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<tr>
<td>28</td>
<td>90.0</td>
<td>96.9</td>
<td>98.4</td>
</tr>
<tr>
<td>30</td>
<td>97.7</td>
<td>97.2</td>
<td>103.2</td>
</tr>
</tbody>
</table>

Once again, the carbon cloth electrode did not yield as much ethanol as the electrochemically enhanced process. This is attributed to the physical properties of the electrode itself despite the fact that it has a large surface area as seen in Figure 3.19. The microbial growth on the carbon cloth electrode is similar to the carbon paper. The microbes grew on the surface of the electrode material.
Graphite electrode allowed for very rapid cell growth, which in turn allowed for high ethanol production rates. This was achieved by large surface area on the electrode itself. Figure 3.20 is an image of the graphite electrode. The electrode was considerable thicker than the carbon paper and carbon cloth electrode. The image shows limited to no growth on the graphite electrode. Since no microbes grew on the surface of the electrode, all of the electrons produced were removed through indirect contact with the electrode in solution.
3.3.4 Effect of Added Resistance

As with any industrial plant, there will be a certain amount of energy that will be needed to perform day to day operations. The transfer of electrons is utilized in the enhancement of cellular growth rates. These electrons could possibly be used for energy production. Addition of an external resistance will provide a simulation for the effect of harvesting the energy produced from electron flow. With the addition of a large resistor, the flow of electron could be inhibited and limit the effect of the electrodes. This part of the study tries to identify the optimal resistor for power

Figure 3.20-SEM image of microbial growth on carbon paper.
generation. Figure 3.21 shows an illustration for the growth of bacteria in the addition of a resistor experimental set-up.

![Figure 3.21-Schematic illustrating the experimental set-up used in the addition of a resistor experimental set-up.](image)

Figure 3.21 provides evidence that the addition of a 10MΩ does not hinder the electron transfer of the system. The cell count is 22.9% higher for the added resistance electrochemically assisted set-up than that of the control without electrodes. The difference in the absorbance at 600nm of the added resistance experimental set-up was 0.004 at 26hrs. Table 3.8 shows growth values normalized to growth of control after 30hrs for the addition of a resistor and control cultures up to 30 hrs.
Figure 3.22- Growth rate of the addition of a resistor experimental set-up. The top curve represents the effect of the added resistance and the bottom the control.

Table 3.8- Shows growth values normalized to growth of control after 30hrs for the addition of a resistor and control cultures up to 30 hrs.

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>Control</th>
<th>Added Resistor</th>
</tr>
</thead>
<tbody>
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<tr>
<td>22</td>
<td>73.4</td>
<td>90.9</td>
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<tr>
<td>24</td>
<td>81.9</td>
<td>100.2</td>
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<tr>
<td>26</td>
<td>89.2</td>
<td>109.6</td>
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<tr>
<td>28</td>
<td>96.3</td>
<td>111.1</td>
</tr>
<tr>
<td>30</td>
<td>100.0</td>
<td>110.6</td>
</tr>
</tbody>
</table>
This was one of two experimental configurations to achieve a growth higher than electrochemically assisted. This shows that not only does the added resistance not inhibit growth, but it appears to enhance the growth rate. The curve in Figure 3.23 confirms the hypothesis.

![Power Generated](image)

**Figure 3.23** Illustrates the power generated by the electrochemically enhanced fermentation process.

The electron flow at the beginning of the growth does not seem to be inhibited, thus allowing for proper growth of the bacteria. As the growth increases, the electron transfer increases until the dissolved oxygen is completely consumed. At 24hrs, the electron flow begins to decrease as the metabolic pathway turns to anaerobic
conditions. Eventually, the cells consume all of the glucose, which will stop cellular respiration and thus stop electron production.

![Normalized Resistance Effect](image)

**Figure 3.24-** Ethanol Production of added resistor experimental set-up. The top curve represents the addition of a resistor and the bottom the control.

Figure 3.24 illustrates the ability of the resistor to further enhance the production of ethanol. The percent enhancement was observed at 26.6% at 26hrs. The difference in the ethanol production of the added resistance experimental set-up was 0.2gpl at 26hrs. The maximum observed production increase was at 22 hrs, 33.3%. Table 3.9 shows ethanol production values normalized to 32hrs of control ethanol production.
Table 3.9- Shows ethanol production values normalized to ethanol production of control after 32hrs for added resistor process and control cultures up to 30 hrs.

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>Control</th>
<th>Exogenous Electron Shuttle</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>16.9</td>
<td>20.1</td>
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<tr>
<td>18</td>
<td>27.1</td>
<td>28.0</td>
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<tr>
<td>20</td>
<td>34.3</td>
<td>37.1</td>
</tr>
<tr>
<td>22</td>
<td>43.3</td>
<td>57.7</td>
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<tr>
<td>26</td>
<td>73.8</td>
<td>93.5</td>
</tr>
<tr>
<td>28</td>
<td>90.0</td>
<td>102.0</td>
</tr>
<tr>
<td>30</td>
<td>97.7</td>
<td>96.2</td>
</tr>
</tbody>
</table>

This achieved a greater ethanol production rate in a reduced time when compared to the electrochemically enhanced process. At 30hrs, the ethanol concentration was greater in the control without electrodes. The benefit to the added resistance is also noticed towards the end of the experiment. The normalized ethanol concentration at 36hrs is the lowest when compared to other experimental set-ups at 93.5% of control concentration.

Electrochemically assisted fermentation shows many advantages over conventional methods. By electrochemically removing the electrons produced during respiration, the growth rate and ethanol production rate can be greatly enhanced. A high surface area electrode is ideal for increased efficiency due to direct electron
transfer as it allows for microbial growth to occur on the electrode as shown in Figure 3.10. The higher bacterial content on the electrode leads to faster electron transfer kinetics. Electrons are transferred from the growth chamber to the un-inoculated chamber. Once in the un-inoculated chamber, the electrons are used by dissolved oxygen.

Figure 3.25 shows the growth of bacteria on the carbon paper electrode in the presence of a 10MΩ resistor. The growth is similar to the growth observed in the electrochemically enhanced reactor. In this image microbes are clearly present on the electrode. The presence of a bio-film is noticeable as well. As discussed above, this bio-film could help with electron transfer.

Figure 3.25- Shows the growth of bacteria on the carbon paper electrode in the presence of a 10MΩ resistor.
3.4 Conclusion

The addition of passive electrodes for the transfer of electrons to dissolved oxygen for the enhancement of the growth rate is evident. Figure 3.26 shows the normalized values of the various configurations in order of enhancement to the growth rate.

Figure 3.26 shows the normalized values of the various configurations in order of enhancement to the growth rate.

In the above figure, it is noticeable that the cloth electrode was the least beneficial. This was due to its lack of rigidity in the presence of the media. The exogenous electron shuttle appeared to limit the growth of bacteria as a result of limited electron removal. This was counter intuitive as the AQDS would allow for the removal of more electrons by serving as an electron storage medium. The graphite electrode showed surprisingly high growth rates for not having microbial growth on the surface.
The effect of the experimental set-ups on the production of ethanol can be observed in Figure 3.27. As expected the electrochemically assisted reactor with a 10MΩ resistor gave the highest ethanol production rate.

In summary, the EFR process can enhance growth kinetics by 22.2% and the ethanol production rate by 22.7%. This study shows how a simple modification by addition of a passive electrode can achieve these results.
Chapter 4- Engineering of an Electrically Conducting Polymer-Microbe Material

4.1 Introduction

A global demand for the increased efficiency for the production of viable alternative fuels has resulted in multiple studies aimed at engineering microbial encapsulation [36-38]. Microbial Encapsulation is currently being investigated as it enhances microbial handling and simplifies processes controls [38]. There are several encapsulation methods that are currently being utilized. The most common encapsulation techniques are spray-drying, emulsifying-crosslinking or coacervation [38-41]. In this research, electrospinning is used. Electrospinning is the use of high-voltage spinning as an effective method to produce polymeric fibers. The polymeric fibers produced from the electrospinning process can range from a few micrometers to nanometers. It is our hypothesis that electrospinning is the microbial encapsulation method that requires the least amount of effort and delivers the most desired characteristics in the encapsulated material. This process ejects a polymer-microbe mixed feedstock through a narrow aperture in the presence of a high-voltage electrostatic field for the formation of bio-polymer fibers. As the electrostatic field surpasses the surface tension of the bio-polymer, a stream of bio-polymer is pulled from the feed side to the charged receiving plate. Once ejected to the receiving plate, polymeric fibers are formed [42, 43].
The focus of this research is to make electrospun polymeric material conductive by adding carbon black concentration all the while maintaining healthy, viable cells. Such a material will be useful to further enhance fermentation as studied in chapter 3.

4.2 Material and Methods

The polymer was created following procedures reported by Liu et al. and Sosnik et al. [44, 45]. The polymer used to encapsulate the bacteria was Pluronic F127 dimethacrylate [FDMA or PEO$_{99}$- polypropylene oxide (PPO)$_{67}$-PEO$_{99}$ DMA]. FDMA was selected as a model membrane material because of its non-biodegradability and non-toxicity. Once electrospinning is complete, the fibers produce a porous sheet thus allowing for ideal fluid flow properties. The porous fiber sheet is comprised of multiple fibers over-lapping and touching one another, this large contact area of the bio-polymer is ideal for electron transfer, which is a requirement for use as an electrode. *Z. mobilis* was chosen as the bacteria for this research. *Z. mobilis* is a rod-shaped bacteria. *Z. mobilis* is among the most efficient fermentors known to produce ethanol from glucose as shown earlier in this dissertation. This work opens an avenue for exploring the use of electrospun fibers for more mainstream applications in separation technology as well as in biofilm reactors.

All solvents were analytical grade or better. 40.1g of Pluronic F127 (Sigma) was added to an Erlenmeyer flask and dried for 2hrs in vacuum at 120 °C. The polymer was removed from the oven and allowed to cool to just above room temperature. Once at temperature, 75ml of chloroform (Sigma) was added. The solution was spun by hand
until the entire polymer was dissolved. Next, the solution was placed in an ice bath and cooled to 0°C. Once cooled, 2.63g of Triethylamine (Mallinckrodt) was added. 2.65g of methacryloyl chloride (Alfa Aesar) was dissolved in 20ml of chloroform and added dropwise to the cooled solution, which was maintained under nitrogen purge and magnetic stirring, for the next 2hrs. After the methacryloyl chloride solution had been added, the reaction was allowed to proceed at room temperature for the next 24hrs, maintaining the nitrogen environment and magnetic stirring. The solution was then dried under vacuum for 2hrs at 120°C. The dried product was then suspended in 100ml of hot toluene. This mixture was magnetically stirred to allow full dissolution of the polymer. The hot mixture was vacuum filtered to remove triethylammonium hydrochloride salt. The solid remaining was washed three times with petroleum ether 50-70° to precipitate F127 dimethacrylate (F127 DMA). Complete removal of the toluene must be achieved to prevent petroleum ethers from reacting and forming unwanted precipitates.

Once dry, the F127 DMA was combined with water and poly(ethylene oxide) (PEO) at a ratio of 13:84:3, respectively. The addition of PEO is required to facilitate the formation of fibers during the electrospinning process [44]. This mixture was placed in a refrigerator until the solution became clear. After the solution had cooled, it was ready for electrospinning.
The electrospinning process, shown schematically in Figure 4.1, utilizes a syringe pump (New Era Pump Sysytems) and a custom-designed high voltage power supply. A distance of 2 to 3 inches between the syringe and the collector was used during the electrospinning process. A voltage of 12-15kV and a flow rate of 0.2ml/min was used to produce fibers of optimum thickness.

Varying quantities of carbon black were added to vials containing 2ml of polymer solution. The resistivity of each polymer-carbon black solution mixture was measured at a distance of 1.3cm. Each resistance measurement was conducted in duplicate. After resistance was measured, *Z. mobilis* was added to the polymer-carbon black solution. *Z. mobilis* was initially grown in separate cultures to stationary phase. 1.5ml of the culture was then centrifuged at 10000rpm for 5min and the supernatant was discarded to concentrate the culture to 0.5ml of liquid volume. The cells were washed 3 times
with a sodium chloride salt solution that was isotonic to growth media. 0.5ml of concentrated cells were added to the 2ml of polymer. A syringe was filled with the mixture and placed on a syringe pump. A sheet of aluminum foil with a glass slide taped to it was used as the collector base. The glass slide allows to immediately view the electrospun polymer-microbe fibers under a microscope.

4.3 Results

Optical microscopy was used extensively throughout this study for the imaging of microbial cells. Figure 4.2 shows an optical micrograph of *Z. mobilis* at a magnification of 400x. Microbial cells appear like clear rods in the micrograph. The green hue in the background of the image is from the filter used in imaging.

![Figure 4.2- Optical microscopy image of *Z. mobilis* at 400x magnification using a halogen light source.](image-url)
Once the polymer had been created and placed in vials, conductivity was measured. The conductivity of the liquid polymer solutions is shown in Figure 4.3. The FDMA polymer is a polyelectrolytic polymer as it has repeating units that contain electrolytes. Once submersed in a liquid media, such as water, the electrolytes are allowed to dissolve allowing charge transfer. This charge transfer will be measured through conductivity. As observed in the figure below, with the increase in carbon black concentration there is an increase in the conductivity of the polymer. This is expected as carbon black is highly conductive component.

![Conductivity Graph](image)

Figure 4.3- Conductivity of liquid polymer solution at varying carbon black concentrations.

Microbial cells were added to the polymeric solution after conductivity was measured. The conductive polymer-microbe solution was electrospun. During the
electrospinning process many variable can affect the formation of polymer fibers. These parameters include; (i) solution properties such as viscosity, elasticity, conductivity and surface tensions; (ii) governing variables such as hydrostatic pressure in the syringe, electric potential at the needle tip and the distance between the tip and the collector plate; (iii) ambient parameters such as temperature, moisture content in the air and the presence of a air cross stream [47, 48]. The electrospinning process focuses the electric field at the end of the needle tip which contains the polymeric solution held in place with surface tension [48]. The surface charges in the polymer undergo a force toward the collector plate which is opposite the surface tension [49]. As the electric potential is increased, the fluid at the tip of the needle deforms into a conical shape known as a taylor cone [50]. Once a critical electric field is achieved, which is greater than the surface tension, the stream of polymer travels to the collector plate. If the polymer fibers are in an optimum range, the bacteria can be held within. Figure 4.4 shows an electron micrograph of pure electrospun polymer without the addition of bacteria or carbon black.

It can be easily seen from Figure 4.4 that the polymer fiber diameter is not constant. Demir et al. discussed the effect of temperature on the uniformity of polymer thread thickness [51]. A more uniform fiber mat was found to form as the temperature of the electrospinning process was increased. Another characteristic observed in Figure 4.4 is the formation of beads as shown by red arrows. The beads are most prevalent at conjunctions between polymer fibers. It has been shown that the
concentration of polymer in the polymer solution has a direct effect on the formation of beads [52, 53]. An increase in the polymer concentration will increase the viscosity of the solution and decrease the formation of beads. The concentration of FDMA and PEO in this study helps limit the formation of beads. Beads were only observed to form at intersections of individual polymer fibers in contrast to most processes where bead formation was observed throughout the fiber [53].

![Electron micrograph of FDMA polymer](image)

**Figure 4.4**- Electron micrograph of FDMA polymer at 1500x magnification. The polymer was electrospun at 12kV, 0.2ml/min pump rate and a distance of 3inches between the syringe and collector. The red arrows represent bead formation.

With the addition of the microbes to the polymer solution, the viscosity will decrease thus leading to an increase in formation inconsistencies. Figure 4.5 shows an electron micrograph of a single microbe encapsulated by the electrospun polymer. The
Electrospun polymer is originally about 400 nm in diameter. With the encapsulation of the bacteria, the polymer stretches to above 1 µm only where the microbe is located. Once cross-linked, this will allow for cellular immobilization and a constant surface force that has been observed to prevent cellular reproduction [44]. The surface roughness observed on the polymer encapsulating the microbe is hypothesized to be stretching of the polymer solution. The electrostatic force of the electrospinning process will allow for the transfer of a given quantity of polymer based on the parameters mentioned above with or without a microbial cell.

Effect of encapsulation on the bacterial viability was compared to that of un-encapsulated bacteria. 15 ml of growth media was placed in a two sterile petri dishes. 0.5 ml of concentrated microbial cells were placed in the first petri dish (control) and an electrospun polymer containing an equivalent quantity of cells was placed in the second petri dish. The control culture exhibited a cell density of 1.6 absorbance using 600 nm after 24 hrs, whereas, the electrospun polymer containing bacteria took 48 hrs to achieve the same amount of growth. This is promising as viable cells remained after the electrospinning process. The lag in the experiment could be due to a lower number of viable microbes in the electrospun mat.
Figure 4.5- Electron micrograph of a microbial cell encapsulated by electrospun FDMA polymer. The electrospinning was conducted at 12kV, 0.2ml/min pump rate and a distance of 3inches between the syringe and collector.

The final part of this study was to illustrate the effects of carbon black on the conductivity of electrospun polymer and viability of cells after electrospinning. Fluorescent microscopy images for the lowest concentration of carbon black (4gpl) are shown in Figure 4.6. Figure 4.6 (a) shows an image of the microbes being held in position with carbon black on the surface of the polymer. The microbes can be seen within the stretched polymer, whereas the carbon black is observed to be on the surface of the polymer. Multiple bacterial cells are observed with a low concentration of carbon black. The conductivity of the electrospun polymer was found to be negligible, partially due to the immobilization of electrolytes in the solid polymer. The viability of this electrospun solution is 5:7. There are 5 living cells to 7 dead cells.
Figure 4.6 (a) An optical micrograph of the electrospun polymer fiber matrix with bacteria and carbon black at 400x magnification. (b) A fluorescent micrograph of the microbial cells from the fixed polymer at 400x magnification. The live cells are green and dead cells are red. Both images are taken with a carbon black concentration of 4gpl.
The carbon black concentration of 8gpl did not have any effect on the conductivity of the electrospun polymer. The carbon black is not at a concentration that allows it to efficiently conduct electric charge. Figure 4.7 shows images of the microbes encapsulated by the electrospun polymer. The image below was conducted at 1000x magnification to illustrate more effectively the encapsulated bacteria and formation of electrospun fibers with carbon black on the surface. It is visually confirmed that the amount of carbon black on the polymer surface has increased with comparison to 4gpl polymer solution. The cell viability decreased from 5:7 to 3:16. This decrease could be attributed to the effect of carbon black on the microbes during the electrospinning process by changing the charge distribution at the end of the taylor cone.
Figure 4.7 (a) An optical micrograph of the electrospun polymeric fiber matrix with bacteria and carbon black at 1000x magnification. (b) Fluorescence micrograph of the microbial cells from the fixed polymer at 400x magnification. The live cells are green and dead cells are red. Both images are taken with a carbon black concentration of 8gpl.
An SEM image of 8gpl carbon black can be observed in Figure 4.8. The carbon black increased the viscosity of the polymer solution, which led to the reduction of bead formation. However, this also leads to an increase in fiber diameter variation. The change in fiber diameter could be attributed to the change in charge distribution on the surface of the polymer solution. Without a uniform charge density, the polymer will be extruded at non-uniform rates. The thick fiber seen in Figure 4.8 shows carbon black (pointed using red arrows) on the surface of the polymer. This further proves the hypothesis that the carbon black remains on the surface, which is essential; to providing conductivity to the material.

Figure 4.8- SEM micrograph of 8gpl carbon black on electrospun polymer. The electrospinning was conducted at 12kV, 0.2ml/min pump rate and 3in gap.
The final concentration of carbon black utilized in this study was 12gpl. Carbon black at this concentration also did not have an effect on the conductivity of electrospun polymer. Figure 4.9a shows images of the microbes encapsulated by the electrospun polymer. With this increase in carbon black, the uniformity of the electrospun polymer was seen to decrease. Fiber sizes varied from large to small and multiple splitting effects and beads were noticed. The high concentration of carbon black also resulted in difficulties with the extrusion of the polymeric solution. The solution became viscous at a faster rate, when exposed to air, and resulted in clogging of the needle tip. Clogging of the needle tip results in inconsistencies in the electrostatic field, which eventually leads to inconsistencies in the physical properties of the electrospun polymer. At 12gpl carbon black concentration there were no viable cells after electrospinning as seen in Figure 4.9b.
Figure 4.9- (a) An optical micrograph of the electrospun polymeric fiber matrix with bacteria and carbon black at 400x magnification (b) Fluorescence micrograph of the microbial cells from the fixed polymer at 400x magnification. The live cells are green and dead cells are red. Both images are taken with a carbon black concentration of 12gpl.
4.4 Conclusion

The conductivity of the polymer solution increased as the carbon black concentration increased. This was due to the polyelectrolytic nature of FDMA and PEO dissolved in water. The FDMA polymer solution with minimal amounts of carbon black can be electrospun to produce viable encapsulated microbial cells. Once the polymer solution was electrospun, the polymeric fibers were less conductive. Through this study, it was confirmed that carbon black is also present on the outside of the polymeric fibers; a characteristic critical to use of these materials as electrodes.
Chapter 5-Microbial Reduction of Iron Oxide

5.1 Introduction
The consensus in the scientific community is first respiratory processes to evolve in the hyperthermal reducing environment of early Earth, over 3.5 billion years ago, would have utilized either Fe(III) or S(0) as electron acceptors [54]. Microbial reduction of Fe(III) and other transition metals is dependent on thermodynamic driving force and an appropriate electron transfer mechanism. Since Fe(III) acts as an electron shuttle there is little need for specific enzymes, it was previously thought that there were no iron reductases operating in the subsurface environment [55]. Iron reduction in subsurface environments was previously hypothesized to be a result of abiotic processes [56]. Microbial reduction of iron oxides is an important process in anoxic soils and sediments as it has many consequences to the chemistry of the environment [57]. Fe(III) oxides are important in organic matter degradation and nutrient cycling in soils and sediments [58]. Fe(III) may serve as an electron acceptor for microbial metabolism. With a redox potential near that of nitrate, Fe(III) should serve as an excellent electron acceptor for anaerobic respiration [59]. Dissimilatory Fe(III) reducing bacteria have been demonstrated to completely oxidize organic matter, with Fe(III) serving as the sole electron acceptor[60].

Two kinds of processes are possible for the microbial reduction of Fe(III); intracellular and extracellular. Intracellular processes are those in which the Fe(III) diffuses into the cell of the organism or adsorbs to the cell membrane, where it is
reduced. Extracellular mechanisms are those in which there is no contact between the Fe(III) and the microbe, and in which the reduction is conducted by means of an electron transferring shuttle which is a molecule that ferries electrons from the microbe to Fe(III). Some examples of extracellular electron shuttles include humic substances, quinines, phenazines and thiol containing molecules [61]. Extracellular electron shuttles can be of two kinds: endogenous and exogenous. Endogenous electron shuttles are those that are secreted by the organism as opposed to exogenous shuttles which are naturally occurring compounds found in the environment that can act as shuttles. Extracellular microbial reduction may be the mechanism behind subsurface electron transfer processes. Until this study, it has been found that direct contact with Fe(III) oxides is required for electron transfer and this reduction might occur at cell membrane via membrane bound proteins or through the use of bacterial conducting pili [62-64].

A single electron shuttle could cycle thousands of times, which will ultimately lead to a significant effect on the turnover of the terminal oxidant (Fe(III))[61]. Amongst various microbial communities, Geobacter is the genus that is well known for dominating subsurface environments where iron oxides are present [65]. Geobacter does not require electron shuttles as it reduces iron through direct contact; however, in the presence of extracellular electron shuttles the reduction of iron occurs at least 27 times faster than in their absence [66, 67]. Unlike Geobacter, organisms such as Shewanella sulfurreducens are unable to transfer electrons through direct contact with the Fe(III), but in the presence of electron shuttling compounds such as flavins, the
reduction progresses at sufficient rates [68]. Figure 5.1 illustrates the process by which a redox shuttle will anaerobically reduce Fe(III) [69].

![Figure 5.1 - Schematic illustrating the electron transfer from an anaerobic bacteria to Fe(III) via a redox shuttle (RS).](image)

Hydrogen can serve as an electron donor for the reduction of Fe(III) [70]. Based on the literature, no study has shown the existence of such an external pathway for iron oxide reduction. *Evidence for a redox active endogenous electron shuttle would be ground-breaking and change the paradigm of environmental biological iron reduction.* This chapter provides evidence for the ability of *Clostridium* sp. BC1. to secrete a soluble redox active protein capable of reducing goethite, a model iron oxide.

### 5.2 Experimental

#### 5.2.1 Materials
All reagents used in this study were of analytical grade or better. Deionized water (>18MΩ) used in this study. Goethite was synthesized according to the protocol described by Schwertmann and Cornell [71] and confirmed via characterization using X-ray diffraction.
5.2.2 Culture

_Clostridium_ sp. BC1 (ATCC No. 53464) was grown in a medium, the recipe of which is provided in Table 5.1. The pH of the medium was adjusted to 6.8 using 1M NaOH.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Amount (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>5</td>
</tr>
<tr>
<td>Ammonium Chloride</td>
<td>0.5</td>
</tr>
<tr>
<td>Glycerol Phosphate</td>
<td>0.3</td>
</tr>
<tr>
<td>Magnesium Sulfate</td>
<td>0.2</td>
</tr>
<tr>
<td>Calcium Chloride</td>
<td>0.5</td>
</tr>
<tr>
<td>Peptone</td>
<td>0.1</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>0.1</td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>0.00278</td>
</tr>
<tr>
<td>Ultrapure water</td>
<td>1000</td>
</tr>
</tbody>
</table>

The medium was pre-reduced by boiling for 15 min while purging with filtered nitrogen gas. It was then cooled and transferred to an anaerobic glove box, and 80 ml and 85 ml aliquots of the medium were dispensed into 150 ml serum bottles. The bottles were sealed with butyl rubber stoppers and aluminum crimps, and autoclaved. Reagents were pre-reduced by boiling and purging with a stream of nitrogen prior to use. All manipulations of the samples were carried out inside an anaerobic glove chamber (Coy Laboratory products, MI) filled with nitrogen containing trace amounts of
hydrogen. Hydrogen was present to catalytically remove any trace oxygen inside the glove box.

5.2.3 Exocellular Electron Transfer Studies

The apparatus used for the reduction experiments consisted of two individual 100ml custom blown flasks. The two chambers were separated by a polycarbonate membrane as shown in Figure 5.2.

![Polycarbonate Membrane](image)

**Figure 5.2** - The 2-cell apparatus Used in these studies is shown above. In this case microbes were cultured in right cell (inoculated) and goethite was added to left cell (un-inoculated). The two cells are separated by a polycarbonate membrane, held in place by O-rings and a stainless steel clip, as shown above.

Experiments were conducted with membrane sizes ranging from 0.01 um to 0.4 um. One side was labeled ‘inoculated’ while the other was labeled ‘un-inoculated’. 80 ml and 85 ml of the autoclaved media were transferred to the ‘inoculated’ and un-inoculated’ sides, respectively. 5 ml of a 24 hour old culture of *Clostridium* sp. BC1 was added to the ‘inoculated’ side and the culture was allowed to grow at 26°C. The mouth
of the cells were covered with cotton and aluminum foil. Screw caps were not a possibility as the microorganism used produced a considerable amount of gas. The cells were placed on a magnetic stir plate and the un-inoculated side was stirred continuously at 300 rpm.

Goethite, a Fe(III) oxyhydroxide (FeOOH), was added to the ‘un-inoculated’ compartment at the end of log phase growth, approximately 18 hours, to be consistent with the previous work conducted in this laboratory. After addition of 10 mg of goethite to the un-inoculated compartment, the concentration of goethite was measured at various time intervals for approximately 96 hrs. Reduction of the ferric ion in solid phase leads to the formation of soluble ferrous ion:

$$\text{Fe}^{3+} \text{ (solid)} + \text{electron} \rightarrow \text{Fe}^{2+} \text{ (soluble)}$$

The rate of reduction of goethite was then followed spectrophotometrically by analyzing the concentration of soluble Fe$^{2+}$ [72].

All experiments were performed in duplicates. For each membrane size, one set of experiments were conducted in which anthraquinone-2,6-disulfonate (AQDS) was added. AQDS, an analog for humic acids and a known electron shuttle, was used to facilitate the reduction of goethite. Experimental controls were performed as shown in Table 4.1 and were designed as follows:

(i) Abiotic control: In these experiments goethite was added to pure media without any inoculation
(ii) Effect of metabolites (spent media): Cellular growth was allowed for 48 hrs following which the membrane was replaced by an impermeable glass slide. Goethite was then added to the un-inoculated side. These controls were conducted to study the effect of microbial metabolites on the reduction of goethite. Cellular growth was not observed in the un-inoculated side for the length of the experiment.

(iii) Effect of oxygen: Cellular growth was allowed for 48 hrs following which the cell was removed from the glove box and the membrane was replaced by an impermeable glass slide. No experiments out-side of the glove-box used the inoculated side, as Clostridium sp. BC1 is an anaerobic bacterium. Goethite was added to the un-inoculated side. These controls were conducted to confirm the need for an anaerobic environment for the reduction of iron.

Table 5.2 - Controls conducted in this study. The “✓” represents the presence or addition of that constituent. The “X” represents the utilization of spent media products and not respirating microorganisms.
5.2.4 Analytical Methods

The soluble ferrous formed from bioreduction of goethite was analyzed colorimetrically at 510nm using the complexation reaction with the organic ligand o-phenanthroline [72]. Multiple reagents were used in the determination of iron concentration. Reagent preparation is as follows. An ammonium acetate buffer solution was created by dissolving 250g of NH₄C₂H₃O₂ in 150ml of water then adding this mixture to 700ml of concentrated glacial acetic acid. Hydroxylamine solution was created by dissolving 10g of NH₂OH-HCl in 100ml water. 100mg of 1,10-phenanthroline monohydrate was dissolved in 100ml water to create the phenanthroline solution. Finally, concentrated HCl was used. When testing ferrous iron, 1ml of ammonium acetate buffer and 0.5ml of phenanthroline was added to 0.5ml of liquid iron sample. When testing for ferric iron, 0.05ml of hydroxylamine solution and 0.1ml of concentrated HCl was added to the mixture above. These mixtures were allowed to react for 10min then quantized in the spectrophotometer.

5.3 Results and Discussion

Figure 5.3 shows the Scanning Electron Microscopic (SEM) image of the goethite. Goethite’s spindle shape is seen in Figure 5.3 [73]. Goethite phase was confirmed using XRD as shown in Figure 5.4 [74]. The red lines in the diffraction pattern show the database values used as standard for goethite. The diffraction pattern confirms the formation of goethite.
Figure 5.3- Scanning electron microscopic image of goethite particles.

Figure 5.4- X-ray diffraction of goethite particles. The black line indicates the results from the XRD as compared to given database lines (red).
In these experiments, *Clostridium* sp. BC1 was allowed to grow on one side of 2-cell apparatus (left side for Figure 4.5), but the other side was maintained un-inoculated. 10mg of goethite was added to the un-inoculated chamber of a 2-cell apparatus as shown in Figure 5.5. In all experiments, the un-inoculated chamber was stirred at 300rpm using a magnetic stirrer. The constant stirring is to enhance the contact between the electron shuttle and the goethite.

![Schematic diagram of experimental apparatus](image)

*Figure 5.5- Schematic illustrating the experimental apparatus for the reduction of goethite.*

Addition of 10mg of goethite to the un-inoculated side when the *Clostridium* sp. BC1 reaches late log phase (OD600nm=0.6; pH~3.2) resulted in gradual reduction of goethite in the un-inoculated side as shown in Figure 5.6.
Figure 5.6 shows the normalized iron concentration in the un-inoculated chamber. Figure 5.6 has been normalized to the total theoretical ferrous iron present in the two-cell apparatus. After 96hrs of exposure to exocellular electron shuttles, the un-inoculated chamber showed 54.9±17.8% of the total possible reduction. Whereas, after 96hrs the 0.01µm and 0.003µm porous membrane showed only 2.8±0.1% and 1.3±0.7% reduction of goethite, respectively. From Figure 5.6 it is clear that an electron shuttle is being utilized to reduce the goethite in the un-inoculated chamber. The exocellular electron shuttle or redox mediator would be synthesized in all three membrane
scenarios; however, the smaller membrane pore sizes prevent the passage of the exocellular electron shuttle. The shuttles/mediators are able to cross the membrane and transfer the electron to the goethite, thus reducing the goethite. Reduction of goethite results in the generation of highly soluble ferrous (Fe$^{2+}$) ion in solution, which can be measured colorimetrically. The reduced iron can diffuse across the membrane, where it can be utilized by the bacteria.

For the next set of experiments, microbial growth was allowed for 48hrs before the insertion of the glass slide. An impermeable glass slide was placed where the porous membrane was located. Glass slide was placed to prevent any interaction with the bacteria. For the spent media experiments, the separation between the inoculated camber and the un-inoculated chamber was a 0.4μm porous membrane. In the experiments exposed to air, growth was allowed for 48hrs before removal from the glove box. Figure 5.7 gives a schematic illustrating the apparatus used in the glass slide experiments.

Figure 5.7- Schematic illustrating the experimental apparatus for the reduction of goethite for glass slide experiments.
Figure 5.8- Ferrous iron concentration in the un-inoculated chamber when normalized to ferrous iron concentration of 0.4µm porous membrane in both chambers.

From Figure 5.8, it can be seen that neither spent media nor abiotic controls result in the reduction of any significant amount of initial goethite. The max reduction of goethite from spent media was realized in the first 24hrs, after which there was no additional reduction. This initial reduction can be attributed to the electrons that were present in the electron shuttle in the spent media. Once all of the electrons had been removed from the shuttles, there was no source to replenish them. Normally the electron shuttles will be replenished with electrons being removed from the microbes. The abiotic controls provide the results for the effect of media on the reduction of goethite. As seen in Figure 5.8, the goethite reduction and thus the effect of media on the reduction of goethite is minimal. The values observed from the abiotic controls and
spent media exposed to oxygen controls are barely higher than the ferrous concentration in media. The media contains a small amount of ferrous, an essential nutrient, as given in Table 5.1.

![Dissolved Iron Concentration Normalized to 0.01µm Membrane](image)

**Figure 5.9** - Comparison of the dissolved iron concentration from the un-inoculated chamber at 72hrs as a ratio of iron concentration in un-inoculated chamber with 0.01µm porous membrane.

Figure 5.9 is useful in explaining the effectiveness of the electron shuttle in the reduction of goethite. The results are normalized to the dissolution seen in the experiment conducted using a 0.01µm porous membrane as the separator. The 0.4µm shows the largest increase of ~14-fold over the 0.01µm porous membrane. This increase over 0.01µm is proof that a electron shuttle is present and is likely larger than
the 0.01µm membrane, but small enough to pass through the 0.4µm porous membrane.

All other experiments resulted in less than 0.5 fold increase in dissolved iron compared to the dissolved iron from experiment with 0.01µm porous membrane.

Table 5.3-Comparison of the dissolved iron in solution as a ratio to dissolved iron from experiment with 0.01µm at 72hrs.

| Experimental Configuration | Reduction Efficiency Compared to 0.01µm (%)
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>No Bacteria</td>
<td>34.9±3.4</td>
</tr>
<tr>
<td>No Bacteria in Air</td>
<td>57.4±1.4</td>
</tr>
<tr>
<td>Spent Media in Air</td>
<td>59.8±0.7</td>
</tr>
<tr>
<td>0.003µm</td>
<td>75.0±22.3</td>
</tr>
<tr>
<td>Spent Media</td>
<td>154.8±8.8</td>
</tr>
<tr>
<td>0.4µm</td>
<td>1407.9±452.2</td>
</tr>
</tbody>
</table>

It is clear from Figure 5.10 that 0.4µm porous membrane is capable of reducing goethite, whereas the other two membrane sizes are not. Figure 5.10 shows a trend similar to Figure 5.6. The ferrous iron measured in the inoculated chamber is also included in the values shown in Figure 5.10. The difference between the inoculated and un-inoculated chambers ferrous iron concentration is approximately 10%. Once goethite is reduced, it is converted into soluble ferrous iron in the un-inoculated chamber. The soluble ferrous then diffuses into the inoculated chamber with time. The difference seen in the concentration of ferrous iron in the two chambers come from the
fact that diffuse is a slow process and it takes time to equilibrate. The bacteria can now utilize the ferrous iron present in the solution to sustain microbial growth.

Figure 5.10- Ferrous iron concentration in both chambers when normalized to ferrous iron concentration of 0.4µm porous membrane in both chambers.

Once again, 0.4µm porous membrane allows for the least inhibited electron shuttle transport, thus allowing for the maximal goethite reduction. 0.01µm and 0.003µm porous membranes allow approximately the same amount of reduction to take place, as they do not allow the transfer of the electron shuttle to the un-inoculated side.
Table 5.4—Comparison for the ability of each pore size to reduce goethite as compared to 0.01µm @ 72hrs

<table>
<thead>
<tr>
<th>Pore Size</th>
<th>Reduction Efficiency Compared to 0.01µm (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.003µm</td>
<td>85.4±3.4</td>
</tr>
<tr>
<td>0.01µm</td>
<td>100</td>
</tr>
<tr>
<td>0.4µm</td>
<td>1359.2±36.1</td>
</tr>
</tbody>
</table>

A relationship between membrane size and iron reduction is provided in Table 5.4. Table 5.4 confirms there is an electron shuttle, the size of which is between 0.01µm and 0.4µm, that is utilized for the reduction of goethite.

5.3.1 Addition of Exogenous Electron Shuttle
An exogenous electron shuttle was used to enhance the electron transfer from the microorganism to the goethite. It is hypothesized that the addition of AQDS is expected to enhance the electron transfer abilities of Clostridium sp. BC1. If the hypothesis can be proven, then it would provide further evidence to the fact that the microbe is able to interact with exocellular electron shuttles. Figure 5.11 shows a schematic of the experimental set-up used. Figure 5.11 is identical to Figure 5.5 except for the addition of AQDS to the un-inoculated chamber.
Figure 5.11 - Schematic illustrating the experimental apparatus used to study the reduction of goethite in the presence of AQDS.

![Figure 5.11](image)

Figure 5.12 - Ferrous iron concentration in the un-inoculated chamber shown as a percent ratio of total dissolved ferrous iron concentration in experiment conducted using 0.4µm membrane with the addition of AQDS.

![Figure 5.12](chart)

**Effect of Membrane Size on Dissolution of Iron in the Presence of AQDS**

1. **0.4µm**
2. **0.01µm**
3. **0.003µm**
Figure 5.12 shows the normalized iron concentration in the un-inoculated chamber in the presence of AQDS. Figure 5.12 has been normalized to the overall ferrous iron measured with the 0.4µm porous membrane in both chambers. After 96hrs of exposure to the endogenous and exogenous exocellular electron shuttles, the un-inoculated chamber showed 54.7±8.9% of the total reduction observed in both chambers. Whereas, after 96hrs the 0.01µm and 0.003µm porous membrane showed only 3.9±0.8% and 1.3±1.6% reduction of goethite, respectively. Once again, the membranes with smaller pore sizes prevent the rapid reduction of goethite.

Figure 5.13- Ferrous iron concentration in the un-inoculated chamber shown as a percent ratio of total dissolved ferrous iron concentration in experiment conducted using 0.4µm membrane with the addition of AQDS.
The addition of the exogenous electron shuttle leads to no marked change in the case of controls as seen in Figure 5.13. The addition of AQDS appears to have no increased effect on the reduction of goethite in any of the controls. The values for the normalized reduction are all minimal and similar to those observed without the addition of AQDS. The observed (minimal) reduction is once again a direct effect of the utilization of the limited number of electrons originally present in the electron shuttles in the un-inoculated chamber.

**Figure 5.14**- Comparison of the dissolved iron concentration in the presence of AQDS from the un-inoculated chamber at 72hrs as a ratio of iron concentration in un-inoculated chamber with 0.01µm porous membrane.
As seen in Figure 5.14 and Table 5.5, the 0.4µm shows the largest increase over the 0.01µm porous membrane at 1783.3±45.5%. This increase over 0.01µm is proof that an electron shuttle is present and is too big for the 0.01µm porous membrane, but small enough to pass through the 0.4µm porous membrane.

Table 5.5—Comparison of total dissolved iron at 72hrs as a ratio to dissolved iron observed in the experiment with a 0.01µm membrane.

<table>
<thead>
<tr>
<th>Experimental Configuration</th>
<th>Reduction Efficiency Compared to 0.01µm (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abiotic control</td>
<td>10.4±2.5</td>
</tr>
<tr>
<td>Abiotic control exposed to oxygen</td>
<td>12.4±3.2</td>
</tr>
<tr>
<td>Spent media</td>
<td>167.8±14.6</td>
</tr>
<tr>
<td>Spent media exposed to oxygen</td>
<td>12.2±0.7</td>
</tr>
<tr>
<td>0.003µm</td>
<td>30.1±24.8</td>
</tr>
<tr>
<td>0.01µm</td>
<td>100</td>
</tr>
<tr>
<td>0.4µm</td>
<td>1783.3±45.5</td>
</tr>
</tbody>
</table>

Thus, it is clear that bacteria separated by 0.4µm porous membrane is capable of reducing goethite, whereas studies in which the bacteria was separated by other two porous membrane sizes do not result in any significant goethite reduction. The difference between the inoculated and un-inoculated chambers ferrous iron concentration is approximately 10%.
Figure 5.15 - Effect of AQDS on goethite reduction shown normalized to similar experiments conducted in the absence of AQDS.

The biggest effect from the addition of AQDS was seen in the case of experiment performed using 0.4µm porous membrane as the separator. A five-fold increase in the reduction of iron was observed in the presence of AQDS, compared to in the absence of AQDS. As the membrane size is decreased the effectiveness of the AQDS is also decreased. Thus, the ability of Clostridium sp. BC1 to utilize exocellular electron shuttles as well as the presence of an exogenous electron shuttle is confirmed. Whether the electron shuttle is exogenous and endogenous shuttle is still not known.
5.3.2 Determination of Endogenous Electron Shuttle

It is hereby hypothesized that the microorganism produces a protein that acts as an endogenous electron shuttle. Cyclic voltammetry (CV) studies were conducted to determine the electron transfer abilities of the synthesized protein. CV is utilized to analyze the redox active components in a sample. During CV, electron are provided to and removed from the species. If the electron is only captured by the species and not removed from the species, then it is referred to as a reducing species. On the other hand, if the species can only donate an electron then the species is said to be oxidizing. If both electron transfer mechanisms are present, the species is classified as redox active. Coupled reactions and electron transfer are explained in the CV. The CV works through the following application, a monolayer of non-interacting molecules are formed at the surface of the electrode. These non-interacting molecules must retain full biological activity and electron transfer abilities for the observation of redox peaks.

A sample was taken from the un-inoculated chamber of the 2-cell apparatus. The first step of the CV was to conduct a broad potential sweep confirm the presence of a redox active species, as seen in Figure 5.16. The next step was to narrow the voltage range to get a more controlled and higher resolution data that provides the exact redox potential of the species. Figure 5.17 shows the redox active peaks from the protein at 75mV and 150mV. Figures 5.16 and 5.17 provide evidence for the production of an endogenous electron shuttle that is capable of passing through a membrane for the reduction of iron.
Figure 5.16- CV representing redox properties of protein – a broad sweep

Figure 5.17- CV representing redox properties of protein – specific potential range
Figure 5.17 shows two peaks; oxidation at 75.43mV and a reduction peak at 145.4mV.

Controls were also conducted to ensure the presence of living bacteria was needed for the reduction of iron in a membrane separated cell. The two controls conducted were media and autoclaved spent media. Figure 5.18 shows the absence of peaks in the potential range used above. Autoclaving the spent media denatures any protein and thus renders it redox inactive. Figure 5.19 shows the CV of pure media. In Figure 5.19, there are no observed peaks. The absence of peaks provides evidence that the redox active constituents are produce microbially.

![Figure 5.18- Cyclic Voltammetry of autoclaved spent media](image)
For the determination of the size of the exogenous electron shuttle Dynamic Light Scattering (DLS) was conducted. DLS is used to determine the size of particles in suspension. The DLS uses light and measures the time dependent scattering of light. The scattered light will provide information to the size of the molecules present. Smaller molecules will elicit a more active time dependant response as the light will interact with more particles more rapidly. On the other hand, a larger particle will provide less intensity spikes for a given time. Once the light is measured, the software will use the gathered information to provide an estimate of the size of the particles.

A spent media sample was taken from a sample in the glove-box and placed in an air tight vial to ensure the endogenous shuttle does not oxidize before the DLS is run.
The sample was placed in the DLS and the size was measured. Figure 5.20 shows the DLS of spent media.

Figure 5.20-DLS of spent media.

Figure 5.20 shows two peaks. The first peak represents the size of the endogenous electron shuttle. The size of the endogenous electron shuttle is approximately 172nm. This size is in agreement with the membrane size used in this study. A protein size of 172nm is large enough to be prevented from crossing the 0.01µm membrane, but small enough to pass through the 0.4µm membrane. This provides further proof that an endogenous electron shuttle is synthesized in the range of 172nm. The larger peak of ~690nm shown is an alternative growth product that is not used in the reduction of goethite. The larger fraction could be cell debris from dead cell and is of no concern to this study as they are too large to pass through 0.4µ, membrane.
Gel electrophoresis was conducted to determine the molecular weight of the exogenous electron shuttle. Gel electrophoresis obtains the molecular weight of proteins in suspensions by applying an electrical current and allowing the proteins to diffuse through the gel. The larger proteins diffuse more slowly, ultimately leading to less distance traveled. Proteins are dyed with marker before electrophoresis takes place. Dyes can be selective for certain compounds, however in this study a broad spectrum dye was used as there was no information on the type of protein synthesized by the microorganism. A gel electrophoresis was conducted on samples from the 2-cell apparatus with a 0.01µm and 0.4µm membrane as shown in Figure 5.21.

Samples were taken from the 2-cell apparatus with two membrane sizes: 0.01µm and 0.4µm. Two cell volumes of 85ml from the un-inoculated chamber were combined into an airtight flask. Protease inhibitor was added to the 170ml of traversed metabolites in the flask. The protease inhibitor is used to prevent the protease in biological systems from degrading the proteins present. The traversed metabolites were centrifuged at 3°C for 5min in a 10kDa membrane centrifuge tube. The supernatant of the 10kDa centrifuge tube will house the proteins of interest, as we already know that the protein is larger than a 0.01µm membrane, which is approximately 10kDa. The centrifuge will contain media chemicals and any other small non-reductive proteins produced by the microorganism.
Figure 5.21 - Shows a gel electrophoresis of the 2-cell apparatus. Slots 1 and 2 represent the supernatant from a 10kDa centrifuge tube of experiment using 0.01µm membrane. Slot 3 represents centrifugate from a 10kDa centrifuge tube of experiment using 0.01µm membrane. Slot 5 represents centrifugate from a 10kDa centrifuge tube of experiment using 0.4µm membrane. Slot 7 represents supernatant from a 10kDa centrifuge tube of experiment using 0.4µm membrane.

The outerbands on the gel are the standards placed in the gel to provide the molecular weights of the observed proteins. The centrifugate did not generate any bands on the gel electrophoresis, as see in Figure 5.21. Also, the lack of any color in slots 3 and 5 from Figure 5.21 prove that there were no proteins present. This shows that all proteins are larger than 10kDa. Slots 1 and 2 from Figure 5.21 show that extremely small proteins were present in the un-inoculated chamber. This is illustrated by the darkening at the bottom of the gel. Slot 7 in Figure 5.21 shows two bands. These
two bands represent 43kDa and 70kDa proteins. The lower band represents the 43kDa and the higher band 70kDa. With this information, we can confirm that at least two proteins were synthesized by the microorganism and are able to traverse to the un-inoculated chamber. One or both of these proteins are able to effectively reduce the goethite in the un-inoculated chamber.

5.4 Conclusion
A novel experimental protocol was developed using a 2-cell apparatus separated by varying membrane sizes, to determine the synthesis of an endogenous electron shuttle by microbes. Sufficient reduction of goethite (~14-fold) in the un-inoculated chamber separated from the inoculated chamber by a 0.4μm membrane, with or without AQDS, is evidence of an electron shuttle produced by Clostridium sp. BC1. This is further confirmed by the cyclic voltammetry and gel electrophoresis. The cyclic voltammetry showed the presence of a redox active constituent in the un-inoculated chamber. The gel electrophoresis showed that the protein was too large for diffuse through the smallest membrane but small enough for diffusion through the larger membrane. Through the controls utilized in this study, it is clear that the presence of respiring Clostridium sp. BC1. is necessary for the enzymatic reduction of goethite. Gel electrophoresis showed the presence of two protein bands. These two bands represent 43kDa and 70kDa proteins. One or both of these proteins are able to effectively reduce the goethite in the un-inoculated chamber. In summary, this is the first instance that a gram positive bacterium has been shown to secrete an endogenous shuttle.
Chapter 6-Microbial Conversion of Glycerol

6.1 Introduction

As fossil fuel prices rise due to limited supply and increasing demand in the world, there is an increasing need for alternative energy sources especially for liquid drop-in transportation fuels. ‘Drop-in’ fuels are fuels that can be used in existing vehicles without engine modifications. Certain bio-fuels have the potential the act as drop-in fuels and thus represent a growing sector in the alternative energies field. Any fuel produced from biological source is considered bio-fuel. The production of diesel fuel from any biologically generated oil (although vegetable oil is the most commonly used source) is considered biodiesel. Biodiesel production has increased from 500,000 gallons in 1999 to 70 million gallons in 2005 [75]. The production of biodiesel yields glycerol, a highly viscous and benign chemical. For every 9 kg of biodiesel produced, there is approximately 1 kg of glycerol produced as shown in Figure 6.1 below [76]. Although glycerol used to be referred to as a value added byproduct, it is now considered a waste product as increased biodiesel production has resulted in a large surplus that now far exceeds the demand [77]. Biodiesel as a fuel source is cost competitive with fossil fuel, however the lack of cost effective means to dispose/utilize glycerol produced has translated to non-economical issues. As a result of the high production rates of glycerol, it makes an attractive alternative for carbon source utilized in fermentation [78].
The research and development of processes to convert glycerol into useful higher value added products is both a necessity for further increase in biodiesel production and a lucrative business opportunity. These processes could be implemented into existing biodiesel processes thus creating an economical solution to the ever expanding glycerol problem. Furthermore, some industries such as oleochemical industries generate waste steams containing 55-90% glycerol [80]. These industries would also benefit from the development of process to convert glycerol to other value-added products.

Glycerol conversion is being pursued through both biological and chemical means. Biological fermentation of glycerol has many advantages over chemical conversion which typically suffers from low product specificity, use of high pressure and/or temperature (thus energy intensive) and inability to use crude glycerol with high
levels of contaminants. Biological fermentation allows for microbes to generate a plethora of products given the single carbon source [77]. Moreover, the biological pathways could be engineered to yield certain product of interest.

Given that US national Policy is to produce ethanol from biomass (fuel termed bioethanol) via fermentation, there are now several plants set-up across the country. Fermentation technology is well understood and implemented across the industry. Thus, the goal of this study is to be able to develop a fermentation process that utilizes glycerol. While such processes are known [81-85], the concentration of glycerol that is tolerated is on the order of 5 gpl and therefore is too dilute for commercial viability. This chapter discusses the isolation and characterization of a bacterial strain capable of utilizing high levels of glycerol. Further, the chapter discusses process development and identification of the metabolites.
Figure 6.2- Metabolic Pathway for the consumption of glycerol [86]. This metabolic pathway is found in primarily anaerobic microorganisms.
6.2 Materials and Methods

The microorganism used was isolated from Galena creek region in Reno, Nevada.

Soil was obtained from moist environments approximately 6 inches below the surface.

The microbes were cultured in two different media containing 0.5g KH$_2$PO$_4$, 0.5g K$_2$HPO$_4$, 0.2g MgSO$_4$, 0.02g CaCl$_2$, 5mg FeSO$_4$, 1g yeast extract, 2ml trace element solution and: (I) 5g glycerol and 3g (NH$_4$)$_2$SO$_4$, which is hereafter referred to as recipe BD5 and (II) 50g glycerol and 5g (NH$_4$)$_2$SO$_4$, which is hereafter referred to as recipe BD6. Media compositions are given in Table 6.1. Yeast extract (Oxoid Lp0021) is a rich composition of amino acids, carbohydrates, vitamins and minerals.

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|}
\hline
Constituent & BD5 & BD6 \\
\hline
Potassium dibasic phosphate & 0.5 (g) & 0.5 (g) \\
\hline
Potassium monobasic phosphate & 0.5 (g) & 0.5 (g) \\
\hline
Ammonium sulfate & 3 (g) & 5 (g) \\
\hline
Magnesium sulfate & 0.2 (g) & 0.2 (g) \\
\hline
Calcium chloride & 0.02 (g) & 0.02 (g) \\
\hline
Iron sulfate & 0.005 (g) & 0.005 (g) \\
\hline
Yeast Extract & 1 (g) & 1 (g) \\
\hline
Glycerol & 5 (g) & 50 (g) \\
\hline
Trace Mineral Solution & 2 (ml) & 2 (ml) \\
\hline
\end{tabular}
\caption{Media recipes used for the culturing bacteria.}
\end{table}
A trace mineral solution (ATCC MD-TMS) was utilized to expedite and limit the errors in the creation of growth media. The trace mineral solution is comprised of the chemicals listed in Table 6.2.

Table 6.2-Composition of Trace Mineral Solution.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Amount g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethylenediaminetetraacetic acid</td>
<td>0.5</td>
</tr>
<tr>
<td>MgSO$_4$ · 7H$_2$O</td>
<td>3.0</td>
</tr>
<tr>
<td>MnSO$_4$ · H$_2$O</td>
<td>0.5</td>
</tr>
<tr>
<td>NaCl</td>
<td>1.0</td>
</tr>
<tr>
<td>FeSO$_4$ · 7H$_2$O</td>
<td>0.1</td>
</tr>
<tr>
<td>Co(NO$_3$)$_2$ · 6H$_2$O</td>
<td>0.1</td>
</tr>
<tr>
<td>CaCl$_2$ (anhydrous)</td>
<td>0.1</td>
</tr>
<tr>
<td>ZnSO$_4$ · 7H$_2$O</td>
<td>0.1</td>
</tr>
<tr>
<td>CuSO$_4$ · 5H$_2$O</td>
<td>0.01</td>
</tr>
<tr>
<td>AlK(SO$_4$)$_2$ (anhydrous)</td>
<td>0.01</td>
</tr>
<tr>
<td>H$_3$BO$_3$</td>
<td>0.01</td>
</tr>
<tr>
<td>Na$_2$MoO$_4$ · 2H$_2$O</td>
<td>0.01</td>
</tr>
<tr>
<td>Na$_2$SeO$_3$ (anhydrous)</td>
<td>0.001</td>
</tr>
<tr>
<td>Na$_2$WO$_4$ · 2H$_2$O</td>
<td>0.01</td>
</tr>
<tr>
<td>NiCl$_2$ · 6H$_2$O</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Deionized water (18.2 MΩ) and ultrahigh purity gases were used throughout this study. Cultures were grown at 28±1°C in an incubator shaker operated at 125 rpm. All chemicals used were analytical grade or better.
Sample aliquots were taken at the following time intervals: 0, 24, 48, 72 and 96 hrs. The growth of the bacteria was measured with a UV-Vis (Shimadzu, UV-1800) @ 600nm. The UV-Vis has a 6-cell changer, so each cuvette retained its own cell-blank value for the entire growth of the bacteria. This feature is important as it limits the variability in the process. Approximately 2ml was taken at each time point and added to a micro centrifuge tube. Samples were centrifuged (RevSci, RevSpin 200T) @10000 RPM for 5min to remove cellular biomass. 1ml of supernatant from the tube containing media 6 was diluted 10 fold to prevent saturation in the HPLC. Approximately 1ml of sample was then placed in an HPLC vial and analyzed using an HPLC (Shimadzu LC-20AB) with a Refractive Index Detector (RID) (RID-10A). All experiments were conducted in duplicates.

6.3 Results

The ability of NGC to convert glycerol to viable products is the main topic of this research. Over the next few sections, the discussions will cover the growth of NGC, glycerol consumption and the production of viable products.

Figure 6.3 shows the growth curve of the bacterium in media recipes BD5 and BD6. The results show that 50gpl glycerol can be tolerated by NGC. This is a significant first step, as bacterial strains used in studies until now have found using concentration above 5gpl to be elusive. In the next step, yeast extract concentration was varied to maximize the amount of glycerol utilization. Figure 6.4 shows the growth of NGC in BD6 media containing 50gpl glycerol and varying amounts of yeast extract.
**Figure 6.3** - Growth curves, as absorbance at 600nm, representing the growth of NGC in media BD5 and BD6.

**Figure 6.4** - Growth curve of NGC, as absorbance at 600nm, cultured in BD6 media containing 50gpl glycerol with varying amounts of yeast extract.
Figure 6.4 shows the NGC exhibits a maximum cell density (as seen by an absorbance of 2.77±0.1 at 600nm) when cultured in BD6 media containing 50gpl glycerol and 20gpl yeast extract concentration. The lowest cell density was observed when NGC was cultured in BD5 media containing 5gpl glycerol.

![Normalized Cell Density After 72 hrs of Growth](image)

Figure 6.5-Cell density (interpreted from absorbance at 600nm) of NGC after 72hrs of growth in BD6 media containing 50gpl glycerol and varying amounts of yeast extract. Results are normalized to cell density observed in BD6 medium with 1gpl yeast extract.

The effect of concentration of yeast extract on the growth of NGC can be observed in Figure 6.5. Since yeast extract contains a cocktail of amino acids, vitamins and other growth factors, it is expected that cultures grown in higher amounts of yeast
extract will have higher cell densities. The results above show that the highest concentration of yeast extract leads to the highest cell density and thus is in-line with expectations [87]. Table 6.3 shows normalized values shown in Figure 6.5.

Table 6.3- Cell density of NGC cultured in media containing 50gpl glycerol as a function of yeast extract content. Results shown are normalized with respect to media containing 1gpl yeast extract.

<table>
<thead>
<tr>
<th>Concentration of yeast extract (gpl)</th>
<th>Normalized percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>151.1±0.1</td>
</tr>
<tr>
<td>10</td>
<td>140.1±2.4</td>
</tr>
<tr>
<td>6</td>
<td>131.3±2.2</td>
</tr>
<tr>
<td>4.75</td>
<td>124.5±2.7</td>
</tr>
<tr>
<td>3.5</td>
<td>119.2±4.6</td>
</tr>
<tr>
<td>2.75</td>
<td>108.4±1.3</td>
</tr>
<tr>
<td>1.75</td>
<td>106.5±5.8</td>
</tr>
<tr>
<td>1</td>
<td>100±1.7</td>
</tr>
</tbody>
</table>

The growth of NGC with 50gpl glycerol and 20gpl yeast extract leads to approximately 150% higher cell density when compared to growth in media containing 50gpl glycerol and 1gpl yeast extract. The cumulative growth of NGC observed in a media containing 50gpl glycerol as a function of yeast extract concentration is shown below in Figure 6.6. It can be seen that as the amount of yeast extract increases, the total growth increases. However, with increasing yeast extract concentration the incremental increase in cumulative growth of the culture starts decreasing.
Figure 6.6- Growth of NGC at 72hrs, as absorbance at 600nm, in media containing 50gpl glycerol with varying yeast extract concentration.

6.3.1 Glycerol Consumption

Figure 6.7- Concentration of glycerol versus time for NGC grown in media BD5 and BD6.
Nearly all of the glycerol (5gpl) provided as part of BD5 was consumed. When the glycerol concentration was increased in the media, an increase in the overall consumption was also observed. NGC was found capable of utilizing 8.2gpl of glycerol in the BD5 media without changing the concentration of other constituents. Thus, glycerol was not the limiting factor in BD6. If glycerol was not the limiting factor, then some other constituent was limiting the consumption or utilization of the entire 50gpl of glycerol in BD6. There are two possible hypotheses that could potential explain these observations: (i) either a metabolic product that is formed during microbial growth inhibits growth when a certain critical concentration is reached or (ii) media contains insufficient nutrients to allot proper growth (i.e. some other media ingredient is the limiting factor). Determining the effect of growth products on the growth of the bacteria is arduous process and is better conducted after testing hypothesis (ii) above. By increasing the amount of nutrients in the media, via the addition of a nutrient rich mixture such as yeast extract, the effect of nutrients can be determined. As mentioned before, yeast extract is a cocktail of nutrients that typically provides the amino acids and vitamins and minerals often necessary for microbial growth. Figure 6.7 illustrates the effect of increasing concentrations of yeast extract on the consumption of glycerol in BD6 media containing 50gpl glycerol.
The maximum glycerol consumption, at the end of 96hrs of growth, was 27.4±0.2gpl as observed in NGC grown in media containing 50gpl glycerol and 20gpl yeast extract.

The effect of concentration of yeast extract on the consumption of glycerol can be observed in Figure 6.9, shown normalized to the initial BD6 media containing 50gpl glycerol and 1gpl yeast extract. Increasing the yeast extract concentration to ~5gpl
leads to a two-fold increase in the utilization of glycerol, while increasing yeast extract to 20gpl leads to a nearly 3-fold increase in utilization of glycerol. As expected, the highest concentration of yeast extract lead to the highest glycerol utilization [87]. Table 6.4 shows normalized values shown in Figure 6.8.

![Normalized Glycerol Consumption @ 72 hrs](image)

Figure 6.9-Effect of yeast extract concentration on glycerol consumption after 72 hrs of growth in BD6 media containing 50gpl glycerol. Data shown is normalized to glycerol consumption in media containing 1gpl yeast extract.
Table 6.4- The table below shows the glycerol consumption (after 72 hours of growth), normalized with respect to glycerol consumption in media containing 1gpl yeast extract.

<table>
<thead>
<tr>
<th>Concentration of yeast extract (gpl)</th>
<th>Normalized percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>288.9±0.4</td>
</tr>
<tr>
<td>10</td>
<td>270.7±2.1</td>
</tr>
<tr>
<td>6</td>
<td>224.8±1.7</td>
</tr>
<tr>
<td>4.75</td>
<td>224.9±27.4</td>
</tr>
<tr>
<td>3.5</td>
<td>150.6±25.3</td>
</tr>
<tr>
<td>2.75</td>
<td>158.3±17.7</td>
</tr>
<tr>
<td>1.75</td>
<td>124.3±22.5</td>
</tr>
<tr>
<td>1</td>
<td>100±13.2</td>
</tr>
</tbody>
</table>

The consumption of glycerol with 20gpl yeast extract is approximately 290% higher than that achieved by 1gpl yeast extract. The increase in the consumption of glycerol with increasing yeast extract concentration shows that nutrients required by the microorganism for efficient growth are found in yeast extract and those were the limiting factor when cultured in media containing lower amounts of yeast extract. Figure 5.10 illustrates the total consumption of glycerol by NGC after 72hrs of growth in media containing 50gpl glycerol with varying yeast extract concentrations. The total consumption increases linearly at lower concentration of yeast extracts and then tends towards a maximum value of glycerol being consumed. Increasing yeast extract concentrations above gpl leads to smaller increases and there does not seem to be much effect on total glycerol consumption once the yeast extract concentration exceeds 10gpl.
6.3.2 Analysis and Identification of Metabolites

In this study, an HPLC was utilized for the qualitative and quantitative determination of metabolites. This study will only be industrially relevant if the metabolites are chemicals that are value-added products and/or can be utilized for other applications. The HPLC paired with a refractive index detector provided qualitative and quantitative information for the metabolites produced. Figure 6.11 illustrates the individual chromatograms for compounds that were initially considered as the metabolites that could potentially be formed by the fermentation of glycerol by NGC.
A standard curve of each metabolite was created by analyzing increasing concentrations of known metabolites utilizing a HPLC. The standard curves were used for the quantitative determination of the fermented metabolites. Glycerol is eluted at 12.5 min and the intensity of the peak represents concentration. Acetic Acid shows the
weakest interaction with the chromatography column used and is thus the first metabolite to be measured. At 14.1min, the acetic acid peak is detected. Following the acetic acid is the elution of propionic acid as observed by the peak at 16.4min. The next two constituents are detected in short time span leading to a small overlap in their peaks. The butyric acid is retained for 19.7min in the HPLC column, whereas the ethanol is retained for 21.1min. These two peaks required simultaneous injections to ensure proper determination of the metabolite concentration. The LCSolutions software package allows for the extension of overlapping peaks to yield full area under the peak. This feature was utilized to calculate peak area values for butyric acid and ethanol. Butanol was the last eluent as observed by the peak at 35.3min. Butanol has the highest retention as it is the largest and most polar compound, which increases its interaction with the column and the retention time.

To identify the metabolites resulting from the consumption of glycerol by NGC, a series of series of aliquots were taken from the culture and analyzed using HPLC. Chromatograms for 0, 24, 48, 72 and 96hrs of growth in media containing 50gpl glycerol and 20gpl yeast extract are shown in Figure 6.12.
Figure 6.12-HPLC chromatograms obtained from NGC culture grown in BD6 medium containing 50gpl glycerol and 20gpl yeast extract. Chromatograms for growth media and cultures after 24, 48, 72 and 96 hrs of growth are shown.
Consumption of glycerol and the formation of metabolites can be observed in the chromatograms shown in Figure 6.12 and Figure 6.13. Glycerol is consumed as NGC grows. This consumption is seen as the decrease in peak height shown in the above figure. Acetic and propionic acid are the first metabolites formed by the bacteria. The acetic and propionic acid peaks can be observed after 24hrs growth, whereas at which instance the other metabolites are yet to form in detectable concentrations. Butyric acid has minimal production at 24hrs, but after 48hrs majority of the butyric acid had
formed. Ethanol can be observed in the chromatogram after 48hrs. Butanol is also produced after 48hrs, but continues to increase in concentration as growth proceeds. The production of metabolites is discussed in the following sections.

6.3.3 Acetic Acid

Acetic acid is a two carbon acid, microbially synthesized through the Acetyl-CoA enzyme after the production of an ATP as shown in Figure 6.2. Each mol of glycerol will yield 1.5 moles each of acetic acid and hydrogen.

\[
2 \text{Glycerol} \rightarrow 3 \text{Acetic Acid} + 3 \text{Hydrogen gas}
\]

(Equation 6.1)

Acetic acid is used in various applications ranging from an additive in the food industry to a solvent for carbocations [88, 89]. Majority of the produced acetic acid is utilized in the production of vinyl acetate monomer [89]. Biological production of acetic acid accounts for 10% of the total global demand of acetic acid [90]. Majority of the biologically produced acetic acid is used in the food industry, as the food industry has stringent controls on the purity of the acetic acid.
Figure 6.14-Formation of Acetic acid versus time by NGC grown in media BD5 and BD6.

Acetic acid production from NGC is shown in Figure 6.14 for media containing 1gpl yeast extract but either 5 or 50gpl glycerol. It is clear that the formation of acetic acid is dependent on the initial glycerol concentration and greatly increases as the initial concentration of glycerol is increased. The amount of acetic acid produced increases from less than 0.2gpl in BD5 medium to over 0.4gpl in BD6 medium. To understand how increasing the concentration of yeast extract affects acetic acid production, the cultures were grown in varying amounts of yeast extract and analyzed using HPLC at various times.
Acetic acid production from NGC is shown in Figure 6.15. It is clear that the formation of acetic acid is dependent on the yeast extract concentration and greatly increases as the concentration of yeast extract is increased. However, as observed below, acetic acid is not the preferential product formed in the fermentation of glycerol. The maximal acetic acid concentration of $1.4 \pm 0.1 \text{gpl}$ was observed after 72hrs growth in BD6 medium containing 20gpl yeast extract concentration. *Clostridium butyricum*
resulted in an acetic acid production of 0.8gpl with the consumption of 20gpl glycerol in 24hrs [91]. This is compatible to that produced by NGC. However NGC is able to produce 1.0±0.1gpl acetic acid with consumption of 17.8±0.5gpl glycerol with a starting yeast extract concentration of 6gpl in 24hrs. NGC also showed improved acetic acid production rates. *C. butyricum* was capable of acetic acid production rates of 0.033 g/L/h within 24hrs. Within the same 24hr growth time frame, the acetic acid production rate observed in NGC culture was 0.042 g/L/h. When the production rates are compared, again, NGC is superior to *C. butyricum*. NGC has ~25% higher production of acetic acid in the same amount of time.

![Normalized Acetic Acid Production at 72hrs](image)

**Figure 6.16-** Effect of yeast extract concentration on Acetic acid production after 72 hrs of growth in BD6 media containing 50gpl glycerol. Data shown is normalized to glycerol consumption in media containing 1gpl yeast extract.
The effect of yeast extract concentration on the production of acetic acid can be observed in Figure 6.16. As expected, the highest concentration of yeast extract yields the highest acetic acid yield. Table 6.5 shows normalized values shown in Figure 6.14.

**Table 6.5** The table below shows the Acetic acid production (after 72 hours of growth), normalized with respect to glycerol consumption in media containing 1gpl yeast extract.

<table>
<thead>
<tr>
<th>Concentration of yeast extract (gpl)</th>
<th>Normalized percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>337.1±11.4</td>
</tr>
<tr>
<td>10</td>
<td>267.5±1.1</td>
</tr>
<tr>
<td>6</td>
<td>275.4±4.7</td>
</tr>
<tr>
<td>4.75</td>
<td>213.0±21.0</td>
</tr>
<tr>
<td>3.5</td>
<td>200.6±28.9</td>
</tr>
<tr>
<td>2.75</td>
<td>193.1±18.4</td>
</tr>
<tr>
<td>1.75</td>
<td>168.9±3.4</td>
</tr>
<tr>
<td>1</td>
<td>100±48.9</td>
</tr>
</tbody>
</table>

The production of acetic acid with 20gpl yeast extract is approximately 340% higher than that observed from culture grown in media containing 1gpl yeast extract. The overall production of acetic acid does not have a 1:1 correlation to yeast extract concentration i.e. the production is not 20x greater for 20gpl when compared to 1gpl. This change in the ratio of yeast extract to acetic acid formation is evidence that the formation of other products is favorable with a higher consumption of glycerol. Figure 6.17 illustrates the effect of yeast extract concentration for acetic acid production at 72hrs of growth. For a given yeast extract concentration an estimate for the acetic acid production can be interpolated.
Figure 6.17- Acetic acid production after 72hrs of growth in a media containing 50gpl glycerol and varying yeast extract concentrations.

Figure 6.18- Molar ratio of acetic acid production over glycerol consumption after 72hrs in a media containing 50gpl glycerol and varying yeast extract concentrations.
Figure 6.18 illustrates the selectivity of NGC for the production of acetic acid at 72 hrs of growth. The molar ratio of acetic acid produced initially increases with increasing concentration of yeast extract, but then remains constant at ~0.06. NGC grown in media containing 50gpl glycerol and 1.75gpl yeast extract showed the highest selectivity at a ratio of 0.08 (moles acetic acid produced)/(moles glycerol consumed). Typically lower yeast extract concentrations resulted in a higher acetic acid selectivity. With the lower amount of nutrients present, the pH does not change sufficiently to allow for the production of alcohols, thus leading to the formation of more acid relative to alcohol.

6.3.4 Propionic Acid

Propionic acid is a three carbon acid, microbially synthesized through the succinic acid route of the metabolic pathway. Each mol of glycerol will yield 1 mol each of propionic acid and water.

\[
\text{Glycerol} \rightarrow \text{Propionic Acid} \quad \text{(Equation 6.2)}
\]

Cellulose-based plastics, herbicides, solvents and perfumes are produced using propionic acid. Further, it can function as an antifungal agent in food and feed [81, 92]. Due to the various applications of propionic acid, there is an ever present need for
propionic acid to be produced biologically. Through the production of propionic acid biologically, the process would be considered renewable.

![Propionic Acid Production](image_url)

**Figure 6.19-** Formation of Propionic acid versus time by NGC grown in media BD5 and BD6.

Propionic acid production from NGC is shown in Figure 6.19 for media containing 1gpl yeast extract, but either 5 or 50gpl glycerol. It is clear that the formation of propionic acid is dependent on the initial glycerol concentration and greatly increases as the initial concentration of glycerol is increased. The amount of propionic acid produced increases from less than 2gpl in BD5 medium to ~4gpl in BD6 medium. At these concentrations, propionic acid is produced at 10 times the concentrations of acetic acid. To understand how increasing the concentration of yeast extract affects
propionic acid production, the cultures were grown in varying amounts of yeast extract and analyzed using HPLC at various times.

![Propionic Acid Production Graph](image)

Figure 6.20- Formation of Propionic acid versus time by NGC grown in media containing 50gpl glycerol with varying yeast extract concentration.

Propionic acid production from NGC is shown in Figure 6.20. It is clear that the formation of propionic acid is dependent on the yeast extract concentration and greatly increases as the concentration of yeast extract is increased. Propionic acid is a preferential product formed in the fermentation of glycerol. Majority of the propionic
acid produced takes place within the first 24hrs of growth. The highest concentration of propionic produced was 8.44±0.32gpl, which was achieved after 48hrs in NGC cultures in a media containing 50gpl glycerol and 20gpl yeast extract. This is a lower amount when compared to that of P. acidipropionici [81]. P. acidipropionici has been shown to be the most efficient strain at producing propionic acid from glycerol when compared to Propionibacterium acnes and Clostridium propionicum [81]. Using NGC, the overall production of propionic acid was 8.4±0.3 gpl at 48hrs with a starting concentration of 50gpl glycerol and 20gpl of yeast extract. When compared to the 13.6gpl of propionic acid produced by P. acidipropionici cultured in a media with a starting glycerol concentration of 20gpl after 73hrs, it is apparent that NGC does not exhibit the same conversion efficiency [81]. However, when the production rates are compared, NGC shows an advantage over P. acidipropionici. P. acidipropionici achieved a propionic acid production rate of 0.18 g/L/h within 73hrs. Within the first 24hrs, the production rate of propionic acid for NGC was observed at 0.33g/L/h, or almost twice that of P. acidipropionici. NGC has a more rapid production of propionic acid, which can correlate to a higher production in a lower amount of time.
Figure 6.21 - Effect of yeast extract concentration on Propionic acid production after 72 hrs of growth in BD6 media containing 50gpl glycerol. Data shown is normalized to glycerol consumption in media containing 1 gpl yeast extract.

The effect of yeast extract concentration on the production of propionic acid can be observed in Figure 6.21. As expected, the highest concentration of yeast extract yields the highest propionic acid yield. Table 6.6 shows normalized values shown in Figure 6.21.

The difference in propionic acid concentration between growth in media containing 1gpl and 20gpl yeast extract is ~220%. The overall production of propionic acid does not have a 1:1 correlation to yeast extract concentration, i.e. the production is
not 20x greater for 20gpl when compared to 1gpl. Therefore, another constituent must be synthesized to account for the stoichiometrically lower production of propionic acid. The constituent change the pH of the system thus changing the metabolic pathway for the formation of products. Figure 6.20 illustrates the effect of yeast extract concentration for propionic acid production at 72hrs of growth.

*Table 6.6- The table below shows the Propionic acid production (after 72 hours of growth), normalized with respect to glycerol consumption in media containing 1gpl yeast extract.*

<table>
<thead>
<tr>
<th>Concentration of yeast extract (gpl)</th>
<th>Normalized percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>221.6±1.9</td>
</tr>
<tr>
<td>10</td>
<td>194.5±2.4</td>
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<tr>
<td>2.75</td>
<td>119.4±1.7</td>
</tr>
<tr>
<td>1.75</td>
<td>105.3±0.9</td>
</tr>
<tr>
<td>1</td>
<td>100±0.1</td>
</tr>
</tbody>
</table>
Figure 6.22- Propionic acid production after 72hrs of growth in a media containing 50gpl glycerol and varying yeast extract concentrations.

Figure 6.23- Molar ratio of propionic acid production over glycerol consumption after 72hrs in a media containing 50gpl glycerol and varying yeast extract concentrations.
Figure 6.23 illustrates the selectivity of NGC for the production of propionic acid at 72hrs of growth. NGC grown in BD6 media containing 50gpl glycerol and 1 gpl yeast extract shows the highest selectivity at a ratio of 0.46 (moles propionic acid produced)/(moles glycerol consumed). The selectivity for propionic acid production appears to be inversely related to the concentration of the yeast extract. The lower concentrations of yeast extract favor the production of propionic acid. Similar to acetic acid, lower amount of nutrients present in the media will lead to lower pH which in turn will limit the production of alcohols and increase the formation of more acid when compared to alcohol. This effect is observed more profoundly with propionic acid as opposed to acetic acid.

6.3.5 Butyric Acid

Butyric acid is a four carbon acid, synthesized microbially by utilizing the Acetyl-CoA enzyme then the Butyrl-CoA enzyme with the formation of an ATP. Each mol of glycerol will yield 0.5mol of butyric acid, 2mol of hydrogen gas and 1mol of carbon dioxide.

\[
\begin{align*}
2 \text{Glycerol} & \rightarrow 4 \text{H}_2 \text{gas} + 2 \text{CO}_2 \\
\text{Butyric Acid} &
\end{align*}
\]

\((\text{Equation 6.3})\)

Butyric acid is utilized in the animal feed stock industry as an additive to reduce pathogenic bacteria colonization [93]. Also due to its powerful, unpleasant odor it is
used as an additive in fish bait [94]. Due to its limited use and nasally offensive nature, butyric acid production is generally not seen as favorable. However, through esterification butyric acid can form butanoates which generally smell and taste pleasant. Butanoates are utilized in the food industry.

![Butyric Acid Production Graph](image)

**Figure 6.24**- Formation of Butyric acid versus time by NGC grown in media BD5 and BD6.

Butyric acid production from NGC is shown in Figure 6.24 for media containing 1gpl yeast extract, but either 5 or 50gpl glycerol. It is clear that the formation of butyric acid is dependent on the initial glycerol concentration and greatly increases as the initial concentration of glycerol is increased. The amount of butyric acid produced during growth in BD5 medium is insignificant. Less than 0.2gpl butyric acid produced during growth in BD6 medium. At these concentrations, butyric acid is produced is lower than
the concentrations of acetic acid. To understand how increasing the concentration of yeast extract affects butyric acid production, the cultures were grown in varying amounts of yeast extract and analyzed using HPLC at various times.

Figure 6.25-Formation of Butyric acid versus time by NGC grown in media containing 50gpl glycerol with varying yeast extract concentration.

Butyric acid production from NGC is shown in Figure 6.25. It is clear that the formation of butyric acid is dependent on the yeast extract concentration and greatly
increases as the concentration of yeast extract is increased. The difference in butyric acid produced in media containing 6gpl yeast extract relative to 10gpl yeast extract is significant when compared to the formation of other fermented acids. As with acetic acid, butyric acid is not the preferred fermentation product. The maximal butyric acid concentration is observed after 72hrs regardless of yeast extract concentration. The highest concentration of butyric acid produced was 1.3±0.1gpl after NGC was cultured for 72hrs in media containing 50gpl glycerol and 20gpl yeast extract. This is an insignificant amount when compared to prolific butyric acid producers such as C. butyricum [84]. C. butyricum was capable of producing 4.6gpl of butyric acid from 50gpl glycerol in only 17hrs. Not only does C. butyricum show improved overall production, it also produces butyric acid at a greater rate than NGC. C. butyricum achieved a butyric acid production rate of 0.27 g/L/h within 17hrs. Meanwhile, NGC was limited to a butyric acid production rate of only 0.029g/L/h with 20gpl yeast extract concentration in 24hrs. C. butyricum has a more rapid production of butyric acid, which can correlate to a higher production in a lower amount of time. This limited production of butyric acid is desirable for fermentation of glycerol by NGC as butyric acid is an unwanted product. The reason for the limited butyric acid could be butyric acid is one of the final products in the metabolic pathway and is not energetically favorable as new hydrocarbon bonds are formed.
The effect of concentration of yeast extract contained in the media on the production of butyric acid can be observed in Figure 6.26. As expected, the highest concentration of yeast extract enhances growth and glycerol utilization leading to the highest butyric acid yield. Table 6.7 shows normalized values shown in Figure 6.26.
Table 6.7- The table below shows the Butyric acid production (after 72 hours of growth), normalized with respect to glycerol consumption in media containing 1gpl yeast extract.

<table>
<thead>
<tr>
<th>Concentration of yeast extract (gpl)</th>
<th>Normalized percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>993.2±17.3</td>
</tr>
<tr>
<td>10</td>
<td>912.7±50.8</td>
</tr>
<tr>
<td>6</td>
<td>371.1±15.4</td>
</tr>
<tr>
<td>4.75</td>
<td>265.1±119.0</td>
</tr>
<tr>
<td>3.5</td>
<td>227.9±2.0</td>
</tr>
<tr>
<td>2.75</td>
<td>180.8±78.8</td>
</tr>
<tr>
<td>1.75</td>
<td>113.6±37.9</td>
</tr>
<tr>
<td>1</td>
<td>100±14.2</td>
</tr>
</tbody>
</table>

The production of butyric acid when NGC is cultured in BD6 media containing 20gpl yeast extract is approximately 10-fold greater than that observed from culture grown in BD6 media with 1gpl yeast extract. The increased overall production of butyric acid at 72hrs does, unlike the other acids, correlate to the increase in yeast extract concentration. Figure 6.27 illustrates the effect of yeast extract concentration on the total amount of butyric acid produced after 72hrs of growth. Since the production of butyric acid increases so greatly with an increase in the consumption of glycerol, to limit butyric acid all that would need to be done is limit the consumption of glycerol. This method would not be desirable as the goal of this research is to more efficiently consume glycerol into viable products. Since butyric acid formation is one of the final processes in the metabolic pathway and requires longer fermentation time to appear,
another strategy would be to shorten the time allowed for fermentation to prevent higher amounts of butyric acid from being produced.

Figure 6.27- Butyric acid production after 72hrs of growth in a media containing 50gpl glycerol and varying yeast extract concentrations.
Figure 6.28- Molar ratio of butyric acid production over glycerol consumption after 72hrs in a media containing 50gpl glycerol and varying yeast extract concentrations.

Figure 6.28 illustrates the selectivity of NGC for the production of butyric acid at 72hrs of growth. NGC grown in BD6 media shows low selectivity at a ratio of 0.04 (moles butyric acid produced)/(moles glycerol consumed) for NGC cultures in media containing 50gpl glycerol and 20gpl yeast extract. Unlike the other metabolic acids, butyric acid shows a higher selectivity when provided with a higher yeast extract concentration.
6.3.6 Ethanol

Ethanol is a two carbon alcohol, microbially synthesized through the reduction of acetaldehyde as shown through the metabolic pathway. Each mol of glycerol yields 1 mol each of ethanol, hydrogen gas and carbon dioxide.

\[
\begin{align*}
\text{HO-} & \quad \text{HO} \\
\text{OH} & \quad \text{H} \\
\text{OH} & \quad \text{O} \\
\text{Glycerol} & \quad \text{Ethanol} + \quad \text{H} \quad \text{Hydrogen Gas} + \quad \text{O} \quad \text{Carbon Dioxide}
\end{align*}
\]

(Equation 6.4)

Ethanol is most popularly produced via fermentation of simple sugars by yeast [95]. A more efficient method for the production of ethanol is described in chapter 1 using \textit{Z. mobilis} in an electrochemical cell. Ethanol can be used as a fuel additive or as a fuel by itself for transportation needs. Global production of ethanol in 2006 was 514.855*10^6 gal, with over 69% of the world supply coming from Brazil and the United States [96]. Using glycerol, the waste byproduct from the production of biodiesel transportation fuel, to produce ethanol, another liquid transportation fuel is a twofold approach for the production of a renewable fuel.
Ethanol production from NGC is shown in Figure 6.29 for media containing 1gpl yeast extract, but either 5gpl (BD5) or 50gpl (BD6) glycerol. It is clear that the formation of ethanol is not directly dependent on the initial glycerol concentration as both 5gpl and 50gpl glycerol leads to production of an insignificant amount (less than 0.2gpl) of ethanol. At these concentrations, ethanol produced is lower in concentrations of organics acid discussed so far. To understand how increasing the concentration of yeast extract affects ethanol production, the cultures were grown in varying amounts of yeast extract and analyzed using HPLC at various times.
Ethanol production from NGC cultured in media containing various amounts of yeast extracts is shown in Figure 6.30. The formation of ethanol greatly increases as the concentration of yeast extract is increased. In the presence of larger amounts of yeast extract, ethanol production is substantially larger than that observed by acetic and butyric acid. However, ethanol is the third highest produced product, with little ethanol...
being produced at low yeast extract concentrations. The maximal ethanol concentration is achieved after 72hrs regardless of yeast extract concentration. The highest concentration for ethanol produced was 2.9±0.3gpl which was observed after NGC was cultured for 72hrs in BD6 medium containing 50gpl glycerol and 20gpl yeast extract. NGC culture results in a better ethanol production when compared to the *Klebsiella planticola* strain isolated from red deer [83]. *K. planticola* was capable of producing 1.75gpl of ethanol in only 96hrs. NGC is also capable of producing ethanol at a much more rapid rate than *K. planticola*. *K. planticola* achieved an ethanol production rate of 0.028 g/L/h within 48hrs. NGC demonstrated an ethanol production rate of 0.054g/L/h, or almost twice that of *K. planticola* when cultured in BD6 medium containing 20gpl yeast extract concentration for 48hrs. NGC has a more rapid production of ethanol, which can correlate to almost twice the production in the same amount of time.
Figure 6.31 - Effect of yeast extract concentration on Ethanol production after 72 hrs of growth in BD6 media containing 50gpl glycerol. Data shown is normalized to glycerol consumption in media containing 1gpl yeast extract.

The effect of concentration of yeast extract on the production of ethanol can be observed in Figure 6.31. As expected, the highest concentration of yeast extract yields the highest ethanol yield. Table 6.8 shows normalized values shown in Figure 6.31.
The table below shows the Ethanol production (after 72 hours of growth), normalized with respect to glycerol consumption in media containing 1gpl yeast extract.

<table>
<thead>
<tr>
<th>Concentration of yeast extract (gpl)</th>
<th>Normalized percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>2114.5±108.9</td>
</tr>
<tr>
<td>10</td>
<td>2051.2±44.6</td>
</tr>
<tr>
<td>6</td>
<td>565.7±65.9</td>
</tr>
<tr>
<td>4.75</td>
<td>489.1±355.6</td>
</tr>
<tr>
<td>3.5</td>
<td>296.2±19.3</td>
</tr>
<tr>
<td>2.75</td>
<td>221.5±112.0</td>
</tr>
<tr>
<td>1.75</td>
<td>158.7±54.1</td>
</tr>
<tr>
<td>1</td>
<td>100±15.5</td>
</tr>
</tbody>
</table>

The production of ethanol by NGC cultured in BD6 medium with 20gpl yeast extract is greater than 20-fold compared to the culture grown in BD6 medium with 1gpl yeast extract. The increased overall production of ethanol at 72hrs does not have a 1:1 correlation to yeast extract concentration. The increase in ethanol production could be attributed to a pH change in the solution thus changing the metabolic pathway [82]. Figure 6.32 illustrates the effect of yeast extract concentration on ethanol production after 72hrs of growth.
Figure 6.32- Ethanol production after 72hrs of growth in a media containing 50gpl glycerol and varying yeast extract concentrations.

Figure 6.33- Molar ratio of Ethanol production over glycerol consumption after 72hrs in a media containing 50gpl glycerol and varying yeast extract concentrations.
Figure 6.33 illustrates the selectivity of NGC for the production of ethanol at 72hrs of growth. NGC grown in BD6 media shows the lowest selectivity at a ratio of 0.026 (moles ethanol produced)/(moles glycerol consumed) for NGC cultures in media containing 50gpl glycerol and 1gpl yeast extract. However, NGC grown in BD6 media containing 50gpl glycerol and 10gpl yeast extract leads 10-fold greater selectivity as seen by the selectivity ratio of 0.20 moles ethanol produced to moles glycerol consumed. Similar to butyric acid, ethanol shows a higher selectivity for a higher yeast extract concentration.

6.3.7 Butanol

Butanol is a four carbon alcohol, microbially synthesized through the reduction of butyraldehyde as shown through the metabolic pathway. Each mol of glycerol will yield 0.5 moles each of butanol and water as well as 1 mol each of hydrogen gas and carbon dioxide. Equation 6.5 shows the chemical structure and stoichiometric coefficients for the production of ethanol from glycerol.

\[
\begin{align*}
2 \text{Glycerol} & \rightarrow 2 \text{Butanol} + 2 \text{H}_2 \text{O} + 2 \text{H}_2 \text{H} + 2 \text{O}_2 \text{C}_2 \text{O} \\
\end{align*}
\]

*(Equation 6.5)*

Butanol is a by-product of the fermentation of sugars and carbohydrates [97]. Butanol is used in perfumes. Butanol is widely used industrially as a solvent for the
extraction of oils [98]. Butanol is gaining traction as a possible renewable transportation fuel as it has nearly double the energy content as ethanol[99].

![Butanol Production](image)

Figure 6.34- Formation of butanol versus time by NGC grown in media BD5 and BD6.

Butanol production from NGC is shown in Figure 6.34 for media containing 1gpl yeast extract, but with either 5gpl (BD5) or 50gpl (BD6) glycerol. It is clear that the formation of butanol directly dependent on the initial glycerol concentration as media containing 5gpl and 50gpl glycerol lead to production of ~0.2 and 1.2gpl of butanol, respectively. At these concentrations, butanol is produced in much higher concentrations than ethanol discussed above. To understand how increasing the concentration of yeast extract affects butanol production, the cultures were grown in varying amounts of yeast extract and analyzed using HPLC at various times.
Butanol production observed in NGC cultured in BD6 media containing varying amounts of yeast extract is shown in Figure 6.35. The concentration of butanol greatly
increases as the concentration of yeast extract is increased. There is a considerable lag in the formation of butanol. This delay is a direct result of the pH of the system [82]. Butanol is produced at a much higher level than all of the previous products except for propionic acid. The maximal butanol concentration is observed after 96hrs regardless of yeast extract concentration. The highest concentration for butanol production of 3.9±0.4gpl was seen after 96hrs with NGC cultured in BD6 medium with 50gpl glycerol and 10gpl yeast extract. Butanol is the only product that showed higher production with a lower than 20gpl concentration of yeast extract. However, based on range observed in the triplicates, the concentration of butanol observed in media with both 10 gpl and 20gpl yeast extract may be in the same range. NGC does not show comparable butanol production when compared to C. pastuerianum. C. pastuerianum was capable of producing 21gpl of butanol in only 72hrs [85]. C. pastuerianum achieved an butanol production rate of 0.29 g/L/h within 72hrs [85]. NGC cultures achieved an butanol production rate of 0.072g/L/h with 10gpl yeast extract concentration in 48hrs. Furthermore, results from Beibel showed a butanol production rate of 0.566 g/L/h within 30hrs [82].
Figure 6.36- Effect of yeast extract concentration on Butanol production after 72 hrs of growth in BD6 media containing 50gpl glycerol. Data shown is normalized to glycerol consumption in media containing 1gpl yeast extract.

The effect of yeast extract concentration on the production of butanol can be observed in Figure 6.36. Table 6.9 shows normalized values shown in Figure 6.36.
Table 6.9: The table below shows the Butanol production (after 72 hours of growth), normalized with respect to glycerol consumption in media containing 1 gpl yeast extract.

<table>
<thead>
<tr>
<th>Concentration of yeast extract (gpl)</th>
<th>Normalized percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>340.8±4.8</td>
</tr>
<tr>
<td>10</td>
<td>344.9±7.5</td>
</tr>
<tr>
<td>6</td>
<td>328.7±0.7</td>
</tr>
<tr>
<td>4.75</td>
<td>275.9±55.4</td>
</tr>
<tr>
<td>3.5</td>
<td>215.6±42.6</td>
</tr>
<tr>
<td>2.75</td>
<td>175.5±14.2</td>
</tr>
<tr>
<td>1.75</td>
<td>123.2±16.8</td>
</tr>
<tr>
<td>1</td>
<td>100±15.3</td>
</tr>
</tbody>
</table>

The production of butanol by NGC cultured in BD6 medium containing 10gpl yeast extract is approximately 340% greater than that produced in BD6 medium containing 1gpl yeast extract. The increased overall production of butanol at 72hrs does not have a 1:1 correlation with the yeast extract concentration. The increase in butanol production could be attributed to a pH change in the solution thus changing the metabolic pathway [82]. Figure 6.37 illustrates the effect of yeast extract concentration on butanol production after 72hrs of growth.
Figure 6.37- Butanol production after 72hrs of growth in a media containing 50gpl glycerol and varying yeast extract concentrations.

Figure 6.38- Molar ratio of butanol production over glycerol consumption after 72hrs in a media containing 50gpl glycerol and varying yeast extract concentrations.
Figure 6.38 illustrates the selectivity of NGC for the production of butanol at 72hrs of growth. NGC cultured in BD6 media containing 1gpl yeast extract shows the lowest selectivity at a ratio of 0.19 (moles butanol produced)/(moles glycerol consumed). The highest selectivity ratio seen is ~0.18 for NGC cultures in media containing 50gpl glycerol and ~6gpl gpl yeast extract. Thus, the selectivity is relatively unaffected by concentration of yeast extract. In fact, butanol and acetic acid are the only metabolic products that show a selectivity that is not dependent on yeast extract concentration.

6.3.8 Gas Production
As discussed in the following section, NGC produces hydrogen and carbon dioxide gas. The production of these gases leads to increases in pressure. Pressure was observed to increase with time, and was monitored, for NGC growth in both BD6 media containing 50gpl glycerol and 10 and 20gpl yeast extract. The pressure shown in the Figure 6.39 below is that of the gas measured in the headspace of a 130ml serum bottle containing 22 ml of culture. As shown in Figure 6.39, majority of the gas production occurs within the first 48hrs of growth.
Figure 6.39-Pressure generated from NGC grown in BD6 media containing 50gpl glycerol and varying yeast extract concentrations.

Figure 6.40-Total molar gas production from NGC cultured in BD6 media containing 50gpl glycerol and varying yeast extract concentrations. Results are normalized to pressure observed from NGC cultured in BD6 media containing 50gpl glycerol and 1gpl yeast extract.
The effect of yeast extract concentration on the production of gas can be observed in Figure 6.40. Table 6.10 shows normalized values shown in Figure 6.40. The production of gas with 20gpl yeast extract is ~5-fold greater than the pressure measured from NGC culture grown in BD6 media with 1gpl yeast extract.

**Table 6.10—Total molar gas production. Results are normalized to gas production observed in BD6 medium with 1gpl yeast extract.**

<table>
<thead>
<tr>
<th>Concentration of yeast extract (gpl)</th>
<th>Normalized percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>503.2</td>
</tr>
<tr>
<td>10</td>
<td>479.2</td>
</tr>
<tr>
<td>1</td>
<td>100</td>
</tr>
</tbody>
</table>

Using the stoichiometric relationship, solved for in the previous section, the amount of gas produced can be determined. Knowing that the stoichiometric relationship between hydrogen and carbon dioxide gas is 1:1, the concentration of gas produced can be determined. In the following two sections the concentrations of the gases will be discussed.

**6.3.8.1 Hydrogen**

Hydrogen is a diatomic, gaseous molecule that is formed as a by-product to metabolism. Hydrogen is an important product as it can be burnt to produce a substantial amount of energy, or can be used in fuel cells to also produce energy. Using the molar ratio, the production of hydrogen can be determined. Figure 6.41 illustrates the effect of yeast extract concentration on the production of hydrogen as a function of time.
Figure 6.41 - Hydrogen produced by NGC cultured in BD6 media containing 50gpl glycerol and varying yeast extract concentrations.

The amount of gaseous hydrogen produced is limited as the culture continues to grow. Based on unpublished results from the laboratory, a negative feedback between headspace gas pressure and continued gas production is hypothesized to exist. However, no studies have been conducted to date to prove such a relationship. As the production of alcohols, increases the production of hydrogen gas is decreased. The production of alcohols requires the hydrogenation of aldehydes. This reduction consumes some of the hydrogen produced thus limiting the production of hydrogen gas.

NGC culture yields competitive hydrogen production when compared to *klebsiella planticola*. NGC produced 0.06gpl of hydrogen gas within 72hrs with NGC cultured in media containing 50gpl glycerol and 20gpl yeast extract concentration. *klebsiella planticola* produced only 0.02gpl of hydrogen after 1008hrs of growth [83].
6.3.8.2 Carbon Dioxide

Carbon dioxide is a gaseous molecule formed as a by-product of glycerol fermentation. Carbon dioxide is useful as the carbon source for methane production while using methanogenic microbes. The amount of production of carbon dioxide can be determined using molar ratio. Figure 6.42 illustrates the effect of yeast extract concentration on the production of carbon dioxide as a function of time.

![Carbon Dioxide Production Vs Time](image)

Figure 6.42- Carbon dioxide produced by NGC cultured in BD6 media containing 50gpl glycerol and varying yeast extract concentrations.

The amount of carbon dioxide gas produced is limited as the culture continues to grow. The carbon dioxide production is stoichimetrically equal to hydrogen and follows the same trend as the hydrogen production curve. Since gas production is almost halted
after 48 hours, carbon dioxide is also not produced in any measureable amount after 48 hours of growth.

6.3.9 Stoichiometry of Metabolites

Molar ratios can be used for elemental balances as a way to determine the amount of a product that is not easily measured. A molar ratio was utilized in this research to determine the amount of gas produced as the GC-TCD was used only as a qualitative analytical tool. An overall equation was developed to determine the amount of hydrogen, carbon dioxide and water. Also, a molar relationship as a function of time will provide an idea as to the time at which each metabolic pathway is utilized. This approach can be done as the concentrations of all of the aqueous phase products are known and determined by the HPLC. The biomass produced was neglected as at ~1.3%, it represented a small percentage of the overall carbon utilized. The overall equation for the consumption of glycerol can be seen in equation 6.6.

\[
\text{Glycerol} \rightarrow n \text{Acetic Acid} + m \text{Propionic Acid} + p \text{Butyric Acid} + q \text{Ethanol} + v \text{Butanol} + w \text{Hydrogen Gas} + s \text{Carbon Dioxide} + t \text{Water}
\]

\text{(Equation 6.6)}

The molar ratio was based on the molar production of each constituent as a function of glycerol consumed. For calculations, the NGC grown in a media containing 50gpl glycerol and 20gpl yeast extract at 72hrs was used. Since all calculations are performed at a total level of 1 liter medium, the magnitude of moles produces and
moles/liters produced are the same and have been used interchangeably. Equation 6.7 was used to determine the moles of glycerol:

$$\text{Mols\_Glycerol}(t) = \frac{(\text{Concentration}_0 - \text{Concentration}(t))}{92.09\frac{g}{mol}} = 0.293\text{mol\_consumed}$$

*(Equation 6.7)*

The above determined moles of glycerol was used in the ratio with the aqueous products. The moles of aqueous products was determined using the format outlined in equation 6.8.

$$\text{Mol\_Produced} (t) = \frac{\text{Concentration}(t)}{\text{MW\_product}} = \frac{1.3\frac{g}{L}}{60.05\frac{g}{mol}} = 0.022\frac{\text{mol}}{L}$$

*(Equation 6.8)*

Equation 6.8 uses acetic acid as a numerical example. The above equation yields the moles of product produced per liter of medium from the consumption of glycerol at a given time. Equation 6.9 gives the molar ratio of aqueous products to glycerol.

$$\text{Molar\_Ratio} = \frac{\text{Mol\_Produced} (t)}{\text{Mols\_Glycerol}(t)} = \frac{0.022\frac{\text{mol}}{L}}{0.293\frac{\text{mol}}{L}} = 0.075\frac{\text{Acetic\_Acid}}{\text{Glycerol}}$$

*(Equation 6.9)*

The above process was used to determine the molar ratio for all of the aqueous products. Table 6.11 shows the molar ratio for the remaining aqueous products.
Table 6.11 - Molar ratios for the aqueous fermentation products

<table>
<thead>
<tr>
<th>Aqueous Product</th>
<th>Molar Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic Acid</td>
<td>0.075</td>
</tr>
<tr>
<td>Propionic Acid</td>
<td>0.386</td>
</tr>
<tr>
<td>Butyric Acid</td>
<td>0.046</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.208</td>
</tr>
<tr>
<td>Butanol</td>
<td>0.167</td>
</tr>
</tbody>
</table>

The overall equation for the consumption of glycerol can now be rewritten as:

\[
\text{Glycerol} \rightarrow 0.075\text{H}_2\text{CO}_2\text{H} + 0.386\text{H}_2\text{C}_3\text{O}_2\text{H} + 0.0046\text{H}_2\text{C}_4\text{O}_2\text{H} + 0.208\text{H}_2\text{C}_2\text{O}_5\text{H} + 0.167\text{H}_2\text{CH}_2\text{OH} + \text{CO}_2 + \text{H}_2\text{O} + \text{H}_2\text{H}
\]

\text{Equation 6.10}

To complete the equation an elemental balance can be conducted. Out of the three products that need to be determined, carbon dioxide is the only one with a carbon molecule. Again, the carbon from the production of biomass was neglect as it represented on 1.3% of the total consumed. A carbon balance solved for the molar ratio of carbon dioxide with glycerol. Equation 6.11 shows the carbon balance.
Solving for \( s \), the obtained value is 0.43 mols carbon dioxide/mol glycerol. Then, oxygen balance was conducted in a manner similar to that of the carbon balance. Equation 6.12 shows the oxygen balance.

\[
3O = 0.075(2O) + 0.386(2O) + 0.046(2O) + 0.208(2O) + 0.167(2O) + t \cdot C
\]

\textit{(Equation 6.12)}

Solving for \( t \), the obtained value is 0.75 mols water/mol glycerol. Then, hydrogen balance was conducted to determine the amount of hydrogen produced during the bacteria metabolism. Equation 6.13 shows the hydrogen balance.

\[
8H = 0.075(4H) + 0.386(6H) + 0.046(8H) + 0.208(6H) + 0.167(10H) + 0.43(0) + 0.75(2H) + w \cdot (2H)
\]

\textit{(Equation 6.13)}

Solving for \( w \), the obtained value is 0.4 mols hydrogen/mol glycerol. With this last elemental balance the entire molar relationship was completed. Table 6.12 shows the molar ratio for all products formed.
Table 6.12 - Molar ratio of fermented products from glycerol.

<table>
<thead>
<tr>
<th>Fermentation Products</th>
<th>Molar Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic Acid</td>
<td>0.075</td>
</tr>
<tr>
<td>Propionic Acid</td>
<td>0.386</td>
</tr>
<tr>
<td>Butyric Acid</td>
<td>0.046</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.208</td>
</tr>
<tr>
<td>Butanol</td>
<td>0.167</td>
</tr>
<tr>
<td>Hydrogen Gas</td>
<td>0.400</td>
</tr>
<tr>
<td>Carbon Dioxide</td>
<td>0.430</td>
</tr>
<tr>
<td>Water</td>
<td>0.750</td>
</tr>
</tbody>
</table>

With information in Table 6.12, a final stoichiometric equation was determined and can be written as shown in equation 6.14.

\[
\begin{align*}
\text{Glycerol} & \rightarrow 0.075\text{H}_2\text{C}(\text{O})\text{OH} + 0.386\text{H}_2\text{C}(\text{O})\text{OH} + 0.0046\text{H}_2\text{C}(\text{O})\text{OH} + 0.208\text{H}_2\text{C}(\text{O})\text{OH} + 0.167\text{H}_2\text{C}(\text{O})\text{OH} + 0.4\text{H}_2\text{C}\text{H}_2\text{OH} + 0.43\text{C}=\text{C}=\text{O} + 0.75\text{H}_2\text{O} \\
\text{Acetic Acid} & \text{Propionic Acid} \text{Butyric Acid} \text{Ethanol} \text{Butanol} \text{Hydrogen Gas} \text{Carbon Dioxide} \text{Water}
\end{align*}
\]

\textit{(Equation 6.14)}

6.3.10 Characterization of Microbial Strain

The characterization of the environmental microbial strain isolated from Galena Creek, NV was conducted using 16s rDNA sequencing method. All bacteria contain the 16s rDNA gene, which makes distinguishing the variations practical. The 16s gene
sequence is sufficiently large at \(~1500\text{bp}\) (base pairs), to provide deterministic and statistically valid comparisons.

Table 6.13 - The Genetic Sequence from the 16s rRNA test to determine the species of bacteria.

<table>
<thead>
<tr>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGTATGGGGGCGAaGCCTGATGCAGCAACCCGCGGTAAGTGAGCTGGACTCTCCGGAATGGTA</td>
</tr>
<tr>
<td>AAGCTCTGTCTTTTGGGACGATAATGACGCTACCAAAGGAGGAAGCCACCGGCTACTAGCTGAAAG</td>
</tr>
<tr>
<td>TGCCAGCAGCCGCGGTATACGCTAGGTTGGCAAGCGGATCTGCTGGGATTTACTGGGCTAAGAG</td>
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<tr>
<td>GATGTGATGGCGGACATTAAAATTGAGATGCTGAAAGCCCGAGCTCAACTTGGGGACTGCAATGCA</td>
</tr>
<tr>
<td>TTTCAAATGTTGTCTAGAGTGCAACAGAAGGAAAGGGAATTCTATGTGAAGGCGTTGAA</td>
</tr>
<tr>
<td>ATGGCTAGAGATTAGGACACATGTCAGGCAAGGCGGTTTCTGGAAGCGTGAAGCTGACGCTGAGCTG</td>
</tr>
<tr>
<td>CTGAGGCATGAAACCGCGGAGCAACAGGAATATTAGATACCCCTGGAAGTGGGCGCTTTAGGAAAG</td>
</tr>
<tr>
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</tr>
<tr>
<td>TACTCCGGCTGGGAAAGTCGCGAGGATTAACCTCAGGATACCTAGCGTCAAAGGCGGGCACGCCCGC</td>
</tr>
<tr>
<td>ACAAGCAGCCGACGATGTTTAAATTGCAAGCAGGAAAGCCTTACCTAGCTAGTTAAGGCGCAGACG</td>
</tr>
<tr>
<td>ATGTTGGATCAGTCAGTGGTGGAGATGTTGGGAGGAAAGTCCGCCAACGAGCGAACCCCT</td>
</tr>
<tr>
<td>TATCATTAGTTGCTACCATTAATGGAGACACCTCTAGGAGACTGCCCCGGGTAAACCACGGA</td>
</tr>
<tr>
<td>GGAAGGCGGGGATAGCTGAAATCTCACTACGCTACCGGTGCTCTGCTAGGCTGCTA</td>
</tr>
<tr>
<td>CAAATGTTGAGAACACCGAGATGCAATACCGGAGGTGAGGACAAACTTTGGAAACACTCC</td>
</tr>
<tr>
<td>CAGTTGCAGATGGTACGCAAATCCGCTACATGAAAGTGGAGGCTAGTAAATGCGGCAA</td>
</tr>
<tr>
<td>TCAGAATGGTCCGCTGGAATACGTCTCCGGGGACTGTCACACCGCCGTACACCATGAG</td>
</tr>
<tr>
<td>AGCTG</td>
</tr>
</tbody>
</table>

The genetic sequence shown above was compared to a database. From the genetic database, it was determined, with a similarity of 0.991, the isolated microbial sample was *Clostridium sensu stricto* spp. *Clostridium sensu stricto* spp. is a nomenclature identifying clostridium strain. Further, GenBank identification for the strain of clostridium was conducted. The GenBank Accession number was found to be EU816420, corresponding to the species *Clostridium arbusti*. Based on literature, *Clostridium arbusti* has not yet been demonstrated to have the ability to ferment glycerol.
6.4 Conclusion

The focus of this study was to compare the effectiveness of NGC, a bacterial strain isolated locally in Reno, NV, to metabolize glycerol to form viable products. NGC was able to utilize increasing amounts of glycerol when provided with increasing amounts of yeast extract. A total of 27.4±0.2gpl glycerol was consumed when 20gpl yeast extract was provided. This is the most glycerol utilization that has been reported. NGC is more capable of fermenting glycerol to viable products in a shorter time than most other bacteria studied thus far. NGC produces the following products: acetic acid, propionic acid, butyric acid, ethanol, butanol, hydrogen and carbon dioxide. The major fermentation products that were formed in media containing high yeast extract concentration were propionic acid and butanol. The products produced in least amounts at high yeast extract concentrations is butyric acid, followed by acetic acid. The comparison of the amounts of fermented product after 72hrs of growth in a media containing 50gpl glycerol and 20gpl yeast extract is shown in Figure 6.43.

The stoichiometric equation for the consumption of glycerol after 72hrs in a media containing 50gpl glycerol and 20gpl yeast extract was determined and is shown below:
Figure 6.43- Amount and type of fermentation products formed after 72hrs of growth in a media containing 50gpl glycerol and 20gpl yeast extract concentration.

At lower concentrations of yeast extract, NGC produces a higher percentage of acetic acid and propionic acid. However, at higher concentrations of yeast extract, the alcohols and butyric acid are formed at a higher percentage.

NGC was characterized using a 16s rRNA sequencing method. This method was able to determine that NGC was *Clostridium arbusti* with a confidence of 0.991. This is the first study to report on a strain of *Clostridium arbusti* to have the ability to ferment glycerol.
Summary

Microbial electrochemistry covers a broad range of research from microbial fuel cells to fermentation of carbon food sources. The ability to harness microbial electron transfer can lead to a greener and cleaner future. This study was focused on microbial electron transfer applications in liquid fuel production, environmental remediation and consumption of unwanted byproducts.

An electrochemical fermentation reactor (EFR) was developed to enhance microbial growth rate by adding passive electrodes for the transfer of electrons from respiring microbes to dissolved oxygen. The EFR process was found to enhance growth kinetics by 22.2% and the ethanol production rate by 22.7%. The addition of an exogenous electron shuttle was found to lead to a 16.2% increased growth kinetics over control, which was minimal when compared to that of the EFR process. The exogenous electron shuttle appeared to limit the growth of bacteria as a result of limited electron removal. This was counter intuitive as the AQDS would allow for the removal of more electrons by serving as an electron storage medium. The graphite electrode showed surprisingly high growth rate enhancement of 19.3% for not having microbial growth on the surface. As expected, the electrochemically assisted reactor with a 10MΩ resistor resulted in the highest ethanol production rate increase of 26.6%.

An encapsulation method, which would also utilize exocellular transfer of electrons through direct contact, was hypothesized for the suspension of viable cells in a conductive polymer substrate. The FDMA polymer solution with minimal amounts of
carbon black was electrospun to produce viable encapsulated microbial cells. This conductive polymer substrate could have applications in bio-fuel production. Carbon black was added to a polymer solution to test electrospun polymer conductivity and cell viability. Polymer morphology and cell viability were imaged in both electron and optical microscopy. Once the polymer solution was electrospun, the polymeric fibers were less conductive. At a carbon black concentration of 12gpl, the conductivity only increased marginally. Cell viability decreased from 41.7% at 4gpl carbon black to 0% at 12gpl carbon black. Through proper encapsulation, higher fuel production efficiencies could be achieved.

Electron transfer through endogenous exocellular protein shuttles was observed, for the first time, in gram positive obligate anaerobes in this study. Existence of redox active exocellular proteins was shown using a novel experimental methodology utilizing a 2-cell apparatus. In the presence of the endogenous protein, the amount of goethite reduced was found to be 18-fold greater than in its absence. Cyclic voltammetry and gel electrophoresis were used to show the presence of the protein. Cyclic voltammetry showed the presence of two peaks; oxidation at 75.43mV and a reduction peak at 145.4mV. DLS determined the protein size was 172nm. The exocellular protein is capable of reducing ferrous iron in a membrane separated chamber. Confirmation of a redox active protein could reshape our understanding of the subsurface redox reactions.
The final topic in this dissertation discussed electron transfer within the cell for production of fermentation products from an unwanted chemical byproduct. Glycerol, which is an unwanted side-product of biodiesel transesterification, was utilized as the carbon source for fermentation. Bacterial samples isolated from Reno Galena Creek are (NGC) were found to be efficient in utilizing glycerol. NGC was characterized through 16s rDNA genetic sequencing and found to belong to the genus Clostridium. Glycerol consumption was observed to reach 27.4gpl within 72hrs when NGC was cultured in a media containing 50gpl glycerol. All observed fermentation metabolites were characterized and quantified through an HPLC and GC-TCD. The most prominent metabolite formed was propionic acid. This study has found that NGC has higher selectivity for low weight acids at lower yeast extract concentration and higher selectivity for larger acids and alcohols at higher yeast extract concentrations. The stoichiometric equation for the consumption of glycerol after 72hrs in a media containing 50gpl glycerol and 20gpl yeast extract was determined and is shown below:
Future Directions

It is suggested that in future, the electrochemical reactor be scaled up to pilot or pre-pilot levels to study if the laboratory observations hold good at larger scales. With a larger cell volume, the effect of electrode size can be determined. Also, with a larger cell volume the effect of electrode separation distance can also be determined. As the electrodes are moved further away from each other, the resistance of the system will increase. This could, as shown in this dissertation, actually improve the kinetics of the fermentation. Another avenue of research for this process would be to find the optimum resistance to achieve the maximum growth rate.

When looking at conductivity removal of electrons generated during fermentation, it is important to provide large surface areas for removing electrons from microbes that are being cultured. An avenue of further research for the encapsulation study would be to find a compound that could coat the electrospun polymer without sacrificing cell viability. Following identification of a conductive compound, electrospinning of the polymer solution should be done at as high a temperature as tolerated by microbes as this will prevent formation defects.

The most important task in understanding subsurface electron transfer would be the characterization of the protein responsible for the reduction of goethite. Once the protein has been characterized, a hypothesis can be created and tested for the mechanisms of subsurface electron transport. Further, such proteins may be used at industrial scales for environmental remediation. The characterization should be done
through protein isolation and crystallization. Another imperative aspect of this research to observe would be the microorganism’s ability to reduce constituents other than ferrous iron.

With respect to NGC, the utilization of crude glycerin formed during biodiesel production is paramount to the successful implementation of the process. This study utilized pure glycerin, purchased from chemical vendors, to conduct laboratory experiments. If NGC is capable of fermenting crude glycerin at high efficiencies, then no added energy would be required to purify glycerin generated during biodiesel production. Also, directed evolution is an effective tool in increasing not only the consumption of glycerol, but the tolerance of metabolites.
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Appendix
Enhanced Fermentation in an Electrochemical Reactor

Jason Hastings and Dev Chidambaram


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Introduction
Globally, there is an increased need to identify cost-effective renewable liquid fuels that can be used as a drop-in replacement to fossil fuels in the transportation sector. Ethanol can be used as a fuel source, production of smog-forming compounds and accumulation of global warming CO2 are limited. Certain classes of microbes convert biomass and carbohydrates to various alcohols and organic acids by fermentation. Fermentation can be used to produce ethanol, which is currently considered as a critical component in the replacement of liquid fossil fuels for transportation according to current government policies. This is clear from the congressional mandate requiring the blending of at least 36 billion gallons of ethanol into transportation fuels by the year 2017. Fermentation of glucose yields two main products: (i) ethanol and (ii) carbon dioxide [1].

Electrochemical engineering of fermentation processes to enhance the production rate of ethanol is yet to be studied in detail. This study demonstrates the advantage of the Enhanced Fermentation in an Electrochemical Reactor (EFER-Green) process, a technology that is currently patent-pending in the United States. While, this study will utilize a model system with glucose as the substrate for fermentation and ethanol as the sole byproduct, it should be noted that the process and principles are generally suited for the production of any desired product of fermentation including butanol and butyric acid, from a variety of substrates including cellulose from biomass.

Experimental
Zymomonas mobilis strains were obtained from ATCC and was cultivated in an incubator at 28°C in media consisting of (l-1) glucose, 20 g; yeast, 10 g; KH2PO4, 2g. This media was used for the sole purpose of maintaining bacterial growth. A one day old culture was used to inoculate the experimental reactor. Experiments were conducted in media consisting of (l-1) glucose, 100g; yeast, 10g; KH2PO4, 1g; MgSO4.7H2O, 0.5g; (NH4)2SO4, 1g. Various electrode materials were evaluated for their effectiveness.

Cultures were grown and aliquots were taken at the following times after inoculation: 16, 20, 24, 26, 28, 30, 32, 36 and 40 hours. The growth of the bacteria was monitored based on absorbance at 600nm obtained using an ultraviolet-visible spectrometer (Shimadzu, UV-1800). Glucose and ethanol concentrations were determined using a High-Performance Liquid Chromatograph (HPLC) equipped with a Refractive Index Detector.

Results and Discussion
The growth of bacteria in an electrochemical reactor (EFER-green process) resulted in an increase of ~35% over growth observed in a traditional batch reactor at 30hrs, as illustrated in Figure #1. The enhanced growth leads to an increase in the rate of production of ethanol. The EFER-green process shows an enhanced ethanol production rate of ~22% over the traditional batch process at 30hrs, also illustrated in Figure #1. It should be noted that while the kinetics of fermentation is enhanced in an electrochemical reactor, the yield of ethanol remains the same as that in a traditional reactor. This is because the yield of ethanol is determined by the initial concentration of glucose (carbon source). However, enhancing the production rate can lower fixed costs and ultimately increase the cost-competitiveness of bioethanol.

In conclusion, the results indicate that fermentation processes can be kinetically enhanced by use of electrochemical reactors.

References:
Bioelectrochemical Degradation of Acid Orange 7

Jason Hastings and Dev Chidambaram


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Bioreduction of Acid Orange 7

The catalytic properties of various metal nanoparticles have led to their use in environmental remediation applications. However, these remediation strategies are limited by their ability to deliver catalytic nanoparticles and hydrogen as a suitable electron donor to large treatment zones. Our aim was to develop and apply an efficient bioremediation method based on in-situ biosynthesis of bio-nanopalladium and hydrogen. C. pasteurianum BC1 cells, loaded with bio-nanopalladium, were used to effectively catalyze the reductive degradation and removal of Acid Orange 7 (AO7). Hydrogen produced fermentatively by the C. pasteurianum BC1 acted as the electron donor for the process, thus eliminating the need to add an external source as required by the process developed by De Windt et al. [1] and Lloyd et al. [2].

Bio-nanopalladium was formed using cultures of C. pasteurianum. The morphology of the bio-nanopalladium was observed using a SEM to confirm the formation of nanoparticles. Samples containing Pd(0) and controls without Pd(0) were inoculated with AO7 and the residual concentration of AO7 was monitored with time. Living bacterial cultures containing bio-nanopalladium showed significantly faster reductive degradation of the azo compound when compared to Pd(0)-free or heat-killed controls. Pd-free bacterial cultures or control kille controls without Pd(0) were inoculated with AO7 and the residual concentration of AO7 was monitored with time. Living bacterial cultures containing bio-nanopalladium showed significantly faster reductive degradation of the azo compound when compared to Pd(0)-free or heat-killed controls. Pd-free bacterial cultures or control experiments conducted with heat-killed cells showed negligible reduction of AO7. Our experiments also showed that the in-situ biological production of H2 by C. pasteurianum BC1 was essential for the degradation of AO7, which suggests a novel process where the in-situ microbial production of hydrogen is directly coupled to the catalytic bio-nanopalladium mediated reduction of AO7. These results are confirmed via analysis conducted using UV-Vis spectroscopy. A full spectrum analysis from 190nm-900nm was done on all samples using UV-Vis to determine absorbance. Concentration of AO7 can be monitored via intensity of peak at 484nm. Experiments containing 5ppm Pd showed a more rapid degradation of AO7 than those containing 1ppm and 3ppm Pd. For 50ppm AO7, the initial rates of reaction increased as Pd concentration increased; 0.63, 1.86 and 2.84hr\(^{-1}\) were the initial rates of reaction for 1, 3 and 5ppm Pd, respectively. A first order kinetic model was used to illustrate the reaction kinetics of samples containing both living microbes and bio-nanopalladium. Zero order curves were not modeled against experimental data, but were used in describing the rate of color change for samples without bio-nanopalladium. AO7 concentrations of 50 and 100ppm were not fully degraded by cultures containing 1ppm Pd. The quickest degradation of 50ppm AO7 was achieved in experiments containing 5ppm Pd and was approximately 2hr. The spectrum showed the gradual decline in the 484nm peak as well as the increase in the 330nm, 280nm and 275nm peaks. The 330nm and 286nm peaks represented absorption peaks of 2-naphtol, which were confirmed from the NIST database [3]. Other studies also found the same degradation products as found in this study [4, 5]. The minor peak at 275nm in the degradation products spectrum could indicate the presence of dibutyl phthalate, which is the autooxidation product of one of the two main hypothesized degradation products. Further, MALDI analysis confirmed the presence of 1-amino 2-naphthol, a constituent of AO7 as a breakdown product, in line with our hypotheses. Also, among 34 studies on microbial remediation of acid azo dyes, the process developed and presented in this thesis appears to have the fastest rate of degradation.

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Nanotechnology and Microbial Electrochemistry for Environmental Remediation

Ashley Johnson, Giorvanni Merilis, Jason Hastings, M. Elizabeth Palmer, Jeffrey Fitts and Dev Chidambaran

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Members of the genus Clostridia can reduce Pd(II) ions to form metallic Pd nanoparticles (bio-Pd). Cultures of C. pasteurianum BC1 were used to generate bio-Pd, which is primarily formed on the microbial cell wall. Batch experiments using C. pasteurianum BC1 cells loaded with bio-Pd showed efficient reduction of the organic azo dyes methyl orange and Evans Blue, while little reduction of dyes was observed in control experiments using Pd-containing heat-killed microbial cultures or Pd-free viable bacterial cultures. Degradation of azo dyes was found to occur via reductive hydrogenation of the azo-linkage. Molecular hydrogen, which is concomitantly generated by C. pasteurianum, is used in the reduction reaction. The process described in this study is a potentially viable alternative to current groundwater and wastewater treatment technologies that fail to adequately degrade the large quantities of hazardous spent textile dyes that are discharged into the environment each year.

Introduction

Metal nanoparticles, by virtue of their catalytic properties, have the potential to be exploited for applications in environmental remediation (1, 2). For instance, numerous studies have explored the use of Fe(0) nanoparticles to remove organic and inorganic contaminants (3). In the presence of a suitable electron donor, these nanomaterials can catalyze the reduction of pollutants, thereby rendering them insoluble. A variety of pollutants such as carcinogenic hexavalent chromium, and hexavalent uranium are soluble in their highly oxidized form and become sparingly soluble on reduction (to trivalent chromium and tetravalent uranium in the above case, respectively). Further, several organic compounds such as organic dyes, fertilizers, pharmaceutical compounds and intermediates undergo reductive degradation. In all these cases, the contamination levels in the subsurface environment and their downstream flow can be significantly controlled and managed. However, these remediation strategies are limited by their ability to deliver catalytic nanoparticles, in addition to a suitable electron donor, to large treatment zones.
A new biologically inspired method to produce a nanoparticulate catalyst involves the precipitation of transition metals such as palladium, gold and iron on bacterial surface, resulting in the formation of bio-nano-Met, where Met = transition metal catalyst. Two gram-negative model organisms have been primarily used to reduce Pd(II) and subsequently induce precipitation of Pd(0) nano-particles: the metal-respiring bacterium *Shewanella oneidensis* (4) and the sulphate-reducing bacterium *Desulfovibrio desulfuricans* (5). It is known that Pd and bio-Pd can catalyze the reduction of various groundwater and soil pollutants (4, 6, 7). However, all these processes utilize two-steps, wherein bio-nano-Met nanoparticles are formed in a separate reaction before contaminant treatment, thus making these processes less suitable to treat subsurface contaminants. Recently, the authors described the proof of concept for a process in which Pd nanoparticles were formed at the zone of interest along with the *in-situ* generation of molecular hydrogen leading to a reductive removal of hexavalent chromium (1). To our knowledge, a one-step nanoparticulate-based reductive process has not been demonstrated for organics. In this study, we present a proof of concept for such a process using two common azo dyes, methyl orange (MO) and Evans Blue (EB). EB is also known as Direct Blue 53. Azo dyes were chosen because they constitute the largest group of synthetic colorants of the more than 280,000 tons of textile dyes that are improperly discharged into the environment each year.

**Experimental**

Deionized water was used to make growth medium and in analysis. Chemicals used in this study were analytical grade or better. All liquid culture experiments were performed in triplicate.

**Culture:** *Clostridium pasteurianum* sp. BC1 (ATCC No. 53464) was grown as previously described (1, 8). The medium contained (per liter) glucose, 5.0 g; NH₄Cl, 0.5 g; glycerol phosphate, 0.3 g; MgSO₄.7H₂O, 0.2 g; CaCl₂.2H₂O, 0.5 g; FeSO₄.7H₂O, 2.8 mg; peptone, 0.1 g; yeast extract, 0.1 g. NaOH was used to adjust the pH of the medium to 6.8. The medium was pre-reduced by boiling for 15 min with simultaneous purging using filtered ultra-high-purity (UHP) nitrogen gas. Growth medium was then cooled and transferred to an anaerobic chamber. 40 mL aliquots of the medium were dispensed into 60 mL serum bottles. The bottles were sealed with butyl rubber stoppers and aluminum crimps, and autoclaved. All manipulations of the samples were carried out inside an anaerobic glove chamber (Coy Laboratory products, MI) filled with nitrogen containing trace amount of hydrogen for catalytic removal of any oxygen. Cultures were grown in an incubator shaker at 28°C and 125 rpm.

**Formation of Bio-Pd:** Cultures were grown until the end of the log phase of growth (O.D.₆₀₀nm = 0.6), which typically required 18 hours. Aqueous Pd(II) (1 g/L Na₂PdCl₄ stock solution) was then added to the cultures to achieve an initial Pd(II) concentration of 100 mg/L. As reported previously, the medium turned black within one minute, due to the reduction of Pd(II) ions to Pd(0), which was confirmed with X-ray diffraction and X-ray absorption spectroscopy (1). Scanning electron microscopy was performed to confirm the nanoparticulate nature of the Pd particles. The bio-Pd morphology and spatial distribution within the biomass were examined using a LEO 1550 SEM equipped with a Schottky Field-Emission Gun.
Batch studies of azo dye reduction: The native ability of *C. pasteurianum* BC1 to degrade the two dyes of interest was examined by studying the amount of dye degradation that occurs in the presence of live cells of *C. pasteurianum* BC1 and heat-killed cells of *C. pasteurianum*. MO and EB were added to bio-Pd suspensions using stock solutions to result in a final concentration of 450 μM and 48 μM, respectively. Dye concentrations and their breakdown products were analyzed using UV-Visible spectroscopy. These studies served as controls.

The following sets of experiments were conducted to monitor the effects of the addition of bio-Pd on the degradation of EB and MO. The bio-Pd was formed *in-situ* by the addition of requisite amount of Pd^{2+} from a stock of Na_{2}PdCl_{4}. The concentrations of bio-Pd used in this study were 1ppm, 5ppm and 10ppm. These results were compared to sets containing no bio-Pd, which were used as control. Starting concentrations of 450 μM and 48 μM were used for MO and EB, respectively.

Analytical Techniques: The concentration of azo dyes and their breakdown products in the cultures at different time intervals was determined spectrophotometrically. Methyl orange and Evans Blue concentrations were monitored using UV-Visible spectroscopy at 479 nm and 610 nm, respectively. Further, the degradation product of MO was qualitatively and quantitatively observed at 244 nm.

Results and Discussion

*C. pasteurianum* BC1 glycolysis produces fermentation products including hydrogen, acetate, butyrate and formate that create a highly reducing environment (1). When a culture of *C. pasteurianum* BC1 was supplemented with aqueous Pd(II), the Pd(II) was immediately reduced forming bio-Pd. Supplementing the medium with an initial Pd concentration of 1, 5 or 10 mg/L changed the color of the medium to gray within one minute and the aqueous Pd concentration was found to be below the detection limit of 1 mg/L. The intensity of the gray color was directly related to added Pd concentration; higher concentrations led to a darker color, as seen in Figure 1. In contrast, no color change was observed in an abiotic control starting with aqueous Pd(II) in sterile growth media. Thus the compounds in the growth medium did not significantly complex with Pd(II) ions or contribute measurably to Pd(II) reduction.. The gray color change has previously been reported under comparable conditions with *Shewanella oneidensis* (3) and *Desulfovibrio desulfuricans* (4) as an indicator of reductive precipitation of metallic Pd nanoparticles. The reduction of over 99% Pd(II) by *C. pasteurianum* BC1 within one minute is notably faster than the rates reported for *S. oneidensis* and *D. desulfuricans*, where black precipitates first appear after 5-10 minutes and complete reduction is only observed after approximately one hour (3, 4).

Scanning electron microscopy images showed bio-Pd to be precipitated mostly on the bacterial cell wall and to a lesser extent in the extracellular region. As shown earlier, particles in both regions were found to be present in nanoparticulate metallic form or Pd(0) (1). An SEM image showing the nanoparticulate matter is shown in Figure 2.
Figure 1: Photograph shows the formation of increasing intensity of color in the cultures with increasing concentrations of added Pd.

Figure 2: A scanning electron microscopic image of the nanoparticulate bio-Pd formed on the cells of *C. pasteurianum* BC1.

**Controls:**

Heat-killed cells of *C. pasteurianum* BC1 did not degrade azo dyes to any significant extent as shown in Figure 3. Further, no degradation of azo dyes was observed in abiotic controls, which showed a constant dye concentration (data not shown). MO was degraded by a living culture of *C. pasteurianum* BC1. An initial MO concentration of 450 μM underwent almost complete reductive degradation in about 90 minutes.

On the other hand, EB was found to be extremely recalcitrant and was degraded only in insignificant amounts. The decomposition of Evans Blue was limited and *C. pasteurianum* BC1 culture containing no bio-Pd was unable to degrade EB at higher concentration. Thus, the amount of EB that was added was lowered to 4.8 μM; an almost 100-fold lower concentration compared to MO. As seen in Figure 4, even that concentration was not degraded completely by both heat-killed and living cultures of *C. pasteurianum* BC1 without bio-Pd.
Figure 3: Concentration of methyl orange, as determined using UV-visible spectroscopy, show that heat-killed cells of *C. pasteurianum* BC1 did not degrade the dye to any significant extent when compared to live *C. pasteurianum* BC1 culture.

Figure 4: Concentration of Evans Blue, as determined using UV-visible spectroscopy, showed that neither living culture or heat-killed cells of *C. pasteurianum* BC1 can degrade the dye to any significant extent.
Bio-Pd catalyzed reduction of azo dyes:
In the presence of bio-Pd, the degradation kinetics of azo dyes was significantly enhanced. Figure 5 shows the concentration of MO over time for a bio-Pd containing culture and a control without bio-Pd. It can be seen that the degradation rate of MO was accelerated over 10 times, as observed from the time taken to degrade 450 μM of MO (7 min with 10 ppm bio-Pd as opposed to nearly 90 min in the absence of bio-Pd).

Figure 5: Concentration of methyl orange, as determined using UV-visible spectroscopy, shows that the in-situ formed bio-Pd (10 ppm) acted as a catalyst for the reduction of MO in the presence of *C. pasteurianum* BC1.

In a similar manner, *C. pasteurianum* BC1 that was unable to entirely degrade 4.8 μM EB in the absence of bio-Pd, was found to be able to degrade 48 μM of EB in the presence of bio-Pd, as shown in Figure 6. Further, it was seen that even a 5ppm concentration of bio-Pd was sufficient to induce a near complete reduction of EB. However, 1 ppm concentration of bio-Pd was not found to lead to any significant reduction of EB (data not shown). Figure 7 shows the color changes that were observed through the course of this set of experiments. The initial addition of Pd in varying concentrations changed the color to varying levels of grey (Figure 7a). The addition of 48 μM of EB led to the formation of an intense blue color at time t=0 (Figure 7b). Visible reduction of EB was catalyzed by 5 ppm and 10 ppm of bio-Pd, as seen by the slow loss of color observed at 10 minutes (Figure 7c) and 6 hours (Figure 7d) after the addition of EB.
Figure 6: Concentration of Evans Blue, as determined using UV-visible spectroscopy, shows that the *in-situ* formed bio-Pd (5ppm and 10 ppm) acted as a catalyst for the reduction of EB in the presence of *C. pasteurianum* BC1.

Figure 7: Photographs show the color changes observed through the course of this set of experiments. A: The initial addition of Pd in varying concentrations changed the color to varying levels of grey. B: The addition of 48 µM of EB led to the formation of an intense blue color at time t=0. Visible reduction of EB was catalyzed by 5 ppm and 10 ppm of bio-Pd, as seen by the slow loss of color observed at (C) 10 minutes and (D) 6 hours after the addition of EB.
Degradation Mechanism:
It is our hypothesis that the degradation of azo compounds occur via reductive breakage of the azo linkage. This proposed reaction mechanism is shown below. The N=N is broken down and hydrogenated, leading to the formation of aminated daughter compounds. In the case of methyl orange, the expected daughter products are sulfanilic acid and N,N-Dimethylbenzene-1,4-diamine as shown below in reaction 1. Breakdown of Evans Blue leads to three daughter products as shown in reaction 2, namely, 3,3'-dimethyl-biphenyl-4,4'-diamine, 6,8-diamino-7-hydroxy-naphthol-1,3-disulfanate and 4,7-diamino-6-hydroxy-naphthol-1,3-disulfanate and neither of which is identifiable using UV-Visible spectroscopy. In this proof-of-concept study, we only evaluated the breakdown of MO.
Sulfanilic acid (SA) formation was monitored using UV-Visible spectroscopy at 244 nm. It can be seen that the degradation of MO led to the formation of SA at a stoichiometric ratio of 1:1. The concentration-time plot for SA is shown in figure 8. The initial concentration of SA was determined to be 0 and the final concentration of which was found to be \(~400\, \mu M\), thereby confirming our hypothesis that MO degradation occurred via reductive degradation of the azo linkage.

![Concentration of sulfanilic acid, as determined using UV-visible spectroscopy, showed that the degradation of MO led to the formation of stoichiometric amounts of SA.](image)

**Figure 8:** Concentration of sulfanilic acid, as determined using UV-visible spectroscopy, showed that the degradation of MO led to the formation of stoichiometric amounts of SA.

**Conclusions**

Biodegradation of azo dyes was found to occur via reductive hydrogenation of the azo-linkage. These results conclusively showed the need for bio-Pd as a catalyst for degradative reduction of azo dye. Concomitantly generated hydrogen was used in the reduction reaction. Current groundwater treatment technologies rely on delivering pre-formed catalytic nanoparticles into groundwater treatment zones as well as the addition of expensive molecular hydrogen to above ground pump and treat systems; two shortcomings that were overcome in this one-step process.

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References

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Nanotechnology and Microbial Electrochemistry for Environmental Remediation

M. Elizabeth Palmer¹, Jason Hastings¹, Jeffrey Fitts² and Dev Chidambaram¹*
¹University of Nevada Reno Chemical and Materials Engineering, 1664 N. Virginia St, Reno, NV 89557-0388
²Brookhaven National Laboratory Environmental Sciences Department, Upton, NY 11973
dcc@ unr.edu

Introduction: Metal nanoparticles, by virtue of their catalytic properties, have the potential to be exploited for applications in environmental remediation [1]. For instance, numerous studies have explored the use of Fe(0) nanoparticles to remove organic and inorganic contaminants [2]. In the presence of suitable electron donor, these nanomaterials can catalyze the reduction of pollutants. A variety of pollutants such as hexavalent chromium and hexavalent uranium are soluble in their highly oxidized form and become sparingly soluble on reduction (to trivalent chromium and tetravalent uranium in the above case, respectively). Further, several organic compounds such as organic dyes, fertilizers and pharmaceutical compounds and intermediates undergo degradation on reduction. In all these cases, the contamination levels in the subsurface environment and their downstream flow can be significantly controlled and managed. However, these remediation strategies are limited by their ability to deliver catalytic nanoparticles plus a suitable electron donor to large treatment zones.

A new biologically inspired method to produce a nanoparticulate catalyst involves the precipitation of transition metals such as palladium, gold and iron on bacterial surface, resulting in the formation of bio-nano-Met, where Met=transition metal catalyst. Two Gram-negative model organisms have been primarily used to reduce Pd(II) and subsequently induce precipitation of Pd(0) nanoparticles: the metal-respiring bacterium Shewanella oneidensis [3] and the sulphate reducing bacterium Desulfovibrio desulfuricans [4]. It is known that Pd and bio-Pd can catalyze the reduction of various groundwater and soil pollutants [3, 5, 6]. However, all these processes consist of two-steps, where bio-nano-Met nanoparticles are formed in a separate reaction before contaminant treatment, thus making these processes less suitable to treat subsurface contaminants. In this study, we demonstrate the proof of concept for a one-step process and subsequently demonstrate its applicability for the improved remediation of both inorganic and organic contaminants.

Experimental: Members of the genus Clostridium were used to reduce transition metal ions such as Pd(II) and Au(III) to form Pd (bio-Pd) and Au (bio-Au) nanoparticles, respectively. The speciation of bio-nano-Met particles was assessed using XANES conducted at the National Synchrotron Light Source. The generated nanoparticles were found to be precipitated on the cell wall and in the cytoplasm in metallic form. The organisms were then challenged with contaminants of interest and the concentration of the contaminant was then monitored. Also, the metabolites were monitored for their potential for reduction reaction.

Results and Discussion: Bio-nano-Met were synthesized rapidly and efficiently using Clostridia. An example is shown in Fig 1. Batch and aquifer mesocosm experiments using Clostridia, loaded with bio-nano-Met, showed efficient reductive removal of soluble Cr(VI) and degradation of azo compounds, and organics. An example of degradation of an organic pollutant is shown in Fig. 2. Our experiments also showed that the in-situ biological production of H₂ by Clostridia was essential for the reduction reaction, which suggests a novel process where the in-situ microbial production of hydrogen is directly coupled to the catalytic reduction of contaminants. Both extracellular microbial electron transfer and H₂ act as the source of electrons for the reduction.

Conclusions: Clostridia can synthesize catalytic nanoparticles in-situ at the zone of treatment. In essence, the microbial surface becomes the support for the catalyst, thus maintaining their high surface area. Concomitantly, microbes generate electron donors that cause reduction of contaminants. This unique one-step process offers significant advantages over the current groundwater treatment technologies that rely on introducing pre-formed catalytic nanoparticles into groundwater treatment zones and the costly addition of molecular hydrogen to above ground pump and treat systems.

References: