Genentech: Cell Fermentation

A thesis submitted in partial fulfillment of the requirements for the degree of Bachelor of Science in Chemical Engineering and the Honors Program

By
Anita Albanese
Ian Stewart
Dr. Alan Fuchs, Thesis Advisor
Dr. Christie Howard, Thesis Advisor
May, 2016
University of Nevada, Reno
The Honors Program

We recommend that the thesis prepared under our supervision by

Anita Albanese
Ian Stewart

entitled

Genentech: Cell Fermentation

be accepted in partial fulfillment of the requirements for the degree of

Chemical Engineering, Bachelor of Science

______________________________

Alan Fuchs, Ph.D., Faculty Mentor, Department of Chemical Engineering

______________________________

Christie Howard, Ph.D., Faculty Mentor, Department of Biochemistry and Molecular Biology

______________________________

Daniel Villanueva, Ph.D., Assistant Director, Honors Program

______________________________

Tamara Valentine, Ph.D., Director, Honors Program
Abstract

A challenge to grow *E. coli* and express a Lucentis-like protein of interest in the laboratories of the Department of Chemical and Materials Engineering at the University of Nevada, Reno is presented by Genentech. A potential model to achieve this goal is created involving a bioreactor hosted in the unit operations lab. Sterile technique is studied, equipment ordered, and a Memorandum of Understanding and Agreement on use of Biological Agents and Recombinant DNA is completed in accordance with this goal. In order to not waste the final reserves of Lucentis-like proteins, an alternative protein, IDOLDH, is used to develop the process. IDOLDH is a protein studied by a faculty member of the University of Nevada, Dr. Tittiger, for commercial use in his start-up company, EscaZyme. Utilizing this protein, standard operating procedures are designed and experiments are conducted to legitimize the use of a bioreactor. These results are then modeled in an effort to develop relevant equations. Potential scale up from lab models to large scale industry is theorized. Economic viability of the process is determined.

Lessons learned during this project are utilized in the prospective start up competition, "Governor's Cup." Utilizing basic concepts implemented throughout multiple design projects, a business plan tackling renewable energy sources in the form of graphite is explored. Key design points are developed, determining the viability of such a venture.
Acknowledgement

We would first like to thank our thesis advisor Dr. Alan Fuchs of the Department of Chemical and Materials Engineering at the University of Nevada. Professor Fuchs was openly communicative throughout every stage in the project and assisted in developing clear goals to achieve throughout the semester. His experience in heuristics and economic scale up played an important role in the development of the later goals in this project. Dr. Fuchs also allowed for key improvements for the project by creating contacts with Genentech and involving the biotechnology masters students.

We would equally like to thank our second thesis advisor Dr. Christie Howard of the Department of Biochemistry and Molecular Biology at the University of Nevada. Professor Howard's undying dedication to the project showed night after night when she came in to help conduct experiments. Her guidance played a key role in the project, and without her constant assistance, the design project group would have been lost from the start.

Thanks must also be given to the gracious engineers at Genentech for providing us not only with the problem statement which lead to this undertaking, but key insights during the process which allowed for pivoting. Without their assistance, the project would have taken a much different path, and not for the better.

We would also like to acknowledge Professor Mike Kivistik of the Department of Chemical and Materials Engineering Department, Dr. Sage Hiibel of the Department of Civil and Environmental Engineering, and Dr. Claus Tittiger of the Department of Biochemistry and Molecular Biology at the University of Nevada. Each staff member
played a large role in developing specific portions of the project, whether it be IDOLDH mechanics with Dr. Tittiger, bioreactor process with Dr. Hiibel, or safety and operating procedure with Professor Kivistik.

We would also like to thank the experts who were involved in the references we consulted during this project. Without the work done by those before us, much of the time spent on this project would have been lost to trial and error.

Finally, we would express our profound gratitude for group members Anthony Ramirez and Eric Lindemann. Though not directly involved in the creation of the honors thesis, each played a major role in the success of our project throughout the semester. They were in it from the start whether they knew it or not, and spent hours in the lab and out of it helping work towards this goal.
Table of Contents

Abstract i
Acknowledgement ii
List of Tables v
List of Figures vi
Introduction 1
Timeline 2
Theory 4
Environmental Health and Safety 12
Modeling and Simulations 13
Experimental Results 20
Equipment Design Problems 31
Scale-Up 32
Conclusions 35
References 38
Appendix A: Memorandum of Understanding and Agreement on Use of Biological Agents and Recombinant DNA 39
Appendix B: Standard Operating Procedure 51
Appendix C: Experimental Data 56
Appendix D: Governor's Cup 61
Appendix E: Heuristics for 2016-2017 81
# List of Tables

<table>
<thead>
<tr>
<th>Title in order of Appearance</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1 Responses to (+)- and (-)-ipsdienol</td>
<td>5</td>
</tr>
<tr>
<td>Table 2 USDA ZYM-5052 media components</td>
<td>15</td>
</tr>
<tr>
<td>Table 3 Shaker flask conditions testing various lactose and glucose levels at 30°C and 37°C using ZYM-5052 media</td>
<td>16</td>
</tr>
<tr>
<td>Table 4 Parameters for the first bioreactor experiment run at 37°C</td>
<td>17</td>
</tr>
<tr>
<td>Table 5 Parameters for the second bioreactor experiment run at 30°C</td>
<td>18</td>
</tr>
<tr>
<td>Table 6 Parameters for bioreactor experiment run at 37°C (growth phase) and 30°C (production phase)</td>
<td>19</td>
</tr>
<tr>
<td>Table 7 Parameters for oxygen uptake rate and volumetric mass transfer coefficient</td>
<td>20</td>
</tr>
<tr>
<td>Table 8 The activity for samples collected at 5.5 hours and at 27.5 hours</td>
<td>26</td>
</tr>
<tr>
<td>Table 9 Parameters to extrapolate a bioreactor of geometric similarity</td>
<td>34</td>
</tr>
<tr>
<td>Table 10 Bioreactor design checklist</td>
<td>81</td>
</tr>
</tbody>
</table>
List of Figures

<table>
<thead>
<tr>
<th>Title in order of Appearance</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1 Timeline of project</td>
<td>4</td>
</tr>
<tr>
<td>Figure 2 Ponderosa pines killed by Ips pini</td>
<td>5</td>
</tr>
<tr>
<td>Figure 3 Reaction mechanism for producing enantiomerically pure ipsdienol beginning with a racemic mixture</td>
<td>6</td>
</tr>
<tr>
<td>Figure 4 Example of a growth curve</td>
<td>7</td>
</tr>
<tr>
<td>Figure 5 Graphical method for determining $\mu_{\text{max}}$</td>
<td>9</td>
</tr>
<tr>
<td>Figure 6 Dissolved oxygen concentration against time</td>
<td>9</td>
</tr>
<tr>
<td>Figure 7 Block flow diagram</td>
<td>11</td>
</tr>
<tr>
<td>Figure 8 Process flow diagram</td>
<td>11</td>
</tr>
<tr>
<td>Figure 9 Piping and instrumentation diagram</td>
<td>12</td>
</tr>
<tr>
<td>Figure 10 Two phase model of IDOLDH production in E. coli</td>
<td>14</td>
</tr>
<tr>
<td>Figure 11 The optical density of each shaker flask over the course of 6.5 hours</td>
<td>21</td>
</tr>
<tr>
<td>Figure 12 The SDS-PAGE gel for the shaker flask experiments at 37°C with no changes to the media and with no lactose added to the initial media</td>
<td>22</td>
</tr>
<tr>
<td>Figure 13 The SDS-page gel for the shaker flask experiments at 30°C with no changed to the media and with no lactose added to the initial media</td>
<td>22</td>
</tr>
<tr>
<td>Figure 14 The cell and oxygen concentration of the first bioreactor experiment run</td>
<td>23</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>Figure 15</td>
<td>Growth curve, glucose depletion, and oxygen depletions for the second bioreactor experiment at 37°C</td>
</tr>
<tr>
<td>Figure 16</td>
<td>The SDS-PAGE gel for the first bioreactor experiments at 37°C with no changes in the media</td>
</tr>
<tr>
<td>Figure 17</td>
<td>Activity assay curve for bioreactor experiment two at 37°C</td>
</tr>
<tr>
<td>Figure 18</td>
<td>Growth curve, glucose depletion, and oxygen depletion for the second bioreactor experiment at 37°C → 30°C</td>
</tr>
<tr>
<td>Figure 19</td>
<td>The SDS-PAGE gel for the first bioreactor experiments at 37°C → 30°C</td>
</tr>
<tr>
<td>Figure 20</td>
<td>Activity assay curve for the bioreactor experiment at 37°C → 30°C</td>
</tr>
<tr>
<td>Figure 21</td>
<td>Oxygen uptake and volumetric mass transfer coefficient study</td>
</tr>
<tr>
<td>Figure 22</td>
<td>Monod kinetics for bioreactor experiment at 37°C</td>
</tr>
<tr>
<td>Figure 23</td>
<td>Scale up variable proportions</td>
</tr>
</tbody>
</table>
Introduction

Problem Statement

The University of Nevada, Reno’s Chemical Engineering seniors have been given the task to build and conduct experiments using a one liter bioreactor. A plasmid was provided from Dr. Tittiger in conjunction with the United States Department of Agriculture (USDA). Using *E. coli* and the provided plasmid we were able to get a large volume of our protein of interest, ipsdienol dehydrogenase (IDOLDH). IDOLDH is a bark beetle enzyme, which is used to produce enantiomerically pure ipsdienol, a pheromone used for traps; therefore, mitigating damage to trees across North America [1]. We believe we will be able to make a far more economical approach making this enzyme compared to traditional organic synthesis. Various parameters such as oxygen, cell growth, substrate concentration, and expression will be studied during the course of the experiments. With the data gathered from the one liter experiments, scale up calculations will be performed to recommend sizing and parameters.

Economic Considerations

Currently, methods to produce bark beetle pheromones for pest control cost over $2,000 per gram using traditional chemistry techniques [2]. This same process can be mirrored using biological pathways. IDOLDH can be used to create enantiomerically pure (+)- or (-)-ipsdienol, reducing the cost of production to $150-200 per gram [2]. This makes pest control a cheaper process than before. The challenge proposed to the fermentation group was to produce large amounts of *E. coli* that expresses IDOLDH for Dr. Tittiger’s start-up company EscaZyme to be used in beetle traps. In total cost, the process can be done for fairly cheaper per run of the bioreactor. This is important because it lowers the
cost of a process by over 90%. This will guarantee a great return on investment. When scaling up like Genentech, making sure that the process is monitored and keeping the equipment within standard operating range maintains the equipment.

Goals

Overall goals within the Department of the Chemical Engineering were outlined to help orient the direction of the project. These were determined in accordance with the multiple variables involved with the cell culture (fermentation) group.

The primary goal, as outlined above, is to produce large amounts of IDOLDH for Dr. Tittiger's start-up company EscaZyme. This is done in a two-fold effort, one in which to express gratitude for his involvement in the project, and the second to achieve another goal; that of providing an experimental protein for next year's senior class. As the reserves of Lucentis-like protein used in the purification design project are running out, they will need a new protein to run experiments. IDOLDH can work as this protein. The third and final goal is to optimize operating conditions of the bioreactor to promote IDOLDH expression. This is done in an effort to better understand the encompassing principles involved in this project.

Timeline

In order to tackle the complexity of such a large undertaking, a comprehensive timeline is needed. In engineering, Gantt charts as seen in Figure 1 are utilized to map out the time needed to accomplish each sub goal in an attempt to complete the project. An overarching goal of the project was writing reports which were due each week, detailing the progress of our experimental group. Beyond this, the cell culture design project was
separated into three main phases as loosely read in Figure 1: preparation, intermediate tests, and bioreactor tests.

**Preparation Phase**

The preparation phase of the project was characterized by a mass amount of learning which needed to be undertaken. During the first week, sterile technique was taught, and shortly after, media preparation was conducted. This time was also spent ordering equipment (Figure 1). From here, preparing the unit operations lab for adequate sterility and experimental process was of the utmost important.

**Intermediate Tests Phase**

Throughout the end of February and up through spring break, the intermediate testing phase of the project was completed. Preliminary shaker flask experiments were conducted. This was largely comprised of complications which arose in the ordering of equipment but still played a large role in the success of the project. As shown in the Gantt chart, tests on the chosen media were undertaken using shaker flasks, as well as running gels to determine expression of the desired enzyme. During this time period, the first presentation to Genentech was given which helped the project team in pivoting proposed tests. This period also allowed a brief period of respite in which to begin working on scale-up calculations. It was ended by a field test with a much more complex bioreactor in Howard Medical Sciences before letting out for spring break.

**Bioreactor Tests Phase**

The last phase of the cell culture project was conducted in April, seen in Figure 1, once the required materials were delivered. This is where the majority of the experimental data was collected. Experiments were done over a short three week period in which
multiple days outside of the specified lab period were used to run tests. This period was concluded by the presentation to Genentech, in which results and future plans for upcoming seniors were discussed with cell culture engineers.

![Gantt Chart - Spring 2016 - Cell Culture](chart.png)

**Figure 1.** *Timeline of project.*

**Theory**

*Ipsdienol Dehydrogenase (IDOLDH)*

Ipsdienol dehydrogenase (IDOLDH) is a bark beetle enzyme isolated from *Ips pini* and characterized in Dr. Claus Tittiger’s lab. This species of bark beetle has been known to be an aggressive tree killer, especially in the western United States (Figure 2) [1]. Thus, traps baited with aggregation pheromones are used in order to mitigate damage.
Bark beetle pheromones are multi-component mixtures [2]. Both enantiomers of ipsdienol are often part of the mix (Table 1) [2]. Therefore, depending on the species of interest, developing a racemic mixture of the corresponding (+)- to (-)- ipsdienol ratio is key. Specifically, Western *Ips pini* has a 5% to 95% breakdown, respectively [2].

**Table 1. Responses to (+)- and (-)-ipsdienol [2].**

<table>
<thead>
<tr>
<th>Species</th>
<th>(+)-ipsdienol</th>
<th>(-)-ipsdienol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Western <em>Ips pini</em></td>
<td>5%</td>
<td>95%</td>
</tr>
<tr>
<td><em>Easter Ips pini</em></td>
<td>65%</td>
<td>35%</td>
</tr>
<tr>
<td><em>Ips typographus</em></td>
<td>95%</td>
<td>5%</td>
</tr>
<tr>
<td><em>Ips calligraphus</em></td>
<td>inhibitory</td>
<td>aggregation</td>
</tr>
<tr>
<td><em>Ips paraconfusus</em></td>
<td>aggregation</td>
<td>inhibitory</td>
</tr>
<tr>
<td><em>Ips acuminatus</em></td>
<td>aggregation</td>
<td>inhibitory</td>
</tr>
</tbody>
</table>

*adapted from [Chang, 2010; Kovalenko, 2012; & Tittiger, 2013]
Presently, using traditional chemistry, producing enantiomerically pure (+)- or (-)-ipsdienol costs approximately $2000 per gram. However, EscaZyme has created a laboratory scale method to produce enantiomerically pure ipsdienol at a cost of approximately $150-200 per gram (Figure 3) [2].

**Figure 3. Reaction mechanism for producing enantiomerically pure ipsdienol beginning with a racemic mixture** [2].

A racemic mixture of ipsdienol can be obtained for approximately $24 per gram [2]. EscaZyme is able to first produce IDOLDH using an insect model [2]. Once the enzyme is extracted from the insects, it is used to convert the racemic mixture of ipsdienol to (+)-ipsdienol and ipsdienone [2]. Liquid chromatography is then used to separate (+)-ipsdienol and ipsdienone [2]. Ipsdienone can be converted back to (-)-ipsdienol [2]. Overall, this method produces enantiomerically pure ipsdienol.

*E. coli* has been genetically engineered to express IDOLDH [3]. Theoretically, *E. coli* can be grown in shaker flasks to produce IDOLDH. However, a bioreactor is used to
manipulate multiple variables such as temperature, pH, oxygen, nutrients, and mixing. Additionally, using an *E. coli* method in a bioreactor can reduce the time of production.

**Bioreactor**

Bioreactors are used in industry for purposes such as mass production of desired proteins and pharmaceuticals. Understanding the basic design and operation of a bioreactor allows one to determine the impact of parameters such as temperature, pH, impeller speed and design, and oxygen levels.

A growth curve is an important part of understanding how a particular culture grows under specific conditions. Therefore, when an experiment in a batch bioreactor is conducted, a growth curve can be generated by plotting cell concentration (cell number/mL) against time (hour) which will indicate the five phases of growth (lag, exponential, deceleration, stationary, and death phase) as seen in Figure 4. (*It is important to note that the scale of the y-axis is log*).

![Figure 4](example.png)  
*Figure 4. Example of a growth curve [4].*
A growth-associated product is ideally harvested during log phase to maximize the quantity and quality of the product. In other words, during log phase for a growth-associated product, the production will be at its highest.

The Monod equation (Equation 1), substrate-limited growth kinetics, takes into account that cells experience limited growth due to their dependency on nutrients such as glucose. Essentially, as the growth-limiting substrate, $S$, is used up, the cell growth plateaus and the culture transitions into stationary phase. The Monod equation describes the growth rate as a function of substrate.

$$\mu_g = \frac{\mu_{\text{max}} S}{K_S + S} \quad (Equation \ 1)[4]$$

When doing a simple growth experiment, we can determine specific growth rate ($\mu_g$) and maximum growth rate ($\mu_{\text{max}}$) which will tell us how different conditions impact the growth rate. To find $\mu_g$, Equation 2 is utilized where $X$ is the cell concentration and $dX/dt$ is the change in cell concentration over change in time.

$$\mu_g = \frac{1}{X} \frac{dX}{dt} \quad (Equation \ 2)[4]$$

Using the linearized form of the Monod equation (Equation 3), $\mu_{\text{max}}$ and half-velocity constant ($K_s$) can be determined. Plotting $1/ \mu$ vs $1/S$ will result in a linear relationship. The $y$-intercept will be equivalent to $1/ \mu_{\text{max}}$ and the slope of the line will be $K_s/\mu_{\text{max}}$ (Figure 5).

$$\frac{1}{\mu_g} = \frac{K_s}{\mu_{\text{max}}} \left(\frac{1}{S}\right) + \frac{1}{\mu_{\text{max}}} \quad (Equation \ 3)[5]$$
To determine the oxygen uptake rate (OUR), one is able to graph the dissolved oxygen (DO) concentration (mg/L) against time (minutes). When the air is turned off, the DO level will rapidly decline as the aerobic culture uses up the available oxygen. Hence, the negative of the slope when air supply is turned off is the OUR. When the air supply is turned back on, levels of DO will rise and the slope of the linear region of $dC_L/dt$ can be determined (Figure 6).

Furthermore, the volumetric mass transfer coefficient, $k_{L,a}$, can be determined using the following relationship where $C^*$ is the DO concentration when the media is completely
saturated with oxygen and \( C_L \) is the DO concentration recorded at each time point (Equation 4).

\[
K_L a = \frac{dC_L}{dt} + \frac{OUR}{C - C_L} \quad (Equation \ 4)[4]
\]

Process Design

In designing a process to tackle the issues outlined, a series of diagrams are constructed to better break down the necessary requirements in instrumentation. These diagrams are outlined below as a block flow diagram, process flow diagram, and piping and instrumentation diagram. These are created in increasing orders of complication, with more details being added per diagram.

Block Flow Diagram (BFD)

The first diagram created is a block flow diagram, as seen in Figure 7. A block flow diagram outlines the basic principles needed in the experimental process. The diagram illustrated in Figure 7 first shows a central batch bioreactor. This is the crux of the process developed, and its one pure outlet is the cell media, which is created once per batch. From here, there are two concurrent processes going on while the batch is being processed: an air feed which enters and exits the bioreactor and a water feed which regulates the temperature of the bioreactor.
From here, the process flow diagram is constructed, as seen in Figure 8. A process flow diagram is created to outline the main experimental apparatus needed in the experimental process. The main apparatus needed is a bioreactor. There are three elements which are being monitored: air feed, water feed, and cell concentration.

**Figure 7. Block flow diagram.**

**Process Flow Diagram (PFD)**

From here, the process flow diagram is constructed, as seen in Figure 8. A process flow diagram is created to outline the main experimental apparatus needed in the experimental process. The main apparatus needed is a bioreactor. There are three elements which are being monitored: air feed, water feed, and cell concentration.

**Figure 8. Process flow diagram.**
**Piping and Instrumentation Diagram (P&ID)**

The final step in the process of constructing diagrams is the piping and instrumentation diagram, as shown in Figure 9. A piping and instrumentation diagram profiles the remaining apparatus needed to run the experiment, breaking down the process completely as shown in Figure 9. The water is driven around the system by a motor powered turbine and circulates acting as a heat exchanger to moderate the temperature of the system. The air feed throughout the system is controlled through a gate valve. A Rushton impeller is used to mix up the media inside the tank of the bioreactor, and the media is collected once per batch reaction.

![Piping and Instrumentation Diagram](image)

**Figure 9. Piping and instrumentation diagram.**

**Environmental Health and Safety**

Safety precautions should always be implemented before starting any experiment. A Memorandum of Understanding and Agreement on Use of Biological Agents and Recombinant DNA was created prior to the start of this project (Appendix A). The use of
microbial agents and recombinant DNA require special precautions compared to using non-biologically active products. Although IDOLDH presents no danger to humans, nitrile gloves, goggles, and a lab coat are required at all times. Biohazard waste should be stored in a designated disposal bin with a proper label. Any culture should be cleaned with a 10% bleach solution when done with experiments, while other areas should be cleaned with 70% ethanol. Lab training in accordance with the standard operating procedure (SOP) is required and a written SOP must be available at all times. When no trained personnel are in the lab, it should be cleaned and locked at all times.

**Modeling and Simulations**

*Two Phase Model*

To optimize any process, it is crucial to understand the mechanisms by which it functions. It was hypothesized that the production of IDOLDH in an E. coli model occurred in two stages: the first being growth and the second being IDOLDH production (Figure 10). Stage one is characterized by the growth of E. coli. Simple respiration was used to model the growth stage with glucose being the primary carbon source. The gene of interest is produced using a classic lac UV promoter. Therefore, when glucose levels are low, then amino acids are able to produce T7 RNA polymerase which is specific in producing IDOLDH. All experiments were designed using this two phase model.
Figure 10. Two phase model of IDOLDH production in E. coli.

Media

The media used for the production of IDOLDH was first published by Studier et al and later adapted by the USDA (Table 2) [6]. It is categorized as an undefined media due to having at least one undefined carbon source which includes yeast extract and tryptone. Additionally, the media is autoinducable because lactose, which induces the production of IDOLDH, is in the media during the entirety of the experiment.
Table 2. USDA ZYM-5052 media components [6].

<table>
<thead>
<tr>
<th>Undefined Components</th>
<th>Carbon Sources</th>
<th>Macronutrients</th>
<th>Micronutrients</th>
<th>Antibiotics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast Extract</td>
<td>2.8 mM Glucose</td>
<td>Magnesium</td>
<td>Trace Metals</td>
<td>Kanamycin (pET vector)</td>
</tr>
<tr>
<td>Tryptone</td>
<td>5.6 mM Lactose</td>
<td>Sulfate (S)</td>
<td>Including</td>
<td>Chloramphenicol 1</td>
</tr>
<tr>
<td></td>
<td>54 mM Glycerol</td>
<td>Phosphate (P)</td>
<td>100uM FeCl₃</td>
<td>(BL-21 DE3 cells)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ammonia (N)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Salts</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>100mM Phosphate buffer</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Standard Operating Procedure for Bioreactor

A standard operating procedure (SOP) was created so that all experiments were conducted using identical procedures. The SOP includes instructions for making the media, streaking a plate, inoculating the media, calibrating the pH meter and dissolved oxygen (DO) meter, using the bioreactor, and clean up procedures (Appendix B).

Experimental Design

Experiment 1: Shaker Flask

An initial experiment in shaker flasks was conducted to test the effect of temperature and sugar (lactose and glucose) on the growth of E. coli and expression of IDOLDH.
Eight different conditions starting with an optical density (OD) of approximately 0.023 at $A_{600}$ were incubated for 24 hours on a shaker table (Table 3). One milliliter samples were taken every hour until the sample reached an OD of approximately 0.4. Then samples were taken every 30 minutes. Samples were obtained for approximately 7 hours. The last sample was taken after approximately 24 hours of incubation. OD, pH, and glucose concentrations were recorded for each sample (Appendix C).

**Table 3.** *Shaker flask conditions testing various lactose and glucose levels at 30°C and 37°C using ZYM-5052 media.*

<table>
<thead>
<tr>
<th>Shaker Flask</th>
<th>Temperature (°C)</th>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>Control</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>2 x lactose</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>4 x glucose</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>No lactose</td>
</tr>
<tr>
<td>5</td>
<td>37</td>
<td>Control</td>
</tr>
<tr>
<td>6</td>
<td>37</td>
<td>2 x lactose</td>
</tr>
<tr>
<td>7</td>
<td>37</td>
<td>4 x glucose</td>
</tr>
<tr>
<td>8</td>
<td>37</td>
<td>No lactose</td>
</tr>
</tbody>
</table>

**Experiment 2: Bioreactor 37°C (One)**

An initial experiment in the bioreactor at 37°C was conducted to determine if *E. coli* could be grown in a bioreactor and express the enzyme of interest—IDOLDH.
The bioreactor was run under constant conditions (Table 4). Samples were taken at the beginning of the experiment and the end of the experiment. OD, pH, DO, and glucose concentrations were recorded for each sample (Appendix C).

**Table 4. Parameters for the first bioreactor experiment run at 37°C.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Impeller Speed</td>
<td>400-600 rpm</td>
</tr>
<tr>
<td>Oxygen Source</td>
<td>House air</td>
</tr>
<tr>
<td>Temperature</td>
<td>37°C</td>
</tr>
<tr>
<td>Sampling Size</td>
<td>20 mL</td>
</tr>
<tr>
<td>Bioreactor Volume</td>
<td>1 L</td>
</tr>
<tr>
<td>Air Flow Rate</td>
<td>undetermined</td>
</tr>
</tbody>
</table>

**Experiment 3: Bioreactor 37°C (Two)**

The bioreactor was run under constant conditions similar to experiment 2 (Table 5). This experiment was conducted due to experiment 2 surpassing growth exponential phase while unattended. Samples were taken from 0 hours to 27.5 hours at various time points. OD, pH, DO, and glucose concentrations were recorded for each sample (Appendix C).
Table 5. Parameters for the second bioreactor experiment run at 37°C.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Impeller Speed</td>
<td>400-600 rpm</td>
</tr>
<tr>
<td>Oxygen Source</td>
<td>House air</td>
</tr>
<tr>
<td>Temperature</td>
<td>37°C</td>
</tr>
<tr>
<td>Sampling Size</td>
<td>20 mL</td>
</tr>
<tr>
<td>Bioreactor Volume</td>
<td>1 L</td>
</tr>
<tr>
<td>Air Flow Rate</td>
<td>undetermined</td>
</tr>
</tbody>
</table>

Experiment 4: Bioreactor 37°C to 30°C

The initial shaker flask experiments resulted in higher expression of IDOLDH during the 30°C condition than the 37°C condition. Therefore, to increase expression, temperature was kept at 37°C during the growth phase and lowered to 30°C during the production phase.

The bioreactor was run under constant conditions (Table 6). The experiment was run at 37°C from 0-4 hours (growth phase) and 30°C from 4-20 hours (production phase). Samples were taken from 0 hours to 20 hours at various time points. OD, pH, dissolved oxygen levels, and glucose concentrations were recorded for each sample (Appendix C).
Table 6. Parameters for bioreactor experiment run at 37°C (growth phase) and 30°C (production phase).

<table>
<thead>
<tr>
<th>Parameter</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Impeller Speed</td>
<td>400-600 rpm</td>
</tr>
<tr>
<td>Oxygen Source</td>
<td>House Air</td>
</tr>
<tr>
<td>Temperature</td>
<td>37°C (0-4 hours) and 30°C (4-20 hours)</td>
</tr>
<tr>
<td>Sampling Size</td>
<td>20 mL</td>
</tr>
<tr>
<td>Bioreactor Volume</td>
<td>1 L</td>
</tr>
<tr>
<td>Air Flow Rate</td>
<td>Undetermined</td>
</tr>
</tbody>
</table>

Experiment 5: Oxygen Uptake Rate and Volumetric Mass Transfer Coefficient

In the first four experiments, oxygen was a limiting factor as DO levels declined from 100% to approximately 2% during the first few hours. Oxygen uptake rate (OUR) and volumetric mass transfer coefficient (kL,a) can be determined to calculate the necessary air flow rate and oxygen source to keep oxygen levels constant. A medical oxygen concentrator was used to increase oxygen levels in the bioreactor.

The bioreactor was run under constant conditions (Table 7). Samples were taken to determine the growth phase of the culture. When the culture reached log phase, the dissolved oxygen concentration and time was recorded as "0 seconds." The air source was turned off. Dissolved oxygen and time was recorded every 30 seconds for 10 minutes (Appendix C). The air source was turned back on and values for dissolved oxygen and time were recorded every 5 seconds for a minute (Appendix C).
Table 7. Parameters for oxygen uptake rate and volumetric mass transfer coefficient.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Impeller Speed</td>
<td>400-600 rpm</td>
</tr>
<tr>
<td>Oxygen Source</td>
<td>Oxygen Concentrator</td>
</tr>
<tr>
<td>Temperature</td>
<td>37°C</td>
</tr>
<tr>
<td>Sampling Size</td>
<td>20 mL</td>
</tr>
<tr>
<td>Bioreactor Volume</td>
<td>1 L</td>
</tr>
<tr>
<td>Air Flow Rate</td>
<td>Undetermined</td>
</tr>
</tbody>
</table>

**Experimental Results**

Three stages of experimentation were ran to understand the different steps that go into operating a bioreactor. The first stage introduced the fermentation group to basic biochemical lab skills. This allowed for the fermentation groups to get acclimated with micro pipetting, making media, autoclaving, sterility, and inoculating. In this stage, data was not collected. However, it was still extremely vital in understanding the concepts needed to operate the bioreactor.

*Experiment 1: Shaker Flasks*

In the second stage of experimentation, shaker flasks were obtained and tests on the effects of nutrients and temperature were ran. The purpose for these tests was to decide whether or not different sugar concentration and temperature levels had any effects on growth and expression. The results for this experiment are seen in Figure 11.
The results from this lab confirmed the conjecture that growth was better at the 37°C compared to 30°C. Different concentrations of glucose and lactose were added to the media as seen in Figure 11. This was done to decide if any changes to the recipe for the media were needed to optimize the growth of *E. coli*. The results on this end were inconclusive. This issue is due to the fact that the optical density values obtained from the spectrophotometer were out of the range of what the spectrophotometer read accurately.

This observation was confirmed during the midpoint checkpoint with Genentech. The engineers at Genentech suggested looking at literature values and comparing the optical densities obtained to theirs. Further examination of the data found in Appendix C resulted in the observation that growth of the *E. coli* was better with the unchanged media and at 37°C.

Two, 2 milliliter aliquots were saved during the shaker flask experiments and then centrifuged to separate the cells and supernatant for further testing. SDS-Polyacrylamide gel electrophoresis, SDS-PAGE, was examined to determine the quality of expression.
Based off of the SDS-PAGE gels seen in Figure 12 and Figure 13 this revealed that the *E. coli* had higher expression at 30°C.

**Figure 12.** The SDS-PAGE gel for the shaker flask experiments at 37°C with no changes to the media and with no lactose added to the initial media.

**Figure 13.** The SDS-PAGE gel for the shaker flask experiments at 30°C with no changes to the media and with no lactose added to the initial media.
Supernatant pH was examined from the aliquots and revealed that the *E. coli* entered anaerobic conditions. This was determined by noticing the decrease in pH. This trend in the decrease of pH was caused by the introduction of lactic acid in the system. The accumulation of lactic acid occurs in the last step of glycolysis when oxygen levels are depleted and pyruvate is reduced. The fermentation group took this into careful consideration when beginning the third stage of experimentation.

*Experiment 2: Bioreactor at 37°C*

In the third and final stage of the experiments the bioreactor was ran multiple times. Heeding the lessons learned in the shaker flask tests, a bioreactor was ran at 37°C. However, only oxygen level and cell concentration were recorded during this run, as the experiment quickly encountered some issues once the oxygen level dropped to 0% within the first two hours as seen in Figure 14. Thus, after four hours it was called off.

![E Coli, Oxygen Concentration vs Time(hr)](image)

**Figure 14.** The cell and oxygen concentration of the first bioreactor experiment run.
Experiment 3: Bioreactor at 37°C

The second run of the bioreactor was run at 37°C, with impeller speed of 300-400 rpm to diffuse oxygen throughout the system. Media provided by Dr. Claus Tittiger was used and air was introduced from the environment to add oxygen to the system—a bioreactor of one liter. This experiment was run for 27.5 even though the same issues were faced as the previous experiment. Data was collected every 30 minutes for the first 5.5 hours as seen in Figure 15.

![Graph](image)

**Figure 15.** Growth curve, glucose depletion, and oxygen depletions for the second bioreactor experiment at 37°C.

The data received from this portion of the experiment complicated the analysis of the models outlined earlier in theory. This data revealed that there are other nutrient sources that the *E. coli* uses to grow in the media used. The yeast extract, tryptone, lactose, and glycerol are contributing to the *E. coli* growth after the glucose is depleted. To determine the enzymatic kinetics, the substrate amount has to be determined. However, in the model
used, the glucose was the only substrate taken into consideration. Oxygen is also depleted, a problem caused by the extremely high growth rate of the *E. coli*.

A key difference between the shaker flask tests and the bioreactor tests is that the pH never descended beyond 6.8. There are two possible causes for the stability of pH; the first being that the *E. coli* never went into anaerobic conditions. This is the most plausible answer, as the dissolved oxygen level stagnated around 3.2%. Because the oxygen stabilized, the *E. coli* received the correct amount of oxygen needed and was able to operate at aerobic conditions. The second possibility is that the *E. coli* went into anaerobic conditions and produced the lactic acid. However, a buffer which was added to combat the drop in pH seen in the shaker flask experiments, did its job and kept the pH relatively constant. Expression was also looked at and an SDS-PAGE was ran as shown by the gel in Figure 16.

![Bioreactor Expression IDOLDH from the 37°C Bioreactor Run](image)

**Figure 16.** The SDS-PAGE gel for the first bioreactor experiments at 37°C with no changes to the media.
This provided a lot of information regarding the second phase of the model that looked at expression. This means that the lactose induced the gene regulatory sequence and allowed for the *E. coli* to produce an ample amount of IDOLDH at the 5.5 hour range and overnight. There are small bands in the earlier time points which are caused from the lactose being introduced from the beginning of the media. Then an activity assay was run to look at the activity of IDOLDH producing the results seen in Table 8.

**Table 8. The activity table for the samples collected at 5.5 hours and at 27.5 hours.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Lysate Volume (ml)</th>
<th>Protein Concentration (mg/ml)</th>
<th>Total Protein (mg)</th>
<th>Activity (Units/ml)</th>
<th>Total Activity (Units)</th>
<th>Specific Activity (Units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5hr</td>
<td>40.00</td>
<td>15.26</td>
<td>610.40</td>
<td>0.48</td>
<td>19.15</td>
<td>7.84E-04</td>
</tr>
<tr>
<td>27.5hr</td>
<td>138.00</td>
<td>10.97</td>
<td>1513.86</td>
<td>0.44</td>
<td>61.13</td>
<td>2.93E-04</td>
</tr>
</tbody>
</table>

This activity data provides information about how active the enzyme measured is per volume. Meanwhile, the specific activity provides further information about the purity of the enzyme in the mixture. Analyzing Table 8 it is revealed that the IDOLDH is extremely active. This relationship can be better observed looking at the activity curve of the assay in Figure 17.
At 27.5 hours as well as 5.5 hours, the activity is higher than the sample obtained from the bark beetle cell. This is an amazing development, and in direct agreement with the issue that Dr. Tittiger brought to the group in the beginning. The chemical process used to produce IDOLDH from insect cells is more expensive than IDOLDH being produced in *E. coli* and so in this regard, the fermentation group accomplished one of the goals outlined earlier.

**Experiment 4: Bioreactor at 37°C to 30°C**

The project group then turned to optimizing the bioreactor conditions by exploring other options. In the final run of the bioreactor, it was determined that at 5.5 hours the temperature would be dropped from 37°C to 30°C. This was done to determine if the drop in temperature had any change on expression and activity. Determining if the growth curve ever entered the stationary or death phase was also an immediate goal of this bioreactor run. Figure 18 show the curve obtained from this "drop in temperature" experiment.
Figure 18. *Growth curve, glucose depletion, and oxygen depletion for the second bioreactor experiment at 37°C → 30°C.*

This growth curve shows that the *E. coli* culture during the second run reached a maximum growth at the 12 hour time point. Then, it started to decrease as it went to the death phase. The stationary phase is not observed in Figure 18. However, make no mistake; this is not because it did not occur but rather that not enough data points were obtained. This observation is further validated by the time point at 18 hours when an increase in oxygen occurred. Nothing in the bioreactor set up was changed apart from the decrease in temperature which would not cause an increase in the oxygen level. Thus, the only possibility left is that the system entered the stationary and death phase, allowing for the oxygen levels to increase in the system. This observation makes a fair amount of sense considering the amount of *E. coli* in the system is constant and/or decreasing. The expression for this bioreactor run is observed in Figure 19.
**Figure 19:** The SDS-PAGE gel for the first bioreactor experiments at 37°C → 30°C.

Figure 19 gel shows that the IDOLDH is not being expressed until it reaches 5.5 hours. This validates the earlier data that from the previous bioreactor run. An activity assay was also run for this experiment. This is seen in Figure 20.

**Figure 20.** Activity assay curve for the bioreactor experiment at 37°C → 30°C.

The activity of the IDOLDH is increased based off of the analysis of Figure 20. This means that IDOLDH is more active once the temperature step occurs. From this assay
the fermentation group determined that the experiment was a success. The bioreactor benefits from being ran at 37°C until 5.5 hours when it is then lowered to 30°C.

Experiment 5: Oxygen Uptake Rate and Volumetric Mass Transfer Coefficient

Previous experiments indicated that oxygen was a limiting factor. This was denoted by the rapid decline in oxygen levels from 100% to approximately 2% within the first few hours. Low levels of oxygen persisted before and during exponential growth phase. Determining the oxygen uptake rate and volumetric mass transfer coefficient is crucial in designing parameters to keep oxygen levels constant within the system. For this study, a medical oxygen concentrator was used to increase the DO level within the bioreactor. When the culture reached growth exponential phase, the air source was turned off for ten minutes (Figure 21). The oxygen uptake rate was found during this time as 0.0372 mg/L/s. The air was turned back on and the volumetric mass transfer coefficient was determined as 0.0012 1/s (Figure 21).

![Oxygen Uptake and Transfer Rate Experiment](image_url)

**Figure 21. Oxygen uptake and volumetric mass transfer coefficient study.**
Oxygen uptake rate and volumetric mass transfer coefficient can theoretically be used to calculate the necessary flow rate of concentrated oxygen into the system to maintain DO levels. However, under further investigation, it was discovered that the DO probe used for experiments was not properly calibrated. Therefore, to obtain conclusive decisions about oxygen flow rate, this experiment should be performed again using a calibrated DO probe. Other recommendations include using a regulator on the air source and using a submersible DO probe.

Equipment Design Problems

When looking at designing different processes, there are various parameters that have to be taken into consideration. Since the bioreactor project is fairly new, the fermentation group had to assess the different parameters that go into running and maintaining it.

One of the biggest problems that the group faced was modeling the *E. coli* growth as a function of a single substrate. This problem can be attributed to using undefined media. In other words, it contains unknown amounts of carbon sources that can be broken down and used as nutrients. This makes it extremely difficult to model and determine the maximum growth rate of the process using Monod kinetics. Figure 22 depicts the data from experiment 3 with the bioreactor at 37°C. Monod kinetics are not observed because the specific growth rate does not approach a maximum growth rate as substrate increase. Instead, it can be seen that specific growth rate peaks and then steadily decreases.
Another difficulty that the group encountered was DO levels rapidly declining. Presently, it was determined that house air does not provide the necessary oxygen to saturate the media. An oxygen concentrator can be used to increased DO levels. Additionally, a regulator should be used to control the flow rate of air. Furthermore, a sparger can be used to break up the air bubbles to increase the surface area which will increase the volumetric mass transfer of oxygen into the media.

Time was another constraint. This caused the group to decide on a couple of different design parameter to address. One of the biggest challenges seen was the response time of the equipment that provide the data to determine if the bioreactor is running properly. Heuristics for next year are found in Appendix E.

**Scale-Up**

When scaling up a bioreactor there are four possible cases to consider: constant power input, constant liquid circulation rate, constant shear at impeller tip, and constant Reynolds number.
A constant power input is most commonly used except under certain conditions. For example, if a cell is shear sensitive, then a constant shear at the impeller tip should be used. A scale up strategy using constant power input was implemented.

We aim to maintain geometric similarity for the 1000 liter bioreactor from the 1 liter bioreactor. Starting with the volume of a cylinder and common heuristics, parameters for the tank diameter, tank height, and impeller diameter can be found. Common heuristics provided by Genentech were tank height is equal to three times the tank diameter, and the impeller diameter is equal to thirty percent of the tank diameter. With these set relationships values for the tank diameter, tank height, and impeller diameter are 7.52 cm, 22.6 cm, and 2.26 cm, respectively. Next the geometric similarity was found to be a factor of ten using the cubic root of the volume ratio. Finally, we multiplied our values for tank diameter, tank height, and impeller diameter found for one liter by this factor of ten to find the values for the 1000 liter bioreactor: 0.752 m, 2.26 m, and 0.226 m, respectively. These variables can be used to extrapolate other parameters such as impeller rotation or shear using the help of variable proportions found in Perry’s Chemical Engineering Handbook [7].

\[
\begin{align*}
P \mu N^3 D_i^5 &= \frac{P}{V} \mu N^3 D_i^2 \\
V \mu D_i^3 &= \frac{Q}{V} \mu N \mu D_i^3
\end{align*}
\]

**Figure 23. Scale up variable proportions [7].**
Using these proportions and the assumption of constant energy input, P/V, the impeller rotation number for the 1000 liter reactor is able to be calculated (Equation 5).

\[ N_2 = N_1 \left( \frac{D_{T1}}{D_{T2}} \right)^{2/3} = 400 \text{rpm} \left( \frac{1}{10} \right)^{2/3} = 86.2 \text{rpm} \] (Equation 5)

The proportions of these variables were experimentally determined in literature and widely accepted. By setting the N and D perimeters of P/V of the small and large scale equal to one another, the large scale parameters can be found. For example this was done for tip speed by isolating \( N_2 \) for \( N_2^3 \cdot D_2^2 \) is equal to \( N_1^3 \cdot D_1^2 \). Using this method the equation for tip speed can be clearly developed. A chart was made to determine parameter scale up factor (Table 9).

**Table 9. Parameters to extrapolate a bioreactor of geometric similarity.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Constant P/V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy input</td>
<td>P</td>
<td>1000</td>
</tr>
<tr>
<td>Energy input/volume</td>
<td>P/V</td>
<td>1</td>
</tr>
<tr>
<td>Impeller rotation number</td>
<td>N</td>
<td>0.215</td>
</tr>
<tr>
<td>Impeller diameter</td>
<td>Di</td>
<td>10</td>
</tr>
<tr>
<td>Pump rate of impeller</td>
<td>Q</td>
<td>215.4</td>
</tr>
<tr>
<td>Pump rate of impeller/volume</td>
<td>Q/V</td>
<td>0.215</td>
</tr>
<tr>
<td>Max impeller speed</td>
<td>ND</td>
<td>2.154</td>
</tr>
<tr>
<td>Reynolds number</td>
<td>ND2p/m</td>
<td>21.5</td>
</tr>
</tbody>
</table>

For example, if the impeller rotation number for the one liter bioreactor is 400 rpm then we would multiply 400 with 0.215, resulting in the 86.2 rpm, as found in the previous
equation. The variable proportions were used to create this table. Using this chart, any of the listed parameters can be determined.

Conclusion

The goals as discussed in the beginning of this project were to produce large amounts of IDOLDH, provide an experimental protein for next year's senior class, and optimize the running conditions of the bioreactor for producing IDOLDH. These objectives were supplemented by other sub goals, such as discussing potential scale up of the project to a large scale process. Over the course of the project, all goals were accomplished in at least some fashion.

As touched upon in the experimental data, the bioreactor experiment was able to produce cheap and active IDOLDH using *E. coli* cells compared to the insect cells previously used by EscaZyme. It was determined to be an effective replacement for producing large amounts of the enzyme, which means that production costs can be reduced a large amount from the previous process. In this regard, the goal which was set out during the onset of the project was completed.

The second goal of the bioreactor tests was to provide a new protein for purification design project groups to use in the future. As the Lucentis-like protein given to the Department of Chemical Engineering in 2008 grows thin, other options need to be explored. During the visit to Genentech, an alternative protein, human growth hormone, a prominent protein in the industry, was discussed. However, after discussion, it was decided that IDOLDH would serve as a fine replacement and will most likely be utilized by purification groups moving forward. This means that the second goal was a success as well.
The final overall goal of the project was to optimize bioreactor conditions in a manner which effectively constructs IDOLDH in the biggest, most efficient batches possible. This entails manipulating a variety of variables involved in the bioreactor project in a manner which effectively expressed the enzyme needed. This goal was perhaps the least fleshed out due to the small amount of time spent with a working bioreactor. While an effective method was discovered in reducing the temperature of the bioreactor at approximately five and a half hours, a variety of other variables can be explored in future iterations. This will likely play a large role in future iterations of the cell culture design project undertaken by future seniors.

Looking back on the final extent of the project, it can be said that it was a success. The main goals set were met, and the engineers at Genentech were excited with the progress and understanding that was made throughout the semester. That being said, there are many improvements that can be made in the future. Major changes to be made lie in converting the cell culture process into two bioreactors rather than one. This allows for one bioreactor to be used for growth, in which the cells feed off a simple media made of glucose. The second bioreactor can then be used for expression of IDOLDH. There are multiple advantages to changing the process in such a manner, but the main one comes from an advanced ability to troubleshoot two distinct processes.
References


[2] Albanese, A. Lindemann, E. Olsen, R. Rameriz, A. Stewart, I. (2016). *E. coli Cell Culture Team* [PowerPoint slides]. Retrieved from https://docs.google.com/presentation/d/1o80ni0GEyyqfqAxxDwnkcgced4aPU5y8G6f_0L1d6FQ/edit#slide=id.g12dc53d0b2_3_0


Appendix A: Memorandum of Understanding and Agreement on Use of Biological Agents and Recombinant DNA

University of Nevada, Reno
Memorandum of Understanding and Agreement on Use of Biological Agents and Recombinant DNA

PRINCIPAL INVESTIGATOR: Alan Fuchs

PI’s TITLE: Associate Professor

DEPARTMENT: Chemical Engineering

TELEPHONE NUMBERS (office): 775-784-4960; (labs):

E-MAIL ADDRESS: afuchs@unr.edu

By signing below, I certify that I agree to conduct the research covered by this protocol in compliance with current guidelines regarding use and disposal of recombinant DNA, biological agents and toxins, specified in the NIH Guidelines for Research Involving Recombinant DNA Molecules; the CDC/NIH Biosafety in Microbiological and Biomedical Laboratories; the UNR Biosafety Manual, and the UNR Bloodborne Pathogens Exposure Control Plan.

The PI accepts responsibility for the health and safety of all personnel involved in this project as it relates to the scope of this work. Additionally, I certify that I have read the following statements and agree that all persons working under this protocol will comply with these statements:

* Personnel have received or will receive appropriate safety training (including lab-specific training) prior to working in the laboratory.
* Notify EH&S (327-5040, 24 hr/dy) as soon as possible of laboratory associated injuries or illnesses and spills or environmental releases of hazardous materials.
* Notify Worker’s Compensation for work-related injuries and illnesses.
* Request approval from the IBC of any modifications to biological agents or toxins, facilities, or procedures by submitting an amended MOUA.

_________________________________________  ______________________________
Signature of Principal Investigator                  Date

_________________________________________  ______________________________
Signature of Department Chair                    Date
PERSONNEL INVOLVED IN THE PROJECT:
Include name and job title (e.g., Associate Professor, Postdoctoral Fellow, Ph.D. student, etc.)
Senior class students in ChE 482
Alan Fuchs, Associate Professor, Chemical and Materials Engineering
Paul Michael Kivistik, Lecture Professor, Chemical and Materials Engineering
Christy Howard, Associate Professor, Biochemistry

1. BIOLOGICAL AGENT(S) AND RECOMBINANT DNA TO BE USED:
Check all that apply:

☐ Microbial agents (bacteria (including eubacteria), archaea, viruses, fungi, parasites)
  ☐ Lentiviral Vectors (if lentiviral vectors will be used. MOUA Supplement 1 must be submitted with this MOUA).

☐ Recombinant DNA (check all that apply)
  Specify the section(s) of the NIH Guidelines that correspond to the proposed recombinant DNA research:
  IBC and NIH Approval Required Before Initiation
  ☐ Section III-A: Transfer of drug resistance to microorganisms
  ☐ Section III-B: Cloning of genes coding for toxins with a LD50 less than 100 ng/kg

IBC, IRB, and NIH Approval Required Before Initiation
  ☐ Section III-C: Transfer of recombinant DNA to humans (gene therapy)

IBC Approval Required Before Initiation
  ☐ Section III-D-1: Use of Risk Group 2 or above agent as host-vector system
  ☐ Section III-D-2: DNA from Risk Group 2 or above agent is cloned into a host-vector system
  ☐ Section III-D-3: Use of infectious viruses (> 2/3 of viral genome) in tissue culture
  ☐ Section III-D-4: Experiments involving whole animals (BL2-N or greater)
  ☐ Section III-D-5: Experiments involving whole plants (BL2-P or greater)
  ☐ Section III-D-6: Greater than 10 liters of culture

Page 2 of 10
IBC Notification (MOUA submitted) Simultaneous With Initiation

☐ Section III-E-1: Formation of recombinant DNA containing less than 2/3 of an eukaryotic viral genome
☐ Section III-E-2: Experiments involving whole plants (BL1 only)
☐ Section III-E-3: Experiments involving transgenic rodents (BL1 only)

Excerpt From NIH Guidelines
☐ Section III-F: Specify applicable section of NIH Guidelines App. C-II
☐ Toxins or toxic products (produced by biological agents)
☐ CDC or USDA Select Agent (Click here for list)
☐ Humans, human blood, human body fluids, human tissues, or human cells/cell lines*
☐ Animals (non-human except insects), animal blood, body fluids, tissues, or cells
☐ Plants, plant tissues, plant cell culture, or eukaryotic algae
☐ Arthropods (e.g., insects, arachnids; insect or arachnid tissues; or insect or arachnid cell cultures)

* The use of unfixed human tissue, or human cell or tissue culture normally requires inclusion in the UMR Bloodborne Pathogens Program. Contact Ben Owens (+5196) or Mike Krstel (+4882) of EH&S for additional information.

List all agents and the biosafety containment level (BSL) assigned to each agent (including toxins). Identify all CDC and USDA Select Agents. Include preserved stocks (even if not currently in use), and organisms and toxins "inherited" from other researchers. Indicate current use status (currently in use or only preserved stock).

E.coli K-12 , strain BL21 (DE3), BSL-1

If introducing foreign DNA or RNA into a gene expression system, indicate the source DNA or RNA, and any expected toxic gene products. If not, indicate "N/A."

Source DNA or RNA:

Expected toxic gene products: N/A

Location of use (indicate lab and site within lab, if restricted):
HMS 207, LME 218
Location of storage:
If biological agents are stored in a freezer that is located in either the Howard Medical Sciences (HMS) freezer room (207 and 217) or the Fleischmann Agriculture (FA) freezer room (229), please indicate here the building and freezer number (as indicated on the freezer room maps for HMS 192 and FA 229). Additionally, please print a copy of the relevant freezer room map, label the appropriate freezer, and attach the map to the completed MOUA form.
HMS 207, LME 218

Period/Frequency of use:
1-3 times per month in the Spring Semester 2016

Quantity (give typical and maximal culture volumes, number of organisms, etc.):
1 L

List any permits required (e.g., CDC or USDA Import or Export, CDC or USDA Select Agent Registration). For each permit indicate the agency or group issuing the permit; the agents, materials, and work covered by the permit, and expiration date (if no permits are required indicate “none”).

N/A

2. PROJECT SUMMARY
Provide a short summary (no more than 2 – 3 sentences) of the work to be conducted that describes the overall purpose and goals.

This project introduces students to a bioreactor and have them develop heuristics for a 1L bioreactor. We will be using E.coli BL21 (DE3) standard cells from Invitrogen (BSL-1) to express IDOLDH gene. IDOLDH is found in Ips Pini (Bark beetle) and was genetically engineered into E.coli. The students might expand to separate the protein.

3. EXPERIMENTAL PROCEDURES:
Provide an overview of the experimental work involving each biological agent and the biosafety practices to be used. Provide sufficient detail to allow the IBC to evaluate the risks and proposed biosafety containment levels and procedures.

The E.coli and IDOLDH gene was obtained from Dr. Claus Tittiger. The bacteria will be inoculated on a plate in Howard Medical Science room 207. The media that the E.coli will be growing in contains ampicillin and kanamycin antibiotics. The reason for this is because we are interested in only the E.coli growing and not any environmental contaminants. The inoculated
plate will then be transported to the unit operations lab, LME 218. All cultures will be placed in a leak proof primary container, then a leak proof secondary container, and finally in an outer transportation container for transport to the unit operations lab. The plate prepared in HMS 207 will then be used to inoculate the culture in the bioreactor. The media contains sugars such as glucose lactose, and glycerol. The primary source of macro nutrients contain yeast extract and tryptone. Samples will be collected to determine growth rate using a spectrophotometer at absorbance of 600 nm. Optical density data will then be collected for further analysis. Homogenizing will occur by adding lysing enzymes into the bioreactor. The lysate will be collected and stored in the refrigerator in the unit operations lab for further analysis. All equipment will be decontaminated using 70% ethanol and then autoclaved in the autoclave in Mackay Sciences upon completion of each experiment. All waste cultured with be inactivated with 10% bleach and let to set for at least 30 minutes contact time before disposal through the sewer. Sharps will be collected in an approved sharps container, and solid waste in an autoclave bag. Solid waste will then be autoclaved at Mackey Science. Autoclaved sharps containers will be submitted to EH&S for pick-up, and autoclaved bags can be disposed in the land fill waste after being placed in an opaque trash bag.

4. BIOLOGICAL HAZARDS

For the biological agents and recombinant DNA to be used describe the following. a) the hazards of the materials to be used with respect to humans, animals, and plants and the potential for an environmental hazard. If agents are considered low risk (BSL1), briefly describe why this is the case (do not simply indicate not applicable); b) The infectivity (or toxicity for toxins), and routes of transmission to humans, the risk to humans from exposure, and symptoms that an infected person may display.

The IDOLDH transgene is used in industry as a sustainable alternative to pesticides so as to control bark beetles. There is no danger to humans, although we will wear nitrile gloves, lab coats and safety eye wear. The E.coli is low risk because it is a standard laboratory strain that is low risk to humans.

5. EXPOSURE CONTROL PROCEDURES:

a. Personal Protective Equipment:

☐ Safety glasses ☑ Face shield ☑ Lab coat

☐ Disposable gloves ☑ Other (specify)

b. Engineered Containment Devices:

☐ Biosafety cabinet (show location on lab map; see Section 10) ☑ Lab hood (toxins) ☑ Centrifuge safety cups/rotors

☐ Other (specify)

c. Facility Controls:

Page 5 of 10
PROTOCOL No. 8016-09  REV. APRIL 11, 2013

Hand washing available* □ Negative room pressure □ One-pass ventilation

□ Room air HEPA filtered exhausted to outside

d. Administrative Controls:

□ Controlled laboratory access and door sign*
□ Lab-specific safety training for lab workers*
□ Written safety procedures (SOPs)*
□ UNR laboratory safety training for lab workers*
□ Procedures for sharps collection and disposal
□ IACUC protocol using any agents listed in sect. 1; provide protocol #:

* Required for all biological agents

Describe any additional controls used to protect personnel, animals, and plants from inadvertent exposure to the agent(s), with consideration of unknown infectious agents (e.g., potential unidentified agents in primary tissue or cells).

Decontamination with 10% bleach performed at end of experiment and autoclaved.

6. SECURITY

a. Describe security procedures used to prevent unauthorized access to the agent(s) or release of viable material to the environment (e.g., laboratory locked when not occupied, storage freezers locked, security swipe cards, security cameras, etc.).

Labs locked when not occupied. The E.coli is kept in the freezer with paraflm around it in a petri dish in a zip lock bag.

b. Describe procedures used to account for the agent(s) and to detect missing material (e.g., monthly inventory of biological materials).

Agents always under supervision of Biochemistry or ChE faculty.

7. DECONTAMINATION AND DISPOSAL:

a. Chemical Disinfectants for Decontamination of Surfaces and Equipment:

□ Ethanol (70%) □ Bleach (10% household bleach)
□ Phenolic* □ Quaternary ammonium*
□ Other* (specify)

* mix and use according to manufacturer’s instructions
b. Biohazardous Waste Decontamination:
   - Autoclave* (indicate location) MS
   - Bleach*  □  Sanitary Sewer (small quantities of blood/body fluids)

# A minimum autoclave time of 60 minutes is required by regulation for decontamination of biohazardous waste.
* The only chemical disinfectant allowed by regulation for treatment of biohazardous waste (liquid microbial cultures and stocks) is sodium hypochlorite at a minimum final concentration of 5,000 ppm and a minimum 30 minutes residence time. A concentration of 5,000 ppm can be achieved by a 1/10 final dilution of consumer bleach (5-6% sodium hypochlorite). Sodium hypochlorite solutions used to treat spore-forming pathogenic organisms must be pH adjusted to 7 prior to treatment (must be freshly prepared).

If toxins are used, describe deactivation and disposal procedures (for chemical treatment include residence time). If no toxins are used, indicate "none."

None

8. EMERGENCY PROCEDURES:
Standard response and reporting procedures for incidents involving personnel exposure to biological agents, or spills or releases of biological agents, are described in chapters 10 and 11 of the UNR Biosafety Manual.

a. Location of nearest eyewash: LME 111

b. Indicate response procedures for personnel exposure incidents involving biological agents covered by this MOU.

□ The standard response procedures described in the UNR Biosafety Manual have been reviewed, are judged to be adequate, and will be implemented as needed.

□ Additional or alternative response procedures are needed and are described below.
indicate response procedures for spills or releases of biological agents covered by this MOUA.

The standard response procedures described in the UNR Biosafety Manual have been reviewed, are judged to be adequate, and will be implemented as needed.

Additional or alternative response procedures are needed and are described below.

9. MONITORING PROCEDURES:
   a. List vaccinations\* and medical surveillance required for the work covered by this MOUA, including animal handlers\#.

Vaccinations:
   \(\square\) hepatitis B  \(\square\) tetanus  \(\square\) other (specify)

Describe applicable medical surveillance and personnel sampling procedures or indicate "not applicable" as appropriate.

\* personnel exposed to human fluids, cells, or unfixed tissue (or other sources of HIV or HBV) must take UNR's bloodborne pathogens training annually and be offered the Hepatitis B vaccination, which they may decline.
\# personnel included in the animal handlers program must be offered the tetanus vaccination, which they may decline.

b. Describe applicable laboratory and environmental monitoring or indicate "not applicable" as appropriate.

10. FACILITY OPERATIONAL PROCEDURES:
    Describe any special facility access control measures, controlled areas, and other special facility/laboratory procedures not normally associated with standard laboratories.

11. LAB MAP:
Attach a floor plan of each laboratory that shows the following: 1) where the agent(s) will be cultured, incubated, and stored; 2) location of biological safety cabinets and other safety equipment; and 3) any special room ventilation features not included in standard laboratories (including items marked in Section 4, Facility Controls).
12. INSTITUTIONAL BIOSAFETY COMMITTEE APPROVAL

The procedures and facilities described in this document have been reviewed by the University of Nevada, Reno (UNR) Institutional Biosafety Committee (IBC). The IBC considers the procedures and facilities listed to be appropriate for the described project, and consistent with NIH and UNR guidelines. The IBC, in conjunction with the Environmental Health and Safety Department, will monitor the procedures, facilities, and training of personnel.

________________________________________
Signature of IBC Chair

________________________________________
Protocol Activation Date          Protocol Termination Date
Paul Laxalt Mineral Engineering 218

Revised 9/19/2015

Chemical Hood

Spectrophotometer Work Station

Bench Flammable Cabinet

HPLC Protein Purification Station

Protein Purification Station

Distillation Column

REF

Bio Reactor Work Station

N
Appendix B: Standard Operating Procedure

Making the Media

1) First, appropriate PPE such as gloves, long pants, and close shoes should be worn at all times.

2) Use a flask that is approximately twice the size of the amount of media you are making. Thus, the flask should be approximately 2 L.

3) Then add a stir bar and distilled water before adding any other chemicals. The water should not be too close to the desired amount.

4) Add the chemicals one at a time measuring out the mass using a balance.

5) Transfer the solution to a graduated cylinder.

6) Water should be added up to the 1000 mL mark.

7) Transfer the solution back to the flask.

8) Tin foil is put over the top of the flask and 1-inch piece of autoclave tape is placed on the foil.

9) Depending on the amount of liquid, autoclave time will vary. Autoclaving is necessary to decontaminate the solid media. *Autoclaving will be performed by instructor.

Streaking a Plate from a Single Bacteria Colony

1) Make sure to be wearing gloves when conducting this procedure.

2) Using a sterilized toothpick, take a single colony and streak as follows:
3) Change toothpicks between each streaking event. In the example above, you will use four sterile toothpicks.

4) The plate will be stored overnight at 37°C before being able to inoculate your media with a single colony.

5) The overnight plate can be sealed with Parafilm and stored upside down in the refrigerator for over one month.

**Inoculating the Media**

1) Add antibiotic to the media

   a. The amount of antibiotic is determined using $C_1V_1=C_2V_2$ (*Ensure that the antibiotic is stored in a sterile and dark place*).

   b. When adding antibiotic to liquid media, the solution should not be too hot. Hot media will degrade the antibiotic and allow for unwanted organisms to grow. To determine if the temperature is correct for adding the
antibiotic, touch the flask to the inside portion of your wrist. It can be quite warm, but should not be painful (~55 C).

c. Prior to inoculating the media, a 10 ml sample of the media with antibiotic should be set aside to use as a zero reference for the spectrophotometer. (*All reading for the OD to determine cell concentration will be at 600 nm. If a sample reads above 0.6, the sample should be diluted with leftover media to read below 0.6 as above that value, the spectrophotometer readings are no longer linear).  
d. If a reading is above the threshold of the spectrophotometer, the sample should be diluted with extra media before obtaining a reading.

2) One colony from the streaked plate of *E. coli* is then added to a 100 mL aliquot of media and placed in the shaker table at 37 C. It should be kept on the shaker table overnight until the experiment is ready to be run. Additionally, set aside 20 mL of media to be used for dilutions if necessary.

3) In the morning, place the remaining media in the bioreactor. Add the 100 mL aliquot of media with *E. coli*.

**Calibrating the pH meter and DO meter**

1) The pH meter can be calibrated used a three buffer solutions of pH 4, 7, and 10. The pH meter should always be kept in solution when not being used in the bioreactor. To adjust the reading of the pH meter to match the known buffers, a screw driver can be used to adjust the reading to the known buffer pH if it does not read the specified pH.
2) The DO meter is calibrated by completely sparging the bioreactor for several hours with nitrogen. The Oxygen concentration should be 0% after approximately 6 hours. (This can be done a day prior to running experiments).

Using the Bioreactor

1) Turn on the water pump that is attached to the heating jacket; set at 37 C. This will ensure that the bioreactor is kept at a consistent 37 C. The temperature can be recorded manually by reading the water pump thermometer.

2) Next the impeller should be turned on to a speed of approximately 400-600 rpm with a fixed 1 L/min air flowing through the system. (*It is important to open air slowly as to not cause the water/air filtration intermediate to explode due to high pressure build up).

3) Take a 2 mL using the syringe connected to the top. Samples should be taken at time 0 minutes, 1 hour, 2 hours (and every 30 minutes once the OD (600 nm) reading has reached 0.4 indicating that the E. coli is in the log exponential growth phase). pH, DO, OD (600 nm) and temperature readings should be recorded at each time a sample is taken from the bioreactor.

4) After a sample is taken from the bioreactor, the syringe should be wiped down with ethanol to reduce contamination.

5) Once the OD reading has reached approximately 0.6, turn off the air supply. Samples should be taken every 30 seconds for a total of 10 readings (5 minutes).

6) Turn the air supply back on at a flow rate of 1 L/min. Samples should be taken every five seconds for a total of 12 readings (1 minutes).
7) Continue taking samples every 30 minutes once the 20 readings described in steps 5-6 have been taken.

8) Readings should be taken for six hours.

9) The Genzyme glucose kit will be used to determine the glucose concentration for each sample.
   a. A 1:100 sample to reagent dilution should be made
   b. The spectrophotometer should be set to read at 505 nm
   c. The blank will be the reagent only
   d. Glucose concentration is reported as mg/dL (mmol/L)

Clean Up

1) Once all recordings have been obtained, the mixer and water pump should be stopped and unplugged. The bioreactor contents should be mixed with bleach before being dumped into the organic waste bucket.

2) All glassware and cuvettes should be washed with a 10% bleach solution, rinsed with ddH2O to remove bleach (which will kill residual E. coli), rinsed again with 70% ethanol solution and left to dry before use.

3) Other pieces (DO meter and pH) meter should be wiped down with a 70% ethanol solution.

4) Autoclaving of all equipment will be handled by the instructor.
### Appendix C: Experimental Data

#### Time Zero

**Sample No.** | **Sample Name** | **Spokes Produced (24)** | **Spokes Produced (24) Rate of Change** | **Time 1** | **Time 2** | **Spokes Produced (24) Rate of Change** | **Time 3** | **Spokes Produced (24) Rate of Change**
---|---|---|---|---|---|---|---|---
1 | T.C. | 20 | 6 | 4.00 | 15.0 | 20.83 | 15.44 | 79.38 |
2 | T.C. | 20 | 4 | 6.00 | 18.0 | 22.25 | 15.70 | 79.38 |
3 | T.C. | 20 | 6 | 4.00 | 15.0 | 20.83 | 15.44 | 79.38 |
4 | T.C. | 20 | 4 | 6.00 | 18.0 | 22.25 | 15.70 | 79.38 |
5 | T.C. | 20 | 6 | 4.00 | 15.0 | 20.83 | 15.44 | 79.38 |
6 | T.C. | 20 | 4 | 6.00 | 18.0 | 22.25 | 15.70 | 79.38 |

#### Time Three

**Sample No.** | **Sample Name** | **Spokes Produced (24)** | **Spokes Produced (24) Rate of Change** | **Time 1** | **Time 2** | **Spokes Produced (24) Rate of Change** | **Time 3** | **Spokes Produced (24) Rate of Change**
---|---|---|---|---|---|---|---|---
1 | T.C. | 20 | 6 | 4.00 | 15.0 | 20.83 | 15.44 | 79.38 |
2 | T.C. | 20 | 4 | 6.00 | 18.0 | 22.25 | 15.70 | 79.38 |
3 | T.C. | 20 | 6 | 4.00 | 15.0 | 20.83 | 15.44 | 79.38 |
4 | T.C. | 20 | 4 | 6.00 | 18.0 | 22.25 | 15.70 | 79.38 |
5 | T.C. | 20 | 6 | 4.00 | 15.0 | 20.83 | 15.44 | 79.38 |
6 | T.C. | 20 | 4 | 6.00 | 18.0 | 22.25 | 15.70 | 79.38 |

#### Time Four

**Sample No.** | **Sample Name** | **Spokes Produced (24)** | **Spokes Produced (24) Rate of Change** | **Time 1** | **Time 2** | **Spokes Produced (24) Rate of Change** | **Time 3** | **Spokes Produced (24) Rate of Change**
---|---|---|---|---|---|---|---|---
1 | T.C. | 20 | 6 | 4.00 | 15.0 | 20.83 | 15.44 | 79.38 |
2 | T.C. | 20 | 4 | 6.00 | 18.0 | 22.25 | 15.70 | 79.38 |
3 | T.C. | 20 | 6 | 4.00 | 15.0 | 20.83 | 15.44 | 79.38 |
4 | T.C. | 20 | 4 | 6.00 | 18.0 | 22.25 | 15.70 | 79.38 |
5 | T.C. | 20 | 6 | 4.00 | 15.0 | 20.83 | 15.44 | 79.38 |
6 | T.C. | 20 | 4 | 6.00 | 18.0 | 22.25 | 15.70 | 79.38 |

#### Time Five

**Sample No.** | **Sample Name** | **Spokes Produced (24)** | **Spokes Produced (24) Rate of Change** | **Time 1** | **Time 2** | **Spokes Produced (24) Rate of Change** | **Time 3** | **Spokes Produced (24) Rate of Change**
---|---|---|---|---|---|---|---|---
1 | T.C. | 20 | 6 | 4.00 | 15.0 | 20.83 | 15.44 | 79.38 |
2 | T.C. | 20 | 4 | 6.00 | 18.0 | 22.25 | 15.70 | 79.38 |
3 | T.C. | 20 | 6 | 4.00 | 15.0 | 20.83 | 15.44 | 79.38 |
4 | T.C. | 20 | 4 | 6.00 | 18.0 | 22.25 | 15.70 | 79.38 |
5 | T.C. | 20 | 6 | 4.00 | 15.0 | 20.83 | 15.44 | 79.38 |
6 | T.C. | 20 | 4 | 6.00 | 18.0 | 22.25 | 15.70 | 79.38 |
### Appendix C: Experimental Data

**Experiment 2: Bioreactor 37°C First Run**

#### 37°C Experiment

<table>
<thead>
<tr>
<th>Conversion (cell/ml)</th>
<th>Time (h)</th>
<th>OD</th>
<th>x (kg cells m⁻³)</th>
<th>r (kg cells m⁻³ h⁻¹)</th>
<th>u = r/ν (kg D⁻¹)</th>
<th>1/α (h)</th>
<th>O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>80000000</td>
<td>0.000</td>
<td>0.097</td>
<td>0.078</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>94.20%</td>
</tr>
<tr>
<td>1E-15</td>
<td>0.500</td>
<td>0.162</td>
<td>0.130</td>
<td>0.061</td>
<td>0.469</td>
<td>2.132</td>
<td>84.20%</td>
</tr>
<tr>
<td>ml to m⁻³</td>
<td>1.000</td>
<td>0.173</td>
<td>0.138</td>
<td>0.113</td>
<td>0.815</td>
<td>1.227</td>
<td>83.20%</td>
</tr>
<tr>
<td>100000</td>
<td>1.500</td>
<td>0.303</td>
<td>0.242</td>
<td>0.255</td>
<td>1.053</td>
<td>0.950</td>
<td>67.10%</td>
</tr>
<tr>
<td>Final Conversion</td>
<td>2.000</td>
<td>0.492</td>
<td>0.394</td>
<td>0.462</td>
<td>1.173</td>
<td>0.853</td>
<td>1.20%</td>
</tr>
<tr>
<td>0.8</td>
<td>2.500</td>
<td>0.880</td>
<td>0.704</td>
<td>1.082</td>
<td>1.536</td>
<td>0.651</td>
<td>2.90%</td>
</tr>
<tr>
<td>$S$ correlation</td>
<td>3.000</td>
<td>1.844</td>
<td>1.475</td>
<td>1.557</td>
<td>1.055</td>
<td>0.948</td>
<td>2.30%</td>
</tr>
<tr>
<td>-2.1486</td>
<td>3.500</td>
<td>2.826</td>
<td>2.261</td>
<td>1.861</td>
<td>0.823</td>
<td>1.215</td>
<td>1.80%</td>
</tr>
<tr>
<td>4</td>
<td>4.17</td>
<td>3.336</td>
<td>2.150</td>
<td>0.645</td>
<td>1.551</td>
<td>2.00%</td>
<td></td>
</tr>
</tbody>
</table>

**Graph:** E Coli, Oxygen Concentration vs Time (hr)

- x (kg cells m⁻³)
- O₂
**Data Experiment #3 37°C**

<table>
<thead>
<tr>
<th>Conversion (volumetric)</th>
<th>1000000</th>
<th>5600000</th>
<th>11650000</th>
<th>17300000</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.000</td>
<td>0.000</td>
<td>0.026</td>
<td>0.087</td>
<td>0.237</td>
</tr>
<tr>
<td>0.050</td>
<td>0.050</td>
<td>0.067</td>
<td>0.187</td>
<td>0.487</td>
</tr>
<tr>
<td>1.000</td>
<td>1.000</td>
<td>1.017</td>
<td>0.793</td>
<td>0.793</td>
</tr>
<tr>
<td>1.100</td>
<td>1.100</td>
<td>0.188</td>
<td>1.250</td>
<td>0.793</td>
</tr>
<tr>
<td>2.000</td>
<td>2.000</td>
<td>0.256</td>
<td>1.540</td>
<td>0.877</td>
</tr>
<tr>
<td>2.334</td>
<td>2.334</td>
<td>0.396</td>
<td>0.425</td>
<td>0.911</td>
</tr>
<tr>
<td>2.533</td>
<td>2.533</td>
<td>0.534</td>
<td>0.866</td>
<td>1.718</td>
</tr>
<tr>
<td>3.147</td>
<td>3.147</td>
<td>1.109</td>
<td>1.900</td>
<td>1.173</td>
</tr>
<tr>
<td>4.000</td>
<td>4.000</td>
<td>2.716</td>
<td>1.199</td>
<td>0.548</td>
</tr>
<tr>
<td>5.000</td>
<td>5.000</td>
<td>3.693</td>
<td>2.279</td>
<td>0.632</td>
</tr>
<tr>
<td>5.500</td>
<td>5.500</td>
<td>3.698</td>
<td>4.227</td>
<td>0.754</td>
</tr>
<tr>
<td>10.000</td>
<td>10.000</td>
<td>9.712</td>
<td>12.140</td>
<td>0.931</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>S g glucose/l</th>
<th>6.18326107</th>
<th>6.52797432</th>
<th>6.531950451</th>
<th>5.968994467</th>
</tr>
</thead>
<tbody>
<tr>
<td>S l mg/l</td>
<td>61.8</td>
<td>66.3</td>
<td>64.5</td>
<td>59.1</td>
</tr>
</tbody>
</table>

**Diagram:**

- **E. Coli, Glucose, Oxygen Concentration vs Time (h)**
- **Time (h): 0.000 to 10.000**
- **OD**: 0.000 to 6.18326107
- **S g glucose/l**: 6.18326107 to 6.52797432
- **S l mg/l**: 61.8 to 65.3
### Appendix C: Experimental Data

#### Experiment #4: Bioelectric 37°C vs 30°C

<table>
<thead>
<tr>
<th>Time(h)</th>
<th>Optical Density</th>
<th>Volts (cells m⁻³)</th>
<th>Volts (dig cells m⁻³)</th>
<th>% = NOx/</th>
<th>I/L (h)</th>
<th>O2</th>
<th>OD160</th>
<th>% (mg/L)</th>
<th>% (mg glucose m⁻³)</th>
<th>L/l (m⁻³, kg glucose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.000</td>
<td>0.097</td>
<td>0.078</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>55.9</td>
<td>0.02</td>
<td>-</td>
<td>0.017</td>
<td>0.00000</td>
</tr>
<tr>
<td>0.000</td>
<td>0.167</td>
<td>0.130</td>
<td>0.065</td>
<td>0.385</td>
<td>2.13</td>
<td>84.75</td>
<td>0.218</td>
<td>0.985</td>
<td>0.155</td>
<td>0.00000</td>
</tr>
<tr>
<td>10.000</td>
<td>0.173</td>
<td>0.188</td>
<td>0.113</td>
<td>0.713</td>
<td>1.292</td>
<td>85.39</td>
<td>0.326</td>
<td>77.6</td>
<td>2.703983718</td>
<td>0.126313236</td>
</tr>
<tr>
<td>1.000</td>
<td>0.303</td>
<td>0.242</td>
<td>0.258</td>
<td>1.053</td>
<td>0.555</td>
<td>67.18</td>
<td>-</td>
<td>-</td>
<td>0.473320445</td>
<td>0.146304488</td>
</tr>
<tr>
<td>2.000</td>
<td>0.446</td>
<td>0.411</td>
<td>0.462</td>
<td>1.171</td>
<td>0.633</td>
<td>71.06</td>
<td>0.42</td>
<td>83.5</td>
<td>0.549081332</td>
<td>0.137553719</td>
</tr>
<tr>
<td>2.500</td>
<td>0.554</td>
<td>0.384</td>
<td>0.632</td>
<td>1.148</td>
<td>0.635</td>
<td>2.906</td>
<td>0.115</td>
<td>52.3</td>
<td>0.23217914627</td>
<td>0.171524948</td>
</tr>
<tr>
<td>3.000</td>
<td>0.684</td>
<td>0.351</td>
<td>0.827</td>
<td>1.151</td>
<td>0.988</td>
<td>3.301</td>
<td>0.077</td>
<td>26.5</td>
<td>2.806048148</td>
<td>0.376602127</td>
</tr>
<tr>
<td>3.500</td>
<td>2.945</td>
<td>2.261</td>
<td>1.892</td>
<td>0.819</td>
<td>1.75</td>
<td>1.936</td>
<td>0.00</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4.000</td>
<td>3.434</td>
<td>3.306</td>
<td>1.658</td>
<td>0.407</td>
<td>2.011</td>
<td>2.306</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4.500</td>
<td>4.100</td>
<td>3.525</td>
<td>1.762</td>
<td>0.222</td>
<td>4.049</td>
<td>0.006</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5.000</td>
<td>5.166</td>
<td>4.228</td>
<td>2.566</td>
<td>0.685</td>
<td>1.569</td>
<td>1.936</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5.500</td>
<td>6.045</td>
<td>4.653</td>
<td>3.287</td>
<td>0.321</td>
<td>6.019</td>
<td>2.306</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6.000</td>
<td>16.006</td>
<td>13.512</td>
<td>1.034</td>
<td>0.076</td>
<td>13.185</td>
<td>2.306</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6.500</td>
<td>18.958</td>
<td>23.160</td>
<td>2.735</td>
<td>1.113</td>
<td>2.598</td>
<td>1.936</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7.000</td>
<td>22.500</td>
<td>18.800</td>
<td>1.257</td>
<td>0.029</td>
<td>16.909</td>
<td>1.936</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7.500</td>
<td>24.600</td>
<td>19.680</td>
<td>0.435</td>
<td>-0.222</td>
<td>45.241</td>
<td>49.99</td>
<td>0</td>
<td>-</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Final Conversion**: 0.8

**S correlation**: 0.146

**mg Hz kg**: 0.0001

**Decelerate to m⁻³**: 0.0001
### Appendix C: Experimental Data

**Experiment 45: Oxygen Uptake and Transfer Rate Experiment**

<table>
<thead>
<tr>
<th>Time (s)</th>
<th>DO (mg/L)</th>
<th>Air</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>21.43</td>
<td>Off</td>
</tr>
<tr>
<td>30</td>
<td>20.32</td>
<td>Off</td>
</tr>
<tr>
<td>60</td>
<td>19.4</td>
<td>Off</td>
</tr>
<tr>
<td>90</td>
<td>18.21</td>
<td>Off</td>
</tr>
<tr>
<td>120</td>
<td>17.04</td>
<td>Off</td>
</tr>
<tr>
<td>150</td>
<td>15.84</td>
<td>Off</td>
</tr>
<tr>
<td>180</td>
<td>14.63</td>
<td>Off</td>
</tr>
<tr>
<td>210</td>
<td>13.47</td>
<td>Off</td>
</tr>
<tr>
<td>240</td>
<td>12.24</td>
<td>Off</td>
</tr>
<tr>
<td>270</td>
<td>11.16</td>
<td>Off</td>
</tr>
<tr>
<td>300</td>
<td>9.96</td>
<td>Off</td>
</tr>
<tr>
<td>330</td>
<td>8.89</td>
<td>Off</td>
</tr>
<tr>
<td>360</td>
<td>7.91</td>
<td>Off</td>
</tr>
<tr>
<td>390</td>
<td>6.79</td>
<td>Off</td>
</tr>
<tr>
<td>420</td>
<td>5.78</td>
<td>Off</td>
</tr>
<tr>
<td>450</td>
<td>4.68</td>
<td>Off</td>
</tr>
<tr>
<td>480</td>
<td>3.65</td>
<td>Off</td>
</tr>
<tr>
<td>510</td>
<td>2.6</td>
<td>Off</td>
</tr>
<tr>
<td>540</td>
<td>1.47</td>
<td>Off</td>
</tr>
<tr>
<td>570</td>
<td>0.49</td>
<td>Off</td>
</tr>
<tr>
<td>600</td>
<td>2.31</td>
<td>On</td>
</tr>
<tr>
<td>620</td>
<td>2.26</td>
<td>On</td>
</tr>
<tr>
<td>630</td>
<td>2.31</td>
<td>On</td>
</tr>
<tr>
<td>640</td>
<td>2.42</td>
<td>On</td>
</tr>
<tr>
<td>645</td>
<td>2.47</td>
<td>On</td>
</tr>
<tr>
<td>650</td>
<td>2.62</td>
<td>On</td>
</tr>
<tr>
<td>655</td>
<td>2.67</td>
<td>On</td>
</tr>
<tr>
<td>660</td>
<td>2.95</td>
<td>On</td>
</tr>
<tr>
<td>649</td>
<td>3.09</td>
<td>On</td>
</tr>
<tr>
<td>650</td>
<td>3.22</td>
<td>On</td>
</tr>
<tr>
<td>655</td>
<td>3.48</td>
<td>On</td>
</tr>
<tr>
<td>660</td>
<td>3.42</td>
<td>On</td>
</tr>
<tr>
<td>665</td>
<td>3.5</td>
<td>On</td>
</tr>
</tbody>
</table>
Appendix D: Governor’s Cup

University of Nevada, Reno

Gap Industries: A Business Plan to Change the World

By
Anita Albanese
Andrew Sorensen
Ben Wallace
Ian Stewart
Dr. Alan Fuchs, Thesis Advisor
May, 2016
Abstract

A business plan competition enamored in entrepreneurship is presented. An analysis of applicable concepts in the chemical engineering industry is sifted through to find suitable fits in the startup field, deciding upon graphene production. Potential products and services are deliberated upon to find an applicable niche. The market industry is analyzed for an advantage, finding no competition. An organizational structure is decided upon to lead this potential venture. Critical risks and operating strategies are pursued in an effort to determine viability. Cash flow, income, and sheet balances are analyzed over a period of five years. Potential offerings to investors are determined.
<table>
<thead>
<tr>
<th>Table of Contents</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Tables and Figures</td>
<td>i</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Executive Summary</td>
<td>1</td>
</tr>
<tr>
<td>Company Overview</td>
<td>3</td>
</tr>
<tr>
<td>Products and Services</td>
<td>4</td>
</tr>
<tr>
<td>Market Analysis and Advantage</td>
<td>5</td>
</tr>
<tr>
<td>Management Team</td>
<td>7</td>
</tr>
<tr>
<td>Operating Strategies</td>
<td>7</td>
</tr>
<tr>
<td>Critical Risks</td>
<td>9</td>
</tr>
<tr>
<td>Cash Flow, Income, and Balance</td>
<td>10</td>
</tr>
<tr>
<td>Award Funding</td>
<td>12</td>
</tr>
<tr>
<td>Offerings</td>
<td>12</td>
</tr>
<tr>
<td>Bibliography</td>
<td>15</td>
</tr>
<tr>
<td>Appendix A: Twelve Month Cash Flow</td>
<td>16</td>
</tr>
</tbody>
</table>
## List of Tables and Figures

<table>
<thead>
<tr>
<th>Title in order of Appearance</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1. The molecular structure of graphene leads to incomparable strength</td>
<td>5</td>
</tr>
<tr>
<td>Table 1. The five branches of GAP Industries, and their respective chiefs</td>
<td>7</td>
</tr>
<tr>
<td>Figure 2. Above is a rough estimate on the milestones by month pushed for by GAP Industries</td>
<td>14</td>
</tr>
<tr>
<td>Figure 3. Business model canvas</td>
<td>14</td>
</tr>
<tr>
<td>Appendix A. Twelve month cash flow</td>
<td>16</td>
</tr>
</tbody>
</table>
Introduction

The Governor’s Cup is an entrepreneurial competition that encourages Nevada’s collegiate students to pursue their business ideas. These ideas then have the opportunity for publicity, disclosure to entrepreneurial networks and investors, business planning, and development activities. There are endless ideas and opportunities for potential businesses in the world of engineering and entrepreneurship, especially when universities are able to facilitate commercialization, funding, and development of new technologies. Thus, the competition allows students to demonstrate their acquired skills and test the feasibility of their ideas. Furthermore, the project promotes the development of professional skills—team work, report writing, and presenting.

The competition is judged based on the executive summary, company overview, products, market and competitive analysis, management team, operating strategies, critical risks, cash flow statement, income statement, balance sheet, funds required, and offering. After a preliminary stage, the semi-finalists are asked to give an oral presentation about their business. Overall the competition awards almost $400,000 in cash prizes divided among undergraduate and graduate competitors. Each team must have a faculty advisor acting as a mentor to guide them through the process. Dr. Alan Fuchs, a professor and researcher in chemical and materials engineering, was the mentor who led to the creation of the proposed business Graphene Applications and Production (G.A.P.) Industries.

Executive Summary

Graphene Applications and Production (G.A.P.) Industries produces the new super material graphene for a variety of consumer applications. The use of our graphene in
technologies such as electronics, filtration, and even body armor results in superior reliability, efficiency, and sustainability of targeted products. Our major objective is to become a leading producer of graphene for real world applications by implementing innovative ideas and creating superior products.

Graphene is currently being considered as a candidate to replace silicon as the leading material for semiconductor technologies. Graphene applications can extend far beyond things like Kevlar and ultra-strong gas cylinders, from bioengineering to optics, photovoltaics, and energy storage. Finding a way to manufacture graphene at reduced cost could potentially change the world.

As of February 2016, there appears to be no company that is mass producing graphene, at least to the scales needed to use in practical applications that require large amounts. If successfully funded, GAP Industries would have a huge advantage in the market being the only mass supplier of graphene. Since we would be only producing bulk material, the potential customers would be very diverse. We could easily sell graphene and its derivatives to research labs, the semiconductor industry, cable manufacturers, clothing manufacturers, the list goes on.

The company aims to follow an open floor organizational structure, keeping things fairly intertwined within the first five years. Further expansion beyond that point will be met accordingly, but as it stands, each of the five founders of GAP Industries will lead an important division within the company.

GAP Industries follows a business to business operating strategy, meaning we sell our products to other businesses that then use it in their own specific application. The foundation of our success is the successful sales of a selected product from our targeted company. Product
testing is a major component to success in the market. Reno is a great place for cheap material resources for ample product testing before the consumer product makes it to the market.

As a large scale venture, GAP Industries is subject to a great many risks that will pursue the company throughout its tenure. That being said, there have been two critical risks identified that need to be dealt with in the early stages of the business if it is to be a successful organization of graphene production, research loops and scaling up too quickly.

GAP Industries will need an initial investment of 500,000 dollars. The investment will be used to buy all the resources needed to make the graphene. In the second year, the company will establish itself as a major producer of graphene. In year three, GAP Industries will become the major producer of graphene in the United States. Year four will see GAP Industries establishing itself as a power player in the energy industry, with year five seeing the company go public.

GAP Industries values being the first company to provide high quality graphene on a large scale. This will allow the company to bring a competitive edge to customers utilizing graphene for the future of the world.

**Company Overview**

Graphene Applications and Production (G.A.P.) Industries produces the new super material graphene for a variety of consumer applications. The use of our graphene in technologies such as electronics, filtration, and even body armor results in superior reliability, efficiency, and sustainability of targeted products. Our major objective is to become a leading producer of graphene for real world applications by implementing innovative ideas and creating superior products. We have a vision of improving several technologies through technological
advancements due to implementation of graphene. GAP Industries is based out of Reno, Nevada, a city close to Silicon Valley and sources of rich metallurgical resources useful for technological integration and cheap material for testing conditions. The company is still in the startup phase, generating quality ideas for production and finding loyal customers. Since the initial foundation in 2015, our mission has been to bridge the gap between the practical applications and affordable production of graphene.

**Products and Services**

Graphene is an allotrope of the element carbon. It is essentially a monoatomic sheet of carbon atoms arranged in a hexagonal pattern. Its structure allows it to be rolled into tubes called carbon nanotubes (or CNT’s). The physical and chemical properties of graphene and CNT’s are phenomenal. They are 207 times stronger than steel per weight. In technical terms, steel has a tensile strength of 4150 MPa, while graphene has a tensile strength of about 130,000 MPa. Numerous applications can be thought of when using graphene and its derivatives. A mere few sheets of graphene can be used to create ballistic armor with unparalleled performance compared to today’s traditional Kevlar soft armors or plated ceramic armors. Combined with resin sealants, graphene can be used to create super strong gas cylinders that are ultra-light. With CNT’s, ultra-high strength cables can be created with enough strength to allow elevators to space possible.
Figure 1. The molecular structure of graphene leads to incomparable strength.

Since it is possible to vary the diameter of the tubes, CNT’s can be used to create membrane separation units with desired parameters easily. CNT’s have many desirable electrical properties. Since it a tube, electrons can flow inside the CNT in only one direction and with no possibility of leakage (which is a problem in today’s semiconductor industry).

Graphene is currently being considered as a candidate to replace silicon as the leading material for semiconductor technologies. Graphene applications can extend far beyond these mentioned, from bioengineering to optics, photovoltaics, and energy storage. Finding a way to manufacture graphene at reduced cost could potentially change the world.

**Market Analysis and Advantage**

As of February 2016, there appears to be no company that is mass producing graphene, at least to the scales needed to use in practical applications that require large amounts. If successfully funded, GAP Industries would have a huge advantage in the market being the only mass supplier of graphene. Since we would be only producing bulk material, the potential customers would be very diverse. We could easily sell graphene and its derivatives to research
labs, the semiconductor industry, car manufacturers, armorers, the military, cable manufacturers, clothing manufacturers, the list goes on.

The current price of graphene makes it one of the most expensive materials in the world (depending on its quality). Current estimates on the price of graphene are about $1000 per gram. Finding a way to mass produce graphene would greatly reduce this price and put us at a significant advantage. Plus, the proposed methods below suggest that any material containing carbon atoms can be used as a starting material. Methane is used below, but graphite may work as well. Given that the cost of graphite is about $2 per kilogram, makes mass producing graphene a viable option (though the capital cost would be high).

Due to recent discoveries in the potential mass production of graphene it is hoped that we could easily implement said discoveries to produce graphene. Traditionally, graphene has been produced using a method called chemical vapor deposition (CVD). This involves heating a copper chamber filled with methane (CH4) to 1000 degrees Celsius. Methane molecules are stripped of their hydrogen atoms and the remaining carbon atoms are allowed to arrange themselves onto the copper substrate. This process is time consuming, very expensive, and does not produce very high quality graphene. Researchers at MIT and Caltech have found ways to reduce the temperature, time, and double the quality of the graphene produced. At Caltech, Boyd et. al. utilized a copper substrate that has been treated with a nitrogen compound, effectively smoothing out the surface and creating a more effective catalytic surface. This allows the temperature down to about 420 degrees Celsius while doubling the quality of graphene and only taking about five minutes (compared to nine hours). MIT researchers have
come up with a continuous CVD method that uses ribbons of copper as the substrate. By potentially combining both Boyd’s and MIT’s methods, we could mass produce graphene.

**Management Team**

The company aims to follow an open floor organizational structure, keeping things fairly intertwined within the first five years. Further expansion beyond that point will be met accordingly, but as it stands, each of the five founders of GAP Industries will lead an important division within the company. While at first spearheading their organizational department, with expansion, each chief will lead a small innovative team to tackle the problem presented, as seen in Table 1.

**Table 1. The five branches of GAP Industries, and their respective chiefs.**

<table>
<thead>
<tr>
<th>Chief Executive</th>
<th>Ian Stewart</th>
<th>The executive branch of the organization will decide on the organization’s goals and future ambitions. This will be a small team.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chief Operations</td>
<td>Ben Wallace</td>
<td>The operations branch of the organization will run the production of the graphene on the day to day basis. This will likely be the largest team one the company ramps up full scale operations.</td>
</tr>
<tr>
<td>Chief Engineering</td>
<td>Andrew Sorenson</td>
<td>The engineering branch of the organization will develop and refine the graphene production process. This will be the largest team in the beginning of the company, and will be downsized accordingly due to growth.</td>
</tr>
<tr>
<td>Chief Financials</td>
<td>Anthony Ramirez</td>
<td>The financials branch of the organization will be in charge of securing future partners and research grants. This will be a small interconnected team that will pursue the profitability of GAP Industries</td>
</tr>
<tr>
<td>Chief Logistics</td>
<td>Anita Albanese</td>
<td>The logistics branch of the organization will be the jack of all trades of GAP Industries, filling in where necessary. This will be a small team of innovative experts who immerse themselves in challenges.</td>
</tr>
</tbody>
</table>

**Operating Strategies**

GAP Industries follows a business to business operating strategy, meaning we sell our products to other businesses that then use it in their own specific application. The foundation
of our success is the successful sales of a selected product from our targeted company. Product testing is a major component to success in the market. Reno is a great place for cheap material resources for ample product testing before the consumer product makes it to the market.

Qualified electrical, materials science, mechanical, and chemical engineering personnel will be required to successfully research and develop specific products for customers. Once a product is tested, these engineers will primarily focus on scaling up for profitable production. The production process will be controlled by operators and managed through proper administration. Administrative personnel in operations, finance, and production will provide leadership for the different sectors of the production facility.

One of the company business objectives crucial to revenue generation is to create an industry wide reputation as experts in the field of graphene production and applications. Our image in the business world is dependent on active marketing, i.e. presenting products at conventions, giving talks about the many benefits of incorporating graphene based components into consumer products, writing new articles from various points of view within the graphene industry, and expanding our professional network as much as possible. A reputable company with a team of experts shown to deliver quality work has a higher chance of getting business than a competing company with no reputation in the same industry.

Because of its material properties, graphene leads our company towards businesses focused around electronics, production, defense, and recreation. The majority of products we work with are already developed and marketed. We work to identify deficiencies in these existing products and troubleshoot how our product, graphene, can enhance their reliability
and performance. Our goal is to be a leader in improvement and innovation of consumer products for future markets.

**Critical Risks**

As a large scale venture, GAP Industries is subject to a great many risks that will pursue the company throughout its tenure. That being said, there have been two critical risks identified that need to be dealt with in the early stages of the business if it is to be a successful organization of graphene production.

**Risk #1 - Research Loops**

GAP Industries runs the very real risk of being stuck perpetually in a constant loop of research, looking for ways to improve the process of production, rather than scaling up and pursuing opportunities. If the process never comes to fruition in a profitable manner, this will result in not only money, but time wasted.

To solve this, a top notch research team will be necessary to constantly stay up to date in the field. It will also need a Board of Advisors directing the company to proceed towards scale up once a certain level of efficiency in graphene production has been achieved.

**Risk #2 - Scaling Up Too Quickly**

As the forerunner in a field soon to explode, GAP Industries would feel unnecessary pressure to scale up operations quickly to meet the growing demand for graphene, once the process proves successful. In a field that is constantly advancing, this could quickly lead to the creation of facilities which will be outdated in not only years, but months.

GAP Industries aims to solve this problem by partnering slowly and deliberately with its customers. Rather than sell to the highest bidder and begin a rat race with itself, the company
plans to develop long lasting relations that will result in mutual relationships in the years to come.

**Cash Flow, Income, and Balance**

**Year One**

GAP Industries will need an initial investment of 500,000 dollars. The investment will be used to buy all the resources we need to make the graphene. The items we need are listed in Appendix B. A substantial amount of the investment will be used to buy the chemical vapor deposition machines. These machines will be used to make graphene, which will be sold to the target audience. This audience is different photovoltaic companies to produce superconductors, filtrations companies, and the military. The first year will be in the negative as the majority of the money will be used on capital cost. GAP industries will also use the funds to travel to further competitions to compete in different business planning contests. Another cost that must be accounted for is the cost of assistants and the engineers. This is a must, because it will help the company in many different aspects. The engineers are needed to maintain and run the equipment; they are also needed in the research and development aspect of the company. The first year of the corporation will be a rough one but it does show promise. The cash flow is seen in Appendix B. Even though it seems like there was a loss this year, it takes some time for GAP Industries to produce a profit. In the second year of the company’s existence innovations will be introduced, opening the door to expand the company’s market.

**Year Two**

GAP Industries had a rough first year, but in the second year the company will establish itself as a major producer of graphene on the west coast. This will be accomplished by
introducing different ways to produce graphene. In the first year the corporation established itself as a producer of graphene for different uses. In the second year the company’s research and development branch will be further expanded which will innovate the production of graphene and the application of graphene. The company will focus and expand the military aspect. GAP Industries will start producing and selling different products which incorporate graphene. The cash flow statement will be similar to that seen in Appendix B. The biggest difference is that more money will go into buying supplies to produce more graphene and into developing graphene for different usages. There will be substantial profit this year.

**Year Three**

GAP Industries is the major producer of graphene in the United States. In this year, the company will become even larger. This will be done by following a similar formula that was taken in year two. The biggest difference is that GAP Industries will be expanding on the energy side of graphene while still maintaining a huge presence in the military sector. Energy will be a focal point because of the huge strides the United States is making in renewable energy. The company will be competing in different Green Energy competitions to get funding for the research and development branch of the company. The cash flow statement for this year will start to show the hard work that the team has put in. There will be an increase in revenue coming in, which will allow for the purchase of new equipment and the expansion of people working at GAP Industries. GAP Industries will also begin to be profitable.

**Year Four**

In this year the company will go off and be the most profitable it has been. Gap Industries has already established itself as a power player in the energy industry. Gap industries
will be a multimillion dollar company by this time. We will expand and compete in the national level. In Graphite appliances are lucrative. The prices will be lowered to allow for people to afford items.

*Year Five*

This will be the year we go public. The reason for this is because it allows for the company to expand. This is extremely important. Companies peak around year 5 if innovation and improvement were to continue this need to happen.

*Award Funding*

The prize for the Sontag Competition is $50,000. This money will be used for travel. To reach our initial investment requirement, GAP Industries needs to enter other competitions. There will also be applications done for grants. NV Energy has a grant for renewable energy research which happens to be one of the long term goals of the company. Money left over will be used to buy and obtain the material needed to get the company up and running. A fair amount of this money will be used to rent out a facility to begin the company operations.

*Offerings*

*Proposal to Investors*

GAP Industries is selling initial investment stocks at 10% of what we expect the company to be worth at the end of five years. For example, if an investor invests $200,000 for start-up costs, the investor should expect at total worth of stock at $2,000,000 after the fifth year. We expect to begin turning a profit at 30 months.
Conception to Full Operation

GAP Industries values being the first company to provide high quality graphene on a large scale. This will allow the company to bring a competitive edge to customers utilizing graphene for the future of semiconductors, energy, electronics, research, and material engineering.

Building a strong team of top chemical engineers, chemists, and material science engineers is the first priority in creating the backbone of GAP Industries. Once the team has been established, producing a viable process for the large scale production of graphene is required before identifying and meeting with potential investors. During this time, the marketing of the company would be in full swing to begin identifying and targeting our potential clients. By the sixth month of GAP Industries, meeting with potential clients to determine their specific needs of graphene would take place to establish clientele. Adjustments can be made to the scale up process based on the specifications desired by the clients. By the twelfth month, large scale production would begin. Adjustments would be made as necessary to improve the process. By the fourteenth month GAP Industries would like to begin distribution of graphene to clients, and begin making a profit by the thirtieth month.
Figure 2. Above is a rough estimate on the milestones by month pushed for by GAP Industries.

Business Canvas Model

Figure 3: Business canvas model.
Bibliography


### Appendix A: Twelve Month Cash Flow

#### Twelve-month cash flow

<table>
<thead>
<tr>
<th></th>
<th>GAP Industries</th>
<th>First Month of Forecast: Jan-15</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cash on Hand (beginning of month)</strong></td>
<td>$50,000</td>
<td>$20,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>February</strong></td>
</tr>
<tr>
<td><strong>CASH RECEIPTS</strong></td>
<td></td>
<td>$71,862</td>
</tr>
<tr>
<td>Cash Sales</td>
<td></td>
<td>$71,862</td>
</tr>
<tr>
<td>A/R Payments</td>
<td></td>
<td>$10,000</td>
</tr>
<tr>
<td>Collections from CR accounts</td>
<td></td>
<td>$10,000</td>
</tr>
<tr>
<td><strong>TOTAL CASH RECEIPTS</strong></td>
<td></td>
<td>$100,900</td>
</tr>
<tr>
<td><strong>CASH FUND OUT</strong></td>
<td></td>
<td>$20,000</td>
</tr>
<tr>
<td>Purchases (COGS)</td>
<td></td>
<td>$2,000</td>
</tr>
<tr>
<td>Payroll expenses</td>
<td></td>
<td>$5,000</td>
</tr>
<tr>
<td>Gross wages (cost withdrawal)</td>
<td></td>
<td>$30,000</td>
</tr>
<tr>
<td>Supplies, office &amp; operating</td>
<td></td>
<td>$10,000</td>
</tr>
<tr>
<td><strong>SUBTOTAL</strong></td>
<td></td>
<td>$58,900</td>
</tr>
<tr>
<td><strong>NON-FIL CASH FLOW DATA</strong></td>
<td></td>
<td>$0</td>
</tr>
<tr>
<td>Loan principal payment</td>
<td></td>
<td>$0</td>
</tr>
<tr>
<td>Capital purchases (specify)</td>
<td></td>
<td>$0</td>
</tr>
<tr>
<td>Other startup costs</td>
<td></td>
<td>$0</td>
</tr>
<tr>
<td>Reserve &amp;/or Escrow</td>
<td></td>
<td>$0</td>
</tr>
<tr>
<td>Owners Withdrawal</td>
<td></td>
<td>$0</td>
</tr>
<tr>
<td><strong>TOTAL CASH FLOW OUT</strong></td>
<td></td>
<td>$58,900</td>
</tr>
</tbody>
</table>
Appendix E: Heuristics for 2016-2017

<table>
<thead>
<tr>
<th>Table 10. Bioreactor design checklist</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diameter</strong></td>
</tr>
<tr>
<td><strong>Liquid level</strong></td>
</tr>
<tr>
<td><strong>Reactor Temperature</strong></td>
</tr>
<tr>
<td><strong>Temperature Effect on Product Production</strong></td>
</tr>
<tr>
<td><strong>Reactor pH</strong></td>
</tr>
<tr>
<td><strong>Oxygen Requirements</strong></td>
</tr>
<tr>
<td><strong>Impeller</strong></td>
</tr>
<tr>
<td><strong>MOC</strong></td>
</tr>
<tr>
<td>Reactor Pressure</td>
</tr>
<tr>
<td>------------------</td>
</tr>
</tbody>
</table>