Sustainable Production of Novel Biomaterials in Escherichia coli

Asif Rahman
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SUSTAINABLE PRODUCTION OF NOVEL BIOMATERIALS IN

ESCHERICHIA COLI

by

Asif Rahman

A dissertation submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biological Engineering

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UTAH STATE UNIVERSITY
Logan, Utah
2014
ABSTRACT

Sustainable Production of Novel Biomaterials in *Escherichia coli*

by

Asif Rahman, Doctor of Philosophy

Utah State University, 2014

Major Professors: Dr. Charles Miller and Dr. Ronald Sims
Department: Biological Engineering

Synthetic biological engineering can be used to program microorganisms to perform a variety of functions such as producing new chemicals, aiding in healthcare, and environmental cleanup. In this study, two biomaterials were produced in *Escherichia coli*: polyhydroxybutyrates (PHBs) and spider silk. While production of biomaterials can be optimized using synthetic biological engineering, one of the bottlenecks to mass production of bioproducts is the cost of the carbon substrate. This study coupled synthetic biological engineering with inexpensive carbon substrates for production of biomaterials.

Currently, there is a need to reduce the dependence on petroleum-derived commodities and a move towards renewable products. PHBs are a group of biodegradable plastics that are produced by a wide variety of microorganisms, mainly as a storage intermediate for energy and carbon. Synthetic biological engineering techniques were used to create export systems for secretion of bioproducts from the cell. PHAs form granules inside bacteria and have associated proteins bound to the granule surface. Phasin, a granule bound protein, was targeted for type I secretion by fusion with a HlyA
signal peptide and thereby facilitating indirect secretion of PHAs. To help understand secretion of PHAs, a green fluorescent protein (GFP) was tagged to the PHA polymerase enzyme encoded by *phaC*. *phaC* is part of a three-gene cassette that includes *phaA* and *phaB*, which are required for PHA production.

Spider silk is a highly versatile biomaterial with a range of potential applications. In this study, spider silk DNA sequences were optimized for production in *E. coli* and assembly of DNA constructs was carried out with the use of synthetic biological engineering. Specific tRNAs were engineered to be compatible with these spider silk sequences for optimized production.

To address the issue of expensive carbon substrate, a wastewater microalgae extract was used. One of the side streams of microalgae biodiesel production is an aqueous phase that contains simple sugars, ideal for recombinant bacteria growth and bioproduct generation. As a demonstration, PHB production was shown from *E. coli* grown on an algae-based medium. Coupling synthetic biological engineering with inexpensive substrates could potentially make bioproduct production economically feasible in the future.
Sustainable Production of Novel Biomaterials in *Escherichia coli*

The biotechnology revenues in the United States exceeded $100 billion in 2010 and the potential impact of synthetic biological engineering has been identified nationally as an emerging technology to further expand the national bioeconomy. Synthetic biological engineering approaches biology from an engineering perspective to make biology easier to engineer. The potential to engineer microorganisms for novel applications can have far-reaching implications and benefits for society. Some of the potential applications range from biosensors, biofuels, therapeutics, and biomaterials.

In this study two biomaterials were produced in genetically engineered *Escherichia coli*: polyhydroxybutyrates (PHBs) and spider silk. PHBs are bioplastics that have similar properties to petrochemical-derived plastics. Synthetic biological engineering can be used to optimize PHB extraction from *E. coli* by secretion of the PHB polymer outside of the cell.

Another biomaterial, spider silk, was also produced in *E. coli*. Spider silk is a unique material with high tensile strength and elasticity and thus could have a wide range of potential applications. Since spider silk is not naturally produced in microorganisms, the DNA sequences were optimized for increased production in *E. coli*.

In addition to optimization of bioproduct production in microorganisms using synthetic biology, another major cost is the carbon substrate. In this study wastewater microalgae were used as an alternative carbon substrate. Coupling synthetic biological
engineering and sustainable engineering could potentially make production of bioproducts economically viable in the future.

Asif Rahman
VISUAL ABSTRACT
DEDICATIONS

This work is dedicated to my parents Naseem and Asheq Rahman, and Nidhi Doshi for their love and support.
ACKNOWLEDGMENTS

When I first started this project I did not realize that by the end of it I would be so vastly enriched by the experience. There are so many people to thank, for without their support and guidance this would not have been possible. Firstly, I would like to thank my advisors, Dr. Charles Miller and Dr. Ronald Sims, for their endless support with this endeavor. Both of you have opened my eyes, inspired me, and pushed me beyond what I thought would be possible. Charlie, your door was always open for which I am extremely grateful and I enjoyed all our conversations both research and non-research related. Ron, your positive energy and passion for research I will never forget. I would also like to thank my committee, Dr. Randy Lewis, Dr. Anthony Turhollow, and Prof. H. Scott Hinton, for all the advice and wisdom you shared with me.

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Asif Rahman
# CONTENTS

<table>
<thead>
<tr>
<th>ABSTRACT</th>
<th>iii</th>
</tr>
</thead>
<tbody>
<tr>
<td>PUBLIC ABSTRACT</td>
<td>v</td>
</tr>
<tr>
<td>VISUAL ABSTRACT</td>
<td>vii</td>
</tr>
<tr>
<td>DEDICATIONS</td>
<td>viii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xiv</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xv</td>
</tr>
<tr>
<td>TERMINOLOGY</td>
<td>xviii</td>
</tr>
</tbody>
</table>

## CHAPTER

### 1. INTRODUCTION

1. Overview ................................................................. 1
2. Significance .............................................................. 3
3. Format of Dissertation ............................................. 4

### 2. LITERATURE REVIEW

1. Motivation for study ................................................ 6
2. Bioplastic companies .............................................. 8
3. The case for polyhydroxyalkanoates .......................... 15
4. Isolation and recovery of non-secreted PHAs ............ 22
5. Development of a PHA secretion system .................... 24
6. Analytical methods to analyze polyhydroxyalkanoates ... 26
7. Polyhydroxyalkanoate imaging techniques ................. 28
8. Using synthetic biology to optimize spider silk production in *E. coli* ............... 31
9. Production of biomaterials from unrelated carbon sources .............................. 33
10. Summary ............................................................... 35
11. References ............................................................. 36

### 3. BIOBRICKSTM TO BIOPRODUCTS: THE CASE FOR SYNTHETIC BIOLOGICAL ENGINEERING ........................................... 53

1. Abstract ........................................................................... 53
7. BIOREMEDIATION OF DOMESTIC WASTEWATER AND PRODUCTION OF BIOPRODUCTS FROM MICROALGAE USING WASTE STABILIZATION PONDS................................................................................................................................. 147
   1. Editorial............................................................................................................. 147
   2. References..................................................................................................... 149
8. EFFECTS OF WASTEWATER MICROALGAE HARVESTING METHODS ON POLYHYDROXYBUTYRATE PRODUCTION................................................................. 151
   1. Abstract ........................................................................................................ 151
   2. Introduction .................................................................................................. 151
   3. Materials and Methods............................................................................... 154
   4. Results and Discussion .............................................................................. 157
   5. Conclusions ................................................................................................. 161
   6. References................................................................................................... 161
9. SUMMARY....................................................................................................... 164
APPENDICES ..................................................................................................... 167
APPENDIX A-FUTURE WORK............................................................................... 168
   A1. Scaling up of PHA production ................................................................. 168
   A2. Polyhydroxybutyrate production using a wastewater microalgae based media ............................................................................................................................... 171
APPENDIX B - PATENTS .................................................................................. 184
APPENDIX C- COPYRIGHTS AND PERMISSIONS .............................................. 187
APPENDIX D- PERMISSION FROM AUTHORS TO REPRINT PUBLISHED MATERIALS.................................................................................................................. 195
APPENDIX E- STRAINS, PLASMIDS, AND OLIGONUCLEOTIDES................. 200
CURRICULUM VITAE .......................................................................................... 206
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1. Properties of some common petrochemically derived plastics compared with polyhydroxybutyrate (Adapted from: [14]).</td>
<td>16</td>
</tr>
<tr>
<td>2.2. Summary of the different techniques used to extract PHAs from various microorganisms (adapted from: [23]).</td>
<td>23</td>
</tr>
<tr>
<td>2.3. Studies using Scanning Electron Microscope (SEM) to visualize polyhydroxyalkanoates</td>
<td>29</td>
</tr>
<tr>
<td>2.4. Different studies that utilize Transmitting Electron Microscope (TEM) for polyhydroxyalkanoate granule visualization.</td>
<td>30</td>
</tr>
<tr>
<td>3.1. Best Manufacturing prize for iGEM projects 2008-2013 (igem.org)</td>
<td>66</td>
</tr>
<tr>
<td>3.2. Best Food and Energy Project prize for iGEM projects 2007-2013 (igem.org)</td>
<td>67</td>
</tr>
<tr>
<td>3.3. iGEM 2013, teams that focused on production of bioproducts from BioBricks™ (igem.org)</td>
<td>68</td>
</tr>
<tr>
<td>4.1. Production of PHB in secreting (pCMEL3+pLG575) and non-secreting (pBHR68+pLG575) strains of <em>E. coli</em> at 24 and 48 hours.</td>
<td>82</td>
</tr>
<tr>
<td>4.2. Strains, plasmids, and oligonucleotides used in this study</td>
<td>88</td>
</tr>
<tr>
<td>5.1. Strains, plasmids, and oligonucleotides used in this study</td>
<td>107</td>
</tr>
<tr>
<td>6.1. Codon usage for one repeat of MaSp2 <em>A. aurantia</em> spider silk containing two elastic units (2E). <em>Escherichia coli</em> B codon usage (DNA 2.0, Menlo Park, CA), Unoptimized DNA sequence (Brooks et al. 2008), Balanced codon usage (this study). Not shown are the codons for the scar region (2 amino acids that are between each of the spider silk subunits): T-Threonine (ACT codon) and R-Arginine (AGA codon).</td>
<td>134</td>
</tr>
<tr>
<td>6.2. Strains and plasmids used in this study</td>
<td>135</td>
</tr>
<tr>
<td>8.1. Total weight of biomass, mass of algae, % dry mass of algae, and amount of coagulant used</td>
<td>158</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1. Total United States Municipal Solid Waste Generation in 2011 (Before Recycling, modified from: EPA report 2013[1])</td>
<td>7</td>
</tr>
<tr>
<td>2.2. Plastic generation and recovery in the United States from 1960 to 2007 (modified from EPA report 2007 [2])</td>
<td>8</td>
</tr>
<tr>
<td>2.3. Worldwide distribution of bioplastic producing companies. Red dots are individual companies with their approximately location in each respective country. 97 bioplastic producing companies are represented on the map</td>
<td>10</td>
</tr>
<tr>
<td>2.4. Location of bioplastic producing companies around the world</td>
<td>10</td>
</tr>
<tr>
<td>2.5. Types of bioplastic polymers produced commercially</td>
<td>11</td>
</tr>
<tr>
<td>2.6. Commercial applications of bioplastics</td>
<td>12</td>
</tr>
<tr>
<td>2.7. Carbon substrates for industrial bioplastic production</td>
<td>13</td>
</tr>
<tr>
<td>2.8. General structure of PHA where the R side chain variation gives the PHA different chemical properties. If R is methyl (-CH\textsubscript{3}) this polymer is known as polyhydroxybutyrate or PHB. If R is an ethyl (-C\textsubscript{2}H\textsubscript{6}) this is known as polyhydroxyvalerate (modified from:[14])</td>
<td>16</td>
</tr>
<tr>
<td>2.9. General pathway used to produce PHB from Acetyl CoA using a three protein system. Proteins (blue) β-ketothiolase (phbA), acetoacetyl-coA reductase (phbB), and PHB polymerase (phbC). Species and compounds (green). Note: CH\textsubscript{3} group can change depending on substrate</td>
<td>19</td>
</tr>
<tr>
<td>2.10. D, E, and F. Catalytic mechanism of PHA synthase part II. (adapted from: [18])</td>
<td>21</td>
</tr>
<tr>
<td>2.11. Characteristic of a PHA granule showing the PHA core and proteins adhering to the granule. PhaC and Phasin are commonly found on the surface of a PHA granule (adapted from: [15])</td>
<td>22</td>
</tr>
<tr>
<td>2.12. 460 acre facultative lagoon system in Logan, UT</td>
<td>35</td>
</tr>
<tr>
<td>2.13. Wet lipid extraction procedure to produce various bioproducts from a wet algal feedstock (adapted from: [118])</td>
<td>36</td>
</tr>
<tr>
<td>3.1. Abstraction—comparing synthetic biological engineering to more established fields such as electrical and chemical engineering</td>
<td>59</td>
</tr>
</tbody>
</table>
3.2. Standard Biological Part in a plasmid ................................................................. 62

3.3. Standard BioBrick™ assembly RFC 10 and RFC 23 ........................................... 63

4.1. SDS polyacrylamide gel and corresponding immunoblot of subcellular fractions for PhaP1:HlyA. C – cytoplasmic fraction, P – periplasmic fraction, M – membrane fraction, S – concentrated supernatant (media) fraction. The position of phasin bands varies from roughly 22-26 kDa. ....................................................... 81

4.2. CFU/mL vs. Time (h) for secreting and non-secreting strains of PHB producing 
E. coli, averaged from triplicate experiments (one standard deviation shown). ........ 82

4.3. SEM images taken from overnight cultures of E. coli XL1Blue harboring 
different plasmid systems: A) pBHR68 (non-secreting), B) pCMEL3 (non-
secreting with phasin overexpression), and C) pCMEL3+pLG575 (complete 
PHB production and secretion system). ..................................................................... 86

4.4. A. Schematic for PHB secretion containing dual plasmid system pCMEL3 and 
pLG575. Phasin with attached signal peptide binds to PHB granule surface and the 
PHB-phaC-phaB signals complex is targeted for type I secretion. B. 
pCMEL3 plasmid consisting of phaC, phaC, and phaB genes from pBHR68. 
pCMEL3 also contains the genes needed for phasin-HylA production. ..................... 90

5.1. Schematic for real time secretion of PHB granules with associated proteins from 
Escherichia coli harboring pAKF01 + pLG575. Direct production of PHB with 
the phaABC operon and expression of the GFP-PhaC fusion protein with the 
phaP1-hylA secretion system both under lactose control. ...................................... 109

5.2. Fluorescence measurements in relative fluorescent units (RFUs) of GFP in the 
media at 24 and 48 h of the E. coli XL1 Blue strains harboring the 
pAKF02+pLG575 (-hlyA/+hlyBD, non-secreting) and pAKF01+pLG575 
(+hlyA/+hlyBD, full secretory system) plasmids respectively. ................................. 112

5.3. Fluorescent microscope images of GFP bound PHB granules in E. coli after 24 h 
of growth A) pAKF01 (+hlyA/-hlyBD) and B/C) pAKF02 (-hlyA/-hlyBD). 
White bar represents 5 µm. .................................................................................... 115

5.4. Fluorescent microscope images of GFP bound PHB granules in pAKF01 + 
pLG575 (+hlyA/+hlyBD, full secretory system). A) E. coli after 24 h of growth, 
cell exhibiting accumulation and secretion, B) E. coli after 24 h of growth cell 
showing secretion and accumulation of PHB at the new septum, and C/D) E. 
coli after 48 h of growth cell showing secretion of GFP tagged PHB. Arrows 
denotes secretion from the polar regions of the cell. White bar represents 5 µm. ... 116
5.5. Fluorescent microscope images of GFP bound PHB granules in pAKF02 + pLG575 (-hlyA/+hlyBD, non-secreting system) *E. coli* after A) 24 h and B) 48 h of growth. White bar represents 5 µm.

6.1. Nucleotide and amino acid sequence for one monomer of MaSp2 dragline spider silk from *Argiope aurantia* (BBa_K844004). Codon usage: glycine (G=2 (ggt, gaa)), tyrosine (Y=1 (tat)), proline (P=2 (cct, cca)), alanine (A=1 (gca)), glutamine (Q=1 (caa)), and serine (S=2 (agt, tct)). GC content 55%

6.2. Spider silk production system constructed using the BioBrick™ assembly standard (pB14). BBa_K208010 is a composite part consisting of a Lac Promoter and Ribosome Binding site. BBa_K84408 is spider silk subunit of the MaSp2 gene from *Argiope aurantia* containing a methionine (atg). 13 repeats of BBa_K844044 are spider silk subunits of the MaSp2 gene from *A. aurantia*. BBa_K844000 is a 10x His-tag with a double stop codon.

6.3. Different repeats of spider silk subunit with GFP fused onto the C-terminal end. Each repeat was tested for fluorescence with and without tRNAs. ‘B’ denotes ‘balanced’ construct and the subsequent number is the number of spider silk gene repeats.

6.4. Fluorescent microscope images of *E. coli* cells expressing different sizes of MaSp2 *Argiope aurantia* spider silk fused to green fluorescent protein. A) 4 repeats, B) 12 repeats, and C) 16 repeats of the MaSp2 subunit.

6.5. AKTA results for spider silk production system. Blue line indicates absorbance at UV 280 nm, where first peak is wash fraction and second peak is elution fraction.

6.6. AKTA results for spider silk production system with tRNA expression. Blue line indicates absorbance at UV 280 nm, where first peak is wash fraction and second peak is elution fraction.

6.7. HPLC on elution fractions from spider silk sample with and without tRNAs. Construct that has tRNAs demonstrates increased production of spider silk. Peak of interest is between 2.45 and 2.55 min.

6.8. SDS polyacrylamide gel (A) and corresponding immunoblot (B) for: Cell lysate, Flow through, wash fractions, elution fraction, and Precision Plus Protein™ Marker.

8.1. CFU/mL (A) and PHB% (% of dcw) (B) of *E. coli* XL1-blue harboring the pbHR68 plasmid 48 h after induction, grown on different WLEP aqueous phases from cationic potato starch (CPS), cationic corn starch (CCS), aluminum sulfate (Alum), and centrifuged mixed culture algae. Numbers are averaged from triplicate experiments.
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA</td>
<td>Polyhydroxyalkanoate</td>
</tr>
<tr>
<td>PHB</td>
<td>Polyhydroxybutyrate</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscope</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>MSW</td>
<td>Municipal Solid Waste</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>DCW</td>
<td>Dry cell weight</td>
</tr>
<tr>
<td>WLEP</td>
<td>Wet lipid extraction procedure</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

1. Overview

Synthetic Biological Engineering holds the promise of benefiting society with the advancement of recombinant technologies in the medical, agricultural, food, environmental, and energy sectors. The production of biomaterials using genetically engineered organisms as a platform is another aspect of synthetic biological engineering that has gained interest recently. With any new technology, there are many challenges that need to be overcome in order for it to be economical and self-sustaining. The purpose of this dissertation is to couple synthetic biological engineering and sustainable engineering to create platforms for production of useful biomaterials such as bioplastics and synthetic spider silk using *Escherichia coli* as a microbial cell factory.

Traditional plastics are derived from petroleum and are non-biodegradable. Currently, there is a need to reduce our dependence on petroleum derived products and move towards ‘greener’ biodegradable materials. Polyhydroxyalkanoates (PHAs) are a group of biodegradable plastics that are produced by a wide variety of microorganisms, mainly as a storage intermediate for energy and carbon. Recombinant *E. coli* have been used to increase PHA yields in bioreactors, however this process is currently not economically viable. There are two main laboratory-to-market bottlenecks in producing PHAs in microorganisms: 1) downstream processing of bacterial cultures to extract the valuable PHA and 2) cost of carbon substrate.

Studies have shown that, in nature, bacteria can accumulate up to 90% of the dry cell weight; unfortunately due to the PHA remaining inside the cell the cost of PHA
isolation can be extremely high. Secreting PHA from *E. coli* could potentially reduce downstream processing costs as it can be easier to separate secreted PHA from the biomass. Since PHAs are polymers they cannot be directly targeted for secretion from *E. coli*, instead an intermediate must be used. PHA granules have a variety of associated proteins. One such protein, Phasin, binds to the PHA granule and can be targeted for type I secretion. Synthetic biological engineering principles can be used to secrete a PHA-bound-Phasin complex outside of the cell and could make PHA production a sustainable process. In conjunction with secretion, visualizing the secretion process with SEM and fluorescence microscopy would further enhance the understanding of the PHA secretion phenomena.

In addition to using synthetic biological engineering for secretion of PHAs from *E. coli*, molecular biology techniques can be used to optimize the production of another biomaterial, synthetic spider silk. Spiders can naturally produce a variety of different silks and one such silk, dragline silk, has mechanical properties similar to that of Kevlar and rubber. Spiders are territorial and cannibalistic which makes farming spiders not possible. *E. coli* can be used as a microbial factory for producing spider silk proteins and production can be optimized using synthetic biological engineering tools to optimize protein expression levels.

To address the issue of expensive carbon substrate, inexpensive alternatives can be used. A cheap carbon substrate is ideally free and requires little or no modification. One such alternative is the use of a wastewater algae-based media for bacterial growth and subsequent production of recombinant products. Algae have been well studied as a production platform for the manufacturing of various bioproducts. From algal-based
biofuel production processes, “waste” streams can be generated and utilized for the production of other value added products, thus reducing the overall production cost of the biofuel. The method of creating a sustainable biorefinery can draw parallels to a traditional chemical refinery system, where production of specialty chemicals is market driven.

In the United States over 7,000 lagoon systems are used to treat domestic wastewater. The city of Logan, Utah processes approximately 15 million gallons per day of its wastewater in a 460 acre open pond facultative lagoon system. Microalgae grown in these ponds can be harvested and processed with a wet lipid extraction procedure (WLEP). The WLEP produces 4 streams, and one stream (aqueous phase) can be used to culture recombinant *E. coli*. As an example, growing PHA producing bacteria in an aqueous phase algal medium can potentially make PHA production sustainable. The approach of utilizing “algae-waste” as a substrate for biomaterial production could potentially make the process of PHA and other recombinant product manufacturing sustainable.

2. **Significance**

The impact of the studies presented in this dissertation could have far reaching implications. Synthetic biological engineering holds the promise for production of bioproducts but the “bench-to-market-barrier” needs to be overcome. If PHAs are able to be successfully secreted with synthetic biological engineering techniques, the process can be further engineered to a larger scale using bioreactors. Preliminary studies have already been completed with scale-up of the process and results thus far are encouraging.
Using synthetic biological engineering to produce other biomaterials such as synthetic spider silk can also create many new applications. Spider silk has highly desirable properties, which from a materials engineering standpoint could replace traditional manmade materials. The goal to engineer bacteria to express a range of sizes of spider silk protein at a higher level can lead to a variety of potential applications.

The production of algae-based bioproducts sustainably has great implications to society as a whole. Remediation of wastewater systems with algae to remove nitrogen and phosphorus, along with the subsequent harvesting and bioproduct generation has the potential to impact everyday lives. The addition of PHA production to an algal biorefinery model could potentially help make algae bioproducts economical and sustainable in the future.

3. Format of Dissertation

This dissertation is structured in a multiple paper format and with the exception of Chapter 2, all subsequent chapters have been formatted as publications. Chapters 4, 7, and 8 were published prior to the completion of this dissertation.

The initial chapters are a review of the literature: Chapter 2- “Literature Review” and an explanation of synthetic biological engineering: Chapter 3- “BioBricks to Bioproducts: The case for Synthetic Biological Engineering.” Chapter 4- “Secretion of Polyhydroxybutyrate in Escherichia coli using a Synthetic Biological Engineering approach” studies the process of type I secretion of polyhydroxybutyrates (PHB) from E. coli (published). Chapter 5- “A Synthetic Biological Engineering approach to understanding type I secretion of polyhydroxybutyrates from Escherichia coli” explores secretion of GFP-tagged PHB granules from E. coli. The focus of Chapter 6-“A Synthetic
Biological Engineering approach to produce *Argiope aurantia* spider silk in *Escherichia coli*" is to biomanufacture spider silk in *E. coli* using synthetic biological engineering techniques.

The next part of the dissertation focuses on production of bioproducts from wastewater microalgae. Chapter 7- “Bioremediation of domestic wastewater and production of bioproducts from microalgae using waste stabilization ponds” is an editorial (published) on the potential of wastewater microalgae to make bioproducts. Chapter 8- “Effects of wastewater microalgae harvesting methods on polyhydroxybutyrate production” is a research article (published) on the conversion of wastewater microalgae to polyhydroxybutyrates (PHB). Finally, Chapter 9- “Summary” reviews the work presented in the dissertation and provides details on potential future work.

The appendix contains an additional manuscript on bioproduct production from algae. Additionally, the appendix also includes front covers of three patent applications related to the work carried out in this dissertation. All strains, constructs, and oligonucleotides used in this study are also provided.
CHAPTER 2
LITERATURE REVIEW

1. Motivation for study

Plastics are a large part of everyday life. Over the last 70 years synthetically made plastics derived from petroleum have been produced in significantly large quantities. It has been well documented that fossil reserves are a finite resource and our dependence on plastics has added to the pressure on petrochemically derived products. In 2010 approximately 190 million barrels of liquid petroleum were used in the United States to produce plastics. Additionally, approximately 400 billion cubic feet of natural gas was used to produce other plastic products [1]. Petroleum and natural gas derived plastics are generally non-biodegradable and can be toxic to the environment.

One of the major concerns with petroleum derived plastics is that they accumulate in landfills. A study by the United States Environmental Protection Agency (EPA) found that plastics account for approximately 13% (or 32 million tons) of all municipal solid waste (MSW) before recycling in 2011, Figure 2.1 [2]. After recycling, the proportion of plastics in the MSW system goes up to approximately 19% as most petroleum derived plastics cannot be recycled. Petroleum derived plastic production has steadily increased since 1960 (Figure 2.2) but as a percentage of MSW has increased from 1% (in 1960) to 13% (2010) which poses problems for both the petrochemical industry and local municipal councils [2].

As worldwide demand for plastic based products continues to grow, production will likely continue to increase, adding stress on our infrastructure. At this current rate, the continued use of petroleum based plastics is not sustainable. While plastic recovery
through recycling (Figure 2.2) has increased since 1990 it is not able to keep up with plastic production levels. Alternative means of producing plastics in large quantities which are both economically and environmentally friendly have gained considerable attention recently [3]. Part of the challenge of alternative plastic production is that the raw plastic product needs to have comparable properties to petroleum derived plastics. If the properties of this new plastic are similar, then the final plastic products could be the same as petroleum derived plastics. This “drop in” technology is currently desired in the alternative energy sector where energy generation needs to fit current systems to avoid additional cost of changing infrastructure [4].

![Figure 2.1. Total United States Municipal Solid Waste Generation in 2011 (Before Recycling, modified from: EPA report 2013 [2]).](image)
Figure 2.2. Plastic generation and recovery in the United States from 1960 to 2011 (modified from EPA report 2011 [2]).

2. Bioplastic companies

Economic and sustainable production of bioplastic is a global problem. Different companies around the world are pursuing the possibilities of a bioplastic economy. In conducting a thorough literature search only one recent study compared the production of ten different commercial polyhydroxyalkanoates [5]. To understand the impact that bioplastic has in the global market, an internet based survey was conducted to evaluate the different bioplastic commercial ventures around the world. The survey explored the geographic location of companies, types of bioplastics produced by the companies,
applications of bioplastics, and primary carbon substrate of the plastic produced. Additionally, intellectual property was also briefly surveyed.

It was found that the word “bioplastic” is a term loosely used by many companies that produce plastics that are either: 1) completely biological in origin, 2) biodegradable, or 3) in part biologically derived. In addition, only information obtained from the company website was used to create the figures and charts seen in this section of the dissertation. Active bioplastic companies were surveyed exclusively and non-active companies were disregarded from the survey.

2.1 Global distribution of companies

A total of 97 companies were surveyed from across the world and it was found that bioplastics companies were active in Europe, Asia, Oceania, North America, and South America. There were no active bioplastic companies found in the African continent when this survey was conducted. Figure 2.3 shows the global distribution of bioplastic companies marked in red. Figure 2.4 shows the share of each continent’s bioplastic companies. North America and Europe have the majority of the bioplastic companies with over 74 companies out of the 97 located in these two regions (76% of total). Not all the companies surveyed were exclusively producing bioplastics, for many of the companies bioplastic were a secondary product that was being manufactured. This is an interesting observation, as it suggests that for a bioplastic company to thrive it needs to take a combined approach (in producing more than one product) in order to be profitable. The value of a combined approach to producing multiple bioproducts on a single manufacturing platform will be discussed in a later section of the dissertation.
2.2 Type of bioplastic manufactured

From the different companies surveyed there was a wide range of biopolymers that each produced. Figure 2.5 shows the variety of bioplastics that the different companies manufactured. This survey found that not all companies reported on their website what type of bioplastic was produced either for proprietary reasons or lack of
large scale manufacturing. Polylactic acids (PLAs) had around 25% share of the bioplastics produced. This is not surprising as many products made from PLAs have been approved by the United States Food and Drug Administration (FDA) for human use [6]. PLAs while biological in origin, (e.g. derived from corn starch) are typically manufactured with an inorganic catalyst. Polyhydroxyalkanoates (PHAs) and polyhydroxybutyrates (PHBs) make up approximately 12% of all bioplastic production and are produced exclusively in bacteria. PHA polymers hold many advantages such as having high diversity of branched side groups and being biodegradable. Having a variety of branched groups gives PHAs a wide range of possible applications as these side groups affect the chemical and physical properties of the biopolymer.

Figure 2.5. Types of bioplastic polymers produced commercially.
2.3 Commercial bioplastic applications

There are many applications observed for currently available bioplastics (Figure 2.6). Of the companies surveyed, the majority had applications in agriculture and packaging. Most packaging plastics have a lifetime of 1 year or less from the time of production to disposal and continue to accumulate in landfills, thus having an alternative biological plastic would seem like an ideal solution. Food related plastics such as cups, dishes, cutlery, and bottles are also plastic products that have a short lifetime therefore landfills would also benefit from a plastic that is biodegradable. In addition, the motivation for the company to produce a particular plastic is two-fold. Either the application of the plastic is the driving force, or the primary carbon substrate locally found is the motivating factor for production. The primary carbon substrate will determine the type of plastic produced and hence the application.

2.4 Carbon substrate

Geographically, bioplastic companies are located close to the primary carbon substrate source used produce the respective bioplastic. For instance, in North America
companies located in the Midwest typically use corn or other starchy crop based carbon substrates for production of bioplastic. In the Pacific Northwest, tree based carbon substrate are used for bioplastic production. Figure 2.7 shows the type of carbon based compounds that are used for bioplastic production purposes. The most common source for bioplastic production is a plant based carbon substrate.

In order for the bioplastic industry to be sustainable, a consistent carbon substrate source is paramount to its success. A carbon substrate that is a finite resource such as crude oil for petroleum products is not sustainable. Additionally, crop based carbon substrates such as corn, potatoes, and sugar beet are not feasible as long term sustainable carbon substrates, as many of these crops are grown on fertile land that would be more suited for food production that plastic production. Furthermore, the cost associated with production of such crop-based plastics will not make the resulting bioplastic cost-competitive with petroleum based plastics. An ideal carbon substrate would be renewable wastes or a waste product of another process, thus effectively making the carbon source free. Some potentially sustainable carbon substrates could be algal based carbon substrates from algae cultivation on wastewater or waste products from cheese manufacturing that could be used to produce bacterial based bioplastics such as PHAs.

Figure 2.7. Carbon substrates for industrial bioplastic production.
2.5  **Intellectual property**

Another discussion point is the number of bioplastic patents issued in each of the respective parts of the world. From the internet based search approximately 2,000 such patents exist. Of these patents, over 50% were issued in Asia, 20% in Europe, and 25% in North America. The remaining percentages of patents were issued in South America, African, and Oceania. While Asia having 50% of the bioplastic patents may come as a surprise seeing that North America and Europe have the majority of the bioplastic companies, it should be noted that patent filing in each respective country is different. Another explanation for the disparity between Asia and the rest of the world is that Asia has a larger population and hence a greater need for alternative sources of plastic material, thus more intellectual property. With the population in Asia and Africa set to expand in the foreseeable future it is expected that the number of patents issued in these regions will also continue to grow.

2.6  **Conclusions and future work**

The survey shown in this section shows the many potential possibilities with regards to bioplastic production. There are different ventures across the world that are pursuing the possibilities of large scale production of bioplastics.

Although the survey carried out was extensive, it was not comprehensive. This survey was insightful and provided motivation for some of the studies conducted in this dissertation, to get a complete understanding of commercial bioplastic ventures, a comprehensive survey will need to be conducted. This was not carried out here as it was beyond the scope of the dissertation and due to significant time constraints in conducting a full survey. A comprehensive study would involve sending a detailed survey to each
company and compiling the results into similar figures seen above. The detailed survey would also ask questions such as: cost of manufacturing, specific type of polymer produced, and quantity of polymer produced per day. Additionally, a statistical analysis could also be performed on the data.

3. **The case for polyhydroxyalkanoates**

Replacing traditional plastic with biodegradable plastic such as polyhydroxyalkanoates (PHA) could potentially reduce total MSW waste by up to 20% [7]. Polyhydroxyalkanoates (PHA) are produced by a variety of microorganisms (e.g. *Ralstonia eutropha*, and Cyanobacteria) as an intercellular storage medium for energy and carbon and can accumulate up to 90% of the cell dry weight [8].

PHAs are biodegradable polymers. The biodegradability can range from approximately several months to several years [8]. There are 155 different confirmed types of PHA monomer subunits, each with varying monomer repeat number and side groups [9]. This means that PHAs have a variety of possible applications, thus if scaled up could replace traditional plastics derived from petroleum [5]. Some of the possible applications of PHAs include: packaging, medical, agricultural uses [10], and more recently in carbon nanotubes [11]. PHAs was first identified by Lemoigne in 1926 [12] and Henry Ford made a vehicle out of plastic derived from soy fibers in 1941, thus demonstrating that applications for biologically derived plastic have been ongoing since before World War II [13].

The general polymer structure of PHA is shown in Figure 2.8. The PHA molecule contains an ester group hence makes this type of polymer biodegradable via hydrolysis. The most studied PHA is polyhydroxybutyrate (PHB), where R is a methyl (CH₃). PHB
has a melting temperature of approximately 179°C [14] and crystallinity between 30-70% [15]. The molecular weight of PHAs can range from $2 \times 10^5$-$3 \times 10^6$ Da, and this weight range is dependent on the microorganism or carbon substrate used [10].

![Figure 2.8](image)

Figure 2.8. General structure of PHA where the R side chain variation gives the PHA different chemical properties. If R is methyl (-CH$_3$) this polymer is known as polyhydroxybutyrate or PHB. If R is an ethyl (-C$_2$H$_6$) this is known as polyhydroxyvalerate (modified from: [14]).

To demonstrate that PHB can be used to replace petroleum based plastics Table 2.1 compares some of the physical properties. The mechanical properties of PHB are comparable to polypropylene and polystyrene in terms of melting temperature, Young’s modulus and tensile strength. This is advantageous in two ways: Firstly, replacing petroleum based plastics or creating hybrid polymers with PHB is possible and secondly, downstream processing equipment such as extruders, molders, and reactors can be used to process the PHB polymer.

Table 2.1. Properties of some common petrochemically derived plastics compared with polyhydroxybutyrate (Adapted from: [14]).

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Melting Point (°C)</th>
<th>Young's Modulus (GPa)</th>
<th>Tensile strength (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHB</td>
<td>179</td>
<td>3.5</td>
<td>40</td>
</tr>
<tr>
<td>Polypropylene</td>
<td>170</td>
<td>1.7</td>
<td>34.5</td>
</tr>
<tr>
<td>Polystyrene</td>
<td>110</td>
<td>3.1</td>
<td>50</td>
</tr>
</tbody>
</table>
3.1 Polyhydroxybutyrate genetic system and pathway

The production of PHB can be carried out in either wild type bacteria or recombinant bacterial systems. Wild type bacterial PHB production is seen in (not limited to) *Cupriavidus necator*, *Rhodopseudomonas palustris*, and *Methylobacterium organophilum* [10]. Recombinant systems could include *E. coli* and Cyanobacteria [16, 17].

For scale up and large scale production of PHB *E. coli* is generally seen as an ideal candidate due to relatively simple cultivation and ease of downstream processing of bioproducts that are produced. The PHB producing genes from *R. eutrophus* (previously known as *Alcaligenes eutrophus*) were successfully cloned, identified, and subsequently expressed in *E. coli* by Schubert et al. [12]. The study by Schubert et al. was able to demonstrate up to 30% of cellular dry mass as PHB [12]. Spiekermann et al. cloned in the three gene PHB cassette from *R. eutrophus* into the plasmid pBHR68 [16]. The pBHR68 plasmid is widely used in recombinant *E. coli* systems for the production of PHB.

The pathway to produce PHB from acetyl-CoA is depicted in Figure 2.9. Three genes encoding for beta-ketothiolase (phbA), acetoacetyl-CoA Reductase (phbB), and PHB polymerase (phbC) (or PHB synthase) are required for production of PHB [9].

Figure 2.9 shows two acetyl-CoAs are condensed to acetoacetyl-CoA with β-ketothiolase, the acetoacetyl-CoA is reduced to 3-hydroxybutyryl-CoA with acetoacetyl-CoA reductase. Polymerization with PHB polymerase occurs as a step-wise process as shown in detail in Figure 2.10. Figure 2.10A shows the PHB polymerase and three conserved amino acids: histidine, aspartic acid, and cysteine. A proposed theory suggests that a catalytic triad is formed when two PHB polymerase come together. The histidine activates the thiol group of the cysteine, which in turn carries out a nucleophilic attack on
the thioester bond of the 3-hydroxybutyryl-CoA, this is now a 3-hydroxy-fatty acid attached to the cysteine as shown in Figure 2.10B. Since there are two PHB polymerase together in a catalytic triad a second 3-hydroxy-fatty acid attached to the cysteine would be present as shown in Figure 2.10C. The aspartic acid of the first PHB polymerase activates the hydroxyl group of the first 3-hydroxy-fatty acid. The first 3-hydroxy-fatty acid then attacks the thioester bond on the second 3-hydroxy-fatty acid attached to the second PHB polymerase (Figure 2.10C). This is the beginning of the first monomer units coming together to start a polymerization process (Figure 2.10D). This process is repeated with new 3-hydroxy-fatty acid molecules adding to the growing polymer chain, while the PHB molecule remains covalently attached to the PHB polymerase (Figure 2.10D and E) [18].

In addition to understanding the genetic mechanism for PHA production, the formation of PHA granules has gained interest recently. As the PHA polymer is being created, the PHA synthase remains covalently attached and forms an amphipathic molecule with hydrophilic (polar) and hydrophobic (non-polar) ends, this aids in the formation of a PHA granule [19]. A typical PHA granule is shown in Figure 2.11, with associated proteins attached [15]. A study by Peters and Rehm 2005 demonstrated that the PHA granule localization begins at the polar regions of the cell [20]. A study by Tomizawa et al. demonstrated that the PhaC synthase can adversely affect the molecular weight of the PHA generated; this is of interest as the molecular weight range can significantly affect the downstream processing of PHA [21]. In addition, the phasin protein is another protein which is bound to the PHA granule. Studies have shown that
Phasin proteins play an important role in PHA granule formation as they are tightly bound to the PHA core as shown in Figure 2.11 [22].

![Figure 2.9](image)

Figure 2.9. General pathway used to produce PHB from Acetyl CoA using a three protein system. Proteins (blue) β-ketothiolase (phbA), acetoacetyl-coA reductase (phbB), and PHB polymerase (phbC). Species and compounds (green). Note: CH₃ group can change depending on substrate.
Figure 2.10. A, B, and C. Catalytic mechanism of PHA synthase part I. (adapted from: [18]).
Figure 2.10. D, E, and F. Catalytic mechanism of PHA synthase part II. (adapted from [18]).
4. Isolation and recovery of non-secreted PHAs

A bottleneck in scaling up PHA production is the isolation of the PHAs. Currently techniques that are used to isolate PHA from \textit{E. coli} are invasive and generally use toxic chemicals. These techniques involve lysing open the cellular membrane prior to isolation of the PHAs. There are a variety of methods that have been suggested in recovery of PHAs from microorganisms such as: organic solvent extraction, enzymatic digestion, and extraction using supercritical \textit{CO}_2. These methods present the problems of cost, scalability, and viability of the biomass after such harsh treatment procedures [3]. A detailed review of the different PHA isolation methods is outlined in Jacquel et al. [23].

There are a number of different methods that have been used to purify and isolate PHAs from microbes. The problem with the use of current methods is that the biomass cannot be reused for PHA production. A review by Jacquel et al. highlighted the techniques that are currently being employed to extract PHAs from microorganisms. The main isolation methods can be placed into four major categories: Solvent extraction,
digestion, mechanical disruption, and others [23]. Table 2.2 provides the major extraction methods.

All the aforementioned PHA isolation methods mentioned in Table 2.2 are invasive methods of PHA isolation. As discussed earlier, it is desired to develop a technique that will not harm the cell culture, rather, allow for it to be recycled. In addition, these methods present further complications such as: the solvent extraction can be highly toxic (due to the use of halogens), digestion methods can degrade the polymer, and enzymatic digestion is expensive. Most of these approaches present major hurdles with regard to scale up of the downstream processes involved in extraction and purification of PHA.

Table 2.2. Summary of the different techniques used to extract PHAs from various microorganisms (adapted from: [23]).

<table>
<thead>
<tr>
<th>Extraction Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent Extraction</td>
<td>[24], [25], [26]</td>
</tr>
<tr>
<td>Digestion</td>
<td></td>
</tr>
<tr>
<td><em>Chemical Digestion - surfactants</em></td>
<td>[27], [28]</td>
</tr>
<tr>
<td><em>Digestion by sodium hypochlorite</em></td>
<td>[29]</td>
</tr>
<tr>
<td><em>Digestion by sodium hypochlorite and chloroform</em></td>
<td>[30], [31], [32]</td>
</tr>
<tr>
<td><em>Surfactant- hypochlorite digestion</em></td>
<td>[33]</td>
</tr>
<tr>
<td><em>Surfactant- chelate digestion</em></td>
<td>[27]</td>
</tr>
<tr>
<td><em>Enzymatic digestion</em></td>
<td>[34], [35], [36]</td>
</tr>
<tr>
<td>Mechanical disruption</td>
<td></td>
</tr>
<tr>
<td><em>Breadmill disruption</em></td>
<td>[37]</td>
</tr>
<tr>
<td><em>High pressure homogenization</em></td>
<td>[38], [37]</td>
</tr>
<tr>
<td><em>Centrifugation and chemical treatment</em></td>
<td>[39]</td>
</tr>
<tr>
<td>Others</td>
<td></td>
</tr>
<tr>
<td><em>Supercritical fluid</em></td>
<td>[40], [41]</td>
</tr>
<tr>
<td><em>Using cell frugality</em></td>
<td>[42], [43], [44]</td>
</tr>
<tr>
<td><em>Dissolved air flotation</em></td>
<td>[45]</td>
</tr>
</tbody>
</table>
5. Development of a PHA secretion system

The development of a secretion mechanism eliminates the need for cell disruption by mechanical or chemical means, and may lead to continuous (or semi-continuous) PHB production systems. In gram-negative bacteria like *E. coli*, compounds are exported via six major secretory pathways [46-49]. Recombinant proteins can be targeted to type I and II secretory pathways through genetic fusion with signal peptide targeting sequences.

PHAs are non-proteinaceous polyesters and therefore cannot be directly targeted for translocation by signal peptide fusion. Phasins are low-molecular weight proteins that play a role in PHA granule formation by binding to the PHA granule surface [22, 50]. Phasins are structural proteins found in organisms that naturally produce PHAs and are similar in function to oleosins which are found in plants [50, 51]. Currently, oleosins are used to purify various compounds such as pharmaceuticals from plants [52].

Translocation of PHAs is possible through optimization of granule size, which reportedly varies from 50 to 1,000 kDa based on growth parameters and host strain [8]. One of the functions of phasin is to increase the surface-to-volume ratio of granules so that higher accumulation levels can be achieved [22, 53, 54]. Therefore, the size of the PHA granule can be decreased significantly through phasin overexpression. In one such study, overexpression of phasin resulted in a decrease of PHA granules from 70-310 nm diameter to 20-60 nm [53].

Type I secretion is a simple one step secretion system that can translocate proteins from the cytoplasm to the extracellular medium without protein interaction with the periplasm [55]. Proteins of nearly 900 kDa (large adhesion protein, *LapA*) have
reportedly been secreted to the extracellular milieu by the type I secretory mechanism of gram-negative bacteria [56, 57]. Specifically using the Hemolysin (HlyA) secretion mechanism proteins such as β-galactosidase (117 kDa) [58], β-gal-OmpF (56 kDa) [59], and green fluorescent protein (GFP) (27 kDa) [60] have been secreted by *E. coli*. The physical characteristics of the secretion channel are approximately 3.5 nm in diameter with a length of 14 nm as reported by Fernández et al., which makes the secretion phenomena of large proteins very interesting [61].

A synthetic biological engineering approach has been previously used to demonstrate the feasibility of HlyA, GeneIII, PelB, and TorA secretion systems in *E. coli* with the use of GFP [60]. From the aforementioned study, the type I secretion system using the HlyA signal peptide yielded the best results for secretion of GFP outside of the cell and into the medium [60].

Secretion of PHA from *E. coli* can reduce downstream processing costs by using less invasive recovery methods, and elimination of the use of toxic chemicals, in addition the biomass can be recycled. Therefore, scale up and downstream processing of bioplastics can potentially be economically viable.

The positive impacts of this new technique of PHA isolation can be summarized as follows:

- Allows the recycling of microbial biomass as it will not be damaged/lysed
- Non-invasive technique used, thus also reducing the damage to the product
- Can potentially be a continuous/semi continuous process
- Minimize use of toxic solvents for extraction
6. **Analytical methods to analyze polyhydroxyalkanoates**

There are a variety of different methods to detect, analyze, and quantify PHB produced from microorganisms. These methods include: Gas Chromatography (GC), Nuclear Magnetic resonance (NMR), Gel-permeation chromatography (GPC), Differential Scanning Calorimetry (DSC), and Thermogravimetric analysis (TGA).

6.1 **Gas chromatography (GC)**

*Purpose: Detection, purity, and recovery*

Gas chromatography is a process used in PHA detection and concentration determination. PHA polymers are hydrolyzed and methylated to produce volatile monomers [62], these volatile monomers are processed through GC and compared with standards. In the case of PHB, it is converted to 3-hydroxybutyrate (bpt 144 °C) and analyzed with GC. Jian et al. used a GC method to determine PHA content and composition. The parameters used were oven temperature of 80°C for 1.5 min, which was changed to 140°C at a heating rate of 30°C/min and then heated to 220°C at a rate of 40°C/min. Temperature of the injection and detector were 200°C and 220°C [63]. Another study by Hahn et al. used GC as a method of determining purity and recovery of PHB. In this study GC was used following the methods outlined in Linton et al. [64]. This GC method was a modification of the Branunegg et al. method using acidified methanol to convert PHB to 3-hydroxybutyrate [65].

6.2 **Nuclear Magnetic Resonance (NMR)**

*Purpose: Detection and structure*
NMR was used by Hahn et al. as a method of detection and structure determination of PHB [66]. In this study quantitative NMR was used following the methods outlined in Linton et al. [64], where standard curves were created linking NMR spectra integration with GC results.

6.3 Gel-permeation chromatography (GPC)

*Purpose: Molecular weight*

Gel-permeation chromatography (GPC) is a standard method used to determine the molecular weight of polymers. C.W. Lo et al. used GPC as a method to determine molecular weight of polymers, especially PHB polymers. C.W. Lo et al. mentions that a polymer/chloroform solution (0.01g/2ml) was used as the loading solvent on two styragel columns. The flow rate used was 1.0 ml/min at 40°C, and the system was calibrated with a narrow range polystyrene standards. The narrow range polymer standards were used since it would then be easier to determine the molecular mass of the PHB [67]. Hahn et al. 1995 also used GPC as a method determining molecular weight of the samples. Polystyrene was used as the molecular weight standards with 100 µl of PHB sample (0.1% wt/vol) was used [66].

6.4 Differential Scanning Calorimetry (DSC)

*Purpose: Melting point and crystallinity*

DSC is used to measure the melting temperature of PHB. C.W. Lo et al. used a DSC with a temperature range of -40 °C to 200 °C and a heating rate of 10 °C/min [67]. Hahn et al. used the DSC as a method for melting point determination, and in addition also uses DSC to determine the crystallinity of the resulting PHB [66].
In this study a TA instruments DSC 2910 Modulated DSC machine was used to determine the melting temperature of the PHA. Nitrogen was used as the coolant and air was used to purge the system. Five milligrams of PHA sample was loaded in a DSC pan with a lid. The initial temperature was set to 25 °C and held for 1 min. The pan was heated at a rate of 5 °C/min and heated to 220 °C where the temperature was held for 1 min. Integration of the peak melting temperature was carried out with the computer software.

6.5 Thermogravimetric analysis (TGA)

Purpose: Decomposition temperature

Hahn et al. used TGA as a means of determining the thermal stability of PHB. The TGA in this study was operated with a nitrogen flow rate of 20 ml/min and a scanning rate of 10 °C/min [66].

7. Polyhydroxyalkanoate imaging techniques

In order to fully understand PHA localization and secretion, different imaging methods can be employed.

7.1 Fluorescence microscopy

As mentioned previously, the PHA synthase remains covalently attached to the PHB granule and phasin is targeted for secretion. A study by Peters et al. demonstrated the fusion of green fluorescent protein to phaC to visualize PHA location in E. coli [20, 68]. If this system can be included in the PHA secreting system it could help understand: 1) from where PHA secretion is occurring and 2) when secretion takes place. E. coli could be visualized using a fluorescent microscope.
7.2 Scanning electron microscope (SEM)

SEM is widely used in material science to visualize surfaces of metals and plastics. Table 2.3 shows SEM’s use with PHA visualization. Clearly SEM is not widely used for PHA analysis as a surface topographical analysis is of little use. In the case of secretion however, SEM can provide an image of what is occurring at the surface of the bacteria when secretion is occurring.

7.3 Transmitting Electron Microscope (TEM)

TEM allows visualization of internal granules of PHB as cross sections of bacteria can be visualized. Table 2.4 demonstrates that TEM is widely used as an imaging method to visualize PHB granule formation in a variety of microorganisms. TEM could also be a useful tool to help understand secretion of PHAs from *E. coli*.

Table 2.3. Studies using scanning electron microscope (SEM) to visualize polyhydroxyalkanoates.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Type of Polyhydroxyalkanoate</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>PHB</td>
<td>[86]</td>
</tr>
<tr>
<td><em>Comamonas sp.</em></td>
<td>PHA</td>
<td>[92]</td>
</tr>
<tr>
<td><em>Pseudomonas Lemoignei</em></td>
<td>PHA</td>
<td>[92]</td>
</tr>
<tr>
<td><em>Pseudomonas Fluorescens GK13</em></td>
<td>PHA</td>
<td>[92]</td>
</tr>
</tbody>
</table>
Table 2.4. Different studies that utilize Transmitting Electron Microscope (TEM) for polyhydroxyalkanoate granule visualization.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Type of Polyhydroxyalkanoate</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cupriavidus necator</em>, <em>Pseudomonas mendocian</em>, and <em>Rhodococcus opascus</em></td>
<td>PHA</td>
<td>[69]</td>
</tr>
<tr>
<td><em>Pseudomonas putida</em> KT2442mmi_7450 402..418</td>
<td>PHA</td>
<td>[70]</td>
</tr>
<tr>
<td><em>Comamonas</em> sp. EB172</td>
<td>PHBV</td>
<td>[71]</td>
</tr>
<tr>
<td><em>Pseudoaltermonas</em> sp. SM9913</td>
<td>PHA</td>
<td>[72]</td>
</tr>
<tr>
<td><em>Rhodobacter capsulatus</em></td>
<td>PHB</td>
<td>[73]</td>
</tr>
<tr>
<td><em>Ralstonia eutropha</em> strain A-04</td>
<td>P(3HB-co-4HB)</td>
<td>[74]</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Unknown biopolymer</td>
<td>[75]</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> 42A2</td>
<td>PHA</td>
<td>[76]</td>
</tr>
<tr>
<td><em>Rhodobacter sphaeroides</em></td>
<td>PHB</td>
<td>[77]</td>
</tr>
<tr>
<td><em>Caryophanon latum</em></td>
<td>PHB</td>
<td>[78]</td>
</tr>
<tr>
<td><em>E. coli</em> MG1655</td>
<td>PHB</td>
<td>[79]</td>
</tr>
<tr>
<td><em>W. eutropha</em> H16</td>
<td>PHB</td>
<td>[80]</td>
</tr>
<tr>
<td><em>W. eutropha</em></td>
<td>PHB</td>
<td>[81]</td>
</tr>
<tr>
<td><em>Wautersia eutropha</em> H16</td>
<td>PHB</td>
<td>[82]</td>
</tr>
<tr>
<td><em>R. eutropha</em></td>
<td>PHB</td>
<td>[83]</td>
</tr>
<tr>
<td><em>P. putida</em></td>
<td>PHB</td>
<td>[84]</td>
</tr>
<tr>
<td><em>E. coli</em> JM109 pBPP1</td>
<td>PMV</td>
<td>[85]</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>PHB</td>
<td>[86]</td>
</tr>
<tr>
<td><em>E. coli</em> JM109 pBPP1</td>
<td>P(3HB-co-5 mol% 3HHx)</td>
<td>[87]</td>
</tr>
<tr>
<td><em>Pseudomonas putida</em></td>
<td></td>
<td>[88]</td>
</tr>
<tr>
<td><em>E. coli</em> (pTZ18U-PHB, pSH2)</td>
<td></td>
<td>[89]</td>
</tr>
<tr>
<td><em>Pseudomonas lemoignei</em></td>
<td>PHB</td>
<td>[90]</td>
</tr>
<tr>
<td><em>Bacteria</em> (unknown)</td>
<td>PHB</td>
<td>[91]</td>
</tr>
</tbody>
</table>
8. **Using synthetic biology to optimize spider silk production in *E. coli***

Synthetic biological engineering could be used to secrete PHAs outside of an *E. coli* cell. Similarly, optimization of spider silk DNA sequences for the purposes of increased production using synthetic biological engineering could also be carried out.

Spider silk is an ancient biomaterial with remarkable properties. Orb-web weaving spiders have seven different types of silk glands, with each gland producing different spider silk protein each with unique characteristics [93]. Interestingly, dragline spider silk has a strength of approximately $4 \times 10^9$ N/m² and energy to break of $4 \times 10^5$ J/kg [94]. These mechanical properties similar or more superior to other more commonly used materials such as Kevlar and rubber [94]. In the future there could be a possibility for these spider silk proteins to replace currently available materials if the spider silk protein can be produced in large qualities and specific application-based downstream processing conducted.

One of the six different fibers that orb weavers can produce is the major ampullate dragline silk. Dragline silk consists of two proteins: the major ampullate spidroin 1 and 2 (MaSp1 & MaSp2) [95] and can be produced in different species of spiders such as *Nepila clavipes* and *Argiope aurantia* [96].

Due to the fact that spiders are territorial, cannibalistic, and that the actual spider web silk which is spun by a spider is an average of at least four different silks, it is not desirable to manufacture spider silk on a large scale using spider farms. These confounding factors mean that an alternative spider silk production mechanism (such as a synthetic biological engineering approach) is necessary.
Dragline spider silk has been expressed in a few different organisms: yeast [97], plants [98], silkworms [99], mammalian cells [100], and *Escherichia coli* [101]. Each host production system has its advantages and disadvantages ranging from yield of protein generated, cost of production, and production time [102].

From the different aforementioned host organisms, *Escherichia coli* is advantageous as it offers a quick and efficient system for DNA propagation and doubling time of approximately 20 min. Additionally, purification of protein from *E. coli* is well established and can be cost effective. Several studies have expressed different dragline spider silk proteins in *E. coli*: Xia and colleagues produced 55-285 kDa MaSp 1 *Nephila clavipes* spider silk protein [103], a study by Lewis et. al demonstrated production of 31-112 kDa MaSp 2 spider silk protein of *N. clavipes* [104], and Brooks et al. produced MaSp 2 protein from *A. aurantia* [101].

*E. coli* was chosen as the organism to express genes from the spider *Argiope aurantia* in this study. Spider silk genes are generally simple, yet highly repetitive in nature so choosing a suitable cloning technique to assembly genes together is an important factor to consider when designing an expression system. An article by Tokareva and colleagues highlight some of the different cloning approaches for recombinant spider silk production systems [102]. A novel cloning method is to use synthetic biological engineering and BioBrick™ standard assembly [105], multiple repeats of the spider silk subunit can be assembled together quickly and easily. Another advantage of using BioBrick™ standard assembly is that different sizes of spider silk repeat units can be assembled together to produce a variety of different synthetic spider silk sizes. Different sized spider silk protein could lead to many potential applications. A
typical MaSp 2 protein from *A. aurantia* only has six amino acids of which, 44% are glycine. With the highly repetitive nature of spider silk sequences, the available tRNA pool in *E. coli* could be diminished, thus engineering the DNA sequences of MaSp 2 with specific tRNAs, could substantially increase the yields of spider silk produced in *E. coli*.

In addition to designing a suitable cloning and expression system for spider silk expression system in *E. coli*, an efficient purification system is required. In previous spider silk expression systems in *E. coli*, cells were lysed and protein purified from a cell lysate. One study used a chemical based method where cell lysate was acid precipitated and spider silk protein was soluble under acidic conditions. An additional step of ammonium sulfate precipitation further enhanced recover with a purity of close to 90% [103]. Another chemical treatment method utilized propionic acid and bacterial proteins were clarified via centrifugation [106]. One of the most commonly used methods for protein purification is immobilized metal ion affinity chromatography (IMAC). Bacterial cells are lysed and the mixed protein solution is passed over a metal immobilized column, typically for spider silk protein purification, nickel is used as the immobilized metal and the protein is tagged with a histidine residue [101, 104, 107]. Histidine affinity tags for protein purification can yield in 90% of protein recovery and can be easily scaled up, thus giving it many advantages over other methods [108, 109].

9. **Production of biomaterials from unrelated carbon sources**

As mentioned previously, the economic production and scale-up of biomaterials is dependent on a cheap carbon substrate. The cheapest carbon substrate is one that is completely free and is a waste product of another process. Previous studies have demonstrated production of PHB from: anaerobic digesters [64], food wastes [110], and
waste glycerol from biodiesel production using a variety of microorganisms [111]. Interestingly, the use of different carbon sources can create PHA polymers with different properties. For example propionate addition can be used to produce co-polymers of PHB and polyhydroxyvalerate (PHV) [112]. To our knowledge, the production of synthetic spider silk from \textit{E. coli} grown in waste carbon substrates has not been previously demonstrated.

Microalgae have been extensively studied as a feedstock for biodiesel production [113]. High growth rates, low fresh water requirement, and high photosynthetic efficiency are few of the important characteristics that have made microalgae the obvious choice as a biodiesel production platform [114]. Microalgae can remediate wastewater by assimilating phosphorus and nitrogen as growth nutrients, thereby achieving tertiary treatment [115]. In the United States over 7,000 lagoon systems are used to treat domestic wastewater (U.S EPA, 2002, Report No. EPA 832-F02-014) [116]. Lagoon systems represent one solution to wastewater treatment and are suitable for developing countries and rural areas. The city of Logan, Utah processes approximately 15 million gallons per day of its wastewater in a 460 acre (~1.9 km$^2$) open pond facultative lagoon system shown in Figure 2.12. This system consists of seven ponds with an average depth of five feet (~1.5 m). Microalgae grown in these ponds can be harvested to provide a sustainable supply of biomass for bioproduct generation [116].

Algae harvested (chemically or mechanically) from the lagoons can be processed through the wet lipid extraction procedure (WLEP) creating four streams (as illustrated in Figure 2.13), three of which have been previously utilized [117, 118]. The first stream (hydrolyzed algal biomass) was used to generate acetone, butanol, and ethanol (ABE) by
Clostridium saccharoperbutylacetonicum N1-4 fermentation [119]. The remaining two side streams, the lipid extract and resultant aqueous phase can be used to produce biodiesel and to grow genetically engineered E. coli respectively.

Figure 2.12. 460 acre facultative lagoon system in Logan, UT treating 15 MGD domestic wastewater.

This aqueous phase from this process is the most useful for culturing E. coli and was demonstrated to give growth up to $10^{12}$ CFU/mL that is comparable to standard E. coli growth media [118]. Evaluating the effects of various wastewater microalgae harvesting techniques on the resulting algae-based media (from WLEP) to support E. coli growth and production of bioproducts will be important moving forward.

10. Summary

To summarize, large scale production of recombinant biomaterials (PHA and spider silk) is currently hindered by two main factors: 1) downstream processing to separate the bioproduct from the biomass, and 2) the cost of the carbon source. These two issues can be address by: 1) using synthetic biology to optimize production and 2) using a wastewater based algae carbon substrate.
Figure 2.13. Wet lipid extraction procedure to produce various bioproducts from a wet algal feedstock (adapted from: [118]).

11. References


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72. Wang Q, Zhang H, Chen Q, Chen X, Zhang Y, Qi Q: A marine bacterium accumulates polyhydroxyalkanoate consisting of mainly 3-


105. *Idempotent Vector Design for Standard Assembly of Biobricks*  
[http://hdl.handle.net/1721.1/21168](http://hdl.handle.net/1721.1/21168)


CHAPTER 3

BIOBRICKS™ TO BIOPRODUCTS: THE CASE FOR SYNTHETIC BIOLOGICAL ENGINEERING

1. Abstract

The foundations of synthetic biological engineering are built on molecular biology and genetic engineering. One of the purposes of synthetic biological engineering is to make biology easier to engineer by the creation of standardized biological parts and devices. There are a wide range of potential applications for synthetic biological engineering and a variety of approaches to constructing parts and systems. The focus of this commentary is two-fold: 1) to explain abstraction and standardization of biological systems, and 2) briefly review the production of bioproducts created from standardized biological parts (BioBricks™) at the annual International Genetically Engineered Machine (iGEM) competition.

2. What is synthetic biological engineering?

Synthetic biological engineering approaches biology from an engineering perspective to benefit society by: making new chemicals, aiding in healthcare, and alleviating environmental concerns [1]. Three major categories involve: 1) DNA-based device construction, 2) Genome-driven cellular engineering, and 3) Protocell creation [2].

The first classification’s major objective is to make biology easier to engineer by designing, building, and testing biological parts and devices. The potential to engineer or program microorganisms for novel applications can have far reaching implications and benefits to society. The problems with existing biological systems are the complexities
with design and assembly. Synthetic biological engineering aims to reduce these issues by making it easier to engineer biological systems [3]. The second category, Genome-driven engineering, aims to synthesize complete genomes and transflect them into cells. By showing just how reprogrammable cells are, novel synthetic organisms with specialized industrial or environmental functions can be created. The third category’s approach is to construct minimal cellular systems and to understand non-synthetic biological engineering. Finding a minimal genome can help to elucidate the origins of microorganisms and provide an insight into the complex interactions that occur within cells. However, the third classification of synthetic biology is not widely used and the first two categories are increasing in popularity due to their immediate impact on societal needs. DNA-based device construction is by far the simplest form of synthetic biological engineering. The relatively simple concepts and decreasing economic barrier to entry allows those not trained in the traditional fields of molecular biology and genetics to create new, functional genetic devices. In fact, do-it-yourself (DIY) synthetic biological engineering laboratories have begun to appear worldwide, where the main focuses of DIY groups are: 1) remain non-profit, 2) use cost effective tools and equipment, 3) demonstrate open source innovation, and 4) be self-empowered [4]. The focus of this manuscript is on this first category of synthetic biological engineering because of its popularity, simplicity, and impact.

There are many practical applications of synthetic biological engineering, ranging from biosensors, biofuels, biomaterials, and biologically-derived therapeutics [5]. Applications of synthetic biological engineering to produce useful bioproducts have already been demonstrated, including microbial production of artemisinic acid [6, 7],
biliverdin [8], biofuels [9-11], bioplastics [12-14], and spider silk [15, 16]. Recently, the first fully synthetic microorganism was created by the J. Craig Venter Institute starting from a digital copy of the (1.08 Mbp) *Mycoplasma mycoides* genome [17]. The possibility to create new life from a simple DNA sequence and ‘booting up’ an organism opens many new possibilities for synthetic biological engineering and the creation of useful bioproducts [18].

The impact of synthetic biological engineering is making waves in the public and private sectors. A non-exhaustive study by Oldham and colleagues in 2012 found that there were close to 3,000 researchers in over 40 countries engaged in research related to synthetic biological engineering [19]. In that same year, the White House released the National Bioeconomy Blueprint stating that in 2010 the revenues from industrial biotechnology were in excess of $100 billion in the United States. On page one of this document, the White House identifies that synthetic biological engineering will play a role as an emerging technology to expand the United States bioeconomy in the future [20].

The International Genetically Engineered Machines (iGEM) competition (http://igem.org) is a worldwide undergraduate synthetic biological engineering competition. iGEM started out as a one month class in 2003 with 16 students at the Massachusetts Institute of Technology, and has since become the showcase event for synthetic biological engineering [21]. In 2004, five university teams participated [22, 23], 2007 saw 60 teams join [24], and with 245 teams registered to compete in 2014, this year’s competition will include thousands of student participants from across the globe.
According to the iGEM website, approximately 15,000 students have participated in the first decade of competitions from 2004-2013 [26].

The basis of the iGEM competition is that during the summer months student-led groups design, build, and test biological circuits and devices, then compete head-to-head in a World Jamboree at the Massachusetts Institute of Technology. Groups submit their standardized biological parts to the Registry of Standard Biological Parts (http://parts.igem.org/), an open source biological parts repository that was started in 2004 [23]. Teams also present their work in formal conference style settings with podium presentations, poster presentations, and the creation of a team website.

The cost of participating in the iGEM competition can be in the tens of thousands of dollars per team due to team and individual registration fees, laboratory materials cost, and traveling expenses [25]. Money spent is not always proportional to success in the competition as there are many factors that are considered when projects are judged and prizes are handed out at the end of the Jamboree. An article by Materi in 2012 suggested that there are various ways in which to maximize resources for a successful iGEM project and that each team is run differently depending on available funding and team objectives [27]. The iGEM competition awards various prizes ranging from Best Poster to Best Human Practices Advance. In many cases teams choose to place emphasis on only a few categories in which they feel present the greatest chance for team success.

Since iGEM is an open source biological engineering competition, safety considerations are important. The iGEM competition promotes safe environments with strict requirements on teams having faculty mentors, institutional support, additional safety checks, and approval of a team’s project by a safety committee. iGEM teams also
develop safety mechanisms themselves such as “kill switches” for destroying bacteria if in the unlikely case the modified organism manages to escape the laboratory. A study by Guan and colleagues in 2013 suggested that while safety and the creation of new biological parts to aid in a safer working environments has improved since the start of iGEM, there are still issues that need to be addressed [28]. It has also been recommended that new biological parts submitted to the aforementioned registry of standard biological parts be put into separate safety categories depending on the part’s properties [29]. Additionally, the intellectual property discussion surrounding this open source field will continue to be discussed in academic and industrial circles [30].

3. Abstraction

Synthetic biological engineering is a fusion science combining concepts from different disciplines [31]. Tom Knight (widely regarded as the Father of standardized assembly and iGEM) published the first document on standardized BioBrick™ assembly in 2003 [32]. The first tangible idea of abstraction was brought to light by Andrianantoandro et al., with the concept that the goals and methods of synthetic biological engineering were similar to those of computer engineering [33]. All engineering disciplines use standards and guidelines. In electrical engineering, for example, there are set parameters for how electronic circuits are built. Resistors, transistors, LEDs, etc. are arranged in a systematic fashion to create circuits. In computer programming, sequential coding of a program is written in order for a program to function. In chemical engineering, unit operations (mass and heat transfer operations) of a refinery are arranged in such a way as to give the best recovery and separation of products. Endy compared synthetic biological engineering to the railway industry where
railroads have standards associated with railway construction and measurements [3]. In every engineering discipline there is a trend to build more complex systems from initially simple devices and parts to complex operations as depicted in Figure 3.1. To further draw parallels to electrical engineering, if the first transistor was developed in 1947, it been suggested that we are currently in the 1960s with synthetic biological engineering [1].

The analogy of synthetic biological engineering compared to chemical engineering is also an interesting concept (Figure 3.1). A biological system is constructed using biological parts similar to how a chemical plant is assembled. Once all the parts are assembled, a microbial cell factory is constructed, which is analogous to a chemical plant, both producing valuable commodities. Additionally, optimization of biological systems occurs in a similar manner to chemical engineering systems. For example, heat transfer operations in chemical engineering systems are optimized to have the greatest energy efficiency and thus greatest economic output. Biological systems can also be optimized to control flux balances by gene additions, knockouts, or via optimization of key metabolic pathways. Feedback loop systems exist both in a chemical engineering context and a synthetic biological engineering one. As an example, buildup of a toxin in a microbial system could trigger a system to start exporting it outside of the cell. This is analogous to a chemical plant where buildup of a toxin in a plant starts a chain reaction to remove it from the factory. While the scale of biological systems and chemical systems are different, there are many intersecting features and thus many overlapping industries and research activities.
4. Assembly Standards

Standardization in synthetic biological engineering is also a key step in maintaining functionality in a genetic circuit. The biological engineer cannot simply assemble a DNA circuit randomly, rather a systematic and standardized approach must be taken. A basic biological circuit consists of a promoter, ribosome binding site (RBS), gene, and transcriptional terminator (Figure 3.1). Just like in an electrical circuit, the order of these components is important for a circuit to function (promoter – rbs–gene of interest– transcriptional terminator). Additionally, the biological circuit is typically placed in a closed loop extrachromosomal circular DNA (plasmid DNA), much like an electrical circuit is arranged in a closed loop system. Plasmid DNA has well-defined cloning regions (where the standard biological circuit is inserted), antibiotic resistance marker (for selection), and origin of replication. The fundamental characteristics of a DNA plasmid can also be modified as reported by Shetty and colleagues, where specific
engineered BioBrick™ vectors are constructed for the sole purpose of BioBrick™
propagation [34]. The completed plasmid system is transformed into a microorganism, or
“chassis,” as the plasmid system is now used to program the cell for a specific function.
Synthetic biological engineering is not limited to cellular production systems as recently
there has been interest in cell-free, *in vitro* systems where competing biochemical
reactions and the inherent variability that a living organism displays can be minimized
[35].

The complexity of a system is not limited to that seen in Figure 3.1, which is a
promoter system driving single gene expression. A complex biological system contains
many different parts and devices that could depend on each other and have feedback
loops typically seen in engineering systems. Voigt in 2006 highlighted the three major
categories for making complex biological systems comprised of many parts: sensors,
genetic circuits, and actuators [36]. Keasling summarizes the major synthetic biological
ingineering tools needed for increased production of bioproducts through metabolic
pathways: chassis, vectors, promoters, coordinated expression of genes, CAD tools, and
debugging systems [37, 38].

Standardization exists in all engineering disciplines, yet recombinant biological
systems did not have any major standards in place until the onset of synthetic biological
engineering. In addition to creating a functional biological circuit, the ability to create
circuits quickly means that microorganisms can be programmed to carry out a specific
task rapidly. Utilizing standard, interchangeable biological parts is akin to the
interchangeable electric components in computer engineering. Furthermore, just like any
system, the use of standards allows other synthetic biological engineers to follow and
understand the assembly process that was employed. If errors are found they can be identified and remediated quickly. Müller and Arndt suggested that in addition to having standardized assembly of DNA parts, there should also be standardized methods of part characterization. Some of these guidelines include: physical composition of the part, experimental environment (host cell/chassis), experimental results, potential implementation, security/safety, and intellectual property claims by authors [39].

Currently there are many different standards for the assembly of synthetic biological systems and devices [40]. One of the most common assembly methods used is the BioBrick™ standard assembly. A BioBrick™ is a standard, interchangeable biological part that performs a specific function and that has been engineered to meet specific design requirements [41]. Tom Knight suggested the first standard biological assembly strategy in 2003 [32], termed the “BioBrick™ standard assembly.” The BioBrick™ standard assembly procedure uses a defined set of rules to first create simple BioBrick™ parts and then, using a systematic approach, assemble these parts together to make more complex devices.

The major guidelines for making these standardized biological parts are as follows: 1) before the BioBrick™ part should be the restriction enzyme sites EcoRI and XbaI (also known as the prefix), 2) after the BioBrick™ should be the restriction sites SpeI and PstI (also known as the suffix), 3) the BioBrick™ coding region cannot have any of these restriction sites, if so, they must be removed, and 4) the plasmid DNA hosting the BioBrick™ part cannot have any of the above mentioned restriction sites [32]. Figure 3.2 outlines the basic characteristics of a standard BioBrick™ part.
One of the fundamental features of the BioBrick™ design is the compatibility of the *XbaI* (TCTAGA) and the *SpeI* (ACTAGT) sites in the prefix and suffix. When the *XbaI* site is cut the overhanging DNA sequence is CTAG, this is compatible with the overhanging sequence of a cut *SpeI* site with a DNA sequence of GATC. This design feature allows for systematic assembly of BioBricks™ together in series and regeneration of the BioBrick™ cloning sites. Figure 3.3 demonstrates an example of standard BioBrick™ assembly. BioBrick™ A could be cut with *SpeI* and *PstI* and BioBrick™ B could be cut with *XbaI* and *PstI*. BioBrick™ B is now just a fragment and it can be cloned behind BioBrick™ A as the restriction enzyme cut sites are compatible. The same orientation would result if BioBrick™ A was cut with *EcoRI* and *SpeI* and BioBrick™ B was cut with *EcoRI* and *XbaI*. If instead it was desired to have BioBrick™ B assembled ahead of BioBrick™ A the steps needed for this would be similarly straightforward. Every time two BioBricks™ are assembled together there is a creation of a BioBrick™ “scar” between each BioBrick™ that is an artifact of cloning using this method. Knight formulated this concept of BioBrick™ assembly in 2003 and it is one of the most robust DNA assembly systems currently used, and is commonly known as assembly standard 10 (BBF RFC 10).

Figure 3.2. Standard Biological Part in a plasmid
The aforementioned BioBrick™ scar from Knight’s original construction (BBF RFC 10) in 2003 was found to cause a frame shift when linking two coding regions together. The RFC 10 scar also creates a premature stop codon (TACTAGAG). In 2006 Phillips and Silver modified the BBF RFC 10 assembly standard to allow for in-frame protein fusions. This new assembly standard (called Silver BBF RFC 23) follows the same rules and requirements as Knight’s BBF RFC 10 and includes the same restriction sites. However, the BioBrick™ prefix and suffix have shortened sequences (one base pair deletions, G proceeding the XbaI site and T before the SpeI site) to allow for an in-frame scar site. This new, RFC 23 6 bp scar site (ACTAGA) codes for the amino acids threonine and arginine [42].

Another widely used standard is the Berkeley RFC 21, or BglBrick standard, developed in 2009. This standard assembly method uses the restriction enzymes EcoRI and BglII in the prefix and BamHI and XhoI in the suffix. The advantage of this system is that in-frame protein expression is possible and the resulting scar site is a glycine-serine (GGATCT) compared to the threonine-arginine (ACTAGA) in BBF RFC 23 assembly.
standard. Glycine-serine fusion is harmless in a protein fusion context in *E. coli* [43, 44]. The major drawbacks of RFC 21 is that the BioBricks™ and vectors used in this system are not compatible with RFC 10 or RFC 23 due to different restriction enzyme sets being used, which makes converting existing parts already produced using RFC 10 or RFC 23 to RFC 21 problematic.

Two other standards currently supported by the Registry of Standard Biological parts are the BioBrick™ BB-2 Standard (RFC 12) [45] and Freiburg Standard (RFC 25) [46]. Since these are not widely used by the synthetic biological engineering community they will not be here. Additional synthetic biological engineering assembly methods can be found in a review by Ellis [40].

An assembly standard that has gained attention recently is the Gibson Assembly method. The Gibson Assembly method is a one tube reaction that combines multiple fragments together at once. The idea is that two pieces of DNA could be assembled together as they share the same end DNA sequence overlap. A T5 exonuclease removes nucleotides from the 5’ end of each of the DNA strands and the complementary strands from each of the pieces of DNA are assembled together with a polymerase to fill in missing gaps in DNA sequence and ligase to assemble the pieces together. The whole procedure takes place in a thermocycler. The advantages of this process are that large DNA fragments can be sequential assembled together in one reaction tube potentially saving time and money for the synthetic biological engineer. It was reported that products as large as 900 kb could be assembled from DNA fragments [47]. iGEM teams have started adopting this assembly system in recent years to assemble more complex biological systems and pathways.
A survey by Kahl and Endy in 2013 found that Gibson Assembly, *de novo* synthesis, and BioBrick™ standards were the three most used assembly standards in synthetic biological engineering. Additionally, in this study it was reported that the iGEM registry (http://parts.igem.org/) was the most widely used public registry for synthetic biological engineers, where researchers both submitted and utilized parts from the registry [48].

5. **BioBricks™ to Bioproducts**

Many of the aforementioned assembly strategies have been used by different iGEM groups to produce a wide range of BioBrick™-based bioproducts. Different microorganisms (chassis) have been used to host these biological circuits including: *Escherichia coli*, Yeast, Cyanobacteria, and *Bacillus subtilis*. Each organism is chosen on the basis of optimization or novelty of the bioproduct production system.

The iGEM competition first began presenting an award for the Best Manufacturing Project in 2008 (Table 3.1). The concept was to reward the team that demonstrated production systems in an organism by either programming the organism to produce a novel bioproduct or, optimization of production systems. The number of teams selecting manufacturing as their track ranged from 11-17 each year from 2008-2013. The 2008 Imperial College project, “Biofabricator Subtilis,” used *B. subtilis* for their chassis to produce self-assembling biomaterials. Cornell University’s “BioFactory” project in 2011 used a cell-free method to produce complex biomaterials from *E. coli*. More recently, Utah State University’s team “Arachnicoli” in 2012 used BioBricks™ to produce synthetic spider silk in *E. coli*. In 2013, the Imperial College team “Plasticity” produced the bioplastic polyhydroxybutyrate (PHB) in *E. coli*. While this award is
presented in a manufacturing context, this is not the only track in the iGEM competition that bioproduct production from BioBricks™ takes place. For example, teams may select the “Food and Energy” or “Health and Medicine” tracks if their bioproduct has implications in any of a number of different research areas.

Many teams build upon previous team’s work, and in doing so advance the field of synthetic biological engineering. As an example, the production of polyhydroxybutyrates (PHBs) in an iGEM context was first suggested by the 2008 Utah State team. However, the team was not able to demonstrate successful production using BioBricks™ at the time. The 2012 Tokyo Tech team demonstrated a functioning PHB production system from BioBrick™ parts. The following year, the 2013 Imperial College team demonstrated an 11x increase in production of PHB compared to the Tokyo Tech team in 2012 with the use of a hybrid promoter system and a BioBrick™ based operon. Interestingly, the Imperial College team also collaborated with the Yale 2013 team as Yale was trying to produce polylactic acid (another plastic) in *E. coli*. Imperial College’s ability to build and improve upon previous team’s work, demonstrate a functional production system, and collaborate followed the iGEM spirit set out almost 10 years prior.

Table 3.1. Best Manufacturing prize for iGEM projects 2008-2013 (igem.org)

<table>
<thead>
<tr>
<th>Year</th>
<th>Team Name</th>
<th>Project name</th>
<th>Bioproduct/process</th>
</tr>
</thead>
<tbody>
<tr>
<td>2013</td>
<td>Imperial College</td>
<td>Plasticity</td>
<td>Bioplastic</td>
</tr>
<tr>
<td>2012</td>
<td>Utah State</td>
<td>Arachnicoli</td>
<td>Spider silk</td>
</tr>
<tr>
<td>2011</td>
<td>Cornell</td>
<td>BioFactory</td>
<td>Cell-free bioproduct synthesis</td>
</tr>
<tr>
<td>2010</td>
<td>MIT</td>
<td>Living Materials</td>
<td>Self-assembly of biomaterials</td>
</tr>
<tr>
<td>2009</td>
<td>Imperial College</td>
<td>The E.ncapsulator</td>
<td>Encapsulation of proteins for therapeutic purposes</td>
</tr>
<tr>
<td>2008</td>
<td>Imperial College</td>
<td>Biofabricator Subtilis</td>
<td>Self-assembling biomaterials</td>
</tr>
</tbody>
</table>
Another category that includes groups using BioBricks™ to generate bioproducts is the “Food and Energy” division. This section started receiving a specialized award in 2007 and out of the 8 awarded teams, 5 teams worked towards energy production from BioBricks™. In 2008 a team from Harvard University used *Shewanella oneidensis* as a microbial fuel cell and in 2013, the Bielefeld-Germany team used *E. coli* in a similar endeavor. Successful drop-in fuel BioBrick™ production systems were constructed by teams from: Alberta in 2007 (Butanol), UNIPV-Pavia in 2009 (Ethanol), and Washington in 2011 (Biodiesel).

Table 3.2. Best Food and Energy Project prize for iGEM projects 2007-2013 (igem.org)

<table>
<thead>
<tr>
<th>Year</th>
<th>Team Name</th>
<th>Project Name</th>
<th>Bioproduct/process</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bielefeld-Germany</td>
<td>Ecoelectricity</td>
<td><em>E. coli</em> as a microbial fuel cell</td>
</tr>
<tr>
<td>2012</td>
<td>Groningen</td>
<td>Food Warden</td>
<td>Spoiled meat detector</td>
</tr>
<tr>
<td>2011</td>
<td>Washington (tie)</td>
<td>Make It or Break It</td>
<td>Diesel Production</td>
</tr>
<tr>
<td>2011</td>
<td>Yale (tie)</td>
<td>Nature’s Antifreeze</td>
<td>Antifreeze protein production</td>
</tr>
<tr>
<td>2010</td>
<td>BCCS-Bristol</td>
<td>agrEco</td>
<td>Soil fertility sensor</td>
</tr>
<tr>
<td>2009</td>
<td>UNIPV-Pavia</td>
<td>Ethanol? Whey not!</td>
<td>Whey to ethanol</td>
</tr>
<tr>
<td>2008</td>
<td>Harvard</td>
<td>bactricity</td>
<td>Electricity production in <em>Shewanella oneidensis</em></td>
</tr>
<tr>
<td>2007</td>
<td>Alberta</td>
<td>Butanerds</td>
<td>Butanol production</td>
</tr>
</tbody>
</table>

In 2013 over 10% of teams focused on production of bioproducts from BioBricks™ (Table 3.3). The bioproducts that each group aimed to produce ranged from biomaterials to additives for food. Also, many groups used different chassis organisms such as *E. coli*, *Bacillus*, and *Clostridia* for their BioBrick™ systems, which opens up many doors for future teams.
Table 3.3. iGEM 2013, teams that focused on production of bioproducts from BioBricks™ (igem.org)

<table>
<thead>
<tr>
<th>Team Name</th>
<th>Project name</th>
<th>Bioproduct</th>
<th>Track</th>
</tr>
</thead>
<tbody>
<tr>
<td>Berkeley</td>
<td>Genes to Jeans: a green solution to blue denim</td>
<td>Indigo</td>
<td>Manufacturing</td>
</tr>
<tr>
<td>Bielefeld-Germany</td>
<td>Ecoelectricity</td>
<td>Microbial fuel cell</td>
<td>Food &amp; Energy</td>
</tr>
<tr>
<td>Biwako Nagahama</td>
<td>AgRePaper&amp;E.coli-ink</td>
<td>Cellulose</td>
<td>Environment</td>
</tr>
<tr>
<td>Bordeaux</td>
<td>The Dairy Planet</td>
<td>Flavored yogurts</td>
<td>Food &amp; Energy</td>
</tr>
<tr>
<td>British Columbia</td>
<td>CRISPR Mediated Automated Design Employed to Bring You Ultrabiotics</td>
<td>Vanillin, caffeine, and cinnamaldehyde</td>
<td>Food &amp; Energy</td>
</tr>
<tr>
<td>Cornell</td>
<td>Organofoam: Genetically Engineering Fungal Mycelium for Biomaterials Development</td>
<td>Biodegradable Styrofoam substitute</td>
<td>Manufacturing</td>
</tr>
<tr>
<td>Costa Rica Cibus</td>
<td>Genetic transformation of <em>Bacillus subtilis</em> for lactose consumption</td>
<td>Biodiesel</td>
<td>Environment</td>
</tr>
<tr>
<td>Edinburgh</td>
<td>WastED</td>
<td>Bioethanol</td>
<td>Environment</td>
</tr>
<tr>
<td>Frankfurt</td>
<td>Steviomyces-sweeter than sugar</td>
<td>Stevia</td>
<td>Food &amp; Energy</td>
</tr>
<tr>
<td>Georgia State</td>
<td>Mamba Juice: Expression of Exogenous Mambalgin Peptide Using the pGAPza Vector System</td>
<td>Mambalgin-1 peptide</td>
<td>Health &amp; Medicine</td>
</tr>
<tr>
<td>Greensboro-Austin</td>
<td>Bioadhesive Production Using an Expanded Genetic Code</td>
<td>Mussel adhesive proteins</td>
<td>Manufacturing</td>
</tr>
<tr>
<td>Groningen</td>
<td><em>Engineering Bacillus subtilis to self-assemble into a biofilm that coats medical implants with spider silk</em></td>
<td>Spider silk</td>
<td>Health &amp; Medicine</td>
</tr>
<tr>
<td>Imperial College</td>
<td>Plasticity</td>
<td>Bioplastic</td>
<td>Manufacturing</td>
</tr>
<tr>
<td>Korea U Seoul</td>
<td>Pearl-coli</td>
<td>Pearl powder (nacre)</td>
<td>Environment</td>
</tr>
<tr>
<td>Manchester</td>
<td><em>E. coli</em>; <em>The Lean, Green, Fat-Producing SynBio Machine</em></td>
<td>Palm oil</td>
<td>Food &amp; Energy</td>
</tr>
<tr>
<td>Minnesota</td>
<td><em>The pMNBB vector system: A toolkit approach for engineering Pichia pastoris</em></td>
<td>Human insulin</td>
<td>Manufacturing</td>
</tr>
<tr>
<td>MSOE Milwaukee</td>
<td>Synthesizing Eucalyptol from Spend Grain Waste</td>
<td>Eucalyptol</td>
<td>Food &amp; Energy</td>
</tr>
<tr>
<td>OU-Norman OK</td>
<td>A shuttle vector for Clostridial Chassis Organisms</td>
<td>Isopropanol, butanol, and ethanol</td>
<td>Food &amp; Energy</td>
</tr>
</tbody>
</table>
While many teams have illustrious aims when first setting out on a team project, many teams are not able to accomplish their goals due to a variety of reasons such as: complexity of the project, lack of experience, short time frame, or insufficient funding. From the bioplastic example mentioned previously, the first team that came up with the idea did not necessarily achieve all their project objectives, but rather provided a foundation for other groups to build upon. The purpose of iGEM is not solely to make a profitable bioproduct from BioBricks™, because regardless of the final product that was created, the students will have gained valuable teamwork and laboratory experience along the way.
6. Conclusions

One the purposes of synthetic biological engineering is to make biology easier to engineer and this idea is seen most prominently in the iGEM competition. Synthetic biological engineering will have a greater role in the global bioeconomy and iGEM will continue to add to this growing field through innovations in projects and training of students. In an iGEM context, there are various assembly standards currently being utilized and there will inevitably be further advancement and new assembly standards seen in the future. With the sustained expansion of the BioBrick™ registry and the iGEM competition, there will be a continued development of BioBrick™-based bioproducts. The field will also see many new applications for BioBrick™-based synthetic biological engineering as we progress towards increasingly complex systems.

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47. Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA, Smith HO:

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CHAPTER 4
SECRETION OF POLYHYDROXYBUTYRATE IN *ESCHERICHIA COLI* USING A SYNTHETIC BIOLOGICAL ENGINEERING APPROACH\(^1\)

1. Abstract

1.1 Background

Polyhydroxyalkanoates (PHAs) are a group of biodegradable plastics that are produced by a wide variety of microorganisms, mainly as a storage intermediate for energy and carbon. Polyhydroxybutyrate (PHB) is a short-chain-length PHA with interesting chemical and physical properties. Large scale production of PHB is not widespread mainly due to the downstream processing of bacterial cultures to extract the PHB. Secretion of PHB from *Escherichia coli* could reduce downstream processing costs. PHB are non-proteinaceous polymers, hence cannot be directly targeted for secretion. Phasin, PhaP1, is a low molecular weight protein that binds to PHB, reducing PHB granule size. In this study PHB is indirectly secreted with PhaP1 from *E. coli* via type I secretion using HlyA signal peptides.

1.2 Results

This study demonstrated the successful secretion of phasin and phasin bound PHB outside of the cell and into the culture medium. The secretion of PHB was initiated between 24 and 48 h after induction. After 48 h of culturing, of the total PHB produced in the secreting strain, 36 % was collected in the secreted fraction and 64 % remained in the

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\(^1\) Coauthors: Elisabeth Linton, Alex Hatch, Ronald C. Sims, and Charles D. Miller
internal fraction. To further support the findings of this study, the PHB secretion phenomenon was observed using SEM.

1.3 Conclusions

From this study, the ability to use type I secretion to: 1) secrete phasin and 2) successfully secrete PHB has been shown.

2. Background

Fossil derived plastics are non-biodegradable and toxic to the environment. Based on an United States Environmental Protection Agency study in 2011, there was an increase in non-biodegradable plastic accumulation in municipal solid waste systems from 0.5 % to 12.4 % during 1960 to 2010 [1]. Alternative means of producing plastics in large quantities that are both economically and environmentally friendly have recently gained considerable attention [2].

Replacing traditional plastic with biodegradable plastic such as polyhydroxyalkanoates (PHAs) can potentially reduce total waste by up to 20 % [3]. PHAs are produced by a variety of microorganisms as an intercellular storage medium for energy and carbon and can accumulate up to 90 % of the cell dry weight [4]. PHAs are biodegradable polymers. The biodegradability can range from days [5] to months [6] with degradation either taking place extracellularly or intracellularly. Extensive review on degradation of PHAs can be found in Jendrossek et al. 2002 and Jendroseek et al. [7, 8]. There are 155 different confirmed types of PHA monomer subunits, each with varying monomer repeat number and side groups [9]. Additionally, PHAs have melting temperatures between 50-180°C and crystallinity of 30-70 % [10]. Thus, PHAs have a
variety of possible applications, that could replace traditional plastics derived from petroleum [11]. Some of the possible applications are highlighted in previous studies [12, 13] and include: packaging, medical uses [14], agricultural uses, and in carbon nanotubes [15].

Polyhydroxybutyrates (PHB) are a short-chain-length (scl) PHA polyester with between 3-5 carbon monomers [9]. The production of PHB in recombinant systems such as *Escherichia coli* has been made possible by the isolation of the phaCAB operon from *Ralstonia eutropha* (*Cupriavidus necator*) and cloning into pBluescript SK- to generate plasmid pBHR68. pBHR68 has been widely used for recombinant production of PHB in *E. coli*. The phaCAB operon is a three-step enzymatic process by which acetyl-CoA is converted to PHB: phaC (PHA synthase), phaA (β-ketothiolase), and phaB (acetoacetyl-CoA reductase) [4, 16]. After production of PHB, the polymer forms spherical granules with a hydrophobic core and attached proteins at the surface, including PHA synthase and phasin, PhaP1 [17-19].

The cost of producing PHAs is approximately US$ 4-6/ kg and one of the major bottlenecks in scaling up recombinant PHA production systems is the isolation of the PHAs [12, 20]. Current techniques that are used to isolate PHB from *E. coli* are invasive, including mechanical, chemical, and biological treatments. These techniques involve lysing the cellular membrane prior to isolation of the PHBs. There are a variety of methods that have been suggested for recovery of PHBs from microorganisms and a detailed review of the different PHB isolation methods is outlined in Jacquel et al. [20].

The development of a secretion mechanism eliminates the need for cell disruption by mechanical or chemical means, and may lead to continuous (or semi-continuous) PHB
production systems. In gram-negative bacteria like *E. coli*, compounds are exported via six major secretory pathways [21-24]. Recombinant proteins can be targeted to type I and II secretory pathways through genetic fusion with signal peptide targeting sequences.

PHBs are non-proteinaceous polyesters and therefore cannot be directly targeted for translocation by signal peptide fusion. Phasins are low-molecular weight proteins that play a role in PHB granule formation by binding to the PHB granule surface [19, 25]. Phasins are structural proteins found in organisms that naturally produce PHAs and are similar in function to oleosins which are found in plants [19, 26]. Currently, oleosins are used to purify various compounds such as pharmaceuticals from plants [27].

Translocation of PHBs is possible through optimization of granule size, which reportedly varies from 50 to 1,000 kDa based on growth parameters and host strain [4]. One of the functions of phasin is to increase the surface-to-volume ratio of granules so that higher accumulation levels can be achieved [25, 28, 29]. Therefore, the size of the PHB granule can be decreased significantly through phasin overexpression. In one such study, overexpression of phasin resulted in a decrease of PHB granules from 70-310 nm diameter to 20-60 nm [28].

Type I secretion is a simple one step secretion system that can translocate proteins from the cytoplasm to the extracellular medium without protein interaction with the periplasm [30]. Proteins of nearly 900 kDa (large adhesion protein, *LapA*) have reportedly been secreted to the extracellular milieu by the type I secretory mechanism of gram-negative bacteria [31, 32]. Specifically using the Hemolysin (HlyA) secretion mechanism proteins such as β-galactosidase (117 kDa) [33], β-gal-OmpF (56 kDa) [34], and green fluorescent protein (GFP) (27 kDa) [35] have been secreted by *E. coli*. The
physical characteristics of the secretion channel are approximately 3.5 nm in diameter with a length of 14 nm as reported by Fernández et al., which makes the secretion phenomena of large proteins very interesting [36].

Our group has previously used a synthetic biological engineering approach to demonstrate the feasibility of HlyA, GeneIII, PelB, and TorA secretion systems in \textit{E. coli} with the use of GFP [35]. From the aforementioned study, the type I secretion system using the HlyA signal peptide yielded the best results for secretion of GFP outside of the cell and into the medium [35]. The objective of this study was to demonstrate that phasin can be used to secrete PHB from \textit{E. coli} using type I secretion machinery.

3. \textbf{Results and Discussion}

Initial studies were carried out to demonstrate expression and then successful translocation of phasin, PhaP1, into the extracellular medium. Once this was demonstrated, PHB secretion experiments were conducted that included: growth studies, PHB production in secreted and non-secreted fractions, and visualization with scanning electron microscopy (SEM).

3.1 \textbf{Analysis of Phasin Translocation}

For \textit{E. coli} cells expressing pCMEL1 and pLG575, a phasin band is observed at 22-26 kDa in the cytoplasmic fraction, the periplasmic fraction, the membrane fraction, and the concentrated extracellular media (Figure 4.1). This polyacrylamide gel and corresponding immunoblot demonstrated: 1) the ability for \textit{E. coli} to produce non-codon optimized Phasin (from \textit{R. eutropha}), a protein not naturally expressed in \textit{E. coli} and 2) translocation of phasin into different fractions of the cell. Compared to other studies that
focused non-translocated phasin, the phasin band sizes in all of the different fractions are similar [19, 29]. Since the translocation of phasin was successful, secretion of PHB using phasin was then attempted.

![SDS polyacrylamide gel and corresponding immunoblot of subcellular fractions for PhaP1:HlyA. C – cytoplasmic fraction, P – periplasmic fraction, M – membrane fraction, S – concentrated supernatant (media) fraction. The position of phasin bands varies from roughly 22-26 kDa.](image)

Figure 4.1. SDS polyacrylamide gel and corresponding immunoblot of subcellular fractions for PhaP1:HlyA. C – cytoplasmic fraction, P – periplasmic fraction, M – membrane fraction, S – concentrated supernatant (media) fraction. The position of phasin bands varies from roughly 22-26 kDa.

### 3.2 Growth studies

The PHB secreting strain consisted of pCMEL3 + pLG575, whereas the non-secreting strain consisted of pBHR68+pLG575. From the CFU/mL vs. time graph (Figure 4.2) stationary phase was reached at approximately 8-12 h for both non-secreting and secreting strains. The non-secreting and secreting strains had the highest overall CFU/mL at approximately $9 \times 10^{12}$ and $5 \times 10^{12}$ CFU/mL, respectively, after 12 h. There was no significant difference between CFU/mL for the non-secreting and secreting strains after 24 and 48 h ($p>0.05$). This statistical analysis on the CFU/mL demonstrated that there were no significant differences in *E. coli* growth between the two samples at times when PHB analysis was conducted (24 and 48 h). Furthermore, this suggests that the secretion of PHB does not affect cell viability.
Figure 4.2. CFU/mL vs. Time (h) for secreting and non-secreting strains of PHB producing E. coli, averaged from triplicate experiments (one standard deviation shown).

Table 4.1. Production of PHB in secreting (pCMEL3+pLG575) and non-secreting (pBHR68+pLG575) strains of E. coli at 24 and 48 hours.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Time (hr)</th>
<th>%mass of PHB in Dry mass</th>
<th>Production g/L PHB</th>
<th>g PHB/g Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Secreted fraction</td>
<td>Non-secreted fraction</td>
<td>Secreted fraction</td>
</tr>
<tr>
<td>Non-Secreting</td>
<td>24</td>
<td>0.31±0.35</td>
<td>41.93±13.5</td>
<td>0.40±0.27</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.72±0.89</td>
<td>47.24±6.0</td>
<td>0.50±0.41</td>
</tr>
<tr>
<td>Secreting</td>
<td>24</td>
<td>0.69±0.18</td>
<td>28.85±0.41</td>
<td>0.40±0.06</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>28.29±7.2*</td>
<td>38.80±15.5</td>
<td>2.57±0.75*</td>
</tr>
</tbody>
</table>

*Indicates statistical significance within column (p<0.05)
3.3 PHB production analysis

PHB production analysis was carried out 24 and 48 h after induction because after 24 h *E. coli* harboring the plasmid systems were in stationary phase. Previous studies demonstrated that PHB did not accumulate to significant levels during the exponential growth phase. Acetyl-CoA is required for cell synthesis during the exponential phase but is diverted to produce PHB in the stationary phase, thus, there is a delay between carbon source utilization and PHB production [37].

PHB measured inside the cell is defined as the internal fraction and PHB collected by the CaCl₂ precipitation method is the secreted fraction. Twenty-four hours after induction, the internal fraction of the cells demonstrated PHB production for both the secreting and non-secreting strains. While the non-secreting strain accumulated approximately 41.93±13.5 % of PHB in the dry cell weight at 24 h, the secreting strain accumulated approximately 28.85±0.41 % (Table 4.1). Forty-eight hours after induction the non-secreting and secreting strains had accumulated 47.24±6.0 % and 38.80±15.5 % PHB, respectively. There was no significant statistical difference seen in internally accumulated PHB in either the non-secreting or secreting strains at 24 and 48 h (p>0.05) after induction. The internal levels of PHB accumulation in the non-secreting and secreting strains are comparable to those seen in other studies such as Kang et al. 2008, where the authors showed accumulation of 42 % PHB in *E. coli* DH5α harboring pBHR68 after 24-48 h [38].

PHB secreted fractions were analyzed at 24 and 48 h after induction for the secreting and non-secreting strains. PHB harvested in the secreted fraction of the non-secreting strains was 0.31±0.35 % and 0.72±0.89 %, respectively. The PHB present in the
secreted phase demonstrated that some lysed cells containing PHB were found in this fraction after CaCl$_2$ precipitation. This is to be expected from a differential centrifugation technique, such as that used in this study. 24 and 48 h after induction the secreting strain produced 0.69±0.18 % and 28.29±7.2 % respectively in the secreted fraction. The level of PHB seen in the secreted fraction of the secreting strain was statistically significant after 48 h post induction, compared to the non-secreting strain (p<0.05). This increase in PHB present in the secreted phase after 48 h indicates that the secretion system is functioning and producing higher amounts of PHB, with a small amount of non-secreted PHB ending up in the secreted fraction. These results demonstrate that PHB secretion is initiated 24 h after induction.

Secretion of PHB can help in downstream processes by aiding in PHB separation from biomass. Of the total PHB produced by the secreting strain after 48 h, 36 % was collected in the secreted fraction and the remaining 64 % was in the internal fraction. The secreting strain had a total PHB production of 7.15 g/L compared to 5.93 g/L for the non-secreting strain 48 h after induction.

It has been demonstrated from previous studies that PHB can accumulate in larger quantities in *E. coli* when using a bioreactor compared to a shaker flask. A fed-batch bioreactor study by Choi et al. 1999, reported accumulation of up to 77 % PHB of dry cell weight [39] and another study reported accumulation of up to 80 % [37]. Future studies will be performed to determine how well the secreting strain performs under similar bioreactor growth conditions.
3.4 SEM analysis

SEM is not widely used for PHB analysis since a surface topographical analysis is typically of little use. SEM has however been used to visualize PHA granules produced from recombinant *E. coli* [40] and PHA degradation from a variety of organisms [41]. In the case of secretion, SEM can provide images of what is occurring at the surface of *E. coli* during the secretion process. Figure 4.3 A shows a PHB non-secreting *E. coli* strain harboring the pBHR68 plasmid. Figure 4.3 B shows *E. coli* that is accumulating PHB and overexpressing phasin. Figure 4.3 C shows the full secretion system in *E. coli* (pCMEL3+pLG757). Figure 4.3 C suggests that PHB is being secreted outside of the bacteria and into the medium. These observations further demonstrate the functioning of the PHB secretion system.

The SEM photo in Figure 4.3 C suggests that secretion of PHB occurs at the polar regions of the cell. Interestingly it has been found in previous studies [42, 43] that PHB granule formation occurs at the cell poles in *E. coli* when both phasin and PHB are being produced. When PHB is being produced the interpretation is that PHA synthase is active, and it has previously been suggested that PHA synthase has polar targeting information [43].

It is interesting that *E. coli* is able to secrete PHB through pore sizes of 3.5 nm [36] in diameter. From the results of this this study not all the PHB produced is being secreted, with a large fraction remaining internally, suggesting that there could be differences between the secreted and non-secreted PHB.
Figure 4.3. SEM images taken from overnight cultures of *E. coli* XL1Blue harboring different plasmid systems: A) pBHR68 (non-secreting), B) pCMEL3 (non-secreting with phasin overexpression), and C) pCMEL3+pLG575 (complete PHB production and secretion system).

4. **Conclusions**

This study demonstrated the successful expression of phasin, PhaP1, and its translocation using type I secretion in *E. coli*. Once translocation of PhaP1 was successful, a recombinant synthetic biological system to secrete PHBs was designed and tested. Initiation of PHB secretion occurs between 24 and 48 h after induction. From the total PHB produced by the secreting strain, 36 % was collected in the secreted fraction and 64 % remained in the internal fraction after 48 h.

Secretion of PHB should help in downstream processing whereby the PHB is separated from the cell mass which can aid in PHB recovery and purification. Future studies will include a detailed comparison of secreted versus non-secreted PHB characteristics.
5. Methods

5.1 Strains and Plasmids

Genetic parts were constructed in accordance with the BioBrick and BioFusion technical standards [44, 45]. The BioFusion standard was specifically used to create phasin and signal peptide parts because this genetic fusion system is compatible with the original BioBrick standard [45, 46].

Descriptions of strains and plasmids used to study PHB and phasin production and translocation are provided as Table 4.2. Completed BioBrick parts were transformed in BL21-Gold (DE3) competent *E. coli* (Agilent Technologies, Santa Clara, CA) for protein expression studies. PHB production and secretion studies were carried out in *E. coli* XL1 Blue (Agilent Technologies, Santa Clara, CA). Plasmid pLG575 includes the coding regions for proteins HlyB and HlyD [47, 48]. Plasmids pSB1AK3, pSB1A3, and pSB3K3 are BioBrick standard vectors for assembly and expression of BioBrick genetic devices [49].

5.2 BioBrick and plasmid construction

All restriction enzymes and related reagents were purchased from Thermo Fisher Scientific Inc. (Glen Burnie, MD). The signal peptide HlyA was made through synthetic design and construction as mention in Linton et al. 2012 (DNA 2.0, Menlo Park, CA) [35]. A BioFusion-compatible phasin BioBrick was constructed by isolating phaP1 from the genomic DNA of *R. eutropha* using PCR with primers PhaP1FOR and PhaP1REV that included the BioFusion prefix and suffix as overhanging ends (Table 4.2).
Table 4.2. Strains, plasmids, and oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant Characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL21-Gold (DE3)</td>
<td><em>E. coli</em> B F^ompT hsdS(rB^- mB^-) dcm^+ Tet^R gal λ (DE3) endA Hte</td>
<td>Agilent Technologies</td>
</tr>
<tr>
<td>XL1 Blue</td>
<td>*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F’ proAB lacIqZAM15 Tn10 (Tetr)]</td>
<td>Agilent Technologies</td>
</tr>
<tr>
<td><em>Cupriavidus necator</em></td>
<td>Wild type, PHA producing strain</td>
<td>ATCC 17699</td>
</tr>
</tbody>
</table>

**Plasmids**
- pLG575: pACYC184 derivative, HlyBD, p15A origin, Cm^R [47]
- pBHR68: pBluescript SK–, phbCAB genes from *R. eutropha* [16]
- pSB1AK3: High copy BioBrick vector, pMB1 origin, Amp^R and Kan^R [49]
- pSB1A3: High copy BioBrick vector, pMB1 origin, Amp^R [49]
- pSB3K3: Medium copy BioBrick standard vector, p15A origin, Kan^R [49]
- pCMEL1: phaP1, C-terminal BioFusion with HlyA signal peptide, Lac promoter (BBa_R0010), RBS(BBa_B0034), in pSB1A3 This study
- pCMEL2: phaP1, C-terminal BioFusion with HlyA signal peptide, Lac promoter (BBa_R0010), RBS(BBa_B0034), in pSB3K3 This study
- pCMEL3: phaP1, C-terminal BioFusion with HlyA signal peptide, Lac promoter (BBa_R0010), RBS(BBa_B0034), in pBHR68 This study

**Oligonucleotides**
- PhaP1FOR: 5’-ggatcggcggtgacactata-3’ [47] This study
- PhaP1REV: 5’-ctgcagcggccgctactagtgcaggcagccgtcgtcttct-3’ [47] This study
- g114t: 5’-gtcctgagctgaaccttcaggtcgtcaagact-3’ [47] This study
- g114t_antisense: 5’-agttcctggcagctgaaggttcagctcgacg-3’ [47] This study
The 620 bp PCR product was isolated by gel electrophoresis, digested with EcoRI and SpeI, and ligated into pSB3K3. A PstI site was removed from phaP1 (while conserving amino acid sequence) using a QuikChange II Site-Directed Mutagenesis Kit and the QuikChange® Primer Design Program (Agilent Technologies, Santa Clara, CA). The designed primers (g114t and g114t_antisense) for site-directed mutagenesis are shown in Table 4.2. The PstI mutation in phaP1 was successfully carried out and confirmed by sequence analysis.

Step-wise assembly of composite BioBrick devices was primarily carried out in pSB1AK3. Completed devices were subsequently ligated into pSB1A3 and pSB3K3. The lac promoter (BBa_R0010) and ribosome binding site (BBa_B0034) were used as described in Linton et al. [35]. The pCMEL1 plasmid was used for studies on phasin translocation because its origin of replication (pMB1) was compatible with the origin of replication of pLG575 (p15A). BL21-Gold (DE3) was co-transformed with pCMEL1 and pLG575. The pCMEL3 plasmid was used for studies on type I secretion of PHA. The composite part containing the promoter, RBS, coding region, and terminator were cloned into pBHR68 from pCMEL2 by digestion with EcoRI and XhoI. XL1-Blue was co-transformed with pCMEL3 and pLG575 for PHA secretion studies. A schematic of the secretion system is presented in Figure 4.4.
Figure 4.4. A. Schematic for PHB secretion containing dual plasmid system pCMEL3 and pLG575. Phasin with attached signal peptide binds to PHB granule surface and the PHB-phasin-signal peptide complex is targeted for type I secretion. B. pCMEL3 plasmid consisting of phaC, phaC, and phaB genes from pBHR68. pCMEL3 also contains the genes needed for phasin-HylA production.
5.3 Cellular Fractionation and Western Blotting

Overnight cultures of *E. coli* BL21-Gold (DE3) harboring pCMEL1 and pLG575 were used to inoculate (1:100 v/v dilution) 100 ml of LB media containing 25 μg/ml chloramphenicol (Acros Organics, Fair Lawn, NJ) and 50 μg/ml ampicillin (IBI Scientific, Peosta, IA) [50]. The lysozyme/EDTA/osmotic shock and chloroform-based cellular fractionation procedures and methods for analyzing supernatant fractions are previously discussed in Linton et al. [35]. Briefly, 50 mL of cell culture were centrifuged and the pellet was resuspended in a buffer and subjected to osmotic shock. Centrifugation was used to separate the periplasm from *spheroplast*. Cytoplasm and membranes were fractionated via ultracentrifugation.

Approximately 40 μg of protein from each of the respective fractioned samples were separated using precast 12 % SDS-polyacrylamide tris-glycine gels (Jule Inc., Milford, CT). Electrophoresis operating conditions were used as specified by the manufacturer. Phasin immunoblotting was carried out using a PhaP1 specific antibody [51, 52]. An Anti-Rabbit IgG HRP conjugated secondary antibody was purchased from Promega (Madison, WI). Respective primary and secondary antibody concentrations of 1:50,000 v/v and 1:2,500 v/v were used.

5.4 PHB Secretion studies

All secretion studies were carried out in triplicate. Secretion studies were conducted with the secreting system (pCMEL3 + pLG575 in XL1Blue) and non-secreting system (pBHR68 + pLG575 in XL1 Blue). The non-secreting system does not contain phasin-HlyA.
5.5 Media formulation and growth conditions

*R. eutropha* was cultured following the methods outlined in Linton et al. [53]. M9 salts (Becton, Dickinson and Co, Sparks, MD) supplemented with 1.5 % (w/v) glucose (ACS grade, Acros Organics, Fair Lawn, NJ) and 0.2 % (w/v) yeast extract (Becton, Dickinson and Co, Sparks, MD) was used for PHB secretion studies in *E. coli* [38]. Overnight *E. coli* cultures harboring specific plasmids were inoculated from freezer stocks into 5 ml of M9 media with chloramphenicol (34 μg/ml), ampicillin (50 μg/ml), and grown in an orbital shaker table at 220 rpm at 37 °C. Overnight cultures were then used to seed larger 250 ml flasks (50 ml media volume) at an initial optical density (OD$_{600}$) of 0.05 at time 0 h. 0.1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Gold Biotechnology, Inc. St. Louis, MO) was added to each flask at time 0 h. Flasks were removed at 24, and 48 h and analyzed for PHB. CFU/mL was measured at time points 0, 4, 6, 8, 12, and 24 h.

5.6 Recovery of secreted PHB

PHB granules can agglomerate together naturally and previous studies have demonstrated the use of Calcium chloride (CaCl$_2$) to enhance this process. A study by Fidler et al. used CaCl$_2$ to purify PHB from lysed cells by selectively aggregating PHB granules, it was observed that PHB granules fell to the bottom of the test tube after addition of CaCl$_2$ [54]. Another CaCl$_2$ method for PHB recovery by Resch et al. used a low speed centrifugation step to further enhance PHB recovery from cell debris [55].

In this study, techniques for secreted PHB recovery were adapted from the methods outlined in Resch et al.. At 24, and 48 h 0.01 M CaCl$_2$ (final concentration, Avantor Performance Materials, Inc. Center Valley, PA) was added to the bacterial
culture and mixed by inverting the tube several times. The tubes were then allowed to sit for 10 min at room temperature and then centrifuged at 54 x g for 5 min. The supernatant was removed and transferred to a fresh tube and the pellet was freeze dried. The supernatant was centrifuged at 3452 x g for 10 min and the pellet was freeze dried. The pellet from the first centrifugation contained secreted PHB with CaCl$_2$ and the pellet from the second centrifugation contained bacterial mass and non-secreted PHB. Secretion studies and PHB analysis were conducted in triplicate.

5.7 PHB concentration determination

PHB concentrations were determined based on a NMR-GC method described in Linton et al. [53]. Briefly, samples were lyophilized after which approximately 15 mg of sample was mixed with equal volumes of sodium hypochlorite and deuterated chloroform. Samples were centrifuged and 1H NMR was carried out on the PHB fraction. PHB concentration was determined from a NMR-GC standard.

5.8 Scanning electron microscopy (SEM)

To visually show PHB secretion from *E. coli*, SEM was performed. SEM protocols were used as mentioned in Mortensen et al. [56]. Briefly, secreting and non-secreting strains were grown up overnight and fixed onto glass cover slips. Samples were mounted on aluminum stubs and sputter coated with 10 nm gold. SEM was carried out using a Hitachi S4000 SEM.
5.9 Statistical analysis

All growth (CFU/mL) and PHB yield studies were carried out in triplicate to show consistency of data. Statistical analysis was conducted with Statistical Analysis Software (SAS 9.3, SAS Institute Inc., Cary, NC). A two-way analysis of variance (ANOVA) with tukey post hoc comparison performed on significant results (confidence level 95%).

6. References


15. Xu C, Qiu Z: **Crystallization behavior and thermal property of biodegradable poly(3-hydroxybutyrate)/multi-walled carbon nanotubes nanocomposite.** *Polym Advan Technol* 2011, **22**:538-544.


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44. Idempotent vector design for standard assembly of Biobricks
   http://hdl.handle.net/1721.1/21168

45. A new biobrick assembly strategy designed for facile protein engineering
   http://hdl.handle.net/1721.1/32535


49. Registry of standard biological parts http://partsregistry.org/


CHAPTER 5

A SYNTHETIC BIOLOGICAL ENGINEERING APPROACH TO UNDERSTANDING TYPE I SECRETION OF POLYHYDROXYBUTYRATES FROM ESCHERICHIA COLI

1. Abstract

Using synthetic biological engineering to secrete bioproducts such as polyhydroxybutyrate (PHB) from Escherichia coli could potentially lead to many cost saving measures upon scale-up. In a previous study it was demonstrated that PHB could be successfully secreted from E. coli using a type I secretion system. In this present study, secretion of PHB is monitored with green fluorescent protein (GFP) fusion to PhaC synthase that is covalently bound to the PHB granule. From this study it was confirmed that PHB accumulation in E. coli occurred at the polar regions of the cell and secretion of PHB via type I secretion machinery also occurs at the cell poles. This study gives a qualitative understanding of type I secretion of PHB from E. coli and the results of this study can be used to understand type I secretion of other synthetic biologically engineered products.

2. Introduction

Polyhydroxybutyrates (PHBs) are biodegradable polyesters that can be recombinantly produced in Escherichia coli. PHBs are one of 155 different polyesters belonging to the group of polyhydroxyalkanoates (PHAs) [1]. Due to their desirable properties and fast bacterial cultivation, PHBs are one of the most studied biopolymers. Widespread production of this polymer in recombinant systems is limited due to high costs of: carbon substrate [2] and purification to separate PHB from cell mass [3].
In recombinant systems such as *E. coli*, PHB can be produced via a three enzymatic pathway converting acetyl-CoA to PHB. Three genes from *Ralstonia Eutropha* encode for beta-ketothiolase (phaA), acetoacetyl-CoA Reductase (phaB), and PHA polymerase (phaC, or PHA synthase) are required for production of PHB [1]. As the PHB polymer is being synthesized the PHA synthase (PhaC) remains covalently attached and forms an amphipathic molecule with hydrophilic (polar) and hydrophobic (non-polar) ends, this aids in the formation of a PHB granule [4]. There are four classes of PHA synthases, where class I synthases are responsible for short chain length (scl) PHA polymerization and class II synthases are used for medium chain length (mcl) PHA polymerization [4, 5]. Additional proteins such as Phasin also attach to the PHB granules and play a role in granule formation [6].

There are a variety of techniques that can be used to separate PHBs from cell mass, such as chemical, mechanical, and biological treatments [3]. However, almost all these methods are invasive and required some form of cell lysis. Secretion of a bioproduct from its host is advantageous as it potentially allows for biomass recycling and also exporting the bioproduct extracellularly helps shift the equilibrium towards bioproduct generation [7].

Previously, a green fluorescent protein (GFP) was used to understand synthetic biological engineering secretion systems in *E. coli*. From this study, type I secretion of GFP fused to HlyA was determined to be the most successful [8]. Type I secretion is advantageous as it offers direct secretion of a protein once the HlyA tag has been expressed at the C-terminal of a protein [9].
Building upon type I GFP secretion, an approach to secretion of PHB from \textit{E. coli} and into the culture medium was developed. Secretion of the PHB outside of the cell could potentially eliminate the need for a solvent or energy intensive means of PHB purification. Phasin, a structural protein, aids in PHB granule formation and was used in a PHB secretion system with Phasin-HlyA protein-signal intermediate to bind to PHB granules. HlyA, a signal peptide, was targeted for secretion using the aforementioned type I secretion system. The results from this study demonstrated that 36% of the total produced PHB was secreted and 64% remained inside the cell [10].

The ability of PHA synthase and proteins such as Phasin to bind to PHA granules allows the polyester granule to be functionalized. Functionalized PHA granules are being recognized for their potential utility in a wide range of biotechnological applications [11, 12]. These applications include: protein purification [13-15], enzyme immobilization [16, 17], vaccines [18], and imaging [19, 20].

Previous studies have used a GFP-PhaC binding partner to demonstrate localization of PHB granules in \textit{E. coli}. These studies demonstrated that PHA begins to localize at the polar regions of the cell [19, 20]. A study by Peters and Rehm used the class I PHA synthase from \textit{R. eutropha} with GFP bound at the N-terminal of the synthase [19]. Previous studies have demonstrated that the N-terminal of the PHA synthase (PhaC) is not crucial for PHA synthase activity and thus a protein fusion at the N terminus of PhaC will not impede PHA production or covalent binding of the PhaC to the PHA granule [4, 21]. Interestingly, an additional study has suggested that PhaC-GFP fusions are also possible due to the fact that the N-terminal of GFP is hydrophobic thus the C-terminal of PhaC can retain its activity [22].
It has previously been demonstrated that secretion of PHB occurs at the polar regions of the cell using a Phasin-HlyA type I secretion system with scanning electron microscopy [10]. Since a synthetic biological engineering approach was demonstrated to be successful in PHB secretion it was also used to create a GFP-PhaC fusion in this present study. The objective of this study was to couple the PHB secretion system used in Rahman et al. with a GFP-PhaC monitoring system similar to that used in Peters et al. to understand real time type I secretion of PHB from *E. coli*.

3. **Materials and Methods**

All reagents and enzymes were purchased from Thermo Fisher Scientific (Pittsburgh, PA) unless stated otherwise. All cloning, BioBrick™ assembly, and expression studies were carried out in *E. coli* XL1 Blue (Agilent Technologies, Santa Clara, CA).

3.1 **Bacterial strains and growth conditions**

Minimal M9 media was used for experiments, containing M9 salts (Becton, Dickinson and Co, Sparks, MD) supplemented with 1.5% (w/v) glucose (ACS grade, Acros Organics, Fair Lawn NJ), 0.2% (w/v) yeast extract (Becton, Dickinson and Co, Sparks MD), and 0.002 M MgSO$_4$ [10, 23]. *E. coli* strains containing specific plasmids were cultured overnight from single colonies in 5 ml LB media [24]. Overnight cultures were then used to seed M9 cultures at an initial optical density ($OD_{600}$) of 0.05. 0.1 mM Isopropyl β-D-1thiogalactopyranoside (IPTG) (Gold Biotechnology, Inc. St. Louis, MO) was added at 0 h. Where necessary, antibiotics were used in the following amounts: 50 µg/mL ampicillin (IBI Scientific, Peosta, IA) and 34 µg/mL chloramphenicol (Acros
Organics, Fair Lawn, NJ). Cultures were grown in an orbital shaker operating at 225 rpm and 37°C.

3.2 Construct assembly

Genetic parts were designed and assembled with BioBrick™ and BioFusion standards [25, 26]. BioFusion standard RFC 23 allows for in frame protein fusions with a threonine and arginine amino acid sequence between the fused proteins. All strains, plasmids, and oligonucleotides used in this study are listed in Table 5.1.

BioBrick™ compatible phaC1 was amplified from the plasmid containing the phaCAB operon (BBa_K934001, partsregistry.org) using the primers KFphaC1F and KFphaC1RStp. PhaC1 was then cloned into pSB1C3 by digesting the PCR fragment with \textit{EcoRI} and \textit{PstI}.

A cycle 3 green fluorescent protein (GFP) was amplified from the plasmid GFPuv with the primers AR5 and AR6 and cloned into pSB1C3, primer design accounted for removing of a stop codon and in frame protein fusion design [8, 27]. GFP had an \textit{XhoI} site removed via mutagenesis (while conserving amino acid sequence) using a QuikChangeII Site-Directed Mutagenesis Kit and the QuikChange® Primer Design Program (Agilent Technologies, Santa Clara, CA), this mutated GFPuv was now called GFPmut. Sequencing of GFPmut was carried out with VF2 (BBa_G00100, partsregistry.org) and VR (BBa_G00101, partsregistry.org) primers to verify mutagenesis. Subsequent cloning of GFPmut to the N-terminal of phaC1 was carried out, \textit{Lac} promoter (BBa_R0010, partsregistry.org), ribosome binding site (BBa_B0034, partsregistry.org), and double terminator (BBa_B0015, partsregistry.org) were cloned sequentially to give pKF26.
The complete GFPmut-phaC1 system was cloned with the phaP1-hlyA (pCMEL2; [10]) into pSB3K3 (pKF29). The plasmid pKF29 was digested with EcoRI and XhoI, the fragment cloned into pBHR68 giving the plasmid pAKF01. pAKF01 was co-transformed with pLG575 into XL1 Blue thereby generating the secreting strain (+hlyA/+hlyBD). Figure 5.1 A shows the genes in the pAKF01 plasmid with associated proteins on the PHB granule surface and Figure 5.1 B illustrates the complete secretion system.

A non-secreting system lacking hlyA expression was also constructed. pKF26 was transferred to the p4MT that contains a lac driven phasin expression system to give pKF30. pKF31 was then made by ligating the insert of pKF30 in pSB3K3. pKF31 insert was then cloned into the pBHR68 plasmid giving pAKF02. pAKF02 was co-transformed with pLG575 into XL1 Blue thereby giving the non-secreting strain (-hlyA/+hlyBD).

Table 5.1. Strains, plasmids, and oligonucleotides used in this study

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<thead>
<tr>
<th>Relevant characteristics</th>
<th>Reference</th>
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<td>Strains</td>
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<td>XL1 Blue</td>
<td>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F’proAB lacIqZΔM15 Tn10 (Tetr)]</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
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<td>pBluescript SK−, phbCAB genes from <em>R. eutropha</em></td>
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<td>pACYC184 derivative, HlyBD, p15A origin, Cm&lt;sup&gt;R&lt;/sup&gt;</td>
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<tr>
<td>pSB1AK3</td>
<td>High copy BioBrick&lt;sup&gt;TM&lt;/sup&gt; vector, pMB1 origin, Amp&lt;sup&gt;R&lt;/sup&gt; and Kan&lt;sup&gt;R&lt;/sup&gt;</td>
</tr>
<tr>
<td>pSB1C3</td>
<td>High copy BioBrick&lt;sup&gt;TM&lt;/sup&gt; vector, pMB1 origin, Cm&lt;sup&gt;R&lt;/sup&gt;</td>
</tr>
<tr>
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</tr>
<tr>
<td>pCMEL2</td>
<td>phaP1, C-terminal BioFusion with HlyA signal peptide, Lac promoter (BBa_R0010), RBS (BBa_B0034), in pSB3K3</td>
</tr>
<tr>
<td>pCMEL3</td>
<td>phaP1, C-terminal BioFusion with HlyA signal peptide, Lac promoter (BBa_R0010), RBS(BBa_B0034), in pBHR68</td>
</tr>
</tbody>
</table>
pKF22
phaC1 in pSB1C3, amplified from BBa_K934001 in pSB1C3
This study

pGFPuv
Green fluorescent protein (GFP), BBa_K208000 in pSB1AK3
[8]

pKF23
Green fluorescent protein (GFP) amplified from pGFPuv. Stop codon removed and XhoI site mutated, in pSB1C3
This study

pKF26
In frame fusion [RFC 23] of GFPmut (pKF23) with phaC1 (pKF22). Lac promoter (BBa_R0010), RBS(BBa_B0034), and double terminator (BBa_B0015), in pSB1C3
This study

pKF27
EcoRI/SpeI fragment pKF26 and pCMEL2 in pSB1C3
This study

pKF28
EcoRI/PstI fragment of pKF27 in pSB1AK3
This study

pKF29
EcoRI/PstI fragment of pKF28 in pSB3K3
This study

p4MT
promoter (BBa_R0010), RBS(BBa_B0034), phaP1, and double terminator (BBa_B0015) in pSB1AK3
[31]

pKF30
EcoRI/Spel fragment of pKF26 in p4MT
This study

pKF31
EcoRI/PstI fragment of pKF30 in pSB3K3
This study

pAKF01
EcoRI/XhoI fragment of pKF29 (+HlyA) in pBHR68.
This study

pAKF02
EcoRI/XhoI fragment of pKF31(-HlyA) in pBHR68.
This study

Oligonucleotides

KFc426g_antisense 5'-gtgagttatatgtgtactccagtttgtgtccgagaatgt-3'
This study

Primer for mutation of XhoI site from GFP

KFc426g 5'-acattctcggacacaaactggagtacaactataactcac-3'
This study

Primer for mutation of XhoI site from GFP

KFphaC1F 5'-tctggaattcgcggccgcttctagaatggctactgggaaaggagc-3'
This study

Forward primer to amplify phaC1 from BBa_K934001

KFphaC1RStp 5'-tctgctgcagcggccgctactagttcacgcttttgcttttacat-3'
This study

Reverse primer to amplify phaC1 from BBa_K934001 conserving stop codon

AR5 5'-tctggaattcgcggccgctctgtagagctcatc-3'
This study

Forward primer to amplify GFP (BBa_K208000)

AR6 5'-tctgctgcagcggccgctctgtagagctcatc-3'
This study

Reverse primer to amplify GFP (BBa_K208000) with no stop codon (taa removed)
Figure 5.1. Schematic for real time secretion of PHB granules with associated proteins from *Escherichia coli* harboring plasmids pAKF01 + pLG575. Direct production of PHB with the *phaABC* operon and expression of the GFP-PhaC fusion protein with the *phaP1-hylA* secretion system both under lactose control. Once PHB granule is generated Phasin-HlyA is bound to the granule and is targeted for secretion. Granule localization is monitored with GFP-PhaC.
3.3 Fluorometry

A fluorescent 96 well plate reader was used to measure GFP fluorescence. Prior to testing samples, OD$_{600}$ was measured and samples will normalized to an OD$_{600}$ of 1 to ensure that approximately the same number of cells were being tested. Samples were loaded into a Costar® 96-well black plate (Corning Incorporated, Corning, NY) and loaded into a Synergy 2 microtiter plate reader (BioTek, Winooski, VT). The cycle 3 GFP mutant has an excitation of approximately 395 nm and an emission of 509 nm, thus the filter set used in this study was a 360/40 nm for excitation and a 528/20 nm for emission.

3.4 Fluorescent microscopy

The microscope setup used in this study was the same as that used in a previous study to monitor GFPuv secretion [8]. This setup consisted of an inverted Nikon Eclipse Ti-U (Melville, NY), Photometrics® CoolSNAP HQ² high-resolution camera, and B-2A Longpass Emission filter set. For the purposes of this study, a 100x oil immersion objective lens and NIS-Elements AR software were used for capturing fluorescence images.

3.5 Recovery of secreted PHB and associated proteins

A calcium chloride (CaCl$_2$) method was used to separate the secreted PHB and protein from the cell fraction developed in a prior study. Briefly, 0.1 M CaCl$_2$ (final concentration, Avantor Performance Materials, Inc. Center Valley, PA) was added to the culture and mixed. Tubes were allowed to sit for 10 min then via differential centrifugation the secreted fraction was separated from the cell mass [10].
3.6 PHB quantification

PHB was quantified for samples to demonstrate that PHB producing strains were capable of PHB generation. Methods of PHB quantification were followed similar to the methods seen in previous studies [32, 33].

3.7 Statistical analysis

Data from fluorometry studies were processed with Statistical Analysis Software (SAS 9.4, SAS Institute Inc., Cary, NC). Two-way analysis of variance (ANOVA) was performed on the data collected at 24 and 48 h after induction. Tukey post hoc comparison was performed on significant results with a confidence level of 95%.

4. Results and Discussion

PHB production and GFP fluorescence was observed for all strains upon IPTG induction. To quantitatively demonstrate secretion of GFP bound PHB granules cultures were subjected to CaCl$_2$ treatment and differential centrifugation to separate secreted PHB from cell mass [10]. 100µL of samples were then loaded onto Costar® 96-well black plate and fluorescence was measured. Fluorescence values were corrected by subtracting the autofluorescence of the background media. The results of this study are shown in Figure 5.2 where the study was carried out at 24 and 48 h for the pAKF02+pLG575 (non-secreting) and pAKF01+pLG575 (secreting) strains respectively. These results suggest that there is significantly more fluorescence observed in the secreting strain’s secreted fractions at 24 and 48 h compared to the non-secreting strain at the same time points (p<0.05). While the average fluorescence from the secreting strain (pAKF01+ pLG575)
was higher at 48 h compared to 24 h, there was no statistical significant difference in fluorescence between these time points.

The non-secreting strain does demonstrate some fluorescence even after background correction, this could be attributed to the differential centrifugation method allowing some whole or lysed cells through. In a previous study it was observed that there was some PHB present in the secreted fraction of a similar non-secreting strain and it was attributed to liberated PHB due to cell lysis or some cells in the secreted fraction [10].

Figure 5.2. Fluorescence measurements in relative fluorescent units (RFUs) of GFP in the media at 24 and 48 h of the *E. coli* XL1 Blue strains harboring the pAKF02+pLG575 (−hlyA/+hlyBD, non-secreting) and pAKF01+pLG575 (+hlyA/+hlyBD, full secretory system) plasmids respectively.

Figure 5.3 A and B shows GFP fluorescence inside *E. coli* XL1 Blue harboring the pAKF01 (+hlyA/-hlyBD) and pAKF02 (-hlyA/-hlyBD) plasmids respectively after 24 h of growth. The cells visually demonstrate accumulation of GFP tagged PHB granules.
inside the cell. From observation this shows approximately 50 % PHB of the cells total volume. There are visible small granules inside the bacteria suggesting that GFP-PhaC is bound to the granule surface and potentially reducing granule size. Figure 5.3 C, pAKF02 (-hlyA/-hlyBD), shows groups of cells containing individual granules of PHB inside the cells. Interestingly, in Figure 5.3 C some cells also have a large accumulation of GFP tagged PHB at one of the cell poles, this phenomena was also observed in another study [34].

Figure 5.4 A demonstrates pAKF01 + pLG575 (+hlyA/+hlyBD, full secretory system) expressed in XL1 Blue after 24 h of culturing. Similar to pAKF01 being expressed alone there are fluorescently tagged spherical PHB granules inside the cell. Previous studies have also demonstrated spherical shaped PHB granules [19, 20]. To the author’s knowledge, the fluorescently tagged PHB granules in this study are smaller than observed in other studies and this could be in part due to the overexpression of both the PhaC and Phasin. It has previously been reported that overexpression of Phasin reduces the PHB granule size considerably [35]. Additionally, there are preliminary signs of secretion occurring from one of the poles of the cell (indicated with arrows).

Figure 5.4 B shows a cell exhibiting early secretion, this cell also has large PHB granules in the middle of the cell. Previous studies reported that larger granules that accumulate in the middle of the cell are actually cells undergoing the process of cell division and that PHB granule was located at the new septum or future cell poles [19, 34]. Figure 5.4 C and Figure 5.4 D show PHB-GFP secretion 48 h post induction. Comparing the fluorescent images seen in Figure 5.4 B and Figure 5.4 C to the SEM image seen in Rahman et al. [10] it can be confirmed that secretion of PHB occurs at the polar regions
of the cell. Also in Rahman et al. it was suggested that secretion occurs between 24 and 48 h, from the GFP labeled PHB it is clear that secretion is occurring between these time points. Cells expressing the pAKF02 and pLG575 plasmids (-hlyA/+hlyBD, non-secreting strain) were also observed under the fluorescent microscope at 24 and 48 h. Figure 5.5 A shows the accumulation of the majority of the PHB at each of the cell poles.

Figure 5.5 B shows a typical non-secreting cell at 48 h post induction. Similar to Figure 5.4B, the cell in Figure 5.5 B suggests that PHB granules are accumulating at the new septum or cell division sites. Interestingly, many of the cells at 48 h were elongated or filamented, with clear regions of internally accumulated fluorescent PHB. The filamented bacteria suggest that accumulation of PHB affects cell division, this was also observed in a previous study [36]. It was suggested that the reason for filamentation was due to stress on the *E. coli* caused by accumulation of a non-native molecules such as PHB. The stress on the cells due to PHB production inhibits the activity of the FtsZ protein that is required for cell division [37]. FtsZ proteins are essential for the beginning of cell division as they form a ring and framework for other division proteins [38]. A study by S.Y. Lee in 1994 overexpressed the ftsZ gene in *E. coli* harboring the PHB operon cells and demonstrated lower likelihood for filamentation [37]. An additional study by the same group also showed that production of PHB was significantly increased when FtsZ was overexpressed thus suppressing filamentation [39]. Interestingly, in the secreting strain (Figure 5.4C/D) at 48 h cells were observed to be slightly elongated when compared to 24 h. Total filamentation was not seen at 48 h in the secreting strain and thus suggests that secretion of PHB from the cell poles potentially reduces the likelihood for filamentation of *E. coli*. 
Figure 5.3. Fluorescent microscope images of GFP bound PHB granules in *E. coli* after 24 h of growth A) pAKF01 (+hlyA/-hlyBD) and B/C) pAKF02 (-hlyA/-hlyBD). White bar represents 5 µm.
Figure 5.4. Fluorescent microscope images of GFP bound PHB granules in pAKF01 + pLG575 (+hlyA/+hlyBD, full secretory system). A) *E. coli* after 24 h of growth, cell exhibiting accumulation and secretion, B) *E. coli* after 24 h of growth cell showing secretion and accumulation of PHB at the new septum, and C/D) *E. coli* after 48 h of growth cell showing secretion of GFP tagged PHB. Arrows denotes secretion from the polar regions of the cell. White bar represents 5 µm.
Figure 5.5. Fluorescent microscope images of GFP bound PHB granules in pAKF02 + pLG575 (-hlyA/+hlyBD, non-secreting system) *E. coli* after A) 24 h and B) 48 h of growth. White bar represents 5 µm.

Future work will be conducted on optimization of the separation of secreted PHB from cellular mass with CaCl₂. Additionally, other groups have demonstrated that by overexpressing the *ftsZ* gene total PHB production was increased, another study would be to overexpress *ftsZ* and observe its effect on cell division and PHB secretion. Furthermore, functionalization of the PHB granule by fusing a different protein to PhaC other than GFP could be carried out to increase the utility of having co-secretion and co-functionalization of the PHB granule.

5. Conclusions

This study used a GFP-PhaC binding partner to fluorescently tag PHB granules and a Phasin-HlyA fusion for secretion. PHB production was observed for all strains
carrying the GFP-PhaC fusion system. In most samples, individual granules could be visualized with a fluorescent microscope. These fluorescently labelled granules can be monitored in real time for internal localization and secretion of PHB and it was found that 24 and 48 h post induction, secretion was highest when compared to a non-secreting control.

6. References


25. Idempotent vector design for standard assembly of Biobricks http://hdl.handle.net/1721.1/21168

26. A new Biobrick assembly strategy designed for facile protein engineering http://hdl.handle.net/1721.1/32535


CHAPTER 6
A SYNTHETIC BIOLOGICAL ENGINEERING APPROACH TO PRODUCE
ARGIOPE AURANTIA SPIDER SILK IN ESCHERICHIA COLI

1. Abstract

Dragline spider silk possesses a combination of tensile strength, elasticity, and biocompatibility unmatched by any other biomaterial. These unique characteristics give spider silk a wide range of potential applications. Farming spiders for production of dragline spider silk is not feasible, thus different host organisms need to be used for large scale manufacturing. This study demonstrates the use of synthetic biological engineering and BioBricks™ to produce a synthetic dragline spider silk MaSp2 protein from Argiope aurantia in Escherichia coli. We constructed fourteen repeats of a modified MaSp2 gene from A. aurantia for recombinant expression in E. coli. Spider silk sequences were codon optimized for expression in E. coli and the corresponding tRNAs were co-expressed to increase spider silk protein production. The spider silk expression system was assembled using standard BioBrick™ assembly techniques and the completed composite system was transformed into BL21 for protein expression. Spider silk protein (85.7 kDa) was purified using 10x His-TagS and a nickel affinity column. To qualitatively demonstrate increased expression with tRNAs, the green fluorescent protein (GFP) was fused to the C-terminal of the spider silk protein and fluorescence was measured with and without tRNAs. It was observed that expression of the specific tRNAs produced higher levels of fluorescence, indicating higher spider silk protein yields.
2. Visual Abstract
3. **Introduction**

Spider silk is an ancient biomaterial with remarkable properties such as extraordinary strength and elasticity [1]. Orb-web weaving spiders have up to seven different types of silk glands, with each gland producing a different spider silk protein with unique properties [2]. Interestingly, the dragline spider silk has a strength of approximately $4 \times 10^9$ N/m$^2$ and an energy to break of $4 \times 10^5$ J/kg [3]. These mechanical properties are similar or superior to other more commonly used materials such as Kevlar and rubber [3]. In the future, there is a possibility for spider silk proteins to replace manmade materials and have a wide range of potential applications if the spider silk protein can be sustainably produced in large quantities [4].

Spiders can produce six different fibers, one of which is the major ampullate or dragline silk. Dragline silk consists of two proteins: the major ampullate Spidroins MaSp1 and MaSp2 [5] and is produced in many different species of spiders such as the *Nephila clavipes* and *Argiope aurantia*. A study by Brooks et al. 2005 reported that *N. clavipes* dragline spider silk consisted of 81% MaSp1 and 19% MaSp2, whereas *A. aurantia* dragline spider silk contained 41% MaSp1 and 59% MaSp2 [6]. The synthetic MaSp2 protein from *A. aurantia* consists of different motifs giving the protein a wide range of potential properties. The GPGXX motif is directly responsible for increase in type II β-turns, with an increasing number of GPGXX repeats reported to have an increase in elasticity of the silk fiber. The polyalanines motif is responsible for the β-sheet formation that correlates to the strength of the spider silk fiber [7].

Spider silk proteins have been expressed in a variety of different organisms, for example: yeast [8], plants [9, 10], silkworms [11], mammalian cells [12], *Salmonella*...
[13], and *Escherichia coli* [7, 14]. Each host production system has its advantages and disadvantages ranging from ease of upstream bioprocessing, yield of protein generated, cost of production, and total production time.

From the different aforementioned host organisms, *E. coli* is potentially the best industrial production host in which spider silk DNA can be cloned and expressed [15]. It was chosen as the organism to express MaSp2 from the spider *A. aurantia* in this study. MaSp2 from *A. aurantia* contains six amino acids in a highly repetitive system, thus allowing for straightforward assembly of the modular gene. There has been a variety of cloning methods and genetic engineering strategies used to assembly large repeats of spider silk monomers in *E. coli* and these are reviewed elsewhere [16, 17].

In this study, a synthetic biological engineering approach known as BioBrick™ standard assembly RFC 23 was used [18]. Using this approach, multiple repeats of the spider silk monomer can be assembled together quickly and easily. Another advantage of using the BioBrick™ standard assembly is that different sizes of spider silk monomers can be assembled together to produce a variety of different synthetic spider silk sizes. This is advantageous as it has been reported that spider silk proteins of higher molecular weight have better overall mechanical properties [19].

BioBrick™ standard assembly RFC 23 is one of the most robust molecular cloning techniques and allows rapid in frame assembly of functioning molecular systems in different organisms. One of the disadvantages of this assembly method is the generation of “scar” threonine and arginine between the two fused proteins. Arginine is not present in abundance in *E. coli* and hence could lead to lower protein yields. Highly repetitive constructs such as those used to produce synthetic spider silk in *E. coli* will
contain many “scar” regions and hence could deplete the available tRNA pool for arginine in *E. coli*.

In this study we demonstrate the effect of additional specific tRNAs to support synthetic spider silk production in *E. coli*. Along with measuring spider silk yields directly, a cycle 3 green fluorescent protein (GFP) mutant fused to the C-terminal of the spider silk protein was used as an indirect means of measuring spider silk production levels.

### 4. Materials and Methods

All chemicals and reagents were purchased from Thermo Fisher Scientific Inc. (Pittsburgh, PA) unless mentioned otherwise. DNA constructs denoted with ‘BBa’ are available at partsregistry.org. Cloning was conducted with the RFC 23 standard assembly [18]. Spider silk producing genes were expressed in pSB1C3 and tRNA supporting constructs were expressed in pSB3K3. *E. coli* XL1-Blue was used for cloning, while protein expression and fluorescence studies were conducted in BL21.

#### 4.1 Media preparation

All initial growth experiments during cloning were carried out in LB media [20] with appropriate antibiotic added at a final concentration of 34 μg/mL chloramphenicol (Acros Organics, Fair Lawn, NJ) and 50 μg/mL Kanamycin (Gold Biotechnology, Inc. St. Louis, MO). Cultures were grown at 37°C on an orbital shaker at 220 rpm.

#### 4.2 Design and optimization of spider silk sequences for tRNA usage

MaSp2 from *A. aurantia* uses only six amino acids (glycine, tyrosine, proline, alanine, glutamine, and serine) and thus overexpression of the silk protein in *E. coli* could
potentially lead to the depletion of charged tRNA molecules in the host cell. Depletion of charged tRNAs molecules could reduce the yields of the spider silk protein. A review by Makrides highlights several studies that demonstrated significant increase in recombinant protein levels when expressing specific tRNAs [21]. Makrides also highlights the consideration of the expression of arginine in *E. coli* as these codons are rarely used [21]. As mentioned, the use of arginine cannot be avoided with a BioBrick™ assembly system as RFC 23 generates arginine (AGA) as a result of restriction enzyme ligation scar [18].

Codon optimization for specific tRNAs has not been well documented in literature, however, a recent study by Xia et al. demonstrated an increase in *N. clavipes* synthetic spider silk protein production in *E. coli* when the glycine-tRNA pool was increased [22].

Codon optimization for specific tRNAs in this study was performed based on the amino acid sequence of MaSp2 from *A. aurantia* in Brooks et al. [7]. A list of tRNA genes in *E. coli* K-12 was acquired from the Genomic tRNA Database (http://gtrnadb.ucsc.edu/Esch_coli_K12/eschColi_K12-tRNAs.fa, accessed September 2012). Finding the specific tRNA to overexpress from the host organism is vital to increasing expression of spider silk proteins. In this study, specific codons were chosen so that they contained the lowest possible GC% for each specific amino acid.

The spider silk construct was designed using a balanced codon construction (BBa_K844004, partsregistry.org). This gene uses two codons for glycine (ggt & gga), two for proline (cct & cca), and two for serine (agt & tct) as these were the only amino acids out of the six that had multiple codons options with low GC%. Alanine also had two codons with low GC%, but additional codons could not be utilized since a *Pst*I restriction site would be created in the construct, which is incompatible with RFC 23
assembly [18]. Thus, a single codon for Alanine (gca) was used in the construct design (BBa_K844004). Table 6.1 shows the codon usage in *E. coli* (DNA 2.0, Menlo Park, CA), MaSp2 in Brooks et al. [7], and MaSp2 in this study. Distribution of the different codons in the BBa_K844004 construct was also taken under consideration where, the two codons for the glycine, proline, and serine amino acids were distributed evenly.

The amino acid sequence and codon usage for MaSp2 *A. aurantia* (BBa_844004) is shown in Figure 6.1. This sequence contains two repeats of the elastic unit (GGYGPGAGQQGPGSQGPGSGGQQGPGGQ) followed by a GPAYGPS linker and 6 polyalanines, modified from the amino acid sequence for MaSp2 from *A. aurantia* [7]. It is well documented that the polyalanines form the β-sheets that are directly related to the strength of the fiber [23].

The tRNA plasmid p6x5x consisted of a pSB3K3 plasmid backbone with BBa_K844012 and BBa_K844013 BioBrick™ parts (partsregistry.org). These two parts consisted of specific tRNAs for each codon optimized amino acid in the MaSp2 sequence. The promoter chosen for tRNA expression of each tRNA was based on the 12x promoter from the study by Bauer et al. [24].

An additional spider silk BioBrick™ was created to contain a start codon (atg) to allow for direct translation of the mRNA. Start codons were added to each BBa_K844004 spider silk piece with the use of a site directed mutagenesis kit (Agilent Technologies, Santa Clara, CA).

### 4.3 BioBrick™ Assembly

All BioBricks™ were constructed in pSB1C3 [25] according to BioBrick™ standards [18, 26]. All the spider silk repeat units as well as the 10x His-Tag were
assembled according to RFC 23, which allows for in frame protein fusions. All completed parts were transformed via electroporation into *E. coli* XL1-Blue (Agilent Technologies, Santa Clara, CA) to generate higher plasmid levels as BL21 has low plasmid copy numbers.

The Lac promoter and ribosome binding site (BBa_R0010 and BBa_B0034, partsregistry.org) were used as it had been previously demonstrated to work in other protein expression systems such as that for green fluorescent protein (GFP) expression and translocation [27]. Furthermore, the ribosome binding site BBa_B0034 was reported as one of the most useful parts from the parts registry [28]. One MaSp2 spider silk subunit, BBa_K84408 (containing atg), was first cloned proceeding the ribosome binding site followed by 13 repeats of BBa_K844004. Finally the C-terminal 10x His-tag (BBa_K844000) and double terminator (BBa_B0015) were subsequently cloned into the system to give pB14. The 10x His-tag was placed at the C-terminal end to ensure that only fully translated spider silk protein was purified. Sequencing was carried out using primers VF2 (BBa_G00100, partsregistry.org) and VR (BBa_G00101, partsregistry.org) to confirm correct construction (partsregistry.org). Detailed descriptions of strains and plasmids used in this study are shown in Table 6.2 and Figure 6.2, respectively.

Another plasmid, pB14GFP, containing the lac promoter, rbs, and 14x spider silk was constructed with green fluorescent protein (GFP) and a 10x His-Tag at the C-terminal. The GFP (BBa_K208000) and was amplified using primers AR5 and AR6. Primers were designed in accordance with RFC 23 and to remove the stop codon in GFP so that a GFP and 10x His-Tag fusion could be formed. In addition, constructs containing 4x, 12x, and 16x spider silk subunits with GFP fused at the C-terminal were also built.
4.4 Fluorescence studies

Cultures were grown in LB media with appropriate antibiotics. 0.1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Gold Biotechnology, Inc. St. Louis, MO) was added at time=0 and cultures were allowed to grow overnight (~15 hours) in an orbital shaker at 220 rpm, 37°C. The IPTG concentration of 0.1 mM was chosen as this demonstrated optimal protein expression in studies with the same promoter system [27, 29]. Overnight cultures were standardized based on OD$_{600}$. 200 μL of each sample was loaded into three separate wells of a Costar® 96-well black plate (Corning Incorporated, Corning, NY). Fluorescence analysis was then conducted using a Synergy 2 microtiter plate reader (BioTek, Winooski, VT) with an excitation of approximately 395 nm and emission of 509 nm for GFP. Filter wheels used in study were: excitation 360/40 nm and emission 528/20 nm. Background fluorescence values for BL21 Gold cells not containing any fluorescence were subtracted from the values obtained from samples containing GFP. The studies were carried out in triplicate.

Fluorescent microscopy was also used to image the cells, using an inverted Eclipse Ti-U (Melville, NY), Photometrics® CoolSNAP HQ$^2$ high-resolution camera, and B-2A Longpass Emission filter set with 100 x objective [27].

4.5 Bioreactor scale-up

To analyze MaSp2 production, pB14 with and without tRNAs (p6x5x) were grown overnight in 100 mL seed cultures on a shaker table at 220 rpm, 37°C with appropriate antibiotics. Seed cultures and bioreactors had the same media composition. Seed cultures were added to 10 L Winpact Bioreactor and Fermentors (GMI, Inc., Ramsey, Minnesota) for protein production.
Figure 6.1. Nucleotide and amino acid sequence for one monomer of MaSp2 dragline spider silk from *Argiope aurantia* (BBa_K844004). Codon usage: glycine (G=2 (ggt, gga)), tyrosine (Y=1 (tat)), proline (P=2 (cct, cca)), alanine (A=1 (gca)), glutamine (Q=1 (caa)), and serine (S=2 (agt, tct)). GC content approximately 55%.

Figure 6.2. Spider silk production system constructed using the BioBrick™ assembly standard RFC 23 (pB14). BBa_K208010 is a composite part consisting of a Lac Promoter and Ribosome Binding site. BBa_K844008 is the spider silk subunit of the MaSp2 gene from *Argiope aurantia* containing a methionine (atg). 13 repeats of BBa_K844044 are spider silk subunits of the MaSp2 gene from *A. aurantia*. BBa_K844000 is a 10x His-tag with a double stop codon. BBa_B0015 is a double terminator.
Table 6.1. Codon usage for one repeat of MaSp2 *A. aurantia* spider silk containing two elastic units (2E). *Escherichia coli* B codon usage (DNA 2.0, Menlo Park, CA), Unoptimized DNA sequence (Brooks et al. 2008), Balanced codon usage (this study). Not shown are the codons for the scar region (2 amino acids that are between each of the spider silk subunits): T-Threonine (ACT codon) and R-Arginine (AGA codon).

<table>
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<th>Amino Acid composition (%) in MaSp2</th>
<th>Amino Acid</th>
<th>% Codon Usage</th>
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<tr>
<td></td>
<td>Ala (A): 11.80</td>
<td>GCT 22, GCC 26, GCA 27, GCG 25</td>
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<td>Gln (Q): 17.6</td>
<td>CAA 35, CAG 65</td>
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<tr>
<td></td>
<td>Gly (G): 44.1</td>
<td>GGT 34, GGC 29, GGA 19, GGG 17, CCT 24</td>
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<td></td>
<td>Pro (P): 14.7</td>
<td>CCT 24</td>
</tr>
<tr>
<td></td>
<td>Ser (S): 7.4</td>
<td>CCT 24</td>
</tr>
<tr>
<td></td>
<td>Tyr (Y): 4.4</td>
<td>TAT 65, TAC 35</td>
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Table 6.2. Strains and plasmids used in this study

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<th>Strains</th>
<th>Relevant Characteristics</th>
<th>Reference</th>
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<tr>
<td>XL1 Blue</td>
<td>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F’ proAB lacIqZAM15 Tn10 (Tetr)]</td>
<td>Agilent Technologies</td>
</tr>
<tr>
<td>BL21 Gold</td>
<td>E. coli B F’ ompT hsdS(rB− mB−) dcm+ Tet+ gal endA Hte</td>
<td>Agilent Technologies</td>
</tr>
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<th>Plasmids</th>
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<tr>
<td>pSB1C3</td>
<td>High copy BioBrick™ vector, pMB1 origin, CMR</td>
<td>[25]</td>
</tr>
<tr>
<td>pB14</td>
<td>Spider silk production vector. Lac promoter +RBS (BBa_K208010), spider silk subunit (BBa_K84408), 13x spider silk subunit (BBa_K844004), 10x His-tag (BBa_K844000), and double terminator (BBa_B0015) in pSB1C3.</td>
<td>This study</td>
</tr>
<tr>
<td>pB14GFP</td>
<td>Spider silk production vector with GFP fusion at C terminal. Lac promoter +RBS (BBa_K208010), spider silk subunit (BBa_K84408), modified GFP with stop codons removed (BBa_K208000), 10x His-tag (BBa_K844000), and double terminator (BBa_B0015) in pSB1C3.</td>
<td>This study</td>
</tr>
<tr>
<td>pB04GFP</td>
<td>Similar to pB14GFP but with 4x repeats of spider silk subunit</td>
<td>This study</td>
</tr>
<tr>
<td>pB12GFP</td>
<td>Similar to pB14GFP but with 12x repeats of spider silk subunit</td>
<td>This study</td>
</tr>
<tr>
<td>pB16GFP</td>
<td>Similar to pB14GFP but with 16x repeats of spider silk subunit</td>
<td>This study</td>
</tr>
<tr>
<td>p6x5x</td>
<td>tRNA plasmid pSB3K3 containing both BBa_K844012 and BBa_K844013 composite BioBrick™ parts.</td>
<td>This study</td>
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<th>Oligonucleotides</th>
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<tbody>
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<td>AR5</td>
<td>5’-tctggaattcgccgctttagaatgtagcaagaga-3’ Forward primer to amplify GFP (BBa_K208000)</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>5’-tctgtgctgctctactagttttagagctctatc-3’ Reverse primer to amplify GFP (BBa_K208000) with no stop codon (taa removed)</td>
<td>This study</td>
</tr>
</tbody>
</table>
Media used for these studies were modified based on Chen et al. [30]. Briefly, the bioreactor medium consisted of 15 g/L Hy-Express™ System II (Sheffield Bio-Science, Beloit, WI), 5 g/L Hy-Yest™ 444 (Sheffield Bio-Science, Beloit, WI), 20 g/L Glucose, 16 g/L Glycerol (Amresco, Solon, OH), 7.1 g/L Na₂HPO₄, 6.8 g/L KH₂PO₄, and 3.3 g/L (NH₄)₂SO₄. Additionally, trace elements were added as mentioned in Chen et al. [30]. A dissolved O₂ level of 40% was controlled and monitored by gassing with air, O₂, and agitation. NH₄OH was used to maintain pH at 6.0 and Himar FGK antifoam (Jeneil Biotech Inc., Saukville, WI) was added automatically via a controller during fermentation. Protein expression was induced with 0.1 mM IPTG and was added at a culture OD₆₀₀ of approximately 20. Cultures were harvested approximately 4-5 h after IPTG induction using a CEPA Z41 continuous flow centrifuge (Eppendorf, Hamburg, Germany) and cell pellets were immediately stored at -80°C.

4.6 Protein purification

Approximately 50 g of wet biomass were sonicated for 10 min in binding buffer (5 mM Imidazole, 0.5 M NaCl, 20 mM tris-HCl, and 4 M Urea) and lysates were centrifuged at 5000 x g for 10 min. The supernatant fractions were passed through an AKTA Avant system (GE Healthcare Biosciences, Pittsburgh, PA) with a 5 mL HisTrap FF crude nickel column (GE Healthcare Biosciences, Pittsburgh, PA) for spider silk protein purification. Non-specifically bound protein, flow through, and lysed cell debris was saved for analysis. Spider silk protein was dialyzed using Fisherbrand regenerated cellulose dialysis membranes, MWCO 3500 (Fisher Scientific, Pittsburgh, PA). Dialysis was conducted over 48 h in ddH₂O similar to a previous study [31]. Once the protein was
desalted, it was frozen to – 80°C and then lyophilized in a FreeZone 4.5 L benchtop freeze dry system (Labconco, Kansas City, MO).

4.7 PAGE/Western

Proteins were mixed with loading buffer, and heat treated for 1-3 min at 90°C. 30 μL of protein and buffer at a 1:1 ratio were loaded into precast 4-20% Precise Protein gels (Thermo Scientific Inc., Rockford IL). Precision Plus Protein™ marker (Bio-Rad, Hercules, CA) was used to visualize protein propagation. Gels were run at 100 V with Tris-HEPES running buffer. For visualization, gels were stained with Bio-Safe Coomassie Stain overnight (Bio-Rad, Hercules, CA) and de-stained in ddH2O.

For Western Blot analysis, proteins were electroblotted from protein gel to nitrocellulose membrane (Bio-Rad, Hercules, CA) and blocked with 5% non-fat milk TBS-Tween-20 for 30-60 min. Primary antibody, Anti-6X His Epitope tag (Mouse) Monoclonal Antibody (Rockland Immunochemicals Inc., Gilbertsville, PA) was used in a 1:1000 ratio. The membrane was then washed 3 times with TBS-Tween-20 and blocked with blocking solution. Secondary antibody anti-Mouse IgG H&L ab6729 1:1000 (Abcam Inc., Cambridge, MA) was added to 2nd block for 30 min, washed 3 times with TBS-Tween-20. 1-Step NBT/BCIP substrate (Thermo Fisher Scientific Inc., Rockford, IL) was added and chemiluminescence was allowed to occur for 10 min.

4.8 Spinning spider silk

Desalted spider silk protein was spun using a DACA fiber spinning system (DACA Instruments, Santa Barbara, CA). Major ampullate MaSp2 silk protein dopes were generated by dissolving different ratios of silk protein into 1,1,1,3,3,3-hexafluoro-2-
propanol (HFIP). The protocol for dissolving and spinning the silk was adapted from Teule et al. [32]. After the silk has been completely dissolved in HFIP the silk mix was extruded through a needle into a coagulation bath containing isopropanol at room temperature. The strand of silk is then wound through a set of godets and collected as a fiber. Fibers are then glued to cards and diameters are calculated before strength testing was carried out.

5. Results and Discussion

5.1 Spider silk expression studies with green fluorescent protein (GFP)

To demonstrate that tRNAs can help increase spider silk protein expression levels in *E. coli*, GFP was tagged to the C-terminus of different sized silk proteins. Proteins with different spider silk repeat numbers with and without additional tRNAs were expressed in *E. coli* and GFP fluorescence was measured. The benefit of having GFP at the C-terminal means that only fully transcribed DNA sequences will have spider silk and GFP expression. From Figure 6.3 it can be observed that fluorescence levels for samples with tRNAs are higher than those samples without tRNAs. Interestingly, spider silk constructs that were shorter in length had higher levels of fluorescence which demonstrates that either: 1) the duration of time to express different spider silk proteins is longer for a larger protein or 2) smaller spider silk proteins are produced at a higher level, thus higher overall GFP fluorescence. This GFP fluorescence study indirectly demonstrates that additional tRNAs increase overall spider silk production. Furthermore this system is also useful for real time analysis of spider silk production in *E. coli*. Figure 6.4 shows fluorescent microscope images of the *E. coli* expressing different sizes of the spider silk fused to GFP.
Figure 6.3. GFP fluorescence for different repeats of spider silk subunit with GFP fused to the C-terminus of the spider silk protein. Each repeat was tested for fluorescence with and without tRNAs. ‘B’ denotes ‘balanced’ construct and the subsequent number is the number of spider silk gene repeats.

Figure 6.4. Fluorescent microscope images of *E. coli* cells expressing different sizes of MaSp2 *Argiope aurantia* spider silk fused to green fluorescent protein. A) 4 repeats, B) 12 repeats, and C) 16 repeats of the MaSp2 subunit.

5.2 Preliminary protein purification results

Lysed cell extracts were passed onto a nickel column for purification of synthetic spider silk protein. Figure 6.5 and Figure 6.6 show chromatographs of protein
purification (nickel affinity chromatography) from *E. coli* strains without and with tRNAs respectively. The blue lines in Figure 6.5 and Figure 6.6 show absorbance at 280 nm. The first blue line peak shows the wash fraction that indicates non-specifically bound protein being washed off the nickel column and the second peak shows the elution fraction from the nickel column.

In order to compare the approximate yield of spider silk protein with and without tRNAs, HPLC was carried out as demonstrated in Figure 6.7. It was observed that when tRNAs were expressed the overall yield of spider silk was increased approximately 20 fold between 2.45 and 2.55 min.

![Figure 6.5. Nickel affinity chromatography results for spider silk production system without tRNA expression. Blue line indicates absorbance at UV 280 nm, where first peak is wash fraction and second peak is elution fraction.](image)
Figure 6.6. Nickel affinity chromatography results for spider silk production system with tRNA expression. Blue line indicates absorbance at UV 280 nm, where first peak is wash fraction and second peak is elution fraction.

Figure 6.7. HPLC on elution fractions from spider silk sample with and without tRNAs. Construct that has tRNAs demonstrates increased production of spider silk. Peak of interest is between 2.45 and 2.55 min.
5.3 Analysis of spider silk protein production

The Coomassie stained SDS polyacrylamide gel (Figure 6.8 A) showed bands at approximately 85.7 kDa in elution fraction. Additionally, the corresponding immunoblot (Figure 6.8 B) showed the presence of a band of approximately 85.7 kDa in elution fraction.

6. Conclusions

A synthetic biological engineering approach can be used to systematically assemble spider silk subunits together to create functioning systems. This step-wise assembly method using BioBrick™ assembly standard RFC 23 opens up many different possible sizes of spider silk repeats that could be assembled together and expressed. Studies with GFP demonstrate that spider silk production levels can be increased with the use of specific tRNAs. This study demonstrated that E. coli harboring the pB14 plasmid can produce 85.7 kDa MaSp2 spider silk proteins from A. aurantia with the use of BioBrick™ standard assembly.
Future work will include comparing results from this study to another system that uses a lower plasmid copy number system, increasing the number of spider silk repeats (hence increase overall protein size), modifying the growth media for increased protein expression, and spinning fluorescent spider silk proteins.

7. References


18. A new Biobrick assembly strategy designed for facile protein engineering

http://hdl.handle.net/1721.1/32535


25. Registry of Standard Biological Parts http://partsregistry.org/

26. Idempotent Vector Design for Standard Assembly of Biobricks
   http://hdl.handle.net/1721.1/21168


CHAPTER 7

BIOREMEDIATION OF DOMESTIC WASTEWATER AND PRODUCTION OF BIOPRODUCTS FROM MICROALGAE USING WASTE STABILIZATION PONDS

1. Editorial

Domestic wastewater treatment and remediation is an expensive process due to significant time and planning needed for successful treatment. Modern wastewater treatment plants are highly mechanized and expensive to build and maintain. In less economically developed parts of the world alternative methods of wastewater treatment are required. Waste stabilization ponds, or lagoons, provide an ideal solution for wastewater treatment in developing countries and rural areas. These ponds facilitate the oxidation of organic matter through complex symbiotic relationships between bacterial consortiums and assimilation of wastewater nutrients with photoautotrophic microalgae [2]. In the United States more than 7,000 lagoon systems are used to treat domestic wastewater (U.S. EPA, 2002, Report No. EPA 832-F02-014) [1]. Most domestic wastewater is considered weak or medium in strength with nitrogen levels between 20-40 mg/L and phosphorus levels between 4-8 mg/L [3]. These concentrations of nitrogen and phosphorus are undesirable as they can lead to considerable pollution and eutrophication of downstream waterways [2].

Open pond lagoon systems have many advantages over mechanicalized methods and are able to remove nitrogen and phosphorus to required EPA levels. Interestingly, nitrogen and phosphorus found in weak domestic wastewater are at an ideal level for

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2 Co-authors: Joshua T. Ellis and Charles D. Miller
microalgae cultivation and growth. Microalgae can grow to high densities by assimilating nitrogen and phosphorus, thus removing these inorganic nutrients from the wastewater. In addition, open pond lagoon systems also allow ideal mixing and adequate light exposure for microalgae growth. Microalgae play a vital role in recycling carbon in the biosphere by converting carbon dioxide into organic compounds through photosynthesis [1], while also producing oxygen via the oxidation of water. Metal compounds such as Cr, Cu, Pb, Cd, Mn, As, Fe, Ni, Hg, and Zn can also be bioremediated by microalgae. Microalgae such as Chlorella and Scenedesmus have shown tolerance and bioremediation capabilities to certain heavy metals [5]. Additionally, microalgae have been used for the bioremediation of textile dyes in wastewater from industrial textile processes. These bioremediation capabilities of microalgae are useful for environmental sustainability and algal biomass can be used as feedstock for the production of high energy compounds [4, 6].

Algal biomass can be processed chemically and biologically to produce high value products such as bioacetone, biobutanol, biodiesel, and biomethane. Microalgae as feedstocks provide high densities of carbohydrates (typically comprising glucose units), triacylglycerides and free fatty acids that can be used to produce biofuels and biodiesel. It has been demonstrated that microalgae can be a promising feedstock and will play a vital role in the future production of clean and renewable energy [1, 4].

The disadvantages to an open pond lagoon system are that the microalgae nutrient requirement may not match the stoichiometric ratio of the microalgae biomass, where the optimum nitrogen to phosphorus ratio for microalgae growth is 16:1. Thus, photoautotrophic bioremediation of inorganic compounds might not be carried out to
adequate levels. To meet nutrient requirements for microalgae growth, additional chemicals (usually nitrogen rich sources) may need to be supplemented to the wastewater, which is undesirable.

Microalgae grown in open pond lagoon systems are at low densities and specialized harvesting technologies need to be implemented in order obtain suitable biomass yields. Harvesting techniques such as a Rotating Algal Biofilm Reactor (RABR) [1], filtration, sedimentation, and dissolved air flotation (DAF) units can be employed to harvest the microalgae from open pond lagoon systems. There are advantages and disadvantages to each method, but the cost of harvesting is currently high and more efficient technologies need to be created [2].

To summarize, waste stabilization ponds provide an active bioremediation system to clean domestic wastewater, and they can also produce microalgal feedstocks for the production of high value bioproducts. Interest in the use of microalgae will continue to grow as rural cities and developing countries look for sustainable and affordable ways to clean domestic wastewater. Processes where wastewater is bioremediated through heterotrophic and photoautotrophic organisms, and in turn high value bioproducts are generated have great potential to stimulate regional and local economic development [2].

2. References


CHAPTER 8
EFFECTS OF WASTEWATER MICROALGAE HARVESTING METHODS ON POLYHYDROXYBUTYRATE PRODUCTION\textsuperscript{3}

1. Abstract

Microalgae have gained considerable attention recently as a sustainable means to produce biofuels and bioproducts. It has previously been demonstrated that single strain microalgae can be harvested and processed—through a wet lipid extraction procedure (WLEP). After WLEP processing, acetone, butanol, ethanol, and biodiesel can be produced, and growth of recombinant *Escherichia coli* can be achieved from the microalgae. This study demonstrates the application of different wastewater microalgae harvesting techniques and processing through WLEP on the production of polyhydroxybutyrate (PHB) by *E. coli*. The harvesting techniques include: cationic potato starch (CPS), cationic corn starch (CCS), aluminum sulfate, and centrifugation. The microalgae-based media were used to grow *E. coli* to \( \sim 10^{13} \) CFU/mL and produce approximately 7.8\% of dry cell weight as PHB. This study demonstrates the feasibility of harvesting wastewater algae to produce PHB and the potential for bioproduct generation.

2. Introduction

Polyhydroxyalkanoates (PHAs) are promising alternatives to petroleum based plastics (Gumel et al., 2012). PHAs have a diverse range of potential applications due to their chemical and physical properties that are comparable to traditional petroleum derived plastics (Rehm, 2010). A wide variety of PHAs exist, including

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polyhydroxybutyrates (PHB), which are short chain length polymers with 3-5 carbon monomers (Agnew et al., 2013). Production of PHB can be accomplished by recombinant *Escherichia coli* containing the *phaCAB* operon (isolated from *Ralstonia eutropha*) that encodes for a three step enzymatic process to convert a carbon substrate into PHB (Spiekermann et al., 1999).

Despite the similar material characteristics to petroleum derived plastic, high costs of PHA production have limited its widespread use. It has been reported that PHA production can cost approximately $2.65-5/kg compared to petroleum derived plastics costing $1.57-1.67/kg (Choi and Lee, 1997). The economic production and scale-up of PHB is dependent on inexpensive carbon substrates. Studies have shown that the carbon substrate accounts for approximately 40% of the total cost of recombinant production of PHB in *E. coli* (Choi and Lee, 1999). Hence there is motivation to use inexpensive carbon substrates, ideally from waste products of other processes. Previous studies have demonstrated production of PHB from: anaerobic digesters (Linton et al., 2012), food wastes (Hafuka et al., 2011), and waste glycerol from biodiesel production using a variety of microorganisms (Dobroth et al., 2011).

Microalgae have been extensively studied as a feedstock for biodiesel production (Chisti, 2007). High growth rates, low fresh water requirement, and high photosynthetic efficiency are few of the important characteristics that have made microalgae the obvious choice as a biodiesel production platform (Christenson and Sims, 2011). Microalgae can remediate wastewater by assimilating phosphorus and nitrogen as growth nutrients, thereby achieving tertiary treatment (Rahman et al., 2012). In the United States over 7,000 lagoon systems are used to treat domestic waste water (U.S EPA, 2002, Report No.
EPA 832-F02-014) (Christenson and Sims, 2012). Lagoon systems are an ideal solution to wastewater treatment compared to modern wastewater treatment plants and are suitable for developing countries and rural areas. The city of Logan, Utah processes approximately 15 million gallons per day of its wastewater in a 460 acre (~1.9 km²) open pond facultative lagoon system. This system consists of seven ponds with an average depth of five feet (~1.5 m). Microalgae grown in these ponds can be harvested to provide a sustainable supply of biomass for bioproduct generation (Christenson and Sims, 2012).

We have previously demonstrated a “biorefinery” approach to harvesting single strain algae (*Scenedesmus obliquus*) and subsequent production of acetone, butanol, ethanol, biodiesel, and growth of genetically engineered *E. coli* (Anthony et al., 2013). *S. obliquus* was grown in Solar Simulated Bioreactors (SSR) and harvested with cationic starches, alum, and centrifugation. The algae were then processed via a wet lipid extraction procedure (WLEP) creating four streams, three of which have been previously utilized (Anthony et al., 2013; Sathish and Sims, 2012). The first stream (hydrolyzed algal biomass) was used to generate acetone, butanol, and ethanol (ABE) by *Clostridium saccharoperbutylacetonicum* N1-4 fermentation (Ellis et al., 2012). The remaining two side streams, the lipid extract and resultant aqueous phase, were used to produce biodiesel and to grow genetically engineered *E. coli* respectively.

In this study, mixed culture microalgae were collected from the Logan lagoon wastewater treatment plant (Logan, UT) using different harvesting methods. After harvesting, algae were fractionated via the WLEP (Anthony et al., 2013; Sathish and Sims, 2012) to obtain an aqueous phase. This aqueous phase was then used as the substrate for growth of genetically engineered *E. coli* and production of PHB. The
objective of this study was to evaluate the effect of various wastewater microalgae harvesting techniques, including flocculation with cationic potato starch (CPS), with cationic corn starch (CCS), with aluminum sulfate, and centrifugation, on the resulting algae-based media’s (from WLEP) ability to support \textit{E. coli} growth and production of PHB.

3. Materials and Methods

All chemicals and reagents were purchased from Thermo Fisher Scientific (Pittsburgh, PA) unless stated otherwise. Reagents used for the processing of algal biomass post harvesting using WLEP included ACS grade sulfuric acid from EMD Chemicals (Gibbstown, NJ) and sodium hydroxide from Avantor Performance Chemicals (Center Valley, PA).

3.1 Cationic starch synthesis and microalgae harvesting

The methods for cationic potato starch (CPS), and cationic corn starch (CCS) synthesis were adopted from a previous study (Anthony and Sims, 2013). Briefly, potato starch and corn starch (acquired locally, Logan, UT) were dissolved in ceric ammonium nitrate (Sigma Aldrich, St. Louis, MO) and heated for 30 min. 3-methacryloyl amino propyl trimethyl ammonium chloride (MAPIAC, Sigma Aldrich, St. Louis, MO) was added, and mixtures were heated for a further 2 h. Final products were washed with ethanol. The total nitrogen content present in CPS and CCS was measured using Hach Test 'N Tube (Loveland, CO) employing the 4500-N B Standard Methods (Clescerl et al., 1998). The degree of substitution (DS) was calculated as described previously (Anthony et al., 2013).
Methods for harvesting single strain *S. obliquus* are described in Anthony et al. 2013 (Anthony and Sims, 2013), similar methods were used in this study for harvesting mixed culture lagoon algae and processing via the WLEP. Briefly, microalgae were harvested from the Logan lagoons (Logan, UT) using CPS, CCS, aluminum sulfate (alum, Sigma Aldrich, St. Louis, MO), and centrifugation (8000 rpm x 10 min) at pH 7.0. Dosage amounts of each compound are provided in Table 8.1. Apart from centrifugation, lagoon microalgae were harvested by the reduction of the negative zeta potential of algae to 0 mV by the addition of CPS, CCS, and alum. Zeta potential measurements were performed using a ZetaPlus zeta meter (Brookhaven Instruments Corporation, Holtsville, NY). Lagoon microalgae were collected by adding predetermined concentrations of the coagulants, flash mixing, and allowing flocculation and settling for approximately 1 h. Samples for zeta potential measurement were collected before and after addition of CPS, CCS, and alum. A sample of the harvested biomass from each method was washed with 0.1 M NaOH to remove associated coagulant and obtain the true weight of microalgae in the biomass.

### 3.2 Processing of harvested algal biomass via WLEP

Once mixed culture algae were harvested via one of the four methods, they were processed through the wet lipid extraction procedure (WLEP) as described in previous studies (Anthony et al., 2013; Sathish and Sims, 2012). The WLEP was performed to produce four side streams of which one, the aqueous phase, was of interest for the purposes of this study as it had been previously demonstrated to allow for *E. coli* growth (Anthony et al., 2013).
3.3 Production of Polyhydroxybutyrate (PHB)

*E. coli* XL1-blue (Stratagene, La Jolla, CA) containing the pBHR68 plasmid (Spiekermann et al., 1999) was cultured on the four different aqueous phase media. The pBHR68 plasmid contains the three genes (*phaA, phaB*, and *phaC*) needed for PHB synthesis and confers ampicillin resistance. Gene expression was controlled by a lac promoter. *E. coli* containing the pBHR68 plasmid was cultured in each of the different aqueous phase media similar to the methods used in a previous study (Anthony et al., 2013). Briefly, aqueous phase media were autoclaved, 100 μg/ml ampicillin (IBI Scientific, Peosta, IA) and 0.1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG, Gold Biotechnology, Inc. St. Louis, MO) were added to each aqueous phase flask at time 0 h. *E. coli* harboring the pBHR68 plasmid was grown in LB media the previous day and was used to seed each aqueous phase flask at an initial OD₆₀₀ of 0.05. Flasks containing the aqueous phase media and cells were placed in an orbital shaker operating at 225 rpm and 37°C. CFU/mL and PHB content were measured after 48 h of growth. Experiments were carried out in triplicate.

PHB analysis was carried out using a direct polyhydroxyalkanoate analysis with $^1$H NMR as described previously (Linton et al., 2012). Briefly, after 48 h of growth in aqueous phase media, PHB producing *E. coli* were centrifuged at 3500 rpm for 20 min and pellets were lyophilized. Approximately 15 mg of lyophilized cells were dissolved in 0.7 mL of 5% sodium hypochlorite and 1 ml of CDCl₃ (0.03% TMS). Samples were then vortexed, placed on a shaker table, and the organic phase was analyzed for PHB in a Jeol ECX-300 NMR spectrometer (Jeol USA, Inc., Peabody, MA). A standard NMR/GC correlation was used to determine PHB concentrations as described in Linton et al. 2012.
3.2 Statistical analysis

The comparisons of CFU/mL and PHB yields obtained from the aqueous phase of microalgae harvested by each of the different harvesting methods were statistically analyzed using one-way analysis of variance (ANOVA) with REGWQ as the post-hoc comparison (confidence level of 95%). Statistical Analysis Software (SAS 9.3, SAS Institute Inc., Cary, NC) was used for the analysis.

4. Results and Discussion

4.1 Cationic starch and microalgae harvesting

The degree of substitution (DS) for CPS and CCS was 0.04 ±0.005 and 0.06 ±0.01, respectively. Higher DS for the starch molecules (CPS and CCS) suggests that they have higher nitrogen content and thus will have better dewatering performance. As mentioned in Anthony et al. (2013), CPS has a lower zeta potential compared to CCS, thus giving CPS a lower DS. Lagoon microalgae harvested via centrifugation contained approximately 96% microalgae with the remainder attributed to undissolved salts and other particulates in the wastewater (Table 8.1). CPS needed 4.56 g to harvest 8.4 g of microalgae and CCS required 3.15 g to harvest 11.5 g algae (Table 8.1). The amount of respective flocculants used in this study to harvest wastewater microalgae was similar to that used in previous studies (Anthony et al., 2013; Vandamme et al., 2010). The amount of alum required for coagulation and flocculation of microalgae was much higher than CPS and CCS. The mass of alum used was similar to that of the mass of lagoon algae harvested, approximately 11.2 g of alum to harvest 9.2 g of microalgae (Table 8.1). Additionally, only 42% of the mass harvested with alum was microalgae, compared to 70% and 82% from CPS and CCS. The reason for the higher amounts of alum needed for
harvesting microalgae is potentially due to alum having a lower zeta potential, and thus higher dosages are needed to harvest similar quantities of microalgae compared to CPS and CCS. Comparing the results in Table 8.1 for wastewater microalgae (this study) to that of harvesting single strain *S. obliquus* in a previous study (Anthony et al., 2013), the mass of CPS and CCS needed was higher for wastewater microalgae than single strain algae. Furthermore, the percent dry weight of algae of total biomass (%) was lower for the mixed algae in this study compared to that seen in the previous single algal strain study. The higher amounts of CPS and CCS used to flocculate mixed culture algae could be due to various types of algae requiring additional coagulant for charge neutralization. Additionally, wastewater contains other additional particulates and salts that could reduce the interaction of the algae with CPS and CCS.

<table>
<thead>
<tr>
<th>Harvesting method</th>
<th>Total weight of biomass (g)</th>
<th>Mass of algae (g)</th>
<th>% dry weight of algae of total biomass</th>
<th>Mass of coagulant used (g)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPS</td>
<td>12</td>
<td>8.4</td>
<td>70 %</td>
<td>4.56</td>
<td>This study</td>
</tr>
<tr>
<td>CCS</td>
<td>14</td>
<td>11.48</td>
<td>82 %</td>
<td>3.15</td>
<td>This study</td>
</tr>
<tr>
<td>Centrifuged</td>
<td>10</td>
<td>9.6</td>
<td>96 %</td>
<td>0</td>
<td>This study</td>
</tr>
<tr>
<td>Alum</td>
<td>22</td>
<td>9.24</td>
<td>42 %</td>
<td>11.2</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Table 8.1. Total weight of biomass, mass of algae, % dry mass of algae, and amount of coagulant used.**

**Wastewater microalgae**

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**Scenedesmus obliquus**

<table>
<thead>
<tr>
<th>Harvesting method</th>
<th>Total weight of biomass (g)</th>
<th>Mass of algae (g)</th>
<th>% dry weight of algae of total biomass</th>
<th>Mass of coagulant used (g)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPS</td>
<td>10.37</td>
<td>9.12</td>
<td>88 %</td>
<td>2.41</td>
<td>(Anthony et al., 2013)</td>
</tr>
<tr>
<td>CCS</td>
<td>14.9</td>
<td>12.96</td>
<td>87 %</td>
<td>2.5</td>
<td>(Anthony et al., 2013)</td>
</tr>
<tr>
<td>Centrifuged</td>
<td>10</td>
<td>10</td>
<td>100 %</td>
<td>0</td>
<td>(Anthony et al., 2013)</td>
</tr>
<tr>
<td>Alum</td>
<td>28.86</td>
<td>17.89</td>
<td>62 %</td>
<td>12.31</td>
<td>(Anthony et al., 2013)</td>
</tr>
</tbody>
</table>

Where CPS: Cationic potato starch, CCS: Cationic corn starch, and Alum: aluminum sulfate
4.2 Bacterial growth and production of Polyhydroxybutyrate (PHB)

The growth of *E. coli* on WLEP aqueous phase from CPS, CCS, alum, and centrifuged wastewater was determined 48 h after induction. Figure 8.1 A shows the highest *E. coli* growth of approximately $10^{13}$ CFU/mL was observed in the aqueous phase media from centrifuged algae, this was higher than that seen in the aqueous phase media from CPS ($\sim 10^5$ CFU/mL), CCS ($\sim 10^5$ CFU/mL), and alum ($\sim 10^6$ CFU/mL).

The PHB yields were highest from the centrifuged algae aqueous phase media (Figure 8.1 B) compared to CPS, CCS, and alum. *E. coli* was able to produce approximately $7.8 \pm 1.5\%$ PHB of its dry cell weight in the centrifuged algae aqueous phase. The PHB produced in the centrifuged aqueous phase was statistically different ($p<0.05$) from the PHB produced in the CPS, CCS, and alum aqueous phases. The PHB produced in the CPS and CCS, were not statistically different from each other ($p>0.05$). No PHB was detected from *E. coli* growing in alum aqueous phase media. Growth (CFU/mL) of *E. coli* in aqueous phase media from CPS, CCS, and alum were not significantly different ($p>0.05$). The higher CFU/mL from centrifuged algae aqueous phase ($\sim 10^{13}$ CFU/mL, Figure 8.1) correlates to higher PHB production. This would be expected as a higher CFU/mL shows that there are more viable cells and hence more PHB produced 48 h after induction. Studies have suggested that the use of alum (or other metal salts such as: ferric chloride or ferric sulfate) for algae harvesting purposes could potentially affect downstream processes (Molina Grima et al., 2003). From this study, the aqueous phase derived from the alum harvested algae did not allow for PHB production.

Alternative genes for the generation of other products could be incorporated into *E. coli* and grown on aqueous phase media, making for a flexible biorefinery platform. From a biorefinery standpoint, production of more than one bioproduct from microalgal
could generate a more economical and sustainable production platform. Future work can also include harvesting waste water microalgae with a rotating algae bioreactor (RABR) (Christenson and Sims, 2012) and processing the algae with the WLEP.

Figure 8.1. CFU/mL (A) and PHB% (% of dcw) (B) of *E. coli* XL1-blue harboring the pBHR68 plasmid 48 h after induction, grown on different WLEP aqueous phases from cationic potato starch (CPS), cationic corn starch (CCS), aluminum sulfate (Alum), and centrifuged mixed culture algae. Numbers are averaged from triplicate experiments. * indicates statistical significance (p<0.05).
5. **Conclusions**

This study demonstrated four different harvesting techniques of mixed wastewater microalgae, and the processing of microalgae using WLEP to create a medium for *E. coli* growth and PHB production. The aqueous phase from centrifuged wastewater microalgae produced the highest *E. coli* growth (~10^{13} CFU/mL) and PHB production (~7.8% of dry cell weight). CPS, CCS, and alum flocculated wastewater microalgae aqueous phase media reduced the potential for *E. coli* growth and PHB production. Understanding the effects of harvesting on the contents of the aqueous phase will be important moving forward.

6. **References**


CHAPTER 9
SUMMARY

Synthetic biological engineering provides the ability to program an organism to carry out a specific function that could potentially lead to many newfound applications in the future. The studies presented in this dissertation added some useful applications to the synthetic biological engineering toolbox in the form of: increasing bioproduct production with secretion and codon optimization for specific tRNA expression. The hope is that this toolbox can be greatly expanded in the future to allow others to improve upon existing parts and devices to further the various bioproduct production systems.

This dissertation showed the successful design, building, and testing of a synthetic biological engineering PHB secretion system in *E. coli*. It was found that 36% of the total PHB produced by the cell could be secreted. As with any recombinantly produced product, downstream processing is a consideration. Secretion of this polymer could potentially reduce the costs associated with separation of PHB from biomass. Additionally, PHBs have similar properties to their petroleum based plastic counterparts, this is beneficial in two ways: 1) downstream processing of PHB would be similar to that seen with petroleum based plastics and 2) there is a potential for hybrid materials to be made by combining PHB and petroleum based plastics. Similar to biofuels, viable bioplastic production systems need to act as ‘drop in’ technologies, and thus can be incorporated into the current infrastructure system without any major hurdles.

Monitoring the PHB secretion system using a synthetic biological engineering approach with GFP also highlights the importance of understanding and improving upon a biological system. As engineers, optimization of existing production systems can go a
long way into making a process more efficient. In this study, GFP was successfully
tagged to PHB granules using a PhaC intermediate and granules were visible under
fluorescent microscopy. The hope is that by understanding type I secretion of PHBs this
information can be applied to other products such as potentially secreting of spider silk
from *E. coli*.

The work of this dissertation also explored the potential to use wastewater
microalgae to create media suitable for *E. coli* cultivation and bioproduct production. As
an example, *E. coli* grown on a wastewater microalgae based media was able to grow to
approximately $10^{13}$ CFU/mL and accumulate up to 7.8% PHB.

**Future work**

In this study it was demonstrated that multiple proteins could be bound to the
PHB granules. Proteins such as phasin and PHA synthase were used as intermediate
proteins for functionalization of the PHB granules for the purposes of secretion and
visualization with GFP. The studies represented in this dissertation only focused on
secretion of short chain length (scl) PHB, an additional study could utilize the same
secretion system to secrete medium chain length PHAs. Building on the success of this
research, the idea to further functionalize the granules by fusion of spider silk protein to
the PHB granules via an intermediate protein partner could open the possibilities for
creation of new novel biomaterials.

Spider silk proteins could be fused to PHB granules using either Phasin or PhaC
as an intermediate. The reason for this would be to enhance the properties of PHB as
spider silk has significantly higher elasticity and tensile strength than PHB. Using Phasin,
the spider silk protein could be fused to the C-terminal and with PhaC spider silk protein
could be fused to the N-terminus. A comparison between the effectiveness of C-terminal and N-terminal spider silk fusion to a PHB granule could then be studied.

Future work could include building upon the microalgae-to-bioproducts procedure to expand the biorefinery model. This could include growing different bioproduct producing *E. coli* strains in various algae-based media. For example, the PHB secreting strain and spider silk production strains could be cultured on the algae-media and monitored for production. Additionally, other algae sources could be utilized to find the greatest levels of bioproduct production. Further work should be conducted to fully characterize the algae-media and optimize it for production purposes. A technoeconomic study could also be conducted to further understand viable recombinant bioproduct generation from a wastewater-microalgae standpoint.
APPENDICES
APPENDIX A-FUTURE WORK

A1. Scaling up of PHA production

Recovery of polyhydroxyalkanoates from microorganisms is currently a bottleneck during scale up. This bottleneck is one of the reasons why the production of polyhydroxyalkanoates from microorganisms is less economically viable when compared to traditional petroleum based plastic production. Currently, manufacturers of biodegradable plastics are able to produce on average 1000-20,000 tons per year, compared to a single petroleum based polyethylene plant which has a capacity of 300,000 tons per year [1]. Two of the major problems are: 1) the cost of the carbon substrate for bacterial PHA production is expensive and 2) recovery of polyhydroxyalkanoate is expensive.

Studies have been carried out with several different PHA producing bacterial strains at the pilot plant scale, some examples follow. Metabolix, Inc. overexpressed the PHB gene in *E. coli* K12 and produced 100 g/l PHB in 40 h. An Austrian company used *Alcaligenes latus* DSM1124 and showed that it could accumulate up to 80% PHB of the cell weight, the biomass density obtained was 60 g/l. An academic group used *Cupriavidus Necator* H16, PHB was accumulated in the cells up to 76% in 50 hours giving a biomass of 164 g/l with a PHB concentration of 121 g/l [1].

There is a number of different scaling up methods that have been used in bioreactor design, including, batch and fed batch reactors. Table A1 provides examples of: different bioreactors, microorganisms, biomass, and polyhydroxyalkanoate concentrations achieved. There are variations of polyhydroxyalkanoate yields from the different types of strains and reactors used. This gives some indication that reactor type is
significant in deciding how to scale up production effectively. A suggested schematic to scale up PHA production from a PHA secreting strain of *E. coli* is shown in Figure A1. As PHA is being secreted it can be collected in a chamber while cell mass is recycled back into the bioreactor in a semi-continuous fashion.

Table A1. Examples of different types of bioreactors used and respective biomass and polyhydroxyalkanoate concentrations achieved.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Process of production</th>
<th>Biomass concentration</th>
<th>PHA concentration</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cupriavidus necator</em>, NCIB 11599</td>
<td>Two-step fed-batch culture</td>
<td>100 g/l</td>
<td>2.5 g/l h PHB</td>
<td>[1]</td>
</tr>
<tr>
<td><em>Escherichia coli</em> K12</td>
<td>Not Mentioned</td>
<td>Not Mentioned</td>
<td>100 g/l PHB</td>
<td>[2]</td>
</tr>
<tr>
<td><em>Alcaligenes latus</em> DSM1124</td>
<td>one-step fed-batch</td>
<td>60 g/l</td>
<td>48 g/l</td>
<td>[3]</td>
</tr>
<tr>
<td><em>Cupriavidus necator</em> H16</td>
<td>fed-batch</td>
<td>164 g/l</td>
<td>121 g/l PHB</td>
<td>[1]</td>
</tr>
<tr>
<td><em>Cupriavidus necator</em> H16</td>
<td>fed-batch</td>
<td>Not Mentioned</td>
<td>110 g/l PHBV</td>
<td>[4]</td>
</tr>
<tr>
<td><em>Cupriavidus necator</em> DSM 545</td>
<td>batch</td>
<td>10.3 g/l</td>
<td>7.3 g/l PHB</td>
<td>[5]</td>
</tr>
<tr>
<td><em>Bacillus flexus</em></td>
<td>batch</td>
<td>2.5 g/l</td>
<td>1.3 g/l PHB</td>
<td>[6]</td>
</tr>
<tr>
<td><em>Bacillus cereus</em> SPV</td>
<td>batch</td>
<td>2.5 g/l</td>
<td>0.725 g/l PHB</td>
<td>[7]</td>
</tr>
<tr>
<td><em>Bacillus cereus</em> SPV</td>
<td>fed-batch</td>
<td>3.0 g/l</td>
<td>1.14 g/l PHB</td>
<td>[7]</td>
</tr>
</tbody>
</table>
Figure A1. Bioreactor design and downstream processing of PHA from *E. coli*. Vessel A is a 5 L bioreactor (with attached pH, DO and temperature probes), Vessel B is a 500 ml glass column packed with a material to remove secreted PHA. V1-V5 are flow control valves. S1-S6 are stream numbers. Stream S1 brings fresh media into the system and stream S5 removes media out of the system in a semi-continuous process.
A2. Polyhydroxybutyrate production using a wastewater microalgae-based media

(Manuscript in preparation)

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Abstract

Bioproduct production from wastewater microalgae has the potential to contribute to societal needs with value added chemicals. Microalgae can remediate wastewater to remove nitrogen, phosphorus, and heavy metals and can be processed to produce biofuels and bioproducts. It was previously demonstrated that recombinant Escherichia coli could produce polyhydroxybutyrates (PHB) when cultured on a wastewater microalgae wet lipid extracted media. In this present study, microalgae were harvested from the effluent of a wastewater treatment facility via centrifugation and hydrolyzed to create a liquid medium for recombinant E. coli growth and PHB production. Standard E. coli growth media was supplemented with various concentrations of hydrolyzed algal extract to produce a maximum of 31% PHB of the E. coli dry cell weight.

Keywords

Wastewater, Microalgae, Bioproduct, Polyhydroxybutyrate
1. Introduction

Microalgae have been well studied for production of biodiesel (Chisti, 2007) and recently microalgae has been proposed to be the basis for a biorefinery model where multiple chemicals can be produced simultaneously (Anthony et al., 2013b). By producing several chemicals from the same algae feedstock, it could potentially make the production of multiple commodity chemicals from a biological resource economically viable. The limitations to microalgae culturing are well-documented, including but not limited to: nutrient supply, water scarcity, and low energy harvesting and dewatering (Christenson et al., 2011).

The City of Logan, UT has a 460 acre seven pond facultative lagoon system to treat weak domestic wastewater. Weak domestic wastewater contains approximately 20 mg/L nitrogen and 4 mg/L phosphorus and is ideal of microalgae growth (Tchobanoglous et al., 1991). Facultative lagoon systems can be used to culture mixed consortia of microalgae to remediate the wastewater by removal of phosphorus and nitrogen. These microalgae can then be used as a feedstock for production of bioproducts (Rahman et al., 2012; Rawat et al., 2011). There are a wide range of methods that have been employed to harvest the microalgae from an open pond system, these include (but not limited to): rotating algal biofilm reactor (RABR) (Christenson et al., 2012; Kesaano et al., 2014), biological and chemical flocculants (Anthony et al., 2013a; Anthony et al., 2013b), and centrifugation (Greenwell et al., 2010).

*Escherichia coli* is a robust microorganism that can be used to produce a wide range of bioproducts such as biofuels and bioplastics. Its ease of culturing and fast doubling time make it an ideal candidate for production of recombinant bioproducts.
Polyhydroxybutyrates (PHB) are bioplastics that can be recombinantly produced in *E. coli* to sufficiently high levels. PHB is a potentially useful polymer, in addition to being completely biodegradable, it has similar properties to traditional petrochemically derived plastics such as polypropylene and polystyrene (Khanna et al., 2005). Three genes are needed for the conversion of acetyl-coA to PHB in *E. coli*. The pBHR68 plasmid contains the lac promoter and three genes (*phaA*, *phaB*, and *phaC*) needed for production of the bioplastic PHB (Spiekermann et al., 1999).

PHB production is not widely produced in part due to the cost of the carbon substrate. It has been estimated that the carbon substrate in a large scale manufacturing context would constitute approximately 37% of the total production cost (Choi et al., 1999). Due to the high cost of carbon, an alternative low cost substitute is needed to culture *E. coli* in order to make PHB production economically viable. In a previous study, it was demonstrated that *E. coli* harboring the pBHR68 plasmid was able to successfully grow on a *Scenedesmus obliquus* algae-based media (Anthony et al., 2013b). In this previous study, different harvesting methods were used to collect the microalgae and then the harvested algae was processed via the wet lipid extraction procedure (WLEP) to generate a variety of side streams and bioproducts (Anthony et al., 2013b; Sathish et al., 2012). One of the side streams, termed ‘aqueous phase’ was used to culture *E. coli* and it was established that the upstream harvesting method of *S. obliquus* affected the growth of the *E. coli* in the aqueous phase media. The most successful microalgae harvesting method for high levels of *E. coli* growth after 48 h ($10^{12}$-$10^{13}$ CFU/mL) was observed when the *S. obliquus* was centrifuged (Anthony et al., 2013b). The same experiment was extended to harvesting wastewater mixed culture microalgae from the City of Logan, UT
treatment plant pond E. Different harvested wastewater microalgae samples were subjected to the WLEP to generate the aqueous phase. It was found that centrifugation gave the best *E. coli* growth ($\sim 10^{13}$ CFU/mL) and PHB production (7.8% PHB dry cell weight) (Rahman et al., 2014).

Since centrifuged microalgae processed via the WLEP demonstrated highest levels of *E. coli* growth and PHB production, it was decided to use this as the preferred harvesting method. Additionally, the previous studies (Anthony et al., 2013b; Rahman et al., 2014) used an unmodified aqueous phase media to culture *E. coli*, and a subsequent study used a fraction of the aqueous phase with standard *E. coli* media and obtained promising PHB yields (Sathish et al., 2014). The main objectives of this current study were to demonstrate *E. coli* growth and PHB production from an effluent wastewater microalgae-based media. Figure 1 depicts the steps conducted in this study to remove microalgae from wastewater effluent of the treatment facility, hydrolysis of dried microalgae, and *E. coli* growth to produce the polymer polyhydroxybutyrate.
2. Materials and methods

All chemicals and reagents were purchased from Thermo Fisher Scientific (Pittsburgh, PA) unless stated otherwise.

Algae harvesting and processing

Wastewater microalgae were harvested from the City of Logan, UT wastewater treatment facility from the effluent stream leaving the facultative lagoons. Algae were centrifuged using a continuous centrifuge and subject to drying in a temperature controlled oven. After drying, algae were stored at -20°C until the hydrolysis step.

A modified algae hydrolysis method was used similar to Ellis et al. 2012, where hydrolyzed microalgae was used to culture *Clostridia* to produce Acetone, Butanol, and Ethanol (Ellis et al., 2012). Briefly, 10 g of dry algae was dissolved into 0.5 M (final concentration) sulfuric acid (H$_2$SO$_4$) with a total volume of 100 ml, the solution was placed on a stir plate and heated to 90°C for 30 min. After cooling to room temperature,
the solution was neutralized to pH 7 with sodium hydroxide (NaOH). This neutralized
solution was then centrifuged at 3500 rpm for 30 min to clarify the solution. The
supernatant was then used for culturing E. coli.

**Bacterial growth**

Supernatant from hydrolyzed algae was used as the sole carbon source and was
substituted into standard E. coli M9 growth media (Sambrook et al., 2001) in 1 and 2%
ratios (weight dry algae: volume culture). An additional study was conducted with
culturing E. coli in a 10% hydrolyzed algae solution (w/v). The hydrolyzed algae
supernatant was not autoclaved to demonstrate that E. coli growth and PHB production
could occur from a non-sterile carbon source. In addition to the liquid algal extract,
growth media also contained M9 salts (Becton, Dickinson and Co, Sparks, MD), 0.002 M
MgSO₄ (Kang et al., 2008; Rahman et al., 2013), and 50 µg/mL ampicillin (IBI
Scientific, Peosta, IA).

The E. coli strain, XL1 Blue (Agilent Technologies, Santa Clara, CA) harboring
the pBHR68 plasmid (Spiekermann et al., 1999) was grown in LB media (Sambrook et
al., 2001) overnight (~15 h). Cultures were then used to start larger 50 mL cultures with
an initial optical density (OD₆₀₀) of 0.05. Isopropyl β-D-1-thiogalactopyranoside (0.1 mM)
(Gold Biotechnology, Inc. St. Louis, MO) was added at 0 h to induce expression of the
*phaCAB* genes. Bacterial growth was measured using optical density (OD₆₀₀) at 0, 4, 8,
12, 24, and 48 h.

**Sugar analysis**

Total sugar analysis was conducted using a modified phenol-sulfuric acid method
(Ellis et al., 2012). Briefly, 3 µL of 85% (w/v) phenol solution and 150 µL of 12 M
sulfuric acid was added to the samples and the mixture was heated for 5 min at 90°C. After cooling to room temperature for 5 min in an ice bath absorbance (A_{490 nm}) was measured using a Synergy 2 microtiter plate reader (BioTek, Winooski, VT). Sugar concentrations were calculated based on a glucose standard.

**Polyhydroxybutyrate analysis**

PHB analysis was carried out on samples after 48 h of bacterial culturing. PHB concentration was determined from a 1H NMR/GC correlation as mentioned previously (Linton et al., 2012). Briefly, 15 mg of lyophilized sample were dissolved in a deuterated chloroform/bleach solution. Samples were vortexed, incubated, and centrifuged. PHB phase was run on a Jeol ECX-300 NMR (Jeol USA, Inc. Peabody, MA).

**Statistical analysis**

Data was processed with Statistical Analysis Software (SAS 9.4, SAS Institute Inc., Cary, NC). One-way analysis of variance (ANOVA) was conducted on the data collected at 48 h for % PHB for the three different media types. REGWQ post hoc comparison was performed on significant results with a confidence level of 95%. All %PHB experiments were conducted in triplicate.

**3. Results and discussion**

*E. coli* harboring the pBHR68 plasmid was grown on 1%, 2%, and 10% algae-M9 media. The maximum optical density (OD_{600}) for *E. coli* grown in 1% media was around 1.3 where stationary phase was reached at approximately 12 h post induction (Figure 2). The 2% and 10% samples reach stationary phase at around 24 h and reached a maximum
OD$_{600}$ of 2.5 and 7.6 respectively. Cultures were allowed to continue growing until 48 h as this allowed time for PHB accumulation.

Results of the total sugar analysis indicated that simple sugars was present in the algae extract and was being consumed during the course of bacterial growth. The 1% sample had 152 mg/L total sugar at time 0 and after 48 h of bacterial growth had 132 mg/L. The 2% sample had 320 mg/L sugar at time 0 and after 48 h had 267 mg/L. The 10% sample had 1890 mg/L total sugar and finished with 1344 mg/L sugar.

![Figure 3. Growth of *E. coli* harboring the pBHR68 plasmid on 1%, 2%, and 10% algae media.](image)

Comparing PHB yields 48 h post-induction, it was observed that the M9 media containing 1% and 2% algae extract (v/v) had the most PHB (as a percentage of dry cell weight). The 1% and 2% samples had 31 ± 8.9 % and 28.2 ± 2.1 % PHB respectively (Figure 3). In comparison, *E. coli* cultured in 10% algae extract media that had an average PHB accumulation of 4.6 ± 0.7 %. From statistical analysis the p-value was 0.0081 indicating significant results (confidence of 95%, p<0.05). Post-hoc comparison using REGWQ indicated that the PHB production levels at 1% and 2% were not
significantly different from each other. The PHB production from 1% and 2% media samples were both significantly different from the PHB yields observed from *E. coli* grown in 10% algal extract.

The percentage PHB accumulated in bacterial cells cultured in the 1% and 2% algae-M9 media are slightly lower than that seen in *E. coli* harboring pBHR68 grown in M9-glucose media (Table 1). In a previous study, it was found that *E. coli* harboring the pBHR68 plasmid grown in M9 media supplemented with 1.5% glucose could accumulate up to 47.24 ± 6.0 %, 48 h post induction (Rahman et al., 2013). Achieving approximately 31 ± 8.9 % demonstrates the potential of using algae as the sole carbon source in media for *E. coli* culturing and bioproduct production. Addition of an external carbon substrate such as glucose to the algae-based media could potential increased the growth and yields of PHB in *E. coli*. It was demonstrated in a previous study that 1% glucose addition to an algae-based media to culture *Clostridia* tripled the yield of solvent production (Ellis et al., 2012).

Comparing the 10% algae extract media (with no M9 addition) to that of a similar study (Rahman et al., 2014) it was found that this 10% media did not perform as well as the previous study. In the previous study, approximately 9.6 g of algae (dry weight equivalent) were extracted (via centrifugation) from the City of Logan, UT wastewater treatment facility pond E and subjected to the wet lipid extraction procedure (WLEP) to produce approximately 7.8% PHB. In the present study, algae extracted from the effluent of the wastewater treatment plant generated 4.6% PHB. The lower PHB yield could be attributed to the fact that in this study the microalgae harvested could have already been lysed and thus lowering the yield of sugars extracted. There could have also been some
inhibitory effect in the media that was more concentrated, thus reducing the production capacity of the *E. coli*.

![Bar graph showing polyhydroxybutyrate production from different algae-based cultures.](image)

**Figure 3.** Polyhydroxybutyrate production from the different algae-based cultures (where %PHB is a proportion of *E. coli* dry cell weight).

**Table 2.** Production and yields of PHB produced from *Escherichia coli* XL1 Blue harboring the pBHR68 plasmid grown in M9 media supplemented with wastewater algae hydrolyzed fraction.

<table>
<thead>
<tr>
<th>Carbon Source in M9 media</th>
<th>PHB%</th>
<th>g PHB /L</th>
<th>g PHB / g carbon substrate</th>
<th>Carbon needed (kg) needed to produce 1 kg PHB</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% Algae media</td>
<td>30.97 ± 8.9</td>
<td>2.30 ± 1</td>
<td>0.232</td>
<td>4.3</td>
<td>This study</td>
</tr>
<tr>
<td>2% Algae media</td>
<td>28.19 ± 5.1</td>
<td>2.09±0.5</td>
<td>0.104</td>
<td>9.5</td>
<td>This study</td>
</tr>
<tr>
<td>10% Algae media</td>
<td>4.60±0.7</td>
<td>0.32 ± 0.1</td>
<td>0.003</td>
<td>305.2</td>
<td>This study</td>
</tr>
<tr>
<td>1.5% Glucose</td>
<td>47.24 ± 6.0</td>
<td>5.43±1.7</td>
<td>0.40 ± 0.12</td>
<td>2.5</td>
<td>(Rahman et al., 2013)</td>
</tr>
</tbody>
</table>

Table 1 shows the yields of PHB obtained from the different experiments carried out in this study compared to that of another study with the same strain of *E. coli*. 
(Rahman et al., 2013). In addition to PHB% as a fraction of dry cell weight, the total carbon substrate needed to produce 1 kg PHB was also estimated. It was predicted that using the 10% algae in M9 media would need approximately 4.3 kg of dry algae to produce 1 kg of PHB. This is comparable to a standard 1.5% glucose M9 media in the previous study that was predicted to need 2.5 kg of glucose to make 1 kg of PHB. This estimate assumed a linear scaling from shaker flask volume of 50 ml to a large scale bioreactor, in order to get more accurate measurement additional parameters would need to be considered.

4. Conclusions

This study built upon the work previous done with production of PHB from a microalgae feedstock. From this study growth of recombinant *E. coli* harboring the plasmid pBHR68 (containing the phaCAB operon) on microalgae-based media was observed for all samples. It was found that the maximum PHB accumulation in *E. coli* was approximately $31 \pm 8.9\%$ seen on the 10% algae-M9 media 48 h post induction. Future work could include determining the effects of production of additional bioproducts using *E. coli* grown on wastewater algae media and the addition of a traditional carbon source to the algae-based media.

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References


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METHODS FOR HARVESTING AND PROCESSING BIOMASS

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ABSTRACT
A system and method for harvesting and processing algae, the system and method including harvesting algae by mechanical or chemical system and processing the harvested algae to produce at least one of biodiesel, biosolvents, bioplastics, biogas, or fertilizer.
METHODS OF BIOPLASTIC PRODUCTION

Applicants: Charles Miller, North Logan, UT (US); Asif Rahman, Logan, UT (US); Ronald Sims, Logan, UT (US); Ashik Sathish, North Logan, UT (US); Renil Anthony, Salt Lake City, UT (US)

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U.S. Cl. CPC ........................................... C12P 7/625 (2013.01)
USPC .............................................. 435/135; 435/253.6

ABSTRACT
A method of producing bioplastics from algae, the method including processing algae to yield an aqueous phase containing glycerol, and fermenting the aqueous phase with a bioplastic-producing bacteria to yield bioplastics.
United States

Patent Application Publication

Lewis et al.


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USPC 435/471; 435/252.33

ABSTRACT

An expression system, including a host cell, a synthetic spider silk polypeptide-encoding nucleotide sequence, at least one synthetic tRNA molecule-encoding nucleotide sequence or a synthetic serine hydroxymethyl transferase (SHMT)-encoding nucleotide sequence.
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To whom it may concern,

My name is Joshua T. Ellis. Asif Rahman and I wrote the manuscript titled:

“Bioremediation of Domestic Wastewater and Production of Bioproducts from Microalgae Using Waste Stabilization Ponds.” This paper was published in the Journal of Bioremediation and Biodegradation in 2012. Asif was first author and contributed equally to the manuscript. I give him permission to reprint the manuscript in its entirety in his PhD dissertation.

Sincerely,

Joshua T. Ellis
To whom it may concern,

My name is Renil Anthony. Asif Rahman and I wrote the manuscript titled:

“Effects of wastewater microalgae harvesting methods of polyhydroxybutyrate production.” This paper was published in Bioresource Technology in 2014. Asif was first author and major contributor to this work. I give him permission to reprint the manuscript in its entirety in his PhD dissertation.

Sincerely,

[Signature]

Renil Anthony
To whom it may concern,

My name is Ashik Sathish. Asif Rahman and I wrote the manuscript titled: “Effects of wastewater microalgae harvesting methods of polyhydroxybutyrate production.” This paper was published in Bioresource Technology in 2014. Asif was first author and major contributor to this work. I give him permission to reprint the manuscript in its entirety in his PhD dissertation.

Sincerely,

Ashik Sathish
July 1, 2014

To whom it may concern,

My name is Elisabeth Linton. Asif Rahman and I wrote the manuscript titled: “Secretion of polyhydroxybutyrate in Escherichia coli using a synthetic biological engineering approach.” This paper was published in the Journal of Biological Engineering in 2013. Asif was first author and major contributor to this work. I give him permission to reprint the manuscript in its entirety in his PhD dissertation.

Sincerely,

[Signature]

Elisabeth Linton
To whom it may concern,

My name is Alex D Hatch. Asif Rahman and I wrote the manuscript titled: “Secretion of polyhydroxybutyrate in Escherichia coli using a synthetic biological engineering approach.” This paper was published in the Journal of Biological Engineering in 2013. Asif was first author and major contributor to this work. I give him permission to reprint the manuscript in its entirety in his PhD dissertation.

Sincerely,

Alex D Hatch

July 23, 2014
## APPENDIX E- STRAINS, PLASMIDS, AND Oligonucleotides

### Table E1. Strains used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Relevant characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>XL1 Blue</td>
<td>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F’ proAB lacIqZAM15 Tn10 (Tetr)]</td>
<td>Agilent Technologies</td>
</tr>
<tr>
<td>DH5α</td>
<td>F− Φ80lacZAM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK−, mK+) phoA supE44 λ− thi-1 gyrA96 relA1</td>
<td>Agilent Technologies</td>
</tr>
<tr>
<td>BL21-Gold (DE3)</td>
<td>E. coli B F−ompT hsdS(rB mB) dcmTε gal(λDE3) endA Hte</td>
<td>Agilent Technologies</td>
</tr>
<tr>
<td>BL21 Gold</td>
<td>E. coli B F−ompT hsdS(rB mB) dcmTε gal endA Hte</td>
<td>Agilent Technologies</td>
</tr>
<tr>
<td>CopyCutter EP1400</td>
<td>F− mcrA Δ(rr-mhsdRMS-mcrBC) Φ80dlacZAM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ− rpsL (StrR) nupG trfA tonA pcnB4 dhfr</td>
<td>Epicentre</td>
</tr>
<tr>
<td>SA01</td>
<td>MG1655 ΔaraBAD ΔfadR ΔfadIJ fadBA::Φ(PBc-BTE)</td>
<td>[11]</td>
</tr>
<tr>
<td>Cupriavidus necator H16</td>
<td>Wild type, PHA producing</td>
<td>ATCC 17699</td>
</tr>
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</table>

### Table E2. Plasmids used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Relevant characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBHR68</td>
<td>pBluescript SK−, phbCAB genes from R. eutropha</td>
<td>[12]</td>
</tr>
<tr>
<td>pLG575</td>
<td>pACYC184 derivative, HlyBD, p15A origin, CmR</td>
<td>[13]</td>
</tr>
<tr>
<td>pSB1AK3</td>
<td>High copy BioBrick™ vector, pMB1 origin, AmpR and KanR</td>
<td>[14]</td>
</tr>
<tr>
<td>pSB1C3</td>
<td>High copy BioBrick™ vector, pMB1 origin, CmR</td>
<td>[14]</td>
</tr>
<tr>
<td>pSB3K3</td>
<td>Medium copy BioBrick™ standard vector, p15A origin, KanR</td>
<td>[14]</td>
</tr>
<tr>
<td>pSB1A3</td>
<td>High copy BioBrick vector, pMB1 origin, AmpR</td>
<td>[14]</td>
</tr>
<tr>
<td>pET-14b</td>
<td>Ampicillin plasmid, T7 promoter, pBR322 origin</td>
<td>Novagen</td>
</tr>
<tr>
<td>pCR2.1-TOPO</td>
<td>Ampicillin plasmid, Plac promoter, pBR322 origin</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>pCMEL1</td>
<td>PhaP1, C-terminal BioFusion with HlyA signal peptide, Lac promoter(BBa_R0010), RBS(BBa_B0034), in pSB1A3</td>
<td>[15]</td>
</tr>
<tr>
<td>pCMEL2</td>
<td>phaP1, C-terminal BioFusion with HlyA signal peptide, Lac promoter (BBa_R0010), RBS (BBa_B0034), in pSB3K3</td>
<td>[15]</td>
</tr>
<tr>
<td>pCMEL3</td>
<td>phaP1, C-terminal BioFusion with HlyA signal peptide, Lac promoter (BBa_R0010), RBS (BBa_B0034), in pBHR68</td>
<td>[15]</td>
</tr>
<tr>
<td>pKF01</td>
<td>Phasin(ns) in pSB1C3</td>
<td>This study</td>
</tr>
<tr>
<td>pKF02</td>
<td>Phasin(ns) GFP in pSB1C3</td>
<td>This study</td>
</tr>
<tr>
<td>pKF03</td>
<td>Phasin(ns) GFP_B0015 in pSB1C3</td>
<td>This study</td>
</tr>
<tr>
<td>pKF04</td>
<td>Promoter + rbs_ Phasin(ns) GFP_B0015 in pSB1C3</td>
<td>This study</td>
</tr>
<tr>
<td>pKF05</td>
<td>Phasin(ns)GFP(ns) in pSB1C3</td>
<td>This study</td>
</tr>
<tr>
<td>pKF06</td>
<td>Phasin(ns)+GFP(ns)+HlyA in pSB1C3</td>
<td>This study</td>
</tr>
<tr>
<td>pKF07</td>
<td>Phasin(ns)+GFP(ns)+HlyA+B0015 in pSB1C3</td>
<td>This study</td>
</tr>
<tr>
<td>pKF08</td>
<td>Promoter + rbs_ Phasin(ns)+GFP(ns)+HlyA+B0015 in</td>
<td>This study</td>
</tr>
<tr>
<td>pSB1C3</td>
<td></td>
<td></td>
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<tr>
<td>---</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>pKF09</td>
<td>PhaC1(ns) in pSB1C3 amplified from BBa_K934001 with KFphaC1F and KFphaC1R</td>
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<tr>
<td>pKF10</td>
<td>PhaC1(ns) GFP in pSB1C3</td>
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<tr>
<td>pKF11</td>
<td>PhaC1(ns)_GFP_B0015 in pSB1C3</td>
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</tr>
<tr>
<td>pKF12</td>
<td>Promoter + rbs_PhaC1(ns)_GFP_B0015 in pSB1C3</td>
<td></td>
</tr>
<tr>
<td>pKF13</td>
<td>Promoter + rbs_PhaC1(ns)_GFP(mut)_B0015 in pSB1C3</td>
<td></td>
</tr>
<tr>
<td>pKF14</td>
<td>pKF13 in pSB3K3</td>
<td></td>
</tr>
<tr>
<td>pKF15</td>
<td>pCMEL2 +pKF14 in pSB3K3</td>
<td></td>
</tr>
<tr>
<td>pKF16</td>
<td>EcoRI/XhoI fragment from pKF15 in pBHR68</td>
<td></td>
</tr>
<tr>
<td>pKF17</td>
<td>EcoRI/XhoI fragment from pKF14 in pBHR68</td>
<td></td>
</tr>
<tr>
<td>pKF18</td>
<td>PhaC1(ns)_GFP(ns) in pSB1C3</td>
<td></td>
</tr>
<tr>
<td>pKF19</td>
<td>PhaC1(ns)_GFP(ns)_HlyA in pSB1C3</td>
<td></td>
</tr>
<tr>
<td>pKF20</td>
<td>PhaC1(ns)_GFP(ns)_HlyA_B0015 in pSB1C3</td>
<td></td>
</tr>
<tr>
<td>pKF21</td>
<td>Promoter + rbs_PhaC1(ns)_GFP(ns)_HlyA_B0015 in pSB1C3</td>
<td></td>
</tr>
<tr>
<td>pKF22</td>
<td>phaC1 in pSB1C3, amplified from BBa_K934001 in pSB1C3</td>
<td></td>
</tr>
<tr>
<td>pGFPuv</td>
<td>Green fluorescent protein (GFP), BBa_K208000 in pSB1AK3</td>
<td></td>
</tr>
<tr>
<td>pKF23</td>
<td>Green fluorescent protein (GFP) amplified from pGFPuv. Stop codon removed and XhoI site mutated, in pSB1C3</td>
<td></td>
</tr>
<tr>
<td>pKF24</td>
<td>GFP(mut)(ns)_PhaC1 in pSB1C3</td>
<td></td>
</tr>
<tr>
<td>pKF25</td>
<td>GFP(mut)(ns)_PhaC1_B0015 in pSB1C3</td>
<td></td>
</tr>
<tr>
<td>pKF26</td>
<td>In frame fusion [RFC 23] of GFPmut (pKF23) with phaC1 (pKF22). Lac promoter (BBa_R0010), RBS(BBa_B0034), and double terminator (BBa_B0015), in pSB1C3</td>
<td></td>
</tr>
<tr>
<td>pKF27</td>
<td>EcoRI/SpeI fragment pKF26 and pCMEL2 in pSB1C3</td>
<td></td>
</tr>
<tr>
<td>pKF28</td>
<td>EcoRI/PstI fragment of pKF27 in pSB1AK3</td>
<td></td>
</tr>
<tr>
<td>pKF29</td>
<td>EcoRI/PstI fragment of pKF28 in pSB3K3</td>
<td></td>
</tr>
<tr>
<td>p4MT</td>
<td>promoter (BBa_R0010), RBS(BBa_B0034), phaP1, and double terminator (BBa_B0015) in pSB1AK3</td>
<td></td>
</tr>
<tr>
<td>pKF30</td>
<td>EcoRI/SpeI fragment of pKF26 in p4MT</td>
<td></td>
</tr>
<tr>
<td>pKF31</td>
<td>EcoRI/PstI fragment of pKF30 in pSB3K3</td>
<td></td>
</tr>
<tr>
<td>pAKF01</td>
<td>EcoRI/XhoI fragment of pKF29 (+HlyA) in pBHR68.</td>
<td></td>
</tr>
<tr>
<td>pAKF02</td>
<td>EcoRI/XhoI fragment of pKF31(-HlyA) in pBHR68.</td>
<td></td>
</tr>
<tr>
<td>pDA-JAC</td>
<td>pDA-JC with PP_0763 cloned between phaJ3 and phaC2</td>
<td></td>
</tr>
<tr>
<td>pET14b_gfp_phaC_MalE</td>
<td>gfp-phaC-malE fusion in pET14b</td>
<td></td>
</tr>
<tr>
<td>pAR2</td>
<td>gfp-phaC-malE fusion from pET14b in pCR2.1</td>
<td></td>
</tr>
<tr>
<td>pARNH1</td>
<td>patgPhasin(ns)hlyAB0015 in pDA-JAC</td>
<td></td>
</tr>
<tr>
<td>pARNH2</td>
<td>KF08 in pDA-JAC</td>
<td></td>
</tr>
<tr>
<td>pB14</td>
<td>Spider silk production vector. Lac promoter +RBS (BBa_K208010), spider silk subunit (BBa_K84408), 13x spider silk subunit (BBa_K844004), 10x His-tag (BBa_K844000), and double terminator (BBa_B0015) in pSB1C3.</td>
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<tr>
<td>pB14GFP</td>
<td>Spider silk production vector with GFP fusion at C terminal. Lac promoter +RBS (BBa_K208010), spider silk subunit (BBa_K84408), 13x spider silk subunit (BBa_K844004), modified GFP with stop codons removed (BBa_K208000), 10x His-tag (BBa_K844000), and double terminator (BBa_B0015) in pSB1C3.</td>
<td></td>
</tr>
</tbody>
</table>
pB04GFP  Similar to pB14GFP but with 4x repeats of spider silk subunit  This study
pB12GFP  Similar to pB14GFP but with 12x repeats of spider silk subunit  This study
pB16GFP  Similar to pB14GFP but with 16x repeats of spider silk subunit  This study
p6x5x  tRNA plasmid pSB3K3 containing both BBa_K844012 and BBa_K844013 composite BioBrick™ parts.  This study

Note: All BioBrick™ constructs are RFC 23 compatible

---

**Table E3. Oligonucleotides used in this study**

<table>
<thead>
<tr>
<th>Name</th>
<th>Relevant characteristics</th>
<th>Reference</th>
</tr>
</thead>
</table>
| VF2          | 5'-tgccacctgaactaagaa-3'  
Fwd sequencing primer for BioBrick vectors                                               | [14]      |
| VR           | 5'-attaaccgctttgtgtaagc-3'  
Rev sequencing primer for BioBrick vectors                                                 | [14]      |
| PhaP1FOR     | 5'- gaattcgagccgcttgtcttaagaa-3'  
tagatctctacccggaacaa-3'  
Fwd primer for mutation of Xhol site from GFP                                              | [15]      |
| PhaP1REV     | 5'-ctcgagccgctgtgagccgcttgctgaagc-3'  
Reverse primer for BioBrick vectors                                                       | [15]      |
<p>| g114t        | 5'-agctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctc |</p>
<table>
<thead>
<tr>
<th>Sequence</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GFP HT to mutate stop codon from GFP Fwd primer</strong></td>
<td>5'-Ggtgtatagtctagtatattagctgtcatctag- 3'</td>
<td>This study</td>
</tr>
<tr>
<td><strong>AR5</strong></td>
<td>5'-tctggaatcgcgcgtctctagatgctgatagccagaagga- 3'</td>
<td>This study</td>
</tr>
<tr>
<td><strong>AR6</strong></td>
<td>5'-tctgatgcgcgtgcctagtagttctagatgtccagctc- 3'</td>
<td>This study</td>
</tr>
<tr>
<td><strong>NH1</strong></td>
<td>5'-Tctgatagcataaagccagaaacc- 3'</td>
<td>This study</td>
</tr>
<tr>
<td><strong>NH2</strong></td>
<td>5'-Tctgatagctttataaaacgagaaacc- 3'</td>
<td>This study</td>
</tr>
<tr>
<td><strong>NH3</strong></td>
<td>5'-Tctgatagcataaagccagaaaccctc- 3'</td>
<td>This study</td>
</tr>
<tr>
<td><strong>NH4</strong></td>
<td>5'-Tctgatagctttataaaacgagaaacc- 3'</td>
<td>This study</td>
</tr>
<tr>
<td><strong>NH9</strong></td>
<td>5'-Tctgatagcataaagccagaaaccctc- 3'</td>
<td>This study</td>
</tr>
<tr>
<td><strong>NH10</strong></td>
<td>5'-Tctgatagcataaagccagaaaccctc- 3'</td>
<td>This study</td>
</tr>
<tr>
<td><strong>CT294</strong></td>
<td>5'-tctgatagcataaagccagaaaccctc- 3'</td>
<td>This study</td>
</tr>
<tr>
<td><strong>CT295</strong></td>
<td>5'-tctgatagcataaagccagaaaccctc- 3'</td>
<td>This study</td>
</tr>
<tr>
<td><strong>CT296</strong></td>
<td>5'-tctgatagcataaagccagaaaccctc- 3'</td>
<td>This study</td>
</tr>
<tr>
<td><strong>CT297</strong></td>
<td>5'-tctgatagcataaagccagaaaccctc- 3'</td>
<td>This study</td>
</tr>
<tr>
<td><strong>CT298</strong></td>
<td>5'-tctgatagcataaagccagaaaccctc- 3'</td>
<td>This study</td>
</tr>
<tr>
<td><strong>CT299</strong></td>
<td>5'-tctgatagcataaagccagaaaccctc- 3'</td>
<td>This study</td>
</tr>
<tr>
<td><strong>CT300</strong></td>
<td>5'-tctgatagcataaagccagaaaccctc- 3'</td>
<td>This study</td>
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<td><strong>CT301</strong></td>
<td>5'-tctgatagcataaagccagaaaccctc- 3'</td>
<td>This study</td>
</tr>
<tr>
<td><strong>CT302</strong></td>
<td>5'-tctgatagcataaagccagaaaccctc- 3'</td>
<td>This study</td>
</tr>
<tr>
<td>Reference</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>-----------</td>
<td>-------------</td>
<td></td>
</tr>
</tbody>
</table>


CURRICULUM VITAE

Asif Rahman
asif.rahman@aggiemail.usu.edu

Education

**Utah State University**, Logan, UT [August 2014]
PhD in Biological Engineering
Dissertation: Sustainable production of novel biomaterials from *Escherichia coli*

**University of Auckland**, Auckland, New Zealand [2010]
Masters, Chemical and Materials Engineering (First Class Honors)
Thesis: Strength testing of microencapsulated phase change materials

**University of Wisconsin-Madison**, Madison, WI [2009]
Bachelors, Chemical and Biological Engineering

Teaching

Teaching Assistant [Jan-May ’13] [Jan-May ’14]
**Biological Engineering 5630/6630, Synthetic Biological Engineering**, Department of Biological Engineering, Utah State University, Logan, Utah.

Grader [Aug-Dec ’11]
**Biological Engineering 5810/6810, Biochemical Engineering**, Department of Biological Engineering, Utah State University, Logan, Utah.

Teaching and Laboratory Assistant [March-May ’10]
**Chemical and Materials Engineering 211, Introduction to Process Engineering**, Department of Chemical and Materials Engineering, University of Auckland, New Zealand.
Ran weekly discussion sections and bi-weekly three hour laboratory sessions (second year Chemical Engineering class).

Chemistry Tutor [Jan-July ’10]
Teaching high school level chemistry (NCEA level 1 & 2), Auckland, New Zealand.

Teaching and Laboratory Assistant [Sep-Nov ’09]
**Engineering Biotechnology 464 /Food Science 704**, Department of Chemical and Materials Engineering, University of Auckland, New Zealand.
Ran a week long Fermentation laboratory for a total of 25 students. Main focus was to teach students basic laboratory biotechnology skills, safety and support the students before and after the laboratory period. Also graded lab reports.
Research experience

Utah State University, Biological Engineering [Aug ’10-Present]

Graduate Researcher with Prof. Ronald C. Sims and Dr. Charles D. Miller
PhD dissertation work focused on sustainable production of polyhydroxyalkanoates from *E. coli* using synthetic biology and production of other useful biomaterials such as synthetic spider silk.
Skills/techniques used: molecular biology/cloning, bioreactor, protein purification, SDS-PAGE/Western Blot, NMR, GC, particle size analysis, DSC, and spectrophotometry.

University of Auckland, Chemical & Materials Engineering [July ’09-July ’10]

Graduate Researcher with Prof. Mohammed M. Farid and Dr. Michelle E. Dickinson
Project aim was to produce microcapsules that contain a phase change material (PCM). The PCM has desirable thermal properties that can be used for in thermal building insulation for energy conservation. After producing microcapsules, the mechanical properties were then tested using a Nanoindentator.

University of Wisconsin-Madison, Chemical Engineering [Jan ’08-May ’09]

Undergraduate Researcher with Dr. Brian F. Pfleger
Worked part-time during three school semesters for degree credit and worked full time during summer months (May-September, 2008) and received support from the Great Lakes Bioenergy Research Center in Madison, Wisconsin, USA.

R.M. Bock Laboratories at University of Wisconsin-Madison [May ’06-Dec ’08]

Laboratory Assistant with Prof. Ching Kung
Media preparation for bacteria and yeast cultures, responsible for lab inventory and supplies.

Publications

Accepted manuscripts


**Manuscripts in preparation**


**Patents**


Presentations

25 total (11 international, 2 invited*)

Underlined indicates primary presenter

1. *Asif Rahman, Neal Hengge, Ryan Putman, Charles Miller, and Ronald Sims. iGEM and IBE from a Students’ perspective. American Society of Agricultural and Biological Engineers. Montreal, Canada, 14 July, 2014. (Podium)
8. Ashik Sathish, Joshua Ellis, Asif Rahman, Reese Thompson, Issa Hamud, Charles Miller, and Ronald Sims. Utilization of wastewater derived algal biomass for the production biofuels and bioproducts via a wet lipid extraction procedure. International BIOMASS Conference & Expo, Minneapolis, Minnesota, USA, 8-10 April, 2013. (Poster)


Awards and Scholarships

- Outstanding Engineering Graduate Scholar-Student, April, 2014.
- Grand Prize for Poster Presentation, Institute of Biological Engineering, March, 2014.
- Utah State University, International Student of the Year 2012-2013, April, 2013.
- 1st Place for Poster Presentation, Institute of Biological Engineering, March, 2013.
- Graduate student professional conference travel award Utah State University, 2012, 2013, & 2014.
- Utah State Department of Biological Engineering Award for Outstanding Achievement in the Field of Excellence, December, 2012.
- 1st Place for Poster Presentation Intermountain Graduate Research Symposium. Logan, Utah, USA, 31 March, 2011.
- Utah Science Technology and Research (USTAR) PhD fellowship August 2010-present.

Professional Affiliations and Service

Reviewer

- Energy, reviewer 2013
- Open Journal of Chemical Engineering and Science, editorial board 2013-2015
- Chemeca conference, proceedings reviewer, 2012

Membership

- Member, American Society of Microbiology
- Member, Institute of Biological Engineering
- Member, American Institute of Chemical Engineers
- Member, American Society of Agricultural and Biological Engineers

Professional Organizations

Elected to serve as Graduate student council representative for the Institute of Biological Engineering (IBE) 2013 & 2014.

Conference Activities

- Student social co-chair. Institute of Biological Engineering conference, Lexington, Kentucky, USA, 7-9 March, 2014.
• Student Conference Chair and abstract reviewer. Institute of Biological Engineering (IBE) Western Regional Conference, Logan, Utah, USA 2012 & 2011.
• Bioprocess Session Chair and abstract reviewer. Institute of Biological Engineering (IBE) Western Regional Conference, Logan, Utah, USA 2010.

Mentoring

• Graduate Advisor, Utah State University iGEM team (International biotechnology competition) 2011, 2012, 2013, and 2014
  iGEM projects:
  2013: AMPed Up E. coli - Won gold medal
  2012: Arachnicoli: Production and Purification of Spider Silk Proteins in Escherichia coli
  -Won gold medal, Best New BioBrick Device-Engineered, Regional finalist-second runner up, Best Manufacturing Project overall-world championship
  2011: CyanoBricks: Expression testing and Bioproduct development -Won gold medal

• I have directly supervised undergraduate research projects and capstone projects at Utah State University in the Department of Biological Engineering. Directly mentored over 24 students.

Other activities

• Engineering State, Utah State University (1 week undergraduate recruiting for faculty of engineering), summer 2011, 2013, 2013, and 2014.
• Discover Biological Engineering, Utah State University (1 week undergraduate recruiting for department), summer 2011, 2012, 2013, and 2014.
• USA Science and Engineering Festival, Institute of Biological Engineering Booth, Washington DC, 2014.