Engineering *Ralstonia eutropha* for the Production of mcl-PHAs from Plant Oils

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// Abstract

With the world's petroleum reserves being depleted at a rapid rate, vast pollution caused by the inability of nature to break down modern day plastics, and global warming greatly affecting the Earth's climate, green alternatives to current industrial practices are becoming more necessary now than ever before. One possible environmentally friendly alternative is the production of poly 3-hydroxyalkanoates (PHAs), biodegradable polymers naturally synthesized by many types of bacteria. PHB is the most common and easiest to produce PHA, but the polymer is quite brittle and holds little commercial value. However, research has shown that a wide variety of monomers can be incorporated into the polymer chains giving a vast array of physical and material properties to the polymer, some with the potential to be very valuable. With the advances in molecular biology techniques and DNA sequencing technology, metabolic engineering has become a quick and powerful tool for constructing recombinant bacteria with gene sets capable of performing tasks previously unattainable.

The main focus of this thesis was to construct a recombinant strain of *Ralstonia eutropha* capable of producing medium chain length (mcl) PHAs using soybean oil as the sole carbon source. Two approaches were used to accomplish this goal. In one approach the mcl-PHA polymerase gene *phaC1* from *Pseudomonas oleovorans* was integrated into the genome of *R. eutropha* in place of the native *phaC* PHB polymerase. Another approach tested was to construct a plasmid based expression system using homologous recombination in yeast to express an engineered mcl-PHA operon containing the *P. oleovorans phaC1* in conjunction with the *phaJ* from *P. oleovorans* or the *fox2* gene from

S. cerevisiae on a polycistronic gene cassette in a mutated strain of *R. eutropha* rendered unable to synthesize PHAs naturally. Both recombinant systems were tested for mcl-PHA production in fed batch bioreactors using minimal media supplemented with soybean oil.

GC-MS analysis of cell samples taken from the reactor experiments indicated the recombinant organism harboring the integrated *phaC1* gene accumulated trace amounts of mcl-PHAs, mostly in the form of 3-hydroxyoctanoate, but the plasmid expression systems showed much higher accumulation of mcl-PHAs with constituents of 3-hydroxyhexanoate, 3-hydroxyoctanoate, 3-hydroxydecanoate, and 3-hydroxydecenoate. This demonstrated the ability of the recombinant *R. eutropha* to accumulate mcl-PHAs when grown on fatty acids. It was also demonstrated that *R. eutropha* has the ability to grow to high cell densities using soybean oil as the sole carbon source, and that foreign gene expression is possible in *R. eutropha* using plasmid based systems and through gene integrations.

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1. General Introduction

1.1 Significance

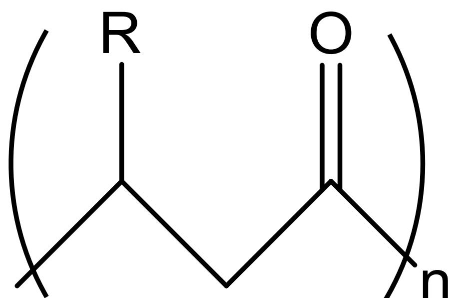
With the growing threat of global warming and dwindling petroleum reserves, there is a great need for alternatives to current petroleum derived products and reducing green house gas emissions. The majorities of plastics made today are synthesized from petroleum and are disposed of by burying them in a landfill or incinerated (Stein, 1992). Plastics in a landfill do not decompose due to the inability of microbes to break down the macromolecules, and incineration creates large amounts of greenhouse gasses, airborne particulates, and other noxious gasses. Plastics also find their way into environments as pollutants such as wastes being carelessly discarded by humans, spillage during transportation, and plastic bags and other light plastic wastes being distributed by wind and rain. Pristine wilderness environments, especially the world's oceans, are being threatened by plastic pollution putting many species on the brink of extinction. These issues pose a real problem that needs to be addressed before irreparable damage is done.

1.2 Polyhydroxyalkanoates (PHAs)

Polyhydroxyalkanoates are simple structured polyesters naturally synthesized by bacteria as forms of carbon storage during times of nutrient stress or excessive carbon supply. They are formed as intracellular hydrophobic granules found in both gram positive and gram negative bacteria. When cultures are grown in the lab for the purpose of producing PHAs, bulk quantities can be made and extracted to observe the physical and mechanical properties which closely resemble the properties of current petroleum derived polymers. PHAs also have the added benefits of being produced using renewable

carbon sources and are biodegradable, even in marine environments.

Polyhydroxybutyrate, the most common PHA, was first discovered in 1926 by M. Lemoigne. Since then over 150 different hydroxyalkanoate monomers have been found as constituents in bacterial PHAs contributing to a wide variety of material properties (Madison & Huisman, 1999; Steinbüchel & Valentin, 1995). Such monomers include polyester chains with straight, unsaturated, and branched chains, aromatic rings, halogenated hydrocarbons, and active groups. These side chains influence the mechanical properties creating crystalline and brittle to soft and rubber-like polymers. Since most PHAs are synthesized by soil bacteria, they are biodegradable in all environments, even after some post production modifications (de Koning et al., 1994; Jendrossek, 1993). If synthesized using renewable carbon sources such as plant oils or CO_2, a completely renewable biodegradable polymer can be produced eliminating our use of petroleum in the production of plastics and the need for incinerating, recycling, or throwing waste plastics into landfills. Recent studies have also demonstrated the ability to use waste streams from urban areas and industries as feedstocks for PHA production (Chua, Takabatake, Satoh, & Mino, 2003; Cromwick, 1996).



R group	3-hydroxyalkanoate	Abbr.
Hydrogen	3-hydroxypropionate	(PHP)
Methyl	3-hydroxybutyrate	(PHB)
Ethyl	3-hydroxyvalerate	(PHV)
Propyl	3-hydroxyhexanoate	(PHHx)
Butyl	3-hydroxyheptanoate	(PHH)
Pentyl	3-hydroxyoctanoate	(PHO)
Hexyl	3-hydroxynonanoate	(PHN)
Heptyl	3-hydroxydecanoate	(PHD)
Octyl	3-hydroxyundecanoate	(PHUD)
Nonyl	3-hydroxydodecanoate	(PHDD)

Figure 1-1 Basic structure of PHA monomer and most common types of bacterial Poly-3-hydroxyalkanoates

The R group can take on many different forms creating a wide range of polymer properties. The table represents the most common types of bacterial PHAs, some of which were created in experiments discussed in the thesis.

There has been some commercial production of PHAs since the technology has become available. Monsanto (St. Louis, MO) produced PHB under the name Biopol® in the 1990's but has since shut down the process due to the inability to compete economically with petroleum derived products. Metabolix (Cambridge, MA) is currently developing a process for PHAs produced using a recombinant *E. coli* strain under the name Mirel ™, and is researching methods to produce bulk quantities in various plant based expression systems.

Almost all green technologies find that their viability is dependent on the price of petroleum and current market conditions, but recent fluctuations in global markets are forcing nations to rethink their dependence on foreign countries for their energy needs. PHAs have the potential to be produced locally which would eliminate our dependence on petroleum for plastic production and remove a major source of pollution to our

atmosphere and wilderness environments.

1.3 Ralstonia eutropha

R. eutropha is a gram negative facultative chemolithoautotrophic soil bacterium capable of accumulating a PHB homopolymer up to 80% of its cell dry weight (Lee, 1995). It has been extensively studied for its ability to accumulate large amounts of PHAs, grow to cell densities over 130 g/L, and grow using H_2, O_2, and CO_2 gasses as the sole energy and carbon sources respectively. It has also been shown to possess a robust metabolic network capable of degrading chemicals toxic to the majority of other living systems (Dursun & Tepe, 2005; Schenzle, Lenke, Fischer, Williams, & Knackmuss, 1997).

R. eutropha possesses a type I PHA synthase capable of polymerizing short chain length (scl) hydroxyacyl CoAs. These include 3 to 5 carbon substrates, propionyl CoA to valeryl CoA, that when polymerized creates a highly crystalline polymer that is relatively strong but quite brittle. Although *R. eutropha* has shown to produce large amounts of PHB using a variety of carbon sources, due to its mechanical properties and cost of production it has been underutilized in the commercial industry. In an attempt to enhance the mechanical properties, copolymers and block copolymers have been developed in *R. eutropha* using controlled feeding (Green, 2002; Pederson, 2006). The copolymers developed showed that mechanical properties and polymer toughness can be tailored by altering the composition of the polymers paving the way for research involving scl-mcl-PHA copolymers.

1.4 Pseudomonas oleovorans and medium chain length-PHAs

P. oleovorans is a gram negative bacterium that was first isolated in the cutting oil of a metal cutting plant. It has since been studied, among other things, for its ability to accumulate medium chain length PHAs when grown on alkanoic acids (Durner, Zinn, Witholt, & Egli, 2001; Huisman et al., 1991; Kocer, 2003; Preusting et al., 1993). Studies have shown that the substrate incorporated into the polyester chain derives from the β-oxidation pathway, and that the constituents found in the polymer are of the same length or closely related to the carbon source fed to the culture (Brandl, 1988; Lageveen et al., 1988).

P. oleovorans contains a type II PHA synthase (*phaC*1) capable of incorporating (R)-3-hydroxyacyl CoAs of 6 to 12 carbons in length, 3-hydroxyhexanoate to 3-hydroxydodecanoate, into the PHA polyester granule (Huisman, 1991). This type of PHA has low crystallinity and behaves like a rubber with increased elasticity over PHB and other short chain length PHAs. This type of polymer is more appealing commercially due to its increased toughness and low crystallinity allowing for longer shelf life without becoming brittle.

Although some Pseudomonads can accumulate PHAs while grown on carbohydrates, *P. oleovorans* cannot due to the lack of genes necessary to make 3-hydroxyacyl CoAs from pathways other than the β-oxidation pathway. Since most alkanes and alkanoic acids are derived from petroleum sources, using these as feedstocks to produce the polymer is even less efficient than current polymer production methods and undesirable. Metabolic engineering holds the key to creating an organism to utilize low cost feedstocks to produce medium chain length polymers that have commercial

value.

1.5 Triglycerides and β-oxidation

Soybean oil was used in experiments described in the final two chapters as the feedstock for PHA production. Soy oil is a triglyceride, a glycerol backbone with three fatty acids connected via ester bonds. The fatty acids most commonly found in soy oil are 54% linoeic acid (18:2), 22% oleic acid (18:1), 10% palmitic acid (16:0), and 8% linoleic acid (18:3). Although the fatty acids found in soy oil contain varying degrees of unsaturation, Figure 1-2 depicts a possible triglyceride found in the plant oil.

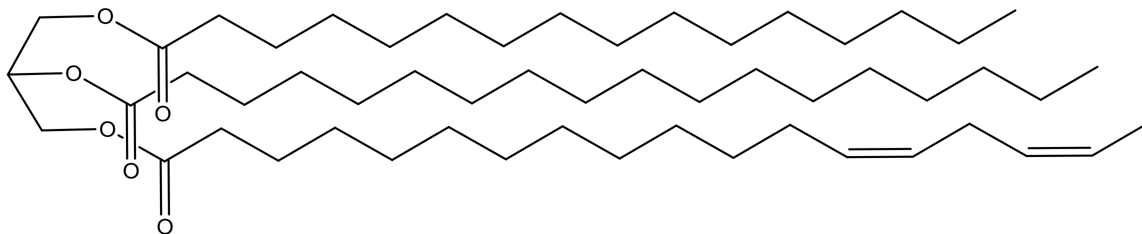


Figure 1-2 Triglyceride molecule with an unsaturated side chain

The above molecule represents a triglyceride that could be found in soybean oil. Three long chain fatty acids are connected to the glycerol backbone via ester linkages. The composition is C16 and C18 fatty acids with varying degrees of unsaturation.

Triacylglycerides are very energy dense molecules because they contain three long chain fatty acids per glycerol molecule that each can be broken down to create a large amount of ATP for the organism. This is done via the β-oxidation cycle shown in Figure 1-3. However, to harvest the energy in the triglyceride the fatty acids must first be hydrolyzed from the glycerol backbone. Some bacteria are capable of secreting an extracellular lipase that can catalyze that reaction creating the free fatty acids which are then able to be taken into the cell for degradation. As occurs in *P. oleovorans*,

intermediates from the β-oxidation pathway were used for mcl-PHA accumulation in recombinant bacteria engineered in chapters 5 and 6. The pathways for PHA production from β-oxidation intermediates are illustrated in Figure 1-3.

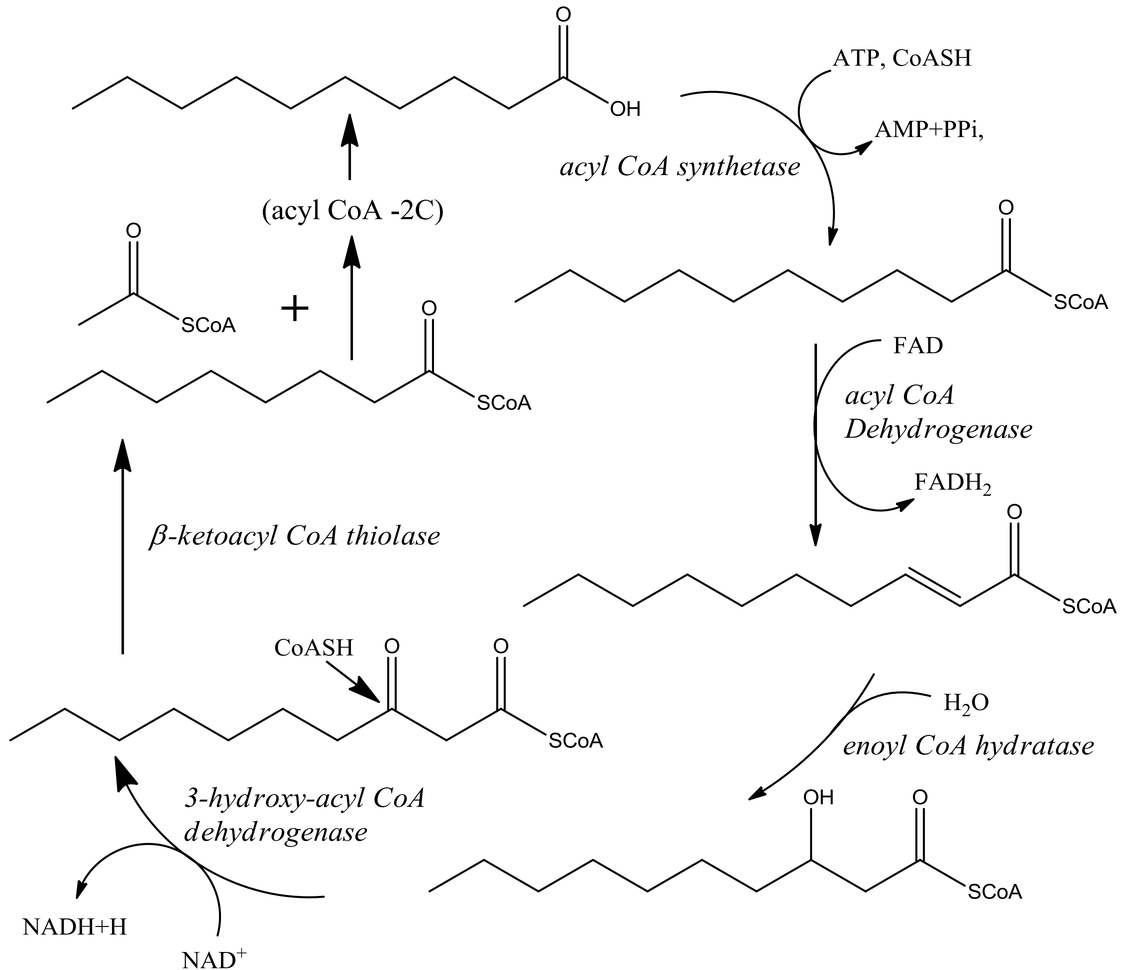


Figure 1-3 β-oxidation cycle in bacteria

The figure above illustrates the β-oxidation cycle in bacteria using decanoic acid as an example. One complete cycle removes two carbons in the form of acetyl CoA turning decanoic acid into octanoic acid. The cycle can repeat itself breaking down the same molecule until all that is left is acetyl CoA.

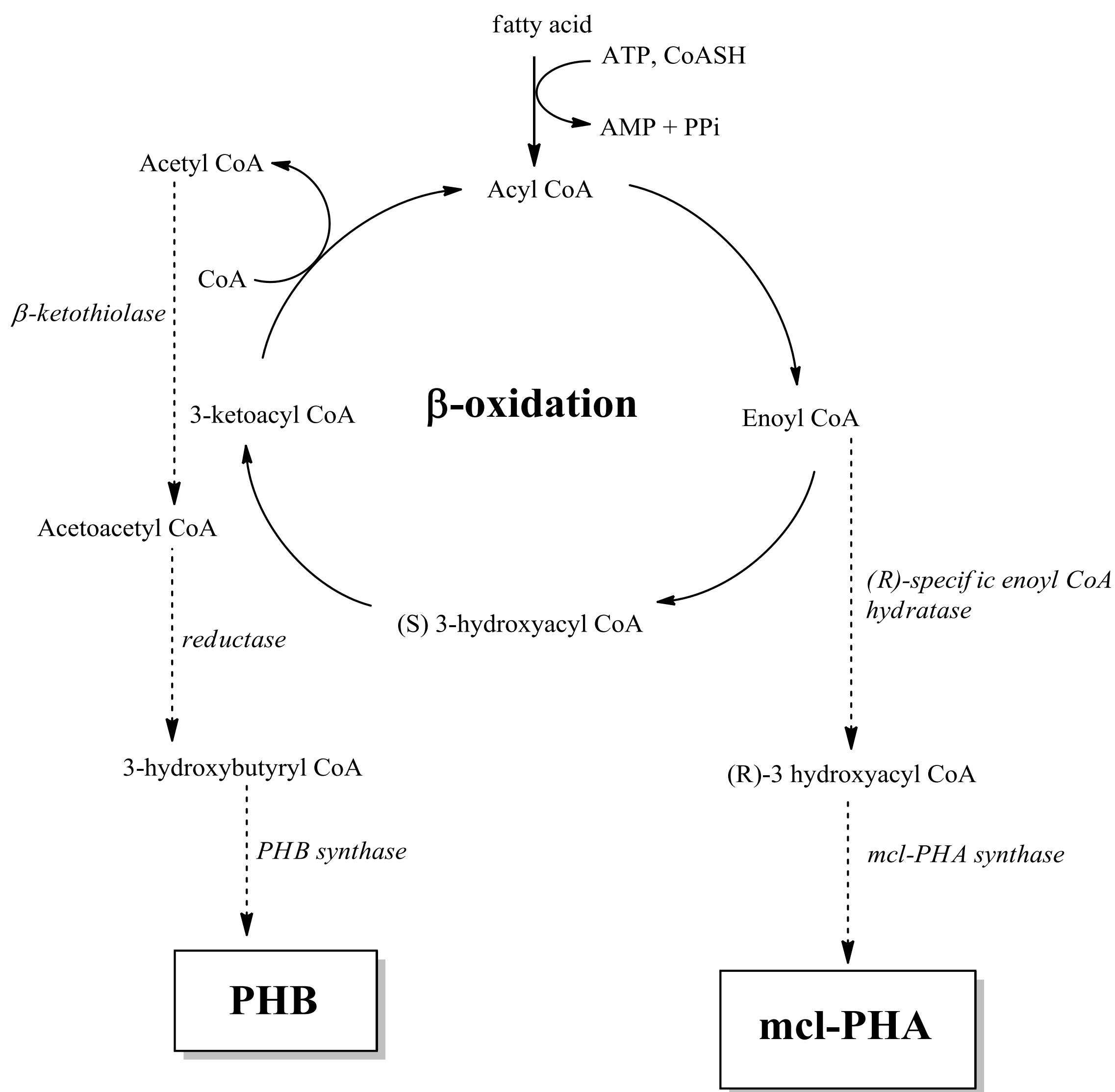


Figure 1-4 Fatty acid β-oxidation routes to PHA synthesis

The diagram above illustrates a simplified metabolic route for the conversion of fatty acids into both short chain length and medium chain length PHAs. The dotted lines indicate the routes taken by intermediates in the degradation pathway to incorporation into PHAs. This is the system used and exploited in the experiments outlined in the following chapters of the thesis.

1.6 Organization of thesis

The first chapter of the thesis serves as a general introduction to the rational for the approach to the work detailed in this thesis, importance of the work performed,

polyhydroxyalkanoates (PHAs), and the bacteria used in the following studies.

The second chapter includes some of the major materials and methods used throughout the experiments detailed in the thesis. The methods presented in chapter 2 are used in more than one experiment discussed in the thesis, and the general procedure is presented here to avoid redundancies in subsequent chapters. Details pertaining to individual experiments (i.e. primers/genes used) are presented in the chapter for which they were used.

Chapter 3 details experiments performed to produce medium chain length PHAs by wild type *Pseudomonas oleovorans* for the purpose of producing bulk quantities of the material. This material was used to compare the PHAs produced in later chapters by recombinant organisms harboring the PHA polymerase gene from *P. oleovorans*.

Chapter 4 details an experiment attempting to produce mcl-PHA block copolymers in *P. oleovorans* using controlled feeding. Previous studies demonstrated that short chain length PHA block copolymers have greatly enhanced material properties over homopolymers, and that the incorporation of double bonds in the side chains of the polymer can be cross-linked extending the temperature range for which the material retains its mechanical properties. These ideas were combined and a method to produce mcl-PHA block copolymers was designed and tested in fed batch reactors.

The following two chapters detail experiments comprising the bulk of the work performed and the main focus of the thesis. Recombinant strains of *Ralstonia eutropha* were engineered to produce medium chain length PHAs using soybean oil as the carbon source for growth and PHA synthesis. Studies have demonstrated the ability of *R.*

eutropha to secrete a lipase capable of releasing the fatty acids from the glycerol backbone in soybean oil and accumulate large amounts of PHAs in fed batch reactors this way (Kahar, 2004). Since the fatty acid degradation pathway can lead directly into the mcl-PHA synthesis pathway, soybean oil is a low cost renewable feedstock, and the encouraging results from other studies, we decided to use soybean oil as the carbon source for our cultures.

Chapter 5 details an experiment where the genome of *Ralstonia eutropha* was manipulated to express the mcl-PHA polymerase gene (*phaC*) from *P. oleovorans* in an attempt to produce medium chain length PHAs from soybean oil. Initial experiments where the *P. oleovorans phaC* gene was expressed on a plasmid in a PHB deficient strain of *R. eutropha* showed no accumulation of mcl-PHAs. However, studies have shown that when antibiotics are used to maintain a plasmid in a culture of *R. eutropha*, only a small fraction of the population maintains the plasmid (Jackson & Srienc, 1999). With this factor combined with the desire to make future gene knock-outs and genomic integrations in *R. eutropha*, it was determined that integrating the mcl-PHA *phaC* gene into the genome of *R. eutropha* would provide good insight as to the ability of the organism to synthesize the necessary precursor molecules to the mcl-PHA pathway.

Chapter 6 details an experiment where two mcl-PHA operons were constructed and individually tested for their ability to produce mcl-PHAs in the PHB-4 strain of *R. eutropha* using a plasmid based expression system. Literature searches revealed two possible genes that may help to produce the correct precursor molecules for mcl-PHA synthesis from intermediates from the fatty acid degradation pathways (Hiltunen, 1992;

Vo, Lee, Jung, & Lee, 2008). These genes were expressed on a plasmid in separate strains of a PHB deficient strain of *R. eutropha* with the mcl-PHA *phaC* used in the previous study, and cultured in fed batch reactors using soybean oil as the carbon source.

Chapter 7 is taken from parts of a paper published from work done by Chris McChalicher and myself studying the ability of 1,2-propylene carbonate to dissolve PHB biopolymers(McChalicher, Srienc, & Rouse, 2010). This work was done to propose a safer, more environmentally friendly, and more easily scalable process than current methods of PHB extraction from biomass. The paper discusses the solubility kinetics, limits, and polymer degradation at different processing temperatures. In this chapter I discuss the portion of the paper in which I was involved including the solubility limits, kinetics, extraction from biomass, and recyclability of the reagents used.

2. Materials and Methods

This section outlines some similar procedures used throughout the experiments described in later chapters of this thesis. The main steps are outlined in this chapter and any details specific to a particular experiment (i.e. primers/DNA used) will be mentioned in the chapter for which it pertains to.

2.1 Strains and media

Multiple species of bacteria and a yeast strain were used during the experiments discussed in this thesis. The following section details the media and growth conditions used for each species used.

2.1.1 *Pseudomonas oleovorans*

Pseudomonas oleovorans wild type was used in the experiments in chapters 3 and 4 for the production of mcl-PHAs from alkanes. Both complex and minimal media were used for culturing the organism.

2.1.1.1 *P. oleovorans* complex media

Table 2.1 Complex media for *P. oleovorans* growth

Component	g/L
Yeast Extract	5
Tryptone	10
NaCl	5

LB media was used as the complex media to grow *P. oleovorans* when culturing from a freezer stock or during growth in seed flasks during the scale up for bioreactor experiments. The media was made in distilled water by dissolving the components into

75% of the final volume, stirring until everything had gone into solution, and then bringing the volume up to the final level. The media was then autoclaved for sterilization.

2.1.1.2 *P. oleovorans* minimal media

Minimal media was used during experiments when control of the amount of nitrogen and the carbon source were required. The recipe was based on previously published work where *P. oleovorans* was cultured on minimal media (Lageveen et al., 1988; Vogel & Bonner, 1956). Minimal media was needed during the fed batch reactor experiments detailed in chapters 3 and 4. The recipe for the minimal media used is shown below.

Table 2.2 *P. oleovorans* minimal media (E media)

Component	g/L
$(NH_4)_2HPO_4$	1.1
K_2HPO_4	5.8
$KHPO_4$	3.7
$MgSO_4*7H_2O$	0.25
Alkane	20
Trace elements	1 mL

The media was made by dissolving the phosphates in 80% of the final volume of distilled water and autoclaved. A 1000X stock of the trace elements solution was made, filtered to sterilize, and added with the remaining components. The remaining components were dissolved in 20% of the final volume in distilled water, filtered to sterilize them, and added once the phosphate solution was allowed to cool. The following table shows the recipe for the trace element solution used.

Table 2.3 1000X trace elements solution for E medium

Component	g/L
$FeSO_4 * 7H_2O$	2.78
$MnCl_2 * 4H_2O$	1.98
$CoSO_4 * 7H_2O$	2.81
$CuCl_2 * 2H_2O$	0.17
$ZnSO_4 * 7H_2O$	0.29
$CaCl_2 * 2H_2O$	1.67
HCl 1M	1 mL

The trace elements solution was made by dissolving all of the components in distilled water and filtering through a 0.2 μm filter to sterilize the solution.

2.1.2 Yeast

A leucine auxotroph of *Saccharomyces cerevisiae* termed S288 was used for plasmid construction via homologous recombination. Both complex and minimal media recipes were used for culturing yeast.

2.1.2.1 Yeast complex media

Complex media was used to grow cultures of S288 in preparation for transformation of linear DNA during the homologous recombination experiments described in detail later in this chapter. YPAD and 2xYPAD were the complex medias used for this purpose.

Table 2.4 YPAD media recipe

Ingredient	g/L
Yeast Extract	10
Peptone	20
Adenine Hemisulfate	0.08
Glucose	20

A 2X solution was used to culture the cells right before harvesting them ensure that the cells were growing very rapidly making them more susceptible to up-taking the linear DNA fragments.

Table 2.5 2X YPAD media recipe

Ingredient	g/L
Yeast Extract	20
Peptone	40
Adenine Hemisulfate	0.16
Glucose	40

Both of the recipes above were made using the same method. The yeast extract and peptone were dissolved in water and autoclaved to sterilize. A 40% glucose solution and 5 mg/mL solution of adenine hemisulfate were made separately, filter sterilized, and added to the media to the concentrations detailed in the tables above.

2.1.2.2 Yeast minimal media

SD media, a yeast minimal media, was used when plasmids were being assembled or maintained. A leucine gene was expressed on a plasmid in S288 which allows only the cells containing the plasmid to continue to grow.

Table 2.6 SD media recipe

Ingredient	g/L
Yeast Nitrogen Base	1.7
Ammonium Sulfate	5
Glucose	20
Adjust pH to 5.6	

The media was made by dissolving the yeast nitrogen base and ammonium sulfate in water, adjusting the pH to 5.6 with NaOH, and autoclaving to sterilize. A 40% glucose solution was made, filter sterilized, and an appropriate amount was added to the media post autoclaving to give a final concentration of 2%.

2.1.3 Escherichia coli

Two different species of *E. coli* were used during the experiments detailed in this thesis. DH5α was used for plasmid production when concentrated DNA was needed for visualization on agarose gels following a restriction digest, and S17-1 was used for transferring plasmid DNA into *Ralstonia eutropha* via conjugation. S17-1 has the genes necessary for this transfer (*tra* genes) integrated on its genome and works well for this application.

2.1.3.1 *E. coli* complex media

The same media recipe, Luria-Bertani (LB), was used for growing both strains and is shown in Table 2-7.

Table 2.7 LB media recipe

Component	g/L
Yeast Extract	5
Tryptone	10
NaCl	5

The media was made by dissolving all of the ingredients in 75% of the final volume in distilled water, and then brought to the final volume after the components are completely dissolved. The media was then autoclaved to sterilize.

2.1.4 *Ralstonia eutropha*

Ralstonia eutropha was used in the experiments described in chapters 5 and 6. The wild type H16 was used during experiments where genes were integrated into the genome, and the strain PHB-4 was used when genes were expressed on plasmids. The PHB polymerase gene was mutated in the PHB-4 strain to eliminate its ability to accumulate PHB (Schlegel, 1970). Both complex and minimal media were used during the course of the experiments.

2.1.4.1 *R. eutropha* complex media

The complex media used for the growth of *R. eutropha* was termed Doi media and was made with the following recipe.

Table 2.8 *R. eutropha* complex media recipe

Component	g/L
Yeast Extract	10
Tryptose	10
Beef Extract	5

The media was made by dissolving in distilled water all of the ingredients in 75% of the final volume, and then brought to the final volume after the components were completely dissolved. The media was then autoclaved to sterilize.

2.1.4.2 *R. eutropha* minimal media

Minimal media was used for multiple purposes while growing *R. eutropha*. In each case the only thing that differed was the carbon source. The minimal media used was termed Mineral Salts Media (MSM) and was based off of one used by Ramsay et al. (Ramsay, 1990).

Table 2.9 Mineral Salts Media recipe

Component	g/L
$Na_2HPO_4 * 7H_2O$	6.7
K_2HPO_4	1.5
$(NH_4)_2SO_4$	4
$MgSO_4 * 7H_2O$	0.2
ferric ammonium citrate	6 mg
$CaCl_2$	7.5 mg
Trace elements (1000x)	1 mL
Carbon source	20

The media was made by dissolving the phosphates in distilled water and autoclaving to sterilize the solution. The rest of the components were dissolved in distilled water, filtered to sterilize, and added after the autoclaved solution was allowed to cool. 1 mL of a filtered trace element solution was added at the same time as the filtered components. The recipe for the 1000X trace elements solution is shown in Table 2-10 below.

Table 2.10 Trace elements for Mineral Salts Media

Component	g/L
H_3BO_3	0.3
$CoCl_2 * 6H_2O$	0.2
$CuSO_4 * 5H_2O$	10 mg
$MnCl_2*4H_2O$	30 mg
$NiCl_2*6H_2O$	20 mg
$NaMo_4*2H_2O$	30 mg
$ZnSO_4*7H_2O$	0.1

The trace elements solution was made by dissolving all of the components in distilled water and filtering with a 0.2 µm filter to sterilize the solution.

2.2 Molecular Biology Techniques

2.2.1 PCR

PCR was routinely used to clone out regions of DNA from the genomes of microbes, or to detect the presence of certain genes in a culture. GoTaq® (Promega, Madison, WI) was used as the master mix for each reaction which contained everything needed for the PCR reaction in a 2X buffer except the primers and template DNA. All reactions were carried out using a Bio-Rad (Hercules, CA) MJ Mini Personal Thermal Cycler. Although the annealing temperatures vary between PCR reactions depending on the melting temperatures of the primers used, the same template was used for each reaction with most steps left unchanged. Table 2.11 below shows the template for the thermal cycler settings used during all of the PCR reactions used in this thesis.

Table 2.11 Template for thermal cycler settings during PCR reactions

Step	Name	Temp (°C)	Time (min)
1	**Initial denaturing**	94	5
2	**Primer anneal**	various	1
3	**Extension**	72	1 min/kb
4	**Denature**	94	0.5
5	**Go to Step 2**	28-32 times	
6	**Primer anneal**	various	1
7	**Final Extension**	72	5

The initial denaturing step was run at 94°C for 5 minutes, the annealing steps were 1 minute, the extension was 72°C for 1 minute per 1000 bases to be cloned, the repeated denaturing step was 94°C for 30 seconds, and the final extension period was run for 5 minutes.

2.2.2 Restriction digest

All enzymes were purchased from Invitrogen (Carlsbad, CA) or NEB (Ipswich, MA) and used according to their respective protocols. 100-1000 ng of DNA were incubated with a 1:10 dilution of the 10X reaction buffer supplied with the enzymes, and 1 μL of the respective enzyme. All digests were incubated at 37°C overnight unless otherwise stated.

2.2.3 Gel electrophoresis

PCR products and plasmid DNA were routinely analyzed by agarose gel electrophoresis on a 0.8% agarose gel run at 120 volts. Pictures of the gel were taken in a UV light box with a transilluminator to be further analyzed by computer. DNA fragments were regularly separated by electrophoresis and purified from the gel to be used in downstream applications such as homologous recombination. Figure 2-1 displays the DNA ladder used on all agarose gels run to measure the size of the DNA fragments analyzed by electrophoresis.

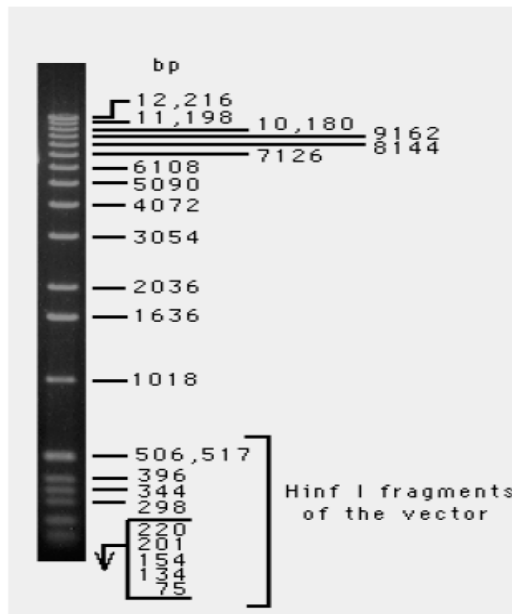


Figure 2-1 DNA ladder

The above figure is from the manual supplied with the 1 Kb ladder purchased from Invitrogen (catalogue #15615-015). It displays the sizes of each band used for measurement during routine DNA fragment analysis. The same ladder was used for all experiments described throughout the thesis.

2.2.4 DNA purification

2.2.4.1 Genomic DNA extraction

Genomic DNA was routinely extracted from a culture to be used as the template for amplifying genes by PCR to be used in downstream applications such as homologous recombination, or used to screen for the insertion of genes into the genome during experiments explained in chapter 6. Genomic DNA was purified from microbial cultures using Purelink ™ Genomic DNA Mini Kits purchased from Invitrogen (catalogue # K1820-01) and used according to the manufacturer's protocol.

2.2.4.2 Plasmid DNA extraction

2.2.4.2.1 Bacterial plasmid extraction

Plasmid DNA was routinely extracted from bacterial cultures to verify the presence of a certain plasmid or to harvest the DNA to be used for downstream applications such as restriction digests. Bacterial plasmid extractions were performed using the Wizard® Plus SV Miniprep kits from Promega (Madison, WI) according to the manufacturer's protocol.

2.2.4.2.2 Yeast plasmid extraction

Plasmid DNA was routinely extracted from yeast cultures following a homologous recombination experiment in order to further analyze the newly constructed plasmid by PCR, and to transform *E. coli* strain S17-1 with the plasmid for downstream applications. Plasmid DNA was extracted from yeast cultures using the Omega bio-tek (Norcross, GA) E.Z.N.A.® yeast plasmid kit according to the manufacturer's protocol.

2.2.4.3 Linear DNA purification

Linear DNA fragments were purified for use in downstream applications such as homologous recombination in yeast from agarose gels or from a PCR reaction mixture. The fragments were purified from PCR mixtures and agarose gels using Qiaquick PCR Cleanup Kits and Qiaquick Gel Extraction Kit respectively according to the manufacturer's protocol (Qiagen, Hilden, Germany).

2.2.5 Homologous recombination in yeast

Homologous recombination was performed in yeast to construct the plasmids used in the experiments described in chapters 5 and 6. The procedure contains three major steps; design of plasmid and primers, PCR amplification of desired fragments, and transformation of linear DNA fragments into yeast cells. The homologous recombination and primer design procedure was adapted from Shanks et al. (Shanks, Caiazza, Hinsa, Toutain, & O'Toole, 2006), and the yeast transformation procedure was adapted from the transformation method published by Schiestl et al. (Schiestl, 1989). The yeast strain S288 was used because of its leucine auxotroph which is complemented with a leucine gene located on one of the fragments used in the recombination.

In order for the plasmid to be recombined in yeast, a yeast origin of replication and a selection marker must be located on the plasmid. The 2 µm ori and leucine gene from yeast satisfied the requirements for the recombination experiments performed in the experiments detailed in this thesis. If the recombined plasmid will be transferred into *E. coli* it must also require a bacterial origin of replication and selectable marker. The bacterial origin of replication changed depending on the experiment and is described in more detail in chapters 5 and 6 where homologous recombination is used, but the selectable marker used for maintaining the plasmid in bacteria was always kanamycin. All of these fragments must be present in the plasmid to be recombined in order for it to be assembled properly and transferred to *E. coli*, so care was taken to first assemble a plasmid containing the necessary fragments, and then use that plasmid as a fragment in the subsequent recombination experiments.

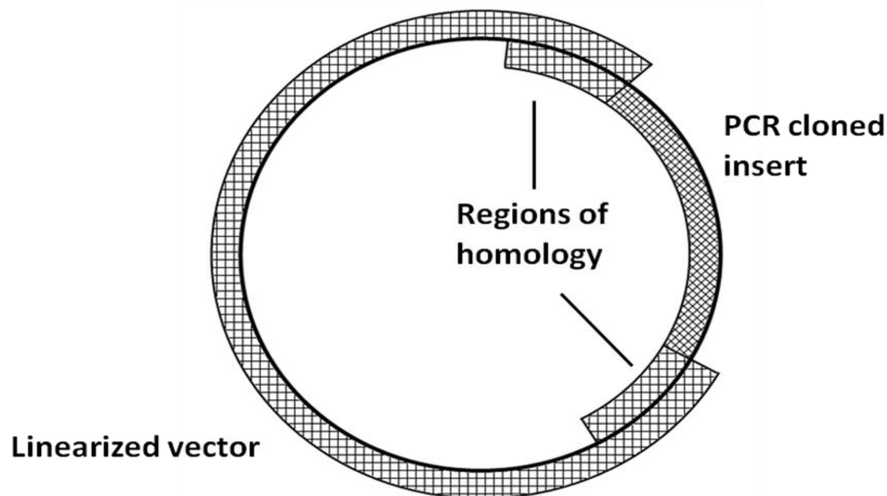


Figure 2-2 Diagram of fragments designed for homologous recombination

The diagram above illustrates how the fragments designed on the ends of the PCR product are homologous to the ends of the fragment adjacent to it. This diagram illustrates a two fragment recombination, but the method was scaled up to recombine up to 5 linear fragments using the same design strategy.

The DNA pieces used in the recombination contain either PCR products or a combination of PCR products and linearized plasmid vector. The first step in the design stage was to create a map of the desired plasmid. Then the DNA fragments necessary to assemble the designed plasmid were determined, and primers were designed to clone the respective fragments from genomic or plasmid DNA. 40 bases of homology to the adjacent DNA fragment on the final plasmid were added to the end of the primer sequence as an overhang to the original DNA template. For the pieces next to a digested plasmid vector, 40 terminal bases of homology were designed to match the vector sustaining the restriction site used to linearize the vector for easier screening downstream.

For the second step, the DNA fragments used in the recombination were amplified via PCR or digested by restriction enzymes and purified using the protocols described previously. Regardless of the protocol used to purify the DNA, the fragments were eluted from the spin columns using sterile nuclease free water at pH 7.

The third step involving yeast transformation of linear DNA proceeded as follows. A 5 mL culture of S288 was grown overnight in YPAD medium at 30°C shaking at 250 rpm. 500 μL of the overnight culture was then transferred to a 250 mL beaker containing 50 mL of pre-warmed 2x YPAD medium. The culture was then grown until a titer of 2 x 10^8 cells was reached (O.D. of 2), which usually took about 3 hours. Cells beginning log phase were found to have the best transformation efficiency.

The cells were harvested by centrifugation at 3000 rpm for 5 minutes using a swing out rotor (Jouan GR 412, Winchester, VA). The supernatant was discarded and the cells were washed twice with 25 mL of autoclaved distilled water, vortexing each time to resuspend the culture. After the second wash the pellet was resuspended by vortexing in 1 mL of autoclaved distilled water and transferred to a 1.5 mL microcentrifuge tube to be centrifuged again at 10,000 rpm in a microcentrifuge for 30 seconds. The pellet was again resuspended in 1 mL of autoclaved distilled water by vortexing and 100 μL aliquots were dispensed into individual 1.5 mL microcentrifuge tubes. The cells were centrifuged again at 10,000 rpm for 1 minute in a microcentrifuge and the supernatant was pipetted off to ensure complete removal of supernatant. The cells were then resuspended in the appropriate transformation solution by breaking the pellet up with a pipette tip and vortexed until a homogeneous solution was attained. The transformation mixture containing the cell suspension was incubated at 42°C for 40 minutes.

The transformation solution was made using the following procedure. Equal molar quantities of the linear DNA fragments dissolved in nuclease free water were added together to make a total volume of 34 μL. Care was taken when preparing the linear

DNA samples to produce as high of a concentration as possible. There was no decrease in efficiency observed from adding too much DNA to the transformation mixture, as long as the volume was capped at 34 µL. To make the transformation mixture the DNA mix described above was combined with 240 µL of a sterile 50% (w/v) PEG 3550 solution, 36 µL of a sterile 1 M lithium acetate solution, and 50 µL of 2 mg/mL denatured salmon sperm DNA (Invitrogen, Carlsbad, CA). Salmon sperm DNA was purchased at a concentration of 10 mg/mL, and prior to use it was diluted 1:5 in TE buffer and placed in a boiling water bath for 5 minutes to denature the double stranded DNA. Immediately after removal from the boiling water bath the denatured DNA sample was placed directly on ice to avoid reannealing of the DNA. The transformation mixture should be a total volume of 360 µL prior to adding it to the cell pellet. Negative controls were made for each fragment used in the recombination by combining the same amount of DNA as used per fragment in the experimental sample and bringing the volume of the mixture to 34 µL with sterile water. Positive controls were made using up to 100 ng of circular plasmid using the same transformation procedure.

After the 40 minute heat shock treatment was over the cell suspension was centrifuged at 10,000 rpm in a microcentrifuge, the supernatant drawn away with a pipette to ensure that nearly all of the transformation solution was removed from the cells, and then resuspended in 1 mL of water by vortexing. 20 and 200 µL of each sample were then plated on separate SD media plates and incubated at 30 °C for 3 to 5 days to allow for colonies to grow up. Colonies that grew on the negative control plates were used to judge the amount of background recombination and the colonies formed on

the experimental plates were screened for the recombined plasmid.

2.2.6 Recombinant DNA transfer

2.2.6.1 DH5α transformation

Chemically competent *E. coli* were purchased from Invitrogen and used according to the published protocol. 50 μL aliquots of competent cells were used per reaction. Typically 1 μL (>50 μg) of plasmid DNA was added directly to the cell suspension and gently mixed by swirling the tip of the pipette around the culture. The cells were incubated on ice for 30 minutes, heat shocked at 42°C for 45 seconds, and then placed back on ice for 1 minute. 500 μL of 37°C LB media was added to the cell suspension and then incubated at 37°C for 1-4 hours. Serial dilutions were then plated on LB agar plates containing the appropriate antibiotic selection marker and incubated overnight at 37°C. Colonies were then screened to contain the appropriate plasmid and saved for downstream applications.

2.2.6.2 S17-1 transformation

Mating strains of *E. coli* termed S17-1 were made chemically competent in the presence of a transformation and storage solution (TSS). TSS solution was made by mixing 15 g PEG 4000, 5 mL of 1 M $MgCl_2$, and adding LB media to 95 mL. The pH was adjusted to 6.5 and then autoclaved. 5 mL of DMSO were added after the solution was allowed to cool, and after mixing the solution aliquots were then stored at -20°C until needed.

Competent cells were prepared by growing S17-1 in complex medium (LB)

overnight in 5mL culture tubes. 500 µL of the overnight culture were used to seed 50 mL of LB and incubated at 37°C to start the cells rapidly growing. The OD was monitored and when the cells reached an OD of 0.4-0.6 they were centrifuged for 10 min at 4000 x g at 4°C. The supernatant was poured off and the cells were carefully resuspended in 2 mL of the cold TSS solution per 50mL of culture and incubated on ice for 5 minutes. The cell suspension was then dispensed into 100 µL aliquots, flash frozen in liquid nitrogen, and stored at -80°C for later use.

The transformation protocol for cells prepared this way is very similar to the procedure used for the Invitrogen DH5α competent cells. Typically 1 µL (>50 µg) of plasmid DNA was added directly to the cell suspension and mixed by gently swirling the tip of the pipette around the cell suspension. The cells were incubated on ice for 45 minutes, heat shocked at 42°C for 1 minute, and then placed back on ice for 3 minutes. 1 mL of 37°C LB media was then added to the cell suspension and incubated at 37°C for 1-4 hours. 50 – 500 µL of the cell suspension were then plated on LB agar plates containing the appropriate antibiotic selection marker and incubated overnight at 37°C to obtain colonies for downstream applications.

2.2.6.3 Bacterial conjugation

Bacterial conjugation is used to transfer plasmid DNA from one species of bacteria to another when other traditional methods do not work (i.e. heat shock/electroporation). The technique used in the experiments detailed in later chapters is called bi-parental mating. This method involves passing the plasmid of interest from one species of bacteria, called the donor strain, to a strain that you wish to accept the plasmid called the

recipient strain through a physical linkage between the two bacteria. The process of bi-parental mating requires that the donor strain has the genes necessary for the linkage of the two bacteria and the transfer of the plasmid to the recipient strain called the *tra* genes. It is also required that the plasmid to be transferred has the *oriT* sequence (origin of transfer) or *mob* region (mobilization element) present. These DNA sequences are recognized by some of the *tra* genes and are necessary to facilitate the transfer of the plasmid to the recipient strain.

The procedure for transferring the plasmid begins with culturing the donor strains overnight in 5 mL of LB medium containing the appropriate antibiotics, and the recipient strain, *Ralstonia eutropha* in these experiments, overnight in 5 mL of Doi media with no antibiotics. 500 μL of the donor strain was removed from the culture and centrifuged at 10,000 rpm in a microcentrifuge for 2 minutes. The supernatant was discarded and the cells were then washed twice with a 0.8% NaCl solution to remove any residual antibiotic, centrifuging each time at 10,000 rpm for 2 minutes to pellet the cells. The supernatant was discarded and 1 mL of the recipient strain was added to the tube containing the donor strain. The cells were centrifuged once more at 10,000 rpm for 2 minutes, the supernatant discarded, and then resuspended in 1 mL of LB or Doi media ensuring a homogeneous mixture of the two bacterial strains. Then 100 μL was transferred to a Doi agar plate, spread in a circle about 2 inches in diameter, and allowed to incubate at the optimal temperature for growth of the recipient strain. When using *R. eutropha* this was done at 30°C overnight.

A large clump of cells was removed from the mating plate and resuspended in a

0.8% NaCl solution creating a homogeneous solution. Serial dilutions of the concentrated cell solution were made and plated on selective media able to preferentially allow the recipient strain harboring the plasmid of interest to grow while suppressing the growth of all other cells in the mixture. For *R. eutropha* Mineral Salt Medias (MSM) plates with kanamycin were made containing no carbon source and incubated at 30 °C in a hydrogen rich atmosphere for up to 5 days. Colonies that formed were picked and re-streaked for isolated colonies on another MSM plate and incubated in the same fashion to ensure no donor cells were able to contaminate the cells of interest. Single colonies were then picked from the secondary screening plates and screened for the plasmid of interest.

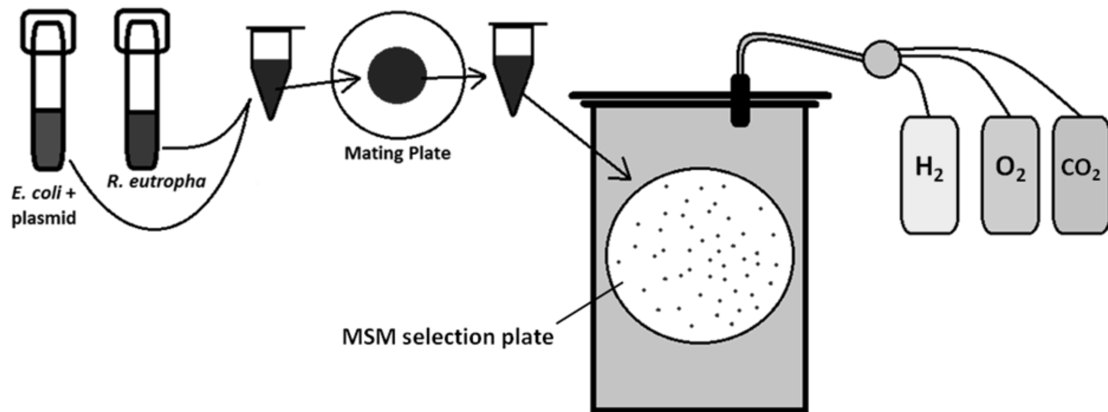


Figure 2-3 Flow diagram of conjugation procedure

The above diagram illustrates the major steps taken during the bacterial conjugation procedure described in the paragraphs above. The donor (*E. coli*) and recipient (*R. eutropha*) strains were grown, mixed together, and plated together on a complex media plate supporting growth of both strains. Some cells were then removed and plated on Mineral Salts Media (MSM) agar plates containing the appropriate antibiotic but no carbon source and placed in a chamber with a regulated atmosphere of 80% H_2, 10% O_2, and 10% CO_2 to select for the recipient strain containing the plasmid of interest.

2.3 Cell Growth

2.3.1 *E. coli*

E. coli was grown mostly for the purposes of plasmid production. Samples were taken from -80°C freezer stocks and streaked for isolation on an LB plate containing 16 g/L agar and incubated at 37°C overnight. From there colonies were grown in 3 – 5 mL of LB broth in 15 mL culture tubes (Fisher Scientific, Waltham, MA) overnight at 37°C in a Lab-Line incubator-shaker at 250 rpm. When large amounts of plasmid were necessary, overnight cultures were diluted 1:50 in 10-50 mL of LB with the appropriate antibiotic and incubated at 37°C in a Lab-Line incubator-shaker at 250 rpm.

2.3.2 *Pseudomonas oleovorans*

P. oleovorans was used in the first two experiments for the production of mcl-PHAs from alkanes. To maintain cultures on plates, LB medium with 16 g/L agar was used and the cells were incubated at 30°C. The strains did not respond well when inoculated from a plate directly into the minimal media (E medium) with alkanes as the carbon source, so overnight or log growth cultures grown on LB broth media were used as the inoculum for experiments requiring minimal media. The inoculum was diluted no more than 1:50 when added into minimal media. Cultures grown in tubes or flasks were grown at 30°C in a Lab-Line incubator-shaker at 250 rpm.

Bioreactor experiments were conducted in 10L Bioreactors (ThermoOnix, Houston USA) with a 5 L working volume. Atmospheric air was sparged into the culture to maintain at least 50% oxygen saturation and the stir rate was 400 rpm. The temperature of the water jacket was controlled to maintain the culture temperature at 30°C.

2.3.3 ***Ralstonia eutropha***

R. eutropha was used in experiments detailed in chapters 5 and 6. Cultures were maintained on Doi media plates containing 16 g/L agar. Cultures grown in tubes or shake flasks were cultured at 30°C in a Lab-Line incubator-shaker at 250 rpm.

2.3.3.1 ***R. eutropha*** **Minimal media growth**

Minimal media was used for several different applications during the experiments using *R. eutropha*. The following three sections describe the different strategies and conditions used for each method.

2.3.3.1.1 Chemolithoautotrophic growth

When grown using Mineral Salts Media described earlier with no carbon source the atmosphere for which the plates were incubated in was defined to be 80% H_2, 10% O_2, and 10% CO_2. MSM agar plates were made by adding 16 g/L of agar to the recipe described earlier. Figure 2-3 diagrams the apparatus used to create and use the hydrogen rich atmosphere required for growth. The minimal media used in conjunction was sufficient in producing colonies on plates in 3-5 days, and the wild type colonies appear opaque due to the internal accumulation of PHB.

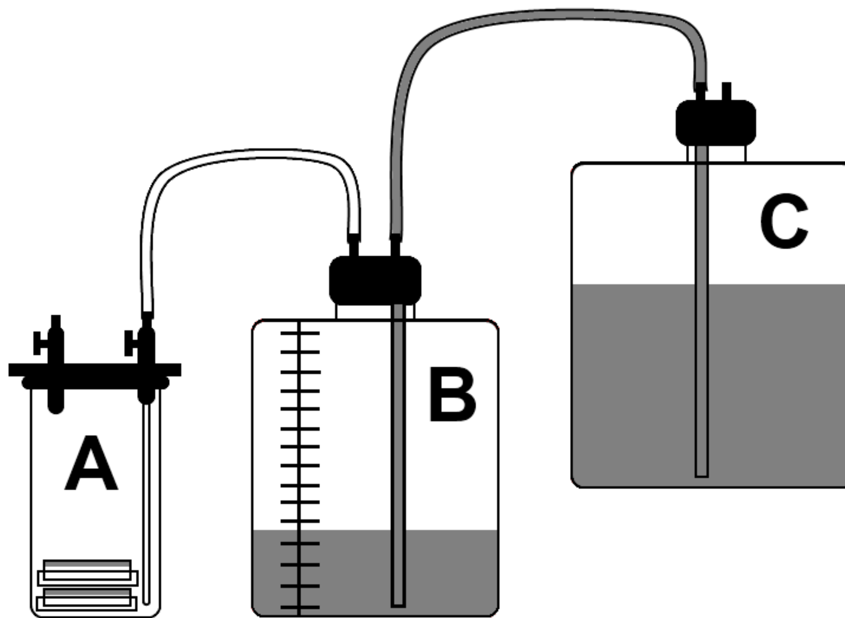


Figure 2-4 Apparatus for chemolithoautotrophic growth

The diagram above shows the system used to control the atmosphere for *R. eutropha* growth on hydrogen. Water from carboy C is first pumped into carboy B until it is full, and then pumped back into carboy C by displacing the water by adding H_2, O_2, and CO_2 gasses into carboy B at the appropriate volumetric concentrations. The differential in water height between carboy B and C was used to pump the hydrogen rich atmosphere into a sealed canister (A) with valves in the cover to allow for air flow through the canister as the hydrogen rich air is pumped in. *R. eutropha* was plated on MSM agar plates containing no carbon source and placed in the canister A. A minimum of three volumes of the hydrogen rich air were pumped through the canister before the valves were closed and it was placed in a 30°C incubator for optimal growth of *R. eutropha*. Fresh hydrogen rich air was flushed through the canister every 24 hours to ensure that none of the gasses in the mixture become limiting.

2.3.3.1.2 R. eutropha growth on fructose

R. eutropha is able to utilize fructose as a carbon source and so the Mineral Salts Media described earlier was supplemented with 20 g/L fructose and made as a liquid broth and solid agar media plates and used for culturing *R. eutropha*. A 40% fructose solution was made in distilled water and sterilized by passing it through a 0.2 µm filter. An appropriate volume was added to the minimal media after it was allowed to cool following removal from the autoclave. Agar plates were incubated at 30°C and colonies formed in 3-5 days. Wild type colonies appear opaque due to the intracellular

accumulation of PHB. Liquid cultures were incubated at 30°C in a Lab-Line incubator-shaker at 250 rpm.

2.3.3.1.2 Culturing on soybean oil

R. eutropha is able to utilize the triglycerides in soybean oil as their sole carbon source, and so the Mineral Salts Media recipe described earlier was supplemented with 20 g/L sterile soybean oil. The oil was autoclaved to sterilize it and added to the media just prior to inoculating a culture. Cultures were grown in either 250 mL or 1 L baffled shake flasks and incubated at 30°C in a Lab-Line incubator-shaker at 250 rpm.

Bioreactor experiments were conducted in 10L Bioreactors (ThermoOnix, Houston USA) with a 5 L working volume. Atmospheric air was sparged into the culture to maintain at least 50% oxygen saturation and the stir rate was 400 rpm. The temperature of the water jacket was controlled to maintain the culture temperature at 30°C. The pH was controlled with NaOH and maintained at 7.

2.4 Analysis of Bacterial Cultures

2.4.1 Biomass measurements

Cell dry weight (CDW) is a measure of the total accumulation of biomass during the growth of a microbial culture. All samples taken during bioreactor experiments were subjected to CDW analysis. 1-5 mL of the culture sample was aliquoted into pre-weighed borosilicate tubes and centrifuged at 3000 rpm for 20 minutes using a swing out rotor (Jouan GR 412, Winchester, VA). The supernatant was discarded and the cells were washed twice with 1 mL of deionized water and then put in an 80°C oven to dry.

The tubes were then dried overnight at 80°C, allowed to cool, and reweighed using the same analytical scale used previously. The difference in the weights between the empty tube and the tube plus dried cell mass was calculated giving the amount of biomass per sample volume. This was then used for many analytical calculations such as determining the amount of biomass per liter, growth rates of cultures, and % PHA accumulation per biomass.

2.4.2 Exhaust gas analysis

A prima δ-B mass spectrometer (MS) (Thermo Scientific, Waltham, MA), was used to analyze the exhaust gas during the bioreactor experiments. The exhaust from the bioreactors was passed through a condenser connected to the headplate and then through a 0.2 μm filter (Acro 50 vents, Pall Corp., Ann Arbor, MI) to ensure sterility inside the reactor and filter any particulates from reaching the MS. The gas was then passed into a 32 port sampling head on the MS at a rate below 1 L/min. The purge time on the sampling loop was at least 5 seconds to prevent mixing between samples. The MS produced a real time analysis of the concentrations of O_2 and CO_2 in the off gas from the reactor. By comparing these values to the concentrations of the house air being sparged into the culture, the MS calculates the rate of carbon evolution (CER) and the rate of oxygen uptake (OUR). An additional value termed the respiratory quotient (RQ) was calculated by dividing the CER by the OUR. This value is unique to the different stages of PHA metabolism and in some cases can be used to detect the type of PHAs being produced.

2.4.3 NH_4 detection assay

The method used for determining the concentration is based on the Berthelot reaction (Russell, 1944). 1 mL of solution A (10 g/L Phenol and 10 mg/L sodium nitroprusside), and 1 ml solution B (67.4 g/L $Na_2PO_4 * 7H_2O$, 6 g/L NaOH, 10 ml/L NaOCl) were mixed with a dilution of filtered supernatant from a centrifuged culture sample, incubated at 37°C for 30 minutes, and the absorbance at 630 nm was read on a spectrophotometer. The absorbance readings were compared to a standard curve created from known standards to calculate the total nitrogen content in the media.

2.4.4 PHA detection

2.4.3.1 Soxhlet extraction of PHAs

A soxhlet apparatus was used to extract PHAs from lyophilized cell mass by chloroform at reflux. Below is a diagram of the soxhlet extraction apparatus.

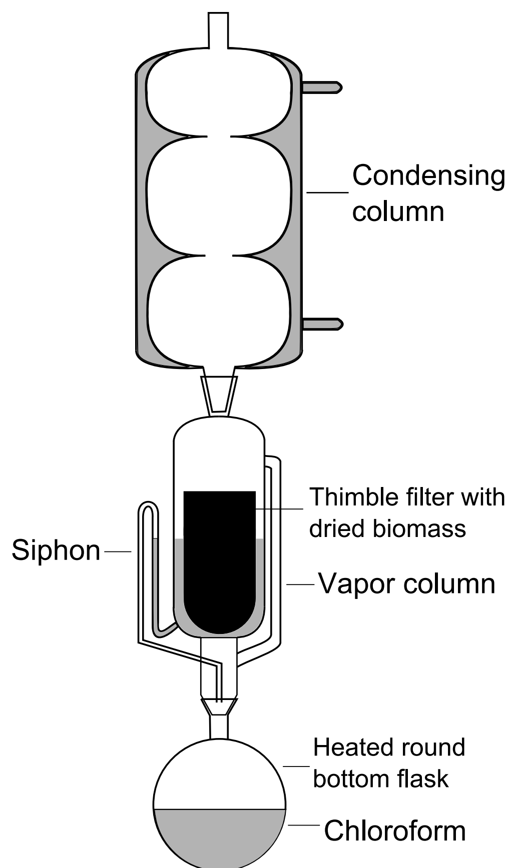


Figure 2-5 Soxhlet apparatus

The above figure illustrates the soxhlet apparatus used to extract intracellular PHAs from lyophilized cell mass. The round bottom flask heater and ring stands used to stabilize the apparatus were omitted on this diagram.

Chloroform was added to a round bottom flask and the flask placed in a round bottom flask heating element turned on a low setting. The middle chamber was fitted on top of the flask and a cellulose thimble filter containing broken up lyophilized cell pellets was placed inside the chamber. A distillation column was then placed on top of the middle chamber and cold tap water was run through the column to keep it cold. As chloroform vapors are liberated from the liquid in the round bottom flask they travel up through the vapor column to the condensing column where they condense and drip

directly on top of the dried cell mass held in the thimble filter. The chamber fills until the level of the liquid reaches the top of the siphon where gravity can pull the liquid down to the siphon exit returning it to the round bottom flask where it originated. The vacuum created by the liquid in the siphon pulls all of the liquid out of the chamber draining it completely allowing a fresh volume of solvent to fill it and continue with the extraction process. This system does not lose any solvent in the process and does not damage the PHAs during the extraction making it very well suited for this application.

2.4.3.2 Propanolysis

The protocol used for analyzing the composition and quantifying the amount of PHAs accumulated by the microbial culture was the propanolysis assay adapted from Riis et al. (Riis & Mai, 1988). This is a transesterification reaction catalyzed by acid hydrolysis of the ester bond in the polymer backbone creating 3-hydroxyacyl propyl esters. These propyl ester monomers are subsequently detected and quantified by gas chromatography (GC) or gas chromatography – mass spectrometry (GC-MS). The propanolysis reaction was carried out in 15 mL borosilicate tubes (Fisher Scientific, Waltham, MA) and is detailed below.

PHA or CDW samples were mixed with 1.5 mL of propanolysis solution consisting of 50% dichloroethane (DCE), 40% 1-propanol, and 10% concentrated hydrochloric acid (HCl). 50 µL of a benzoic acid internal standard solution (40 g/L Benzoic Acid in 1-propanol) was also added to the reaction mixture to act as an internal standard for the GC analysis. The tubes were capped and briefly vortexed to thoroughly mix the reagents. The samples were then placed in an 80°C water bath for 16-20 hours with periodic

vortexing to break up the cell pellet. The samples were then removed from the water bath and allowed to cool to room temperature. About 2 mL of water was added to the mixture and vortexed to quench the reaction and remove residual HCl from the mixture. The organic phase was then removed with a pasteur pipette and injected into a GC vial for analysis by GC or GC-MS.

2.4.3.3 PHA analysis

Propanolysis samples were analyzed by gas chromatography and gas chromatography-mass spectrometry to determine the composition and total mass of the accumulated PHAs. Our lab has a Shimadzu GC-17A (Shimadzu Corp., Kyoto, Japan) which was used to quantify the amount of polymer after the peaks were identified using a Varian Saturn 3400CX gas chromatograph – Varian Saturn 3 mass spectrometer (Agilent Technologies, Santa Clara, CA) in the Center for Mass Spectrometry and Proteomics in the Biotechnology Institute at the University of Minnesota. The same DB WAX GC column (I.D. 0.32mm, 0.5 µm film thickness) (Agilent Technologies, Santa Clara, CA) was used in both instruments to keep the retention times similar for both machines. This helped to make peak identification easier when using the GC only. The injector temperature was 258°C and the detector was 55°C on the GC-MS, and the injector and FID were both held at 250°C on the Shimadzu GC. The following oven temperature program was used for analysis of PHAs on both instruments.

Table 2.12 Method for oven temperature on GC and GC-MS

The oven temperature method above was used during PHA analysis by GC and GC-MS to maximize the distance between the mcl-PHA peaks and finish at a hot enough temperature to be sure all residual long chain fatty acids are removed from the column before the next sample is analyzed.

Step	Temp. (°C)	Rate (°C/min)	Time (min)	Σ Time (min)
1	125	0	1	1
2	135	5	2	3
3	165	1	30	33
4	230	10	6.5	39.5
5	230	0	3	42.5

2.5 Bioinformatics tools

2.5.1 Plasmid Design Software

A plasmid editor (ApE), a freeware plasmid editing program, was used to construct plasmids and their maps on a computer prior to doing any lab work involving DNA manipulation. The program was used to design the plasmids and primers required in the experiments detailed in chapters 5 and 6. Virtual restriction digests were performed giving the expected fragment pattern and size of the linear DNA fragments. ApE was also used for aligning parts of the plasmids designed using the built in alignment software, and used to read abi files received from DNA sequencing reactions. A window showing the fluorescence chromatogram from the sequencing reaction is displayed with the ability to create a FASTA formatted sequence from the basscalls. This program proved to be a very powerful tool when performing any work with DNA.

2.5.2 DNA translation tool

In some instances the DNA sequences obtained from published sources needed to be translated into the corresponding amino acid sequences to identify mutants or to find start/stop codons. When this was necessary the translate tool sponsored by the Swiss Institute of Bioinformatics located online on the ExPASy Proteomics Server (http://ca.expasy.org/tools/dna.html) was used. The DNA sequence can be pasted into an open text box and the program will translate the DNA into the amino acid sequence for every frame possible in the DNA sequence.

2.5.3 DNA Alignment

DNA alignments allow easy visualization of the similarity between two or more DNA sequences. Pairwise sequence alignments were performed using the EMBOSS pairwise sequence alignment tool sponsored by the European Biotechnology Institute and housed on their servers (http://www.ebi.ac.uk/Tools/emboss/align/). The sequences to be aligned were pasted in the text boxes and a global alignment was performed with the Blosum62 matrix and the default Gap Extend score and Gap Open score set at 0.5 and 10 respectively.

2.5.4 DNA Alignment Search

The BLAST tool sponsored by the National Center for Biotechnology Information (NCBI) was used to search through the entire bank of published DNA sequences for highly similar or homologous sequences to my query sequence. This tool is very powerful and allows a researcher to detect the presence of a particular gene in different bacterial strains and find the amount of similarity between the sequences found in different bacteria. This tool was used in the experiments detailed in chapters 5 and 6 to

search for genes involved in the fatty acid metabolism of different bacteria and to identify the identity of DNA sequence data obtained from DNA sequencing reactions.

3. mcl-PHA Accumulation by *Pseudomonas oleovorans*

3.1 Introduction

The purpose of this experiment was to produce medium chain length PHAs using *P. oleovorans* utilizing alkanes as the sole carbon source. Since the polymerase enzyme (*phaC*) used in other experiments was cloned from *P. oleovorans*, the PHAs synthesized by the wild type organism should be very similar to the PHAs made by the recombinant *Ralstonia eutropha* expressing the polymerase enzyme. By producing bulk quantities of the polymers it will be possible to study the physical and mechanical properties allowing for a more accurate estimation of its viability as a commercial plastic. The PHAs will also serve as a comparison to those created with the recombinant strains harboring genes from *P. oleovorans*.

3.2 Methods

3.2.1 Reactor Growth

A colony of *Pseudomonas oleovorans* was picked off of an LB agar plate and inoculated into a 5 mL culture of LB broth media and incubated overnight at 30°C. The culture was then used to inoculate a 2L baffled flask containing 500 mL of LB media containing 1% alkane for 16 hours at 30°C to stimulate the β-oxidation pathways while scaling-up the size of the culture. It was then inoculated into a 10L reactor with 5L of E medium and 2% alkane as the sole carbon source. The cultures were grown from 48 to 56 hours depending on the ability of the organism to efficiently utilize the carbon source given. The cultures were stirred at 500 rpm with air saturation above 40%. The off gas

was measured by mass spectrometry to determine the carbon evolution rate (CER) and the oxygen uptake rate (OUR). Additional alkanes were added to the culture when the mass spectrometer showed a decrease in the CER and OUR indicating that the metabolism of the culture is slowing down due to nutrient limitation. The cultures were maintained until they no longer responded positively to additional feeds of carbon, usually around 50 hours.

3.2.3 Casting PHA films

The cells were harvested by centrifugation using 500 mL containers and spinning at 10,000 rpm for 20 minutes. The resulting cell pellet was flash frozen using liquid nitrogen and lyophilized. The PHAs were then extracted from the cell pellet using a soxhlet apparatus as described in chapter 2. The apparatus was then taken down and the volume of chloroform harboring the dissolved polymer was reduced by evaporating off roughly half of the volume using a rotovap, thus concentrating the polymer dissolved in the chloroform. Films of the PHAs were then cast by pouring the concentrated PHA/chloroform solution into glass petri plates and allowing the chloroform phase to evaporate in a fume hood. As the liquid in the dish would evaporate, more of the PHA/chloroform mixture was poured in to create a thicker polymer film. Once all of the chloroform was evaporated the films were allowed to crystallize for up to 5 days or until they were durable enough to be removed from the dish while remaining intact.

3.2.4 Analysis of PHAs Produced

Both dried cell samples and extracted PHAs were subjected to a propanolysis reaction to hydrolyze the ester bond linking the monomer units together and replacing it with a propyl group as described previously. The organic phase was extracted and analyzed by GC and GC-MS as described in chapter 2.

3.3 Results

3.3.1 Total Biomass Accumulation

The wild type *P. oleovorans* was able to grow using saturated alkanes as their sole carbon source. Their biomass accumulation is represented below.

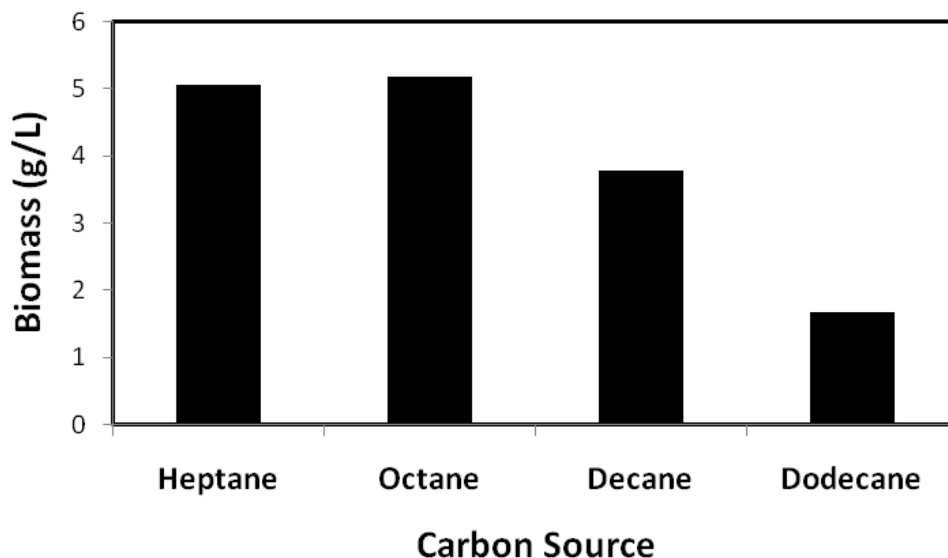


Figure 3-1 *P. oleovorans* Biomass Accumulation

Biomass accumulation by *P. oleovorans* grown on E medium supplemented with 2% alkane as the sole carbon source. The alkane used for each run is indicated on the bottom of the graph. The data represents the total biomass accumulated per liter at the time the reactor was taken down.

P. oleovorans demonstrated the ability to grow best using octane as the sole carbon source. Reaching a maximum of 5.18 g of total biomass, octane proved to be better than the longer chain alkanes used in the fed batch experiments. The second best carbon source was heptane with a maximum of 5.065 g/L biomass, followed by decane at 3.79 g/L, and dodecane with only 2.04 g/L biomass accumulation. This corroborates data from Durner et al which showed higher total biomass and % PHA in cells grown on octane than alkanes and alkanoic acids with both longer and shorter chain lengths (Durner et al., 2001).

3.3.2 PHA Extraction

PHAs accumulated by *P. oleovorans* during the reactor experiments were extracted using a soxhlet apparatus. Gram quantities of PHAs were extracted from all samples except the sample grown on dodecane. No measurable accumulation of PHAs was extracted from the dodecane sample, and therefore no sample could be analyzed by GC-MS.

3.3.3 Analysis of PHAs

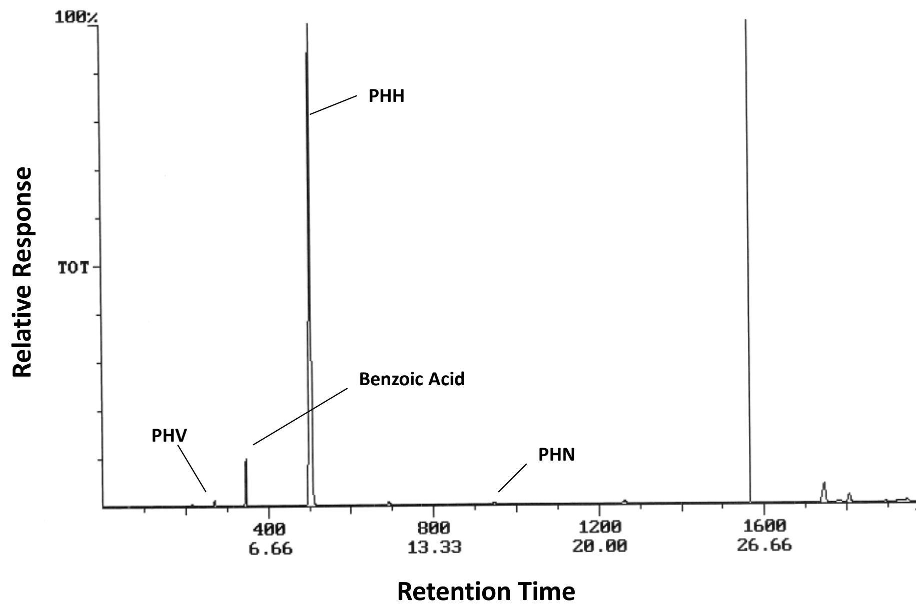


Figure 3-2 Gas chromatograph of PHA from *P. oleovorans* grown on heptane

PHAs synthesized from *P. oleovorans* grown on heptane analyzed by GC-MS to identify peaks. The identified peaks in order of elution are; polyhydroxyvalerate (PHV), benzoic acid (internal standard), polyhydroxyheptanoate (PHH), polyhydroxynonanoate (PHN).

When grown on heptane as the sole carbon source, *P. oleovorans* can incorporate 3-hydroxyheptanoate and 3-hydroxyvalerate into the PHA granule creating a copolymer. There are only trace amounts of PHV in the polymer, but the peak appears and is validated by the mass spectra plots located in the appendix.

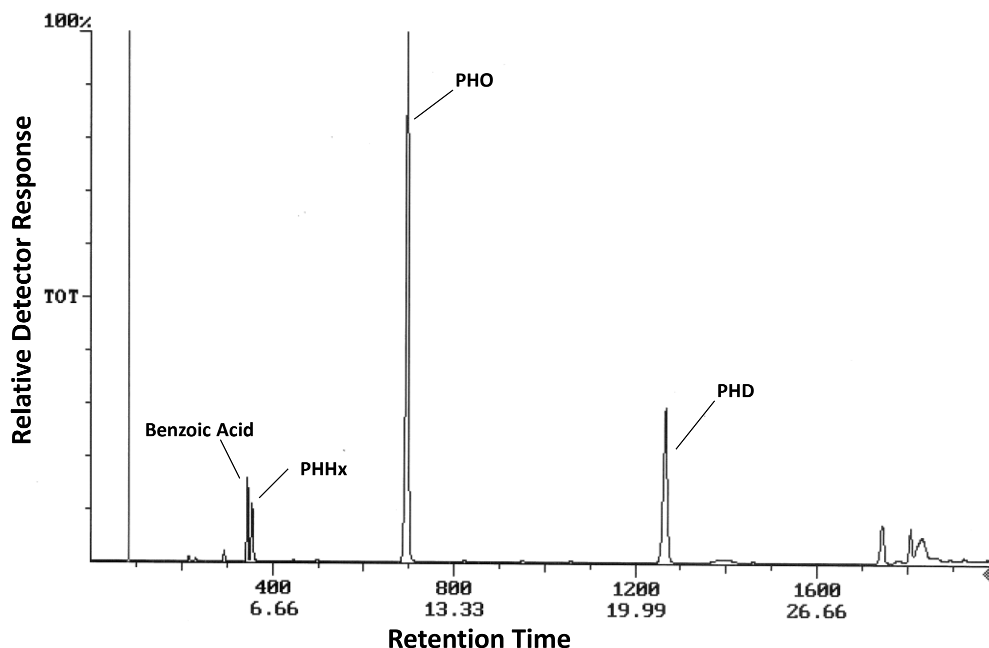


Figure 3-3 Gas chromatograph of PHAs from *P. oleovorans* grown on decane

Organic phase of propanolysis reaction of PHAs synthesized from *P. oleovorans* cultured on decane. The peaks identified in order of elution time are benzoic acid (internal standard), polyhydroxyhexanoate (PHHx), polyhydroxyoctanoate (PHO), and polyhydroxydecanoate (PHD).

The PHAs accumulated using decane as the sole carbon source was a terpolymer with 3-hydroxyhexanoate, 3-hydroxyoctanoate, and 3-hydroxydecanoate constituents. The largest fraction of the polymer as indicated by the GC trace in Figure 3-4 is 3-hydroxyoctanoate, followed by 3-hydroxydecanoate and finally 3-hydroxyhexanoate.

The chromatogram for the polymer produced from octane looks very similar to the ones produced from the other conditions and is omitted to avoid redundancies. The PHA produced was a copolymer mainly 3-hydroxyoctanoate with a smaller population of 3-

hydroxyhexanoate units.

Although the exact percentage of the biomass accumulated as PHAs could not be calculated at this time using the GC traces, rough calculations were made by weighing the amount of PHAs extracted using the soxhlet apparatus and taking the difference between that and the amount of biomass initially used for the extraction. The octane reactor was the highest with 30% PHA, followed by heptane with 28%, and decane with 16% PHAs.

3.3.4 Casting Films of extracted PHAs

As previously stated, the chloroform used in the soxhlet extraction containing dissolved PHAs was poured into glass petri plates and allowed to evaporate at room temperature. After the polymer was allowed to crystallize it was tough enough to be removed from the casting plates and analyzed by touch. Below is a picture of a film cast and a roll of extracted PHAs from the *P. oleovorans* culture using octane as its carbon source.

Figure 3-4 Extracted PHA from *P. oleovorans* growth on Octane

A film casted of polymer extracted using the techniques described previously. The piece on the left was molded from a sample that was attempted to be removed from the casting tray before the polymer was crystallized enough to be removed intact.

3.4 Discussion

The fed batch reactor experiments demonstrated that *P. oleovorans* is capable of growing on alkanes of lengths C7-C12 as their sole carbon source. The best growth, based on total biomass accumulation, occurred while using octane as the sole carbon source, and the poorest growth judging by the same criteria occurred while using dodecane as the sole carbon source. The conditions allowing for the greatest accumulation of PHAs mirrored the results for highest biomass accumulation. At the time of the experiments there was no method for accurately quantifying the amount of PHA in the CDW samples available, so therefore no data for the % PHA per cell dry weight was calculated using the GC traces. However, one of the goals of the experiment was accomplished by producing polymers that will aid in identifying the constituents of other polymers made by recombinant organisms in later experiments.

The analysis by GC-MS revealed that PHAs synthesized by *P. oleovorans* consists of monomers of similar size as the carbon source provided for growth. All of the PHAs produced were copolymers consisting of medium chain length 3-hydroxyalkanoates. This shows that there is little processing of the alkanes provided prior to being incorporated into the PHA granule, which makes sense due to the inability of *P. oleovorans* to synthesize PHAs from any method other than using intermediates from the β-oxidation pathways, and the constraints of the PHA polymerase (*phaC*) which will only accept medium chain length (R) 3-hydroxy acids (C6-12). The cells accumulate much of their PHAs during stationary growth phase. Since energy in the cell is not wasted at this time, there is little processing of the precursor molecules used for PHA synthesis. Therefore, the constituents found incorporated into the polymer are usually of the same

size as the carbon source fed to the organism.

Further analysis on the physical properties of the PHAs made during these experiments will provide a good comparison to future polymers produced using recombinant bacteria to see if there is an improvement on the toughness or elasticity of the polymers thereby increasing their appeal for commercial uses.

4. Controlled feeding for Mcl-PHA Block Co-polymer Production from *P. oleovorans*

4.1 Introduction

In previous experiments it was demonstrated that mcl-PHAs are accumulated as intracellular granules by *Pseudomonas oleovorans* when grown on alkanes. It was also demonstrated that growth on octane allowed for the highest accumulation of biomass and PHAs by *P. oleovorans*. Koning et al demonstrated that when grown on a mixture of octane and octene, *P. oleovorans* will incorporate an alkene into the PHA granule (de Koning et al., 1994). They also demonstrated that the double bonds incorporated in the polymer can be cross-linked by exposure to radiation with the effect of creating a material that retains its physical and material properties across a much wider temperature scale than mcl-PHAs with saturated side chains.

PHA block copolymers have also been shown to have enhanced flexibility and toughness over homopolymers and random copolymers with the same constituents (Pederson, 2006). For this experiment the concept of using a mass spec to control the feeding during batch fermentation (Pederson, 2004) was combined with the ability to incorporate double bonds in PHA granules as described earlier in *P. oleovorans*. This experiment is testing the theory that the *phaC* polymerase gene will incorporate 3-hydroxyalkenoate precursors into the PHA granule, and that microstructures created in the block copolymer combined with cross linking the double bonds post extraction would create a polymer that has both increased toughness and able to tolerate a wider range of temperatures while maintaining its physical and material properties.

4.2 Methods

4.2.1 Fed Batch Reactor Growth

Pseudomonas oleovorans wild type was grown overnight at 30°C in 5 mL of LB medium. The 5 mL culture was then transferred into a 1 L baffled flask containing 250 mL of LB media plus 1% octane and grown for an additional 16 hours at 30°C. The entire seed culture was transferred into a 10L Bioreactor (ThermoOnix, Houston USA) with 5L of E medium and 2.5% octane. The activity of the culture was monitored in real time by observing the oxygen uptake rate (OUR) and carbon evolution rate (CER) as described in chapter 2.

At the first indication that the culture was losing activity, a sharp drop in the CER and OUR, 100 mL of additional octane was added to ensure enough carbon would be present until the depletion of nitrogen in the media activating PHA synthesis in the cells. When the culture started to lose activity for the second time indicating the depletion of the current carbon source octane, 10 mL of 1-octene was added to the culture so the cells would be forced to utilize the octene and incorporate a block of 3-hydroxyoctenoate in the polymer chain. Following the depletion of the octene as indicated by the CER and OUR levels again, 50 mL octane was added to the culture. This switching between the two carbon sources continued every time the culture began to show signs of carbon source depletion for 14 periods until there was no positive response from the culture following a pulse of carbon source. Samples were taken throughout the life of the culture, usually just prior to the addition of fresh a carbon source.

When the culture no longer responded positively to additional carbon, the culture was centrifuged as described previously to harvest the cells and the cell pellet flash frozen in liquid nitrogen. The pellet was then lyophilized and the PHAs extracted using the soxhlet extraction method described in chapter 2. Films of the extracted PHAs were cast using the same method as described in chapter 3. After the chloroform was completely removed from the PHAs, small samples were taken and subjected to a propanolysis reaction and analyzed by GC or GC-MS to determine the composition of the polymer as described in chapter 2.

4.3 Results

4.3.1 Biomass accumulation in Fed Batch Reactors

P. oleovorans again demonstrated its ability to grow on alkanes and alkenes as the sole carbon source.

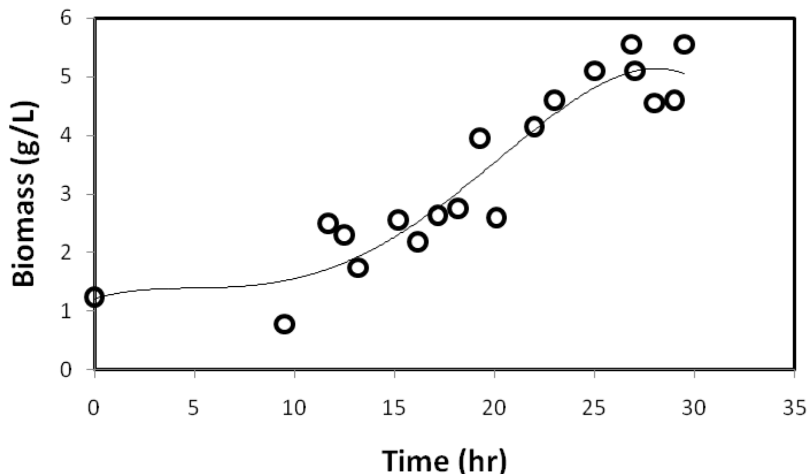


Figure 4-1 Total biomass accumulation from *P. oleovorans* growth on intermittent feeding of octane and octene

Samples taken during the life of the culture were analyzed for biomass accumulation by cell dry weight analysis.

The growth curve of the culture shows the cells endured a lag phase after being inoculated into the minimal media, but gradually started to use the octane as their carbon source. The graph also shows the culture reached a maximum of 5.55 g/L of biomass accumulation, and began to decline slightly right before the reactor was taken down.

4.3.2 Mass spectrometry analysis of off gas

A mass spectrometer was used to monitor the metabolism of the culture in real time as described in chapter 2. When the CER and OUR rates go up, the culture is actively metabolizing the available carbon source. When the values drop it indicates there is no more carbon source available and more must be added in order for the cells to continue growing or producing PHAs. The periodic feeding switching between octane and 1-octene creates defined peaks on the graph showing the CER and OUR values. The figures below represent the CER and OUR values from the culture during the entire length of the experiment (Figure 4-2) and a 5 hour window enlarged to show the periods in better detail (Figure 4-3).

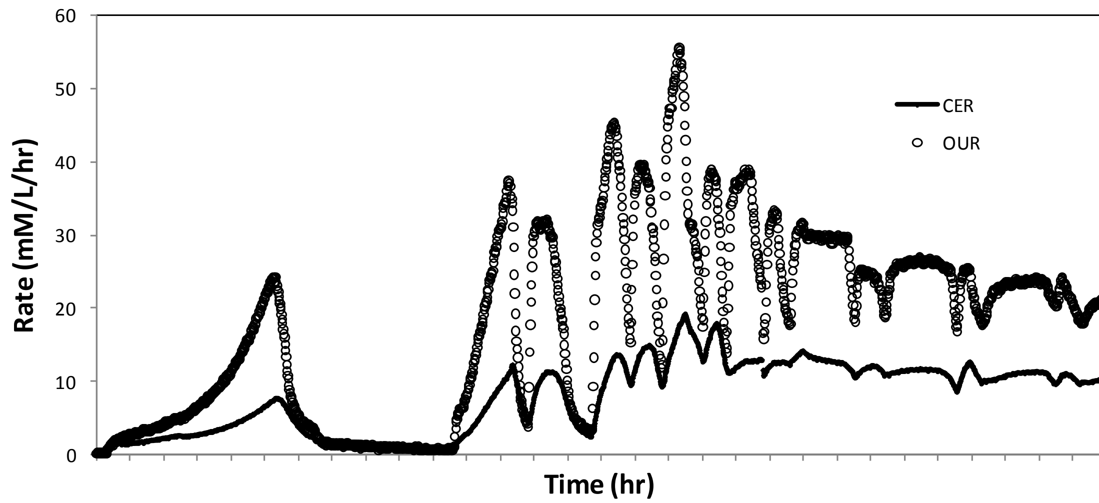


Figure 4-2 Plot of CER and OUR for entire life of culture

The figure above displays the carbon evolution rate (CER) and oxygen uptake rate (OUR) from the time the culture was inoculated to the time the reactor was taken down. The lower line of lighter circles is the CER and the OUR is the upper line indicated with darker squares. The periodic switching of feed sources produces the rapid rise and fall in the rates as the carbon is pulsed in and quickly metabolized by the culture.

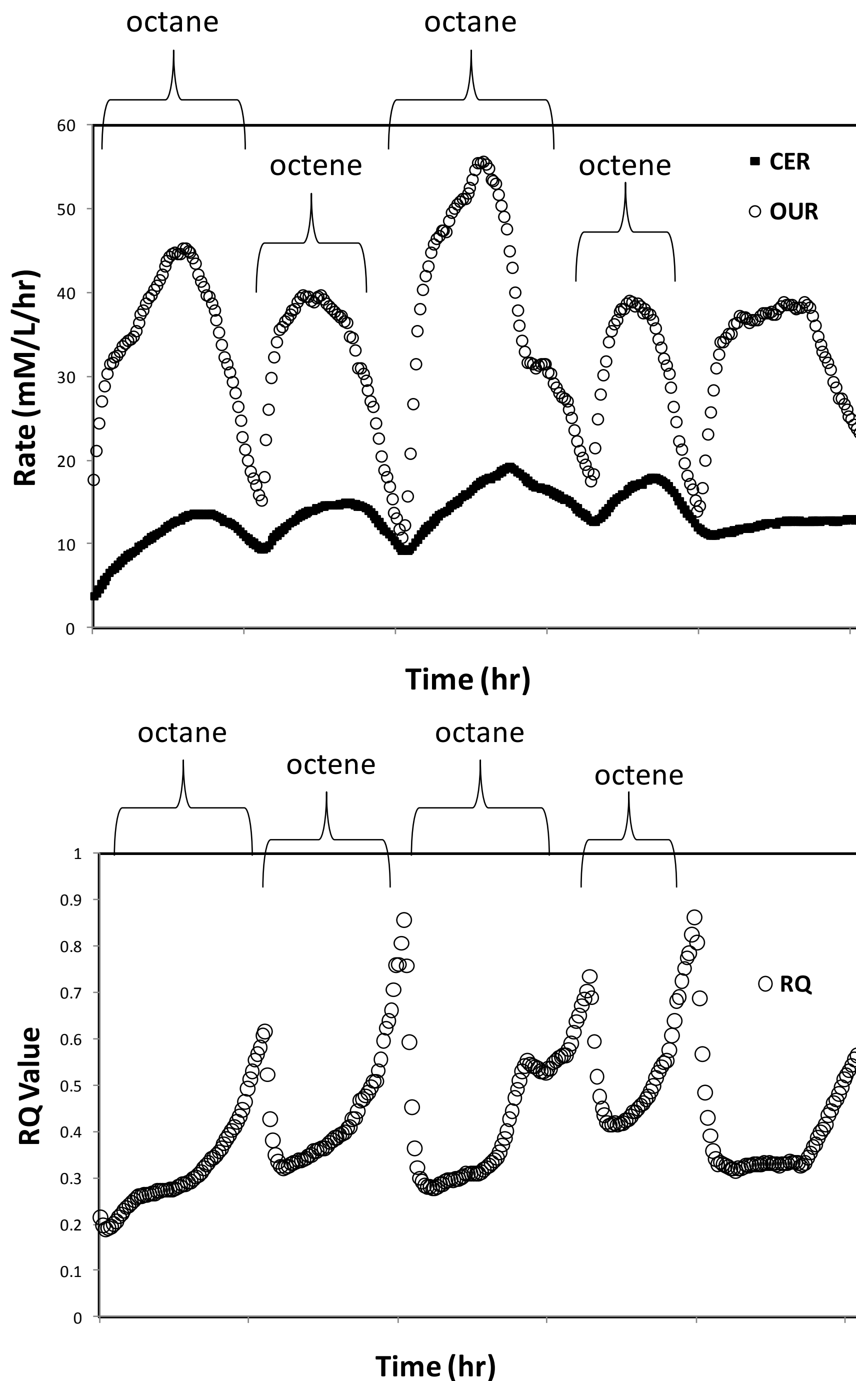


Figure 4-3 CER/OUR (top) and RQ (lower) values from exhaust gas measurements by mass spectrometry

The graphs above is represent a 5 hour period of the life of the culture and the CER, OUR, and RQ values calculated from measurements taken by the mass spectrometer of the carbon dioxide and oxygen levels in the exhaust gas from the culture during that time. The peaks are labeled octane or octene to indicate what carbon source was being fed to the culture at that time. The exact same time period is represented by the two graphs.

The CER and OUR were calculated using the difference between measurements taken of the house air being sparged into the reactor and the off gas coming out of the exhaust port in the head plate. The RQ is the respiratory quotient, a value calculated by dividing the CER by the OUR. This value changes throughout the different stages of a culture, and is unique to the particular metabolism occurring in the cell. The graph above indicates a slightly higher average RQ value when the culture is using octene as opposed to when it is using octane for PHA synthesis. The graphs also show a positive response from the addition of fresh alkane or alkene indicating active metabolism by the culture upon the additional carbon source. This gives a good indication that both carbon sources supplied were incorporated into the PHA chains.

A drop in the rates indicated the current carbon source was depleted, allowing for precise timing for a switch in carbon sources to be made, which forces the organisms to incorporate the new carbon source into the growing PHA chain. The carbon sources were switched between octane and 1-octene for 14 periods during the PHA accumulation phase as indicated by Figure 4-2.

4.3.3 GC-MS analysis of PHAs

PHAs accumulated during the growth study were extracted using a Soxhlet apparatus as described previously. Gram quantities of PHAs were extracted from the lyophilized cell pellet using this method. Milligram quantities were subjected to a propanolysis reaction and analyzed by GC-MS as described in chapter 2.

Analysis of PHAs extracted from lyophilized cells revealed that *P. oleovorans* has incorporated the double bond from the 1-octene into the polymer granules in the form of

3-hydroxyoctenoate and 3-hydroxyhexenoate. The mass spectrum for each peak was analyzed and used to identify the identity of the peaks displayed in the chromatogram. Figure 4-4 below shows what 3-hydroxyacyl monomers were identified in the polymer sample.

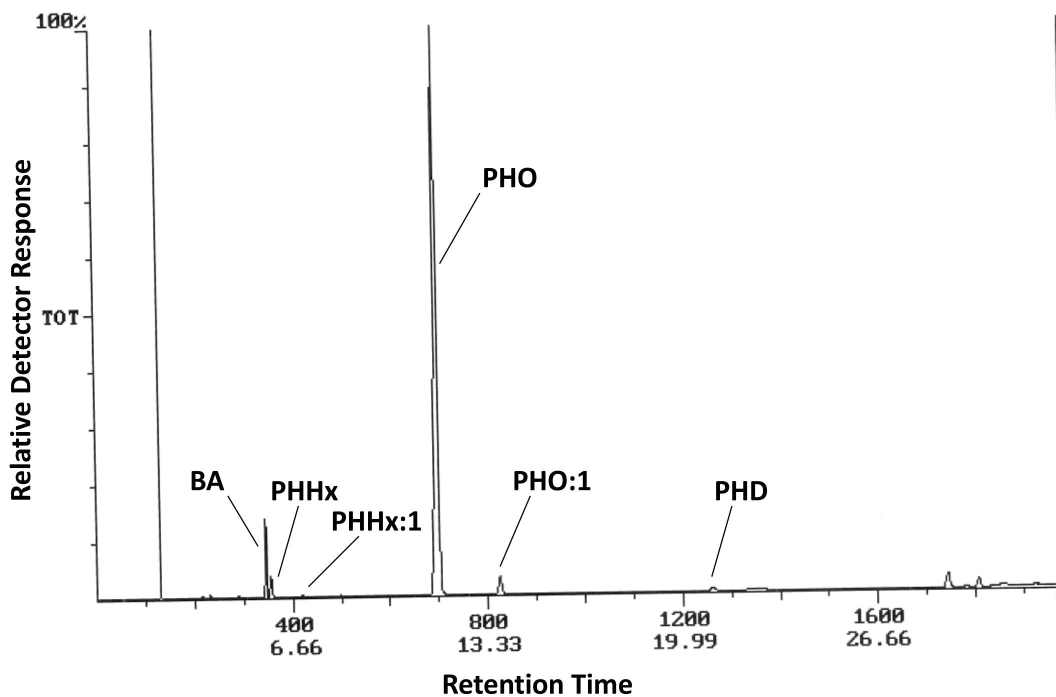


Figure 4-4 Gas chromatogram of PHAs from *P. oleovorans* grown on octane and 1-octene

Extracted PHAs subjected to propanolysis reaction and analyzed by GC-MS. Peaks identified in order of elution time; benzoic acid (internal standard), 3-hydroxyhexanoate (PHHx), 3-hydroxyhexenoate (PHHx:1), 3-hydroxyoctanoate (PHO), 3-hydroxyoctenoate (PHO:1), 3-hydroxydecanoate (PHD).

The gas chromatogram shows a distinct peak indicating the presence of 3-

hydroxyoctenoate, and a small peak indicating trace amounts of hydroxyhexenoate. Further analysis is needed to verify the presence of distinct blocks of alkanes and blocks of alkenes indicating the synthesis of a block copolymer.

4.3.4 Analysis of PHA Accumulation in culture

Cell dry weight (CDW) samples taken throughout the experiment were analyzed for their PHA content as described in chapter 2. Figure 4-5 shows the amount of PHA produced during the life of the culture plotted on the same graph as the total biomass accumulation.

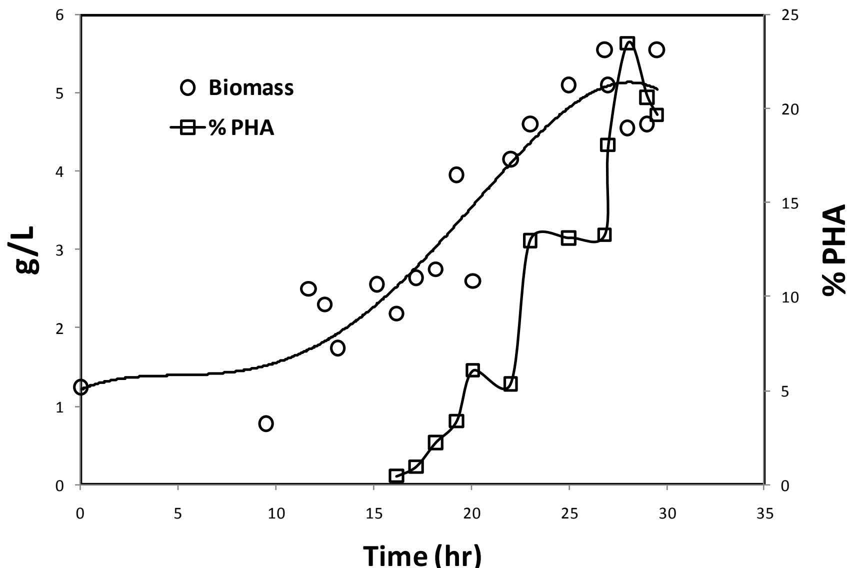


Figure 4-5 Total biomass and PHA production from *P. oleovorans* cultured on octane and 1-octene

The graph above represents the total biomass and mcl-PHA accumulation during the life of the culture when grown on intermittent feeding of octane and 1-octene. The total biomass is represented by the circles and the % PHA accumulation is represented by the squares.

The PHA accumulation is first detected 16 hours after the culture was inoculated. The total biomass at this point changes from exponential growth to linear growth to coincide with the elimination of nitrogen from the media and the accumulation of PHAs.

The analysis of the composition of the PHAs produced from the growth conditions used in this experiment is displayed in Table 4-1.

Table 4.1 Composition of mcl-PHA produced by *P. oleovorans*
The data presented in the table above represents analysis done on the final time point sample taken just before the reactor was taken down. The % PHA is the weight %.

			% Composition				
Biomass (g/L)	PHA (g/L)	% PHA	PHHx (6:0)	PHHX (6:1)	PHO (8:0)	PHO (8:1)	PHD (10:0)
5.55	1.09	19.7	4.2	<0.1	89.5	6.1	<0.1

4.4 Discussion

By measuring the gasses in the exhaust from the reactors with a mass spectrometer and correlating it with the concentrations of the gasses being sparged into the reactor, it is possible to calculate the rate that CO_2 is produced termed the Carbon Evolution Rate (CER), and the rate in which O_2 is used by the cells termed the Oxygen Uptake Rate (OUR). This gives accurate insight into the metabolism of the culture and allows one to know when a culture is actively growing or not. By observing this data in real time, it was possible to determine when the existing carbon source was depleted from the media so one can be sure that when adding in a new carbon source that it will be the only one available to the culture. Incorporating this technique into batch fermentation for the goal of PHA production, this system confers strict control of the molecules that are being incorporated into the growing PHA chain, and the ability to create block copolymers as opposed to random copolymers leading to PHAs with enhanced physical properties.

By switching the feedstock between octane and 1-octene, *P. oleovorans* incorporated both the saturated alkane and unsaturated alkene into the polyester chain.

The analysis showed that double bonds were present in the form of 3-hydroxyhexenoate and 3-hydroxyoctenoate. GC analysis revealed that the cells present in the culture at the end of the experiment contained about 20% of their dry weight as PHA, and the PHA composition was 4% PHHx (C6), 89% PHO (C8), and 6% PHOene (C8:1) with trace amounts of PHHXene (C6:1) and PHD (C10). This confirms that alkenes present in the media with a terminal double bond will be incorporated into the PHA granule by the *P. oleovorans* PHA polymerase. Since it has been shown that double bonds are present in the polymer, the next step will be to crosslink the double bonds by radiation and test the polymer for enhanced material properties. This result also indicates that *P. oleovorans* or a recombinant strain harboring the *phaC* polymerase gene should not have an issue with incorporating unsaturated alkenes into the PHA chain if presented with unsaturated fatty acids as a carbon source.

Further analysis needs to be performed to determine if the polymer produced is in fact a block copolymer, or if the polymer chains were terminated when the cells sensed carbon source depletion and started a new polyester chain upon the addition of more alkanes. Additional experiments should cross-link the incorporated double bonds by irradiating the polymer and testing to see if the properties are enhanced compared to other PHAs produced from octane and those produced from a mixture of octane and octene without cross-linking.

5. Manipulation of *R. eutropha* Genome for mcl-PHA Production From Soybean Oil

5.1 Introduction

This chapter describes an experiment where wild type *Ralstonia eutropha* H16 was engineered to produce medium chain length PHAs using soybean oil as the sole carbon source. Initial experiments where the mcl-PHA polymerase gene from *P. oleovorans* (*phaC*) was expressed in *R. eutropha* on a plasmid based expression system showed no signs of mcl-PHA accumulation when grown on fatty acids. Studies have shown that when antibiotics are used to maintain a plasmid in a culture of *R. eutropha*, only a small fraction of the population maintains the plasmid (Jackson & Srienc, 1999). To overcome this issue, and to establish a method for integrating genes or producing knock-outs of particular genes in *R. eutropha*, a suicide plasmid was designed to integrate the *P. oleovorans phaC* gene into the chromosome of *R. eutropha* in place of the native *phaC* PHB polymerase gene. The ability of the recombinant organism to accumulate mcl-PHAs was then tested in batch fermentation using soybean oil as the sole carbon source.

5.2 Methods

5.2.1 Suicide Plasmid Design

Pex-100T, a suicide vector used in previous studies for chromosomal integration in *R. eutropha* (Voss, 2006), was purchased from ATCC (ATCC ™, Manassas, VA). This plasmid is useful due to the inability of *R. eutropha* to recognize the origin of replication on the plasmid. When challenged with antibiotics in the surrounding media, the organism is forced to integrate the plasmid into its genome in order to retain the antibiotic

resistance gene located on the vector. Another feature of the plasmid is the *sacB* gene from *Bacillus subtillis*. This has been demonstrated to code for the peptide *levansucrase*, an enzyme catalyzing the reaction of Sucrose → β(2,6)-linked fructan, otherwise known as levan, a polysaccharide toxic to most bacteria (Gay, Le Coq, Steinmetz, Ferrari, & Hoch, 1983). Below is a map of the plasmid as it was received from ATCC.

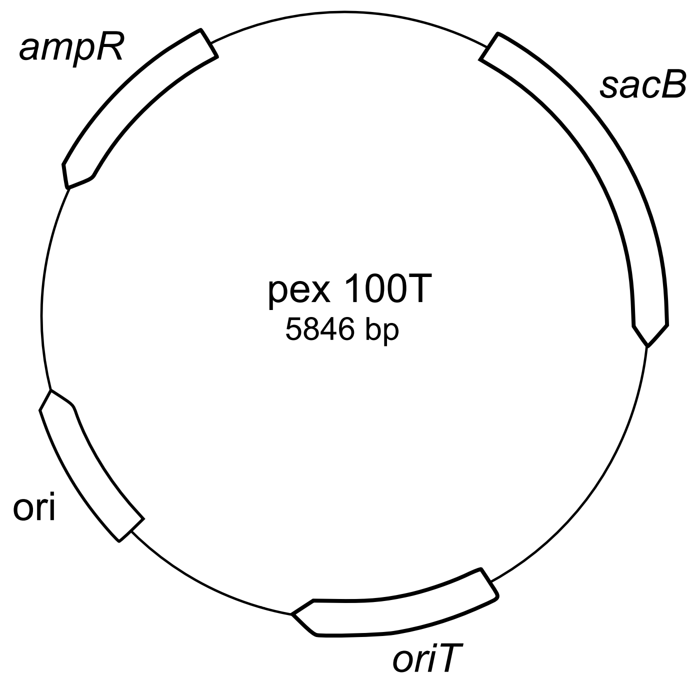


Figure 5-1 Map of plasmid pex100T with important features highlighted
Pex-100T is a suicide vector purchased from Invitrogen and used to create a suicide vector capable of integrating into the genome of *R. eutropha*.

Parts of the plasmid pGP-564 (Thermo Scientific, Waltham, MA), a yeast – *E. coli* shuttle vector, were combined with the pex100T plasmid to gain all of the fragments necessary to assemble a suicide vector by using homologous recombination in yeast. Four regions were needed from pGP-564 to add to the pex-100T vector to construct a suicide vector for *R. eutropha* that can be assembled in yeast. These regions were; the 2 μm ori for plasmid replication in yeast, the leucine gene to complement the leucine

auxotroph in the yeast strain used to put selective pressure to maintain the plasmid, the kanamycin resistance gene to put selective pressure on the bacteria harboring the plasmid, and the multiple cloning site for easier cloning during downstream applications. The bacterial origin of replication that will be used will come from the pex-100T vector. Figure 5-2 below is a map of the pGP-564 plasmid as it was received from Thermo Scientific.

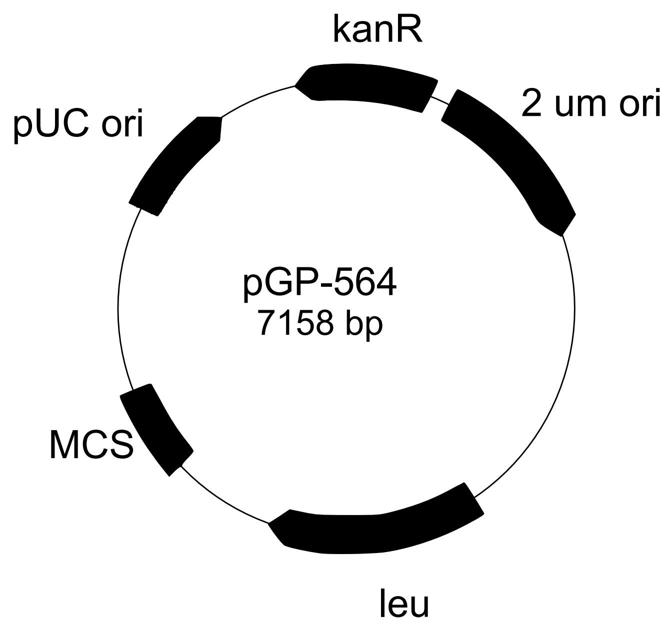


Figure 5-2 Plasmid map of pGP-564 plasmid with important features highlighted
The plasmid pGP-564 is a yeast/*E. coli* shuttle vector. Parts of it were used to create the plasmids used in experiments in chapters 5 and 6.

5.2.2 Suicide Plasmid Construction

Homologous recombination in yeast was used to construct the plasmids necessary for this experiment using the protocols outlined in chapter 2. To ensure the plasmid assembled acted as a suicide plasmid in *R. eutropha*, the origin of replication from the pex-100T plasmid was retained. Also, in order to do the construction of the plasmid in yeast, the 2 μm ori and the leucine gene must be present on the plasmid. For these

reasons the pex-100T plasmid was combined with major parts of the pGP-564 plasmid to construct the suicide vector termed pex-pGP. The multiple cloning site (MCS) and the leucine gene were cloned in one fragment and the 2 μm ori and the kanamycin resistance gene were cloned in another fragment from the yeast – *E. coli* shuttle vector pGP-564. The primers used to clone the fragments are shown in Table 5-1 and Figure 5-4 illustrates how the fragments will be recombined using their regions of homology to one another.

Table 5.1 Primers used for cloning fragments for assembly of pex-PGP plasmid by homologous recombination in yeast

The table below shows the primer sequences used for PCR amplifying the fragments used to assemble the suicide plasmid pex-pGP using homologous recombination in yeast. Fragments 2 and 3 were PCR amplified from the vector pGP-564 with the primer pairs 2μmkanFor - 2μmkanRev and LeuMCSFor – LeuMCSRev respectively. The bold faced portion of the primer sequence indicates the region of the primer that binds to the original DNA template strand in the PCR reaction, and the regular type face sequence is the portion of the primer designed to add 40 base pairs of homology to the fragment to be assembled adjacent to that PCR product on the final plasmid.

Location on gene	Frag. #	Sequence
Upstream	**2**	**GCGTAACTCACGTTAAGGGA** GGCCAGTGCCAAGCTCATTACCCTGTTATCCCTACCCGGG
Downstream	**2**	TGAAAGTTCCCTCAAGAATTTTACTCTGTCAGAAACGGCC **AATCCAATTGAGGAGTGGCA**
Upstream	**3**	**ACTATCCTCCTTTTTCTCCT** GGCCGTTTCTGACAGAGTAAAATTCTTGAGGGAACTTTCA
Downstream	**3**	TGATTACGCCAAGCTCTAGGGATAACAGGGTAATCCCGGG **ATGTGCTGCAAGGCGATTAA**

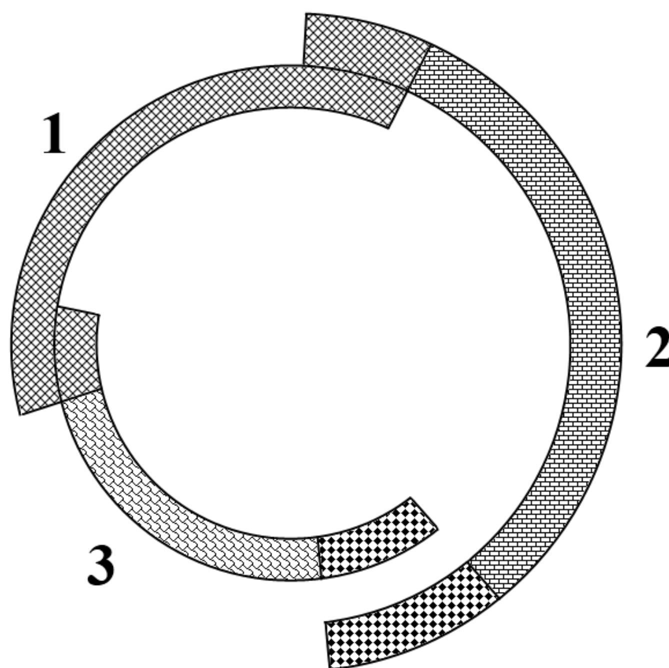


Figure 5-3 Map of fragments used for homologous recombination

The diagram above, using the same numbering scheme as Table 5.1 earlier, illustrates the fragments used during the recombination and the homologous regions engineered into the PCR products. Fragment 1 represents the pex-100T plasmid linearized with a SmaI digest, fragment 2 represents the fragment cloned from the pGP-564 vector containing the 2 µm ori and kanR genes with terminal homology to fragment 1 and 3, and fragment 3 is the fragment containing the leucine gene and the multiple cloning site with terminal homology to fragments 2 and 1.

The fragments were cloned by PCR reaction with an annealing temp of 62°C and run on a 0.8% agarose gel to verify successful cloning. Both PCR derived products were amplified from the pGP-564 vector. The pex-100T vector was linearized by digestion with the unique restriction enzyme SmaI (NEB, Ipswich, MA) and run on a 0.8% agarose gel to verify complete digestion of the circular plasmid.

The samples were then cut out of the agarose gel and purified as described in chapter 2. Equal molar quantities of the purified DNA fragments were then mixed together and transformed into the yeast strain S288 as described in chapter 2 to be recombined into a circular plasmid via homologous recombination. Several colonies that

formed on the experimental plates were then screened for the presence of the fragments used in the recombination by colony PCR. The primers 2umkanFOR and 2umkanREV, the primers used to PCR amplify fragment 2 in Figure 5-3, were used for screening the colonies using the same PCR conditions as before. Colonies were picked and resuspended individually in the PCR tubes containing the buffered PCR solution. Some cells will rupture during the first denaturing step at 94°C in the PCR reaction releasing plasmid DNA that will be used as the template for the PCR reaction. The PCR products were then run on an agarose gel to check for the presence of the amplified fragment indicating the presence of the recombined plasmid in the colony.

One of the colonies that displayed a positive PCR result was grown overnight in SD media and the plasmids were extracted as described in chapter 2. The extracted plasmid was transformed into *E. coli* DH5α as described previously and grown in liquid LB media with kanamycin. This was necessary for producing a high enough concentration of plasmid to be used for downstream applications. The plasmid was then extracted using the *E. coli* plasmid extraction kit as described in chapter 2. The concentrated plasmid was digested with the multiple cutting restriction enzyme ClaI to verify the proper construction of the plasmid by the yeast recombination.

5.2.3 Suicide Plasmid Design for *phaC* Integration

Genomic integration uses a strategy where cells will integrate large pieces of DNA into their chromosome using homologous recombination if they find it necessary to retain the new DNA, and recognizes that the "foreign DNA" shares sequence homology to regions of its own genome. This method was exploited in this experiment by surrounding

the gene we would like to integrate into the genome with fragments homologous to the regions naturally flanking the site we are targeting in the genome. The next phase of plasmid construction involved PCR amplifying regions of the *R. eutropha* genome flanking the native *phaC*, and PCR amplifying the *phaC* gene from the genome of *P. oleovorans*. The primers used for the PCR amplification reaction are shown in Table 5-2 which displays the sequence of the primer designed to bind to the template and PCR amplify the region of interest, and the 40 base pairs of homology to the adjacent fragment on the plasmid added as an overhang to the original DNA template. Figure 5-4 illustrates how the fragments will be recombined using their regions of homology to one another to assemble the plasmid pex-pGP *phaC*.

Table 5.2 Primers used for PCR amplification of fragments used in homologous recombination in yeast to assemble pex-pGP *phaC*

The table below shows the primer sequences used for PCR amplifying the fragments used to assemble the suicide plasmid pex-pGP *phaC* using homologous recombination in yeast. Fragment 2 was PCR amplified with the primers UphomFor and UphomRev from the genome of *R. eutropha* with terminal homology to regions to fragments 1 and 3, fragment 3 was PCR amplified from the genome of *P. oleovorans* using the primers Po*phaC*1For and Po*phaC*1Rev, and fragment 4 was PCR amplified with the primers DownhomFor and DownhomRev from the genome of *R. eutropha* with terminal homology to fragments 3 and 1. The bold faced portion of the primer sequence indicates the region of the primer that binds to the original DNA template strand in the PCR reaction, and the regular type face sequence is the portion of the primer designed to add 40 base pairs of homology to the fragment to be assembled adjacent to that PCR product on the final plasmid.

Location on gene	Frag. #	Primer Sequence
Upstream	2	GCTCCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCC **CGATGAACAGGTCGCGGTTG**
Downstream	2	CTGAGACGAGTACCGGTTGCTGTGGCGGGTGCACAGTTCT **GATTTGATTGTCTCTCTGCC**
Upstream	3	**AGAACTGTGCACCCGCCACA**
Downstream	3	**AAATGAACACCGTGGCGTCC**
Upstream	4	CAGCGCCGTGGCCACCTGCGGGACGCCACGGTGTTCATTT **TGAGTGCCGGCGTGCGTCAT**
Downstream	4	CGATAAGCTTGATATCGAATTCCTGCAGCCCGGGGGATCC **CTTGTCGAAGGCGGGCTTGA**

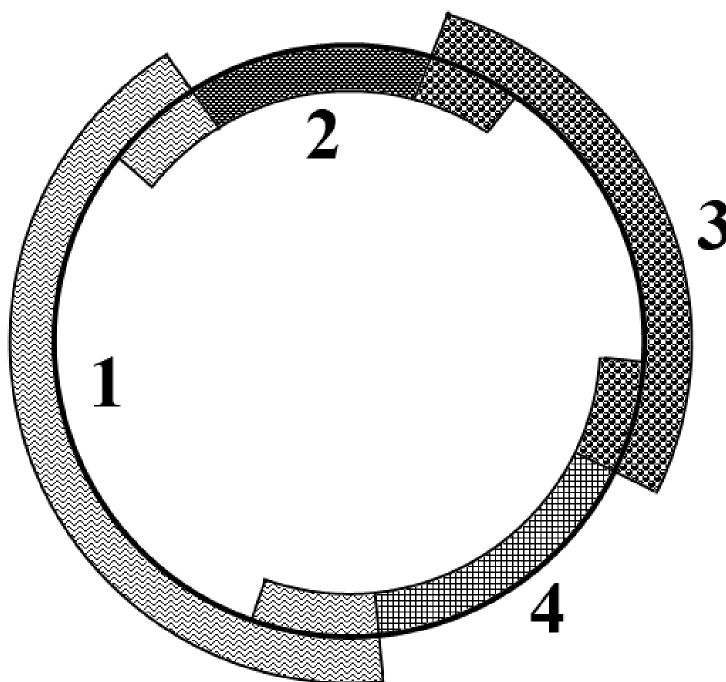


Figure 5-4 Diagram showing the fragments used during the homologous recombination

The diagram above displays the fragments used in the recombination and their associated homologous regions designed into certain PCR amplified products used. Fragment 1 represents the pex-PGP vector linearized with a BamHI digest, fragment 2 indicates the region of the *R. eutropha* genome directly upstream of the targeted *phaC* with terminal homology to fragment 1 and 3, fragment 3 represents the *phaC* gene cloned from the *P. oleovorans* genome with no added terminal homologous regions, and fragment 4 represents the region of the *R. eutropha* genome directly downstream of the targeted *phaC* gene with terminal homology regions to fragments 1 and 3.

Fragment 2 containing the region of the *R. eutropha* genome upstream of the targeted *phaC* gene is an 884 base pair sequence that contains the entire promoter sequence for the PHB operon and ends right before the start codon for the *phaC*, the first gene in the *phbCAB* operon. Fragment 3 containing the region of the *R. eutropha* genome downstream of the targeted *phaC* gene is a 790 base pair fragment starting with the first base following the stop codon of the *phaC*. This region contains part of the *phaA* gene, the second gene in the *phaCAB* operon. Fragment 3 contains the PCR amplified

phaC gene from the genome of *P. oleovorans*. The fragment begins 78 bases upstream of the start codon to ensure to capture the ribosome binding site and ends 47 base pairs past the stop codon.

5.2.4 Suicide Plasmid Construction for *phaC* Integration

The plasmid vector constructed in the previous recombination event was linearized with the restriction enzyme BamHI (NEB, Ipswich, MA). This was also run on an agarose gel to check that the sample was fully digested. All of the fragments to be used in the recombination reaction were then purified from agarose gels as previously described, mixed in equal molar quantities, and transformed into yeast using the same protocol as before. Colonies that formed on the experimental plates were then screened using the primers Po*phaC*1For and Po*phaC*1Rev, primers specific for the *phaC* gene from *P. oleovorans*, using the same PCR conditions as before. The PCR products were run on a gel to verify the presence of the gene fragment in the yeast strain. A colony containing the gene was identified, grown in liquid SD media, and the plasmid extracted as done previously. The extracted plasmid was screened by PCR for all of the fragments used in the last recombination before it was transformed into *E. coli* as part of the next step of the process.

5.2.4 Transfer of Plasmid to *R. eutropha*

The plasmid was then transferred into a mating strain of *E. coli* (S17-1) as described in chapter 2, which was then used to perform the transfer of the plasmid into *R. eutropha* by conjugation using the mating strategy described previously. Following the screening process after the transfer of the plasmid via conjugation, one colony of *R.*

eutropha growing on the secondary screening plates was picked and grown overnight in Doi media with kanamycin. A genomic prep was done as described previously and used as the template for a PCR reaction screening for the *P. oleovorans phaC* integrated into the genome of the bacteria.

5.2.5 Genomic Integration of Suicide Vector

Pending a positive result of the PCR amplification of the *P. oleovorans phaC* gene from the genome of *R. eutropha* indicating the successful integration event into the genome, a liquid culture was then serially diluted in sterile 0.8% NaCl and plated on Doi agar plates supplemented with 5% sucrose and incubated at 30°C for 2 days for the secondary recombination event to take place. This event removes all parts of the plasmid outside of the homologous regions to the genome of the targeted bacteria designed into the plasmid. The counter-selection event will create two populations, one that has reverted back to the wild type and one that keeps the new DNA creating a recombinant organism. Below is a simplified diagram illustrating the recombination events that take place in the genome of *R. eutropha* during this integration event.

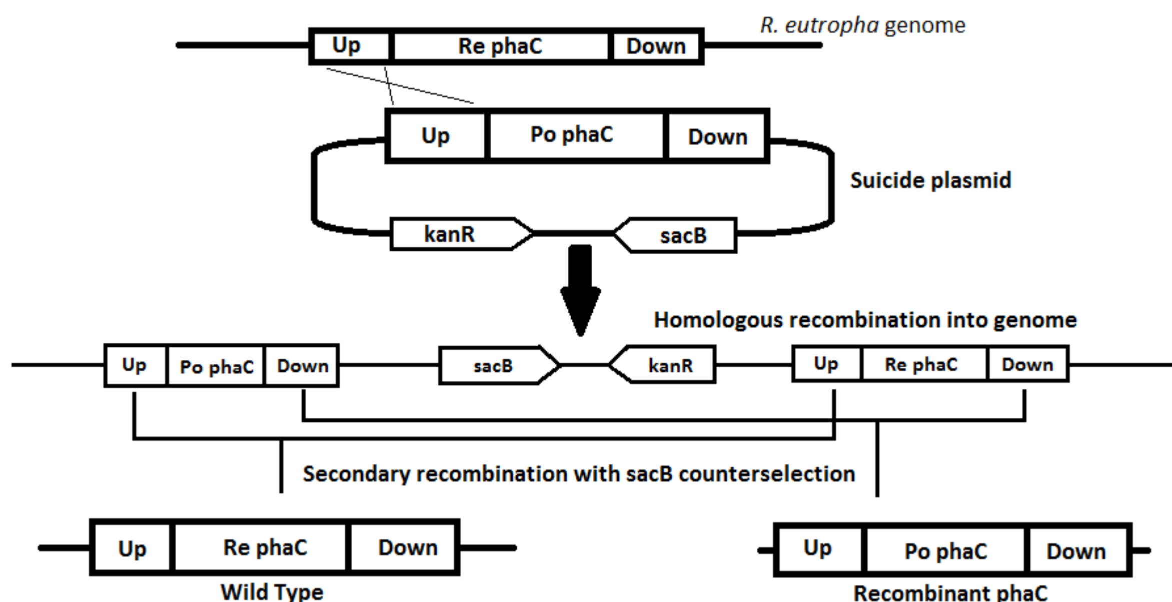


Figure 5-5 Diagram of genomic integration event and counter-selection using suicide plasmid pex-pGP *phaC*

The above figure is a general diagram showing how the suicide plasmid pex-pGP *phaC* is integrated into the genome of *R. eutropha* by a homologous recombination event, and subsequently counter-selected using the *sacB* gene on the plasmid to generate a secondary recombination event removing the DNA outside of the homologous regions that recombine. Up and down stand for the regions upstream and downstream of the genomic region targeted for deletion during the recombination. These regions were PCR amplified from the genome of *R. eutropha* and assembled on the plasmid via homologous recombination in yeast. Re *phaC* stands for the PHB polymerase gene located in the *R. eutropha* genome, and Po *phaC* indicates the mcl-polymerase gene from the *P. oleovorans* genome. The arrow indicates the flow of the diagram.

5.2.6 Screening for *P. oleovorans phaC1* Integration into Genome Following the Secondary Recombination Event

Since wild type *R. eutropha* accumulates large amounts of PHB, the colonies appear opaque when grown under conditions capable of becoming nitrogen limited which induces PHB accumulation. This meant that colonies that did not appear opaque on the agar plates containing sucrose most likely had their native *phaC* removed and replaced with the recombinant gene during the integration process. Three colonies were picked

and screened for the presence of the *P. oleovorans phaC* and absence of the *R. eutropha phaC* by colony PCR. The primers for the *P. oleovorans phaC* were the same as before and the primers for *R. eutropha* were ResynFOR1 ATGCCTTGGCTTTGACGTAT and ResynREV1 CCAACCATTCAAGGTCACG. These primers bind to the very beginning and end of the gene. To be sure that the *P. oleovorans* is integrated in the genome and in the proper location, a primer further upstream of the upstream homology region (Re*phaC*upFOR CCTGAACCTGGGCACC) was paired with the reverse primer for the recombinant *phaC* gene (Po*phaC*1Rev), and the forward primer for the recombinant *phaC* (Po*phaC*1For) was paired with a primer further downstream than the downstream region (Re*phaC*downREV TCGTGCAGCAGCGTCAC) and used in a PCR reaction using the same genomic DNA as the previous test as the template. Figure 5-4 is a diagram illustrating the location the primers bind to in the genome of the recombinant organism and the expected fragments from the PCR reaction.

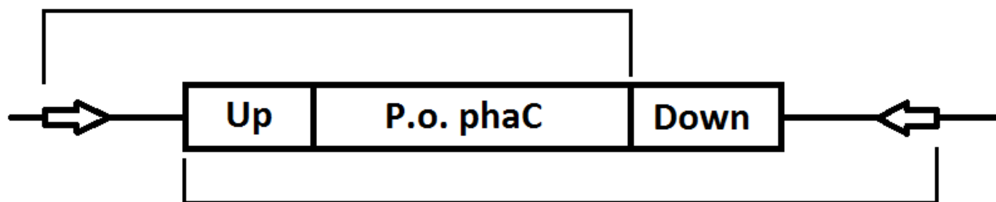


Figure 5-6 Diagram of recombinant *R. eutropha* genome following secondary recombination event and PCR test to verify integration

Up and down represent the upstream and downstream homology regions designed on the suicide plasmid and maintained during the genomic homologous recombination events. The arrows represent the primers paired with the *phaC* primers in the PCR reaction and show how they bind outside of the homologous regions designed on the plasmid. The lines above and below the diagram, indicate the region amplified by each PCR reaction.

5.2.7 Fed Batch Soybean Oil Reactor Growth

The recombinant organism *R. eutropha*Δ*phaC* was tested for its ability to accumulate mcl-PHAs when grown on soybean oil as the sole carbon source. A colony of the wild type H16 and the recombinant *R. eutropha*Δ*phaC* were picked from a Doi agar plate and inoculated into separate tubes containing 5 mL of Doi media and incubated overnight at 30°C. 5 mL of the cultures were then used to inoculate 250 mL of Doi media supplemented with 1% soybean oil to induce lipase production while scaling up the size of the culture in preparation for inoculating the bioreactor, and were incubated for 12 hours at 30°C in a 1 L baffled shake flask. The seed cultures were then added to 5 L of Mineral Salts Media supplemented with 2% soybean oil in 10 L reactors. The cultures were kept at 30°C, stirred at 400 rpm, sparged with atmospheric air, and sampled regularly during the 85 hour life of the culture.

5.2.8 Analysis of Cell Cultures

Samples taken throughout the life of the culture were analyzed for their cell dry weight (CDW), soybean oil concentration in the media, and PHA content using methods described in chapter 2. At the end of the experiment the remaining cells were harvested by centrifugation and the PHAs extracted using the soxhlet apparatus as described previously. Samples of the extracted PHA were poured into glass petri plates to form films for further study of their physical and mechanical properties. Both extracted PHA samples and CDW samples were subjected to propanolysis and analyzed by GC and GC-MS for their polymer content.

5.3 Results

5.3.1 DNA fragments used in Suicide Plasmid Construction

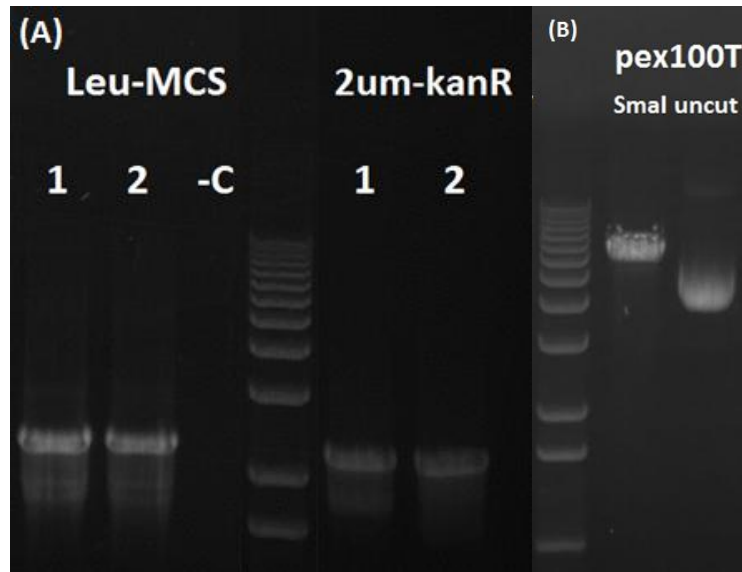


Figure 5-7 0.8% agarose gels displaying fragments used in homologous recombination to construct pex-pGP

Gels contained 0.8% agarose and ethidium bromide for visualization of DNA under UV light. (A) Cloned fragments (2 and 3 from Figure 5-3) from pGP-564 using LeuMCSFOR-LeuMCSREV and 2umkanFOR-2umkanREV. (B) Sample of pex-100T digested with unique cutter SmaI run alongside an undigested sample to verify complete digestion.

Both of the PCR products in Figure 5-5 showed strong bands indicating the desired fragments were successfully amplified from the pGP-564 plasmid. The Leu-MCS region matches the expected size of 2147 base pairs, and the 2μm-kanR fragment matches the expected size of 2376 base pairs. The plasmid appears linear and at the correct size of 5846 base pairs.

5.3.2 Colony PCR for Constructed pex-pGP Plasmid Screening

Figure 5-8 PCR products from colony PCR of yeast colonies following homologous recombination

Eight yeast colonies picked from the SD selection plates after the transformation of linear DNA fragments during the homologous recombination event to construct the plasmid pex-pGP and were used as the DNA template for screening yeast colonies to verify the presence of the recombined plasmid in one of them.

All eight colonies screened showed a positive band indicating a successful recombination of the linear DNA fragments. The colony corresponding to the lane with the brightest band was picked (#2) and used for the next step of the experiment.

5.3.3. Verification of Properly Assembled pex-pGP Plasmid

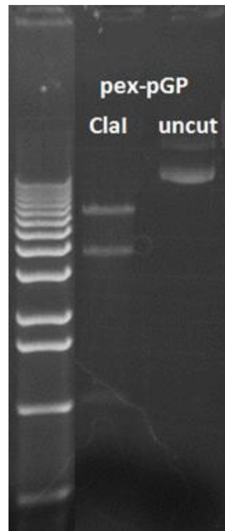


Figure 5-9 ClaI digest of pex-pGP plasmid from *E. coli* plasmid extraction

The pex-pGP plasmid constructed using homologous recombination in yeast was digested with the enzyme ClaI. The fragment pattern matched the expected sizes determined by digesting the plasmid in silico; 5834 bp, 3340 bp, 904 bp, and 337 bp. The 337 bp fragment is not visible due to low amounts of digested DNA loaded into the gel and the lack of ethidium bromide in the lower half of the gel resulting from migration during the electrophoresis.

The DNA fragment pattern created by the restriction digest matched the expected fragment pattern indicating that all of the fragments in the transformation mixture were assembled into a circular plasmid correctly. The plasmid map below illustrates the newly constructed suicide plasmid.

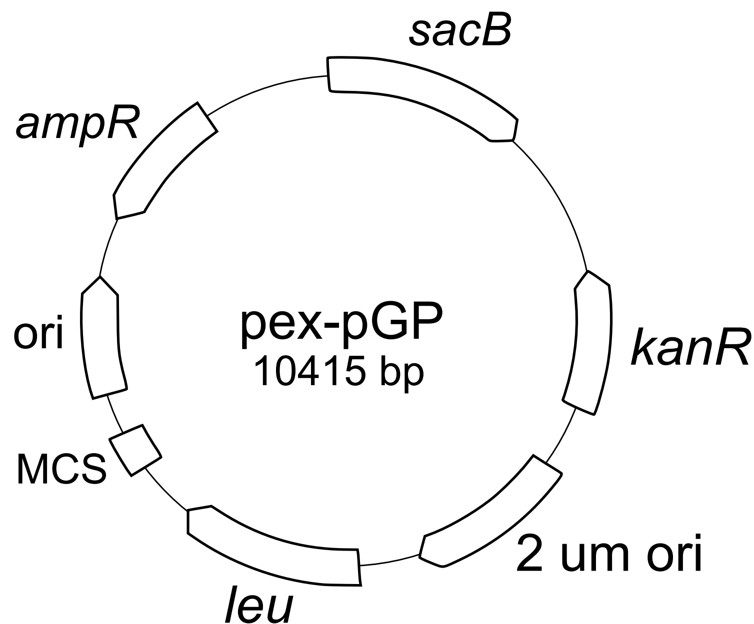


Figure 5-10 Plasmid map of pex-pGP plasmid

The map above shows the plasmid assembled by the first round of yeast homologous recombination. The fragments used for the recombination were linearized pex100T plasmid (SmaI), a 2147 base pair fragment containing the leucine gene and MCS from pGP-564, and a 2376 base pair fragment containing the kanR gene and the 2 μm yeast ori from pGP-564.

5.3.5 Screening for *phaC* Integration into *R. eutropha* Genome

Figure 5-11 Agarose gel from S288 colony screening of second round of homologous recombination event

Colony PCR was used to screen for the presence of the *P. oleovorans phaC* in the yeast colonies formed after the homologous recombination event inserting the upstream, downstream, and *phaC* regions into the pex-pGP plasmid.

All five of the colonies picked for screening were positive for the presence of the

phaC gene indicating this fragment was assembled into a circular plasmid, and most likely the entire plasmid was assembled correctly. The colony corresponding to the brightest band (#4) was picked and used for the downstream applications.

5.3.5. Screening pex-pGP *phaC* Plasmid

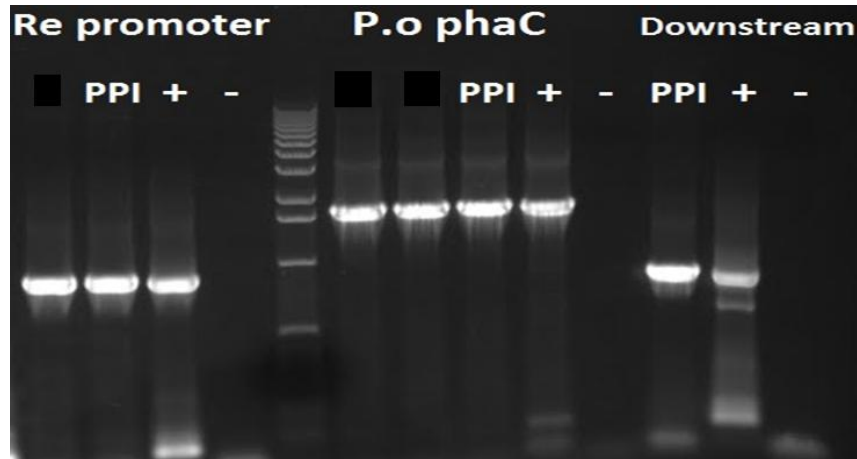


Figure 5-12 PCR screening of plasmid extracted from *E. coli* strain S17-1

The PCR screening of the extracted plasmid indicates that all of the fragments used in the transformation reaction are present on the plasmid. PPI stands for pex-pGP Integration, the integration plasmid constructed for this experiment (pex-pGP *phaC*). Re promoter represents the upstream region homologous to the *R. eutropha* genome, P. o *phaC* represents the *P. oleovorans phaC* gene, and downstream represents the downstream region homologous to the *R. eutropha* genome.

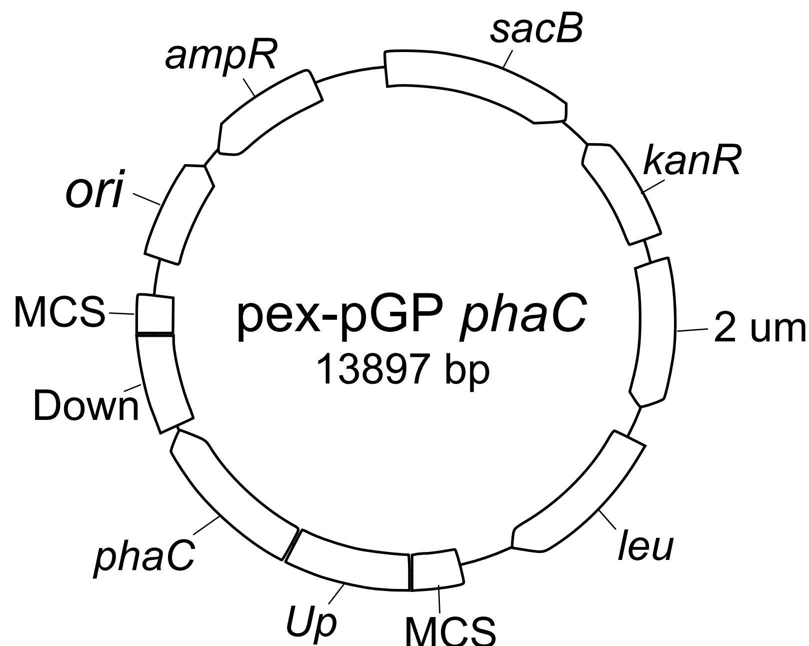


Figure 5-13 Plasmid map of pex-pGP *phaC* suicide vector

Above is a map of the completely assembled suicide vector was constructed from the two rounds of homologous recombination. The new fragments added to the pex-pGP plasmid are the upstream (Up) and downstream (Down) regions homologous to the *R. eutropha* genome flanking the region targeted for integration, and the *P. oleovorans phaC* gene. These genes were added into the vector at the BamHI site splitting the multiple cloning site (MCS) into two fragments.

5.3.6 Colony Screening for *phaC* Following *sacB* Counter-selection

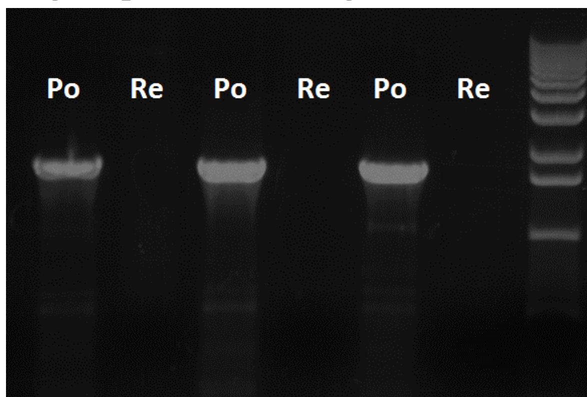


Figure 5-14 Screening for presence of *P. oleovorans phaC* and Absence of *R. eutropha phaC* in *R. eutropha* genome

Genomic DNA samples of colonies picked from Doi sucrose plates were screened for the presence of the *P. oleovorans phaC* and absence of the native *R. eutropha phaC* gene. The primers Po*pha*CFOR and Po*pha*CREV were used in the Po lanes to PCR amplify the *P. oleovorans phaC* gene, and the primers ResynFOR and ResynREV were used in the Re lanes to PCR amplify the *R. eutropha phaC* gene.

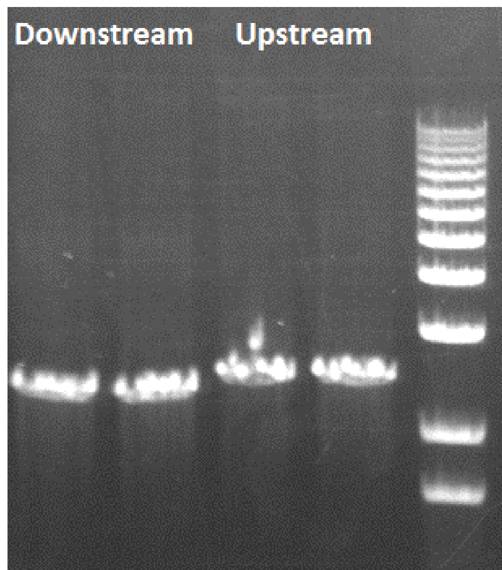


Figure 5-15 Verification of Proper Integration of *P. oleovorans* into Genome of *R. eutropha*

The primers Re*phaC*upFOR and Po*phaC*REV, and Re*phaC*down and Po*phaC*FOR were paired together in PCR reactions to verify the integration of the *P. oleovorans phaC* gene into the place previously occupied by the native *R. eutropha phaC* as shown in the Figure 5-4.

Both PCR tests, one for the presence of the *P. oleovorans phaC* (Fig. 5-12) and the other verifying that the gene is located in the proper site in the genome (Fig. 5-13) were a positive result. This validates the plasmids proper construction, the procedure for integrating genes into the genome of *R. eutropha*, and the secondary recombination event using the sacB marker on the plasmid.

5.3.7 Analysis of PHAs From Fed Batch Reactors

5.3.7.1 *R. eutropha* Δ *phaC*

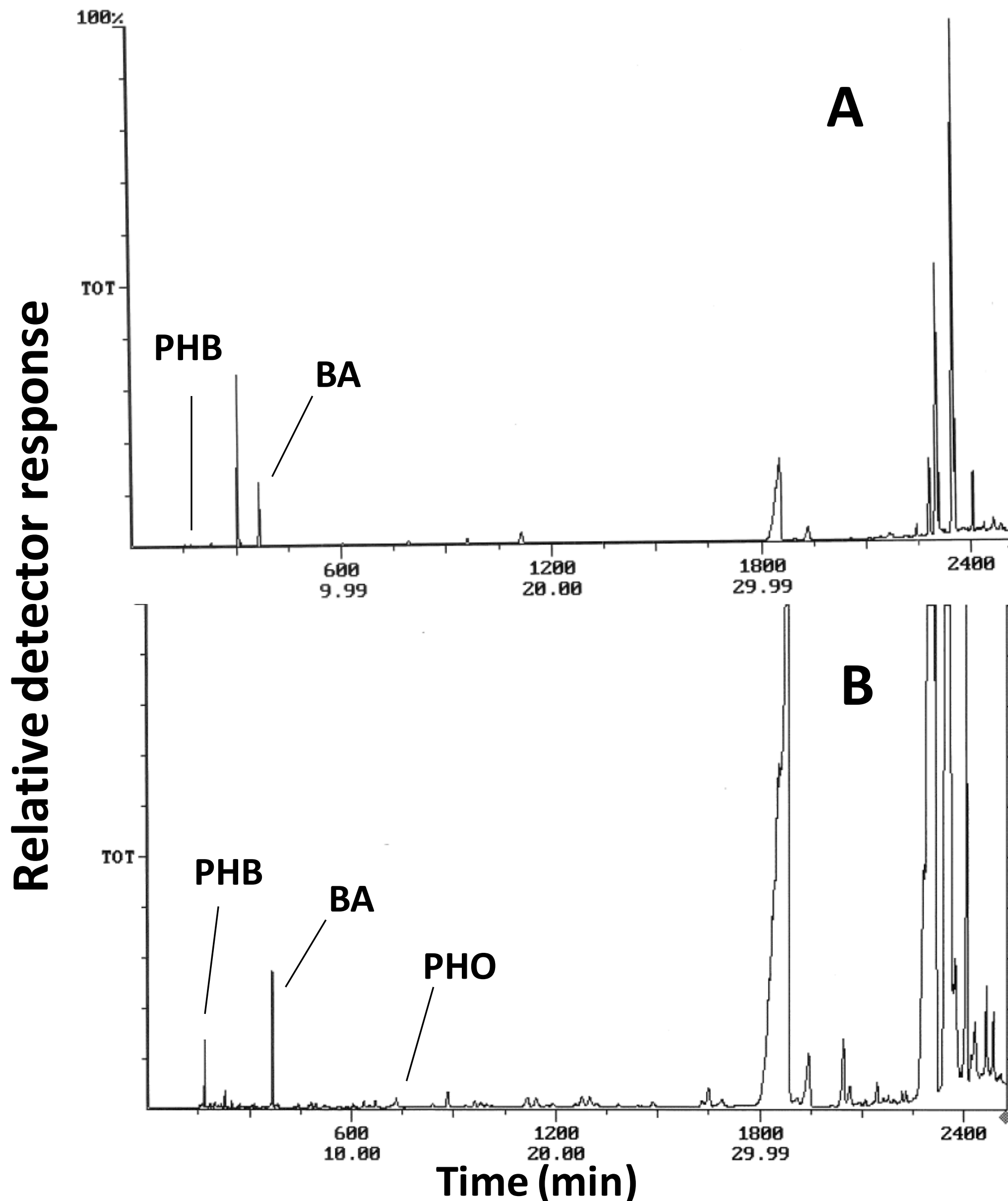


Figure 5-16 Gas chromatogram for *R. eutropha*Δ*phaC* lyophilized cell and PHA samples

The graphs above represent the gas chromatograms for the lyophilized cell (A) and extracted PHA (B) samples from the recombinant *R. eutropha*Δ*phaC* culture when analyzed by GC-MS. The peaks identified were done so by analyzing the mass spectra taken during peak formation. The peaks identified are; PHB – polyhydroxybutyrate, BA - Benzoic acid, PHO – polyhydroxyoctanoate.

The amount of polymer extracted from the *R. eutropha*Δ*phaC* cell pellet during the soxhlet extraction was very minimal, but enough was collected to use for a propanolysis reaction for further analysis. The mass spectra indicated the presence of PHB in both the lyophilized cell mass and the extracted polymer, and trace amounts of hydroxyoctanoate in the extracted polymer.

5.3.7.2 Analysis of Wild Type *R. eutropha* H16 Culture and PHAs

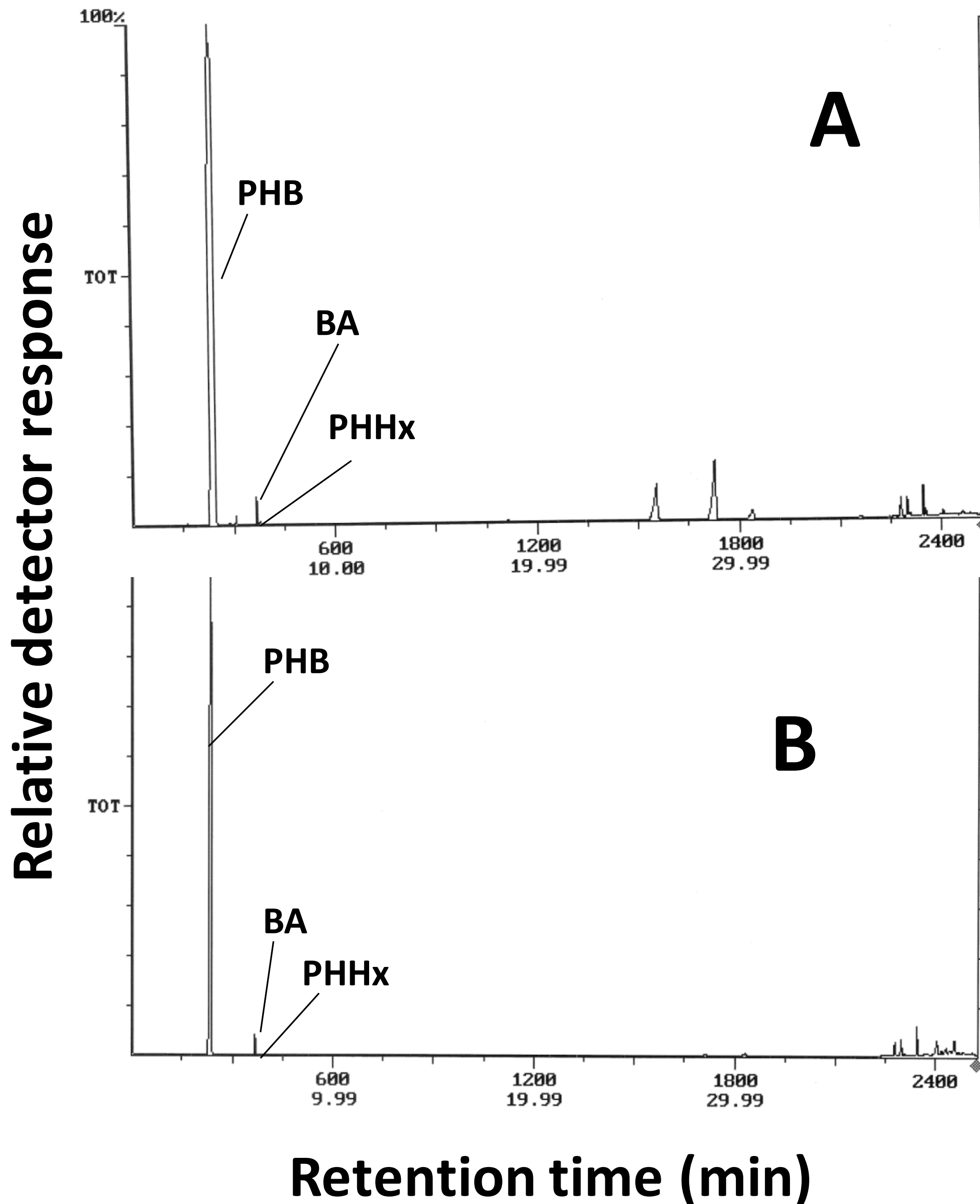


Figure 5-17 Gas chromatogram for *R. eutropha* H16 lyophilized cell and PHA samples

The graphs above represent the gas chromatograms for the lyophilized cell (A) and extracted PHA (B) samples from the *R. eutropha* H16 culture analyzed by GC-MS. The peaks identified were done so by analyzing the mass spectra taken at that time point. The peaks represented on the figure in order of elution time are PHB – 3-hydroxybutyrate, BA - benzoic acid (internal standard), PHHx – 3-hydroxyhexanoate.

Large amounts of PHB were extracted from *R. eutropha* H16 cell pellet during the soxhlet extraction method. Multiple PHA films were able to be poured as described previously and plenty of samples were available for analysis by GC-MS. Both the lyophilized cell and PHA samples indicated a majority presence of PHB with small traces of 3-hydroxyhexanoate.

5.3.8 Summary of culture productivity

By implementing the effective carbon number (ECN) method described earlier, the traces produced from the Flame Ionization Detector (FID) on the GC were used to calculate the amount of PHA produced in relation to the amount of biomass. The method was also used to determine the composition of the PHAs produced and the relative amounts of the different monomers incorporated into the polymer chain.

Table 5.3 PHA content and composition in culture samples

			PHA Composition (%)		
Sample	Biomass (g/L)	% PHA	PHB	PHHx	PHO
H16	33.6	61.2	99.4	0.65	-
H16Δ*phaC*	3.4	0.1	24	22	54

5.4 Discussion

In this experiment, the method designed to integrate foreign DNA into the genome of *R. eutropha* was validated, indicating that a suicide vector for *R. eutropha* was constructed and that regions of about 500 base pairs of homology to the genome flanking the target site are sufficient for the organism to recognize the homologous regions and integrate the DNA into its genome. It was also demonstrated that supplementing complex media with 5% sucrose is sufficient for removing the antibiotic resistance

markers or any part of the plasmid outside the designed homology regions using the *sacB* counter-selection method in *R. eutropha*. This is very useful when multiple integrations or knock-outs are needed so that the same antibiotic can be used for each additional integration event.

Summarizing the data presented in the GC-MS traces and Table 5-5, small traces of medium chain length 3-hydroxyalkanoates were found in the PHA formed from the recombinant *R. eutropha*Δ*phaC* in the form of hydroxyoctanoate. Both cell and PHA samples showed low amounts of hydroxybutyric acid, however this is a short chain length hydroxyalkanoate and is not supposed to appear in PHAs produced with the *P. oleovorans* mcl-PHA polymerase. The lyophilized cell samples of the same organism showed no traces of mcl-PHAs due to the concentration dropping below the detection limit of the instrument. The small amount of PHA accumulated in the recombinant *R. eutropha* included medium chain length constituents of 3-hydroxyhexanoate (C6) and 3-hydroxyoctanoate (C8). This indicates that the molecules must be created in the cell using native pathways, but at a very low rate.

PHB is a short chain length hydroxyalkanoate and is not supposed to be incorporated into polymers synthesized by type II polymerases such as the *phaC1* from *P. oleovorans* used in this study. However, since the genes responsible for synthesizing the precursors for PHB synthesis are still active in the recombinant strain, large quantities of the 3-hydroxybutyral CoA are still being made. With large pools available in the cell, it is possible that the type II enzyme was forced to incorporate small amounts of it into the polymer chain even though it is not the preferred substrate.

The wild type *R. eutropha* H16 demonstrated that it can grow very well using soybean oil as the sole carbon source. The mass spectra from the lyophilized cell mass and the extracted PHAs indicated large amounts of PHB produced with trace amounts of 3-hydroxyhexanoate incorporated into the polymer chains. This corroborates a recent publication indicating that the *R. eutropha phaC* gene can be forced to incorporate some mcl-hydroxyalkanoates if large pools of the substrates are accumulated (Green, 2002).

One theory for the lack of mcl-PHA accumulation when grown on soybean oil is the (S)-isomer of 3-hydroxyalkanoate is being formed in the β-oxidation pathway and that an additional enzyme is needed to convert the monomers to the (R) conformation. Since the polymerase enzyme (*phaC*) only accepts the (R) form of the 3-hydroxyalkanoate, without the correct genes *R. eutropha* may not be able to efficiently utilize the intermediates from the degradation pathway. With this theory in mind, the small amount of mcl-PHAs produced from the recombinant strain could have also resulted in the incorporation of the (S) 3-hydroxyalkanoate monomers into the polymer chains at a much reduced rate. Co-expressing genes to synthesize the correct isomer of the substrates needed for PHA synthesis with the mcl-PHA polymerase gene might boost production of PHAs in a recombinant organism.

6. Synthesized Mcl-PHA operon tested for mcl-PHA accumulation in PHB-4 strain of *R. eutropha*

6.1 Introduction

Previous experiments demonstrated that expression of the *phaC* from *Pseudomonas oleovorans* is insufficient by itself to accumulate significant amounts of mcl-PHAs when grown on soybean oil. One theory explaining this effect is that the polymerase is not able to properly utilize the intermediates from the β-oxidation pathways due to the wrong stereoisomer. The appropriate substrates for the polymerase enzyme are (R) 3-hydroxyacyl CoAs, and bacteria synthesize the (S) 3-hydroxyacyl CoA during fatty acid degradation. Some bacteria possess an epimerase or hydratase gene that can convert intermediates from the β-oxidation pathways into (R) 3-hydroxyacyl CoA, but due to the inability of the recombinant strain used in the previous chapter to produce significant amounts of mcl-PHA's, and the inability to detect the presence of the genes through BLAST searches, it is likely that *R. eutropha* does not possess these genes. It is thought that while expressing the mcl-PHA polymerase from *P. oleovorans* simultaneously with an enzyme to convert the (S) 3-hydroxyalkacyl CoAs to the (R) 3-hydroxyalkacyl CoAs, or an enzyme that creates the (R) 3-hydroxyalkacyl CoAs as part of the β-oxidation pathway that the overall production of mcl-PHAs will increase.

Recent studies have shown that over expression of the *phaJ* gene in *P. putida* increases the amount of PHAs accumulated during growth on sodium octanoate (Vo et al., 2008). Another study demonstrated the ability of the *phaJ* from *P. oleovorans* to synthesize (R) 3-hydroxyacyl CoA from enoyl CoA and increase the amount of mcl-PHA

accumulated when grown on sodium decanoate (Davis, 2008; Fiedler S., Steinbuchel A., Rehm B., 2002). These results indicate that co-expressing the *phaC* and *phaJ* genes might boost the accumulation of mcl-PHAs over the system used in previous experiments.

Another study has showed that the multifunctional yeast enzyme *fox2* is involved in fatty acid degradation and creates (R) 3-hydroxyacyl CoA before performing the following step of the degradation cycle converting the (R) 3-hydroxyacyl CoA into 3-ketoacyl CoA (Hiltunen, 1992). It was thought that the *phaC* will be able to use a fraction of the (R) 3-hydroxacyl CoA intermediates before the next step is catalyzed. This theory was investigated in the same method as with the *phaJ* stated above.

6.2 Methods

6.2.1 Design and Construction of Mobilizable Plasmid

To create a mobilizable plasmid capable of being transferred via conjugation, the *oriT* region from the pex-100T plasmid was cloned into the pGP-564 yeast-*E. coli* shuttle vector. The shuttle vector has the 2 μm ori for plasmid replication in yeast, a leucine gene for plasmid maintenance in yeast, kanamycin resistance gene for plasmid maintenance in bacteria, and a pUC ori for high copy number replication in bacteria, but lacks an oriT or mob region allowing the plasmid to be recognized by the transfer genes (*tra*). These genes facilitate the attachment and transfer of plasmid DNA between the two bacterial strains involved in the conjugation procedure. With the addition of the *oriT* region we will be able to construct a plasmid in yeast that can be transferred into a mating

strain of *E. coli* and subsequently into *R. eutropha* via conjugation. The primers designed to PCR amplify the *oriT* region with the appropriate regions of homology are shown in Table 6.1.

Table 6.1 Primers used to PCR amplify *oriT* region for homologous recombination of mobilizable plasmid pGP-oriT

The table below shows the primer sequences used for PCR amplifying the fragments used to assemble the mobilizable vector pGP-oriT using homologous recombination in yeast. Fragment 2 represents the *oriT* region amplified using the primers OriTFor and OriTRev with terminal homology to the ends of fragment 1. The bold faced portion of the primer sequence indicates the region of the primer that binds to the original DNA template strand in the PCR reaction, and the regular type face sequence is the portion of the primer designed to add 40 base pairs of homology to the fragment to be assembled adjacent to that PCR product on the final plasmid. The primer designed to bind downstream is shown in the 3' to 5' direction.

Location on gene	Frag. #	Primer Sequence
Upstream	2	**AATATGTAAGGGGTGACGCC** TTCTCCTTACGCATCTGTGCGGTATTTCACACCGCATATG
Downstream	2	TGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATG **CGCTCTCCTGAGTAGGACAA**

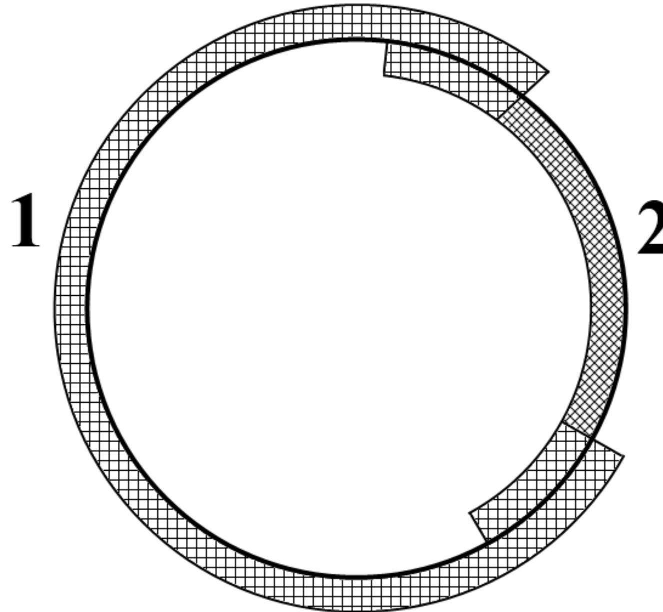


Figure 6-1 Diagram of designed fragments used for homologous recombination of mobilizable plasmid pGP-oriT

The diagram above represents the linear DNA fragments used to construct a mobilizable vector pGP-oriT by homologous recombination in yeast. Fragment 1 represents the NdeI linearized pGP-564 plasmid and fragment 2 represents the *oriT* region with terminal homology to the ends of fragment 1.

The *oriT* fragment was PCR amplified from the pex-100T plasmid using the above primers OriTFor and OriTRev shown in Table 6.1 with an annealing temperature of 64°C and a 1 minute 15 second elongation period. The upstream primer binds 372 base pairs upstream of the beginning and the downstream primer binds 197 base pairs downstream of the end of the *oriT* region as determined from the information about the plasmid published by Invitrogen. The fragment amplified is a total of 1392 base pairs.

The pGP-564 plasmid was extracted from a culture of *E. coli* DH5α and linearized by digesting the plasmid with the unique restriction enzyme NdeI. The PCR product and linearized fragment were checked on an agarose gel and purified from the gel using the DNA purification method described in chapter 2. The fragments were mixed in equal molar quantities and transformed into yeast strain S288 as described previously.

6.2.2 Design of mcl-PHA operon

Bacteria are capable of expressing multiple genes transcribed from one mRNA molecule, called polycistronic gene cassettes. This was taken advantage of by designing an operon where the *P. oleovorans phaC* gene will be directly upstream from either the *P. oleovorans phaJ* or the *S. cerevisiae fox2* gene, both under the expression of the native *R. eutropha* promoter for the *phaCAB* operon. The native *R. eutropha* promoter is induced upon essential nutrient limitation such as nitrogen or phosphorus and is natively located directly upstream of the *phaCAB* operon. The native ribosome binding sites for the PCR amplified genes were maintained in the amplified fragments by designing the forward primer upstream of the putative sites. This ensured that each gene on the polycistronic mRNA would be transcribed.

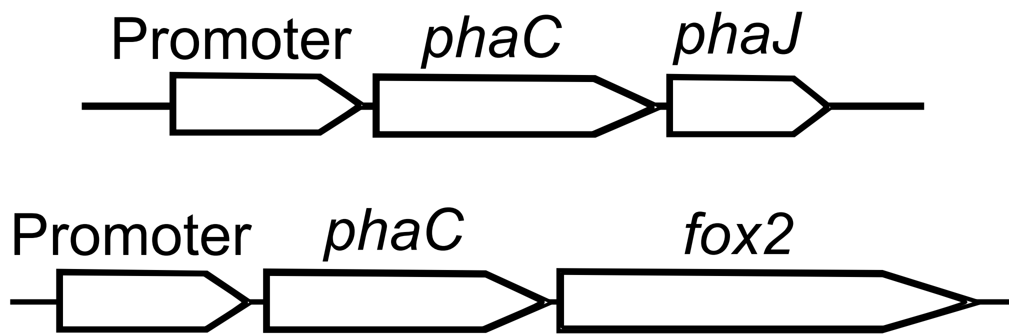


Figure 6-2 Diagram of Designed mcl-PHA Operon

The maps above shows the operon designed for expressing genes involved in mcl-PHA synthesis in *R. eutropha*. The promoter is the promoter from the *phaCAB* operon in *R. eutropha*, *phaC1* is the mcl polymerase gene from *P. oleovorans*, *phaJ* is an enoyl CoA hydratase from *P. oleovorans*, and *fox2* is a multifunctional protein involved in the β-oxidation of yeast.

6.2.3 Construction of Mobilizable Plasmid Containing mcl-PHA Operon

The plasmid constructed in the first round of recombination (pGP-oriT) is capable of being transferred via conjugation from a mating strain of bacteria to another bacterial species. Fragments were designed for homologous recombination in yeast to assemble the mcl-PHA operons discussed in the previous section in the same fashion as earlier experiments. Tables 6.2 and 6.3 and Figures 6-3 and 6-4 show the primers used to PCR amplify the fragments and diagrams of the designed fragments and how they were recombined to form the plasmids pGP-oriT 700*phaJ* and pGP-oriT 700*fox2*.

Table 6.2 Primers used for PCR Amplifying Fragments Used in Homologous Recombination to Assemble the Plasmid pGP-oriT *phaJ*

The table above shows the primer sequences used for PCR amplifying the fragments used to assemble the mobilizable vector pGP-oriT 700*phaJ* using homologous recombination in yeast. The bold faced portion of the primer sequence indicates the region of the primer that binds to the original DNA template strand in the PCR reaction, and the regular type face sequence is the portion of the primer designed to add 40 base pairs of homology to the fragment to be assembled adjacent to that PCR product on the final plasmid. The primer designed to bind downstream is shown in the 3' to 5' direction.

Name	Frag. #	Sequence
Upstream	2	GGAATTCGATATCAAGCTTATCGATACCGTCGACCTCGAG **CGATGAACAGGTCGCGGTTG**
Downstream	2	CTGAGACGAGTACCGGTTGCTGTGGCGGGTGCACAGTTCT **GATTTGATTGTCTCTCTGCC**
Upstream	3	**AGAACTGTGCACCCGCCACA**
Downstream	3	**GGACGCCACGGTGTTCATTT**
Upstream	4	CAGCGCCGTGGCCACCTGCGGGACGCCACGGTGTTCATTT **CAATAAGGAGATATACATAT**
Downstream	4	CTCACTATAGGGCGAATTGGGTACCGGGCCCCCCCTCGAG **CTTCAGCTCGCCACAAAGTT**

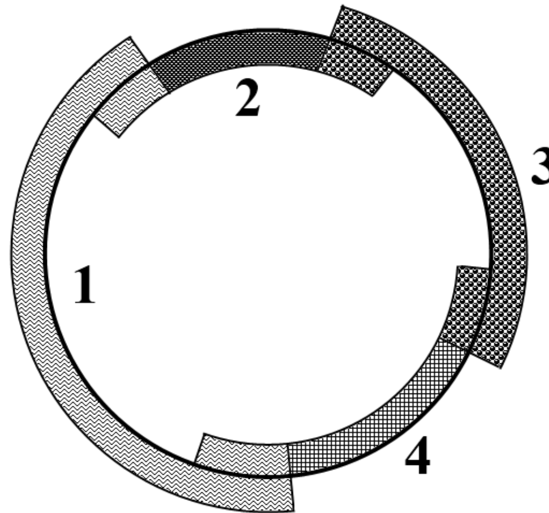


Figure 6-3 Chart showing primers used and diagram showing fragments designed and assembled during homologous recombination of plasmid pGP-oriT 700*phaJ*

The diagram above represents the linear DNA fragments used to construct pGP-oriT 700*phaJ* by homologous recombination in yeast. Fragment 1 represents the linearized pGP-oriT plasmid, fragment 2 represents the PCR product containing the promoter region and terminal homology to the fragments 1 and 3, fragment 3 represents the PCR product containing the *phaC* gene from *P. oleovorans*, fragment 4 represents the PCR product containing the *phaJ* gene from *P. oleovorans* with terminal homology to fragments 1 and 3. The chart above displays the primers used to clone the PCR fragments, the sequence that binds to the template DNA, and the terminal homology regions designed as overhangs to the template DNA.

Fragment 2 containing the *R. eutropha* promoter was PCR amplified from the genome of *R. eutropha* with the primers RepromFOR and UphomREV using an annealing temperature of 62°C. The promoter used is the promoter that regulates expression of the PHB operon in *R. eutropha*. The primer that binds to the upstream region binds 884 base pairs upstream of the start of the PHB operon, and the primer that binds downstream on the fragment binds directly in front of the start of the PHB operon ensuring the entire promoter sequence is included in the fragment. The region amplified is 884 bases long.

Fragment 3 containing the *phaC1* gene was PCR amplified from the *P. oleovorans* genome with the primers Po*phaC*1FOR and Po*phaC*1REV using an annealing temperature of 58°C. The upstream primer binds 78 bases upstream of the start codon and the downstream primer binds 47 bases downstream of the stop codon for the *phaC1* gene. The fragment amplified is a total of 1802 base pairs.

The primer sequences used to PCR amplify the *phaJ* gene from the *P. oleovorans* genome, Po*phaJ*FOR and Po*phaJ*REV, were taken from Vo et al. (Vo et al., 2008). A second set of primers, *PhaJ*homFOR and *PhaJ*homREV, were designed for PCR amplifying the *phaJ* fragment to be used in the homologous recombination reaction. The second set of primers was insufficient for directly cloning the *phaJ* gene from genomic DNA, so the gene was first PCR amplified using the first set of primers, and that fragment was used as the template for the PCR reaction using the second set of primers.

The first PCR was run for only 20 cycles with an annealing temperature of 60°C to minimize possible mutations caused with taq instability. 1 μL of the product from that

reaction was then used as the template for a PCR using the second set of primers and run with an annealing temperature of 62°C. This proved sufficient for cloning the fragment with the appropriate terminal homology sequences for the homologous recombination.

Table 6.3 Primers Used for the Homologous Recombination Assembly of the Plasmid pGP-oriT 700*fox2*

The table below shows the primer sequences used for PCR amplifying the fragments used to assemble the mobilizable vector pGP-oriT 700*fox2* using homologous recombination in yeast. The bold faced portion of the primer sequence indicates the region of the primer that binds to the original DNA template strand in the PCR reaction, and the regular type face sequence is the portion of the primer designed to add 40 base pairs of homology to the fragment to be assembled adjacent to that PCR product on the final plasmid. The primer designed to bind downstream on the gene is shown in the 3' to 5' direction.

Location on gene	Frag. #	Primer Sequence
Upstream	2	GGAATTCGATATCAAGCTTATCGATACCGTCGACCTCGAG **CGATGAACAGGTCGCGGTTG**
Downstream	2	CTGAGACGAGTACCGGTTGCTGTGGCGGGTGCACAGTTCT **GATTTGATTGTCTCTCTGCC**
Upstream	3	AGAACTGTGCACCCGCCACA
Downstream	3	GGACGCCACGGTGTTCATTT
Upstream	4	CAGCGCCGTGGCCACCTGCGGGACGCCACGGTGTTCATTT **GAAGGACTTATCAGCTAATT**
Downstream	4	CTCACTATAGGGCGAATTGGGTACCGGGCCCCCCCTCGAG **CAAAAAAGTTGCGTCCGTA**

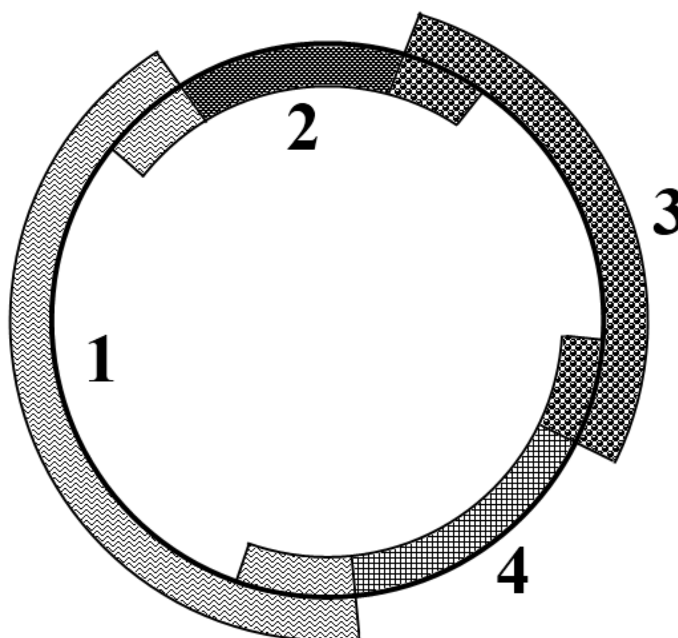


Figure 6-4 Diagram of fragments designed and assembled during homologous recombination of the plasmid pGP-oriT 700*fox2*

The diagram above represents the linear DNA fragments used to construct pGP-oriT 700*fox2* by homologous recombination in yeast. Fragment 1 represents the XhoI linearized pGP-oriT plasmid, fragment 2 represents the PCR product containing the promoter for the PHB operon PCR amplified from the genome of *R. eutropha* with terminal homology to the ends of fragments 1 and 3, fragment 3 represents the PCR product containing the *phaC* gene PCR amplified from the genome of *P. oleovorans*, fragment 4 represents the PCR product containing the *fox2* gene PCR amplified from the yeast genome with terminal homology to the ends of fragments 1 and 3.

Fragments 1,2, and 3 are the exact same for this plasmid as they were for the pGP-oriT 700*phaJ* plasmid described earlier. The only difference between the two plasmids is the addition of the *fox2* gene in the place of the *phaJ* gene to create the plasmid pGP-oriT 700*fox2*. The *fox2* gene was cloned from the genome of *S. cerevisiae* using the primers *Fox2*homFOR and *Fox2*homREV with an annealing temperature for the PCR reaction of 63°C. The primers used to amplify the fragment bind 85 bases upstream of the start codon and 149 bases downstream of the stop codon creating a 2930 base pair fragment.

All of the PCR products amplified were run on an agarose gel to verify successful

cloning and purified from the gel using Qiaquick spin columns as described in chapter 2. Equal molar quantities of the fragments were mixed together and transformed into S288 to complete the homologous recombination as described in chapter 2.

6.2.4 Screening Yeast Recombination Colonies

S288 yeast colonies formed on the selective SD plates following the transformation of linear DNA fragments were picked and screened by colony PCR using the primers that bind to the *P. oleovorans phaC1* gene using the same conditions as before. The lanes displaying the brightest and cleanest band from the colony PCR were picked for downstream applications.

6.2.5 Verification of Mobilizable Vector Containing mcl-PHA Operon

Plasmid DNA was extracted from the yeast colony picked and grown overnight in liquid SD media following a positive colony PCR reaction performed above, and the DNA was transformed into the mating strain of *E. coli* S17-1. A colony was picked from the transformation plate and grown overnight in LB media with kanamycin. 5 mL of the overnight culture was used for extracting the plasmid DNA for further screening. The plasmid DNA was used as the template for additional PCR reactions screening for all of the fragments assembled during the last homologous recombination reaction (*phaC1*, *phaJ*/*fox2*, promoter) using the same primers and reaction conditions as used previously. The plasmids were also separately digested with the restriction enzymes XhoI and EcoRV (NEB, Ipswich, MA, USA) to create a map of the digested fragments giving a better overall picture of the location of the features on the plasmid. Both enzymes are double cutters for this plasmid.

6.2.6 Transfer of Mobilizable Vector to *R. eutropha*

The mating strategy described in chapter 2 was implemented to transfer the plasmid into the PHB-4 strain of *R. eutropha*. *R. eutropha* colonies formed on the secondary selection plates were grown up and the plasmid extracted to verify that their resistance to kanamycin in the media is due to the presence of the plasmid. Following a positive PCR result indicating the presence of the plasmid in the PHB-4 strain of *R. eutropha*, freezer stocks were made of the recombinant strain and frozen at -80°C for storage and later use.

6.2.7 Fed Batch Reactor Experiments using PHB-4 Harboring mcl-PHA Operon

A colony of the recombinant organism was picked from a Doi agar plate containing kanamycin and inoculated into 5 mL of Doi media plus kanamycin and incubated overnight at 30°C. 5 mL of the culture was used to inoculate 250 mL of Doi media supplemented with 1% soybean oil and incubated at 30°C for 16 hours in a 1 L baffled flask. The entire culture was used to inoculate 5L of mineral salts media supplemented with 2% soybean oil and 50 mg/mL kanamycin in a 10 L reactor. The culture was stirred at 400 rpm, air sparged in at 2 L/min, and the pH and temperature maintained at 7 and 30°C respectively. 6 M NaOH was used to maintain the pH in the culture.

A mass spectrometer was hooked up to the exhaust gas to analyze the concentrations of CO_2 and O_2 as described in previous experiments. The first time the culture showed signs of nutrient depletion (about 11 hours), the original concentrations of ammonia, soybean oil, trace elements, and magnesium sulfate were added along with 4 g/L KH_2PO_4. The second time the culture showed signs of nutrient depletion, half the original concentration of ammonia, trace elements, and magnesium sulfate, were added

along with 80 grams of soybean oil. Following the depletion of the second addition of nutrients, only fresh soybean oil was added to the culture.

After about 85 hours the media in the reactor was centrifuged for 25 minutes at 10,000 rpm in 500 mL containers to pellet the majority of the cells. The cell pellets were then flash frozen in liquid nitrogen and lyophilized. The accumulated PHAs were extracted using the soxhlet apparatus and films of the extracted polymers were cast as described previously.

6.2.8 Analysis of PHAs Produced by Cultures

Samples were taken throughout the life of the culture and analyzed for CDW, soybean oil concentration, and ammonia concentrations in the media as described in chapter 2. A sample of the PHAs extracted during the soxhlet extraction procedure along with lyophilized cells were subjected to the propanolysis reaction and analyzed by GC-MS to determine the identity of the 3-hydroxyalkanoate monomers incorporated into the polymer as described earlier.

6.3 Results

6.3.1 Construction of Mobilizable Vector

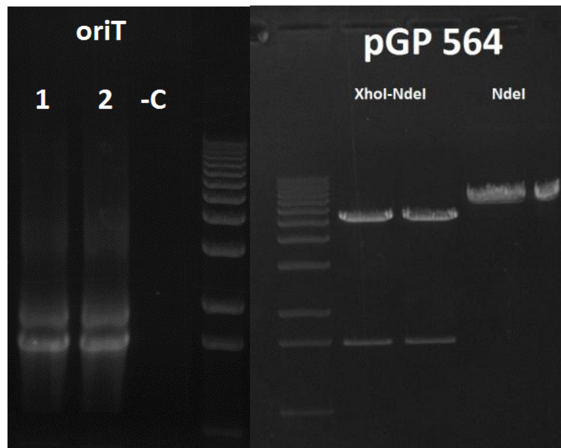


Figure 6-5 Agarose gel of PCR amplified *oriT* fragment

The *oriT* fragment from pex100T was PCR amplified to be inserted into the pGP-564 plasmid at the NdeI site. The predicted fragment size was 823 base pairs which would indicate the bottom brighter band is the *oriT* fragment. The pGP-564 digest with NdeI appears as a solid band at the appropriate size of 7700 base pairs.

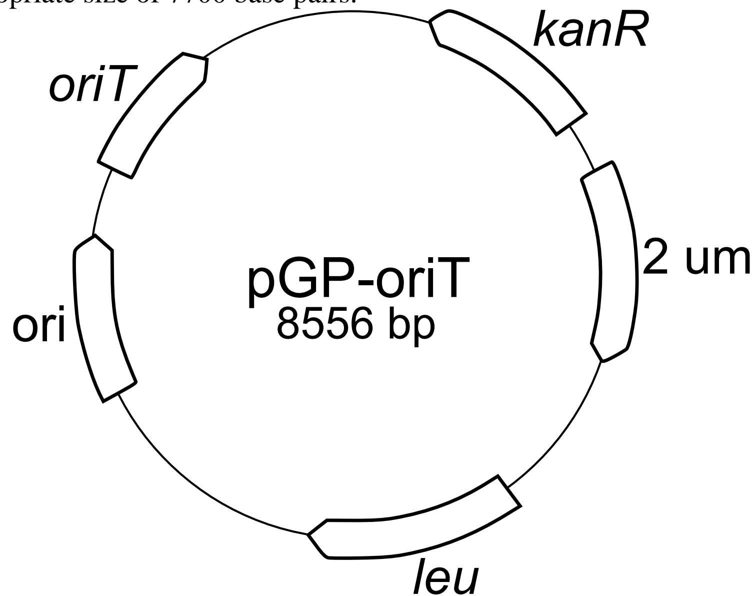


Figure 6-6 Plasmid map of pGP-oriT

Plasmid map showing the product from the homologous recombination of NdeI linearized pGP-564 vector and the *oriT* region cloned from the pex100T plasmid.

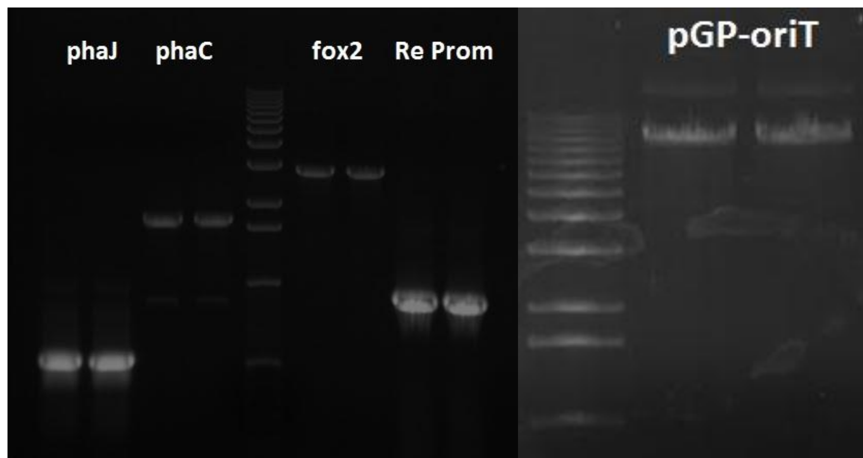


Figure 6-7 0.8% agarose gel with fragments used for homologous recombination

The above gels were used to verify the fragments were properly cloned and digested in preparation for the homologous recombination step. The *phaC* and *phaJ* are PCR products amplified from the *P. oleovorans* genome, *fox2* is a PCR product amplified from the *S. cerevisiae* genome, Re prom is the promoter for the *phaCAB* operon and was cloned from the *R. eutropha* genome, and the pGP-oriT plasmid was digested with XhoI to linearize the vector.

The fragments cloned from preparations of genomic DNA match the expected sizes. The *phaJ* is 554 base pairs, *phaC1* is 1782 base pairs, *fox2* is 2937 base pairs, the promoter fragment is 884 base pairs, and the pGP-oriT vector is 8500 base pairs. Since the lanes look relatively free from nonspecific bands and match the expected size fragments, they were used for the homologous recombination reaction in yeast.

6.3.2 Colony Screening with PCR

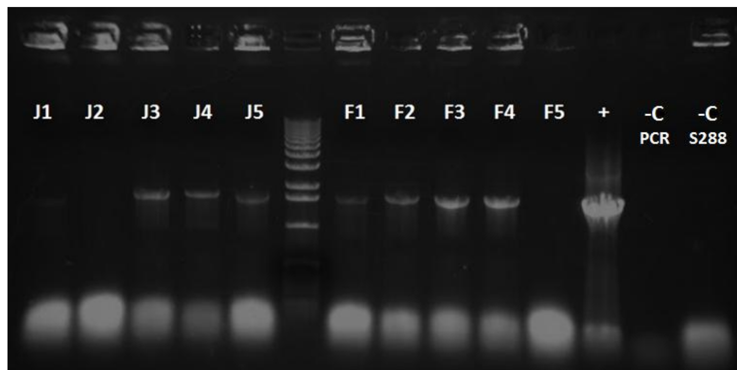


Figure 6-8 Screening S288 colonies from homologous recombination selection plates

The yeast colonies formed on the SD selection plates following the transformation of linear DNA fragments were screened using the primers that bind to the *phaC1* gene, Po*phaC*1FOR and Po*phaC*1REV. J1-J5 corresponds to colonies picked from the cells harboring the plasmid pGP-oriT 700*phaJ*, and F1-F5 corresponds to colonies picked from the cells harboring the plasmid pGP-700 *fox2*. The positive control (+) template was a *phaC1* PCR product, the negative control PCR (-C PCR) is a negative control for the PCR reaction with no template DNA added, and the negative control for nonspecific amplification from the yeast genome (–C S288) used S288 colonies from the positive control of the homologous recombination, S288 harboring the pGP-564 plasmid.

6.3.3 Verification of Plasmid Construction

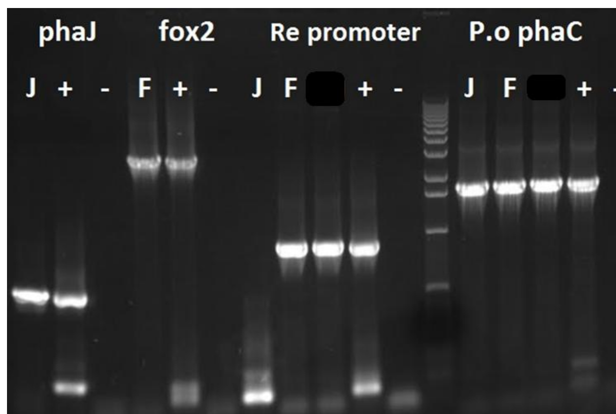
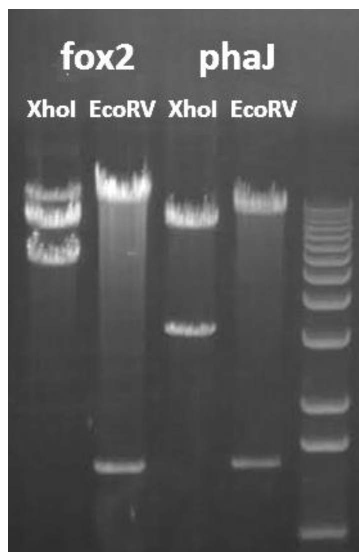


Figure 6-9 0.8% agarose gel of PCR fragments from plasmid verification steps

The above gel contains fragments from the PCR reactions set up using plasmid DNA extracted from *E. coli* S17-1 transformed with the yeast plasmid DNA extracted after the homologous recombination reaction. The PCR was a screening step looking for all of the PCR fragments involved in the homologous recombination. The fragments used in the homologous recombination reaction mixture were used as the templates for the positive control in the PCR reactions. Every fragment is accounted for by the PCR screening except for the promoter in the *phaJ* plasmid. This test was repeated and a band appeared at the expected size, 884 base pairs.



pGP-oriT 700*phaJ*		pGP-oriT 700*fox2*	
EcoRV	**XhoI**	**EcoRV**	**XhoI**
10293	8556	12676	8556
1489	3226	1489	5609

Figure 6-10 Agarose gel displaying fragment patter and table for expected fragment sizes

The gel on the left shows the fragment pattern from digesting the plasmids individually with the double cutting restriction enzymes XhoI and EcoRV. *Fox2* represents the plasmid pGP-oriT 700*fox2*, and *phaJ* represents the plasmid pGP-oriT 700*phaJ*. The table on the right displays the expected size of the fragments generated from the digests performed. The fragment sizes were calculated by performing a digest in silico using ApE©.

The fragments created by the restriction digest match the expected sizes calculated from the in silico digest of the plasmids. This in combination with the positive PCR results earlier indicate that all of the fragments were properly recombined and the plasmid was assembled as it was designed.

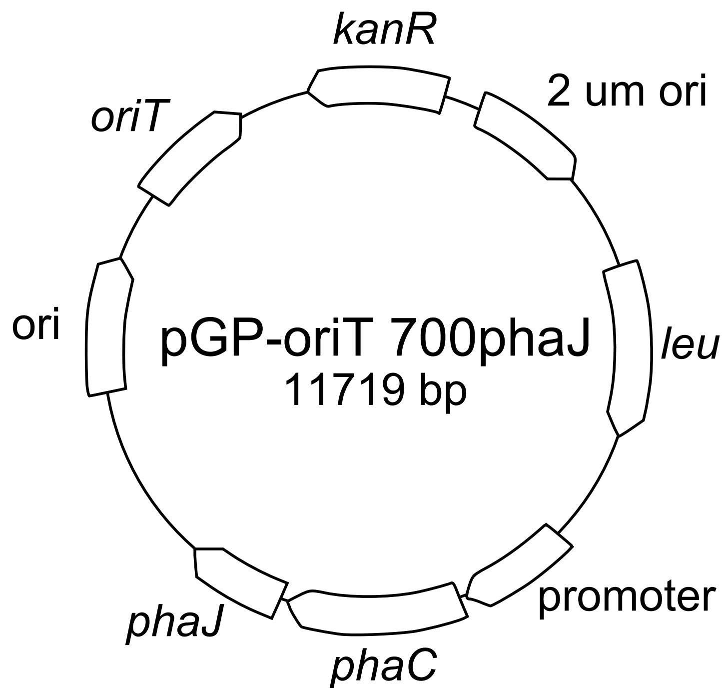


Figure 6-11 Plasmid map of pGP-oriT 700*phaJ*

Above is a map of the plasmid pGP564-oriT 700*phaJ* which was assembled by homologous recombination in yeast and will be used for mcl-PHA production in *R. eutropha*.

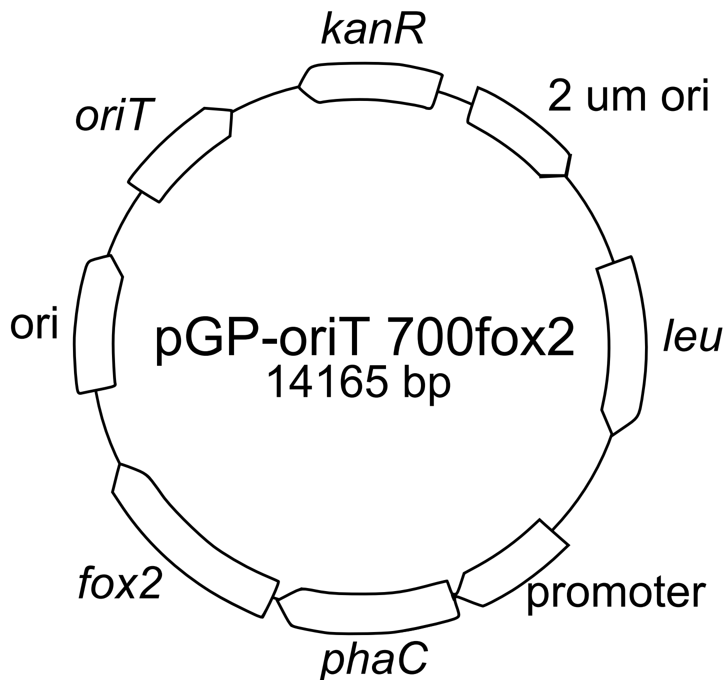


Figure 6-12 Plasmid map of pGP-oriT 700*fox2*

Above is a map of the plasmid pGP-oriT 700*fox2* assembled by homologous recombination in yeast and will be used for mcl-PHA production in *R. eutropha*.

6.3.4 Analysis of Culture from Fed Batch Reactors

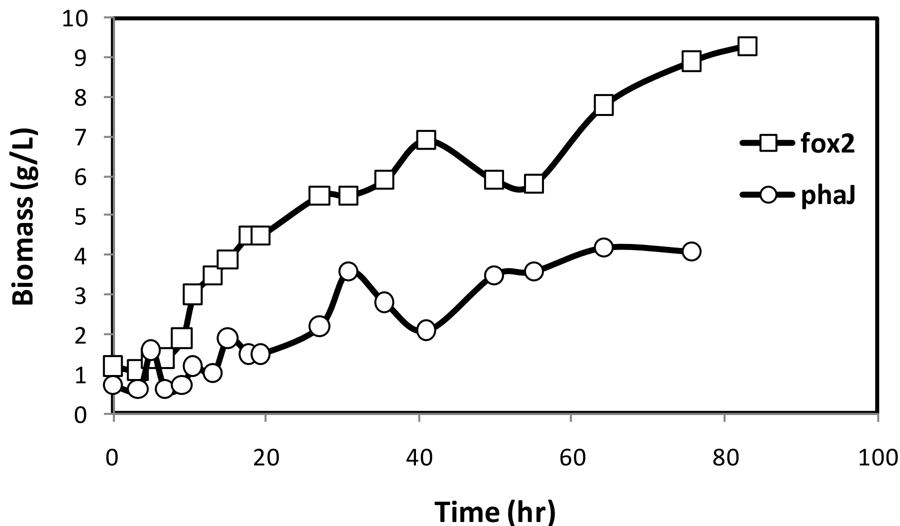


Figure 6-13 CDW samples taken from reactor during life of culture
The graph above shows the amount of total biomass accumulated throughout the life of the culture as determined by cell dry weight analysis. The closed circles represent *R. eutropha* PHB-4 harboring the plasmid pGP-oriT 700*phaJ*, and the closed squares represent *R. eutropha* PHB-4 harboring the plasmid pGP-oriT 700*fox2*.

Both cultures show somewhat exponential growth until about 40 hours where the growth appears to change into more of a linear growth state. This is consistent with cultures that accumulate PHAs where the culture grows and divides until an essential nutrient becomes limiting and the cells begin to accumulate PHAs changing the rate at which they accumulate biomass to a linear as opposed to an exponential rate.

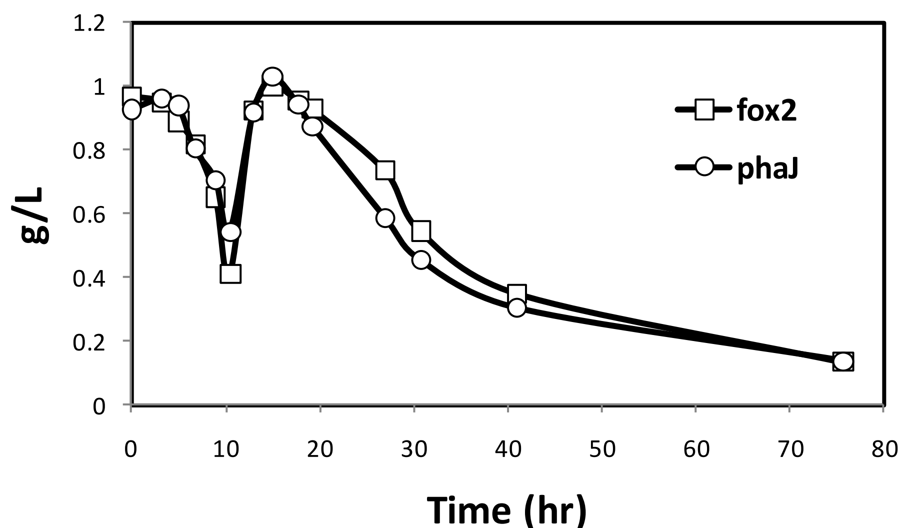


Figure 6-14 Concentration of NH_4 in reactor media

The above graph represents the concentration of NH_4 in the reactor media. The closed squares represent the *R. eutropha* PHB-4 harboring the plasmid pGP-oriT 700*fox2*, and the closed circles represent *R. eutropha* PHB-4 harboring the plasmid pPG-oriT 700*phaJ*. The graph shows two points where the slope of the line abruptly changes corresponding to the time points where additional NH4 was added to the culture.

The concentration of NH4 in the media drops very rapidly indicating rapid growth from the culture upon inoculating the reactor. With the additional feeds of NH_4 supplied to the culture the NH_4 levels never get completely exhausted, but if the carbon source was always in excess the high C:N ratio would have been enough to turn on the promoter and up-regulate the transcription of the mcl-PHA operon.

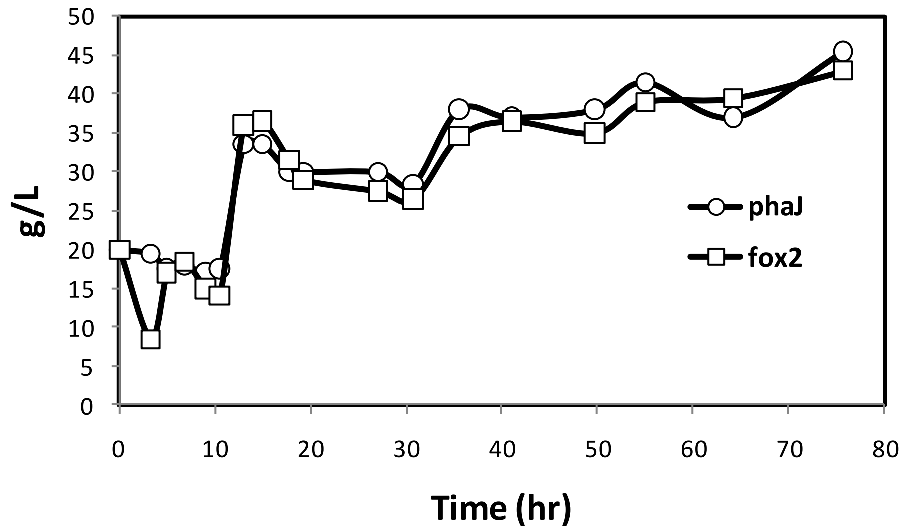


Figure 6-15 Soybean oil concentrations in culture media

Above is a graph representing the concentration of soybean oil in the media during the life of the culture. The closed squares represent the *R. eutropha* PHB-4 pGP-oriT 700*fox2* reactor and the closed circles represent the *R. eutropha* PHB-4 pPG-oriT 700*phaJ* reactor.

The graph above tracking the concentration of soybean oil in the reactor shows an overall increasing amount of soybean oil over the life of the culture. This is caused by the addition of soybean oil at various time points faster than it is being depleted. It is evident that right away, and after each addition of oil that the concentration goes down until the next addition. It was calculated that about 400 g total of soybean oil was added to each reactor throughout the life of the culture. Since the last point shows about 45 g/L (225 g total) left in the culture media it is evident that the soybean oil was used by the culture as their carbon source, and that the carbon was never limiting in the culture producing conditions ripe for PHA production.

6.3.5 Analysis of PHAs from fed batch reactors

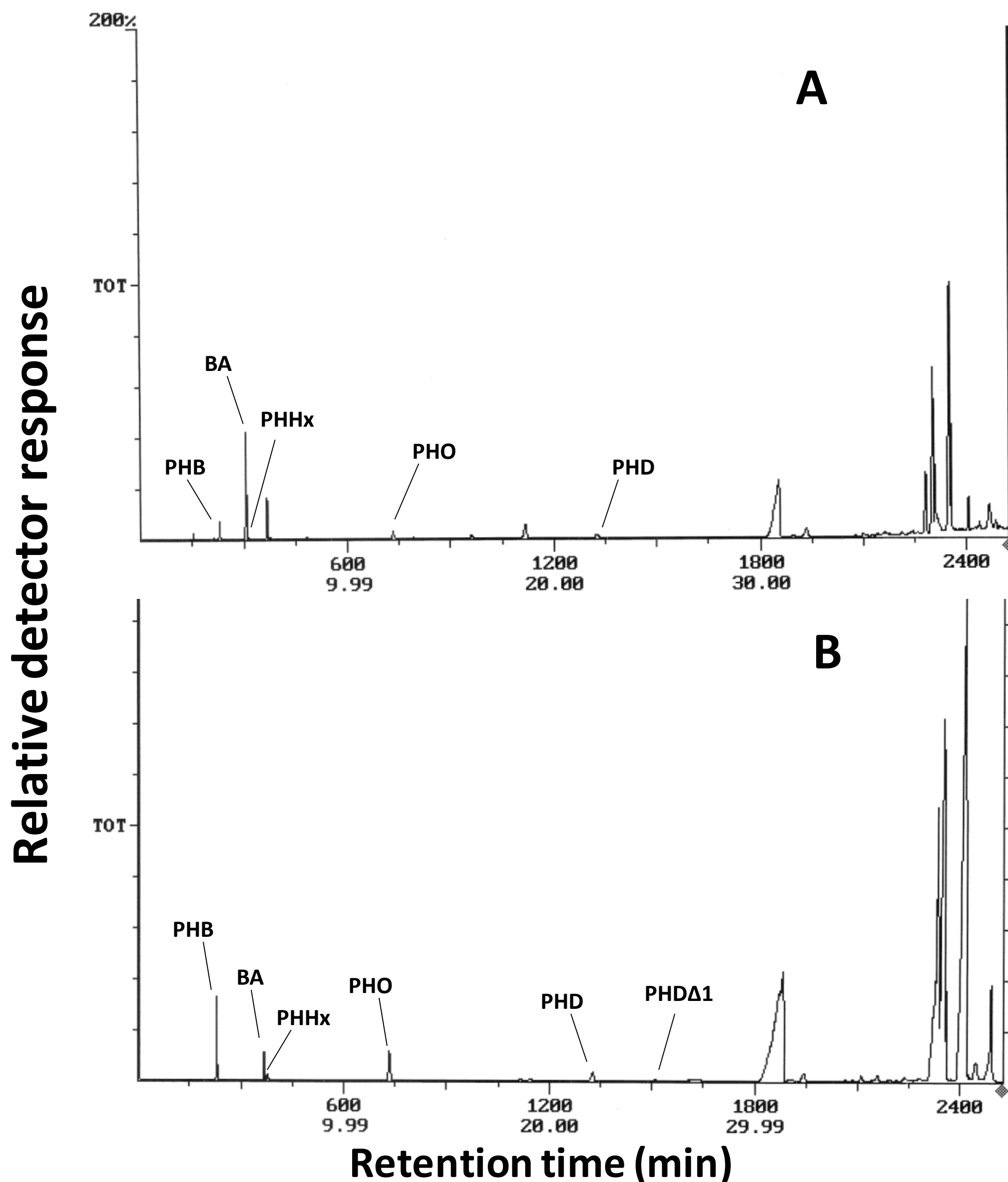


Figure 6-16 Gas chromatogram of *R. eutropha* PHB-4 pGP-oriT 700*fox2* lyophilized cells and extracted PHA samples

The above graph is the gas chromatogram from the GC-MS analysis of lyophilized cell (A) and extracted PHA (B) samples following a propanolysis reaction of the samples. The peaks were identified by looking at the mass spectrum of samples taken during the peak elution time. The peaks identified in order of elution time are PHB – 3-hydroxybutyrate, BA – benzoic acid (internal standard), PHHx – 3-hydroxyhexanoate, PHO – 3-hydroxyoctanoate, PHD – 3-hydroxydecanoate, PHDΔ1 – 3-hydroxydecenoate (10:1).

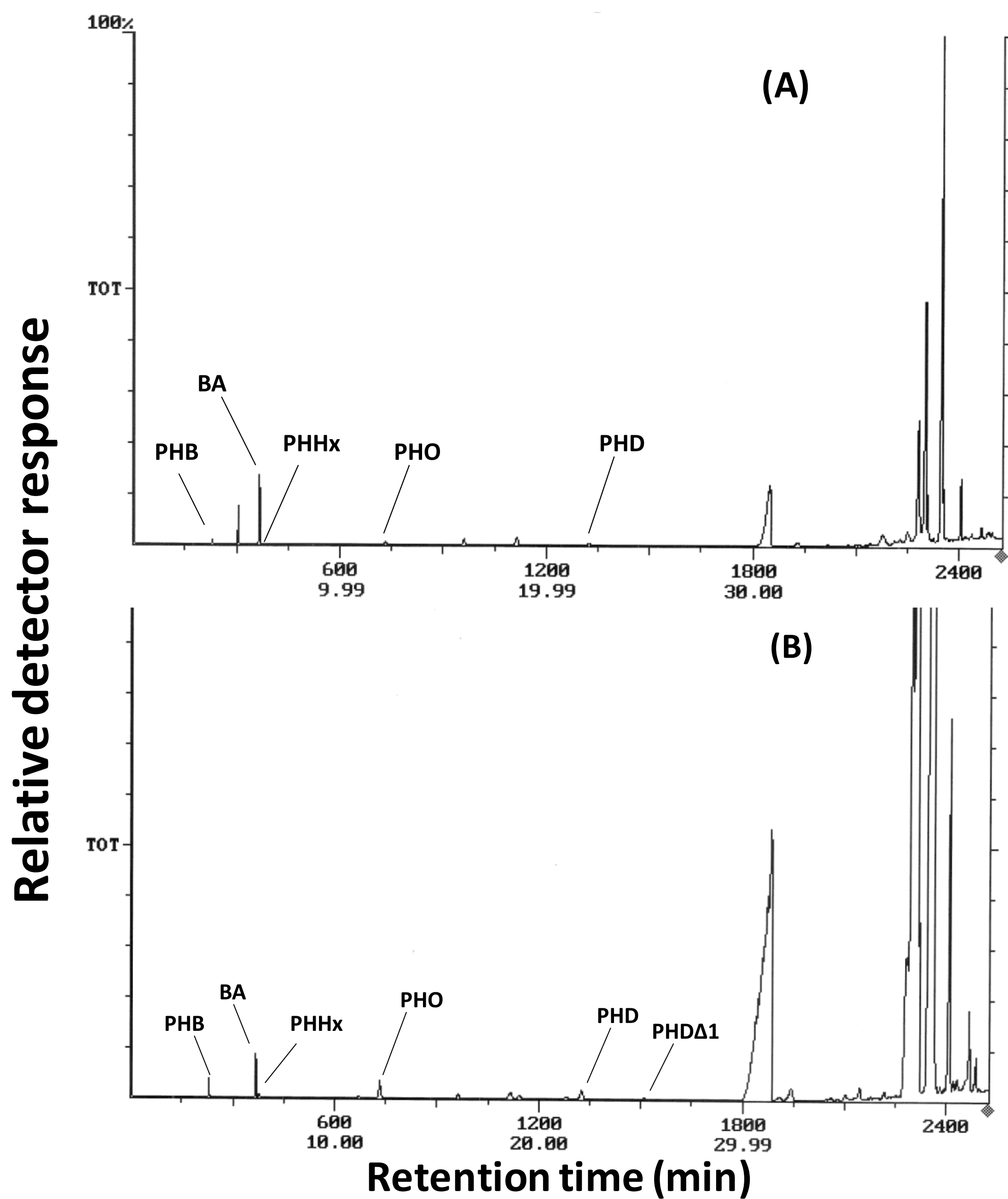


Figure 6-17 Gas chromatogram of *R. eutropha* PHB-4 pGP-oriT 700*phaJ* lyophilized cells and extracted PHA

The above graph is the gas chromatogram from the GC-MS analysis of lyophilized cells (A) and extracted PHA (B) samples following a propanolysis reaction of the samples. The peaks were identified by looking at the mass spectrum of samples taken during the peak elution time. The peaks identified in order of elution time are; PHB – 3-hydroxybutyrate, BA – benzoic acid (internal standard), PHHx – 3-hydroxyhexanoate, PHO – 3-hydroxyoctanoate, PHD – 3-hydroxydecanoate, PHDΔ1 – 3-hydroxydecenoate (10:1).

6.3.6 Analysis of biomass and PHA production by recombinant organisms

Lyophilized cells and extracted PHA samples were analyzed by GC to determine the PHA content in the cells, and the composition of the PHAs accumulated. The method for calculating the PHA content and composition is described in chapter 2. Table 6-1 displays the data gained from this analysis.

Table 6.4 Analysis of PHAs accumulated by recombinant organisms

The table above displays the data gathered from the GC analysis of PHAs accumulated from the recombinant organisms designed. 700*phaJ* represents PHB-4 harboring the pGP-oriT 700*phaJ* plasmid, and 700*fox2* represents PHB-4 harboring the pGP-oriT 700*fox2* plasmid.

			PHA Composition (%)			
Sample	**Biomass (g/L)**	**% PHA**	**PHB**	**PHHx**	**PHO**	**PHD**
700*phaJ*	4.2	2.5	9.7	0	65	25.2
700*fox2*	9.3	2.2	20.6	6.3	42.5	30.6

Both recombinant organisms demonstrated their ability to accumulate mcl-PHAs by using β-oxidation intermediates. However, the strain harboring the *fox2* gene showed a much higher percentage of PHB incorporated into the polymer. The strain harboring the *phaJ* gene showed a preference towards the longer chain hydroxyalkanoates with the most common chain length being 3-hydroxyoctanoate (C8).

6.4 Discussion

A new plasmid was constructed in the beginning of this experiment to combine a yeast/*E. coli* shuttle vector with the *oriT* region of another plasmid (pex100T) to create a mobilizable plasmid capable of being transferred to *R. eutropha* via conjugation. This was then combined with one of two newly designed mcl-PHA operons made to utilize intermediates from the β-oxidation pathways in bacteria, specifically for, but not limited to, *R. eutropha*. The plasmid was demonstrated to be transferred via conjugation and

remained stable in *R. eutropha* conferring resistance to high concentrations of kanamycin in the surrounding media.

The plasmid was then tested for its ability to produce medium chain length (C6-C12) PHAs when expressed in *R. eutropha* grown in a fed batch reactor under nutrient limiting conditions using soybean oil as the sole carbon source. Although the cell dry weights indicate that the total biomass accumulation was far less than the wild type strain used in previous experiments, there were small amounts of mcl-PHA accumulation. PHB-4 harboring the pGP-oriT 700*phaJ* plasmid accumulated 2.5 % of its dry weight in PHAs, and PHB-4 harboring the pGP-oriT 700*fox2* plasmid accumulated 2.2% of its dry weight in PHAs.

The negative control, *R. eutropha* PHB-4 with the backbone plasmid pGP-oriT not containing the mcl-PHA operon showed trace amounts of PHB production, but no mcl-PHA propyl esters were detected by GC or GC-MS. The low levels of PHB detected could be from internal accumulation of 3-hydroxybutyral CoA since only the *phaC* gene is mutated in the PHB-4 strain and the *phaB* and *phaA* remain active creating large pools of 3-hydroxybutyal CoA. The result could also be due to the mutation rendering the *phaC* gene inactive being reverted or allowing leaky activity by the enzyme allowing for some PHB accumulation.

Since the experimental strains harboring the plasmid containing the mcl-PHA operon showed distinct peaks not present in the wild type or negative control, it can be concluded that the expression of the *P. oleovorans phaC* in conjunction with the *P. oleovorans phaJ* or *S. cerevisiae fox2* gene is sufficient for mcl-PHA accumulation. The

plasmid pGP-oriT 700*phaJ* accumulated PHAs with constituents of 3-hydroxybutyrate (9.7%), 3-hydroxyhexanoate (>1%), 3-hydroxyoctanoate (65%), and 3-hydroxydecanoate (25%). The pGP-oriT 700*fox2* accumulated PHAs with constituents of 3-hydroxybutyrate (20%), 3-hydroxyhexanoate (6%), 3-hydroxyoctanoate (43%), 3-hydroxydecanoate (30%), and 3-hydroxydecenoate (>0.1%).

Further tests to optimize the production of mcl-PHAs using the recombinant model developed in this experiment are optimizing the growth of the culture and expression of the genes to create a strain and conditions more favorable to PHA production using this pathway. One such test would be to integrate the operon into the genome of *R. eutropha* in the same location as was picked for the previous study where the *P. oleovorans phaC* was swapped with the native *phaC* gene in *R. eutropha*. Another study could involve integrating the engineered operon in place of the entire *phaCAB* operon in *R. eutropha* knocking out the function of the ketothiolase (*phaA*) and reductase (*phaB*). This could eliminate the possibility of making the precursors for PHB synthesis which could have the added benefit of creating healthier cells which would boost biomass production and possibly PHA production as well.

Part of this chapter has been published in AIChe (McChalicher et al., 2010)*

7. Solubility of PHA Biopolymers in 1,2-Propylene Carbonate

7.1 Introduction

Since PHAs are accumulated as intracellular granules they must be extracted and purified before being used in commercial plastics. This process remains a major hurdle in the efficient scale-up of commercial PHA production. Typically organic solvents such as chloroform are used at reflux to extract the PHAs from dried cells, and are subsequently precipitated using a non-solvent such as methanol. At larger scales this process becomes undesirable due to the high cost, residual solvent contamination in the end products, and safety during processing.

In an attempt to develop a safer, more economical, and more environmentally friendly process we have investigated the use of 1,2-propylene carbonate as a solvent for the extraction of PHAs. A thesis (Heinzle, 1980) and two patents (Lafferty R. M., Heinzle E., 1978; Lafferty R.M., 1978) have shown that propylene carbonate may be an ideal candidate for a greener and safer alternative to current lab scale methods of PHA extraction. The wide gap in the boiling points between propylene carbonate (240°C) and non-solvents such as methanol (≈65°C) also shows promise that the solvents used could be separated and recycled additional extraction processes. This will greatly reduce the amount of solvents needed further reducing cost and environmental impacts from this process.

In this study we investigated the ability of 1,2-propylene carbonate to dissolve PHAs, and measured the kinetics of the extraction process at different temperatures. This

chapter will discuss aspects of the paper for which I was involved in such as PHA solubility, PHA extraction from biomass, and solvent recovery and recyclability. Other topics in the paper not discussed here involve polymer chain degradation kinetics and estimations discussing scalability of the process. They can be found in the complete article published by AIChE (McChalicher et al., 2010).

7.2 Methods

7.2.1 Polymer samples

Most experiments used a stock of powdered Biopol® polymer (PHB) received by our laboratory in 1995 from Monsanto Corporation (St. Louis, MO). Freshly prepared PHB homopolymer and PHB-PHBV bioblock copolymer were also used. These polymers were synthesized using a two-step cultivation method described previously.

7.2.2 Determination of dissolution kinetics

The stocks of Biopol® powder suspended in 1,2-propylene carbonate (0.5 g PHB per 5 mL solvent; (Sigma, St. Louis, MO)) were prepared so that the final concentration was 100 g/L. This stock was then split three ways into open top glass tubes and used for replicates of a single-temperature experiment. The tubes were placed into a mineral oil bath held at constant temperature by use of feedback control. The samples were mixed before sampling by bubbling air through a glass Pasteur pipette. The samples were withdrawn at defined times and diluted with same-temperature propylene carbonate so that the undissolved fraction was less than 2 g/L. These dilutions were analyzed by spectrophotometry at 400 nm to determine the percent transmission. Standard solutions were analyzed by the same sampling method to determine a suitable calibration curve.

7.2.3 Determination of maximum solubility

Various concentrations (between 1 and 350 g/L) of Biopol® suspended in 1,2-propylene carbonate were used to determine the maximum solubility of PHB in the solvent at temperatures ranging from 100 to 140°C. 5 mL of each concentration tested was incubated at the desired temperature in a glass test tube for 15 min. The extent of dissolution was determined by the spectrophotometric method described earlier.

7.2.4 PHA extraction from dried biomass

PHA block copolymer was extracted from freeze dried *R. eutropha* cell mass. The PHAs were made using a method described by Pederson et al (Pederson, 2006). Known amounts of dried cell mass were crushed using a mortar and pestle and poured into a 100 mL beaker containing 25 mL of 120°C 1,2-propylene carbonate. The mixture was stirred using a magnetic stir plate while samples were taken at regular intervals. The absorbance of the propylene carbonate was read as described previously and compared to a curve made from known standards to determine the concentration of PHA dissolved in the 1,2-propylene carbonate.

7.2.5 Solvent recovery

Filter permeate containing propylene carbonate and methanol were recovered using a rotary evaporator (rotovap). The boiler was placed in a water bath at 90°C, and then a vacuum was applied using a vacuum aspirator. Cold tap water was passed through the condensing stage to recover the methanol. A propylene carbonate-rich phase was recovered from the boiler.

7.3 Results

7.3.1 Determination of solubility kinetics

The kinetics of dissolving highly crystalline Biopol® PHB powder in 1,2-propylene carbonate was investigated at various temperatures. In this system, a spectrophotometer was used at 400 nm to determine the extent of dissolution for this polymer powder by measurement of the residual turbidity of the solution. This rapid assay permitted frequent and reliable sampling, which resulted in detailed monitoring of the dissolution process. Figure 7-1 illustrates the absorbance at several wavelengths (400, 500, and 600 nm) for PHB powder suspended in cold propylene carbonate (Panel A) and PHB powder fully dissolved in hot propylene carbonate (Panel B).

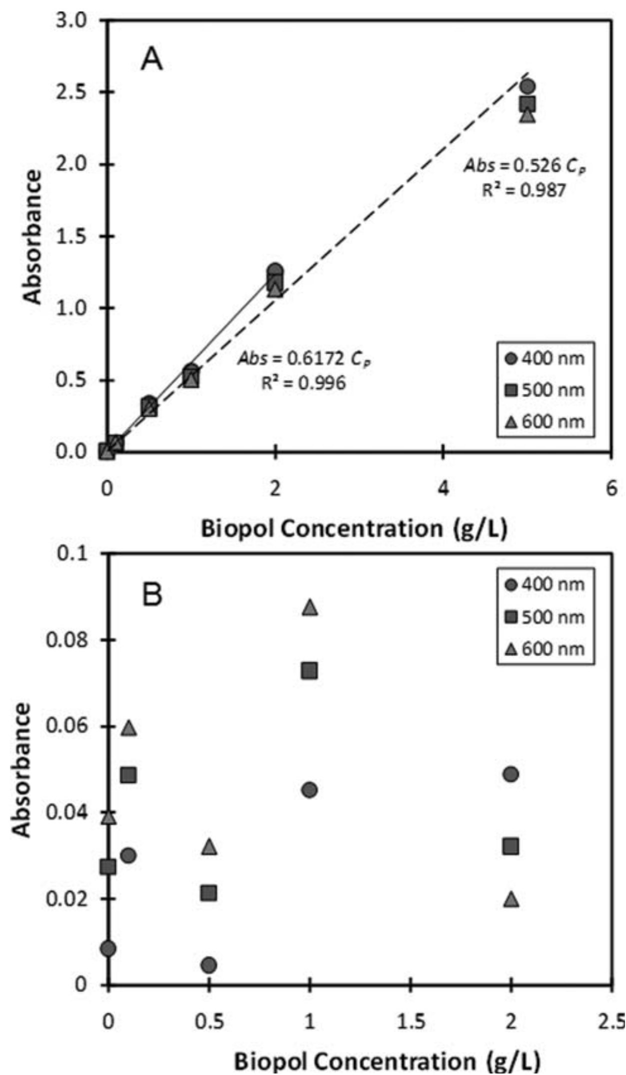


Figure 7-1 Absorbance of undissolved and dissolved Biopol® powder in 1,2-propylene carbonate at various wavelengths as determined by residual turbidity measurements.

(A) Calibration curve for the residual turbidity measurements for various wavelengths for undissolved PHA biopolymer suspended in propylene carbonate. (B) Residual turbidity for PHA polymers dissolved in propylene carbonate measured by various wavelengths.

In Panel A, we see that a linear dependence exists between absorbance and the concentration of the PHB powder for the three wavelengths. Also, we observed that above concentrations of 2 g/L, this dependence is no longer linear. Linear regressions are

presented for the calibration curve from 0 to 2 g/L and from 0 to 5 g/L concentrations. Inclusion of the 5 g/L data point decreases the fitness of the regression and results in a bias of the residual errors (systematic under calibration) at lower concentrations. Therefore, samples were diluted so that the non-dissolved fraction was less than 1 g/L in the sample during analysis to ensure that the sample readings were within the linear region. The corresponding dilution factor was used to determine the real concentration of solid-phase PHB at that time point. Furthermore, Panel B illustrates a low-level background for dissolved polymers at these concentrations. From this data, we chose to use 400 nm as our analysis wavelength because of the generally lower background readings for the dissolved polymer and because of the more sensitive slope for the solid-phase PHB particulates.

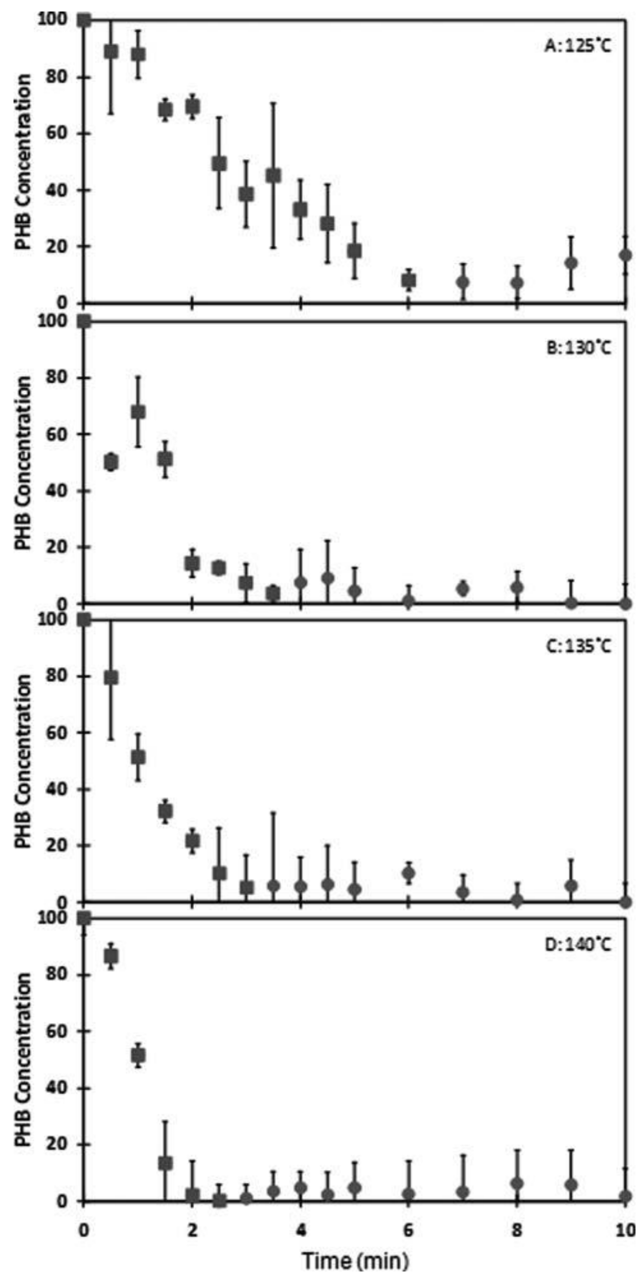


Figure 7-2 Dissolution kinetics of crystalline PHB powder in 1,2-propylene carbonate at several temperatures as determined by residual turbidity measurements.

Error bars represent standard deviation of three replicates. (A) 125°C; (B) 130°C; (C) 135°C; (D) 140°C.

The panels of Figure 7.2 represent the dissolution kinetics of PHB vs. time for several temperatures. Panels A–D contain results for temperatures of 125, 130, 135, and 140°C, respectively. The amount of solid-phase PHB remaining as determined by the spectrophotometric method is plotted vs. time in minutes. Each sample was initially charged with 100 g/L Biopol® PHB in 1,2-propylene carbonate. Each curve represents an average of three runs, and the vertical error bars represent the standard deviation for each sample time. For all temperatures, full dissolution was observed within 6 min. Full dissolution is marked in Figure 2 by the transition from data points marked by squares (still dissolving) to circles (fully dissolved) in the time courses. Error bars are reported, and they are the standard deviations of three repetitions.

7.3.2 Determination of solubility limits at different temperatures

Table 7.1 Dissolution of Crystalline PHB in 1,2-Propylene Carbonate at Various Temperatures

Temperature (°C ± 2°C)	Last Concentration Dissolved (g PHB L^{-1})	First Concentration Not Fully Dissolved (g PHB L^{-1})	Approximate Solubility Limit (g PHB L^{-1})
100	N/A*	N/A	–
110	N/A	N/A	–
115	1	10	5
120	10	25	15
125	100	150	130
130	250	300	280
140	350	–	>350

*N/A indicates that no dissolution was observed for any concentration.

We also investigated the maximum solubility of Biopol® PHB in 1,2-propylene carbonate. Table 7.1 shows the solubility range for temperatures ranging from 110 to

150°C. Several concentrations were tested at each temperature to determine the range of the maximum solubility. The temperature of the solvent, the highest concentration of Biopol® PHB which fully dissolved, and the lowest concentration which failed to fully dissolve are reported in Table 7.1. The maximum solubility at a given temperature is therefore bounded by those concentrations and is estimated in Table 7-1 based on the observations of the undissolved sample.

7.3.3 PHA extraction from dried biomass

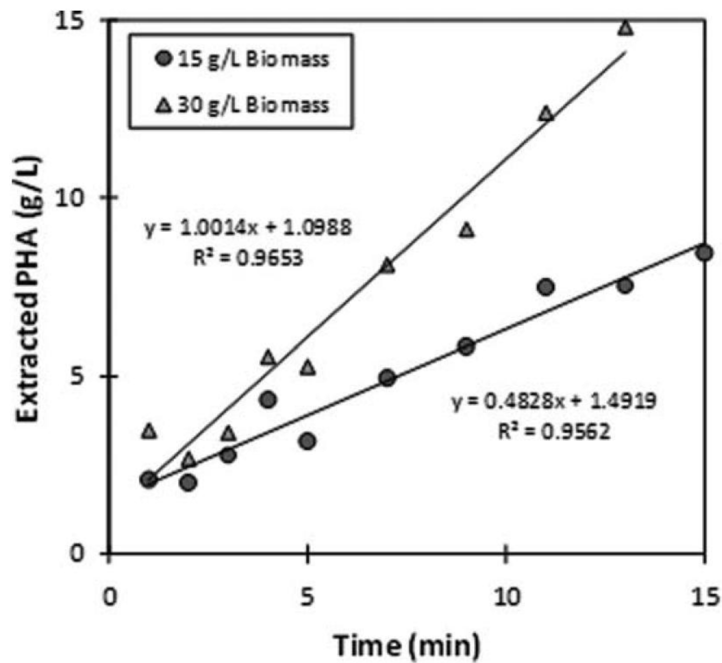


Figure 7-3 Extraction of PHA block copolymer from dried biomass using 1,2-propylene carbonate at 120°C

The previous data sets suggest that the extraction of PHAs from dried biomass should be efficient using the solvent system presented here. Figure 7.3 shows the recovery of PHA block copolymer from biomass vs. time at 120°C. Two concentrations

of freeze-dried biomass were investigated, 15 g/L and 30 g/L. This biomass was determined by GC analysis to hold 72% PHA by weight, indicating that the polymer concentrations of these trials should be 10.8 g/L and 21.6 g/L, respectively. We have presented the kinetics of PHA extraction in Figure 7.3. After 15 min, nearly 75% of the PHA had been extracted from the cells. Because PHA block copolymer is less crystalline than the PHB powder studied earlier, a temperature of 120°C was sufficient to dissolve this material. Other data presented in the paper but not described in this chapter shows that little degradation of the polymer chains occurs at 120°C, and it was estimated that less than 15% degradation would result from a 15 minute extraction process at 120°C.

7.3.4 Solvent recovery and recyclability

As described earlier a rotovap was used to recover the solvents 1,2-propylene carbonate and methanol that were used in the extraction process. The two liquids were able to be separated into propylene carbonate and methanol rich phases respectively. The recovered propylene carbonate and methanol were tested for their abilities to dissolve Biopol® and precipitate it out of solution, respectively, in the same manner as tested previously. Qualitative tests showed that the solvents were able to perform the same as they had done using fresh reagents.

7.4 Discussion

We have investigated the use of 1,2-propylene carbonate for the use in PHA extraction and other processing occurring at ambient pressures. Solubility kinetics have been determined using a notoriously difficult to dissolve PHA: highly crystalline PHB. Because of the highly crystalline character of the Biopol® PHB powder, this material is

the proper choice for the determination of solubility kinetics as this PHA presents the slowest dissolution kinetics. More favorable kinetics should be obtained from less crystalline material (PHB or otherwise); however, systems designed with these parameters may not be robust in processing all grades of desired PHAs. We have shown that temperatures more than 125°C are required to dissolve this material. Once above 125°C, the crystalline PHB dissolves rapidly at high concentrations.

Finally, we have demonstrated the recovery of PHA block copolymer from dried biomass at 120°C. After 15 min, up to 75% of the PHA was extracted from the cells. The reagents used also demonstrated their ability to be recycled and reused for additional extraction processes. These demonstrations suggest that 1,2-propylene carbonate is a useful solvent for the recovery and processing of short-chain-length PHAs. For extraction purposes, balancing the rate of dissolution, the diffusion and movement of the PHA chains away from the bulk cellular material, and the rate of chain degradation is necessary in designing effective large-scale recovery of PHAs.

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8. Appendix

8.1 Mass Spectra used to identify the identity of peaks on gas chromatogram

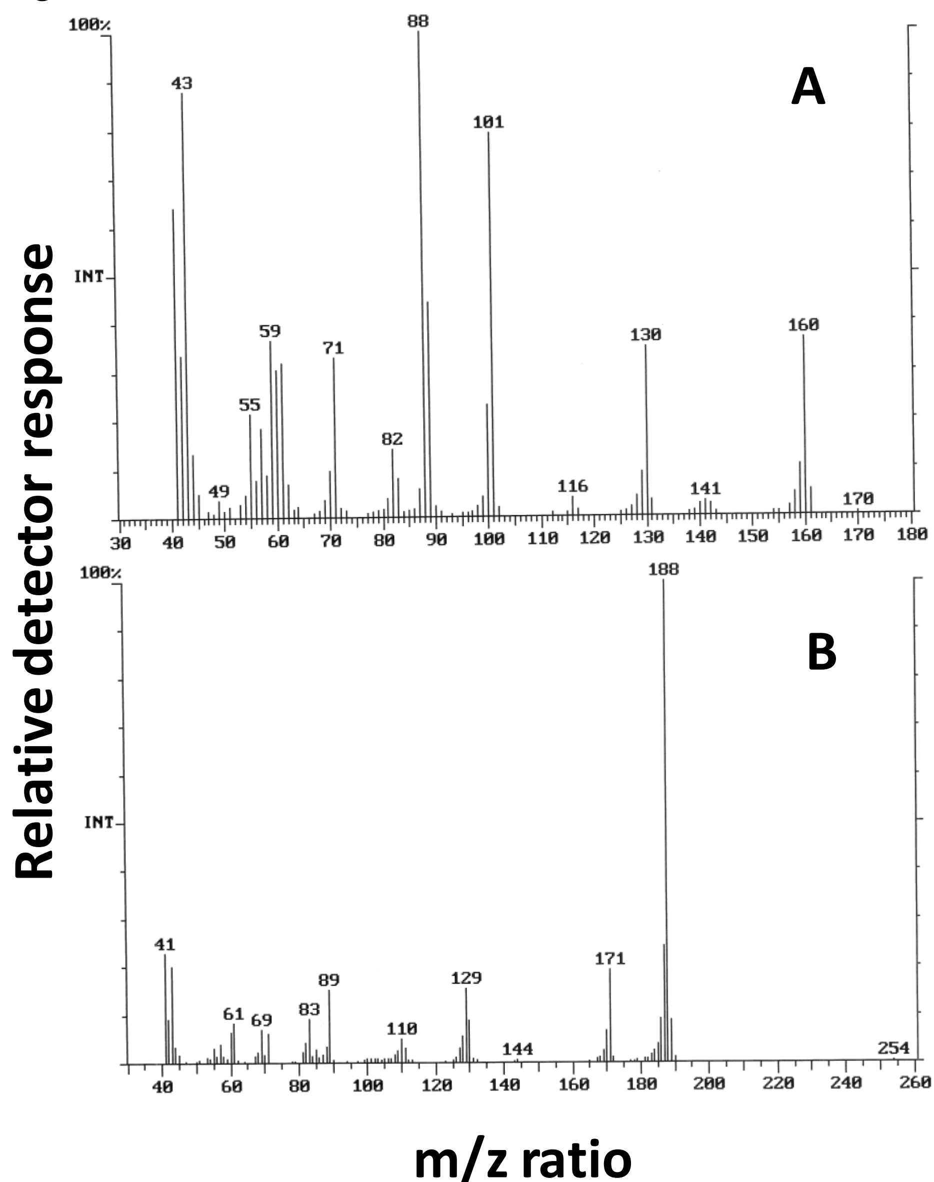

Figure 8-1 Mass spectrum of major peaks identified from GC-MS analysis of PHAs made from *P. oleovorans* grown on heptane

Analysis of major peaks from the GC trace to identify the constituents of the PHAs made by *P. oleovorans* when grown on heptane as the sole carbon source. The x axis represents the molecular weight detected and the y axis represents the relative response from the detector. Identification of the graphs above and the time of MS sampling (retention time in minutes) are as follows: (A) 3-hydroxyvalerate (4.49), (B) 3-hydroxyheptanoate (8.46).

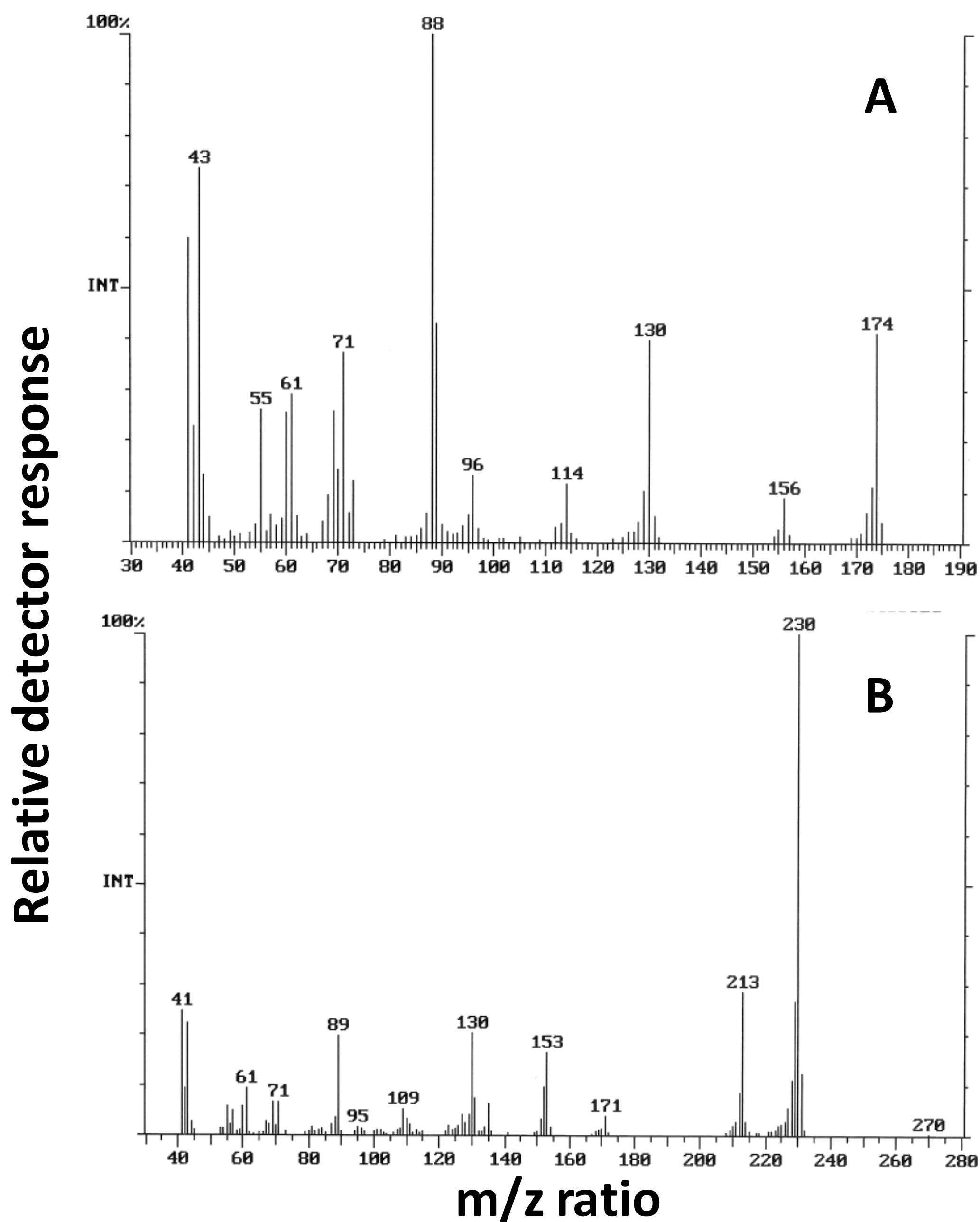

Figure 8-2 Mass spectrum of major peaks identified from GC-MS analysis of PHAs made from *P. oleovorans* grown on decane

Analysis of major peaks from the GC trace to identify the constituents of the PHAs made by *P. oleovorans* when grown on decane as the sole carbon source. The x axis represents the molecular weight detected and the y axis represents the relative response from the detector. Identification of the graphs and the time of MS sampling (retention time in minutes) are as follows: (A) Polyhydroxyhexanoate (5.86), (B) Polyhydroxydecanoate (21.26).

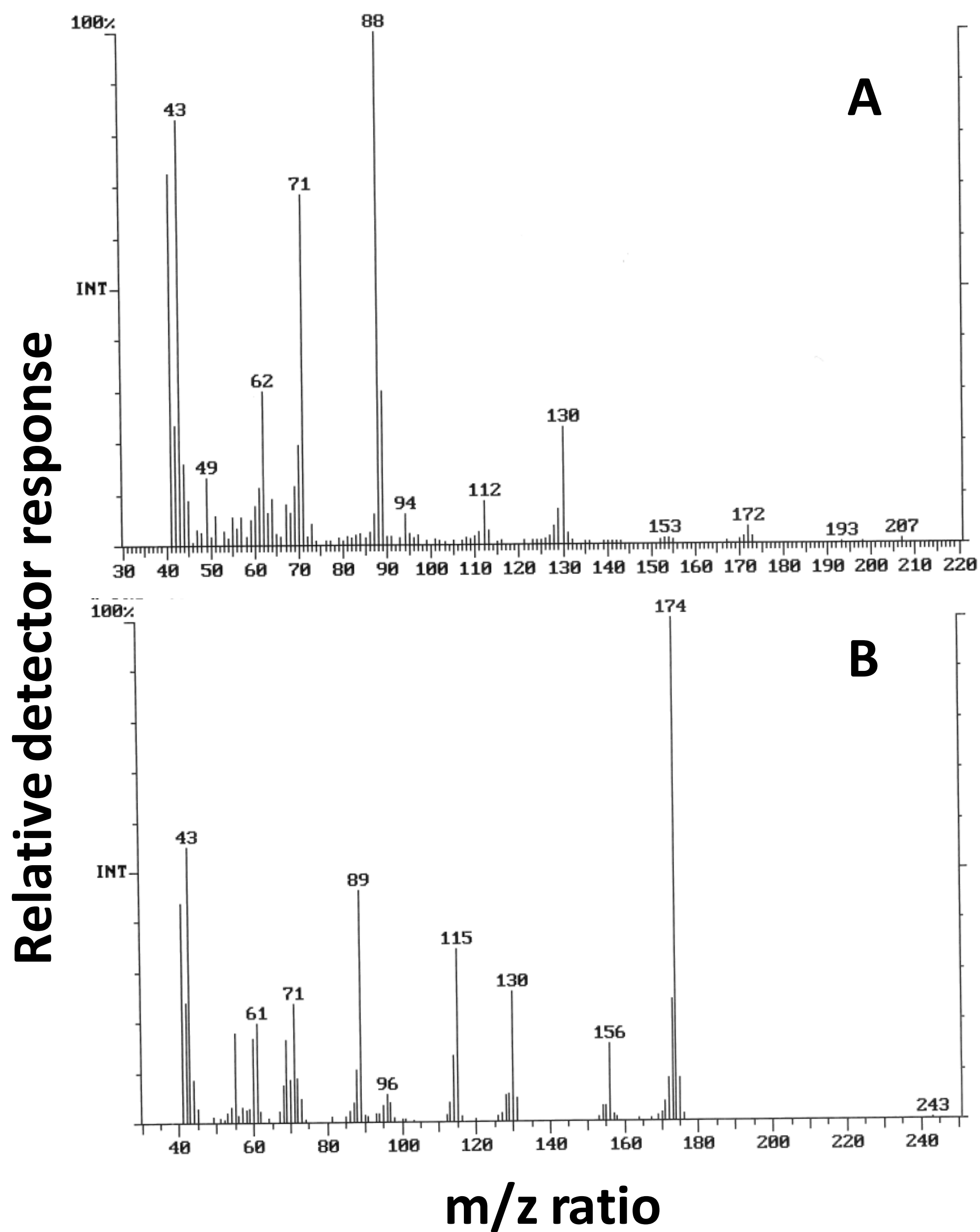

Figure 8-3 Mass spectrum of major peaks identified from GC-MS analysis of PHAs made from *P. oleovorans* grown on octane/octene

Analysis of two peaks from the GC trace to identify the constituents of the PHAs made by *P. oleovorans* when grown on intermittent feeding of octane and octene. The x axis represents the molecular weight detected and the y axis represents the relative response from the detector. Identification of the graphs and the time of MS sampling (retention time in minutes) are as follows: (A) 3-hydroxyhexanoate (5.91), (B) 3-hydroxydecanoate (7.01).

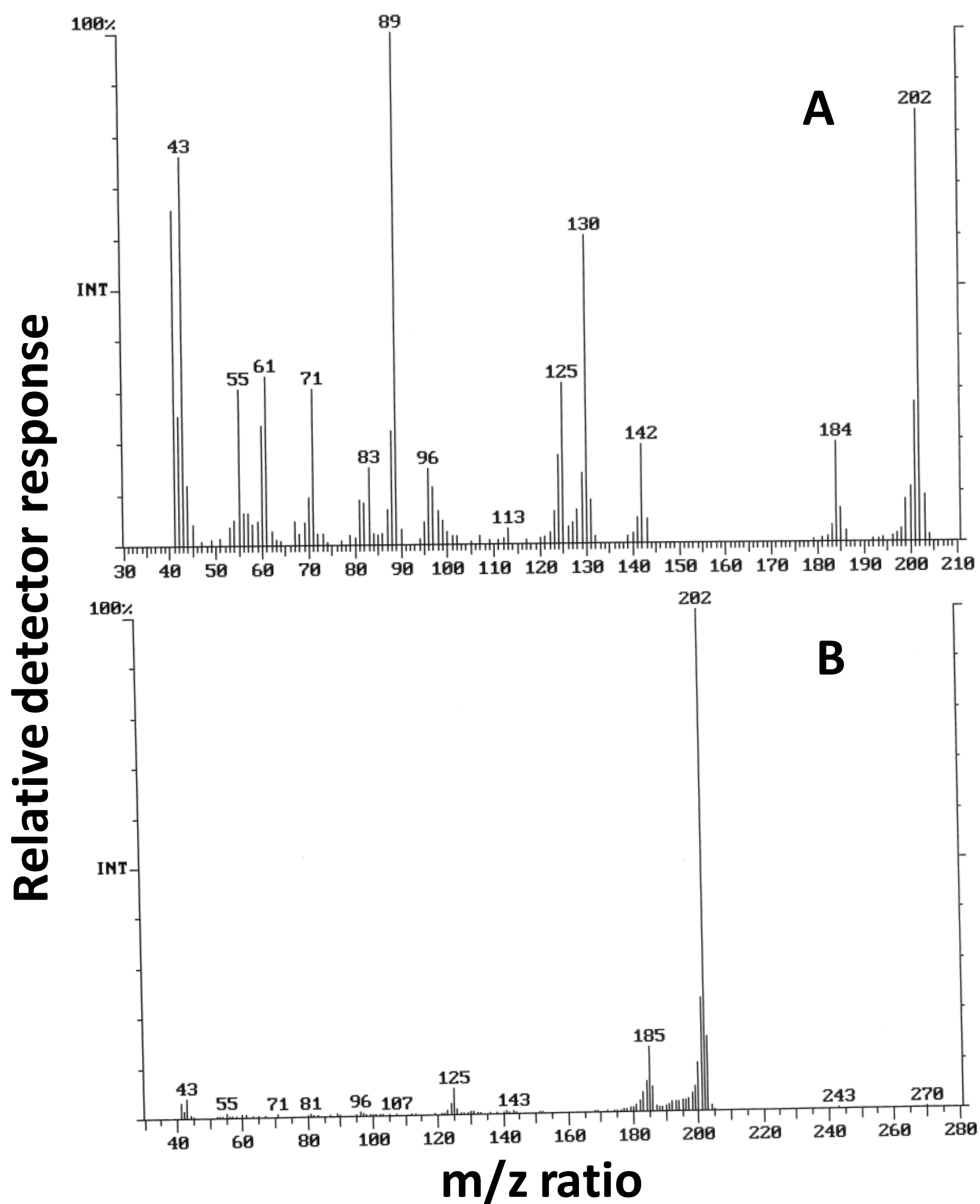

Figure 8-4 Mass spectrum of major peaks identified from GC-MS analysis of PHAs made from *P. oleovorans* grown on octane/octene

Analysis of two peaks from the GC trace to identify the constituents of the PHAs made by *P. oleovorans* when grown on intermittent feeding of octane and octene. The x axis represents the molecular weight detected and the y axis represents the relative response from the detector. Identification of the graphs and the time of MS sampling (retention time in minutes) are as follows: (A) 3-hydroxyoctanoate (5.91), (B) 3-hydroxyoctenoate (7.01).

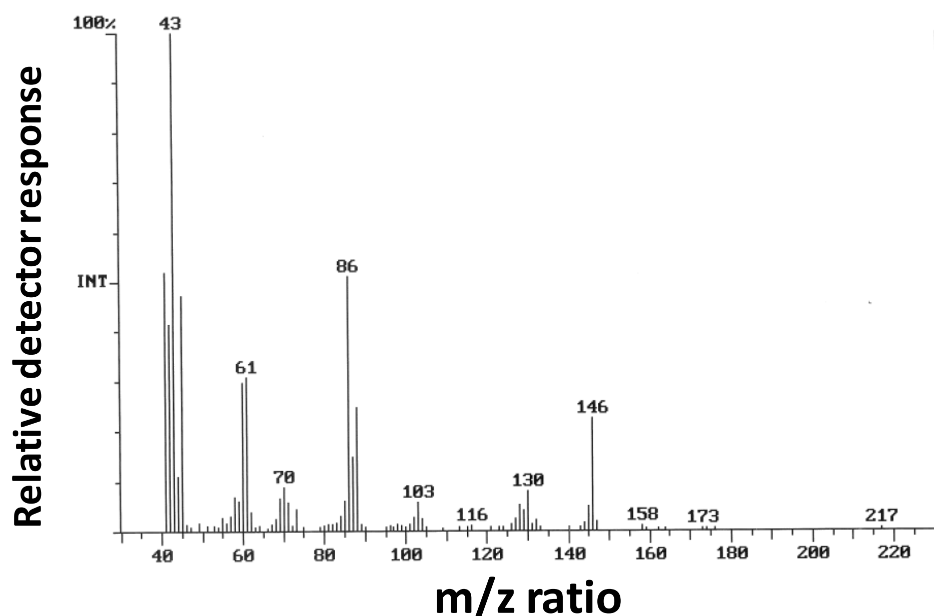

Figure 8-5 Mass spectra for *R. eutropha* Δ *phaC* lyophilized cells

The above graph represents the mass spectra used to identify the PBH peak on the gas chromatograph.

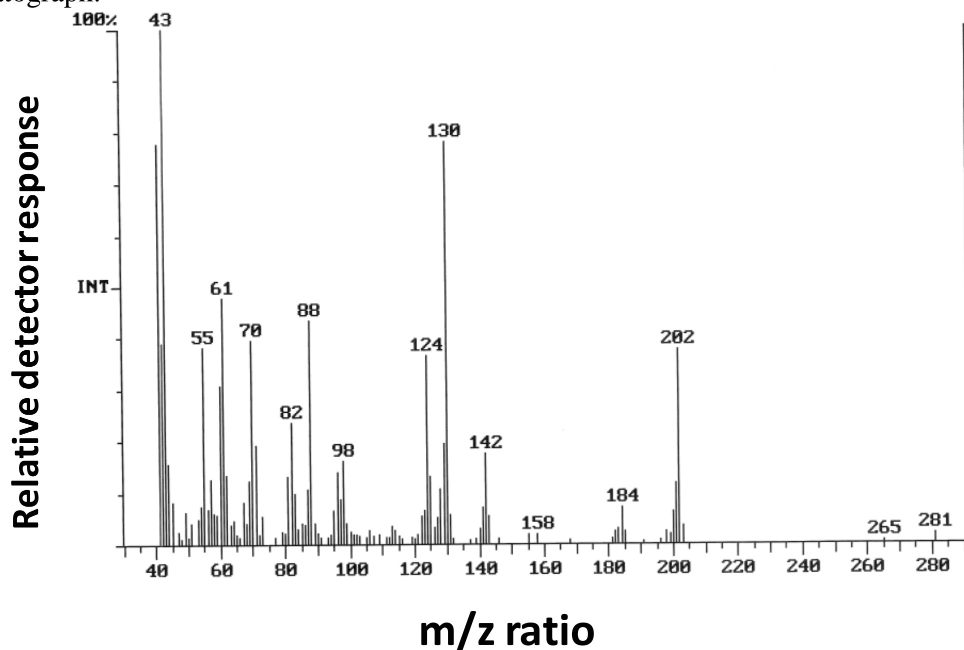

Figure 8-6 Mass spectra for *R. eutropha* Δ *phaC* PHA sample

The graph above represents the mass spectra from a major peak identified by GC-MS analysis of the PHA extracted from the *R. eutropha*Δ*phaC*. The spectrum represents the 3-hydroxyoctanoate peak identified in the gas chromatogram.

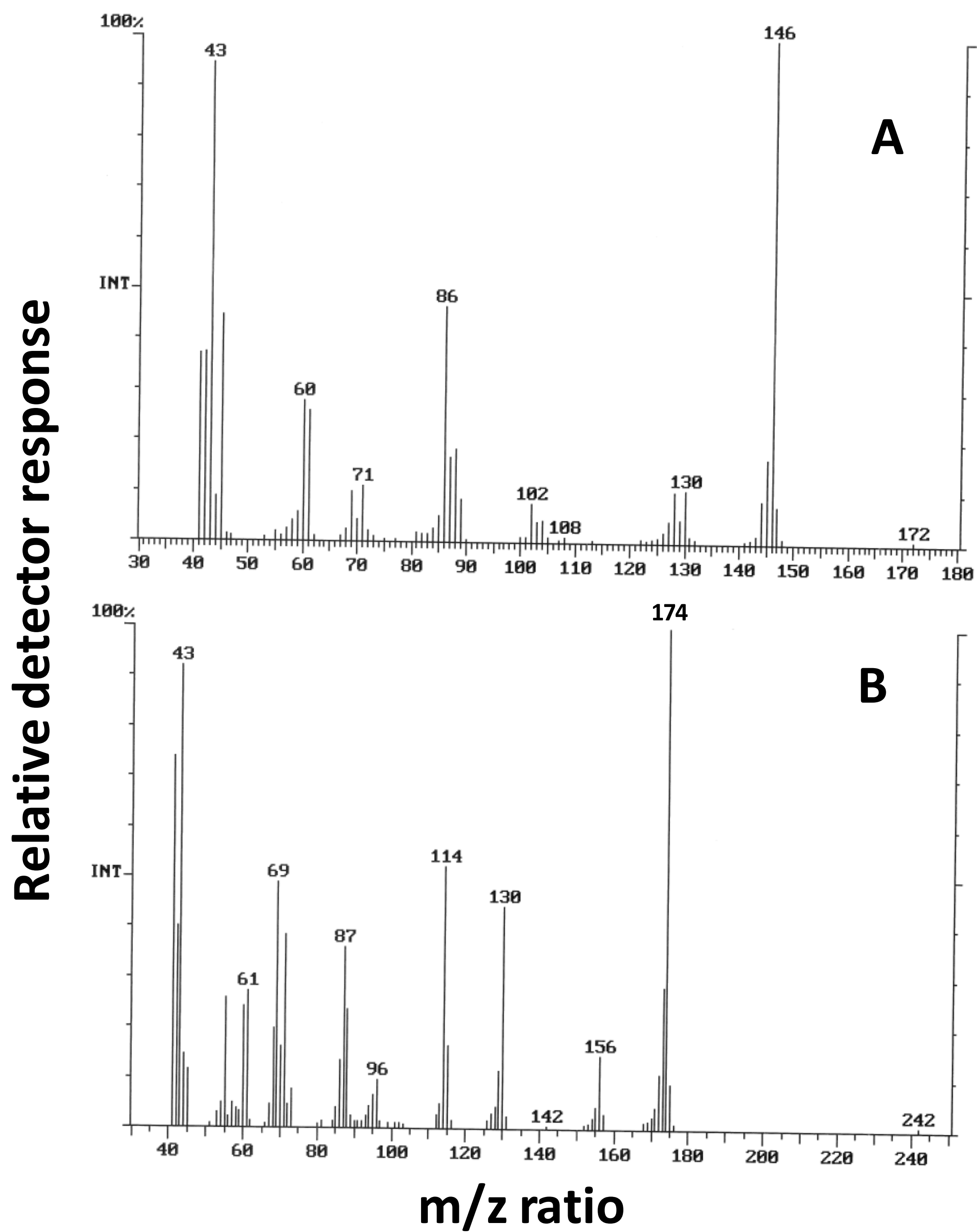

Figure 8-7 Mass spectra for *R. eutropha* H16 lyophilized cell sample

The above graph represents the mass spectra used to identify the peaks in the gas chromatograph. The spectra represent (A) 3-hydroxybutyrate, (B) 3-hydroxyhexanoate.

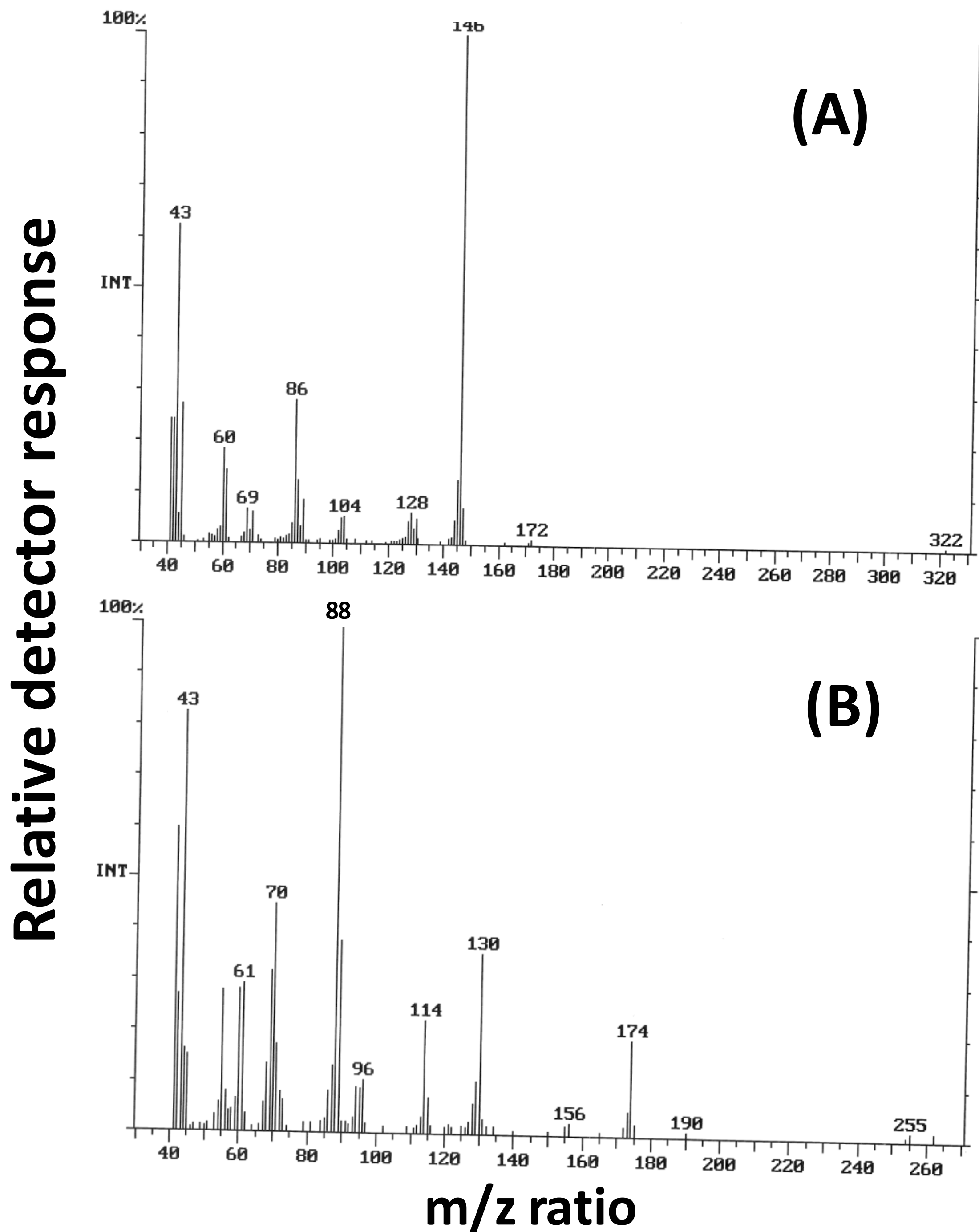

Figure 8-8 Mass spectra for *R. eutropha* H16 extracted PHA sample

The above graph represents the mass spectra used to identify the peaks in the gas chromatograph. The spectra represent (A) hydroxybutyrate propyl ester, and hydroxyhexanoate propyl ester, the only two major constituents of the polymer.

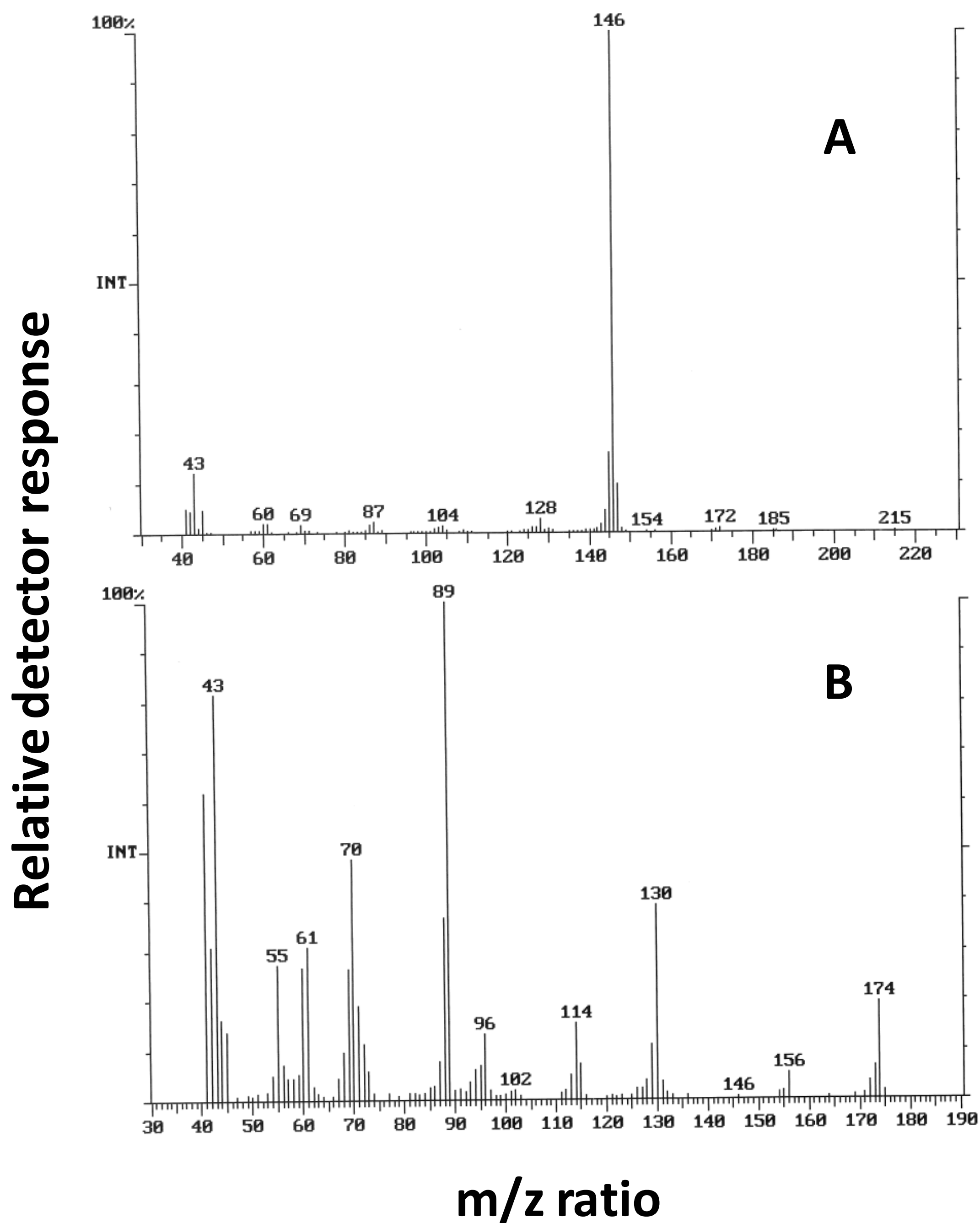

Figure 8-9 Mass spectra of *R. eutropha* PHB-4 pGP-oriT 700*fox2* lyophilized cells

The above graphs are mass spectra of samples taken representing major peaks in the gas chromatogram. (A) is the 3-hydroxybutyrate propyl ester spectra, and (B) is the 3-hydroxyhexanoate propyl ester spectra.

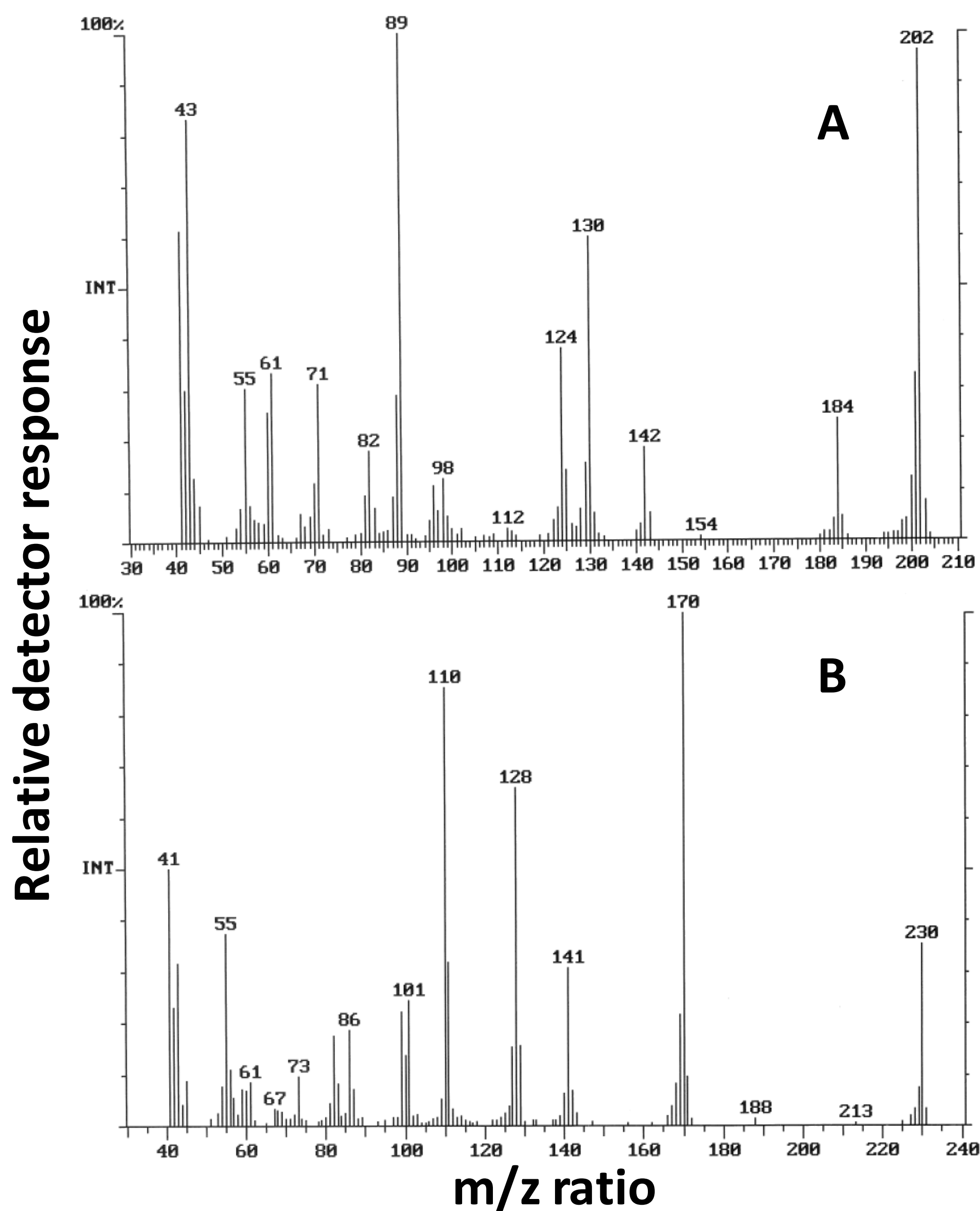

Figure 8-10 Mass spectra of *R. eutropha* PHB-4 pGP-oriT 700*fox2* lyophilized cells

The above graphs are mass spectra of samples taken representing major peaks in the gas chromatogram. (A) is the 3-hydroxyoctanoate propyl ester spectra, and (B) is the 3-hydroxydecanoate propyl ester spectra.

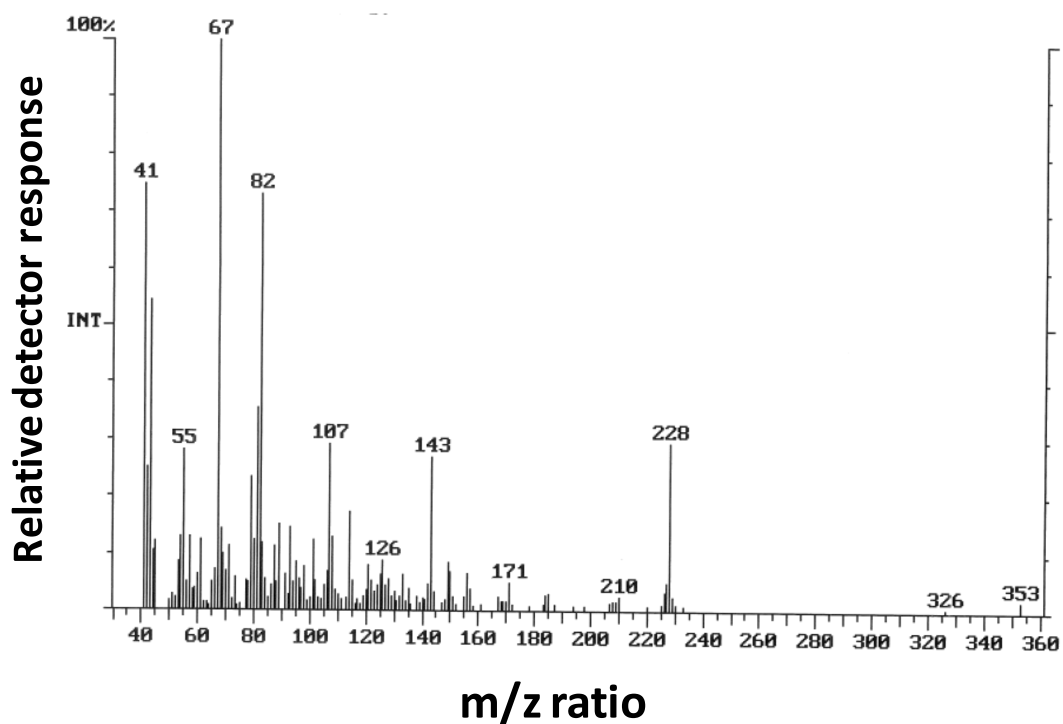

Figure 8-11 Mass spectra of *R. eutropha* PHB-4 pGP-oriT 700*fox2* extracted PHAs

The above graph is the mass spectra of samples unique to the extracted PHAs made from the strain pGP-oriT700*fox2* taken from major peaks in the gas chromatogram. Other constituents found in the PHAs are represented in the previous two figures.

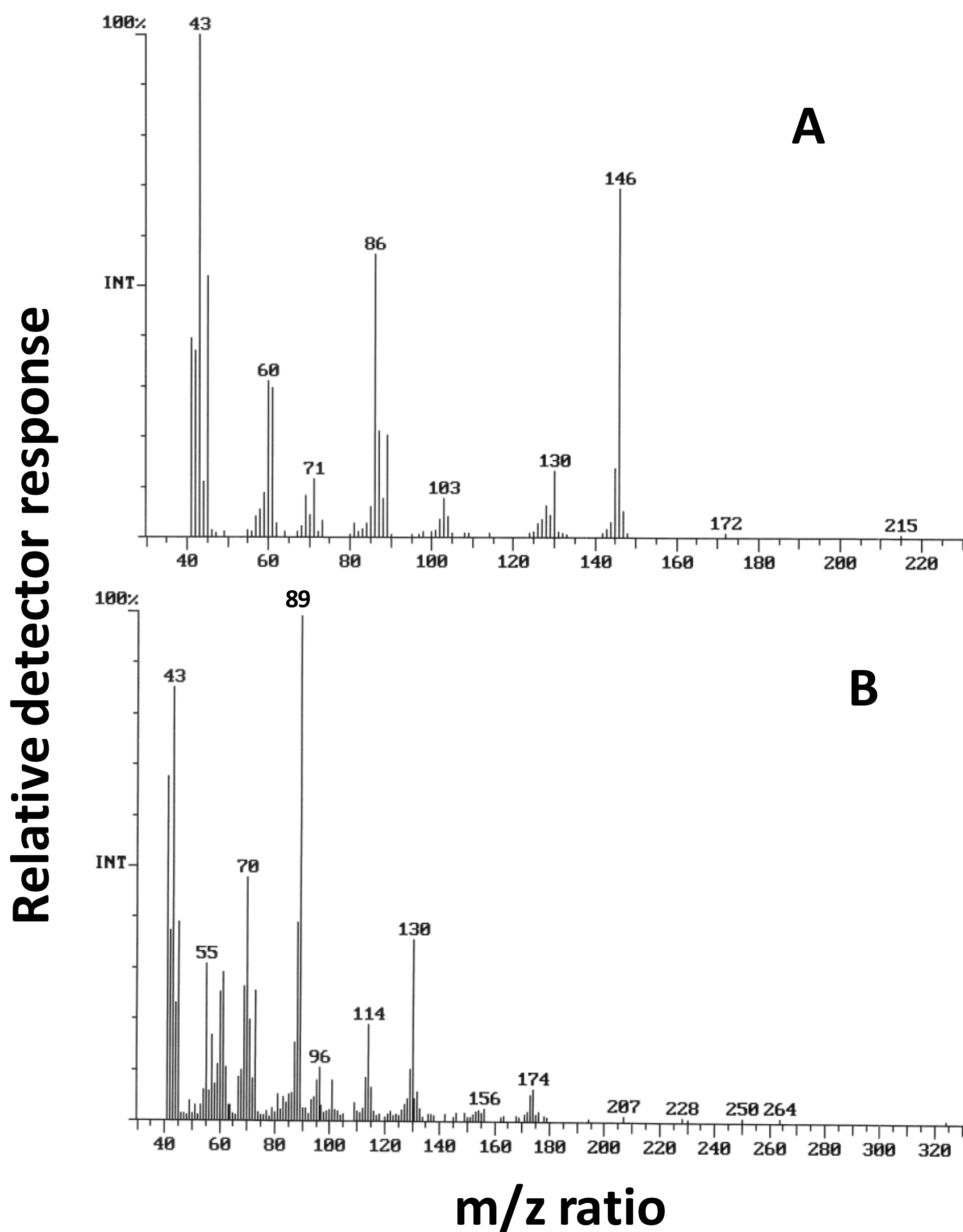

Figure 8-12 Mass spectra of *R. eutropha* PHB-4 pGP-oriT 700*phaJ* lyophilized cells

The above graphs are mass spectra of samples taken from the first two major peaks in the gas chromatogram for this sample. (A) is the 3-hydroxybutyrate propyl ester spectra, (B) is the 3-hydroxyhexanoate propyl ester spectra.

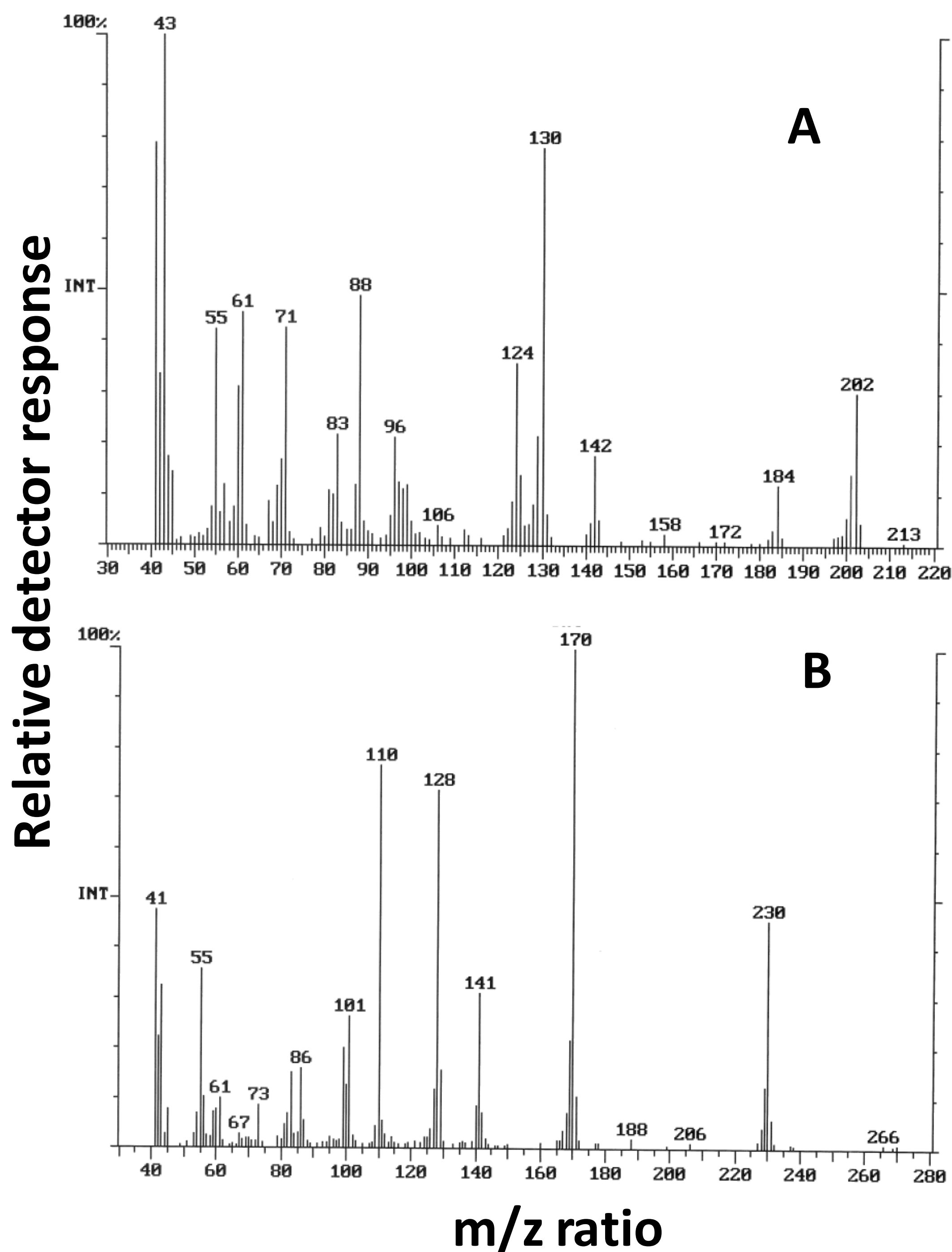

Figure 8-13 Mass spectra of *R. eutropha* PHB-4 pGP-oriT 700*phaJ* lyophilized cells

The above graphs are mass spectra of samples taken from the last two major peaks in the gas chromatogram for this sample. (A) is the 3-hydroxyoctanoate propyl ester spectra, (B) is the 3-hydroxydecanoate propyl ester spectra.

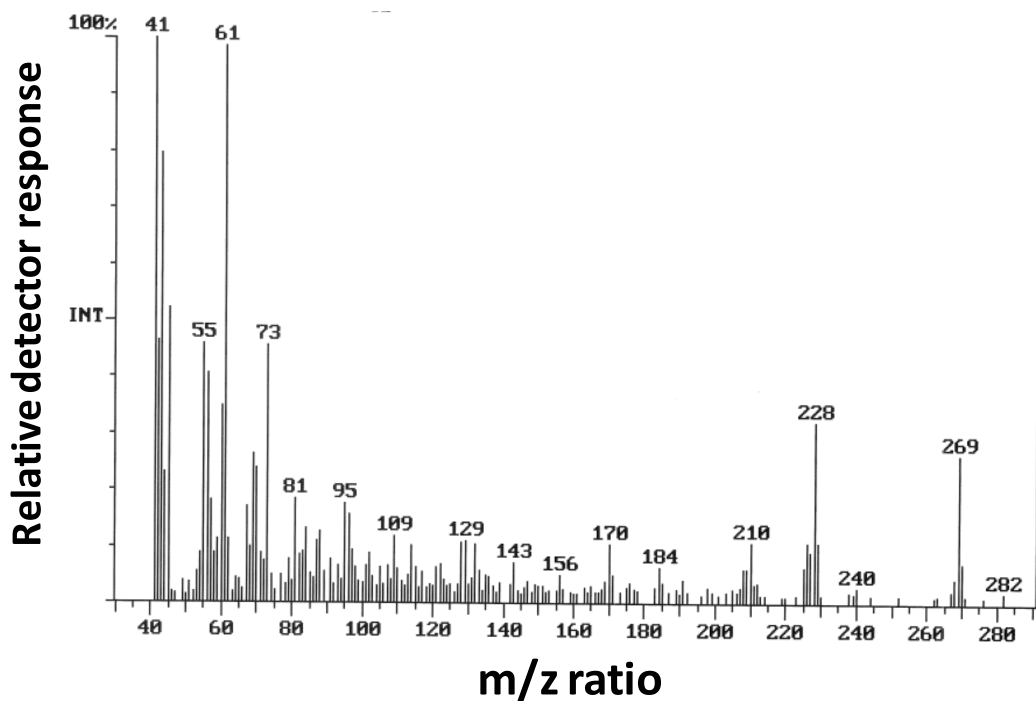

Figure 8-14 Mass spectra of *R. eutropha* PHB-4 pGP-oriT 700*phaJ* extracted PHAs

The above graph is the mass spectra of samples unique to the extracted PHAs made from the strain pGP-oriT700*phaJ* taken from major peaks in the gas chromatogram. Other constituents found in the PHAs are represented in the previous two figures.