INVESTIGATIONS OF POLYHYDROXYALKANOATE SECRETION AND PRODUCTION USING SUSTAINABLE CARBON SOURCES

by

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ABSTRACT

Investigations of Polyhydroxyalkanoate Secretion and Production Using Sustainable Carbon Sources

by

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Utah State University, 2018

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Polyhydroxyalkanoates (PHAs) are promising bioplastic polymers with the potential to replace petroleum-derived plastics in diverse applications. Production costs for PHAs are still very high, however, largely due to the cost of carbon feedstocks for the bacteria that produce the polymers and the cost of extracting the polymers after production. Waste streams that could be diverted to PHA production as inexpensive carbon sources include food wastes and methanol. Secretion-based recovery of PHA granules was studied in the methylotrophic bacteria Methylobacterium to decrease the cost of downstream processing of PHAs, while using methanol as the sole carbon source. The hemolysin type I secretion pathway from Escherichia coli was transformed into a novel isolate of Methylobacterium. A synthetic biology approach was used to create a phasin fusion protein that binds to the surface of PHA granules and the hemolysin
secretion signal peptide. This genetic construct was used in conjunction with the secretion system with the intent of inducing the cell to secrete PHA into the extracellular media. As currently constituted, however, the secretion system made no significant difference in the amount of PHA produced and secreted by the *Methylobacterium* isolate. The information gathered in this work can be further optimized and applied to other methylotrophic bacteria to reduce costs in PHA manufacturing systems.
PUBLIC ABSTRACT

Investigations of Polyhydroxyalkanoate Secretion and Production
Using Sustainable Carbon Sources

Chad L. Nielsen

Polyhydroxyalkanoates (PHAs) are a type of biologically-produced plastic known for their biocompatibility and biodegradability. They have the potential to replace petroleum-based plastics as an environmentally-friendly alternative. This is beneficial because the release of plastics into environments such as the ocean and the buildup of plastics in landfills are major concerns facing society today. Currently, however, PHAs are significantly more expensive than their petroleum-based counterparts. This is largely due to the cost of carbon sources and of extracting the bioplastics from bacteria. The goal of these studies was to examine replacing traditional carbon sources used in PHA production like sugar and oils with sustainable carbon sources and to improve extraction procedures by inducing secretion of PHAs in bacteria.

A few sustainable carbon sources were examined for use in PHA production. First, studies focused on the conversion of food waste into PHAs were reviewed. It was shown that utilizing food wastes as carbon sources may be a viable approach to producing PHAs. A second carbon source examined was methanol. A novel isolate of *Methylobacterium* that demonstrated the ability to produce PHAs from methanol was identified. A system of secreting PHAs that was constructed using synthetic biological engineering approach was introduced to this isolate. This secretion system was not shown to improve extraction of PHAs in *Methylobacterium* in its current form.
ACKNOWLEDGMENTS

This research would not have been possible without the help of many individuals. First, I am grateful to my wife, Elise, for her continued support and encouragement. I am also grateful to my parents for their interest and support as I have pursued a graduate degree in biological engineering. My gratitude goes to Dr. Charles Miller for his guidance, mentoring, and patience throughout this process. I would also like to thank Dr. Asif Rahman for his mentoring, support, advice, and contributions that helped make this research possible. My gratitude is also due to Dr. Ronald Sims for taking me under his wing as a research assistant many years ago, and continuing to provide support throughout my time at Utah State University. This research is also a result of contributions and input made by Dr. Asad-ur-Rehman, Dr. Marie K. Walsh, and Dr. Randy Lewis. In addition, this research was made possible by the Research Catalyst grant program of the Utah State University Office of Research and Graduate Studies. Lastly, I would like to thank my fellow students who have worked with me and kept me company as I have worked and studied in the lab, including Alaric Siddoway, Anna Doloman, Gabby Nielson, Alex Beeston, AJ Walters, James VanderMeyden, Emily Jesgarz, Sierra Julander, and Timothy Kerns. Their friendship and support have made this process more enjoyable than it otherwise would have been.

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<td>ABC transporter</td>
<td>ATP-binding cassette transporter</td>
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<td>AFEX</td>
<td>Ammonia fiber expansion</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>BTU</td>
<td>British thermal units</td>
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<td>cp8</td>
<td>Constitutive promoter 8</td>
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<td>DCW</td>
<td>Dry cell weight</td>
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<tr>
<td>EPA</td>
<td>Environmental Protection Agency</td>
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<td>FBP</td>
<td>Fructose 1,6-bisphosphate</td>
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<td>FF</td>
<td>Feast-famine</td>
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<td>FISH</td>
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<td>FMP</td>
<td>Fructose-6-phosphate molecules</td>
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<td>FP</td>
<td>Flavoproteins</td>
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<td>GAP</td>
<td>Granule-associated protein</td>
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<td>GC</td>
<td>Gas chromatography</td>
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<td>GFP</td>
<td>Green fluorescence protein</td>
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<td>Hly</td>
<td>Hemolysin</td>
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<td>HPH</td>
<td>High pressure homogenization</td>
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<td>HPR</td>
<td>Hydroxypyruvate reducates</td>
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<tr>
<td>iGEM</td>
<td>International Genetically Engineered Machine</td>
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<td>JGI</td>
<td>Joint Genome Institute</td>
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<tr>
<td>KDPG</td>
<td>2-keto 3-deoxy 6-phosphogluconate</td>
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<td>mcl</td>
<td>Medium chain length</td>
</tr>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>MCL</td>
<td>Malyl coenzyme A lyase</td>
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<td>MFP</td>
<td>Membrane fusion protein</td>
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<td>MMC</td>
<td>Mixed microbial culture</td>
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<td>MMO</td>
<td>Methane monooxygenase</td>
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<td>MTK</td>
<td>Malate thiokinase</td>
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<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PEP</td>
<td>Phosphoenolpyruvate</td>
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<td>PhaP</td>
<td>Phasin proteins</td>
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<td>PHB</td>
<td>Polyhydroxybutyrate</td>
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<td>PHBV</td>
<td>Polyhydroxybutyrate-co-hydroxyvalerate</td>
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<td>PHD</td>
<td>Polyhydroxydecanoate</td>
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<tr>
<td>PHH</td>
<td>Polyhydroxyhexanoate</td>
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<td>PHO</td>
<td>Polyhydroxyoctanoate</td>
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<tr>
<td>PHV</td>
<td>Polyhydroxyvalerate</td>
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<tr>
<td>pMMO</td>
<td>Particulate methane monooxygenase</td>
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<td>RABR</td>
<td>Rotating algal biofilm reactor</td>
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<td>REGWQ</td>
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<td>RFU</td>
<td>Relative fluorescent units</td>
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<td>RND</td>
<td>Resistance-nodulation-division</td>
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<td>Abbreviation</td>
<td>Description</td>
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<td>--------------------------------------------------</td>
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<tr>
<td>RuMP</td>
<td>Ribulose monophosphate</td>
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<td>SAS</td>
<td>Statistical Analysis System</td>
</tr>
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<td>SBR</td>
<td>Sequencing batch reactor</td>
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<tr>
<td>SC</td>
<td>Supercritical</td>
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<td>scCO₂</td>
<td>Supercritical fluid extraction with CO₂</td>
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<td>SCG</td>
<td>Spent coffee grounds</td>
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<tr>
<td>scl</td>
<td>Short chain length</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<td>Sec</td>
<td>General secretory</td>
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<td>SEM</td>
<td>Scanning electron microscopy</td>
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<td>sfGFP</td>
<td>Strongly fluorescent protein</td>
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<td>sMMO</td>
<td>Soluble methane monooxygenase</td>
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<td>STHM</td>
<td>Serine hydroxymethyl transferase</td>
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<td>SWBEC</td>
<td>Sustainable Waste-to-Bioproducts Engineering Center</td>
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<td>T1SS</td>
<td>Type I Secretion System</td>
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<td>USTAR</td>
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<td>VFA</td>
<td>Volatile fatty acids</td>
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CHAPTER 1
INTRODUCTION

1.1 Overview

Petroleum-based synthetic plastics have a wide range of applications in industrial and consumer products, and increasingly affect every sphere of human life. As a result of this—according to an United States Environmental Protection Agency study in 2011—there was an increase of non-biodegradable plastics accumulation in municipal solid waste systems from 0.5% to 12.4% over the period from 1960 to 2010 [1]. This increase in accumulation is problematic because the amount of waste being generated has made acquiring sufficient area for landfill sites difficult, especially in urban areas. Synthetic plastics are recalcitrant to microbial degradation and remain in landfill sites semi-permanently, shortening the life of landfill sites. In addition, once in the landfill, harmful chemicals that have been added to the plastics leach into the soil and can spread into groundwater [2].

Renewable, biodegradable plastics are of great interest as an environmentally-friendly replacement for traditional fossil fuel-derived plastics [3]. Polyhydroxyalkanoates (PHAs) are one type of bioplastics that have the potential to replace traditional plastics in many applications. PHAs are intracellular energy- and carbon-storage polyesters produced by a variety of microorganisms. Once PHAs are extracted from bacterial cells, they show material properties that are similar to synthetic polymers such as polypropylene [4]. They are biodegradable [5,6] and biocompatible [7], breaking down into non-toxic...
products—primarily carbon dioxide and water in aerobic conditions or methane in anaerobic conditions [8]. PHAs are known to have melting temperatures between 50-180°C and crystallinity of 30-70% [9]. As such, some potential applications of PHAs could include single-use commercial packaging (food packaging, hygiene and cosmetic product containers, etc.) [10,11], agricultural purposes (single-use plastic coverings and carriers for slow-release of herbicide and insecticide) [8], and medical uses (matrixes for tissue repair and artificial organ construction, implants, capsules or coatings for slow-release of drugs, and nutritional and therapeutic composites) [12–14]. Although it is hoped that PHAs will be able to replace petrochemical plastics in these areas as production processes improve, the cost of production is currently prohibitive to all applications except higher-value medical applications [3,15,16].

There are currently two significant bottlenecks that make PHA production expensive: the cost of downstream processing and the cost of carbon feedstocks for microorganisms that produce PHAs. The development of inexpensive and scalable processes that allow recovery of intracellular PHAs is necessary [17]. It is estimated that extraction and purification costs represent as much as 50% of the total process expense [18]. Current processes are invasive and involve lysing the cellular membrane prior to PHA isolation using mechanical or biological treatments and the use of caustic chemicals. In addition to being costly, these processes also account for >90% of the adverse environmental impact of PHA production [19]. Several alternative methods have been developed for PHA
recovery, but none of them have resulted in significant economic improvements [20].

A promising alternative is using synthetic biology to induce the microorganisms to secrete the bioplastic, which would eliminate the need for cell disruption by mechanical or chemical means. One such system has previously been successfully demonstrated in *Escherichia coli* using the type I secretion system to translocate PHAs from the cytoplasm to the extracellular medium [21]. Phasin proteins that bind tightly to PHAs were tagged for translocation via the Hemolysin (HlyA) pathway. The phasins bound tightly to and carried the PHA granules with them as they were secreted from the cell. Using this system, 36% of the total PHA produced in the secreting strain was collected in the secreted fraction and 64% remained in the bacterial cell after 48 hours. In addition, total productivity was increased relative to the non-secreting strain [21].

The secretion system was constructed using standardized BioBrick assembly. BioBricks are a synthetic biological engineering concept of standardized biological “parts.” Essentially, they are characterized DNA sequences that can be synthesized and pieced together using restriction enzyme sites found in the regions surrounding the gene of interest in standardized DNA plasmid vectors [22]. Using standardized BioBrick parts and assembly methods provides a flexible and powerful platform for constructing and testing options for type I secretion of PHAs in bacteria.

The cost of feedstock for the microorganisms producing PHAs is the other primary factor in the cost of PHA production [17,19]. In fact, techno-economic
analysis estimates that carbon feedstock accounts for as much as 40% of the product cost in pure culture production [23]. Many waste streams have been studied for use as carbon sources with reduced costs, including wastewater microalgae [24,25], agricultural waste [26], syngas [27], traditional plastic waste [28], and others [29]. Currently, however, most of the feedstock sources used to produce PHAs are plant-derived sugars and oils. The disadvantage of these sources is that they tend to be expensive and they are tied to our food supply.

Food wastes can be used as one type of inexpensive carbon sources to produce PHAs. In Europe, it is estimated that approximately 88 million tons of food is wasted per year [30], while in the United States, the Environmental Protection Agency (EPA) estimated that in 2013 approximately 37 million tons of food ended up in municipal solid waste system [31]. Several food wastes have been tested for PHA production, including dairy whey, starches, oils, lignocellulosic materials, legume wastes, and sugar wastes. Mixed microbial cultures have also been used to digest complex food wastes such as food scraps, spentwashes and wastewaters, and molasses into PHAs or PHA precursors. Food wastage provides another opportunity to valorize wastes by utilizing them as carbon sources for PHA production.

Another promising alternative carbon source is methanol. It is an inexpensive and widely-available carbon source that can be utilized as a carbon source by methylotrophic bacteria, many of which are known to produce PHAs [23,32]. Species of *Methylobacterium* are among the best-characterized examples of methylotrophic bacteria that naturally produce PHAs. Its PHB production is
reported to be as high as 57% of cell dry weight in pure culture with a yield of up to 0.59 g PHB/g Methanol [33–35]. Since sequenced genomes indicates that *Methylobacterium* generally have at least one phasin protein gene and precursors for TolC (an outer membrane protein used in Type I secretion), it is an ideal organism for incorporation of the PHA secretion system.

Methane is another potential sustainable carbon source. The bacteria strain *Methylosinus trichosporium* OB3b is an obligate methanotroph that produces PHA and has many of the same advantages as *Methylobacterium* [23,36]. The ability of these bacteria to metabolize methane gas would allow the production of PHAs to be connected to a proposed wastewater treatment biorefinery (see Figure 1.1). Initially, strain OB3b was the target organism of this study, however, difficulties in culturing the bacteria caused the focus to shift to *Methylobacterium*. The two species of bacteria are close enough in relation, however, that is likely that a secretion system that is functional in *Methylobacterium* would also be functional in *Methylosinus*. Thus, research conducted in the *Methylobacterium* provides foundational information that can be applied to *Methylosinus trichosporium* and other related methanotrophs, linking PHB production to the biological engineering concept of a biorefinery.
1.2 Hypotheses

This research is being conducted to obtain foundational information for secretion-based recovery of PHAs produced by methylotrophic bacteria using synthetic biological methods. The hypothesis of the study is that by using a trial-and-error synthetic biological engineering approach with phasin proteins fused to a HlyA signaling peptide will lead to an optimized system for membrane translocation of PHAs. It is believed that producing PHAs using a methylotroph that secretes the bioplastics will provide a more stable and economically-viable method for PHA production by reducing downstream processing complexity and cost.
1.3 Objectives

The overall objective of this study is to investigate whether a method of recovering PHA bioplastics via secretion through a phasin protein/PHA granule interaction can be successfully applied to methylotrophic bacteria. More specific project objectives include:

1. Analyze the potential of utilizing food waste as a carbon source for PHA production
2. Characterize a novel isolate of *Methylobacterium*
3. Design and construct a BioBrick library of phasin proteins, signal peptides, hemolysin transport proteins, and GFP parts, as well as composite devices for investigating secretion in *Methylobacterium*
4. Monitor the translocation of PHAs in *Methylobacterium* using gas chromatography (GC)

The completion of the outlined objectives would contribute novel information to PHA research. It would provide a foundation for minimizing the obstacles to more widespread use of biodegradable bioplastics, which are currently not an economically viable alternative to synthetic plastics due to feedstock costs and separation/purification costs.

1.4 Thesis Outline

Chapter II is a comprehensive overview of using food wastes as inexpensive carbon sources for PHA production. This chapter was previously published as a review entitled “Food Waste Conversion to Bacterial Polyhydroxyalkanoates” in the journal Microbial Biotechnology [37]. Chapter III provides a comprehensive review of PHA production in methylotrophic bacteria.
Chapter IV describes phasin and PHA production in *Methylobacterium*, as well as the genetic systems constructed for bioplastic translocation. Chapter V summarizes general conclusions for the entire study, while Chapter VI provides insight into future directions in PHA secretion and production from inexpensive carbon sources.

References

CHAPTER 2
MICROBIAL BIOTECHNOLOGY PAPER
REVIEW

FOOD WASTE CONVERSION TO MICROBIAL
POLYHYDROXYALKANOATES

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2.1 Summary

Polyhydroxyalkanoates (PHAs) are biopolymers with desirable material properties similar to petrochemically-derived plastics. PHAs are naturally produced by a wide range of microorganisms as a carbon storage mechanism and can accumulate to significantly high levels. PHAs can potentially be an environmentally friendly alternative...
to their petroleum counterparts because they can be easily degraded, potentially reducing the burden on municipal waste systems. Nevertheless, widespread use of PHAs is not currently realistic due to a variety of factors. One of the major constraints of large scale PHA production is the cost of carbon substrate for PHA-producing microbes. The cost of production could potentially be reduced with the use of waste carbon from food-related processes. Food wastage is a global issue and therefore harbors immense potential to create valuable bioproducts. This article’s main focus is to examine the state of the art of converting food-derived waste into carbon substrates for microbial metabolism and subsequent conversion into PHAs.

**Keywords**

Food waste, polyhydroxyalkanoates, microbes, bioplastics, polyhydroxybutyrate

**2.2 Introduction**

Microbially produced polyhydroxyalkanoates (PHAs) are among the most well-studied biologically derived plastics. This is due to their suitability as potential replacements for petrochemically-derived plastics because they are biodegradable and biocompatible [1,2]. PHAs are carbon-based polymers naturally created to store excess carbon sources and maintain energy balances [3]. Under certain conditions, such as nitrogen, phosphorus, or oxygen limitation in the presence of excess carbon sources, some microorganisms accumulate high concentrations of PHAs [4,5].

There are over 155 confirmed unique PHA monomer subunits, which demonstrates the diversity of potential PHA polymers that can be produced using microorganisms [6]. The diversity of available monomers could lead to many different applications, as each resulting polymer has different material properties. For example, the
melting temperatures of PHAs range from 50 °C to 180 °C and crystallinities of PHAs range from 30-70% [7]. Polyhydroxybutyrate (PHB), a short chain length (scl) PHA, is by far the most well-studied PHA polymer and is able to accumulate to high concentrations in cells growing on a variety of carbon substrates [8]. For example, *Cupriavidus necator* has been recorded to have as high as 74% of its cell weight as PHB and recombinant *Escherichia coli* have been recorded to accumulate up to 85% of their dry cell weight as PHB [9,10]. Some potential applications of PHAs could include: commercial packaging [11,12], agricultural purposes [13], and medical uses [14–16]. Although it is hoped that PHAs will be able to replace petrochemical plastics in these areas as production processes improve, the cost of production is currently prohibitive to all applications except higher-value medical uses [17–19].

While the properties of PHAs seem suitable as potential petrochemical plastic replacements, there are still bottlenecks for scaling up microbial production systems. One of the major bottlenecks is the cost of carbon substrates, which have been estimated to be 28-50% of the total production process [20–22]. There are a number of complex waste streams that can potentially act as carbon substrates for microbial PHA manufacture, such as waste streams from biodiesel production [23,24], municipal wastewater [25,26], agricultural waste [27], syngas production [28], traditional plastic waste [29], and others [30]. Food waste is a prime candidate for an inexpensive carbon source, due to its wide availability and the potential to solve a significant waste problem when used to produce PHAs.

Food wastage is a global problem and occurs at different stages of food production systems, starting from the harvesting of food to storage, packaging, and end of
life [31]. In Europe, it is estimated that approximately 88 million tons of food is wasted and of this, 57 million tons is from households and food service [32]. In the United States, the Environmental Protection Agency (EPA) estimated that in 2013 approximately 37 million tons of food ended up in municipal solid waste system, which was approximately 14% of all waste in the United States [33]. Another factor that is coupled to food waste is the energy that is lost in producing, processing, and transporting the waste. This energy has been reported to be upwards of 2000 trillion British thermal units (BTU) in the United States, equivalent to 2.11x10^{12} megajoules (MJ) [34]. If an alternative means of transforming food waste into value-added products are developed, then energy is essentially being transformed into useful products. Food waste conversion to tangible bioproducts has gathered plenty of attention, with systems being developed to produce a wide range of value-added products such as biofuels, materials, and a variety of additional feedstock chemicals [35–37].

The main objective of this review is to summarize the current state of PHA production from food waste using microbes, as depicted in Figure 2.1. More specifically, the purpose of this investigation is to study systems that convert a variety of food wastes into microbe-derived PHAs. Some considerations taken into account were as follows: food waste pre-treatment steps, scalability, bioreactor design, microorganisms used, and final PHA polymer produced.
Figure 2.1. Conversion of food waste into PHAs using PHA-producing microbes. Conversion of food waste often requires a pre-treatment step where complex food waste is broken down into subcomponents. PHA-producing microbes can then metabolize the carbon substrate and accumulate the biopolymer PHA.

2.3 PHAs from food waste using pure cultures

There are many different systems that have been proposed to convert food waste into PHAs as there are many different waste streams generated by food production, processing, and use. Each food source has its own complexities and requires different pretreatments, bacterial strains, culturing conditions, and downstream processing. Often, the organics associated with food wastes are complex compounds that cannot be directly used by PHA-producing organisms [4]. In these cases, a pretreatment or processing method is necessary to convert the complex molecules found in food waste into PHA precursors. Precursors include simple sugars like glucose or lactose and fatty acids like
acetic or propionic acids. Many of the simpler food wastes are hydrolyzed to convert the food waste into suitable precursor molecules and then fed directly to a pure culture of an appropriate microorganism. Whey, starch, oils, lignocellulosic materials, legume, and sugar wastes each have methods proposed to create PHAs.

### 2.3.1 PHAs from whey

One of the food wastes of interest is dairy whey. Whey is a by-product of the cheese-making process that consists of lactose, proteins, fats, water-soluble vitamins, mineral salts, and other essential nutrients for microbial growth. Although whey may be used as a source for producing lactose, casein, and protein powder, it is estimated that approximately 50% of whey is still disposed of in wastewater treatment plants or used in animal feed [38]. One study indicated that $1.15 \times 10^8$ to $1.60 \times 10^8$ tons of whey were produced worldwide, surpassing the requirements for whey powder production [39]. When whey is used to produce proteins like lactoferrin or lactoferricin, lactose-rich whey retentate remains as a waste material that must be disposed. Furthermore, acid whey is a by-product of cottage cheese, cream cheese, and Greek yogurt manufacturing. While acid whey can be used as animal feed, it is difficult to process into traditional whey protein concentrates due to the high acid content [38,40–42]. Disposal of whey is currently a notable problem facing the dairy industry, making it a potentially inexpensive carbon source for PHA production [43].

In addition to being a low cost carbon source, whey has the advantage of not requiring extensive pretreatment for use in fermentation via hydrolysis using enzymes or acid methods [30]. A life cycle assessment demonstrated that optimized production of
PHAs from whey has a comparable ecological footprint to that of producing petroleum-based plastics and is superior to producing whey powder [39]. This life cycle assessment was based producing PHAs using the archaeal production strain *Haloferax mediterranei* DSM 1411. Producing whey powder was inferior because it was a low market value product that used high amounts of energy to concentrate whey by evaporation. The main limitations on PHA production were found to be energy requirement for the fermentation process and a low amount of PHA output per kg whey input.

Most studies that used whey to produce PHAs have involved recombinant *Escherichia coli*, which will be described in a subsequent section. *E. coli* was selected because many of the traditional PHA-producing microorganisms cannot directly metabolize whey. Studies have also examined using organisms such as the halophile archaeon *H. mediterranei* [44,45], an unidentified highly osmophilic organism [46]; *Thermus thermophiles* HB8 [47]; and *Cupriavidus necator* DSM 545 transformed to include the *lacZ*, *lacI* and *lacO* genes of *E. coli* (becoming *C. necator* mRePT) [48] (see Table 2.1). Particularly notable is the fact that *H. mediterranei* produces the copolymer poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), while *T. thermophiles* HB8 produced a unique combination of 3-hydroxyvalerate (3HV) with medium-chain length (mcl) PHAs. Both of these polymer blends have improved ductility compared to PHB, making them preferable for industrial use [7,8]. Apart from *C. necator* mRePT, these organisms are capable of utilizing whey to produce PHAs with more desirable properties than PHB.
2.3.2 PHAs from starch

Starch is another carbon source derived from food wastes that has been studied for production of PHAs. Starch is a glucose polymer produced by plants such as rice, wheat, potatoes, maize, and cassava. Although starch is readily consumed by humans, there are some significant starchy waste streams from food production that can be used by PHA-producing microorganisms. In the work of Poomipuk et al. [49], Cupriavidus sp. KUU38 isolated from cassava starch wastewater was used to produce PHAs from cassava starch hydrolysate. This study demonstrated that under optimal conditions and nitrogen starvation, Cupriavidus sp. could produce a moderately high biomass concentration of 5.97 g/L, with a PHA content of 61.6% (Table 2.1). A study by Kim avoided using expensive enzymes to hydrolyze starch by using Azotobacter crorococcus, a type of bacteria that can digest starch directly [50]. In this study, 54 g/L dry cell weight with 46% PHB was obtained with oxygen limitation (Table 2.1). These studies indicate that high concentrations of cells containing PHAs are possible on starchy food wastes, even with minimal pretreatments in the case of the Kim study.

2.3.3 PHAs from waste oil

Waste oils from both household and industrial applications are potential carbon sources for producing PHAs. These oils generally require no pretreatment and may be added directly to media as a carbon substrate. A study by Taniguchi, et al. investigated the use of Cupriavidus necator H16 to convert waste oils and tallow to PHAs [51]. The highest amount of PHA produced in this study came from using palm oil and lard as carbon sources, achieving a dry cell weight of 6.8 g/L and a PHB accumulation of 83%.
Another notable finding is that when tallow was used as a carbon source, the copolymer PHBV was produced instead of pure PHB (Table 2.1).

Obruca and colleagues also tested waste frying rapeseed oil, waste frying palm oil, and waste frying sunflower oil as carbon sources for PHA production via *C. necator* H16. They demonstrated dry cell weights (g/L) and PHB contents (%) of 10.8 and 67.9%, 11.9 and 58.0%, and 10.8 and 52.4%, respectively for the different oil types [52]. Chaudhry *et al.* used corn oil and found that a *Pseudomonas* strain could achieve a dry cell weight of 12.53 g/L with a mcl-PHA content of 35.63% (Table 2.1) [53]. Although lower quantities of PHA were produced by the *Pseudomonas* species used by the Chaudhry group when compared to the *C. necator* H16 used by the Obruca group, the mcl-PHA produced by the *Pseudomonas* strain when it was fed corn oil is more desirable than PHB. These studies indicate that using waste oils as a carbon source allow cells to produce high concentrations of PHAs relative to dry cell weight in low titers of cells.

### 2.3.4 PHAs from spent coffee grounds

An alternative oil waste from food is spent coffee grounds (SCG) oil. Spent coffee grounds are produced during coffee processing and consumption. Approximately 9-15% of the grounds is oil that can be extracted for use [54]. The remaining portion of the spent coffee grounds is primarily lignocellulosic materials that can be combusted for heat or hydrolyzed and converted into PHAs by *Burkholderia cepacia* [55]. Obruca, Petrik, *et al.* directly compared use of SCG oil to other waste oils in *C. necator* H16 and found that the SCG oil was superior for PHB production [52]. In a shake flask experiment, the SCG oil produced a dry cell weight of 14.2 g/L with a PHB content of 70.3% compared to the
values for the waste oils discussed previously (Table 2.1). When scaled up, the SCG oil achieved an impressive dry cell weight of 55 g/L with a PHB concentration of 89.1% in fed-batch mode (Table 2.1). The main difficulty encountered is that the SCG oil is a natural foaming agent, however, other plant oils, such as waste frying oils, can be added to serve as both carbon sources and as antifoaming agents.

One study by Cruz et al. used supercritical fluid extraction with CO₂ (scCO₂) to extract the SCG oil, then fed it directly to C. necator DSM 428 in fed-batch mode [56]. The culture reached a dry cell weight of 10.7 g/L with a PHB content of 78.4% (Table 2.1). In the same study, batch mode operation was also used to produce PHB from spent coffee ground oil, but this approach produced lower amounts of PHB in comparison with the fed-batch mode. The maximum biomass accumulation observed in batch mode was up to 55% (w/w) of PHB, which yielded a polymer concentration of 6 g/L, in comparison with a polymer concentration of 13.1 g/L observed in the fed-batch mode. The main difference between the study by the Cruz group and the study by the Obruca group was that the Cruz group used an extraction method that avoided the use of hazardous organic solvents like n-hexane. As such, the Cruz process may be superior for mass production processes despite achieving lower dry cell weights. Both studies indicate that spent coffee ground oils are a carbon source with great potential for PHA production.

2.3.5 Lignocellulosic waste conversion to PHAs

Lignocellulosic materials are tough plant-based materials that are made of cellulose, pectin, hemicellulose, and lignin. Examples of this type of waste from food industry include bagasse, rice straw, wheat straw, and bran. Waste streams of
lignocellulosic compounds generally require hydrolysis to convert them into fermentable sugars, then detoxification to remove inhibitory compounds produced during hydrolysis, as reviewed in Obruca et al., 2015 [21]. A variety of lignocellulosic materials have been investigated for PHA production, including oil palm empty fruit bunch [57], wheat and rice straw [58,59], wheat bran [60], sugarcane bagasse [61], and tequila bagasse [62] (see Table 2.1). Despite pretreatments, the lignocellulosic materials often resulted in low levels of cell growth. One of the more promising lignocellulosic processes, however, was investigated by Cesário et al. [58]. In this study, an Ammonia Fiber Expansion (AFEX) process was used as pretreatment followed by an enzymatic hydrolysis of the cellulose and hemicellulose fractions of ground wheat straw to produce glucose, xylose, and arabinose. The hydrolysate was fed to Burkholderia sacchari DSM 17165 in a fed-batch fermentation process. A biomass concentration of 146 g/L with a PHA concentration of 72% was achieved using this method (Table 2.1). While lignocellulosic materials generally require extensive pretreatment, they do offer some potential as carbon substrates.

2.3.6 PHAs from sugar industry waste

Several waste streams from the sugar industry have been investigated for their PHA-producing potential. One example is low-grade molasses, which is a residual syrup generated in sugar-refining mills that is high in sucrose, but not suitable for food [30]. Most studies using molasses indicate that cell production and polymer content are not currently cost competitive. For example, Chaudhry, et al. used a Pseudomonas species to convert sugar industry wastes to PHAs, and found that the dry cell weight and PHA
contents were 7.02-12.53 g/L and 20.63-35.63%, respectively, with molasses functioning the best overall (Table 2.1) [53]. One promising study by Kulpreecha et al. [63], however, used sugarcane molasses as the main carbon source for *Bacillus megaterium* BA-019 to achieve a dry cell weight of 72.7 g/L in 24 h, with a PHB content of 42%. This latter study does indicate that molasses may be used to produce a considerable amount of PHB.

Sugar beet is another industrial waste with high sucrose content. *Alcaligenes latus* (ATCC 29714) was demonstrated to grow in sugar beet juice with supplemental nutrients to achieve optimal growth of 10.3 g/L and a PHB content 38.66% (Table 2.1) [64]. The Italian company, Bio-on, has also developed a range of PHA polymers using local sugar beet juice [65]. The company now uses sugar beet and sugar cane wastes from around the world to produce PHAs for use in cosmetics and pharmaceuticals [66].

Bagasse, the lignocellulosic residue of crushed sugar beets or sugar cane stalks, has been examined as a source of xylose for PHA production [67,68]. As mentioned, bagasse needs pretreatment to convert it to digestible sugars and to remove inhibitory compounds like formic acid, acetic acid, and furfural. A study by Lopes et al., for example, acid treated sugarcane bagasse at 120°C and produced 3.264 g/L PHB in *Burkholderia* sp. In addition, PHBV was produced when levulinic acid was added [69]. Yu and Stahl also used acid and moderate heat (100-130 °C) to pretreat sugarcane bagasse for use as a carbon source for *C. necator*. The inhibitory effects of the solution was overcome by using a large inoculum of a tolerant strain of *C. necator* and a diluted hydrolysate solution which yielded PHB to 57% dry cell weight [61]. This demonstrates
that bagasse can not only be used as a fuel for boilers and as a raw material for paper, but also as a carbon source for PHA production.

Another significant sugar industry waste is vinasse, an acidic compost with a pH of 3.5-5.0, and high organic content. Recent research into using vinasse as a carbon source for PHA production has focused on using extremely halophilic archaea like *Haloarcula marismortui* and *H. mediterranei* [70,71]. These organisms have the advantage of not requiring a sterile environment due to the high salinity of fermentation broth and are also notable for being able to produce PHBV without the addition of organic acids as precursors. One of the main drawbacks of using halophilic organisms is disposing the saline solution after fermentation, which Bhattacharyya and colleagues addressed by using a two-stage desalination of spent stillage medium to reuse medium salts, tested with rice-based ethanol stillage as carbon source [72]. In addition, vinasse contains polyphenolic inhibitory compounds that make pretreatment such as adsorption on activated carbon necessary to use vinasse as a carbon source in concentrations above 10% [70,71]. After pretreating vinasse, concentrations of up to 50% were used with *H. mediterranei* to produce 17.4-19.7 g/L PHA [70] and 100% vinasse was used with *H. marismortui* to produce 4.5±0.2 g/L PHA [71].

**2.3.7 PHAs from legume waste**

Legumes have also been demonstrated as suitable carbon sources for PHA production. In a study by Kumalaningsih *et al.* [73], liquid bean curd waste supplemented with an initial sucrose concentration of 25 g/L was fed to *A. latus*. A dry cell weight of 3.73 g/L with a PHA content of 66.56% was observed after 60 h of culturing (Table 2.1). Soy waste was used by Yu and colleagues to produce PHAs using *Alcaligenus eutrophus*
DSM 1124, but it was found that the organism was more successful at converting malt waste to PHAs than soy, with 32.57% PHA accumulated out of 18.42 g/L dcw for soy compared to 70% PHA out of 32.36 g/L dcw for malt [74,75] (Table 2.1).

Pure cultures grown on food wastes tend to promote high cell growth and accumulation of PHAs. Some substrates, such as starch or spent coffee grounds oil, are more promising than others, such as molasses or waste oils. While using waste food substrates to feed these cultures reduces costs due to carbon source, pretreatments are often necessary. Most of the traditional bacteria used for PHA production produce the PHB polymer, and the cost of sterilization and oxygen supply for cultures are prohibitive. Scale-up and optimization of processes may be able improve these difficulties. Using processes that require less energy expenditure may also reduce costs. For example, use the halophilic organism \textit{H. mediterranei} has the dual benefits of making sterilization unnecessary and producing PHBV polymer instead of PHB alone. Even with these drawbacks, using pure cultures to produce PHAs from food wastes has promise.

\textbf{Table 2.1.} Production of PHAs from food waste using pure microbial cultures.

<table>
<thead>
<tr>
<th>Food Waste Source</th>
<th>Microorganisms</th>
<th>PHA polymer type</th>
<th>Cultivation</th>
<th>Dry cell weight (g/L)</th>
<th>Maximum PHA production reported (g PHA/g dcw)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whey</td>
<td>Highly osmophilic organism</td>
<td>PHBV</td>
<td>Fermenter, batch</td>
<td>NA</td>
<td>8-10%</td>
<td>[46]</td>
</tr>
<tr>
<td>Whey</td>
<td>\textit{Haloferax mediterranei}</td>
<td>PHBV</td>
<td>Fermenter, fed-batch</td>
<td>10.91</td>
<td>66%</td>
<td>[44]</td>
</tr>
<tr>
<td>Whey</td>
<td>\textit{Haloferax mediterranei}</td>
<td>PHBV</td>
<td>Fermenter, batch</td>
<td>7.45</td>
<td>53%</td>
<td>[45]</td>
</tr>
<tr>
<td>Whey</td>
<td>\textit{Thermus thermophiles HB8}</td>
<td>PHV and mcl-PHAs</td>
<td>Flask, batch</td>
<td>1.6</td>
<td>35.60%</td>
<td>[47]</td>
</tr>
<tr>
<td>Whey permeate</td>
<td>\textit{Cupriavidus necator mRePT}</td>
<td>PHB</td>
<td>Flask, batch</td>
<td>8</td>
<td>25%</td>
<td>[48]</td>
</tr>
<tr>
<td>Food Waste Source</td>
<td>Microorganisms &amp; PHA polymer type</td>
<td>Cultivation</td>
<td>Dry cell weight (g/L)</td>
<td>Maximum PHA production reported (g PHA / g dcw)</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>-------------------</td>
<td>----------------------------------</td>
<td>-------------</td>
<td>-----------------------</td>
<td>-------------------------------------------------</td>
<td>-----------</td>
<td></td>
</tr>
<tr>
<td>Cassava starch wastewater Starch</td>
<td>Cupriavidus sp. Kku38 PHB</td>
<td>Flask, batch</td>
<td>9.69</td>
<td>61.60%</td>
<td>[49]</td>
<td></td>
</tr>
<tr>
<td>Soy bean and rapeseed oil</td>
<td>Azotobacter chroococcum Cupriavidus necator H16 PHB</td>
<td>Fermenter, batch</td>
<td>54</td>
<td>46%</td>
<td>[50]</td>
<td></td>
</tr>
<tr>
<td>Soy bean, rapeseed and corn oil and lard</td>
<td>Cupriavidus necator H16 PHB</td>
<td>Fermenter, two-stage batch</td>
<td>6.1</td>
<td>57%</td>
<td>[51]</td>
<td></td>
</tr>
<tr>
<td>Palm oil and lard</td>
<td>Cupriavidus necator H16 PHB</td>
<td>Fermenter, two-stage batch</td>
<td>6.5</td>
<td>79%</td>
<td>[51]</td>
<td></td>
</tr>
<tr>
<td>Tallow</td>
<td>Cupriavidus necator H16 PHB</td>
<td>Fermenter, two-stage batch</td>
<td>6.8</td>
<td>83%</td>
<td>[51]</td>
<td></td>
</tr>
<tr>
<td>Waste frying rapeseed oil</td>
<td>Cupriavidus necator H16 PHB</td>
<td>Flask, batch</td>
<td>10.8</td>
<td>67.90%</td>
<td>[52]</td>
<td></td>
</tr>
<tr>
<td>Waste frying palm oil</td>
<td>Cupriavidus necator H16 PHB</td>
<td>Flask, batch</td>
<td>11.9</td>
<td>58.00%</td>
<td>[52]</td>
<td></td>
</tr>
<tr>
<td>Waste frying sunflower oil</td>
<td>Cupriavidus necator H16 PHB</td>
<td>Flask, batch</td>
<td>10.8</td>
<td>52.40%</td>
<td>[52]</td>
<td></td>
</tr>
<tr>
<td>Corn oil</td>
<td>Psuedomonas species mcl-PHA</td>
<td>Flask, batch</td>
<td>12.53</td>
<td>35.63%</td>
<td>[53]</td>
<td></td>
</tr>
<tr>
<td>Spent coffee grounds oil</td>
<td>Cupriavidus necator DSM 428 PHB</td>
<td>Fermenter, fed-batch</td>
<td>16.7</td>
<td>78.40%</td>
<td>[56]</td>
<td></td>
</tr>
<tr>
<td>Spent coffee grounds oil</td>
<td>Cupriavidus necator H16 PHB</td>
<td>Fermenter, fed-batch</td>
<td>55.4</td>
<td>89.10%</td>
<td>[52]</td>
<td></td>
</tr>
<tr>
<td>Spent coffee grounds hydrolysate</td>
<td>Burkholderia cepacia PHBV</td>
<td>Flask, batch</td>
<td>4.91</td>
<td>54.79%</td>
<td>[55]</td>
<td></td>
</tr>
<tr>
<td>Oil palm empty fruit bunch</td>
<td>Bacillus megaterium R11 PHB</td>
<td>Flask, batch</td>
<td>24.29</td>
<td>51.60%</td>
<td>[57]</td>
<td></td>
</tr>
<tr>
<td>Wheat straw</td>
<td>Burkholderia sacchari DSM PHB</td>
<td>Fermenter, fed-batch</td>
<td>146</td>
<td>72%</td>
<td>[58]</td>
<td></td>
</tr>
<tr>
<td>Rice straw</td>
<td>Bacillus firmus NIH 0830 PHB</td>
<td>Fermenter, batch</td>
<td>1.9</td>
<td>89%</td>
<td>[59]</td>
<td></td>
</tr>
<tr>
<td>Wheat bran</td>
<td>Halomonas boliviensis LCI Saccharophagus PHB</td>
<td>Fermenter, batch</td>
<td>1.08</td>
<td>34%</td>
<td>[60]</td>
<td></td>
</tr>
<tr>
<td>Tequila bagasse</td>
<td>Psuedomonas species PHA</td>
<td>NA</td>
<td>NA</td>
<td>&gt;0%</td>
<td>[62]</td>
<td></td>
</tr>
<tr>
<td>Molasses</td>
<td>Psuedomonas species PHA</td>
<td>Flask, batch</td>
<td>10.54</td>
<td>20.63%</td>
<td>[53]</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.1. (Continued)

<table>
<thead>
<tr>
<th>Food Waste Source</th>
<th>Microorganisms</th>
<th>PHA polymer type</th>
<th>Cultivation</th>
<th>Dry cell weight (g/L)</th>
<th>Maximum PHA production reported (g PHA / g dcw)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spent wash</td>
<td><em>Psuedomonas species</em></td>
<td>PHA</td>
<td>Flask, batch</td>
<td>7.02</td>
<td>23.56%</td>
<td>[53]</td>
</tr>
<tr>
<td>Sugar beet juice</td>
<td><em>Psuedomonas species</em></td>
<td>PHA</td>
<td>Flask, batch</td>
<td>8.56</td>
<td>25.46%</td>
<td>[53]</td>
</tr>
<tr>
<td>Sugarcane molasses</td>
<td><em>Bacillus megaterium BA019</em> Alcaligenus latus</td>
<td>PHB</td>
<td>Flask, batch</td>
<td>72.2</td>
<td>42%</td>
<td>[63]</td>
</tr>
<tr>
<td>Sugar beet juice</td>
<td><em>Burkholderia sp.</em></td>
<td>PHB</td>
<td>Flask, two-stage batch</td>
<td>4.01</td>
<td>38.66%</td>
<td>[64]</td>
</tr>
<tr>
<td>Sugarcane bagasse</td>
<td><em>Burkholderia sp.</em></td>
<td>PHB</td>
<td>Fermenter, fed-batch</td>
<td>6.8</td>
<td>48%</td>
<td>[69]</td>
</tr>
<tr>
<td>Sugarcane bagasse</td>
<td><em>Cupriavidus necator</em></td>
<td>PHB</td>
<td>Flask, batch</td>
<td>NA</td>
<td>57%</td>
<td>[61]</td>
</tr>
<tr>
<td>Sugarcane vinasse</td>
<td><em>Halorécula marismortui</em></td>
<td>PHB</td>
<td>Flask, batch</td>
<td>12</td>
<td>23%</td>
<td>[71]</td>
</tr>
<tr>
<td>Sugar beet juice</td>
<td><em>Halofex mediterranei</em></td>
<td>PHBV</td>
<td>Flask, batch</td>
<td>28.1</td>
<td>70%</td>
<td>[70]</td>
</tr>
<tr>
<td>Rice-based ethanol stillage</td>
<td><em>Halofex mediterranei</em></td>
<td>PHBV</td>
<td>Flask, batch</td>
<td>23</td>
<td>71%</td>
<td>[72]</td>
</tr>
<tr>
<td>Malt waste</td>
<td><em>Alcaligenus eutrophus DSM1124</em></td>
<td>PHB</td>
<td>Fermenter, batch</td>
<td>32.36</td>
<td>70%</td>
<td>[75]</td>
</tr>
<tr>
<td>Soy waste</td>
<td><em>Alcaligenus eutrophus DSM1124</em></td>
<td>PHB</td>
<td>Fermenter, batch</td>
<td>18.42</td>
<td>32.57%</td>
<td>[74]</td>
</tr>
<tr>
<td>Bean curd waste</td>
<td><em>Alcaligenus latus</em></td>
<td>PHB</td>
<td>Flask, batch</td>
<td>3.73</td>
<td>66.56%</td>
<td>[73]</td>
</tr>
</tbody>
</table>

2.4 Recombinant microbes for PHA production

Natively accumulating microbial strains are most commonly utilized for conversion of food based carbon substrates into PHAs. The use of recombinant organisms, however, could be advantageous as the microorganisms can be triggered to produce PHAs without inducing stressed conditions, such as nitrogen or phosphorus starvation, which could potentially lead to cost savings. In addition, recombinant microorganisms are well-defined and thus could be further engineered for optimization. Bacteria known to be able to utilize certain substrates that the native PHA producers
cannot use may also be transformed with PHA-producing genes to produce PHA from food wastes like whey, starch, or oils. Furthermore, culturing recombinant microbes could allow faster growth and turnaround times for bioreactors.

*E. coli* is the standard organism used in genetic engineering and has been shown to be advantageous for producing PHAs. Some strains of *E. coli* are known to be able to utilize lactose, a substrate that many PHA-producing organisms like *C. necator* are not able to metabolize. As such, most studies using high lactose containing dairy whey as a carbon source have used *E. coli* with the PHA-producing genes (the *pha* operon) from *C. necator*. Traditional laboratory strains of *E. coli* like XL1-Blue, JM, or DH5α often lack the ability to utilize lactose as a nutrient source, which has made developing alternative strains from wild-type *E. coli* cells necessary. When nine different strains derived from wild-type cells were tested for their ability to produce PHA using lactose as a sole carbon source, it was documented that strains GCSC4401 and GCSC6576 transformed with a high-copy-number plasmid, pSYL107 containing the *A. eutrophus* PHA biosynthesis operon, were best able to produce PHAs. The maximum PHB concentration and PHB content obtained were 5.2 g/L and 81% of dry cell weight, respectively (Table 2.2) [76].

Initial studies using recombinant *E. coli* showed potential, and subsequent studies focused on improving production. The same group that developed *E. coli* strains GCSC4401 and GCSC6576 published another study focusing on scale-up. *E. coli* GCSC6576 (pSYL107) was grown on a high concentration of whey in a fed-batch system, where a dry cell weight of 87 g/L and PHB content of 79% was achieved (Table 2.2) [77]. A follow-up study improved on these methods by controlling the timing of PHB biosynthesis in recombinant *E. coli* using lactose concentrations. This allowed *E.
coli strain GCSC6576 (pSYL107) to accumulate PHA concentrations up to 80% of the dry cell weight without removing culture broth (Table 2.2) [50]. As such, E. coli has been successfully used to produce PHAs from whey using the pha operon from C. necator.

Other studies have also used E. coli to produce PHAs on whey using a PHB operon from A. latus rather than C. necator. Several strains of E. coli that were known to be able to utilize lactose were transformed with plasmid pJC4 with A. latus pha genes. It was found that strain CGSC 4401 was the ideal strain and dry cell weight and PHB content of 119.5 g/L and 80.5% were achieved (Table 2.2) [78]. This study and other early studies had problems with volumetric limitations of fermenter due to the low solubility of lactose in water and low PHB productivity. The former was partially addressed by using highly concentrated whey solution. A subsequent study by Ahn and colleagues used a cell recycle membrane fed-batch system to increase PHB productivity, achieving a final cell concentration and PHB content of 194 g/L and 87%, respectively (Table 2.2) [79]. In a third study, the same recombinant strain grown in whey in a 30 L fermenter (26 h) and 300 L fermenter (20 h) produced 70% and 67% PHB respectively, demonstrating the process of using whey with this strain was scalable. [80].

Two other studies used recombinant E. coli to produce PHAs from whey. Pais et al. used proton suicide methodology to select for a recombinant strain of E. coli that synthesized a low amount of organic acids after it was transformed with the C. necator pha operon. The results indicated that the lower organic acid production resulted in slower growth, but a higher production of PHB (18.88 g PHB/L vs 7.8 g PHB/L in the original transformed strain) [81]. Another study used recombinant E. coli harboring the PHB biosynthetic genes from Azotobacter sp. strain FA8 to produce PHB from whey and
corn steep liquor as the main carbon and nitrogen sources. The maximum cell density and PHB concentrations attained were 70.1 g/L and 73% (Table 2.2) [82]. These studies demonstrate the use of metabolic engineering to improve production of PHA and another option for PHA producing genes that can be used in *E. coli*.

While whey is the primary food waste substrate investigated for recombinant *E. coli*, other nutrient sources have been pursued. A study by Hong and colleagues successfully cloned the *pha* operon from *C. necator* into *E. coli* XL1-Blue and demonstrated PHB production from soy waste. Soy waste was hydrolyzed in NaOH for 8 h then fed into a batch 3L fermenter and 27.83% PHB accumulation was observed after 9 h of culturing (Table 2.2) [83]. The same group later demonstrated recombinant production of PHBV using *E. coli* HMS174 with a plasmid containing the *pha* operon. Malt and soy waste were obtained locally and recombinant *E. coli* accumulated up to 16% and 23% PHAs respectively (Table 2.2). As a comparison, when this strain was grown in glucose, it produced approximately 43% dry cell weight PHAs [84]. This demonstrates that soy and malt waste are potential carbon sources for PHA production.

In addition to malt and soy waste, another study used locally-procured restaurant waste that was anaerobically digested to produce lactic and acetic acids. Similar to processes mentioned previously, these precursors were fed to recombinant *E. coli* pnDTM2, which accumulated 44% PHB (Table 2.2) [85]. While using restaurant waste required an extra step to convert the complex mixture into organic acids, it provided an opportunity to use an inexpensive and widely-available carbon source.

Many studies that use recombinant bacteria to produce PHAs from food waste focus on using *E. coli*, which is the common workhorse of molecular biology. In addition
to *E. coli*, another *Aeromonas* sp. (strain KC007-1) has also been used. The strain was chosen for its ability to directly use starch as a carbon source, and the *pha* operon from *C. necator* H16 was added to increase production rates of PHAs. In this case, the bacteria were able to accumulate 32.7% PHA [86], indicating that *E. coli* is not the only organism to be successfully modified to produce PHAs from food waste.

The examples mentioned here showed that non-native microorganisms were able to successfully produce high amounts of PHAs. In all cases, pathways for PHA production were transferred from a native host to the non-native microorganism. As genetic tools increase, it will be possible to optimize pathways for non-native hosts to consume substrates and produce PHAs. The idea to genetically engineer microbial strains with a dual purpose of consuming inexpensive substrates and producing valuable bioproducts has been mentioned previously [87], though relatively few studies in the past ten years have used recombinant bacteria to produce PHAs from food waste. Future research into scale-up and maintaining high productivity, cell concentrations, and PHA content are still necessary to make PHA production from food wastes using recombinant bacteria feasible on an industrial scale.
Table 2.2. Production of PHAs from food waste using recombinant bacteria.

<table>
<thead>
<tr>
<th>Food waste source</th>
<th>Strain</th>
<th>Plasmid</th>
<th>PHA operon origin</th>
<th>PHA polymer type</th>
<th>Cultivation</th>
<th>Dry cell weight (g/L)</th>
<th>Maximum PHA production reported (g PHA / g dcw)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whey</td>
<td><em>E. coli</em> GCSC 657</td>
<td>pSYL107</td>
<td><em>C. necator</em></td>
<td>PHB</td>
<td>Shake flask</td>
<td>6.4</td>
<td>81% [76]</td>
<td></td>
</tr>
<tr>
<td>Whey</td>
<td><em>E. coli</em> GCSC 657</td>
<td>pSYL107</td>
<td><em>C. necator</em></td>
<td>PHB</td>
<td>Fermenter, fed-batch</td>
<td>87</td>
<td>80% [77]</td>
<td></td>
</tr>
<tr>
<td>Whey</td>
<td><em>E. coli</em></td>
<td>pSYL107</td>
<td><em>C. necator</em></td>
<td>PHB</td>
<td>Fermenter, fed-batch</td>
<td>31</td>
<td>80% [50]</td>
<td></td>
</tr>
<tr>
<td>Whey</td>
<td><em>E. coli</em> CML3-1</td>
<td>pMAB26</td>
<td><em>C. necator</em></td>
<td>PHB</td>
<td>Fermenter, fed-batch</td>
<td>33.09</td>
<td>28.65% [81]</td>
<td></td>
</tr>
<tr>
<td>Whey</td>
<td><em>E. coli</em> CGSC 4401</td>
<td>pJC4</td>
<td><em>Alcaligenes latus</em></td>
<td>PHB</td>
<td>Fermenter, fed-batch</td>
<td>119.5</td>
<td>80.50% [78]</td>
<td></td>
</tr>
<tr>
<td>Whey</td>
<td><em>E. coli</em> CGSC 4401</td>
<td>pJC4</td>
<td><em>Alcaligenes latus</em></td>
<td>PHB</td>
<td>Fermenter, fed-batch</td>
<td>194</td>
<td>87% [79]</td>
<td></td>
</tr>
<tr>
<td>Whey</td>
<td><em>E. coli</em></td>
<td>pJC4</td>
<td><em>Alcaligenes latus</em></td>
<td>PHB</td>
<td>Fermenter, fed-batch</td>
<td>14.5</td>
<td>71% [80]</td>
<td></td>
</tr>
<tr>
<td>Whey</td>
<td><em>E. coli</em> K24K</td>
<td>pJP24K</td>
<td><em>Azotobacter</em> sp. FA8</td>
<td>PHB</td>
<td>Fermenter, fed-batch</td>
<td>70.1</td>
<td>72.90% [82]</td>
<td></td>
</tr>
<tr>
<td>Malt waste</td>
<td><em>E. coli</em></td>
<td>pUC19/PHA</td>
<td><em>C. necator</em></td>
<td>PHBV</td>
<td>Fermenter, fed-batch</td>
<td>NA</td>
<td>16% [84]</td>
<td></td>
</tr>
<tr>
<td>Soy waste</td>
<td><em>E. coli</em></td>
<td>pUC19/PHA</td>
<td><em>C. necator</em></td>
<td>PHBV</td>
<td>Fermenter, fed-batch</td>
<td>NA</td>
<td>23% [84]</td>
<td></td>
</tr>
<tr>
<td>Soy waste</td>
<td><em>E. coli</em> XL1-Blue</td>
<td>pKS</td>
<td><em>C. necator</em></td>
<td>PHB</td>
<td>Fermenter, batch</td>
<td>3.025</td>
<td>27.83% [83]</td>
<td></td>
</tr>
<tr>
<td>Organic acids</td>
<td><em>E. coli</em> pnDT M2</td>
<td>NA</td>
<td>NA</td>
<td>PHB</td>
<td>Fermenter, batch</td>
<td>2.9</td>
<td>45% [85]</td>
<td></td>
</tr>
<tr>
<td>Starch</td>
<td><em>Aeromonas</em> Sp. KC007-R1</td>
<td>pRK415 H16</td>
<td><em>C. necator</em></td>
<td>PHB</td>
<td>Fermenter, batch</td>
<td>1.83</td>
<td>32.70% [86]</td>
<td></td>
</tr>
</tbody>
</table>

2.5 Production of PHAs from food waste using anaerobic digestion

One method that has been used to biologically convert complex food wastes to PHA precursors is anaerobic digestion using open systems of mixed microbial cultures
(MMC). The general process that has been followed in most studies using food waste has involved three steps: 1) acidogenic fermentation; 2) culture selection; and 3) PHA accumulation. The primary advantage to this approach is that MMCs have lower investment and operating costs, because substrate pretreatment processes are not required, sterilization is typically not necessary and less expensive carbon sources can be used [88,89]. This is important, since analyses have indicated that the high energy expenditure used in sterilization, aeration, and agitation to produce PHAs in pure cultures causes these bioplastics to have little advantage over traditional synthetic plastics in environmental impact [30,39]. In addition, due to the greater variety of organisms working together with complex substrates in MMCs, more diverse PHAs, such as polyhydroxyhexanoate (PHH), polyhydroxyoctanoate (PHO), and polyhydroxydecanoate (PHD) are produced [90]. This indicates that MMCs are a potentially viable option for producing PHAs from food waste.

The first step of producing PHAs from mixed cultures is acidogenic fermentation. During this step, complex wastes, such as food scraps, spentwash, and wastewater are broken down into simpler and smaller fermentative acids, mainly C2-C4 acids such as acetic, propionic, butyric, and lactic acids [91,92]. A variety of feedstocks have been used for anaerobic production of volatile fatty acids (VFAs) from food waste, including food scraps from restaurants or kitchens [85,93–95], whey [96–98], jowar grain spentwash and rice spentwash [99], tomato cannery wastewater [100], olive oil mill pomace and wastewater [101,102], palm oil mill effluent [103,104], sugarcane molasses [91,105,106], pea shell waste [107], condensate of food waste [90], and fermented brewery wastewater [108,109] (see Table 2.3 and Table 2.4). Applying pretreatments such as filtering and
deproteinization to these waste streams has been examined, with mixed results [99,100]. Another pretreatment is buffering the waste solution to keep pH between 5.5-7.0, which has been shown to improve VFA production [85,101,110]. A variety of food waste sources have been used to produce VFAs for use by cultures of bacteria that accumulate PHAs.

The second step of producing PHAs from mixed cultures is culture selection. During this step, bacteria are subjected to alternating conditions to obtain a microbial community where almost all microorganisms have a high PHA storing capacity and production rate. This is often carried out in sequencing batch reactors (SBRs), compact systems where the full feast and famine cycle may be performed in one single reactor, and the length of each phase may be varied. The cycle may be either alternating conditions of external substrate excess (feast) and limitation (famine) in aerobic conditions or alternating anoxic and aerobic microenvironments. In both cases, limiting cell growth (through famine or anoxic conditions) increases PHA production and presents pressures that allow PHA-producing strains to become predominant [92]. By using microautoradiography and fluorescence in situ hybridization (FISH), Albuquerque et al. (2012) found that in a culture grown on molasses, bacteria that their process selected for were primarily from the genera *Azoarcus*, *Thauera*, and *Paracoccus*. Each of these populations specialized in digesting specific products of the acidogenic fermentation. *Azoarcus* and *Thauera* primarily consumed acetate and butyrate, respectively, while *Paracoccus* consumed a broader range of substrates. Other studies on culture selection have indicated that PHA-storing bacteria in MMC are predominantly from the classes of
Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria, as has been reviewed elsewhere [92,111].

The third step of producing PHAs from mixed cultures is PHA accumulation. This step is designed to maximize PHA production in cells harvested from the enrichment bioreactor. The feast-famine cycle (FF) is known to increase synthesis and storage of PHA granules [112]. Alternating anoxic and aerobic conditions have also been used in SBRs to improve PHA production from food waste. It has been documented that anoxic microenvironments tend to promote higher PHA accumulation due to better access to VFAs and lack of an electron acceptor, while aerobic environments tend to promote PHA degradation, but better nutrient removal [113]. The alternating conditions pressure the cells to uptake nutrients and convert them to PHAs.

Several problems exist with MMCs. First, they generally have lower performance than pure cultures (when measured by volumetric productivity). This is due, in part, to lower cell concentrations that are usually found in MMC cultures. Often, concentrations are less than 10 g/L compared to values greater than 100 g/L that can be found in pure culture studies. Further, information on the quality of PHAs produced in MMCs is scarce [105]. Another concern is that many of the organic acids produced during MMC fermentation inhibit bacterial growth, causing decreased productivity. These issues must be overcome to make MMCs a viable approach to producing PHAs from inexpensive carbon sources.
Table 2.3. Production of PHAs from anaerobically digested food waste.

<table>
<thead>
<tr>
<th>Food Waste Source</th>
<th>Microorganisms</th>
<th>PHA polymer type</th>
<th>Cultivation</th>
<th>Maximum Production Reported (g PHA / g dcw)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whey</td>
<td>Wastewater microbes</td>
<td>PHBV</td>
<td>Flask, batch</td>
<td></td>
<td>[97]</td>
</tr>
<tr>
<td>Whey</td>
<td>Pre-selected mixed microbial culture</td>
<td>PHBV</td>
<td>Flask, batch</td>
<td>81%</td>
<td>[98]</td>
</tr>
<tr>
<td>Whey</td>
<td>Activated sludge consortium</td>
<td>PHBV</td>
<td>Three-stage reactors system</td>
<td>65% [96]</td>
<td></td>
</tr>
<tr>
<td>Sugarcane molasses</td>
<td>Activated sludge consortium</td>
<td>PHBV</td>
<td>Three-stage reactors system</td>
<td>56% [96]</td>
<td></td>
</tr>
<tr>
<td>Brewery wastewater</td>
<td>Activated sludge consortium</td>
<td>PHBV</td>
<td>SBR</td>
<td>39%</td>
<td>[109]</td>
</tr>
<tr>
<td>Food processing wastewater effluent</td>
<td>Activated sludge consortium</td>
<td>PHB</td>
<td>Flask, batch</td>
<td>60.70%</td>
<td>[99]</td>
</tr>
<tr>
<td>Jowar grain-based distillery spentwash</td>
<td>Activated sludge consortium</td>
<td>PHB</td>
<td>Flask, batch</td>
<td>42.30%</td>
<td>[99]</td>
</tr>
<tr>
<td>Rice grain-based distillery spentwash</td>
<td>Activated sludge consortium</td>
<td>PHB</td>
<td>Flask, batch</td>
<td>40%</td>
<td>[99]</td>
</tr>
<tr>
<td>Condensate of food waste</td>
<td>Enriched activated sludge consortium</td>
<td>PHBV and mcl-PHAs</td>
<td>VSMBR</td>
<td>1.80%</td>
<td>[90]</td>
</tr>
<tr>
<td>Olive oil mill pomace</td>
<td>Activated sludge consortia</td>
<td>PHBV</td>
<td>SBR</td>
<td>39%</td>
<td>[101]</td>
</tr>
<tr>
<td>Olive oil mill wastewater</td>
<td>Wastewater microbes</td>
<td>PHBV</td>
<td>SBR</td>
<td>11.30%</td>
<td>[102]</td>
</tr>
<tr>
<td>Tomato wastewater</td>
<td>Activated sludge consortium</td>
<td>PHA</td>
<td>Fermenter, batch</td>
<td>20% [100]</td>
<td></td>
</tr>
<tr>
<td>Fermented food waste</td>
<td>Wastewater microbes</td>
<td>PHA</td>
<td>Fermenter, anaerobic/aerobic</td>
<td>51% [114]</td>
<td></td>
</tr>
<tr>
<td>Fermented molasses</td>
<td>Mixed microbial culture</td>
<td>PHBV</td>
<td>Fermenter, pulse feed</td>
<td>56% [105]</td>
<td></td>
</tr>
<tr>
<td>Fermented molasses</td>
<td>Mixed microbial culture</td>
<td>PHBV</td>
<td>Fermenter, batch</td>
<td>60.50% [91]</td>
<td></td>
</tr>
</tbody>
</table>

2.6 Indirect coupling of MMC to PHA production

One approach to overcoming some of the difficulties associated with MMCs is using an indirect coupling approach where complex food wastes are digested in an MMC
to produce VFAs, which are then harvested and fed to a pure-culture fermentation (see Table 2.4). This increases the chance that PHA polymers will be consistent in their quality and produced in a high concentration [115]. Like the MMCs, complex food wastes and other inexpensive carbon sources like food scraps from restaurants or kitchens [85,93,94,115,116] or pea shells [107] can be used as nutrient sources for PHA production with minimal pretreatment steps.

One of the main difficulties of the indirect coupling process is efficiently harvesting the VFAs and transferring them to a pure culture fermenter. This was initially achieved using evaporation and ion exchange, but the processes were costly. In one of the earliest alternative approaches, Du and colleagues compared using a silicone membrane with a dialysis membrane to diffuse the acids into an air-bubbling reactor, while preventing solids mixing. The dialysis membrane worked considerably better, allowing a maximum dry cell weight and PHBV concentration of 22.7 g/L and 72.6%, respectively, compared to a maximum of 11.3 g/L and 60.2% PHB (as opposed to the PHBV) when a silicon membrane was used [115,116]. The primary problem was that the process still consumed a high amount of operational energy due to small membrane pore sizes. A subsequent study saw success with harvesting slurry once a week and filtering the VFAs using a 0.45 μm filter [94]. Another difficulty with indirect coupling is that the VFAs are inhibitory to growth in high concentrations, which means that it is often desirable to use fed-batch approaches to keep concentrations low, while ensuring that VFAs are available for consumption [93]. Despite these problems, using MMC to produce VFAs that are utilized by pure cultures is a way to combine many of the best aspects of pure culture and MMC production of PHAs.
Table 2.4. Production of PHAs from organic acids derived from anaerobically digested food waste.

<table>
<thead>
<tr>
<th>Food waste source for organic acids</th>
<th>Microorganism / Strain</th>
<th>PHA polymer</th>
<th>Cultivation type</th>
<th>Dry Cell Weight (g/L)</th>
<th>Maximum PHA Production Reported (g PHA/g dcw)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Restaurant waste</td>
<td>Recombinant E. coli pnDTM2</td>
<td>PHB</td>
<td>Fermenter, batch</td>
<td>2.9</td>
<td>45%</td>
<td>[85]</td>
</tr>
<tr>
<td>Restaurant waste</td>
<td>C. necator H16</td>
<td>PHB</td>
<td>Fermenter, continuous feeding</td>
<td>1.4</td>
<td>87%</td>
<td>[94]</td>
</tr>
<tr>
<td>Food scraps from cafeteria</td>
<td>C. necator</td>
<td>PHBV</td>
<td>Fermenter, batch</td>
<td>22.7</td>
<td>72.60%</td>
<td>[115]</td>
</tr>
<tr>
<td>Kitchen waste</td>
<td>C. necator CCGUG 52238</td>
<td>PHB</td>
<td>Fermenter, batch</td>
<td>4.6</td>
<td>52.79%</td>
<td>[93]</td>
</tr>
<tr>
<td>Pea shells</td>
<td>Bacillus cereus Strain EGU3</td>
<td>PHB</td>
<td>Fermenter, batch</td>
<td>1.32</td>
<td>71%</td>
<td>[107]</td>
</tr>
</tbody>
</table>

2.7 Conclusions and outlook

Utilizing food waste to create PHAs has potential for long term applications, but is not without some hurdles. In most cases, many of the food waste sources were locally procured and this is an important consideration since transportation of food waste to the source of the microbial PHA production systems could be cost prohibitive. Furthermore, the contributions of food waste pre-treatment to overall process costs of microbial PHA synthesis need to be examined in-depth. Ideally pre-treatment of food waste should be kept to a minimal to reduce time and cost. PHA extraction from microbial biomass should also be considered in a technoeconomic analysis. The most common PHA extraction methods are solvent based extraction, however there are a variety of different methods that can be used [117–119]. In addition, a biorefinery concept could be realized with food waste being the feedstock to producing PHAs and additional products, as has been proposed by others [35,36,65]. Biorefineries offer the advantages of not depending on a single product to be produced and thus are flexible and potentially sustainable.
The microbes used to convert food waste to PHAs are diverse, ranging from known, well-defined microorganisms to mixed microbial consortia. As mention, both natively accumulating PHA strains and genetically engineered strains could be used as platforms for PHA production. Strain selection is important aspect of PHA production and bioprospecting could lead to the discovery of additional microbes that can be used as PHA production strains in the future [120]. In addition to engineering microbes to produce PHAs, microbes could also be optimized to use specific food substrates and generate defined chain-length PHAs.

Food wastage is a global issue, the ability to upgrade complex carbon substrates into a tangible product such as PHAs could help reduce the burden of waste processing by municipalities. As described here, many different routes on food waste conversion to PHAs exist. There is not a one single solution to a specific type of food waste, rather there are multiple paths that could be taken, each with different pros and cons. New and innovative methods of food waste processing to PHAs will continue to grow in the future and these emerging technologies could make the economic production of microbial PHAs a reality in the not-to-distant future.

**Conflict of interest**

The authors declare no conflict of interests.

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3.1 Methanotrophic Bacteria

Methanotrophic bacteria, or methanotrophs, are gram-negative bacteria known for their unique ability to utilize methane as a sole carbon and energy source. They are a subset of a physiological group of aerobic bacteria known as methylotrophs that utilize one-carbon compounds. The general category of methylotrophs utilize a wide variety of different one-carbon compounds including methane, methanol, methylated amines, halomethanes, and methylated compounds containing sulfur. Methanotrophs are unique in their use of the enzyme methane monoxygenase to catalyze the digestion of methane [1].

The discovery and exploration of methanotrophic bacteria has been an ongoing process over the last century, as reviewed in Howard Dalton’s work [2]. Humans have known about the existence of methane since Alessandro Volta collected samples of it at Lake Maggiore, Italy in 1778. It wasn’t until 1906, however, that the first report of a bacterium capable of growth on methane was published. Dutch microbiologist N. L. Söhngen argued that since vast quantities of methane were produced on earth but only trace amounts of the gas could be found in the atmosphere that there must be organisms that consume methane. Through a series of experiments, he isolated *Bacillus methanicus* from aquatic plants and pond water. Despite this discovery, research on methanotrophic bacteria lagged for nearly six decades after Söhngen’s work. The next major study came in 1966, when Foster’s Texas laboratory published a report that detailed the isolation of a few methanotrophic bacteria [2].
The major breakthrough in studies of methanotrophic bacteria came with the Whittenbury group’s research, published in 1970. Using plate microscopes to detect tiny colonies on plates, they isolated over 100 new species of methanotrophic bacteria. Whittenbury and colleges devised a classification scheme for methanotrophs based on morphological type, pathway of carbon assimilation, ability to fix dinitrogen, cyst or spore formation and mol% G+C content [3,4]. Since that time, many more strains of methanotrophic bacteria have been isolated from a variety of habitats, indicating that they are likely ubiquitous in nature [2]. There is significant interest in using methanotrophs in biomanufacturing in order to avoid using a carbon source tied to the human food supply, resulting in an increasing number of studies into the physiology and capabilities of methanotrophs in recent decades.

As stated previously, the defining characteristic of methanotrophic bacteria is their use of methane monooxygenase enzymes (MMOs). The first step in assimilating methane as a carbon source is oxidation to methanol, which is catalyzed by MMOs. There are two known types of MMOs—particulate methane monooxygenase (pMMO) and soluble methane monooxygenase (sMMO). The particulate variety is the more common type—all but one known species of methanotrophs produce pMMO. This enzyme is contained in the membrane of the cells and is thought to use copper active sites to catalyze methane oxidation. Under conditions of copper starvation, however, there are a few species of methanotrophs that express sMMO. This enzyme is contained in the cytosol of the cell and uses a di-iron active site instead of copper to catalyze methane oxidation.[5] The two types of MMOs (pMMO and sMMO) are what allow methanotrophic bacteria to metabolize methane.
Methane is assimilated as a carbon source over the course of several steps. Methane is oxidized by methane monooxygenases, creating methanol. Methanol is then converted into formaldehyde, which is the central intermediate in anabolism and catabolism in methanotrophs (see Figure 3.1). Two main formaldehyde assimilation pathways are possible for methanotrophic bacteria—the serine pathway and the ribulose monophosphate (RuMP) pathway. Through these pathways, methane is assimilated by methanotrophs.

![Diagram of Methane Assimilation](image)

**Figure 3.1.** Pathways for the oxidation of methane and assimilation of formaldehyde. Abbreviations: CytC, cytochrome c; FADH, formaldehyde dehydrogenase.

In the RuMP pathway, one molecule of $C_3$ compound (generally pyruvate or dihydroxyacetone phosphate) is synthesized from three molecules of formaldehyde. The first part of the cyclic sequence (*fixation*) is the aldol condensation of formaldehyde with three molecules of ribulose 5-phosphate to give three hexulose-6-phosphate molecules. These molecules then undergo isomerization to fructose-6-phosphate molecules (FMP).
The second part of the sequence (*cleavage*) begins with the conversion of one molecule of FMP into either fructose 1,6-bisphosphate (FBP) by phosphofructokinase, or to 2-keto 3-deoxy 6-phosphogluconate (KDPG) by the Entner/Doudoroff enzymes. These molecules are then cleaved by aldolases to glyceraldehyde-3-phosphate plus the C₃ molecule that is the “product” of the pathway. During the final part of the cycle (*rearrangement*) the other two molecules of FMP and the remaining glyceraldehyde-3-phosphate molecules undergo a series of reactions to regenerate three molecules of ribose-5-phosphate (see Figure 3.2) [6]. The summary equation for the RuMP is as follows:

$$2HCHO + ATP \rightarrow glyceraldehyde - 3 - phosphate + ADP \quad (1)$$
In the serine pathway of formaldehyde assimilation, two molecules of formaldehyde plus two molecules of glyoxylate yield two molecules of 2-phosphoglycerate. One of the 2-phosphoglycerate molecules is incorporated into cell material by way of 3-phosphoglycerate, while the other is converted to phosphoenolpyruvate (PEP). The PEP molecule undergoes carboxylation, which yields oxaloacetate and subsequently malyl-CoA. The malyl-CoA is cleaved to glyoxylate plus acetyl-CoA, whose oxidation to glyoxylate completes the cycle (see Figure 3.3) [6]. The summary equation for the serine pathway is as follows:

\[
2HCHO + CO_2 + 3ATP + 2NADH \rightarrow 2 - \text{phosphoglycerate} + 2 ADP + Pi + NAD^+
\]

(2)

**Figure 3.3.** Serine pathway for formaldehyde fixation. Unique reactions catalyzed by serine hydroxymethyl transferase (STHM), hydroxypyruvate reducates (HPR), malate thiokinase (MTK), and malyl coenzyme A lyase (MCL) are identified.
Methanotrophic eubacteria are sorted into three main groups, largely based on the pathway used for formaldehyde fixation. Type I methanotrophs, or Methylococcaceae—which include the genera *Methylomonas, Methylococcus, Methylomicrobium*, and *Methylobacter*—utilize the RuMP pathway. They are also distinguished by a membrane arrangement of bundles of vesicular disks and a G+C content of 49-60% (mol/mol). The cell morphology of type I methanotrophs is generally short rods with some cocci or ellipsoids. Group Ia methanotrophs are found in the gamma division of proteobacteria, while group Ib methanotrophs are found in the beta division of proteobacteria.

Type X methanotrophs, such as *Methylococcus capsulatus* (Bath)—form an intermediate group that shares characteristics with both type I and type II methanotrophs. They are often classified as a subdivision of type I methanotrophs, due to use of the RuMP pathway. What sets them apart from type I methanotrophs is their possession of low levels of serine-pathway enzymes, the presence of ribulose-1,5-biphosphate carboxylase, a G+C content of 59-65% (mol/mol), and a membrane structure of bundles of vesicular disks. Type X methanotrophs are found within the gamma division of proteobacteria.

Type II methanotrophs, or Methylocystaceae—which include the genera *Methylosinus, Methylocystis*, and *Methylopila*—utilize the serine pathway for formaldehyde fixation. They are distinguished by a membrane arrangement of paired membranes aligned to the periphery of cells, a G+C content of 62-67% (mol/mol), and the ability to fix nitrogen. Cell morphology of type II cells is generally crescent-shaped rods, rods, and pear-shaped cells and all type II methanotrophs are found within the alpha subdivision of proteobacteria.[1] Types II methanotrophs have different survival
strategies than type I methanotrophs. Type I methanotrophs grow rapidly under favorable conditions, but die off quickly under stress conditions. Type II methanotrophs grow slower, but are adapted to survive under stressed conditions. For example, type II methanotrophs outcompete type I methanotrophs under oxygen- and nitrogen-limiting conditions [7]. A few strains of type II methanotrophs are great interest for biological engineering applications.

Three methanotrophic bacteria have been the primary focus of efforts to characterize methanotrophs. *Methylosinus trichosporium* OB3b is a type II methanotroph that has been called the “work horse” organism for research on the physiology, biochemistry, and molecular biology/genetics of methanotrophy.[8] A draft genome has been published for OB3b [8], and it has been shown that it is capable of producing fairly high amounts of polyhydroxybutyrate (PHB) [9–12]. *Methylocystis* are also type II methanotrophs. They tend to dominate mixed microbial cultures, and the genus is most often studied for non-sterile production of the bioplastic polyhydroxybutyrate (PHB) using methanotrophic bacteria [12–14]. Optimized protocols exist for *Methylocstis parvus* OBBP [15]. *Methylococcus capsulatus* Bath is a type X methanotroph that has also been studied. In the European Union, a mixed culture dominated by *M. capsulatus* Bath has been approved for production of proteins for nutritional feeds for salmon and livestock [16]. Together, these three bacteria are among the most notable methanotrophs.

A significant genus of methylotroph that is related to the type II methanotrophs is *Methylobacterium*. Although most strains lack the ability to metabolize methane due to the absence of MMO genes, *Methylobacterium* species are considered facultative methylotrophs that can metabolize a variety of C₁ feedstocks, including methanol.
Methylobacterium are ubiquitous, simple and inexpensive to culture, and have been studied extensively. The many studies that have been performed have provided optimized fermentation protocols [17–19], and genomic information and genetic tools are available [20–26]. They have been shown to produce valuable products like bioplastics, vitamin B12, pyrroloquinoline, and carotenoids [22]. Thus, Methylobacterium are considered an ideal organism for biomanufacturing and are one of the best-characterized types of methylotrophs.

3.2 PHA Biosynthesis in Methylotrophic Bacteria

Polyhydroxyalkanoates (PHAs) are a class of intercellular energy- and carbon-storage molecules produced by a variety of microorganisms. In general, PHAs are linear, head-to-tail polyesters composed of 3-hydroxy fatty acid monomers (see Figure 3.4). Poly(3-hydroxybutyrate) [P(3HB)] is by far the best-characterized and the most common type of PHA [27]. PHA accumulation is a response to an essential nutrient limitation such as nitrogen, phosphorus, or oxygen limitation in the presence of excess carbon sources [28,29]. PHAs are a way for the cell to store carbon and reducing potential in a way that doesn’t affect osmotic pressure when growth is limited [30,31].

![Chemical structure of PHAs](image)

**Figure 3.4.** Chemical structure of PHAs. PHAs are generally composed of (R)-β-hydroxy fatty acids, where the pendant group (R) varies from methyl to tridecyl. In P(3HB), the R group is methyl.
PHB serves as a model compound of how PHAs are assembled. The basic pathway for PHB monomer construction starts with the condensation of two acetyl-CoA by β-ketothiolase followed by the formation of a hydroxyl group by acetoacetyl-CoA reductase, producing the monomer unit of PHB. These monomer units are then linked together by PHB polymerase into a polymer [32]. There are three genes that work to code the enzymes that convert acetyl-CoA into PHB: phbC (polymerase), phbA (β-ketothiolase) and phbB (acetoacetyl-CoA reductase), which form the phbCAB operon (see Figure 3.5). The carbon source has an influence on which type of PHA is produced by the cells, since different S-CoA-containing molecules may be condensed with acetyl-CoA by β-ketothiolase, becoming incorporated into the PHA molecules [32].

![Figure 3.5](image)

**Figure 3.5.** Dedicated pathway for P(3HB) monomer synthesis. This pathway involves the condensation of two acetyl-CoA by β-ketothiolase (PhaA) and formation of a hydroxyl group by acetoacetyl-CoA reductase (PhaB).

There are several methanotrophs that are known to produce PHB. The production of PHB in type I methanotrophs has been reported in literature [33,34], but these reports are controversial. Usually, only qualitative analysis of PHB production was reported.
Moreover, the genes that are generally required to create PHB are not present in type I or type X methanotrophs [35]. Type II methanotrophs of the genera *Methylocystis* and *Methylosinus*, however, have consistently tested positively for both the presence of genes associated with PHB production and quantitative analysis of PHB [7,13,35–40]. Thus, most studies involving PHA production from methanotrophs utilize Type II methanotrophic bacteria.

It is thought that PHB production in methanotrophs may be linked to the serine cycle. Babel hypothesized that serine-pathway methylotrophs produce PHB as a carbon storage polymer, while RuMP-pathway methylotrophs instead produce exopolysaccharides under unbalanced growth conditions [41]. Korotkova and Lidstrom found that when PHB producing genes were mutated in *Methylobacterium extorquens* AM1 that growth on C₁ and C₂ compounds was defective. Further research indicated that β-hydroxybutyryl-CoA (an intermediate in PHB production) may also be an intermediate in the unknown pathway that converts acetyl-CoA to glyoxylate in methylotrophs [42]. These results point towards the theory that PHB is essential to assimilation of C₁ carbon sources in methylotrophs.

Further research has shown that PHB is utilized primarily as a sink for reducing power in type II methanotrophs instead of energy. Pieja and colleagues observed PHB consumption in the type II methanotroph *Methylocystis parvus* OBBP in a variety of conditions. They found that when *Methylocystis* was starved of methane, the bacteria did not consume significant amounts of PHB. Instead, the bacteria only consumed PHB when methane and nitrogen were also present. When formate (a source of reducing power) was added, PHB consumption was delayed. They theorized that PHB was used as a source of
reducing power to assimilate nitrogen in type II methanotrophs [14]. Likewise, Xin and colleagues found that when *Methylosinus trichosporium* IMV 3011 accumulated higher amounts of PHB that cells had increased capacity to produce methanol from CO₂ because PHB provided intracellular reducing equivalents for the cell [43]. In these cases, PHB primarily served as a sink for reducing potential rather than as a source of C₂ units.

The overall equation for PHB accumulation for methanotrophs that use the serine cycle was calculated by Asenjo and Suk as follows:

\[ 8\text{CH}_4 + 12\text{O}_2 + FP \rightarrow C_4\text{H}_6\text{O}_2(\text{PHB monomer}) + 4\text{CO}_2 + 12\text{ATP} + \text{FPH}_2 \]  

(3)

where FPH₂ is reduced form of flavoproteins (FP) succinate dehydrogenase. This equation has a theoretical yield for bioconversion of methane to PHB that can be estimated to be 67% (86g/128g) [40]. Yamane suggested that if the regeneration of NADP⁺ of acetoacetyl-CoA reductase in the PHB biosynthetic pathway was taken into consideration the equation for PHB accumulation would become:

\[ \text{CH}_4 + 4\text{O}_2 + (5q - 3)\text{ADP} + (5q - 3)\text{Pi} + \text{NADH} + H^+ \rightarrow \]

\[ \frac{1}{n}C_4\text{H}_6\text{O}_2(\text{PHB monomer}) + \text{CO}_2 + 4\text{H}_2\text{O} + (5q - 3)\text{ATP} + \text{NAD}^+ \]  

(4)

Where \( q \) is the number of moles of ATP assumed to be produced through the conversion of CH₃OH to HCHO. The theoretical yield for this equation is 54% (kg/kg).[44]

Studies have been performed to determine the amount of PHB accumulated in methanotrophs and the yield of PHB from methane or methanol. On average, those studies that used methanol tended to display higher levels of PHB Content (% of total biomass). This is likely due to methanol dissolving into water more easily, making it more accessible to bacteria. These findings are summarized in Table 3.1.
Table 3.1. Summary of the methylotrophic and methanotrophic production of PHB. Adapted from Strong et al.[39]

<table>
<thead>
<tr>
<th>Microorganisms (% in Mixed Culture)</th>
<th>Carbon Source</th>
<th>PHB Content (% of Total Biomass)</th>
<th>Yield (g PHB/g Carbon Source)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Methylocystis</em> sp. GB25 DSMZ 7674 (&gt;90%)</td>
<td>Methane</td>
<td>28.3–51.3</td>
<td>0.54</td>
<td>[13,37,45]</td>
</tr>
<tr>
<td><em>Methylocystis</em> sp. GB25 DSMZ 7674 (&gt;86%)</td>
<td>Methane</td>
<td>10.4–33.6</td>
<td>0.45</td>
<td>[36]</td>
</tr>
<tr>
<td><em>Methylosinus/Methylocystis</em> (percent unknown) + Type I genera</td>
<td>Methane</td>
<td>7—46</td>
<td>n/a</td>
<td>[35]</td>
</tr>
<tr>
<td>Mixed consortium including <em>Methylobacterium organophilum</em> (percent unknown)</td>
<td>Methane</td>
<td>34</td>
<td>0.8</td>
<td>[46]</td>
</tr>
<tr>
<td><em>Methylocystis</em> (~77%)</td>
<td>Methane</td>
<td>39</td>
<td>0.64</td>
<td>[47]</td>
</tr>
<tr>
<td><em>Methylocystis</em> (&gt;76%)</td>
<td>Methane</td>
<td>40</td>
<td>0.49</td>
<td>[48]</td>
</tr>
<tr>
<td><em>Methylomicrobium</em> (96%)</td>
<td>Methane</td>
<td>23</td>
<td>n/a</td>
<td>[49]</td>
</tr>
<tr>
<td><em>Methylocystis</em> sp. (71.6%)</td>
<td>Methane</td>
<td>2.5–8.5</td>
<td>n/a</td>
<td>[50]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Microorganisms (Pure Culture)</th>
<th>Carbon Source</th>
<th>PHB Content (% of Total Biomass)</th>
<th>Yield (g PHB/g Carbon Source)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Methylocystis parvus</em> OBBP</td>
<td>Methane</td>
<td>68</td>
<td>n/a</td>
<td>[40]</td>
</tr>
<tr>
<td><em>Methylosinus trichosporium</em> OB3b</td>
<td>Methane</td>
<td>30—50</td>
<td>n/a</td>
<td>[11]</td>
</tr>
<tr>
<td>Type II methanotrophic strain MTS</td>
<td>Methane</td>
<td>3</td>
<td>n/a</td>
<td>[7]</td>
</tr>
<tr>
<td><em>Methylosinus trichosporium</em> OB3b</td>
<td>Methane</td>
<td>30</td>
<td>n/a</td>
<td>[9]</td>
</tr>
<tr>
<td><em>Methylosinus trichosporium</em> OB3b</td>
<td>Methane</td>
<td>25</td>
<td>n/a</td>
<td>[46]</td>
</tr>
<tr>
<td><em>Methylocystis</em> spp.</td>
<td>Methane</td>
<td>38—57</td>
<td>0.48-0.59</td>
<td>[46]</td>
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<tr>
<td><em>Methylocystis</em> spp.</td>
<td>Methane</td>
<td>7—36</td>
<td>n/a</td>
<td>[35]</td>
</tr>
<tr>
<td><em>Methylosinus</em> spp.</td>
<td>Methane</td>
<td>9—38</td>
<td>n/a</td>
<td>[35]</td>
</tr>
<tr>
<td><em>Methylocystis hirsuta</em></td>
<td>Methane</td>
<td>51.6</td>
<td>n/a</td>
<td>[52]</td>
</tr>
<tr>
<td><em>Methylocystis parvus</em> OBBP</td>
<td>Methane</td>
<td>60</td>
<td>0.88</td>
<td>[12]</td>
</tr>
<tr>
<td><em>Methylosinus trichosporium</em> OB3b</td>
<td>Methane</td>
<td>29</td>
<td>1.13</td>
<td>[12]</td>
</tr>
<tr>
<td><em>Methylocystis parvus</em> OBBP</td>
<td>Methane</td>
<td>49.4</td>
<td>n/a</td>
<td>[15]</td>
</tr>
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</table>
Table 3.1. (Continued)

<table>
<thead>
<tr>
<th>Microorganisms (Pure Culture)</th>
<th>Carbon Source</th>
<th>PHB Content (% of Total Biomass)</th>
<th>Yield (g PHB/g Carbon Source)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas</em> sp. K</td>
<td>Methanol</td>
<td>66</td>
<td>0.18</td>
<td>[54]</td>
</tr>
<tr>
<td><em>Methyllobacterium rhodesianum</em></td>
<td>Methanol</td>
<td>45-55</td>
<td>n/a</td>
<td>[41]</td>
</tr>
<tr>
<td><em>Methyllobacterium extorquens</em> K</td>
<td>Methanol + n-amyl alcohol</td>
<td>44</td>
<td>0.11</td>
<td>[55]</td>
</tr>
<tr>
<td><em>Paracoccus denitrifans</em></td>
<td>Methanol + n-amyl alcohol</td>
<td>57</td>
<td>0.97</td>
<td>[55]</td>
</tr>
<tr>
<td><em>Pseudomonas 135</em></td>
<td>Methanol</td>
<td>55</td>
<td>n/a</td>
<td>[56]</td>
</tr>
<tr>
<td><em>Methyllobacterium extorquens</em> NCIMB</td>
<td>Methanol</td>
<td>7—21</td>
<td>n/a</td>
<td>[57]</td>
</tr>
<tr>
<td><em>Methyllobacterium extorquens</em> ATCC 55366</td>
<td>Methanol</td>
<td>40—46</td>
<td>0.09–0.12</td>
<td>[19]</td>
</tr>
<tr>
<td><em>Methyllobacterium organophilum</em></td>
<td>Methanol</td>
<td>52—56</td>
<td>0.19</td>
<td>[58]</td>
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### 3.3 Current Methods for PHA Production in Methylotrophic Bacteria

Bacteria that naturally produce PHAs generally do so under stress conditions such as nitrogen or phosphorous limitation in the presence of an excess carbon source. Due to this, most approaches to increase PHA production in microorganisms generally follow a pattern of growing a high concentration of cells and then creating a stressed environment that favors PHA production. This is largely true of methylotrophic bacteria. Much
research has also gone into optimizing nutrient conditions to produce high-quality and high-quantities of PHB in methylotrophs.

Economic analyses of PHA production using methane show that it is a more promising carbon source than sugars or oils. Raw material costs are reduced from 30-50% of production costs to 22%, though temperature removal, air compression, and biomass drying remain significant factors in production costs [64]. A cradle-to-gate life cycle assessment by Rostkowski’s group indicated that using biogas methane is a preferable source of methane and that PHB recovery and demands for energy are the biggest challenges to production [65]. Utilization of biogas for PHB production also has the advantage of increasing revenue from anaerobic digesters used in wastewater remediation [16]. For these reasons, methanotrophs are of great interest in PHB production, though some hurdles remain.

Methanol is another compound that shows great promise for use as a carbon source in producing PHAs. Like methane, methanol is widely available and independent from seasonal variations and weather conditions. It can also be produced using biological organisms or manufactured from waste products like glycerol. While methane has economic advantages as a carbon source for PHA production, there are practical limitations that make methanol a superior carbon source for actual production. In one of the earliest studies on utilizing methanotrophs for PHA production, Asenjo and Suk observed that methane has a low solubility in water and that conversion of methane leads to high oxygen demands. Their suggestion was to use methanol, which has a higher solubility in the liquid phase, or to operate the system under pressure [40]. Methane is also expensive to store and transport, while methanol is easier to work with in those
Both the amount and the molecular weight of PHB produced by methanotrophs fed with methanol or a combination of methane and methanol have been shown to be superior to those fed by methane alone [11,67,68]. In addition, *Methylobacterium* species have been intensely studied, but are not able to metabolize methane directly. They, like most methylotrophs, are capable of metabolizing methanol, adding incentive to using methanol instead of methane.

One method that has been used to reduce energy-associated costs for methanotroph production is using non-sterile mixed microbial cultures (MMC). Wentlandt et al., found that using *Methylocystis* sp. GB 25 DSM 7674 in a short non-sterile process allowed accumulation of high-quality PHB that neared theoretical yields at 51% [37]. Another study found that in an open system of MMC, that *Methylocystis* sp. GB 25 was dominant, with 7 other species present (2 methylotrophs and 5 other species). This MMC was stable for long periods of time and produced high-quality PHB. It was theorized that mixed cultures were beneficial because they allowed some bacteria to remove toxic byproducts of methylotrophic metabolism, while also providing essential vitamins or growth supplements to the PHB-producing methylotrophs [13]. It has been suggested that investigation of a mixed culture of PHB-producing methanotrophs and PHB-producing cyanobacteria may result in a particularly viable co-culture for PHB production [10]. MMCs of methanotrophic bacteria are likely to be viable for industrial production of PHB.

A large amount of research has gone into optimizing media for PHB production using a variety of methanotrophic bacteria. Much of this has been reviewed elsewhere [10,39,66], but it has been discovered that several environmental factors affect
accumulation and molecular weight of PHAs. Among these factors are pH, temperature, CH₄ concentration, and trace elements. Variations in optimizing media exist across specific strains, and attempts at optimizing media must focus on individual species. For example, a few major attempts have been made to optimize cell growth and PHA production in *Methylobacterium extorquens* over the years, and have found that EDTA is inhibitory to growth [18], and that starvation of both magnesium and nitrogen have been shown to increase PHA production [17]. Optimization of media and protocols for cell growth and production in methylotrophic bacteria is an important step to industrial use of these species.

Most studies that have tried to optimize the parameters of these nutrients have done so in small-scale batch reactors, though a few have examined the use of larger bioreactors. At a larger scale, Stottmeister’s group used pressurized bioreactors at 7 L and 70 L volumes to produce PHAs in a mixed consortia of bacteria [13,36,37,45] and Pfluger *et al.* used a fluidized bed reactor with a total volume of 15.2 L under non-sterile conditions to produce PHB in a mixed culture of methanotrophs [69]. Both of these mixed-culture conditions were shown to be viable. Rahnama *et al.* examined using a pure culture of *Methylocystis hirsuta* in a 1 L bubble column reactor and a forced liquid loop reactor to produce PHB [52], Daniel *et al.* produced PHB with *Pseudomonas* 135 in a 15 L bottle with a 10 L initial working volume in fed-batch mode [56], and Bourque *et al.* utilized a 14 L bioreactor [19]. Finally, three studies looked at using pure cultures of methylotrophic bacteria in 5 L stirred tank reactors, including Song *et al.* [53], Ghatnekar *et al.*, [59], and Ueda *et al.* [55]. Of the larger-scale pure cultures, PHB concentrations (% of total biomass) of greater than 40% were observed in all the studies listed except
Ghatnekar *et al.* (see Table 3.1). Thus, some work has been done with PHB production using methanotrophic bacteria in larger bioreactors.

One problem with PHA production in methylotrophic bacteria is that they often produce low-quality PHAs. It is reported that PHA granule size varies from 50 to 1,000 kDa based on growth parameters and host strain [27]. In general, longer polymer chains of PHAs yield higher molecular weights and better properties. Early research into methylotrophic production of PHAs using *Methylobacterium extorquens* and *Pseudomonas* 135 indicated that methylotrophic bacteria were unable to produce PHAs of a sufficient molecular weight to be usable in commercial applications [56,57]. More recently, however, it has been reported that in certain growing conditions, methanotrophic bacteria *Methylocystis* sp. GB 25 DSM 7674, *Ms. trichosporium* OB3b, and *Ms. trichosporium* IMV 3011 can produce high molecular weight PHBs (above 1x10⁶ Da) [36–38,67]. Studies in *Mtb. extorquens* indicated that molecular weights of PHB produced were lower when methanol was used as a carbon source, compared to succinate (at best 6 x 10⁵ Da compared to 1.7 x 10⁶ Da). Anderson and colleagues discovered, however, that when lower concentrations of methanol were available that the molecular weights of PHB produced were higher and had a lower polydispersity compared to those produced when methanol was abundantly available (6 x 10⁵ Da compared to 2 x 10⁵ Da) [70]. Thus, methylotrophic bacteria are capable of producing high-quality PHB under certain conditions.

It is known that many types of PHAs can be created when different carbon sources are metabolized. When propionate is introduced as a feedstock, it is possible to produce 3-hydroxyvalerate (3HV) using the PHB pathway. This occurs due to one of the
two acetyl-CoA units that are condensed into PHB being replaced with propionyl-CoA, producing polyhydroxybutyrate-co-valerate (PHBV) [32]. Until recently, only PHB production was reported in obligate methanotrophic bacteria, but ongoing research by Myung and colleagues has shown that a greater variety of PHAs may be synthesized in methanotrophs. In a mixed microbial culture that was dominated by *Methylocystis*, they found that when valerate was added, PHBV was produced [48]. It was later confirmed that both *M. parvus* OBBP and *Ms. trichosporium* OB3b were capable of producing PHBV when valerate and propionate were added to pure cultures with methane [71]. When other ω-hydroxyalkanoate monomers were added to pure cultures of *M. parvus* OBBP, PHAs containing repeating units beyond 3-hydroxybutyrate (3HB) and 3HV were found [72]. These studies represent the first times that PHAs other than PHB have been reported in obligate methanotrophic bacteria.

Multiple types of PHAs have also been detected in *Methyllobacterium* strains. PHV has been detected in *Mtb. extorquens* and *Methyllobacterium* sp. GW2 when propionate or valerate are added as co-substrates [24,73]. Zuñiga et al. discovered that the *Mtb. organophilum* CZ-2 strain produced six different monomers when methane was used along with either citrate or propionate as co-substrates. These monomers included 3HB, 3HV, 4HV, 4-hydroxyheptanoate (4HH), 3-hydroxyoctanoate (3HO), and 4HO [74]. Höfer and colleagues have investigated using genetic engineering to transform the *phaC1* or *phaC2* gene from *Pseudomonas fluorescens* GK13 into *Mtb. extorquens* ATCC 55366 to introduce double bonds into PHAs produced. They found that they were able to do so, which allowed functional groups to be added to the PHAs using chemical steps
after production, creating more desirable properties [24,75]. Thus, *Methylobacterium* can produce multiple types of PHAs when specific substrates are made available.

These findings are significant because variations of PHAs may have greater value and use. PHAs vary in chemical structure and in number of monomer subunits. There are 155 different confirmed types of PHA monomer subunits, each with varying monomer repeat number and side groups [32]. The variations among these polymers impact their crystallinity, mechanical strength, and biodegradability, making some types of PHAs more suitable for commercial use than others [67,76]. For example, on its own, PHB is brittle (glass transition temperature \(T_g\)=4°C, melting temperature \(T_m\)=175°C), but when synthesized into a copolymer 3HV, a polymer with better properties for consumer products is created [32]. Each type of PHA has its own advantages and disadvantages, and the variety that exists allows final bioplastic products that can be tailored for many different applications.

Very little literature has been devoted to economic analyses of PHA production using methane and methanol as carbon sources. As previously mentioned, Rostkowski’s group did some analyses on types of methane and indicated that using biogas methane is a preferable. They also indicated that PHB recovery and demands for energy are the biggest challenges to production [65]. Further cost analyses on the influence of methane gas, methanol, and sugar as carbon sources on the final cost of production would be important to demonstrate the viability of these feedstocks for PHA production.
3.4 Current Methods for PHA Recovery

Recovery of PHAs from bacteria is a costly process. PHAs are intracellular molecules that accumulate inside of the cell and are usually not ejected into the surrounding environment. Thus, the cells must be disrupted to recover PHA. Three general steps are involved in the process of recovering and purifying PHA (see Figure 3.6). First, a pretreatment step is applied to help lyse the cell. Second, the PHA is extracted from the cells. Third, further purification or polishing is often necessary after extraction to obtain high purity. The goal of optimizing these processes is to achieve a high purity and low cost.

Figure 3.6. Purification strategy of PHAs. The recovery of PHAs could be composed of three steps: pretreatment, extraction, and purification.
The first step is pretreatment, which is applied after centrifugation to make cell disruption easier. This step is not always used; however, it can lead to a better recovery of PHA granules. One pretreatment option is heat treatment, which destabilizes the outer cell membrane while denaturing genetic material and proteins. [77,78]. A second option is alkaline treatment, often using a solution of sodium hydroxide. For example, one study using 0.12 kg NaOH/kg biomass of *Alcaligenes latus* found that most of the PHB could be released within three passes through a bead mill, compared to at least ten passes with untreated cells [79]. Salt pretreatment is another option that has been investigated. For this treatment, cells are placed in saline solution, putting osmotic pressure on the cells and causing them to shrivel and dehydrate [79,80] Finally, a freezing pretreatment can be used to rupture cells. This is often performed by performing a deionized water washing and then freezing. The important disadvantage to this method is that it has high energy demands [81–83]. Each of these pretreatment methods is intended to loosen up cell membranes to make extraction more successful.

The second, and most important, step in PHA recovery is extraction. One classic approach to extraction is to use solvents to disrupt the cell membrane and solve PHAs. Chlorinated hydrocarbons solvents are one category that has been used, including chloroform, 1,2-dichloroethane, and methylene chloride [84], sometimes in combination with methanol or ethanol. Liquid halogenated solvents, such as chloroethanes and chloropropanes, have also been used [85], as have diols, acetalized triols, di- or tricarboxylic acid esters, butyrolactone [86], and many other compounds [87,88]. The PHAs are generally separated from the solvent by evaporation of the solvent or precipitation in a non-solvent, such as cool water [84,89,90]. Benefits of using solvents
include removal of endotoxins [91] and minimal degradation of the polymer. Problems facing solvent extraction include cost [92,93], viscosity of the extracted polymer solution and a resulting difficulty of separation from cell debris [94], destruction of the natural morphology of PHA granules [95], and hazards to operators and the environment [96]. Thus, solvent extraction is often used in lab studies, but not in industrial extraction of PHAs.

Another extraction method is digestion. Chemical digestion using surfactants like sodium dodecyl sulfate (SDS) or synthetic palmitoyl carnitine breaks up the cell membrane and solubilizes proteins and other cell material [97–100]. While this allows recovery of PHAs from high cell densities, surfactant digestion does not lead to high purities on its own and can cause problems in wastewater treatment and reuse [101]. Alternatively, digestion using sodium hypochlorite breaks down non-PHA cellular material, allowing for high-purity levels of PHAs [81,82]. Unfortunately, sodium hypochlorite is known to degrade PHB, resulting in low molecular weights [81,102,103]. Combining chloroform with sodium hypochlorite overcomes some of this problem, since the chloroform solves and sequesters the PHAs (reducing degradation) while the sodium hypochlorite breaks down cell materials [82]. Lytic enzymes like bromelain or pancreatin have also been used for digestion. Methods of enzyme digestion generally lead to good recovery levels, but high costs [77,104]. Digestion techniques are often used to extract PHAs from bacteria.

Mechanical disruption techniques like bead mills and high pressure homogenization are also widely used in extraction. Bead mills involve grinding cell slurries in a rotating chamber filled with beads. The performance of the chamber is
consistent and predictable, making the process easy to scale-up [101]. A second approach to mechanical disruption is high pressure homogenization (HPH). An air-driven positive displacement pump forces cell slurry through two parallel slots under high pressure. The two streams impinge on a vertical plate, recombine into one stream and then flow out. The shear forces on the liquid during this process disrupt cells. This approach works particularly well with higher concentrations of biomass, but often has issues with blockages [59]. Ultrasonication has also been used on a lab scale, often in combination with centrifugation or chemical treatments [105]. Bead mills and HPH, however, remain the most common mechanical disruption techniques used of PHA extraction.

Biological extraction methods for PHAs have been investigated in recent years. One group investigated using the predatory bacterium *Bdellovibrio bacteriovorus* HD100 to lyse the cells of three PHA-producing species of bacteria (*P. putida*, *C. necator*, and *E. coli*). A strain of *B. bacteriovorus* was mutated to knock out a PHA depolymerase gene in order to prevent unwanted breakdown of PHA granules, and up to 80% of the accumulated PHA was recovered from the prey bacteria [106]. In another novel biological approach, Murugan and colleagues fed PHA-containing *C. necator* to mealworm beetle (*Tenebrio molitor*) larvae and purified the bioplastic granules from the insect’s feces using water, detergent and heat. They observed almost 100% pure PHA with minimal loss of molecular weight as a result of their process [107]. These cases demonstrate that biological methods can be used to release PHAs from bacterial cells.

Other methods of extraction exist, such as supercritical fluid or utilization of cell fragility. Supercritical (SC) fluids are substances held at a temperature and pressure above their critical point, where distinct liquid and gas phases do not exist. The most
widely-used SC fluid is CO₂, since it is widely available and inexpensive, has low toxicity, is nonflammable, and has moderate critical temperature and pressure (31 °C and 73 atm) [80,108]. One study used SC fluids to recover PHB and found that the amount recovered was similar to other studies, at 89% [108]. Cell fragility is another approach to extraction of PHAs. Some bacteria become fragile after accumulating large amounts of PHA, and addition of compounds like NaOH or NH₃ at raised temperatures released PHA into the medium [109,110]. These approaches represent a few other techniques that have been used for extraction.

One other significant method of extraction is spontaneous liberation or secretion of PHAs. One study found that when *E. coli* cells harboring *Alcaligenes phbCAB* genes were grown in a 2 x LB medium containing 21% glucose, that up to 80% of the cells spontaneously secreted PHB granules [111]. Resch’s group used a combination of the *phaCAB* genes and the cloned lysis gene *E* of bacteriophage PhiX174 from plasmid pSH2 in another study. They showed that small PHB granules exited the cells through the E-lysis tunnel structure [112]. Sabitova et al. discovered a mutant of the marine oil-degrading bacterium *Alcanivorax borkumensis* SK2 that hyperproduced PHAs. The result of the hyperproduction was that the bacteria pushed PHA into the extracellular fluid when grown on alkanes [113]. Finally, Rahman and colleagues created fusion proteins of a haemolysin secretion tag with phasin proteins (PhaP) that bind to PHAs. When transformed *E. coli* that contained the fusion proteins, *C. necator phaCAB* genes, and a plasmid with the hemolysin BD pore proteins were cultivated, it was found that up to 36% of PHB was secreted after 48 hours [114]. Thus, secretion is a possible method for liberation of PHAs.
The third step of that may be applied to PHA recovery is a final purification or polishing step. Most often, methods combine a hydrogen peroxide treatment combined with the action of enzymes of chelating agents. These methods have drawbacks, including high operation temperature (80-100 °C), instability of peroxides in the presence of high levels of cellular biomass and a decrease of polymer molecular weight [101,115]. One process proposed by Horowitz and Brennan overcame some of these problems by using an ozone treatment to increase the purity of the polymer [115]. When applied, polishing results in bioplastics suitable for applications that require high purities, such as coating.

Many different approaches to purifying PHAs from the cells that produce them have been developed. Unfortunately, they are generally still too expensive and inefficient to produce sufficient quantities of PHA to be widely used. Although many alternative methods have been developed for PHA recovery, none of them have resulted in significant economic improvements in downstream processing [116]. It is estimated that extraction and purification costs represent as much as 50% of the total process expense [111]. In addition to being costly, these processes also account for >90% of the adverse environmental impact of PHA production [65]. The development of inexpensive and scalable processes that allow recovery of intracellular PHAs is still deemed necessary [117,118].

3.5 Genetic Engineering in Methylobacterium species

Many of the genetic tools used with methylotrophic bacteria were developed for use in Methylobacterium. In the early 2000s, Figueira and colleagues developed a
protocol for electroporation in *Methylobacterium* using wide-host expression vectors pJB3KmD, pRK310, and pVK101 and were able to express GFP proteins [23]. Soon afterwards, Marx and Lidstrom developed a set of improved plasmids for use in *Methylobacterium*. Expression vectors for use in *Methylobacterium extorquens* AM1 used the strong methanol dehydrogenase (P$_{maxF}$) promoter isolated from *Methylobacterium*, since *lac* promoters from *E. coli* proved ineffective [26]. Using one of these plasmids, Choi and colleagues were able to express an esterase gene from *Lactobacillus casei* CL96 in *Mtb*. They did so at levels of enzyme activity 1.2- to 1.5-fold higher than those found in *E. coli* [21]. The same group were also able to use the mini-Tn7 transposon system to integrate three different genes into a specific locus on the bacterial chromosome of *Methylobacterium*. These tools have formed a basis for genetic engineering in *Methylobacterium*.

Further research has continued to provide tools for metabolic engineering of *Methylobacterium*. The P$_{maxF}$ promoter that is commonly used in *Mtb.* is regulated by the presence of methanol, causing it to effectively be a constitutive promoter when used in *Methylobacterium*. To increase flexibility of use, Choi’s group developed an inducible regulatory system to the P$_{maxF}$ promoter using a regulatory element from *Pseudomonas putida* F1. The cym element was added to the P$_{maxF}$ promoter, creating a cumate-inducible promoter for *Methylobacterium* [22]. Research by Höfer and group used this system to clone *phaC1* and *phaC2* genes from *Pseudomonas fluorescens* GK13 to produce PHAs with C-C double bonds [24,75]. Another recent study worked to create a library of standardized plasmid vectors and promoters for use in *Mtb. extorquens* AM1
3.6 Recombinant Proteins

3.6.1 HlyA Secretion Pathway

Secreted proteins are the primary means by which bacteria interact with their external environments. Functions of secreted molecules are vital to cells, and include adhesion, pathogenicity, adaptation, and enzymes related degradation in some cases. Due to this, bacteria have developed a variety of methods used to secrete proteins into the extracellular matrix. For example, there are six secretory pathways by which Gram-negative bacteria are known to translocate proteins to the extracellular matrix [120,121]. Proteins secreted via these pathways tend to have three fates: to remain associated with the bacterial outer membrane, to be released into the extracellular space, or to be injected into a target cell. The difficulty associated with secretion in Gram-negative bacteria compared to Gram-positive bacteria is that Gram-negative strains have two lipid bilayers (making them didermic bacteria) while Gram-positive only have one lipid bilayer (making them monodermic bacteria). Each of the six known secretion systems in didermic bacteria handle this issue in different ways.

Type I Secretion Systems (T1SSs) are composed of three segments that interact together to transport proteins into the extracellular matrix. In this system, proteins with a glycine-rich C-terminus signal peptide are transported across the inner membrane via an ATP-binding cassette transporter (ABC transporter), then fed through a membrane fusion protein (MFP) to an outer membrane polypeptide known as the TolC protein, which
secretes the proteins into extracellular space (see Figure 3.7) [122]. These systems are closely related to the resistance-nodulation-division (RND) family of multidrug efflux pumps, and are often used to secrete toxins and virulence factors [120]. An example of a T1SS found in *E. coli* is the hemolysin system, in which the toxin protein HlyA is secreted through the HlyB (ABC transporter), the HlyD (MFP), and the TolC into the extracellular environment, where the HlyA can lyse red blood cells [123–126]. Several other double-membrane-spanning systems are also classified as T1SSs.

![Figure 3.7. Diagram of the type I secretion system (T1SS). OM is outer membrane, IM is inner membrane, MFP is membrane fusion protein, and IMC is inner membrane channel.](image)

Type II secretion systems (T2SSs) are one of the most ubiquitous secretions systems known in Gram-negative bacteria. They are two-step systems involving four parts wherein a cytoplasmic ATPase, such as the SecYEG complex, exports proteins with
the proper N-terminus recognition sequence into the periplasmic space [127,128]. Following this, a periplasmic pseudopilus mounted on an inner membrane platform transports the protein to an outer membrane complex that secretes the protein into extracellular space [120,129]. The two most well-known T2SSs are the Sec (general secretory) pathway and the twin arginine translocation (TAT) protein export pathway [127,130,131]. Of the two, the Sec pathway is more common, but limited to transporting unfolded peptides into the periplasm [131]. The TAT export pathway is capable of transporting folded proteins across the inner membrane, sparking a greater amount of interest for use in biological engineering than the Sec pathway [130,132,133]. The T2SSs are well-characterized and have potential for use in secreting proteins.

Type III, IV, and VI secretion systems allow direct transport of cell materials into a host cell. Type III secretion systems (T3SSs) are double-membrane embedded nanomachines that allow transfer of bacterial effector proteins to the cytoplasm or plasma membrane of a target cell. They are formed primarily as a needle-like filament on a base that forms a secretion conduit from the cell membrane of the bacterial cell to the interior of the host cell.

Type IV secretion systems (T4SSs) mediate the conjugation of plasmid DNA and some proteins between bacterial cells via pili. These systems are found across the spectrum in bacteria, and are famous as a means of conferring antibiotic resistance in bacteria. Type VI secretion systems (T6SSs) are cell envelope-spanning machines that translocate toxic effector proteins into target cells, and are important in pathogenesis. They are built of two main complexes: a membrane complex made of inner membrane proteins and a tall, bacteriophage tail-like structure that can contract when necessary
These three types of secretion systems (T3SSs, T4SSs, and T6SSs) are known for forming a conduit to transport proteins and DNA from the bacterial cell of origin to a host cell.

Type V secretion systems (T5SSs) are different from other secretion systems in that they are single-membrane-spanning secretion systems, while the other five system types are double-membrane-spanning secretion systems. Most famously, the T5SSs include autotransporter systems in which the substrate and the outer membrane pore are a single peptide, though there are other types of T5SSs. These systems are often involved in bacterial virulence [120,135]. T5SSs are often simpler in composition than the other secretion systems, due to the fewer individual proteins involved in secretion.

The T1SSs and T2SSs are the most commonly-used secretion systems in biological engineering research. The fact that each system recognizes a known sequence on one terminus of the target peptide means that the signal sequence may be fused to other proteins, causing the cell to target the new fusion protein for secretion. This is advantageous for downstream processing of proteins because they are subjected to less degradation via protease activity outside of the cytoplasm, the oxidative environment of the periplasm allows the correct formation of disulfide bonds, and when secreted into the extracellular environment, disruption of the cell membrane is no longer necessary to purify the protein [133]. The use of secretion tags in recombinant protein production has been reviewed extensively elsewhere [133,136]. In short, the T1SSs and T2SSs are most commonly used with recombinant proteins in *E. coli* due to their ability to export proteins to the periplasm or extracellular environment.
Fusion proteins involving secretion peptides have been used to indirectly secrete PHB molecules. A foundational study was performed by Linton and colleagues that tested the efficacy of four of the most common secretion tags in *E. coli*. The signal peptides utilized were the HlyA (T1SS), TorA (T2SS, TAT), GeneIII (T2SS, Sec), and PelB (T2SS, Sec) peptides. The PelB secretion system was ineffective at translocating GFP, while the other two T2SSs successfully exported GFP to the periplasm. They HlyA system was able to successfully secrete GFP into the extracellular media [137]. Building on this research, the Miller group used a fusion protein of the HlyA signal peptide and a phasin protein that associates with PHB granules to link the signal sequence to PHB. This resulted in the secretion of PHB granules into extracellular media via a T1SS in *E. coli* [114].

### 3.6.2 Phasins

PHA granules are not purely composed of the bioplastic polymer, but are covered with a layer of granule-associated proteins (GAPs). This proteinaceous surface layer of GAPs makes up approximately 2% (w/w) of the granules and is a network-like surface of structural, metabolic and regulatory polypeptides (see Figure 3.8) [138]. The GAPs that are involved in PHA granules have been divided into four categories: PHA synthases, which catalyze the formation of ester linkages between the monomeric units of PHAs; PHA depolymerases, which degrade PHAs; phasin proteins (which will be discussed in greater detail); and other proteins (transcriptional regulators, hydrolases, reductases, etc.) [139]. Phospholipids are also potentially included in the external layer, though it is possible that their involvement is an experimental artefact of PHA extraction and
preparation rather than a part of PHA granules \textit{in vivo} \cite{138}. The most abundant polypeptides in PHA granules are the phasin proteins.

\textbf{Figure 3.8.} Schematic of the structure of PHA granules.

Phasin proteins are known to play an important role in the regulation of PHA synthesis, morphology, and degradation. Phasin proteins are amphiphilic molecules that bind to the surface of PHA granules. The seven phasin proteins in the \textit{Cupriavidus necator} H16 (formerly \textit{Ralstonia eutropha}) have been studied most intensely, particularly PhaP1\textsubscript{Reu}. Mutant \textit{C. necator} cells that lack PhaP1\textsubscript{Reu} have been observed to produce larger granules and less PHB compared to the wild type strains \cite{140–142}, while overexpression of PhaP1\textsubscript{Reu} results in a high number of smaller granules \cite{143}. The PhaM has specifically been identified as an activator of PHB synthase activity in \textit{C. necator}
It has also been noted that phasin protein deletion mutants of *C. necator* exhibit some PHB autodegradation *in vivo*, indicating that phasin proteins are important to granule stability [142]. Ironically, PhaP1*Reu* is also known to be essential to PHB degradation [145,146]. Thus, many functions have been observed for phasin proteins relative to PHA granules.

Phasin proteins are also known to regulate PHA granule distribution during cell division. In *Pseudomonas* species, the PhaF phasin protein is composed of two domains, one like PhaP1*Reu* in structure and function on the N-terminal, and a highly-charged, histone-like domain on the C-terminal [147,148]. Working together, these two domains link PHA molecules to nucleoid DNA during cell division, ensuring an equal distribution between daughter cells [149,150]. Similar behavior has been observed with the phasin-like polypeptide PhaM and the Pha5 protein in *C. necator* [151,152]. These studies demonstrate the capability of phasin proteins and phasin-like proteins linking PHA granules to other molecules in the cytosol.

Most PHA-producing organisms are known to produce some form of phasin proteins. In addition to *C. necator* and *Pseudomonas* species, *Paracoccus denitrificans* [153,154], *Rhodococcus ruber* NCLMB 40126 [155,156], *Azotobacter* species [157,158], *Aeromonas* species [159,160], *Rhodospirillum rubrum* [161,162], *Magnetospirillum* [161,163], *Bradyrhizobium* [164,165], *Sinorhizobium meliloti* [166], *Haloflex* *mediterranii* [167], and *Herbaspirillum serepedicae* [168,169] have all been noted for phasin protein or phasin-like protein production related to PHA synthesis. In most cases, phasin protein production has a positive correlation to PHA production and have been
noted for similar functions to those discussed previously relative to *C. necator* and *Pseudomonas*.

Phasin proteins have been used for a few different functions in biological engineering. In addition to their importance in producing PHAs, the amphiphilic nature of phasins allows them to function as natural surfactants. The PhaP<sub>Ah</sub> from *A. hydrophila* 4AK4 has been shown to form emulsions with lubricating oil, diesel, and soybean oil that are relatively stable when compared to those formed by bovine serum albumin, sodium dodecyl sulfate (SDS), Tween 20, and sodium oleate [170]. Taking advantage of the native abilities of PhaF from *Pseudomonas* to link PHA molecules to DNA, one study used the phasin to anchor proteins to PHA granules, immobilizing the enzymes [148]. As has been discussed previously, phasin proteins have also been used to anchor signaling peptides to PHB granules in recombinant *E. coli*, inducing secretion of PHB into the extracellular milieu [114]. The amphiphilic nature of phasin proteins has been utilized in several biological engineering applications.

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CHAPTER 4

AN INVESTIGATION OF PHASIN TRANSLOCATION AND POLYHYDROXYALKANOATE PRODUCTION IN A NOVEL ISOLATE OF METHYLOBACTERIUM

4.1 Abstract

Background

Polyhydroxyalkanoates (PHAs) are a type of biodegradable plastic that are produced by several microorganisms as a carbon and energy storage molecule when the cells are under stress. They are a promising alternative to synthetic plastics but are currently too expensive for widespread use. The two largest hurdles to economic production are the cost of carbon feedstock and the cost of isolating and purifying PHAs. Using methylotrophic bacteria that can utilize methanol as their sole carbon sources may reduce the cost of carbon feedstock. Indirectly secreting PHAs using phasin proteins that bind to PHAs and type I secretion system signal peptides to translocate PHAs across the cell membrane may reduce the cost of downstream processing.

Results

A novel strain of Methylobacterium was isolated and tested to show its ability to produce PHAs using methanol as a sole carbon source. A constitutive promoter known as cp8 was tested for use with a synthetic biology system in the Methylobacterium. The promoter demonstrated expression of superfolding green fluorescent protein (sfGFP) and was used in phasin protein genetic constructs to create a secreting strain of Methylobacterium. When tested, there was no significant difference in the amount of PHB secreted by the secreting and non-secreting strains. This may be because the
promoter was not strong enough to produce a noticeable amount of the secretion-inducing fusion proteins, issues with the protocol used to prepare samples for analysis, or due to an unidentified secretion mechanism in the bacteria.

4.2 Background

Polyhydroxyalkanoates (PHAs) are a class of biodegradable, biologically-produced plastics that accumulate in many microorganisms [1]. In native organisms, they act as carbon reserves that accumulate in response to nutrient deficiency or environmental stress [2]. When extracted, PHAs display properties similar to synthetic plastics, such as polypropylene [3]. Unlike synthetic plastics, however, PHAs are non-toxic, renewable and biodegradable [4,5]. In addition, the chemical structures of PHAs are variable, allowing them to be tailored for a range of applications in packaging, the medical field, agriculture, and elsewhere [6]. Consequently, there is great interest in using PHAs as an environmentally-friendly alternative to synthetic plastics [7].

Currently, the most significant hurdles to industrial-scale production of PHAs are the costs associated with carbon feedstock for the microorganisms producing the PHAs and the cost of extracting and purifying the PHAs from the microorganisms once they have accumulated in the cell cytoplasm [8,9]. One possible solution to the cost of carbon feedstock is to use methanol or methane rather than the traditional approach of using plant-derived oils and sugars. Both methane and methanol are inexpensive, stable and readily available feedstocks that are independent from seasonal variations and weather conditions. They can be utilized by methylotrophic bacteria, many of which are known to
produce PHAs [10,11]. Thus, methane and methanol have been suggested as an alternative, sustainable carbon sources for PHA production.

One well-known type of methylotrophic bacteria is *Methylobacterium*. *Methylobacterium* are serine-pathway utilizing facultative methylotrophs that are simple and inexpensive to culture. There are optimized fermentation protocols [12–14], genomic information, and genetic tools [15–21] available for use with these bacteria. They have also been shown to produce valuable products like PHAs, vitamin B12, pyrroloquinoline, and carotenoids [17]. Strains of the most well-characterized species, *Methylobacterium extorquens*, have been known to achieve PHA concentrations as high as 46% dry cell weight (DCW) [14]. *Methylobacterium* strains normally produce polyhydroxybutyrate (PHB), but can produce polyhydroxyvalerate (PHV) when propionate or valerate are added as co-substrates [19,22]. These characteristics make *Methylobacterium* strains organisms of interest for methanol-based biomanufacturing [23].

Current approaches to extracting and purifying PHAs are invasive, involving the disruption of cell membranes by mechanical, biological, or chemical means [24,25]. It is estimated that extraction and purification costs represent as much as 50% of the total process expense [26] and that these processes account for >90% of the adverse environmental impact of PHA production [8]. Although many alternative methods have been developed for PHA recovery, none of them have resulted in significant economic improvements in downstream processing [27]. Thus, there is a need for discovering a system that can improve isolation of PHAs.

One process for extraction of PHA granules that has been investigated in recent years is spontaneous liberation or secretion of PHAs. One study found that when *E. coli*
cells harboring *Alcaligenes phbCAB* genes were grown in a 2 x LB medium containing 21% glucose, that up to 80% of the cells spontaneously secreted PHB granules [26]. Another study used a combination of the *phaCAB* genes and the cloned lysis gene *E* of bacteriophage PhiX174 from plasmid pSH2. It was shown that small PHB granules exited the cells through the E-lysis tunnel structure [28]. Sabitova and colleagues discovered a mutant of the marine oil-degrading bacterium *Alcanivorax borkumensis* SK2 that hyperproduced PHAs. The hyperproduction caused the bacteria to push PHA into the extracellular fluid when grown on alkanes [29]. Secretion of PHA granules, such as was achieved in these studies, would negate the need to use harsh chemicals or energy-intensive processes to lyse the cells open during an extraction process.

Another secretion system was developed by our group for *E. coli* by taking advantage of a type I secretion system to translocate PHAs from the cytoplasm to the extracellular medium [30]. Type I secretion systems, such as the hemolysin system in *E. coli*, are composed of ATP-binding cassette transporter (ABC transporter) and a membrane fusion protein (MFP) that interact with an outer membrane polypeptide known as the TolC protein [31]. Proteins, often toxins and virulence factors, that have a glycine-rich C-terminus signal peptides are transported from the cytosol of the bacteria into extracellular milieu [32]. Type I secretion systems are of interest as a method to secrete molecules produced within the cell.
The hemolysin system has been used to secrete proteins and PHA granules. Previously, our group demonstrated that when fusion proteins of green fluorescent proteins (GFP) and the C-terminus signal peptide of the hemolysin A (HlyA) protein were expressed in a strain of *E. coli* that also expressed the appropriate ABC transporter (HlyB) and MFP (HlyD), GFP was secreted [33]. In a subsequent study, a fusion protein of phasin proteins that bind to the surface of PHA granules and the HlyA signal was created. The phasins bound tightly to and carried the PHA granules with them as they were secreted from the cell (see Figure 4.1). Using this system, 36% of the total PHA produced in the secreting strain was collected in the secreted fraction and 64% remained in the bacterial cell after 48 hours. In addition, total productivity was increased relative to the non-secreting strain [30].

The secretion system was assembled using a synthetic biological engineering method. Synthetic biological engineering is an approach to genetic engineering that seeks to improve our ability to engineer life. A scientific understanding of the nature of DNA, restriction enzymes, ligation, and polymerase chain reactions (PCR) combined in the late 20th century to make genetic engineering of organisms possible. Generally, however, the process of combining genes of multiple organisms has involved expensive, unreliable, and *ad hoc* approaches. A push to integrate engineering principles more fully into genetic engineering since the early 21st century has led to ideas for simpler, more accessible
One of the most significant ideas put forward is that of creating standardized, biological “parts” that can be assembled into genetic circuits [35]. Standardization allows streamlined research, as information related to DNA sequences can be shared rapidly and DNA can be synthesized to order. This allows for testing our understanding of biological systems; the creation of novel molecules and molecular systems for diagnostic assays and drugs; and development of new technologies that make it easier and more efficient to satisfy human needs [34,36]. One example of this type of standardization in biotechnology is the concept of BioBricks.

BioBricks are defined sequences of DNA used as biological parts. The standardized BioBrick assembly uses a DNA sequence flanked by the restriction sites EcoRI and XbaI upstream and BcuI (formerly SpeI) and PstI downstream with no repetition of the restrictions sites in the DNA sequence. Since BcuI and XbaI are compatible, BioBrick segments may be pieced together sequentially due to the regeneration of the prefix and suffix restriction sites during each step of the process (see Figure 4.2). The BioBrick system enables systematic assembly of DNA sequences, making it a convenient approach for the construction of the DNA for the PHA secretion systems used in this study [37]. To facilitate sharing of information, sequences of BioBrick parts are available in an online registry maintained by the International Genetically Engineered Machine (iGEM) Foundation at http://parts.igem.org/Main_Page. The current study investigates the possibility of using a secretion system constructed from BioBricks in Methylobacterium.
4.3 Materials and Methods

4.3.1 Strains and Plasmids

Descriptions of strains and plasmids that were used to study PHA and phasin production and translocation are provided in Table 4.1. Electrocompetent XL 1-Blue *Escherichia coli* (Stratagene, La Jolla, CA) were used as the host for assembly of BioBrick parts and devices. Plasmids pSB1C3, pSB1A3 and pSB3K3 are BioBrick standard vectors for assembly and expression of BioBrick genetic devices [38]. The
broad-range vector plasmid pCM66 was used to transfer the competed BioBrick devices into *Methylobacterium* via electroporation. Cultures of the *Methylobacterium* were isolated from samples discovered in our laboratory.

**Table 4.1. Strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain/Plasmid</th>
<th>Relevant Characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XL1-Blue</td>
<td>endA1 gyrA96(nalR ) thi-1 recA1 relA1 lac glnV44 F'[ ::Tn10 proAB+ lacIq Δ(lacZ)M15] hsdR17(rK- mK+ )</td>
<td>Stratagene</td>
</tr>
<tr>
<td><strong>Methylobacterium Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pSB1C3</td>
<td>High copy BioBrick vector, pMB1 origin, Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Registry</td>
</tr>
<tr>
<td>pSB3K3</td>
<td>Medium copy BioBrick standard vector, p15A origin, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Registry</td>
</tr>
<tr>
<td>pSB1A3</td>
<td>High copy BioBrick vector, pMB1 origin, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Registry</td>
</tr>
<tr>
<td>pLG575</td>
<td>pACYC184 derivative, HlyBD, p15A origin, Cm&lt;sup&gt;R&lt;/sup&gt; [39]</td>
<td></td>
</tr>
<tr>
<td>pBHR68</td>
<td>pBluescript SK-, phbCAB genes from <em>C. necator</em>, Amp&lt;sup&gt;R&lt;/sup&gt; [40]</td>
<td></td>
</tr>
<tr>
<td>pRK2073</td>
<td>Helper plasmid, Spec&lt;sup&gt;R&lt;/sup&gt; []</td>
<td></td>
</tr>
<tr>
<td>pCM66</td>
<td>High copy broad host range cloning vector in bacteria, ColE1 origin, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Addgene #45827</td>
</tr>
<tr>
<td>pCMHS1</td>
<td>pCM66 with constitutive promoter and superfolder GFP (sfGFP)</td>
<td>This study</td>
</tr>
<tr>
<td>pCMHS2</td>
<td>pCM66 with constitutive promoter, sfGFP, and HlyA</td>
<td>This study</td>
</tr>
<tr>
<td>pCMHS3</td>
<td>pCM66 with constitutive promoter, PhaP1&lt;sub&gt;Re&lt;/sub&gt;, and HlyA</td>
<td>This study</td>
</tr>
<tr>
<td>pCMHS4</td>
<td>pCM66 with constitutive promoter, PhaP4&lt;sub&gt;MtO&lt;/sub&gt;, and HlyA</td>
<td>This study</td>
</tr>
</tbody>
</table>

### 4.3.2 BioBrick Design and Assembly

BioBrick parts utilized in this study included two phasin proteins (PhaP<sub>MtO</sub> from the methanotrophic bacteria *Methylosinus trichosporium* OB3b and PhaP<sub>Re</sub> from *Cupriavidus necator* H16 (formerly *Ralstonia eutropha*)), one signal peptide (HlyA); two secretion system proteins (HlyB and HlyD), one reporter protein (sfGFP), a constitutive promoter, a ribosome binding site, and a terminator (see Figure 4.3). The PhaP<sub>MtO</sub>
The phasin protein gene was obtained using PCR by annealing forward and reverse oligonucleotides to isolate DNA from *Ms. trichosporium* OB3b. PCR was also used to add the BioFusion prefix and suffix to the phasin gene as overhanging ends on a second set of primers (see Table 4.2 for primers used). The PCR product was isolated by gel electrophoresis and extracted using a Thermo Scientific GeneJET Gel Extraction Kit. The DNA product was digested with EcoRI and BcuI, and ligated into pSB1C3. The ribosome binding site and terminator (BBa B0030 and BBa B0015, respectively) were obtained from the 2015 iGEM distribution kit. The promoter (cp8) was a constitutive promoter created for use in *Lactococcus lactis*, but has been shown to be active in multiple gram-negative bacteria [41]. Step-wise assembly of composite BioBrick devices was primarily carried out in pSB1C3.

**Figure 4.3** Schematic of PHA and sfGFP secretion genetic circuits. Promoter is a constitutive promoter; RBS is ribosome binding site; PhaP4MtO and PhaP1Re are phasin protein coding sequences from *Ms. trichosporium* OB3b and *Cupriavidus necator*, respectively; HlyA is the hemolysin A secretion tag; B0015 is a double-stop terminator.
Table 4.2. Oligonucleotides (prefix/suffix overhangs are bolded; restriction enzyme sites are underlined)

<table>
<thead>
<tr>
<th>Oligo/Primer</th>
<th>Description 5'-&gt;3'</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>PhaP4MtO_FOR</td>
<td>Atggcaacccatttcaaagg</td>
<td>This study</td>
</tr>
<tr>
<td>PhaP4MtO_REV</td>
<td>Tcacgcgacgaaagtgagag</td>
<td>This study</td>
</tr>
<tr>
<td>PhaP4MtO_FOR_P</td>
<td>Tcttgactgcggcgcgtctctactagaatggcaacccatttcaaagg</td>
<td>This study</td>
</tr>
<tr>
<td>PhaP4MtO_REV_S</td>
<td>Tctgctgcgcggcgcgtactagttgctacgcgaaagtgagag</td>
<td>This study</td>
</tr>
</tbody>
</table>

4.3.3 Media Formulation and Growth Conditions

Overnight cultures of *E. coli* for genetic transformation were grown in a standard LB Media (containing, per liter: 10 g Bacto-Tryptone, 5 g Bacto-yeast extract, 10 g NaCl). Cultures of *Methylobacterium* were grown in 150-ml serum bottles with 50 mL of NMS media solution (containing, per liter: 1.0 g KNO₃, 1.0 g MgSO₄ x 7H₂O, 0.23 g CaCl₂ x 2H₂O, 0.0005 g Na₂MoO₄ x 2 H₂O, 0.002 g Ferric III ammonium citrate, 0.004 g EDTA, 0.006 ml HCl, 0.0014 g CuCl₂ x 2 H₂O, and 1 mL trace solution); trace solution (containing, per liter: 0.5 g FeSO₄ x 7 H₂O, 0.4 g ZnSO₄ x 7 H₂O, 0.02 MnCl₂ x 7 H₂O, 0.05 g CoCl₂ x 6 H₂O, 0.01 g NiCl₂ x 6 H₂O, 0.250 g EDTA, and 0.015 g H₃BO₃); and 0.5 mL of phosphate solution (containing, per liter: 26 g KH₂PO₄ and 62 g Na₂HPO₄ x 7 H₂O). To sterilize, the base NMS solution, trace solution, and phosphate solution were all autoclaved at 121°C for 30 minutes. For solid media plates, 15 g of agar per 1 L of media solution was added to trace solution prior to autoclaving. The media solution and phosphate solution were mixed in a laminar flow hood after cooling. As a carbon source, 250 μL of methanol were added to the *Methylobacterium* cultures. Cultures were incubated at 30°C, shaking at 200 rpm.
4.3.4 PHA Production in Methylobacterium

To assess PHB concentrations, cell growth was measured via optical density at 600 nm using a spectrophotometer. Cultures that enter the exponential growth phase and achieve an optical density of greater than 0.30 OD$_{600}$ were assayed for PHB production. PHB production was induced by incubation with 0.6% methanol in the absence of nitrogen and magnesium. Cultures were centrifuged at 3452 rcf for 10 min, washed once with nitrogen- and magnesium-free medium, re-centrifuged, and re-suspended in 50 mL of nitrogen- and magnesium-free NMS medium. After 24 h of incubation, cultures were harvested, immediately frozen at -80°C, and then lyophilized for PHA analysis.

4.3.5 Recovery of Secreted PHA

Techniques for secreted PHA recovery followed as described in Rahman et al. 2013 [30]. At 24 h after starvation was initiated, 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 mins at room temperature and then centrifuged at 54 rcf 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 rcf for 10 mins and the pellet from the second centrifugation contained bacterial mass and non-secreted PHA. Secretion studies and PHA analysis were conducted in triplicate samples. Statistical tests were performed using the Statistical Analysis System (SAS) software.
### 4.3.6 PHA Concentration Determination

PHA concentrations were determined using gas chromatography (GC). Sample preparation was carried out in accordance with reported acid methanolysis procedures, as described in Linton, et al. 2012 [42]. Dried cell pellets of approximately 15 mg constituted a sample. Equal volumes (0.7 mL) of acidified methanol (0.03% H₂SO₄) and chloroform were added to each sample. These mixtures were vortexed and incubated at 100 °C for 3 h. After cooling to room temperature, 0.4 mL of distilled water was added, followed by vortexing for 10 min. Following a phase separation time of 20 min, the organic phase was transferred to a new vial for GC analysis.

GC instrument parameters were as follows. An HP 6890 Series II gas chromatography system was used with an HP-INNOWax cross-linked polyethylene glycol capillary column with dimensions of 30 m x 0.25 mm ID x 0.25 µm film thickness. The injection ratio was split to 1:20, with carrier gas argon flow rate of 3.8 mL/min. The initial temperature of 60 °C was held constant for 4 min, followed by a 15 °C/min increase to a final temperature of 250 °C, which was held constant for 5 minutes. The flame ionization detector and injection port temperatures were maintained at 250 °C.

### 4.3.7 Scanning Electron Microscopy (SEM)

SEM protocols were followed as mentioned in Mortensen 2012 [43]. Briefly, bacterial cultures were grown until they achieved exponential phase 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.
4.3.6 Fluorescence Studies

Cultures of bacteria were grown to an OD$_{600}$ of approximately 0.3 A. 1 mL samples were harvested from each culture. As a control, three 300µL samples of the uninoculated media were placed into wells of a 96-well plate. Three 300µL samples each of the bacterial cultures were also placed in wells of the same 96-well plate. Fluorescence was read using a BioTek Synergy 2 well plate reader instrument, with an excitation wavelength of 485/20 and an emission wavelength of 528/20. To adjust for bacteria levels and auto fluorescence in the media, the following equation was used to produce a corrected value:

$$F_{\text{Final}} = \frac{F_{\text{Sample}} - F_{\text{Media}}}{OD_{\text{Sample}} - OD_{\text{Media}}}$$

Where F is the level of fluorescence in relative fluorescence units (RFU) and OD is the optical density of the sample at 600 nm.

4.4 Results

4.4.1 Isolation and Analysis of Methylobacterium sp.

During our research, we isolated a novel strain of Methylobacterium. The isolate was initially found as a contaminant in cultures of another type of methylotrophic bacteria, Methylosinus trichosporium OB3b. The OB3b strain was initially used due to its ability to metabolize methane gas, but difficulties in culturing led to a greater focus on Methylobacterium instead. Cell cultures that were contaminated with Methylobacterium were identified by a red color in the cell pellet (see Figure 4.4) and a lack of the particulate methane monoxigenase (pMMO) gene when tested using PCR. It is likely that the Methylobacterium survived by metabolizing methanol produced by Ms.
trichosporium OB3b. The Methylobacterium was isolated from the initial culture using streaking on agar plates. Analysis of the 16s-region narrowed the isolate down to a few different species, including Methylobacterium extorquens (see Figure 4.5). Several SEM images of the new isolate were generated to demonstrate the morphology of the bacteria. The images indicated that the isolate often exists in clusters of the bacteria, though it also grows as individual cells (see Figure 4.6). It is believed that this naturally-occurring strain of Methylobacterium can serve as a platform for production of PHAs from methanol.

Figure 4.4. Cell pellet of Methylobacterium isolate.
**Figure 4.5.** Phylogenetic tree showing the relationship of the *Methylobacterium* isolate used in the current study to other methylotrophic bacteria based on 16s sequencing. All species displayed except *E. coli* are from the rhizobiales order.

**Figure 4.6.** SEM images of *Methylobacterium* in various sizes of clusters.

The *Methylobacterium* isolate was analyzed for PHB production. The bacteria’s ability to synthesize PHB was initially confirmed using Sudan Black staining (see Figure 4.7). It is known that some strains of *Methylobacterium* have been reported to accumulate PHAs in association with cell growth [22], while most require induction through nitrogen and magnesium limitation [14,44–49]. Initial analysis of PHB production indicated that higher quantities of PHB were accumulated with 24 hours of nitrogen and magnesium...
limitation compared to nitrogen limitation and unstressed conditions (see Table 4.3). This indicates that the isolate is capable of producing PHB when placed in stressed conditions, most successfully when starved of both nitrogen and magnesium.

![Image of Methylobacterium stained with Sudan Black to demonstrate the presence of PHB.](image)

**Figure 4.7.** Image of *Methylobacterium* stained with Sudan Black to demonstrate the presence of PHB.

**Table 4.3.** Concentrations of PHB in cultures of *Methylobacterium* with nitrogen starvation, nitrogen and magnesium starvation, and normal (unstressed) conditions. Differences between the amount of PHB produced between all conditions were statistically significant (p < 0.05).

<table>
<thead>
<tr>
<th>Condition</th>
<th>% mass of PHB in dry mass</th>
<th>Production mg/L PHB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstressed</td>
<td>0.30 ± 0.55</td>
<td>0.03 ± 0.05</td>
</tr>
<tr>
<td>Nitrogen Starved</td>
<td>4.54 ± 4.20</td>
<td>9.02 ± 13.097</td>
</tr>
<tr>
<td>Nitrogen and Magnesium Starved</td>
<td>13.46 ± 5.14</td>
<td>100.95 ± 38.72</td>
</tr>
</tbody>
</table>

### 4.4.2 BioBrick Design and Construction

Initially, the methanotrophic bacteria *Ms. trichosporium* OB3b was identified as a candidate for use in this study. Four phasin or phasin-like proteins were identified in the sequences of the OB3b strain available on the Joint Genome Institute (JGI) and the National Center for Biotechnology Information (NCBI) websites (see Figure 4.8), and PCR primers were designed to isolate each of the four phasin proteins. One of four phasin protein candidates from the type II methanotroph *Ms. trichosporium* OB3b was
successfully isolated from the genomic DNA and tagged with the BioBrick prefix and suffix using PCR. This phasin protein gene (PhaP4<sub>MtO</sub>) was then cloned into the vector plasmid pSB1C3 and transformed into <em>E. coli</em> using electroporation (see Figure 4.9).

**Figure 4.8.** Schematic of the third contiguous region of the <em>Ms. trichosporium</em> OB3b genome with locations of the four phasin proteins indicated.
The genetic circuits that include a phasin protein from *Ms. trichosporium* OB3b (PhaP4\textsubscript{MtO}) and a phasin protein from *C. necator* (PhaP1\textsubscript{Re}) were constructed using step-wise assembly in *E. coli* plasmid pSB1C3 and then were inserted into pCM66 (see Figure 4.10). Two other genetic circuits were constructed with the same constitutive promoter and ribosome binding site as the phasin constructs, one with a sfGFP gene to test the strength of the promoter in *Methylobacterium* and one with the sfGFP gene fused to the HlyA secretion tag. These four plasmids constitute the pCMHS series utilized in this study (see Figure 4.11).
Figure 4.10. A) Gel electrophoresis depicting PhaP1Re genetic circuit (987 bp) digested from pCM66 using NotI and EcoRI (digested fragment size is 3068 bp). B) PhaP4MtO genetic circuit (1015 bp) digested from pCM66 using NotI and EcoRI (digested fragment size is 3096 bp). MW – Molecular Weight Marker (GeneRuler 1 k DNA Ladder, Thermo Fisher Scientific, Waltham, MA).

Figure 4.11. Schematic of PHA and sfGFP secretion genetic circuits. The promoter is a constitutive promoter (cp8); RBS is ribosome binding site; PhaP4MtO and PhaP1Re are phasin protein coding sequences from *Ms. trichosporium* OB3b and *Cupriavidus necator*, respectively; HlyA is the hemolysin A secretion tag; B0015 is a double-stop terminator.

4.4.3 Promoter strength analysis

The pCMHS1 and pCMHS2 plasmids were used to investigate whether the constitutive promoter cp8 was functional in methylotrophic bacteria. The cp8 promoter is
part of a series of synthetic promoters constructed for use in *Lactococcus lactis*, but which were shown to function in a variety of bacteria [41]. Fluorescence levels were measured in wild-type strains and in transformed strains of *Methylobacterium*. Statistical analysis of three separate cultures of each type indicated that there was a significant difference between the wild-type bacteria and the transformed strain (p < 0.05). This indicates that the constitutive promoter is functional in *Methylobacterium* (see Figure 4.12). Levels of sfGFP were significantly lower in *Methylobacterium* when compared with *E. coli* transformed with the same plasmid, however, with fluorescence registering at an average of 1,378,000 relative fluorescence units (RFU) in *E. coli* compared to an average of 4512 RFU in *Methylobacterium*. Thus, the cp8 promoter is active in *Methylobacterium*, but not as effective at generating protein products as it is in *E. coli*.

**Figure 4.12.** Fluorescence comparison between *Methylobacterium* not transformed with sfGFP (PhaP1_hlyA [pCMHS3]) and those with sfGFP (sfGFP [pCMHS 1] and sfGFP_hlyA [pCMHS 2]). Different letters indicate groups of data that were statistically significant in differences (p < 0.05)
In addition, growth rates of transformed and wild-type strains of *Methylobacterium* were investigated using optical density. This was performed to determine if the plasmids would drain a significant amount of cell resources, affecting growth. The results indicated that there was no significant difference between strains of cells that were transformed and the wild-type strain of *Methylobacterium* (see Figure 4.13).

**Figure 4.13.** Cell growth curve of *Methylobacterium* comparing cell densities of wild type strains and transformed strains.

### 4.4.4 PHB Production Analysis

To investigate secretion of bioplastics from the methylotrophic bacteria, the pCMHS3 plasmid was co-transformed with the pLG575 plasmid into the new isolate and PHB production was measured. This was compared with the strain containing an alternative plasmid (pCMHS1) that did not include the secretion machinery. PHB
measured inside the cell is defined as the internal fraction and PHB collected by the CaCl$_2$ precipitation of the media is defined as the secreted fraction. The non-secreting strain and secreting strain produced similar amounts of PHB in the internal fraction at 16.20 ± 1.78% of PHB in the dry cell weight (DCW) for the non-secreting strain compared to 12.09 ± 6.06% DCW PHB for the secreting strain (see Table 4.4). This was not a statistically significant difference (p > 0.05) in the amount of PHB present. The amount of PHB present in the internal fraction of both strains was comparable to previous studies with *Methylobacterium* strains [44,46,50].

**Table 4.4.** Production of PHB in secreting (pCMHS3 + pLG575) and non-secreting (pCMHS1) strains of *Methylobacterium* at 24 h

<table>
<thead>
<tr>
<th>Strain</th>
<th>% mass of PHB in dry mass</th>
<th>Production mg/L PHB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Secreted fraction</td>
<td>Non-secreted fraction</td>
</tr>
<tr>
<td>Non-secreting</td>
<td>12.36 ± 2.56</td>
<td>16.20 ± 1.78</td>
</tr>
<tr>
<td>Secreting</td>
<td>10.68 ± 2.36</td>
<td>12.09 ± 6.06</td>
</tr>
</tbody>
</table>

The PHB secreted fractions were analyzed for the secreting and non-secreting strains. PHB harvested in the secreted fractions of the non-secreting strains was 12.36 ± 2.56% DCW PHB, while the secreting strain produced 10.68 ± 2.36% DCW PHB in the secreted fraction. The amount of PHB present in the secreted fraction of the secreting strain was not statistically significant compared to the non-secreting strain (p > 0.05). Relative to the total amount of PHB produced (mg/L PHB), 30% of the PHB in the secreting strain was collected in the secreted fraction while the remaining 70% was collected in the internal fraction. This was similar to the percentage of PHB collected in the secreted fraction in *E. coli* during our previous study, at 36% [30]. However, 35% of
the PHB in the non-secreting strain of *Methylobacterium* was collected in the secreted fraction. This also indicates that the secreting and non-secreting strains had similar amounts of PHB in the secreted fraction.

There are at least two viable explanations for why the secreting and non-secreting strains of *Methylobacterium* were similar. First, although it has not been reported in literature thus far, it is possible that the cells have a native system for secretion of PHAs. Second, it could be that all PHB detected in the secreted faction was contained inside of cells that were lysed during preparation for GC analysis. A previous study using the same secretion system also observed PHB released by lysed *E. coli* cells in the non-secreting strain’s secreted fraction [30]. During this study, pellets of samples collected using CaCl₂ precipitation and low speed centrifugation were a vivid red, the color of the cell pellets in the non-secreted fraction. This likely means that the majority of matter collected was cell biomass. In addition, it is possible that the *Methylobacterium* cell walls may be more fragile than the cell walls of the *E. coli* strains used in previous studies, rendering the harvesting procedure ineffective at discerning differences between secreted and non-secreted fractions due to cell lysis. Thus, PHB in the secreted faction of non-secreting strains were mostly likely either present due to lysed cells or because of native secretion systems.

If it is the case that all PHB was present due to lysed cells, then it would indicate that the secretion system is not effective in *Methylobacterium* in its present form. Future studies would need to evaluate if using promoters known to have a higher efficiency in *Methylobacterium*, like the P₄₅₅₆₆F promoter, would prove more effective for expressing both the HlyBD secretion system and the fusion proteins of phasins and HlyA signaling
peptides. Since the phasin gene used in this study was originally obtained from *E. coli*, codon optimization for optimal protein expression in the new isolate could also lead to improved production of phasin proteins. This is achieved by altering the DNA sequence so that the mRNA produces the same amino acid sequence, but uses the tRNAs that the species of bacteria favors. Thus, the resources for producing the protein are more likely to be available, allowing higher quantities of protein to be assembled. Improved expression could lead to a more visible demonstration of secretion.

Using native phasin proteins could also improve secretion in methylotrophic bacteria. Since these proteins are naturally produced by the bacteria, they may be produced more effectively than non-native proteins, or may interact more successfully with PHA granules in their native environment. The Joint Genome Institute (JGI) database indicates that there is at least one native phasin protein present in *Methylobacterium extorquens* AM1, which is closely related to the isolate characterized in this study. A phasin protein (PhaP4MtO) from the related type II methanotroph *Methylosinus trichosporium* OB3b was isolated using PCR and converted into a BioBrick. Testing of the hlyA- PhaP4MtO fusion protein in a well-characterized system like *E. coli* will give greater insight into the protein’s ability to induce secretion of PHAs. Isolation or synthesis of other potential phasin proteins from *Mtb. extorquens* AM1 or the *Methylobacterium* isolate used in this study would also allow characterization of their interactions with PHA granules and analysis of their effectiveness in inducing secretion. It is possible that the native phasin protein would prove more effective than phasin proteins from *C. necator* at linking the hlyA secretion tag to PHA granules.
4.5 Conclusion

A novel strain of *Methylobacterium* was isolated and tested for PHB producing capabilities. A constitutive promoter (cp8) was tested in the isolate using the pCM66 plasmid and a sfGFP protein. Gene expression was measured by fluorescence levels, which indicated that the promoter worked in *Methylobacterium*, though not as well as in *E. coli*. Once expression of recombinant proteins was confirmed, a recombinant synthetic biology system was assembled and tested with the intent of causing secretion of PHB granules. There was, however, no significant differences between PHB produced and secreted by the secreting and non-secreting strains, necessitating future work to improve the secretion system in *Methylobacterium*. If achieved, secretion of PHB should help in downstream processing, negating the need to use expensive methods to lyse cells, reducing costs in downstream processing.

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CHAPTER 5

GENERAL CONCLUSIONS

The overall objective of this research was to investigate the viability of alternative carbon sources, like methanol and food waste, and a novel secretion system for use in production of polyhydroxyalkanoate (PHA) bioplastics. Specifically, we aimed to analyze the potential of utilizing food waste as a carbon source for PHA production; characterize a novel strain of Methylobacterium; design and construct a BioBrick library of phasin proteins, signal peptides, hemolysin transport proteins, green fluorescent protein (GFP) parts, and composite devices for investigating secretion in Methylobacterium; and monitor the translocation of PHAs in Methylobacterium using gas chromatography (GC). These aims were all addressed.

A novel strain of Methylobacterium was isolated and characterized for PHA production. The isolate was identified as a Methylobacterium using 16s region sequencing and comparison. SEM images were generated to demonstrate cell morphology. Assays were performed to analyze the bacteria for PHA production and conditions necessary to produce noticeable amounts. It was discovered that the bacteria produce polyhydroxybutyrate (PHB) under stressed conditions of nitrogen and magnesium starvation.

A series of BioBricks and composite parts were constructed to test secretion in Methylobacterium. These were constructed in E. coli, then transformed by electroporation into Methylobacterium in the wide-host vector plasmid pCM66. First, the sfGFP constructs indicated that the cp8 promoter that was used in these constructs could
drive expression of proteins in *Methylobacterium*, though not as well as in *E. coli*. Then, the PhaP1Re and HlyA signal peptide fusion protein construct was used in conjunction with the secretion system plasmid pLG575 to test for secretion in *Methylobacterium*. There was no significant difference between the amount of PHB produced and secreted by the secreting and non-secreting strains. These results indicate that the phasin protein-based secretion system made no noticeable difference in PHA secretion in *Methylobacterium* as it is currently constituted. Future work is necessary to evaluate if improvements in the secretion system will render it viable in *Methylobacterium*. 
CHAPTER 6

ENGINEERING SIGNIFICANCE AND FUTURE RESEARCH

This section outlines specific ideas for working with food wastes as carbon sources for polyhydroxyalkanoate (PHA) production and improving our understanding of the new isolate of *Methylobacterium* and secretion of PHAs with this isolate. Some of the most promising food wastes and processes for producing PHAs from them are discussed and some future directions are pointed out for studies using these carbon sources. As for the methylotrophic bacteria, characterization of the novel isolate of *Methylobacterium* used in this study will allow us to evaluate its potential for bioplastic production. Improving the efficiency of the phasin protein secretion system is important in engineering applications because it could lead to improved economic viability of an important class of bioplastic. There are several ways that cellular product recovery by secretion can be improved and that the isolate of *Methylobacterium* can be characterized.

Production of PHAs using food waste is likely to prove both beneficial and challenging. Associated costs of production using the food wastes discussed are difficult to determine, since most studies did not perform life cycle assessments or other economic analyses. The best-characterized food waste carbon source for PHA production was dairy whey, which was the subject of one life cycle assessment [1]. Some of the most promising studies using pure cultures involved digesting whey with the halophilic archaeal species *Haloferax mediterranei*. This species was shown to thrive in pure cultures without sterilization due to a high saline content in the media used, producing moderate to high amounts of polyhydroxybutyrate (PHB) and polyhydroxybutyrate-co-
valerate (PHBV) [2,3]. When whey was used to feed strains of recombinant \textit{E. coli}, very high levels of PHB production were observed, including 96.2 g PHB / L in a 6.6 L bioreactor during one study [4]. The primary disadvantages of using whey were generally the high energy requirement for fermentation and a relatively low yield of PHB compared to the amount of whey available (see Table 6.1). Use of \textit{Haloferax mediterranei} and recombinant \textit{E. coli}, however, did seem to be capable of producing high yields and may warrant further consideration for PHA production from dairy whey.

\textbf{Table 6.1.} Advantages and disadvantages of different food wastes in PHA production.

<table>
<thead>
<tr>
<th>Food waste</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dairy whey</td>
<td>Extensive pretreatment not required</td>
<td>Energy requirement for fermentation</td>
</tr>
<tr>
<td></td>
<td>Comparable ecological imprint to petroleum plastics</td>
<td>Relatively low amounts of PHA output per kg whey input</td>
</tr>
<tr>
<td></td>
<td>Some studies showed high volumetric productivities of PHAs</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Some studies resulted in production of PHAs other than PHB</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tested at larger scales</td>
<td></td>
</tr>
<tr>
<td>Starch</td>
<td>Minimal pretreatments are necessary</td>
<td>Moderate levels of biomass</td>
</tr>
<tr>
<td>Waste oil</td>
<td>No pretreatment necessary</td>
<td>Moderate to low levels of biomass</td>
</tr>
<tr>
<td></td>
<td>Tallow resulted in PHBV production</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Natural antifoaming agents</td>
<td></td>
</tr>
<tr>
<td>Spent coffee grounds oil</td>
<td>High volumetric productivity</td>
<td>Natural foaming agent</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Use of \textit{n}-hexane in most extraction procedures</td>
</tr>
<tr>
<td>Lignocellulosic waste</td>
<td>One study showed high productivities</td>
<td>Extensive pretreatment necessary</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Generally experienced low levels of cell growth</td>
</tr>
<tr>
<td>Sugar industry waste</td>
<td></td>
<td>Moderate levels of biomass</td>
</tr>
<tr>
<td>Legume waste</td>
<td></td>
<td>Moderate levels of PHA production</td>
</tr>
<tr>
<td>Food scraps</td>
<td>Can produce PHAs and their precursors in anaerobic cultures</td>
<td>Moderate levels of biomass</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Composition varies</td>
</tr>
<tr>
<td>Food processing wastewater</td>
<td>Can produce PHAs and their precursors in anaerobic cultures</td>
<td>Complex waste--cannot be used directly by pure cultures</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Complex waste--cannot be used directly by pure cultures</td>
</tr>
</tbody>
</table>
There were a few other carbon sources and processes that showed good potential. One promising food waste was spent coffee grounds oil, which was used to feed strains of *Cupriavidus necator*. Production as high as 49.3 g PHB / L was observed in 2 L bioreactors during one study [5], among the highest amount observed with native-PHA producing organisms using food waste. The main challenges with spent coffee grounds oil was that the oil is a natural foaming agent and that hazardous materials, like *n*-hexane, were generally used during the oil extraction process (see Table 6.1). Antifoaming agents can be used to deal with the foaming tendencies of the oil, and one study demonstrated that supercritical fluid extraction could be used to extract the oil using CO2 instead of *n*-hexane, which reduces both concerns. Producing PHAs using *Azotobacter chroococcum*, a bacteria that could digest starch directly, negated the need for pretreatments on starch, and displayed relatively high volumetric productivities, thus showing promise for future investigation [6]. Lignocellulosic wastes proved to generally be the least promising food waste carbon sources for PHA production, due to the need for intense pretreatments to reduce toxicity and low levels of cell growth (see Table 6.1). One study, however, used sugars extracted from ground wheat straw and saw production levels as high as 105.1 g PHB / L. Though promising, extensive pretreatment was still required to render the straw digestible to the *Burkholderia sacchari* strain used in the study [7]. While spent coffee grounds, starch, and ground wheat straw show promise for producing PHAs and deserve further examination, most other studies using pure cultures demonstrated that a variety of food sources could be used to produce PHAs, but that relatively low total amounts of PHAs were produced.
Studies in anaerobic mixed microbial cultures (MMCs) were valuable because they did not consume large amounts of energy for sterilization and because they could digest complex wastes without extensive pretreatment. Lower cell concentrations (resulting in lower volumetric productivity) and concerns over the quality of PHAs produced in anaerobic digestion are the major concerns with MMC production of PHAs. An approach to blending the benefits of both pure cultures and MMCs involved using anaerobic digestion in MMCs as a pretreatment for food wastes. During the initial stages of anaerobic digestion, food wastes were converted into volatile fatty acids (VFAs). Those VFAs were then harvested and fed to pure cultures, generally resulting in higher volumetric productivities compared to those seen in MMCs. This indirect coupling approach faces challenges in harvesting VFAs and transferring them to the pure culture fermenters without incurring prohibitive expenses in energy and time. It is also challenged by the fact that VFAs in high concentrations are toxic to most bacteria. Fed batch fermentation helped alleviate this latter concern by making only small amounts of VFA available at first, then occasionally adding more VFAs via the feed media as the previous VFAs were consumed by the bacteria. By so doing, concentrations of VFAs were kept low, but consistently present throughout the fermentation process. Using indirect coupling approaches to digest food wastes and producing PHAs shows promise and deserves further attention, particularly fed-batch approaches and attempts to implement this approach at larger scales.

An important aspect of creating PHAs using food wastes that needs further investigation is scale-up of production. Most pure-culture studies examined used either shaking flasks or bioreactors that contained less than 10 L of media. Among the native-
PHA-producing pure culture studies, the largest volume used was with the halotolerant bacteria *Haloferax mediterranei*, in a 330 L bioreactor with a working volume of 220 L [2]. The only other study using native PHA producers to use a larger volume than 10 L involved an unidentified highly osmophilic organism in a 42 L fermenter [8]. Among studies using recombinant bacteria, most were performed in bioreactors, but at sizes less than 10 L. One study, however, did scale up production using recombinant *Escherichia coli* with dairy whey, operating at volumes of 30 L and 300 L. Volumetric production as high as 10.3 g PHB / L was observed, demonstrating that the process could successfully be scaled up [9]. Another study used recombinant *E. coli* with malt waste and soy waste as the carbon sources in a 15 L fermenter, though relatively low concentrations of PHAs were produced [10]. In general, more studies need to be performed at larger scales using food wastes as carbon sources for PHA production with pure cultures.

Mixed microbial cultures (MMCs) faced similar issues with scale. Sequencing batch reactors (SBRs) generally were operated at a 1 L working volume [11,12], as were at least two other reactor types [13,14]. One SBR used to digest olive oil mill pomace was 12 L, however, which saw moderate success in producing PHAs [15]. The MMC reactor with the largest volume was a vertical submerged membrane bioreactor operating at a working volume of 1,333 L [16]. Otherwise, all MMCs were operated in bioreactors with a capacity less than 5 L or in shaking flasks, as were all studies using volatile fatty acids produced by MMCs to feed pure cultures. More studies with larger bioreactors should be performed with MMCs.

Shifting discussion to the production of PHAs from methane and methanol, studies to further characterize the isolate of *Methylobacterium* and its capabilities need to
be performed to assess its usefulness in bioplastic production. In-depth DNA fingerprinting could clarify the isolate’s relationship to other strains of *Methylobacterium*. In addition, it would be advisable to test the bacteria’s ability to metabolize alternative carbon sources and examine their impact on polyhydroxyalkanoates (PHA) production. Many strains of *Methylobacterium* are capable of synthesizing hydroxyvalerate when propionate or valerate are added as co-substrates [17,18]. One strain has even been shown to also produce hydroxyheptanoate and hydroxyoctanoate when citrate or propionate were added as co-substrates [19]. Similar studies could be conducted with our isolate to test what types of PHAs are produced, particularly once its relationship to well-characterized strains of *Methylobacterium* is established.

Continuing the development of a synthetic biology toolbox for alphaproteobacteria based on *Methylobacterium* species will provide an improved platform for a methanol-based biomanufacturing. Promoters are a sequence that regulates transcription of DNA into RNA. In this study, we utilized the cp8 constitutive promoter, which had not previously been used in methylotrophic bacteria. Characterizing promoters for use in *Methylobacterium* species, such as the constitutive promoters from the same set as cp8 or native promoters from *Methylobacterium* and other methylotrophic bacteria would increase our ability to control levels of production of desired compounds. Inducible promoters have been developed using the P_{mxaf} promoter from *Methylobacterium extorquens* AM1, however, more could be developed. Having an array of inducible promoters would be useful for fine tuning control over production in methylotrophs. Promoters that proved highly effective in *Methylobacterium* could then be
tested with our secretion study to see if improved production results in a noticeable amount of PHA secretion.

Another useful component for use in a synthetic biology toolkit would be the discovery and characterization of more origins of replication beyond the phage-based options currently in use. Origins of replication are a part of plasmid vectors that allow plasmid DNA to replicate in bacteria. Most often, origins of replication that are based on viral DNA sequences have been used in methylotrophic bacteria, since they work in many different types of bacteria. The pLG575 plasmid used in this study and at least one previous study used the *E. coli* p15A and pBR322 origins, respectively, indicating that *E. coli* origins of replication may be utilized in methylotrophic bacteria [20]. Since *E. coli* is a model Gram-negative bacterium for synthetic biology, plasmid vectors that can work in both *E. coli* and methylotrophic bacteria would allow construction of genetic constructs in the former and an easy transition to use in the latter.

Improvement of secretion could be achieved through codon optimization. Codon optimization is a method of DNA design where sequences for the same amino acids are changed to match the codon option that the specific organism in question is most likely to use. This has been shown to improve protein expression and functionality, and could increase the production of foreign phasin proteins like the *Cupriavidus necator* phasin protein PhaP1Re in *Methylobacterium*. Type I secretion systems have not been investigated in methylotrophic bacteria before, and so the hemolysin secretion system components are also a target for codon optimization that could improve the bacteria’s ability to secrete PHA granules. Both the phasin protein and the type I secretion system
components have an impact on the functionality of the secretion system, and codon optimization could improve expression of these important proteins.

In addition to codon optimization, characterization of native phasin proteins could lead to optimized secretion in methylotrophic bacteria. In *Methylobacterium extorquens* AM1, which is closely related to the isolate characterized in this study, the JGI database indicates that there is at least one native phasin protein present. The type II methanotroph *Methylosinus trichosporium* OB3b has four coding sequences that may potentially be for phasin proteins. Strain OB3b was originally the target organism of this study, and one phasin protein (PhaP4_{MtO}) has already been successfully isolated from this organism and converted into a BioBrick. Further testing of the hlyA- PhaP4_{MtO} fusion protein in a well-characterized system like *E. coli* would give greater insight into its ability to induce secretion of PHAs. Isolation or synthesis of other potential phasin proteins would also allow characterization of their interactions with PHA granules and analysis of their effectiveness in inducing secretion. It is possible that the native phasin protein would prove more effective than phasin proteins from *C. necator* at linking the hlyA secretion tag to PHA granules.

If achieved, mastery of the type I secretion system in *Methylobacterium* will enable application in other methylotrophic bacteria. *Methylobacterium* cannot digest methane gas, however, limiting their flexibility. Methanotrophic bacteria that produce PHAs can utilize methane gas, including methane from waste sources like biogas, that *Methylobacterium* cannot use to produce bioplastics. Using their methanotrophic capabilities in combination with the secretion system would create another sustainable platform for PHA production that would be connected to the concept of a biorefinery (see
The type II methanotrophs *Methylosinus trichosporium* OB3b and *Methylocystis* species are potential targets that utilize methane gas and produce PHAs. If the secretion system works in both *Methylobacterium* and either *Methylosinus* or *Methylocystis*, further studies could be carried out with co-cultures of multiple secreting methylotrophic bacteria, which could be a possible approach for improved production of PHAs from methane and methanol.

**Figure 6.1.** Biorefinery proposed by the Sustainable Waste-to-Bioproducts Engineering Center (SWBEC) at Utah State University. Section highlighted in red is the potential area that production of PHAs using methanotrophic bacteria would fit into the biorefinery.

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APPENDICES
## APPENDIX A

### BIOBRICK PARTS AND SEQUENCES

**Table A.1.** Nucleotide sequences of codon optimized protein coding regions for *Ms. trichosporium* OB3b

<table>
<thead>
<tr>
<th>Gene</th>
<th>Nucleotide Sequence</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pha1</td>
<td>ATGGTTGCCACTTTTCGAGACCCTTACAGCAGCTGGGCAA</td>
<td><em>Ms. trichosporium</em> OB3b</td>
</tr>
<tr>
<td></td>
<td>GGAGCAGTTCGAGGCGGTGTCCGCGGCTACCGCCGCCG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TCACCCAAGGCTTGCAGACGATCGCCGGCGGCAGGGCCACC</td>
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</tr>
<tr>
<td></td>
<td>GATTATTCTCGAAGAGAGTCGAGAAGAGCCGCCTGCT</td>
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</tr>
<tr>
<td></td>
<td>TATTCCGAGCTCCGAAGGAAGCCTTCAAGCCGGCTCA</td>
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APPENDIX B

PROTOCOLS, REAGENTS, AND MISCELLANEOUS

PCR

Add the following reagents to a tube (50 µl reaction) in the following volumes and order:

- 29µl sterile H2O
- 5 µl 10X buffer
- 2µl dNTP Mix
- 6µl MgCl2
- 6µl cells/DNA
- 0.25µl Taq Polymerase
- 1µl Primer 1
- 1µl Primer 2

These can be adjusted as necessary. Higher MgCl2 concentrations mean less specific binding, while lower concentrations mean more specific binding. DMSO may be added to lower the annealing temperatures of the primers (usually 0.5-2 µL). DNA is based on concentration of the sample being used. Adjust the amount of water added to bring the total to 50µL.

The thermocycler is setup beforehand with the desired protocol. Typically: 94°C for denaturing, 50-60°C for primer annealing, and 72°C for polymerase extending.

Example setup:
1. 94°C 2 min 1x
2. 94°C 45 sec
3. 55°C 45 sec
4. 72°C 1min 15 sec
5. Repeat Step 2. thru Step 4. 35x
6. 72°C 5min 1x
7. 4°C indefinitely

Restriction Enzyme Digestion

1. Prepare and label 1.5 µl tubes for each reaction to be carried out
2. Add the following to each tube (keeping all materials on ice):
   a. 3 µl 10x FD Green buffer
   b. 1 µl FD restriction enzyme 1
   c. 1 µl FD restriction enzyme 2
   d. 4 µg DNA
      i. Calculate the volumes of DNA to add based on the stock concentration using the digest calculation spreadsheet on the lab computer
e. Bring the solution to 30 µl using sterile ddH₂O (the volume of DNA and H₂O will be 25 µl when added together)

3. Incubate the restriction digests at 37°C for 1 hour

4. Run the digest on a gel to isolate the desired pieces or to check fragment lengths.

**Electrophoresis**

1. Gel Preparation:
   a. Weight out agarose
      i. For large gel, add 200 ml 1X TAE;
      ii. For small gel, add 50 ml 1X TAE.
      iii. For 1% gel, add 2 g agarose to large and 0.5 g agarose to small gel.
      iv. For 2% gel, add double the agarose.
   b. Heat in 20 second increments in microwave until boiling, stir carefully
   c. Let cool until comfortable to touch and add 1 µl of EtBR for every 10 µl of soln.
   d. After cooling period, pour into electrophoresis chamber and let solidify

2. Gel Loading:
   a. Add 10 µl of DNA ladder (GeneRuler™ DNA Ladder Mix, ready-to-use)
   b. Add 6X loading dye (6X = sample volume + X; solve for X)

3. Use Thermo Scientific GeneJET gel extraction kit to isolate DNA bands

**TAE (50X) Buffer:**

242 g Tris base to around 600 ml
57.1 ml glacial acetic acid (1 mole)
100 ml 0.5 M EDTA (pH 8.0)
ddH₂O to 1000 ml

**Ligation Reaction**

1. Prepare and label 1.5 ml tubes for each reaction to be carried out
2. Calculate the amount of insert and vector DNA to add using the ligation spreadsheet on the lab computer.
3. Add the following to each tube:
   1. Insert DNA
   2. Vector DNA
   3. 5µl 10X ligation buffer
   4. 1µl T4 DNA ligase
   5. H₂O to bring the solution to 50 µl
4. Allow reaction to occur at room temperature for 30 min to 1 hour. Alternatively, reactions may be placed on ice and allowed to carry out over a longer period of time (an afternoon, overnight) before use in transformation
5. Ligation reactions not used in transformations may be stored in the fridge for a day or two.

**Transformation using XL1 Blue E. coli cells**
1. Take competent cells out of the -80 °C freezer and thaw on ice.
2. Add 25 µL chilled 10% glycerol to each competent cell tube.
3. Add 5 µL of DNA to the cell solution.
4. Incubate on ice for 5-8 minutes.
5. Turn on the electroporation machine. It should be set to 2500 V, 200 O, and 25 µF for *E. coli*.
6. Carefully, add one of the cell/DNA/glycerol solutions into a cuvette.
7. Before electroporation, lightly tap the cuvette down on the counter to ensure that the solution is filling the bottom portion of the cuvette.
8. Dry the cuvettes off with a KimWipe.
9. Place the cuvette so that the metal sides are touching the metal sides of the electroporator.
10. Pulse the cells, and then quickly add 1 ml pre-warmed LB media with no antibiotic.
11. Remove the solution and transfer it to the original competent cell tube
12. Incubate the cell solutions at 37°C for 1-2 hours.
13. Plate the cells on plates containing the correct antibiotic. Add 500 µL-1000 µL of solution to one plate, spread with the spreading stick.
14. Let the plates dry in the bio-safety hood for no longer than 30 minutes with the cap off.
15. Invert the plates and place them in the 37°C incubator overnight

**CTAB Plasmid DNA Extraction**

1. Inoculate 5 ml of LB media with antibiotics contained in a 12 ml tube with one colony or with a flake from a freezer stock. Grow overnight.
2. Centrifuge the 12 ml tubes containing the 5 ml cultures in the large centrifuge at 3500 RPM for 10 min. Discard supernatant liquid.
3. Re-suspend cells in 200 µl of “STET for CTAB” buffer. Transfer to 1.5 ml tubes.
4. Add 10 µl Lysozyme (50 mg/ml) and incubate at room temperature for 5 min.
5. Boil for 45 seconds in blue plastic holder and then centrifuge for 20 min at 13,000 RPM in the bench top centrifuge (or until pellet gets tight).
6. Use a sterile toothpick or pipette tip to remove the pellet by dragging it (it should be somewhat slimy, but if pelleted well enough, it will hold together), if it doesn’t hold together, re-centrifuge and retry.
7. Add 5 µl RNase A (10 mg/ml) and incubate at 68°C for 10 minutes.
8. Add 10 µl of 5% CTAB and incubate at room temperature for 3 min.
9. Bench top centrifuge for 5 min at 13,000 RPM, discard supernatant, and re-suspend in 300 µl of 1.2 M NaCl by vortexing.
10. Add 750 µl of 95% ethanol to precipitate DNA.
11. Incubate for 30 mins in -20°C freezer (or 15 min in -70°C freezer) to help DNA precipitate.
12. Bench top centrifuge for 5 min at 13,000 RPM to compact DNA pellet. Make sure the hinge is away from the center of the rotor, this will make the pellet form on the bottom of the tube on the side of the hinge (it might be hard to see or invisible, so this way you know where it should be).
13. Discard supernatant, rinse pellet in 750 µL 80% ethanol, and let tubes dry upside down with caps open.
14. Re-suspend DNA in ddH₂O (50 µl). Vortex or pipette up and down to ensure re-suspension of DNA.
15. Measure DNA concentrations using the NanoDrop 1000 instrument and record them on the tube holding the DNA and in the lab notebook.

**BioBrick Assembly**

![BioBrick Assembly Diagram](image)

Figure B.1. BioBrick assembly process. Samples are cut with restriction enzymes. Fragments are purified using gel electrophoresis and gel extraction. Isolated DNA is mixed and ligated together. This process is repeated for each added part.

**References**

APPENDIX C

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To whom it may concern,

My name is Asif Rahman. Chad L. Nielsen and I wrote the manuscript entitled: “Food waste conversion to microbial polyhydroxyalkanoates.” This paper was published in the Journal of Microbial Biotechnology in 2017. Chad was first author and contributed equally to this work. I give him permission to reprint the manuscript in its entirety in his master’s thesis.

Sincerely,

Asif Rahman
To whom it may concern,

My name is Asad-ur-Rehman. Chad L. Nielsen and I wrote the manuscript entitled: "Food waste conversion to microbial polyhydroxyalkanoates." This paper was published in the Journal of Microbial Biotechnology in 2017. Chad was first author and major contributor to this work. I give him permission to reprint the manuscript in its entirety in his master's thesis.

Sincerely,

Asad-ur-Rehman
22 February 2018

To whom it may concern,

My name is Marie K. Walsh. Chad L. Nielsen and I wrote the manuscript entitled, "Food waste conversion to microbial polyhydroxyalkanoates." This paper was published in the Journal of Microbial Biotechnology in 2017. Chad was first author and major contributor to this work. I give him permission to reprint the manuscript in its entirety in his master’s thesis.

Sincerely,

Marie K. Walsh
The article entitled “Polyhydroxybutyrate production using a wastewater microalgae based media,” presented in Appendix D, was published in the journal Algal Research, with Elsevier as the publisher. According to the Copyright Clearance Center, “as the author of this Elsevier article, you retain the right to include it in a thesis or dissertation, provided it is not published commercially. Permission is not required, but please ensure that you reference the journal as the original source.”
To whom it may concern,

My name is Asif Rahman. Chad L. Nielsen contributed to research published in the manuscript entitled: “Polyhydroxybutyrate production using a wastewater microalgae based media.” This paper was published in Algal Research in 2015. I give him permission to reprint the manuscript in its entirety in his master’s thesis.

Sincerely,

Asif Rahman
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 (PHBs) 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.

Introduction

Microalgae have been well studied for production of biodiesel [1] and recently microalgae have been proposed to be the basis for a biorefinery model where multiple
chemicals can be produced simultaneously [2]. Producing several chemicals from the same microalgae feedstock 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, harvesting, and dewatering [3].

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 for microalgae growth [4]. Facultative lagoon systems can be used to culture mixed consortia of microalgae to remediate the wastewater by removal of phosphorus and nitrogen. There are a wide range of methods previously employed to harvest microalgae from an open pond system that include: rotating algal biofilm reactor (RABR) [5,6], biological and chemical flocculants [2,7,8], and centrifugation [9]. Harvested microalgae can then be processed and used as a feedstock for production of bioproducts [10,11]. It has been demonstrated that *Escherichia coli* can be cultured on microalgae based substrates for production of biofuels and bioplastics [12,13].

*E. coli* can be easily cultured and has a fast doubling time making it an ideal candidate for production of recombinant bioproducts. Polyhydroxybutyrate (PHB) are bioplastics that can be recombinantly produced in *E. coli* [14] and cyanobacteria [15]. 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 [16]. 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 short chain length (scl) polymer PHB [17].

Bacterial PHB production is not widespread 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 [18]. 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 microalgae based media [2]. In a different study, various harvesting methods were used to collect microalgae grown in photobioreactors [19] and then the harvested microalgae was processed via the wet lipid extraction procedure (WLEP) to generate a variety of side streams and bioproducts [2,20]. 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 (1012–1013 CFU/mL) was observed when the S. obliquus was centrifuged [2]. 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 (approximately 11% lipids as fatty acid methyl esters, FAME [20]) were subjected to the WLEP to generate the aqueous phase. Results showed that harvesting via centrifugation gave the best E. coli growth (~1013 CFU/mL) and PHB production (7.8% PHB dry cell weight) [13].
Centrifugation was selected as the preferred harvesting method because centrifuged microalgae processed via the WLEP demonstrated the highest levels of E. coli growth and PHB production. Additionally, the previous studies [2,13] 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 [21]. The main objectives of the study reported here were to demonstrate E. coli growth and PHB production using hydrolyzed microalgae from wastewater effluent of the City of Logan, UT facultative pond treatment facility.

**Materials and Methods**

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

*Microalgae harvesting and processing*

Wastewater microalgae were harvested from the City of Logan, UT wastewater treatment facility from the effluent stream leaving the facultative lagoon system. Microalgae were centrifuged using a continuous centrifuge (Alfa Laval Clara 80, Lund, Sweden) and dried in a temperature controlled oven. After drying, microalgae were stored at −20 °C.

A modified microalgae hydrolysis method was used similar to Ellis et al., where hydrolyzed microalgae was used to culture Clostridia to produce Acetone, Butanol, and Ethanol [22]. Briefly, 10 g of dry microalgae was dissolved into 0.5 M (final concentration) sulfuric acid (H2SO4) with a total volume of 100 mL. The solution was
placed on a stir plate and heated to 90 °C for 30 min with constant stirring. 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 microalgae was used as the sole carbon source and was added to standard *E. coli* M9 growth media [23] in 1%, 2%, and 3% ratios (weight dry hydrolyzed microalgae to culture volume ratio, where 1% is 0.5 g dry algae in 50mL). An additional study was conducted that consisted of culturing *E. coli* in a 10% hydrolyzed microalgae solution (w/v). The hydrolyzed microalgae supernatant was not autoclaved in order to demonstrate that *E. coli* growth and PHB production could occur from a non-traditional 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, and 50 μg/mL ampicillin (IBI Scientific, Peosta, IA) [24,25].

The *E. coli* strain, XL1 Blue (Agilent Technologies, Santa Clara, CA) harboring the pBHR68 plasmid [17] was grown in LB media [23] 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-1thiogalactopyranoside (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 nm) at 0, 4, 8, 12, 24, and 48 h.
Sugar Analysis

Total sugar was determined using a modified phenol–sulfuric acid method [22]. Briefly, 3 μL of 85% (w/v) phenol solution and 150 μL of 12 M sulfuric acid were 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 (A490 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 conducted on samples after 48 h of bacterial culturing. PHB concentration was determined from a 1H NMR/GC correlation as described previously [26]. Briefly, approximately 15mg of lyophilized sample were dissolved in 1 mL deuterated chloroform (CDCl3 with 0.03% TMS (v/v), Cambridge Isotope Laboratories, Inc. Andover, MA) and 5% sodium hypochlorite solution. Samples were vortexed, incubated, and centrifuged to promote phase separation. PHB phase was run on a Jeol ECX-300 NMR (Jeol USA, Inc. Peabody, MA) and a standard NMR/GC correlation was used for PHB quantification.

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.

**Results and Discussion**

The steps conducted in this study to remove microalgae from wastewater effluent of the City of Logan, UT facultative pond treatment facility, to hydrolyze the dried microalgae, and to grow *E. coli* to produce the PHB polymer are depicted in Fig. D.1. *E. coli* harboring the pBHR68 plasmid was grown on 1%, 2%, 3%, and 10% microalgae-M9 media (w/v). The maximum optical density (OD600) for *E. coli* grown in 1% media was approximately 1.3, where stationary phase was reached at approximately 12 h post-induction (Fig. 2). The 2%, 3%, and 10% samples reached stationary phase at 24 h and achieved a maximum OD600 of 2.5, 3.61, and 7.6 respectively. *E. coli* growth in microalgae-M9 media was typical of that observed in traditional glucose-M9 media in other studies [25]. Cultures were allowed to continue growing until 48 h to allow time for PHB accumulation.
Results of the total sugar analysis indicated that simple sugars were present in the microalgae extract and were consumed during the course of bacterial growth. The 1% sample had 152 mg/L total sugar at 0 h and after 48 h of bacterial growth had 132 mg/L. The 2% sample had 320 mg/L sugar at 0 h and after 48 h had 267 mg/L. The 3% sample had 445 mg/L sugar at time 0 and 287 mg/mL after 48 h. The 10% sample had 1890 mg/L total sugar and finished with 1344 mg/L sugar. The amount of sugar consumed by the bacteria in each experiment (1%, 2%, 3%, and 10%) was comparable to the growth observed in Fig. D.2.
Comparing PHB yields 48 h post-induction demonstrated that the M9 media containing 1% and 2% microalgae extract had the most PHB (as a percentage of *E. coli* dry cell weight). The 1% and 2% samples produced $31 \pm 8.9\%$ and $28.2 \pm 2.1\%$ PHB, respectively (Fig. D.3). In comparison, *E. coli* cultured in 3% and 10% microalgae extract media that had an average PHB accumulation of $11.2 \pm 2.6\%$ and $4.6 \pm 0.7\%$, respectively. The statistical analysis yielded a p-value of 0.0026 indicating significant results (confidence of 95%, $p < 0.05$). Post hoc comparison using REGWQ showed that the PHB production levels at 1% and 2% were not significantly different from each other (indicated with ‘a’ in Fig. D.3).
Figure D.3. Polyhydroxybutyrate production from different microalgae based cultures (where % PHB is a proportion of E. coli dry cell weight). Data sets with same letter (a or b) demonstrated no statistically significant difference (p N 0.05), error bars represent standard deviation (n= 3).

Additionally, the statistical analysis also demonstrated that PHB accumulation in 3% and 10% samples were not statistically different from each other (indicated with ‘b’ in Fig. D.3). The PHB production from 1% and 2% media samples was both significantly different from the PHB yields observed from E. coli grown in 3% and 10% algal extract. These results demonstrate that there is a drop in PHB production from the 2% to the 3% microalgae media. The drop in PHB production at the higher percent microalgae media could be attributed to the increase in salts present in the media. In this study microalgae media was neutralized from a low pH with sodium hydroxide, thereby generating sodium sulfate. In a previous study, wastewater microalgae harvested with Aluminum Sulfate (Alum) and used to culture E. coli yielded no PHB [13].

As described, E. coli growth in the 3% and 10% samples reached stationary phase later than then 1% and 2% samples, suggesting that E. coli in the 3% and 10% samples
could also have had less time to accumulate PHB. It has been suggested previously that acetyl-CoA is required for cell synthesis in log phase but is diverted to PHB production in stationary phase [27]. Future work could be conducted to best determine if extending the culturing time of the 3% and 10% experiments improves yields of PHB and whether or not this is economically viable.

The percentage of PHB accumulated in bacterial cells cultured in the 1% and 2% microalgae-M9 media was 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 [24]. Achieving approximately 31 ± 8.9% demonstrates the potential of using microalgae 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 microalgae based media could potentially increase the growth and yields of PHB in _E. coli_. It was demonstrated in a previous study that 1% glucose addition to a microalgae based media to culture _Clostridia_ tripled the yield of solvent production [22].

Comparing the 10% microalgae extract media (with no M9 addition) to that of a similar study [13], it was found that this 10% media did not perform as well as the media used in the previous study. In the previous study, approximately 9.6 g of microalgae (dry weight equivalent) was 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 [13]. In the present study, microalgae 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, resulting in a lower yield of sugars extracted. There could have also been some inhibitory effect from the media that was more concentrated, thus reducing the production capacity of the *E. coli*.

Table D.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* [24]. 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 determined that using the 1% microalgae in M9 media would need approximately 4.3 kg of dry microalgae 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, however, in order to get a more accurate measurement additional parameters would need to be considered.

**Table D.1.** Production and yields of PHB after 48 h from *Escherichia coli* XL1 Blue harboring the pBHR68 plasmid grown in M9 media supplemented with wastewater microalgae hydrolyzed fraction. Standard deviations are based on triplicate (n=3).

<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) to produce 1 kg PHB</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% microalgae 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% microalgae 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>3% microalgae media</td>
<td>11.24 ± 2.6</td>
<td>0.77 ± 0.5</td>
<td>0.026</td>
<td>38.7</td>
<td>This study</td>
</tr>
<tr>
<td>10% microalgae 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>0.4</td>
<td>2.5 [24]</td>
<td></td>
</tr>
</tbody>
</table>
Data sets with same letter (a or b) demonstrated no statistical significant difference (p > 0.05).

Conclusions

This study built upon work previous work that demonstrated the production of PHB from a microalgae feedstock. From this study, growth of recombinant *E. coli* harboring the pBHR68 plasmid (containing the phaCAB operon) on wastewater microalgae based media was observed for all samples. It was found that the maximum PHB accumulation in *E. coli* was approximately 31 ± 8.9% seen on the 1% microalgae-M9 media 48 h post-induction. Future work could include determining the effects of production of additional bioproducts using recombinant *E. coli* grown on wastewater microalgae media and the addition of a traditional carbon source such as glucose or a carbon-rich waste to the microalgae based media. Additionally, a technoeconomic analysis could be conducted to determine the most cost effect means of production of various recombinant products in *E. coli* from a wastewater microalgae feedstock.

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