Analyzing polyhydroxyalkanoate accumulation and identifying the PHA synthase gene, *phaC*, in *Thermus* sp.

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Polyhydroxyalkanoate (PHA) is a cellular storage polymer with similar properties to some plastics when extracted from the cell and acts as a renewable and biodegradable plastic alternative. The objective of this research was to identify thermophilic PHA producing microorganisms and characterize their PHA accumulation, by which the process may become more competitive to current petroleum-based plastic production. This study characterizes growth and PHA production of *Thermus thermophilus* HB8 and three strains of *T. filiformis* (MOK, NGM and WRT), while simultaneously performing a genetic analysis for the presence of a *phaC* gene that encodes the PHA synthase.

The results from the genetic analyses, together with attempts to produce PHA in *T. thermophilus* HB8 and *T. filiformis* strain (WRT, NGM and MOK) infer an absence of PHA accumulation. Contrary to our expectations, *Thermus* sp. is not a strong candidate for the thermophilic production of PHA.

Contents

List of Abbreviations

3-HA	3-Hydroxyalkanoic acid
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
C4, C6, C8. C10	Number of carbon units in the backbone of a PHA monomer (C4 = 4 carbon units)
CoA	Coenzyme A
COG	Cluster of Orthologous Groups
dcw	dry cell weight
diH_2O	deionized water
DNA	Deoxyribonucleic Acid
EC	Enzyme Commission Numbers
GC	Gas Chromatograph
lcl	long-chain-length
mcl	medium-chain-length
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
ND	Not Determined
OD_{600}	Optical Density measured at 600 nm
PCR	Polymerase Chain Reaction
PHA	Polyhydroxyalkanoate
rDNA	ribosomal deoxyribonucleic acid
scl	short-chain-length

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Chapter 1: Polyhydroxyalkanoate and its production by thermophilic microorganisms

1.1 PHA as a sustainable and biodegradable replacement for petroleum plastic

One of the many uses for petroleum is in plastic production. According to the Canadian Plastics Industry Association, products made from plastics occupy 9% of landfills by weight, and prices for disposal continue to rise. In modern landfills, very little degradation actually occurs to prevent greenhouse gas emission (Canadian Plastics Industry Association). The economic and environmental impact associated with plastic production highlights the current and future needs for a sustainable and biodegradable substitute.

Known bio-polymers that can act as substitutes for conventional plastics include starch-based polymers, polylactides, polyhydroxyalkanoates, bio-derived polyethylene, and polyamide 11. Starch-based polymers are derived from starch extracted from some plants or microorganisms. The production of starch-based polymers uses renewable resources and the resulting plastics are biodegradable (Tokiwa, et al., 2009). Lactic acid can be obtained as a fermentation product in bacteria using renewable substrates, which can then be synthesized into polylactide and is ultimately biodegradable. One of the current uses of polylactide polymers are for medical implants, stitches and screws, since the polymer will be dissolved in the body after the healing is finished (Plackett, et al., 2005). Polyhydroxyalkanoates are obtained directly from microbial fermentation which allows for the use of renewable resources, and PHAs can be decomposed by microbes in the environment. Due to the varying nature of polyhydroxyalkanoates (discussed below)

many conventional plastic substitutes can be produced. Finally, bio-derived polyethylene has the same properties and polyamide-11 has similar properties as their namesakes however both are non-biodegradable. However an alternative pathway is utilized so they are produced from renewable resources (Tokiwa, et al., 2009).

Polyhydroxyalkanoate (PHA) is a microbial storage polymer that is stored when certain microorganisms are growing in carbon-rich environments, but are stressed due to a deficiency in other nutrients. The polymer may later be consumed as a source of energy (ATP) and reducing power (NADH/NADPH) if the external carbon source is depleted (Madison, et al., 1999). Some microorganisms encode an external PHA depolymerase likely to utilize polymer granules from lysed PHA producers (Jendrossek, et al., 2002). When extracted from the cells, the polymer has similar characteristics to petroleum-derived plastics, and taken together with its metabolism, presents itself as a biodegradable alternative (Madison, et al., 1999). PHA production varies depending on the species and depends on which enzymes are present, the specificities and affinities of those enzymes and the substrates provided. As a result, accumulation can vary from 10% to 90% PHA per cell dry weight, and the polymer may be composed of four to twelve carbon subunit monomers, resulting in polymers that vary in average molecular weight. Moreover, depending on the type of carbon chain subunits, the polymer can be uniform or co-polymers.

Despite the environmental consequences associated with current plastic production, the cost of producing PHA prevents mainstream commercial use. The main cost factors in PHA production are associated with the growth medium, energy expenditure in the fermentation process, and the polymer recovery from the cell. The

growth medium needs to promote growth and PHA accumulation. There is a relationship between environmental stress applied to the microorganism and accumulation of PHA, therefore, media using limited phosphorous, magnesium, or oxygen have been used (Kadouri, et al., 2005). Typically a nitrogen-limiting medium is used to promote PHA accumulation. The choice of substrate will affect growth rates, the composition of the polymer and governs 70 to 80% of the raw material cost. Growth estimations at a scale of one million tonnes per year and using a surfactant-hypochlorite digestion for recovery showed that the carbon substrate was up to 50% of the production cost (Choi et al., 1997; LeMeur et al., 2012). Choi et al. (1997) estimated that the cost of utilities was between 8-9% and the surfactant-hypochlorite digestion cost was around 14% of the production cost. The rest of the production costs were attributed to the factory maintenance and labour cost, and the final cost for one year for processing, to produce one million tonnes of PHB, using *Cupriavidus necator* H16, was US$3.84/kg (Choi, et al., 1997).

There exist many different physical, chemical and enzymatic methods of recovering PHA from the microorganism. Each method has its advantages and disadvantages when time, cost, and product quality are considered. Extraction plays a major role in the market viability of PHA, therefore, consideration must be placed on using the proper method. Although beyond the scope of this review, Sudesh and Kunasundari provided a fairly up-to-date assessment of the different PHA extraction methods and their respective differences (Sudesh, et al., 2011). The estimated production costs for PHB and other biodegradable polymers can be as low as US$2.20/kg. However, compared to the price of synthetic plastics, which ranges from US$0.62-0.96/kg biodegradable polymers are not yet economically competitive (Steinbüchel, et al., 1998).

1.2 The Structure and Variability of Polyhydroxyalkanoate

Polyhydroxyalkanoates are formed in microorganisms using a PHA synthase, which enzymatically condenses hydroxyalkanoate-CoA monomers to form ester bonds. The result is a linear carbon-backbone polymer with a general repeating unit (Figure 1.1). With n=1 and a methyl-group side-chain located uniformly along the polymer formed is called polyhydroxybutyrate (PHB). PHB is a well characterized polymer that is very brittle, since it has a high crystallinity after extraction. More desirable plastic products are being achieved using copolymers and longer carbon side-chain monomers, providing more flexible polymers, which can be tailored to resemble a wider variety of plastics (Madison, et al., 1999).

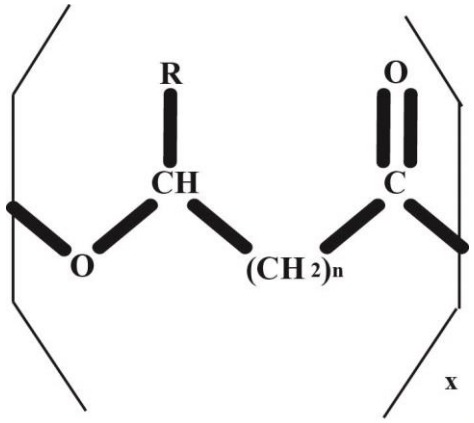


Figure 1.1. The general repeating unit of PHAs. Commonly the incorporated unit is a 3-hydroxyacyl monomer where n=1; however 4- or 5-hydroxyacyl monomers (n=2 and n=3 respectively) are sometimes incorporated. The R-group can be hydrogen, a methyl group, or longer carbon chains which are not necessarily saturated (Madison, et al., 1999).

The monomer specificity of PHA synthase depends on the class of synthase and the monomer biosynthesis, which varies based on species and substrate. In this manner, general repeating units with variances among the backbone length and side-chain composition are created. A PHA polymer can be classified by the monomer chain lengths, which are defined as short-chain-length (scl) or medium-chain-length (mcl) PHAs. Scl-PHAs consist of 3-5 carbon units, while mcl-PHAs have 6-14 carbon units (Lee, 1996). Long-chain-length (lcl) monomers are classified as anything longer than a 14 carbon chain monomer subunit (Steinbüchel, et al., 1992). The majority of PHA studies involve scl- and mcl-monomers, but rare incorporation of lcl-monomers has been reported (Nagamani, et al., 2011). The side-chain can be chemically modified and has been reported to be aromatic, halogenated, epoxidized, or branched in some cases (Madison, et al., 1999). Using the above mentioned variability, PHA polymers can be produced to replace various types of conventional plastics.

1.3 Biosynthesis of Polyhydroxyalkanoate

The following information will provide insight into the common pathways for obtaining different PHA monomers, which along with the substrate specificity of the species' PHA synthase, will ultimately determine the composition of the final PHA polymer. PHA accumulating microorganisms can be grouped by their PHA synthase gene, which is essential as it polymerizes the monomers and is quite variable among the different classes of this enzyme.

1.3.1. Pathways and the importance of the *phaC* gene

The production of PHA occurs using a multitude of enzymes to produce the hydroxyacyl monomers from the given substrate. Depending on the microorganisms'

biosynthetic routes, the resulting polymer composition can vary between small or medium chain-length, and could be uniform or have a mixed monomer composition. Scl-PHAs, such as PHB, are prevalent among most microorganisms. Production can be achieved via several biosynthetic routes starting with carbohydrate substrates or fatty-acid β-oxidation depending on the microorganism. When growing in the presence of excess carbohydrates such as glucose, the substrate enters glycolysis which eventually becomes acetyl-CoA, at which point it can be diverted away from respiration to be stored as PHA.

Only three enzymes are required for the conversion of acetyl-CoA to PHB. The first enzyme, *phaA*, is a β-ketoacyl-CoA thiolase, which converts acetyl-CoA to acetoacetyl-CoA. The second enzyme, *phaB*, is an acetoacetyl-CoA reductase. This yields 3-hydroxybutyrate which is then finally polymerized by the third enzyme a PHA synthase (*phaC*) (Peoples, et al., 1989). Some microorganisms are capable of producing PHA straight from alkanoic acids or from lipids provided as substrates. In the case of scl-PHA monomers, four to six carbon fatty acids are converted into respective length 3-hydroxyacyl monomers by an (R)-specific enoyl-CoA hydratase (*phaJ*). These monomers are then utilized by the PHA synthase to produce PHA (Fukui, et al., 1998).

Mcl-PHA production is linked to three main pathways. When grown on lipid substrates PHA can be produced and then stored via the β-oxidation pathway. When grown on carbohydrates it is possible to produce mcl-PHA through fatty acid *de novo* synthesis. Finally, acetyl-CoA can be condensed directly into mcl-monomers (Hoffmann, et al., 2000). When growing on fatty acids, the substrate is converted to shorter chains releasing acetyl-CoA for respiration. These shortened chains can be modified into a 3-

hydroxyacyl (3-HA) for PHA accumulation in several fashions. First, an epimerase can use S-3-hydroxyacyl-CoA from the β-oxidation cycle and convert its configuration to yield an R-3-hydroxyacyl-CoA which is a suitable monomer for the PHA synthase (Mittendorf, et al., 1998). Secondly, an enoyl-CoA hydratase (*phaJ*), as seen for the production of scl-monomers, modifies six to sixteen carbon enoyl-CoA from the β-oxidation cycle into suitable 3-HA monomers (Madison, et al., 1999). Lastly, a set of ketoacyl-CoA reductases are capable of using β-oxidation cycle intermediates to form 3-HA monomers. Two such reductases, RhlG and FabG, can independently covert 3-ketoacyl-CoA into R-3-hydroxyacyl CoA (Ren, et al., 2000).

The presence, substrate affinities and enzyme kinetics of any or all of these enzymes will determine the relative amount of mcl-monomers available for polymerization (Madison, et al., 1999). When given a carbohydrate substrate some microorganisms are able to produce PHA via the fatty acid *de novo* synthesis pathway. As 3-hydroxyacyl-ACP intermediates become available through fatty acid synthesis, a (R)-3-hydoxyacyl-ACP:CoA transacylase (*phaG*) converts it into 3-hydroxyacyl-CoA for PHA production. (Rehm, et al., 1998).

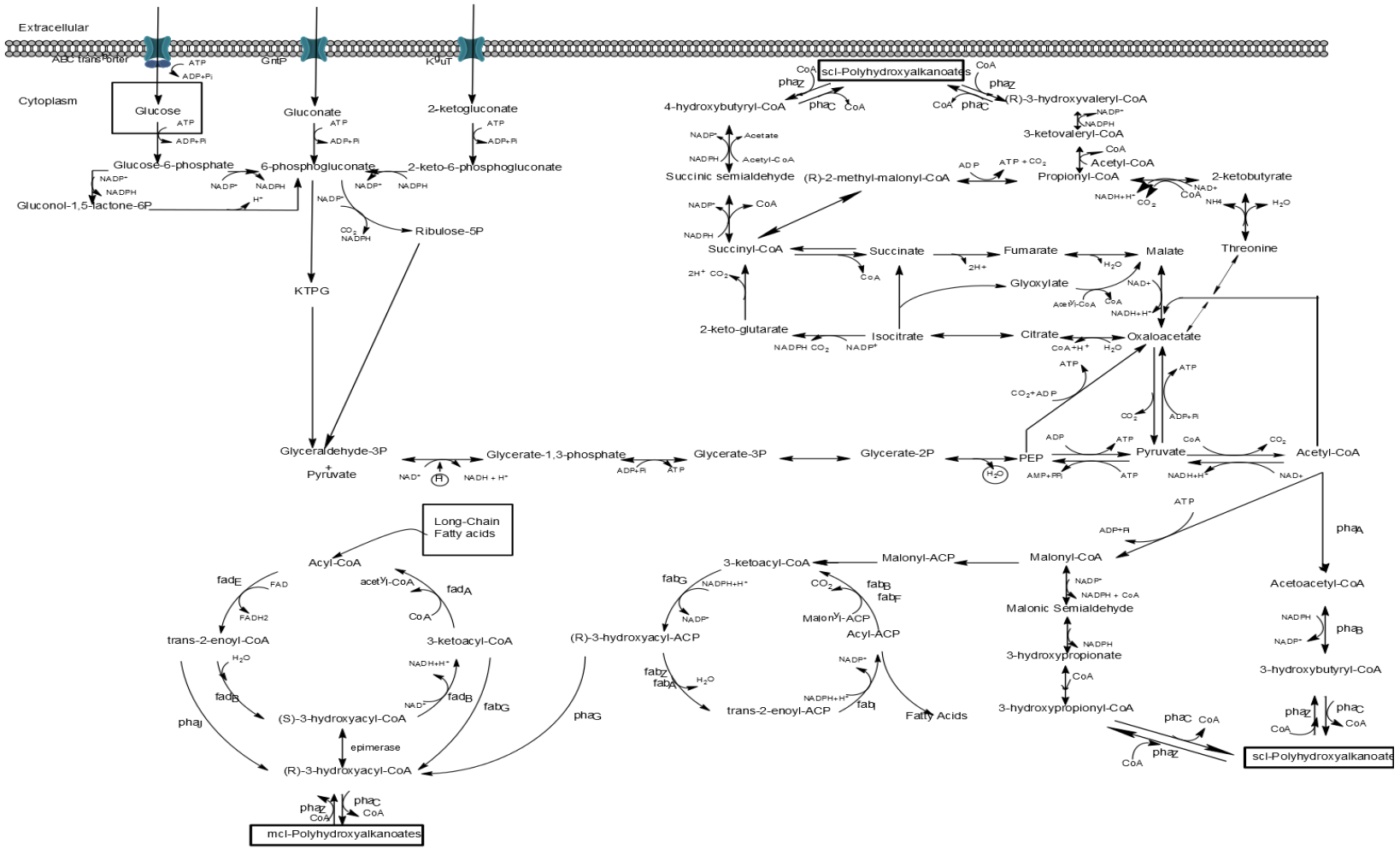


Figure 1.2. Pathway of PHA production starting with carbohydrates or long-chain fatty acids. (Adapted from various sources: Fu et al., 2012; Andreessen et al., 2010; Lehninger et al., 1993; Vakentin & Denis, 1997.)

The PHA pathways illustrated in Figure 1.2 depicts the simplest pathways to obtain the common hydroxyalkanoate monomers. However, depending on the organism and substrate provided, countless other directions may be taken and other monomers formed. There are also factors to PHA production that are not seen in the pathway. Table 1.1 outlines the known enzymes involved in the production of PHA, some of which have been highlighted in the above pathway, and include regulating enzymes involved in PHA kinetics and stability.

While the aforementioned pathways illustrate the various ways of obtaining monomers, it is the PHA synthase that is essential. In all reported cases of PHA synthesis, a PHA synthase is responsible for the polymerization of the monomers. In this manner, the PHA synthase and its respective gene (*phaC*) are indicators of the presence of PHA pathways, and the enzyme is used to help classify microorganisms based on their PHA accumulation.

Table 1.1. The genes associated with PHA synthesis and accumulation of their products.

Gene	Product
phaC	PHA synthase (Madison, et al., 1999)
phaE	Subunit of PHA synthase in Class III (Rehm, 2003)
phaR	Subunit of PHA synthase in Class IV (McCool, et al., 2001)
phaA	B-ketothiolase (Madison, et al., 1999)
phaB	Acetoacetyl-CoA reductase (Madison, et al., 1999)
phaZ	PHA depolymerase (Huismann, et al., 1991)
phaP	Phasins (McCool, et al., 2001)
phaQ	Regulation of phasins (Lee, et al., 2004)
phaI, phaF, phaD	Regulators (Klinke, et al., 2000)
phaG	Hydroxydecanoyl-ACP-CoA transacylase (Link from Fatty Acid *de novo* synthesis to 3-HA-CoA) (Hoffmann, et al., 2000)
phaJ	Enoyl-CoA hydratase (Fatty Acid β-oxidation intermediate to 3-HA-CoA) (Vo, et al., 2008)
fabG, RhlG	Ketoacyl-CoA Reductases (Fatty Acid β-oxidation intermediate to 3-HA-CoA) (Qi. Q., 2001)

1.3.2 Classes of PHA accumulating microorganisms

There are four classes of PHA accumulating microorganisms, classified by their PHA synthases and whose general configurations are shown in Figure 1.3. Class I microorganisms produce scl-PHA polymers using a single sub-unit PHA synthase, and PHA synthase polypeptides range in size from 60-70 kDa (Rehm, 2003; Peoples, 1989; Qi, 2001). An example microorganism is given for each class of PHA synthase in the following. Refer to Figure 4.1 for other sequenced PHA producing microorganigms.

Although various pathways have been studied for scl-PHA accumulation, as described above, some of these microbes have PHA operons where *phaA*, *phaB*, and sometimes *phaC* are clustered together. These have been found in an operon often co-localized with other PHA regulatory genes (Rehm, B., 2003; Steinbüchel et al.,1992). The PHA synthase from the class I microorganism *Cupriavidus necator* (previously known as *Ralstonia eutropha* and *Alcaligenes eutrophus*) was found to have Cys-319, His-508, and Asp 480 that were directly involved in catalysis. These three conserved catalytic residues, based on mutagenesis experiments performed on conserved residues were identified through the sequence alignment of several class I microorganisms (Jia, et al., 2001).

Class II microorganisms generally consists of species in the genus *Pseudomonas* and produce mcl-PHA polymers. These microbes have a single polypeptide PHA synthase of approximately 60-65kDA (Rehm, 2003). However, they often contain two PHA synthase genes, *phac1* and *phaC2*, which are 50-60% similar and both polymerize medium-chain-length monomers. These genes are found in an operon typically separated by *phaZ*, the PHA depolymerase. Other regulatory genes associated with PHA production

can be co-localized with this operon as well (Madison, et al., 1999). Recombinant *Escherichia coli* expressing either *phaC1* or *phaC2* of *Pseudomonas oleovorans* demonstrate that both PHA synthases have similar substrate specificities and polymer composition. When *phaC1* was solely expressed, it produced polymers of greater molecular weight then when *phaC2* was solely expressed. Recombinant *E. coli* expression the *phaC1* gene also produced polymers of greater molecular weight than the wild-type *P. oleovorans* (Matsusaki et al. 2000; Qi et al., 1997). Although it can be generally said that Class II microorganisms produce medium-chain-polymers, evidence exists of scl-monomer and mcl-monomer co-polymers. The PHA synthases of *Pseudomonas* sp.61-3 were cloned into *C. necator* and shown to produce such copolymers depending on which substrate was provided (Matsusaki, et al., 1998). The class II catalytic mechanism has similar conserved catalytic residues, but remains unique from other classes of PHA synthases. Mutagenesis experiments of *P. aeruginosa* identified four catalytic residues: Cys-296, Asp-452, His-453, and His-480. The neighbouring histidine residues seem to substitute for one another since the knock-out of only one barely affects enzyme activity. The knock-out of the cysteine residue considerably affects activity although some activity remained which is inconsistent with Class I or Class III mechanisms. In the case of Class II PHA synthases, the knock-out of the aspartate residue halted activity and appears to be the residue directly involved with catalysis (Amara, et al., 2003). The difference in catalytic mechanisms from other classes is vindicated due to the major substrate differences, in the way of Class II microorganisms producing mcl-PHA.

Class III microorganisms differ from the above classes in that their PHA synthase is a hetero-dimer. The polypeptides of the two subunits are both around 40 kDa in size and are encoded by the genes *phaC* and *phaE*. The Class III *phaC* gene shares some homology with the *phaC* genes of Class I and Class II, whereas the *phaE* genes are significantly different (Aneja, et al., 2009). These two genes are found together in the genome, with *phaE* being encoded up-stream of *phaC* and often located on the complementary strand to *phaA* and *phaB* (Madison, et al., 1999). Class III microorganisms have typically been reported to produce scl-PHA, although in a study in which kanamycin selection was placed on a PHB negative mutant *R. eutropha* containing the *phaE-phaC* of *Allochromatium vinosum,* production of mcl-PHA occurred. This suggests that this Class III PHA synthase is capable of polymerizing mcl-hydroxyacyl monomers if provided by the host (Aneja, et al., 2009).

The catalytic mechanism of Class III PHA synthases has been likened to the Class I PHA synthases due to their similar substrate specificities and conserved residues (Yuan, et al., 2001). The conserved catalytic residues which were identified through mutagenesis experiments on Class III microorganism *A. vinosum* were Cys-149, His-331 and Asp 302; the same motif found in Class I PHA synthases (Jia, et al., 2001). Class I and III are both hypothesized to form a catalytic dyad of cysteine and histidine where the cysteine directly acts in catalysis (Jia, et al., 2000). Due to the likeness of Class I and Class III catalytic mechanisms, the above motif appears responsible for the polymerization of scl-hydroxyacyl monomers despite the major physical differences between the synthases.

Class IV microorganisms belong to the *Bacillus* genus and were once lumped in with Class III microorganisms because they both have two polypeptide sub-units and

produce scl-PHA. The genes encoding the polypeptide subunits are *phaC and phaR*, which are 40 and 22 kDa, respectively. In *B. megaterium*, the synthase is encoded in an operon along with *phaB* and other supporting genes as *phaR-phaB-phaC* (Rehm, et al., 2002). The *phaC* gene showed most homology to synthases of Class III, and like the class III *phaE* gene, the *phaR* gene showed no homology to other synthases. The PHA synthase of *B. megaterium* requires the PhaR sub-unit for PHA production which is a characteristic not seen with the PhaE subunit of Class III synthases (McCool, et al., 2001).

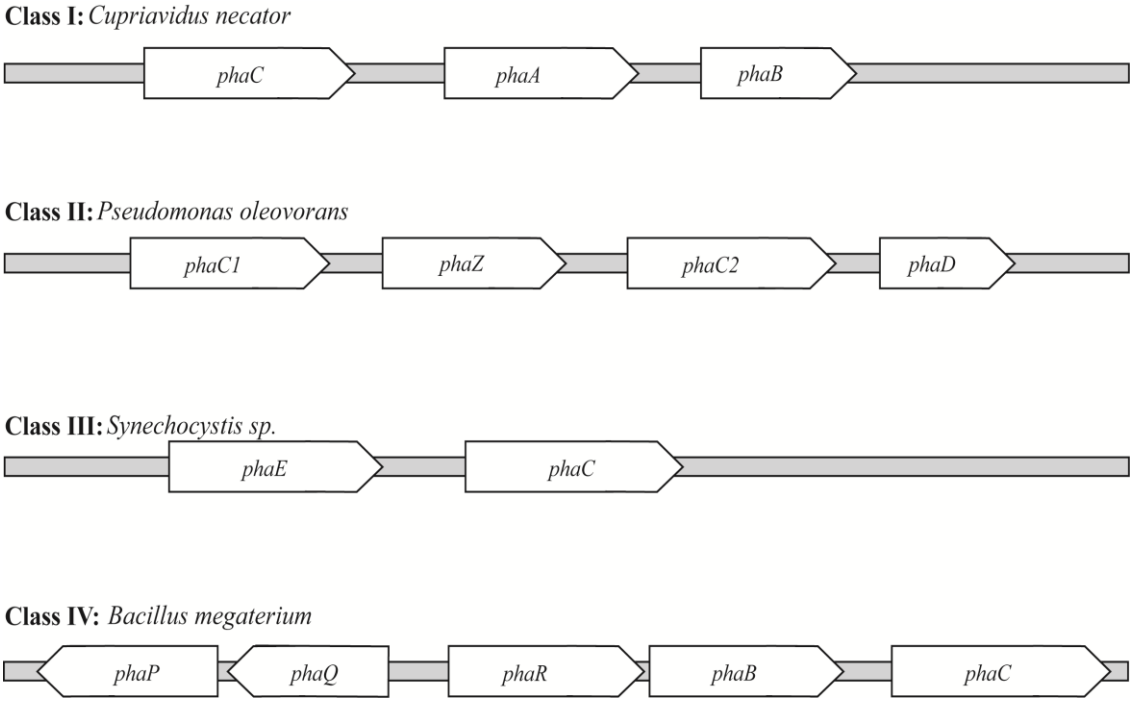


Figure 1.3. Operon configurations for representatives of the four classes of PHA accumulating microorganisms. (Adapted from Madison et al., 1999; Rehm, 2003).

1.4. Detecting PHA accumulating microorganisms

The following describes the techniques for detecting PHA accumulating microorganisms and the analytical methods used to determine the average molecular weight, monomer composition and percent cell dry weight of the polymer product along with detecting and measuring the activity of the PHA synthase.

1.4.1. Colony visualization, microscopy, staining procedures and lipid selection

The simplest form of detecting PHA accumulation is the observation of colony morphology changes as the microorganism reaches stationary phase on a PHA inducing medium. As first observed with *Alcaligenes eutrophus* (*Cupriavidus necator*), a *phaC*- mutant will be opaque, whereas the wild-type appeared white under the same conditions (Peoples, et al., 1989). This method is best used as a quick indicator for PHA production in a species, but requires that the morphological differences be characterized in advance. This method is consequently ineffective for screening for new species of PHA accumulators. The cells can alternatively be viewed using an electron microscope, in which the PHA granules appear as large white clumps (Pantazaki, et al., 2003). Electron microscopy would not be a viable method of screening for PHA accumulation microorganisms due to the time and cost.

An alternative method for visualizing the accumulation of PHA is to dye the cells with a lipophilic dye. Lipophilic dyes such as Sudan Black, Nile Blue, and Nile Red bind to the hydrophobic regions in the cell, highlighting the cell wall and PHA granules. The dye is primarily added to the cells after being grown in PHA-inducing medium and viewed with a light- microscope for Sudan Black, or a fluorescent-microscope in the case of Nile Blue or Nile Red (Kitamura et al., 1994; Kranz et al., 1997). Nile Red can also be

added into the medium and plated prior to cell growth. Upon colony formation, the plate can be quickly irradiated to distinguish PHA producing colonies by their fluorescence (Spiekermann, et al., 1999). In comparison to other qualitative measurements, this can be a more accurate indicator of PHA accumulation and can screen unknown species for production of PHA.

Microorganisms that produce PHA can also be detected using a lipid selection process. The cells are grown on a plate with a high concentration of lipids. The high lipid concentration is toxic to many cells; however, many PHA producers can grow on such a medium and convert the lipids to PHAs, which are stored intracellularly. The medium surrounding the colonies will change consistency as the lipid is withdrawn by the cells, thus acting as an indicator that PHA accumulation may be occurring (Kranz, et al., 1997). The lipid selection protocol can be used to screen for new PHA-accumulating microorganisms however there is the chance of false positives and false negatives.

1.4.2. Polymerase Chain Reaction using primers designed for the *phaC* gene

As previously described, the PHA synthase enzyme is used to classify PHA accumulating microorganisms, as it is essential in the production of PHA and polymerizes the hydroxy-acyl monomers. Thus, the presence of the *phaC* gene can be used as an indicator for the production of PHA. Alignments of *phaC* genes can be used to design primers for the screening of PHA synthesis in a variety of microorganisms using polymerase chain reaction (PCR) amplification of the target genes. This strategy, where primers designed for the *phaC* gene were used for PCR using genomic DNA, has been employed to detect PHA producing microorganisms in soil samples (Sheu, Wang & Lee, 2000).

The difficulty with this technique lies in the strategy taken to identify the *phaC* gene, and thereby how primers are designed. As mentioned above, there are four classes of PHA accumulating microorganisms with differences in everything from size, number of sub-units and catalytic activity. This great variety results in major dissimilarities among *phaC* genes from organism to organism, which only increases when comparing one PHA Class to another. This makes primer design very difficult, and depending on the nature of the experiment and the microorganisms of interest, a variety of approaches could be taken. There are examples of broad-range primers which have the potential to detect *phaC* from any class, other primers have been designed based on PHA classes of closer similarity or of a single PHA class to a subset of microorganisms within a PHA class (Romo et al., 2007; Solaiman et al., 2005; Hai et al., 2004, Hein et al., 2001). Each primer design approach has its advantages and disadvantages, from broad-range primers that may produce many false-positives, to more specific designs which won't amplify the other classes of PHA accumulating microorganisms. The advantage of using PCR is that it allows you to screen many microorganisms at the same time fairly quickly, and the product could be sequenced for verification. This also allows for the detection of the PHA machinery without having to troubleshoot for satisfactory PHA inducing medium. The major disadvantage lies in the possibility of false positives and false negatives.

1.5 Analytical Methods for Characterization of PHAs

1.5.1. Methanolysis and gas chromatography

The process of methanolysis breaks down the PHA polymer into its respective monomers by chemically cleaving the ester bond formed by the PHA synthase. After the process is complete, the solution can be injected into a gas chromatograph (GC) to

measure the concentration of the various monomer sizes. Using this data, the percent of PHA to cell dry weight can be calculated along with the relative percent composition of monomer size in the polymer. The advantages of this method are that it is an accurate quantitative measure for PHA accumulation and the type of polymer can be inferred (Braunegg et al., 1978; Timm et al., 1990). The main disadvantage of this method is that the integrity of the polymer is removed, rendering no way to determine the polymer's average molecular weight or the configuration of the methods. This method requires expensive instrumentation and is quite laborious.

1.5.2. Gel Permeation

To determine the average molecular weight of the PHA polymer it must first be extracted from the cell, in this case typically by the dissolution in chloroform. The polymer can then be run on the chromatography column to separate the molecules by size. The larger molecules will travel faster through the column than the smaller molecules which are hindered by the pores in the column, thus the molecular weight can be obtained by comparing to retention time of known standard sizes (Pantazaki, et al., 2003).

1.5.3. SDS-PAGE and Spectrophotometric assay

Sodium dodecyl sulphate polyacrylamide gel electrophoresis is a common technique for the separation of proteins. To determine where the PhaC enzyme is located, a western blot with an anti-PhaC antiserum and a secondary detection antibody can be performed. This would require developing a PhaC antibody based on a known protein sequence (Langenbach, et al., 1997). Alternatively the gel can be cut into fractions and tested for PHA synthase activity using the spectrophotometric assay to determine the location of the

PhaC enzyme. The spectrophotometric assay operates under the principal that as Coenzyme A is released from the hydroxyalkanoate-CoA monomers being polymerized it reacts with dithionitrobenzoic acid and can be measured at 412nm (Valentin, et al., 1994). The assay can be performed continuously or discontinuously (Müh, et al., 1999). This method is a new alternative to the earlier radiometric assay and is now commonly preferred for the determination of PHA synthase activity (Lowry, et al., 1951). Despite being a quicker and easier method than the radiometric assay, the only disadvantage is the readings are less accurate at low protein concentration (Pantazaki, et al., 2003).

1.6. Thermophilic PHA production

One consideration for improving the fermentation of PHA by microorganisms is to use a thermophilic bacterium as a platform for the process in place of the commonly used mesophiles. Polymerase chain reaction requires a thermostable DNA polymerase, originally isolated from *Thermus aquaticus*, which exemplifies the importance of thermo-tolerant enzymes. There may be several advantages to fermenting at higher temperatures, including the isolation of a thermo-tolerant suite of PHA enzymes, and the production of a more thermo-tolerant PHA product which would ultimately affect the quality of this plastic substitute. A comparison of the *phaC* genes from a thermophilic and mesophilic *C. necator* strain showed an overall homology of 81% with greater differences at the terminal ends. Creating and testing chimeric genes, a series of mutations were determined which increased the thermo-stability and activity of the PHA synthase in the mesophilic *C. necator* H16 (Sheu, et al., 2012). The data presented in this experiment validates the investigation into thermo-tolerant PHA synthases. Using a thermophilic PHA accumulating bacterium may allow for coupling to other reactions such as lignocellulosic

digestion and provide the opportunity for PHA production second or third generation, non-food, feedstocks. Working with thermophiles has proven to alleviate operating costs due to increased diffusion rates, increased ionization rates, increased solubility of chemicals at high temperatures, a decreased chance of contamination and may avoid heating and cooling cycles which are often required with mesophilic fermentations (Ibrahim, et al., 2010).

A handful of thermophilic microorganisms have been isolated which accumulate PHA, in most cases having an upper threshold of approximately 55 °C and showing a drop of production when fermented at its threshold. Table 1.2 illustrates the production of PHA among thermophilic microorganisms in comparison to some well characterized mesophiles. The literature suggests, however, that thermophilic microorganisms may be able to match the PHA production specifications of mesophiles. Of note is the *Thermus thermophilus* HB8 species which reportedly produces PHA at extreme thermophilic temperatures with a medium chain length product similar to mcl-PHAs reported from *Pseudomonas* species.

Table 1.2. Conditions and results of PHA production among various different thermophiles. A mesophilic example of class I (*R. eutropha)*, class II (*P. putida*), class III (*Synechocystis* sp.) and class IV (*B. megaterium)* have been provided for reference

PHA Accumulating Microorganism	Temperature	Substrate	Percent PHA of CDW[1]	Average Molecular Weight	Most abundant monomer
Thermus thermophilus HB8 (Pantazaki, et al., 2011)	75^{0}C	Gluconate	35%	480,000 g/mol	3-hydroxydecanoate (C10)
Caldimonas taiwanensis	55^{0}C	Starch	71%	-	3-hydroxybutyrate (C4)
Pseudomonas sp. SG4502 (Satoh, et al., 2011)	45^{0}C (55^{0}C)	Dodecanoate	36.1% (24%)	70,800 g/mol	3-hydroxyoctanoate (C8)
Chelatococcus sp. MW10 (Ibrahim, et al., 2010)	50^{0}C	Glucose	73%	-	3-hydroxybutyrate (C4)
Bacillus sp. INT005 (Tajima, et al., 2003)	37^{0}C	Glucose	35.30%	525,000 g/mol	3-hydroxybutyrate (C4)
Ralstonia eutropha (Madison, et al., 1999)	30^{0}C	Glucose	90%	-	3-hydroxybutyrate (C4)
Pseudomonas putida (Madison, et al., 1999)	30^{0}C	Octanoate	37%	240,000 g/mol	Medium-chain-length
Synechocystis sp.UNIWG (Yew, et al., 2005)	28^{0}C	Acetate	14%	-	3-hydroxybutyrate (C4)
Bacillus megaterium DSM 32 (Faccin, et al., 2009)	30^{0}C	Sucrose	70%	-	3-hydroxybutyrate (C4)

[1]w/w% of Polyhydroxyalkanoate (PHA) weight versus total Cell Dry Weight (CDW)

1.7. PHA accumulation in Thermus thermophilus HB8

T. thermophilus Hb8 is the only recorded organisms to produce PHA above 60°C. The polymer product is a mixture of scl- and mcl-hydroxyalkanoate monomers when grown on gluconate or octanoate (Pantazaki, et al., 2003). The PHA synthase and B-ketothiolase enzymes have been isolated and tested for activity. The PHA synthase is unlike those of other organisms and its gene sequence remains to be elucidated (Pantazaki, et al., 2005). Primers designed for class II PHA synthase indicate that the PHA synthase would fall into this class (Papi, et al., 2008). Due to the unique nature of the PHA polymer and the production of PHA at extreme thermophilic temperatures, *T. thermophilus* HB8 is a novel organism for PHA production. *Thermus filiformis* is a filamentous, thermophilic bacterium isolated from hot springs in New Zealand (Hudson, et al., 1987). Although it is not known for its PHA accumulation, due to its relation to *T. thermophilus* HB8 and its robustness in the environment, it could be screened for similar PHA accumulation as a novel platform organism.

1.8. Objectives of this research

The objectives of this research are to identify thermophilic microorganisms which produce polyhydroxyalkanoate and screen for new thermophilic PHA producers. Candidates for thermophilic PHA accumulation are to have their optimum growth conditions and substrates characterized and ultimately tested for the accumulation of PHA. Bioinformatic analyses along with PCR designs for amplifying a possible phaC gene are to be conducted to identify and sequence any novel thermotolerant PHA synthase gene.

Chapter 2: Materials and Methods

2.1. Materials, microorganisms and incubation

All reagents and substrates for media production were obtained from Sigma-Aldrich and Fisher Scientific. The strain *Thermus thermophilus* HB8 27634 was obtained from the American Type Culture Collection (ATCC). The strains *Thermus filiformis* WRT, *T. thermophilus* NGM, and *T. thermophilus* MOK were isolated from New Zealand hot springs by Dr. Matthew Stott (a Senior Scientist, with the Extremophile Research Group, GNS Science Wairakei Research Centre, Wairakei Taupo, New Zealand) who kindly provided these strains. *Pseudomonas putida* LS46 was isolated from sewage sludge and used as a positive for Class II, mcl-PHA production (Sharma, et al., 2012). *Cupriavidus necator* H16 was used as a positive for Class I, scl-PHA production.

The strains were grown in 697 *Thermus* medium (Oshima, et al., 1974), which consisted of 8 g/L BBL™ Polypeptone™ peptone, 4 g/L yeast extract, and 2 g/L NaCl corrected to a pH of 7.0, and autoclaved for 20 minutes. The cells were incubated in culture tubes for 24 hours at their respective optimum temperatures, shaking at 120 rpm in an SWB25 shaking water-bath from Thermo scientific. An inoculum of 5% was transferred into fresh culture tubes or 125 mL Erlenmeyer flasks containing 697 *Thermus* medium or Ramsay's minimal medium depending on the experimental design. Ramsay's minimal medium consisted of 6.7 g/L Na_2HPO_4, 1.5 g/L KH_2PO_4, 1 g/L $(NH_4)_2SO_4$, 0.2 g/L $MgSO_4.7H_2O$, 60 mg/L $FeNH_4$Citrate, 10 mg/L $CaCl_2$ and 1 mL of 1000X stock of trace elements. The 1000X minerals stock contained 2.78 g/L $FeSO_4.7H_20$, 1.98 g/L $MnCl_2$, 2.81 g/L $CoSO_4.7H_20$, 0.17 g/L $CuCl_2.7H_20$, 1.47 g/L $CaCl_2.2H_20$, and 0.29 g/L

$ZnSO_4.7H_20$. (Ramsay, et al., 1991) Ramsay's minimal medium was supplemented with a desired carbon substrate and the entire solution was filter sterilized using Millipore Express™PLUS 0.22 μm filters into a 1000 mL Pyrex screw-top bottle. This was done to avoid the negative effects of the autoclave on the medium which otherwise impeded growth.

2.2. PHA composition determination by Gas Chromatography (GC)

To determine the monomer composition and percent cell dry weight (% dcw) of PHA accumulated within the bacteria, 10-30 mg of dried cells were measured and placed in a screw-cap vial. To this vial, 2 mL of 15% sulfuric acid in methanol and 2 mL of 1 mg/mL benzoic acid in chloroform were added, and then boiled in this vial for 8 hours. One (1) mL of distilled-deionized (di)H_20 was then added to the vial and the layers allowed to separate (Braunegg, et al., 1978). The organic layer was analyzed by gas chromatography (GC) using an Agilent 7890A GC system equipped with a HP-5 capillary column (30 m X 0.37 mm), a flame ionization detector, and a CTC Analytics CombiPal autosampler.

2.3. Fluorescent detection of PHA granules

To confirm the presence of PHA granules in viable cells, 0.1% (*m/v*) of a 0.5g/L Nile blue in ethanol solution was applied to 200 μL of culture and loaded into a 96-well plate. Fluorescence was measured using a Biotek Synergy 4 multimode micro-plate reader using an excitation wavelength of 550 nm and observation wavelength of 580 nm (Berlanga, et al., 2006).

2.4. Fluorescent microscopy image capture

Images of PHA accumulation in bacteria were captured by placing, 5 µL of the Nile blue stained cells (prepared in 2.3) on a standard microscopy slide. The cells were heat-fixed and viewed with a Nikon Eclipse T*i*–U inverted microscope using a green light filter.

2.5. Amplification of *16S* rDNA and *phaC* genes using Polymerase Chain Reaction

16S ribosomal (r)DNA genes were amplified by polymerase chain reactions (PCR) using universal primers, 928F and 336R, as well as custom designed primers (Table 2.1). Specific *16S* rDNA gene primers were designed based on *Thermus thermophilus* HB8. Primers for amplification of Class I *phaC* genes (Table 2.2) were designed by aligning the *phaC* genes from the following microorganisms: *A. caviae, A. hydrophila, A. ehrlichii, Azoarcus sp., Cupriavidus necator, C. violaceum, M. populi, M. radiotolerans, N multipartita, R. pomeroyi,* and *V. paradoxus.* Primers for amplification of Class II *phaC* genes were designed by aligning the *phaC* genes from the following microorganisms: *Pseudomonas aeruginosa, P. oleovorans,* and *P. putida.* Primers for amplification of Class III *phaC* genes were designed by aligning the *phaC* genes of the following microorganisms: *A. vinosum, A. prevotii, C. fritschii, D. autotrophicum, Magnetococcus sp., R. xylanophilus,* and *Synechocystis sp.* Primers for amplification of Class IV *phaC* genes were designed by aligning the following microorganisms: *Bacillus megaterium, B. anthracis, B. cereus, B. thuringiensis,* and *B. tusciae.* Primers were synthesized by AlphaDNA. RedTaq ReadyMix® was used in gene amplification. Primer concentrations of 1 µM to 10 µM and DNA concentrations from 97 ng/µL to 240.52

ng/µL were tested for gene amplification. Annealing temperatures were commonly 52 ^{0}C, however, gradients from 47 ^{0}C to 58 ^{0}C were used to optimize the amplification results. A 0.1-10 kb DNA Logic ladder was added to each gel. DNA concentrations were measured using a NanoDrop ND1000 UV-Vis spectrophotometer. Bands were excised and a gel extraction was performed using a Zymoclean™ Gel DNA recovery kit. Amplicon sequencing was performed by MacrogenUSA.

Table 2.1. Designed oligonucleotide primers for identification of *phaC* in *Thermus* sp. Class 3 primers were modified from previous designs (Hai, et al., 2004). Aligned to a) *T. thermophilus* HB8 b) *C. necator* B5786 c) *Azospirillum* sp. 8510 d) *P. putida* KT2440 e) *A. vinosum* DSM 180 f) *B. cereus* FRI-35

Primer Name	Sequence	Gene Target	Position
T.t.3-F	5'-GGCATTGTAGCACGTGTGTC-3'	*T.t. 16S*	280[a]
T.t.3-R	5'-TTAGGAATCTTCCGCAATGG-3'	*T.t. 16S*	1151[a]
Class I-F	5'-AACTTCSTKSYSACCAAYCCCGA-3'	Class I *phaC*	475[b]
Class I-R	5'-GTTCCAGWRSAGCAGRTCGAASG-3'	Class I *phaC*	1308[b]
STI-L	5'-GTTCCAGTCAGCAGGTCGAAGG-3'	Class I *phaC*	493[c]
STI-R	5'-ATCAACAAGTTCTACATCCTCGAC-3'	Class I *phaC*	1017[c]
Class II-F	5'-CCRCCNCAGATCAACAARTWCTA-3	Class II *phaC*	664[d]
Class II-R	5'-GACYTGATCTGGAAYTACTGGGT-3'	Class II *phaC*	1142[d]
Class III-F	5'-GTNTAYMTGMTYGAYTGGGG-3'	Class III *phaC*	746[e]
Class III-R	5'-TCRDADABCCAYTTBTYCAT-3'	Class III *phaC*	317[e]
Class IV-F	5'-GGTTATTGCATGGGTGGAAC-3'	Class IV *phaC*	623[f]
Class IV-R	5'-CTCCAATCGTCGGATACGTT-3'	Class IV *phaC*	27[f]

Table 2.2. Microorganisms and the corresponding ascension numbers for the genes for the alignment of the *phaC* gene for classes I-IV of PHA producers.

Class I Microorganisms	Class II Microorganisms	Class III Microorganisms	Class IV Microorganisms
Aeromonas caviae gi|2335048	*Pseudomonas aeruginosa* gi|115583796	*Allochromatium vinosum* gi| 118132678	*Bacillus megaterium* gi|294799901
Aeromonas hydrophila gi|20372707	*Pseudomonas oleovorans* gi|151441	*Anaerococcus prevotii* gi|256797400	*Bacillus anthracis* gi|229264291
Alkalilimnicola ehrlichii gi|114225560	*Pseudomonas putida* gi|24987239	*Chlorogloeopsis fritschii* gi|18644659	*Bacillus cereus* gi|218534755
Azoarcus sp. gi|119668705		*Desulfobacterium autotrophicum* gi|224367124	*Bacillus thuringiensis* gi|49328240
Cupriavidus necator gi|58045496		*Magnetococcus sp.* gi|117607074	*Bacillus tusciae* gi|295410288
Chromobacterium violaceum gi|3820508		*Rubrobacter xylanophilus* gi|108764099	
Methylobacterium populi gi|179342784		*Synechocystis sp.* gi|47118304	
Methylobacterium radiotolerans gi|170652972			
Nakamurella multipartita gi|258553496			
Ruegeria pomeroyi gi|5667665			
Variovorax paradoxus gi|239799596			

Chapter 3: Characterization of Extreme Thermophilic Growth and PHA Accumulation using *Thermus sp.*

3.1. Introduction

The following sets of experiments were designed to optimize growth of *Thermus* sp. under nitrogen-limited conditions to ultimately measure PHA production in these strains. Due to reports on PHA production by *Thermus thermophilus* HB8, it was used a positive for thermophilic PHA production in order to improve methods of detecting PHA in the three cultured strains (MOK, NGM, and WRT) of *Thermus filiformis* (Pantazaki, et al., 2003).

3.2. Experimental design and Methods

3.2.1. Optimum growth conditions

Optimum growth temperatures for the three strains of *T. filiformis* were determined by monitoring the change in optical density (OD_{600}) while incubated in three water baths at different temperatures. The cells were grown in triplicate culture tubes containing 5 mL of Ramsay's minimal medium supplemented with 1% sucrose.

A variety of carbon sources were used as substrates for *T. filiformis* NGM in Ramsay's minimal medium to determine the best possibility for future testing for PHA accumulation. The first experiment was designed to test sucrose, glucose, lactose, sodium gluconate, and fructose. Triplicate culture tubes containing 5 mL of minimal medium supplemented with 1% of these substrates were measured at 600 nm for optical density at time-points spanning almost four days. The inoculum was a 5% allotment from 697 *Thermus* medium. This was followed with a second experiment which compared the

effects of filter sterilizing the entire medium versus autoclaving using sucrose, glucose and sodium gluconate using the same methods.

The growth of *T. thermophilus* HB8 was characterized using different substrates in the same manner as *T. filiformis* NGM was characterized above. In this case, six different conditions were used to compare the growth in nitrogen rich medium (697 *Thermus* medium) or nitrogen-limited medium (Ramsay's minimal medium containing 1 g\L ammonium). Sucrose, octanoic acid, and sodium gluconate were used as supplemental carbon sources to compare the growth on these substrates to growth on nitrogen-rich medium and minimal medium with no added carbon source. The inoculum was 5% from 697 *Thermus* medium.

3.2.2. Growth and PHA production comparing nitrogen-limited to nitrogen-excess conditions

To test for PHA production by *T. thermophilus* HB8 in nitrogen-rich conditions, triplicate culture tubes of 697 *Thermus* medium were incubated and removed after t = 0, 12, 18, 22, 26, 30, 40, and 48 hours, along with a negative control at each time-point. More frequent time-points were taken between hour 18 and 30 since PHA production was expected to occur predominantly in this time frame. The growth was profiled using optical density reading at 600 nm and then the triplicates were combined, centrifuged at 4195 X g, and the cells were dried and weighed. The dried cells were prepared for GC analysis of PHA composition by methanolysis as per section 2.2. This experiment was repeated twice.

To determine the optimum concentration of nitrogen for good biomass and PHA production, *T. filiformis* WRT was grown in triplicate tubes of Ramsay's minimal

medium containing 1% sucrose. Three different ammonium sulfate concentrations were applied: 5.0 g/L, 1.0 g/L and 0.2 g/L. Seven time-points up to 91 hours were taken and their absorbance at a 600 nm was recorded, then the samples were prepared for residual ammonium analysis using flow injection analysis (Diamond, 2001).

As described in the results of section 3.3.1, our strain of *T. thermophilus* HB8 was not capable of growing in Ramsay's minimal medium with 1% sodium gluconate, contrary to previous reports (Pantazaki, et al., 2003). The interpretation of the methods in that paper suggest that cells growing in nitrogen-rich 697 *Thermus* medium was used to inoculate a minimal medium with 1.5% sodium gluconate to promote PHA production. Since we could not grow *T. thermophilus* HB8 on gluconate and early attempts to extract PHA failed, the medium and inoculum conditions used in the paper by Pantazaki et al. (2003) were reproduced. Furthermore, a new hypothesis was developed for the production of PHA. The results by Pantazaki et al. (2003) described two curious behaviours regarding the growth and PHA production on *T. thermophilus* HB8 on both octanoic acid and sodium gluconate. The cells only reached an optical density slightly above one, which is lower than might be expected. Our previous results indicate that grown in shaking flasks *T. thermophilus* HB8 can grow well above an optical density of three. Secondly, PHA was detected in high amount after 20 hours but depleted just as quickly shortly after. This finding is not consistent with the behaviour of PHA accumulating microorganisms grown in carbon-excess conditions, which typically store the polymer for much longer as it metabolizes the readily available substrate first. Taken together, it seems that *T. thermophilus* HB8 was alternatively growing on carry-over from the rich inoculum and not using sodium gluconate as a substrate for growth. We suggest

that as the remaining peptones became scarce the cells began storing PHA, but when completely depleted of soluble substrate the PHA polymer was metabolized for energy.

To test this hypothesis, conditions were replicated from the Pantazaki et al. (2003) experiment. In this case, a 5% inoculum from 697 *Thermus* medium grown cells was transferred into shaking flasks of minimal medium with 1.5% sodium gluconate. Two conditions of varying ammonium sulfate concentrations were compared (1 g/L versus 5 g/L) to determine whether growth was being limited by minimal nitrogen. Optical density was measured every 24 hours. After 48 hours, a 5% inoculum of these flasks was transferred into flasks of identical medium to measure growth without 697 *Thermus* medium carry-over. A 5% inoculum was also transferred into 697 *Thermus* medium to ensure the inoculum was still viable. Growth was measured by optical density at 600 nm. Flasks were incubated at 73^0C in a shaking water bath.

3.2.3. Growth and PHA production comparing oxygen availability in rich medium

The interpretation of previously obtained fluorescent microscopy images of *T. thermophilus* HB8, which had been grown in 697 *Thermus* medium and stained with Nile Blue, led to the hypothesis that perhaps oxygen availability could play a role in PHA production under these conditions. The observation that growth and fluorescent images were improved when incubated in shaking baffled flasks as opposed to test tubes led to the above interpretations. To address this hypothesis, thirty test-tubes containing 5 mL 697 *Thermus* medium were inoculated with *T. thermophilus* HB8 with ten more tubes remaining sterile as negative controls then placed into a test tube rack and incubated in a 73 ^{0}C shaking water bath. Half of the tubes were placed standing straight in the rack, the other half of the tubes were placed on approximately a thirty degree angle to improve

oxygen transfer into the medium. Three tubes and a negative control tube from each the tilted and standing tubes were removed after t = 0, 9.5, 17.5, 21.5, and 25.5 hours. Each tube had its optical density measured at 600 nm, fluorescent detection of PHA granules was carried out as described in chapter 2.3, fluorescent images were captured as per chapter 2.4, and the pH was measured. This experiment was carried out twice.

Growth of *Thermus sp.* was tested under anaerobic conditions by reducing 10 mL tubes 697 *Thermus* medium with 0.1 mL of a 200 mM Na_2S solution followed by gassing and degassing (1:4 min) stoppered tubes and inoculating from an aerobic culture. The experiment was set up with ten triplicate time-points, each with its own negative. Tubes were incubated in a 69 ^{0}C shaking water bath. The experiment was repeated twice.

3.2.4. Measuring for PHA production by transferring viable cells into PHA-inducing medium

To further test the hypothesis that *Thermus* sp. could produce and store PHA under nitrogen- limiting conditions, cells were to be grown until the beginning of stationary phase in a rich medium then transferred into nitrogen-deficient medium. This bypassed any previous issue with obtaining growth on minimal medium with select substrates such as octanoic acid. This process also ensured that no nitrogen was present when the cells reach a suitable biomass. In this manner, we hoped to determine whether *Thermus* sp. is capable of producing PHA.

Cells were first grown in 12 flasks of 50 mL of rich 697 *Thermus* medium for 24 hours to achieve high biomass. A small sample was taken to measure the optical density. The cells were then spun down at 4,195 x g for 5 minutes; the supernatant was removed using a biosafety cabinet and sterile technique applied to wash the cells once in 50 mL of

nitrogen-deprived Ramsay's minimal medium supplemented with glucose, octanoic acid, or sodium gluconate. The cells were once more centrifuged at 4,195 x g then transferred into six 50 mL flask of the same Ramsay's medium and further incubated at their respective optimum temperatures. Three flasks of each were removed after 10 hours, the final three flasks of each removed after 20 hours. Each flask was sampled for fluorescent microscopy images. After removing the final flasks, a small sample was taken to measure optical density and the cells were spun-down as above. The supernatant was removed then dried and cell dry weight was measure. The dried cells were then transferred into screw-cap vials for methanolysis as described in section 2.2. for GC analysis.

This experiment was performed twice with *Pseudomonas putida* LS46 as a positive for PHA production. The first of the two was performed without triplicates using glucose and octanoic acid supplemented Ramsay's medium and 697 *Thermus* medium. The second experiment had six flasks of 697 *Thermus* medium transferred into gluconate supplemented Ramsay's, the other six flasks into octanoic acid supplemented Ramsay's. These exact conditions were also applied to *T. filiformis WRT*. The experiment was also performed with *T. thermophilus* HB8 to test the reputed thermophilic PHA production in this organism, in which six flasks were transferred into glucose supplemented and the other six transferred into octanoic acid supplemented flasks. The choice of substrates were chosen to test for PHA production with either the *de novo* synthesis pathway (glucose or gluconate) or the β-oxidation cycle (octanoic acid) while using substrates that were shown to work with *T. thermophilus* HB8 (Pantazaki, et al., 2003).

3.3. Results

3.3.1. Optimum growth conditions

The optimum growth temperatures were determined to be approximately 69 ^{0}C for *T. filiformis* NGM, 75 ^{0}C for *T. filiformis* WRT, and 67-75 ^{0}C for *T. filiformis* MOK. All *Thermus sp.* strains used grew easily on 697 *Thermus* medium medium however nitrogen-rich medium are typically undesirable for PHA production. Growth needed to be optimized using a nitrogen-limited Ramsay's minimal medium. Initial attempts to characterize *T. filiformis* NGM showed growth when supplemented sucrose or sodium gluconate. However, no growth occurred on glucose, lactose or fructose. Glucose, lactose and fructose cause the medium to turn brown in the autoclave which has adverse effects on growth. The medium with sucrose or sodium gluconate only started turning brown after several days of thermophilic incubation. To address these negative effects, particularly seen with reducing sugars, the growth profile (Figure 3.1) was repeated comparing autoclaved medium to filter sterilization of the substrates.

The data suggest that in the first two days of growth, there was only a statistically significant difference in growth when comparing the autoclaved glucose, which instantly turned brown and did not seem to grow, to the rest of the substrates. Moderate growth was achieved on all filtered substrates and none of the filtered media turned brown over the course of inoculation, whereas the autoclaved sucrose and sodium gluconate eventually turned brown. The moderate growth in the first two days was seemingly from the inoculum carry-over whereas sucrose was the only substrate that was seemingly utilized by *T. filiformis* NGM. Despite the fact that growth may only have occurred due

to carry-over carbon, all further experimentation for PHA production with Ramsay's minimal medium was filter sterilized due to these findings.

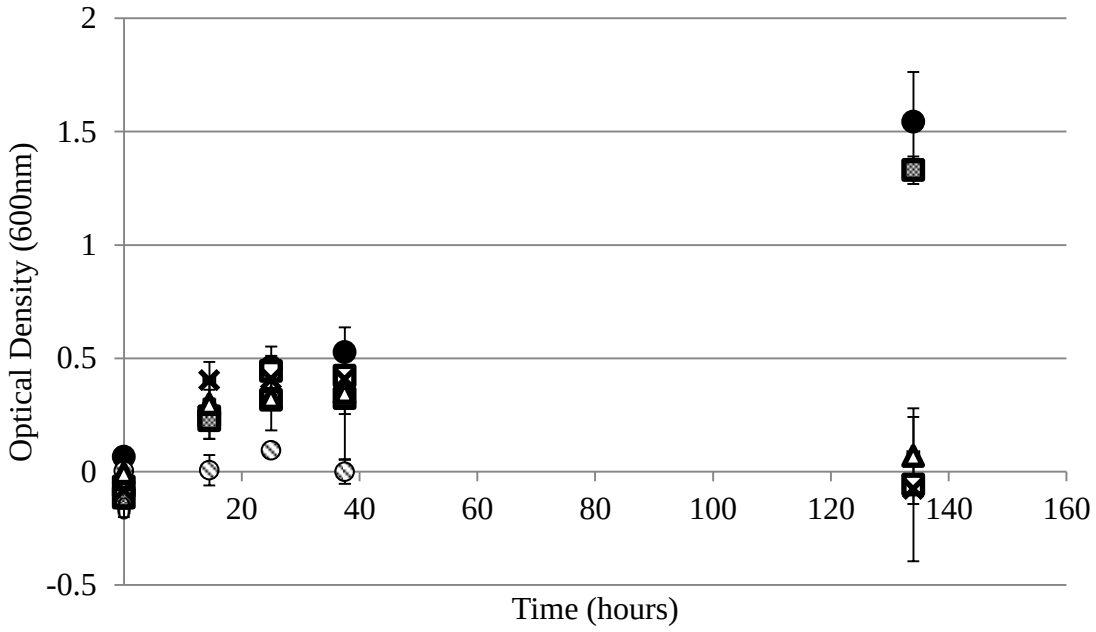


Figure 3.1. Growth profile of *T. filiformis* NGM comparing the effects of filter sterilizing the substrates separately compared to autoclaving the entire medium. Autoclaved medium containing sucrose (●), glucose (▨), and sodium gluconate (■) were compared against filter sterilized sucrose (▩), glucose (△), and sodium gluconate (✖). Growth was measured using 600 nm wavelength light to determine OD. Culture tubes were placed in a shaking water bath at 69 ^{0}C.

Thermus thermophilus HB8 can be grown on Ramsay's minimal medium containing 1 g/L NH_4 as a nitrogen source at rates comparable to nitrogen rich 697 *Thermus* medium medium. (Figure 3.2) After 14 and 24 hours the 697 *Thermus* medium samples were not statistically differentiable from Ramsay's minimum medium with 1% sucrose, although they did not reach the same final OD, possibly due to the depletion of nitrogen. Very little growth was measured on the minimal medium as expected since no additional carbon source was present. This indicates that any growth was likely carry-over from the inoculum. There was no statistical difference between the minimal medium and the medium supplemented with sodium gluconate, suggesting gluconate is not an acceptable substrate for growth of *Thermus thermophilus* HB8. Finally these findings suggest that octanoic acid may inhibit growth of *T. thermophilus* HB8 since optical density readings were below that of the minimal medium and decreases from the original inoculation.

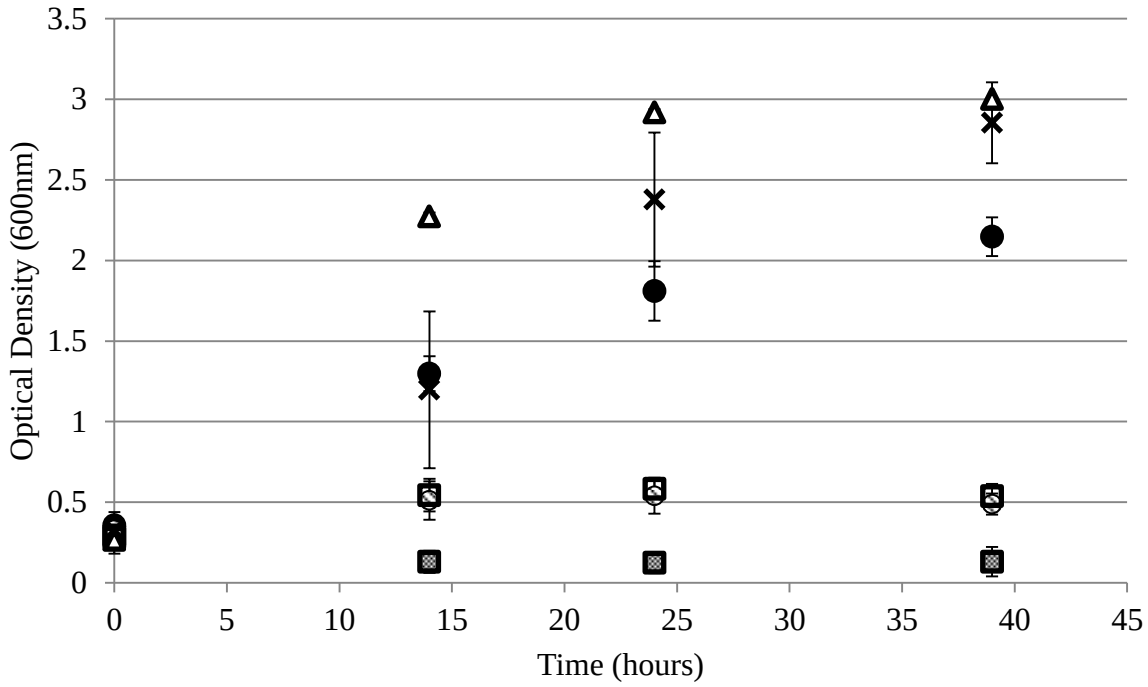


Figure 3.2. Growth profile of *T. thermophilus* HB8 on nitrogen-rich medium, 697 *Thermus* medium (✖) and 697 *Thermus* medium supplemented with 1% sucrose (△), and nitrogen-limited Ramsay's minimal medium with no added carbon source (□), or supplemented with 20 mM octanoic acid (▩), 1% sucrose (●) or 1% sodium gluconate (▨). Culture tubes were incubated in a shaking water bath at 73^{0}C.

3.3.2. Growth and PHA production comparing nitrogen-limited to nitrogen-excess conditions

When grown in culture tubes of 697 *Thermus* medium, *T. thermophilus* HB8 reached stationary phase by the 18 hours post-inoculation (Figure 3.3). Analysis by gas chromatography did not indicate the presence of PHA in any of the time-points. The data presented here suggest that *T. thermophilus* HB8 will not produce PHA when grown under nutrient-rich conditions. Further experiments would be needed to determine what conditions would promote any synthesis of polyhydroxyalkanoate.

Comparing the influence of nitrogen concentrations on the growth of *T. filiformis* WRT, there were no statistical differences between 5 g/L and 1 g/L (Figure 3.4). There was a decrease in growth when only 0.2 g/L ammonium sulfate was provided which indicates that there is insufficient nitrogen. When analyzed by flow injection analysis, the readings for 5 g/L and 1 g/L were oversaturated so an accurate reading could not be attained. The residual nitrogen in the 0.2 g/L conditions was 0.77 +/- 0.057 mM compared to a theoretical starting ammonium concentration of 3.03 mM and a measured starting ammonium concentration of 2.36. Although no PHA was observed in this experiment, the 0.2 g/L ammonium sulfate condition is preferred to the other conditions as it still allows for good biomass production while up to 75% of the ammonium gets utilized which should promote PHA fermentation.

Inoculating minimal medium supplemented with 1.5% sodium gluconate directly from 697 *Thermus* medium resulted in slight growth (Figure 3.5). The range of concentrations used for ammonium did not affect growth. When these test flasks were used as inoculum, no growth was achieved in minimal medium with sodium gluconate

with 1 g/L or 5 g/L ammonium sulfate concentration. The inoculum did, however, grow to an optical density of three in 697 *Thermus* medium indicating that the inoculum cells were viable. These results support the hypothesis that *T. thermophilus* HB8 does not grow in minimal medium with sodium gluconate but instead grows on the carry-over from the rich inoculum.

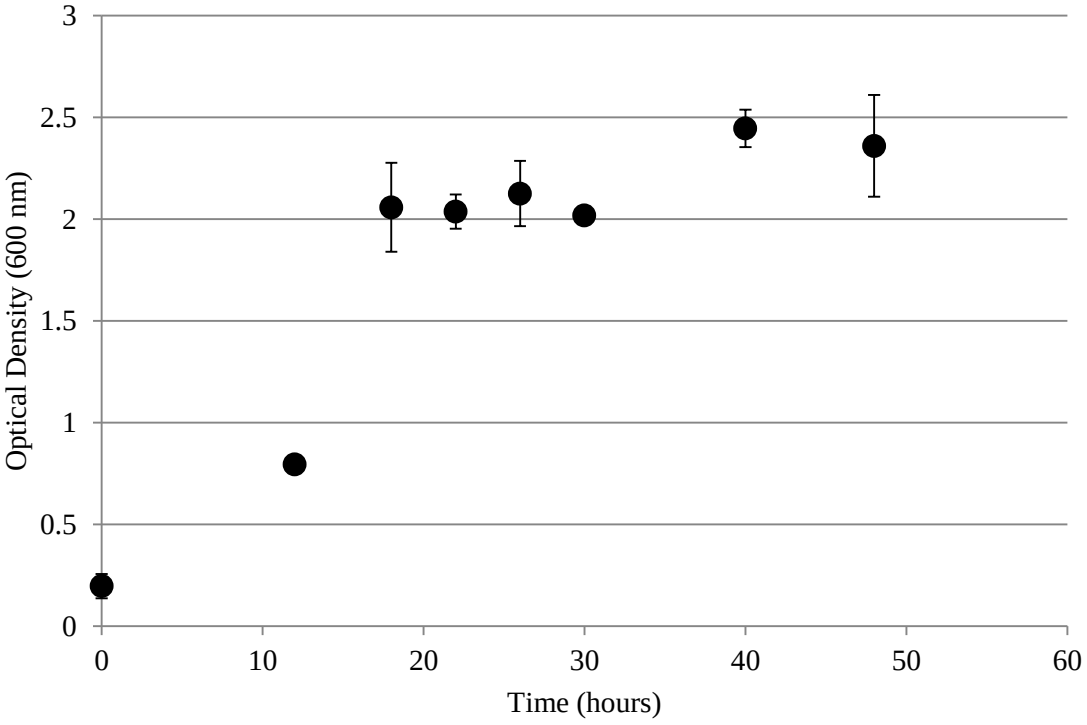


Figure 3.3. Growth profile of *T. thermophilus* HB8 in culture tubes of 697 *Thermus* medium shaken at 73^0C.

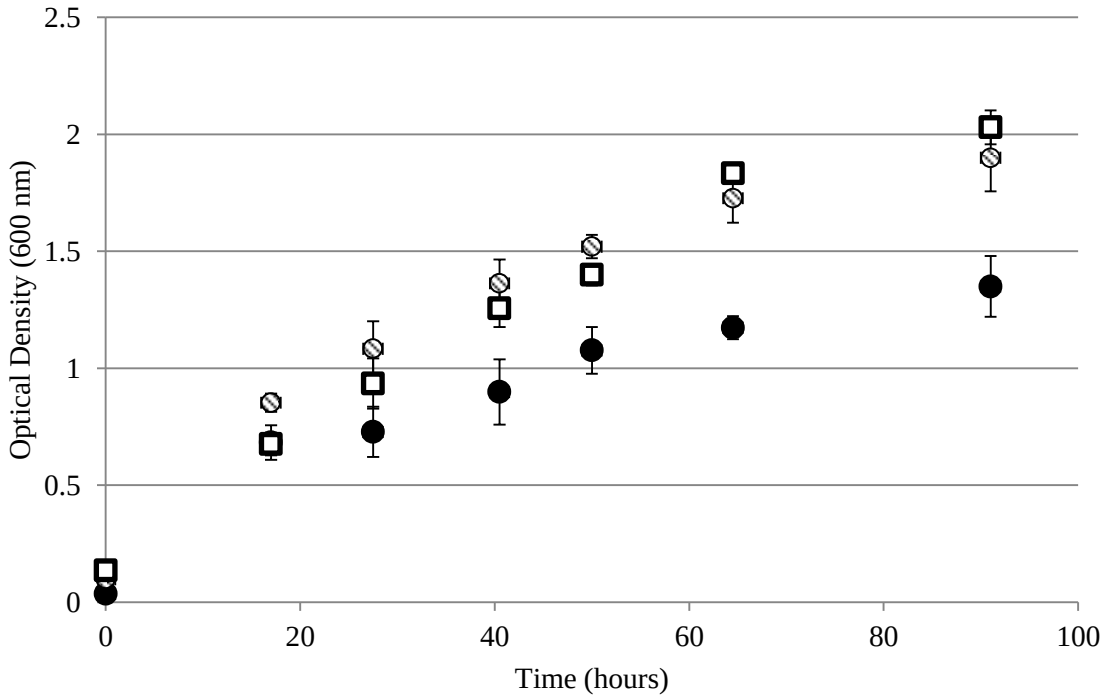


Figure 3.4. Growth profile of *T. filiformis* WRT in Ramsay's minimal medium supplemented with 1% sucrose. A nitrogen concentration of 5.0 g/L (■), 1.0 g/L (▨), and 0.2 (●) g/L were compared. Tubes were incubated at 67 ^{0}C in a shaking water bath.

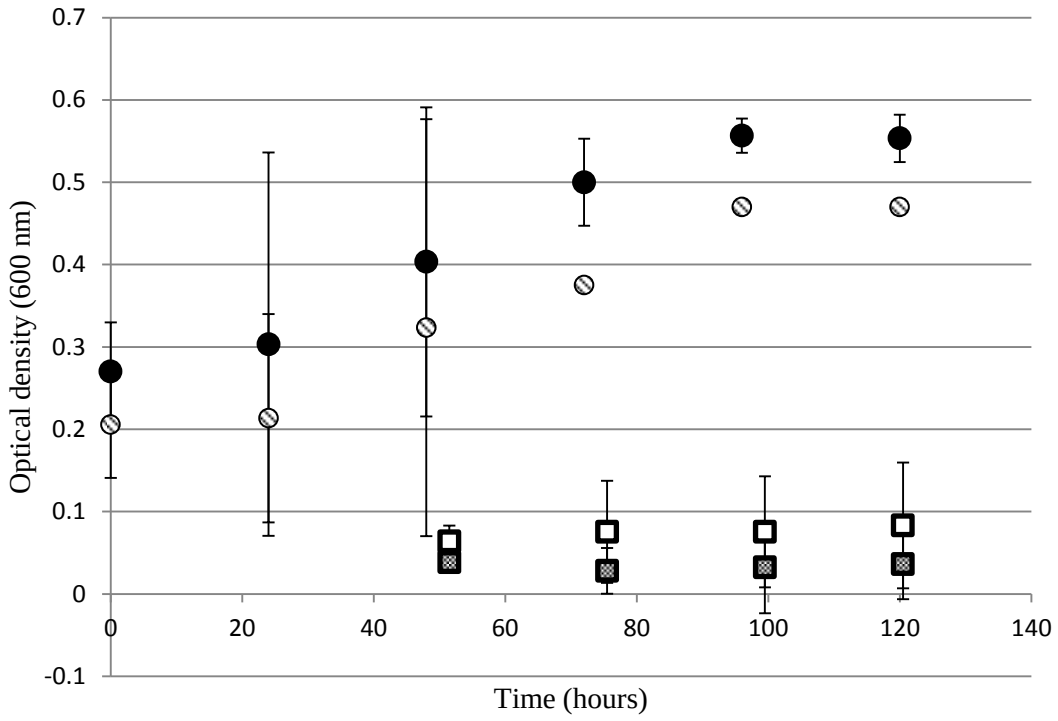


Figure 3.5. Growth profile of *T. thermophilus* HB8 in Ramsay's minimal medium supplemented with 1.5% sodium gluconate. A nitrogen concentration of 1 g/L (⦸) and 5 g/L (●) were compared. A 5% inoculum was transferred from the 48 hour time-point into fresh medium of 1 g/L (▩) and 5 g/L (□). Flasks were incubated at 73^0C.

3.3.3. Growth and PHA production comparing oxygen availability in rich medium

The growth of *T. thermophilus* HB8 was improved when grown with conditions that promoted oxygen diffusion into the medium (Figure 3.6). The culture tubes that were tilted grew to maximum optical density by 9.5 hours post-inoculation, whereas the tubes with limited mixing did not attain the same optical density and grew slowly over the entire period of the experiment. The pH increased with cell growth and the two conditions had only one time-point where the pH was significantly different (at 9.5 hours post-inoculation). The fluorescence data trends with the optical density, which is likely due to the Nile Blue stain binding to the cell membrane. Although there was significant difference in fluorescence readings between the two conditions, there is no evidence that oxygen availability affects PHA accumulation when grown in nitrogen-rich conditions (Figure 3.7). Accumulation of PHA occurrs predominantly at the end of log-phase through stationary-phase, therefore the fluorescence readings would be expected to continue to increase after the absorbance readings reached their peak. Fluorescent microscopy images of Nile Blues stained cells are provided for each time point (Figure 3.8). The cells in some of these images appear to contain small fluorescent inclusion bodies consistent with PHA granules. However, since no PHA monomers were detected by gas chromatography, the brightly staining granules within the cells may be artifacts. The contradictory GC and fluorescent microscopy images suggest that previous assumptions based on these images should be discarded. The data represents the effect of limited oxygen impeding growth while not influencing PHA production under these conditions.

T. filiformis MOK, *T. filiformis* NGM, *T. filiformis* WRT, and *T. thermophilus* HB8 did not grown under anaerobic conditions in culture tubes. The optical densities did not change in either of the replicated experiments. An oxygen deficiency resulted in reduced growth and did not appear to affect PHA production. Therefore future directives would ensure proper oxygen transfer into the medium.

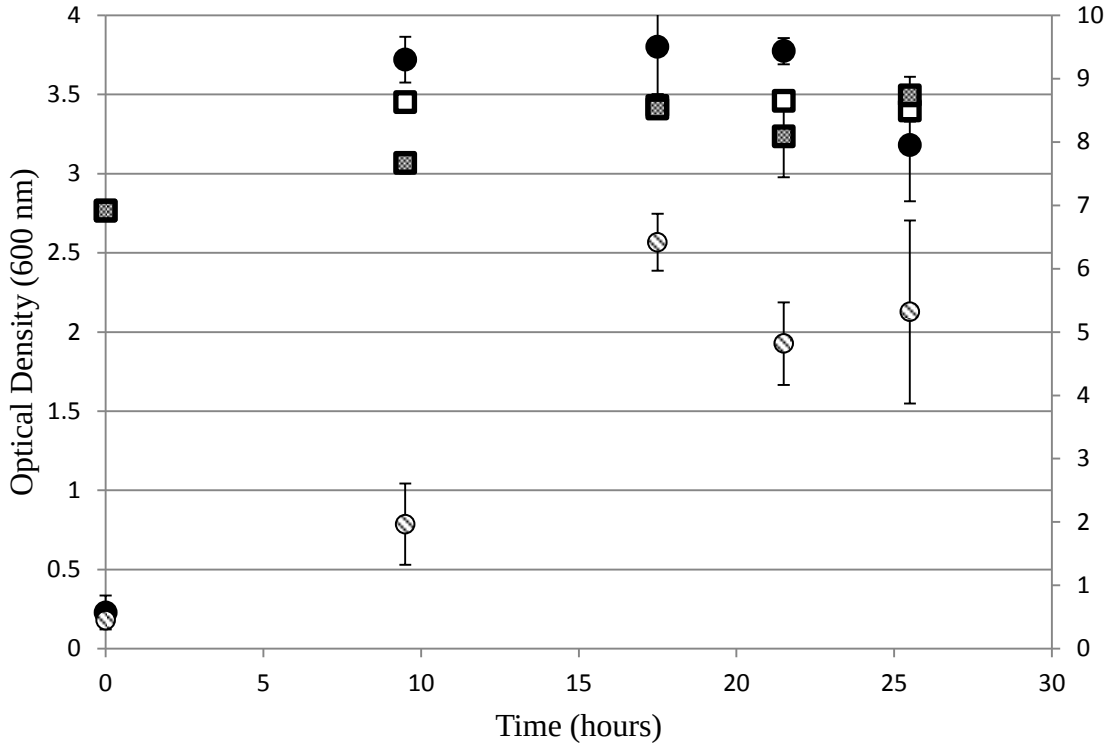


Figure 3.6. Comparison of oxygen diffusion rates on the growth and pH profiles *T. thermophilus* HB8 in 697 *Thermus* medium. Culture tubes that were "standing" in the rack had lower oxygen availability then the tubes that were "tilted" since tilted tubes mix better when shaken. The pH was measure for the tilted (▣) tubes and for the standing (▩) tubes. Optical density was measured for tilted (●) tubes and standing (◍) tubes. Culture tubes were incubated in a shaking water bath at 73 ^{0}C.

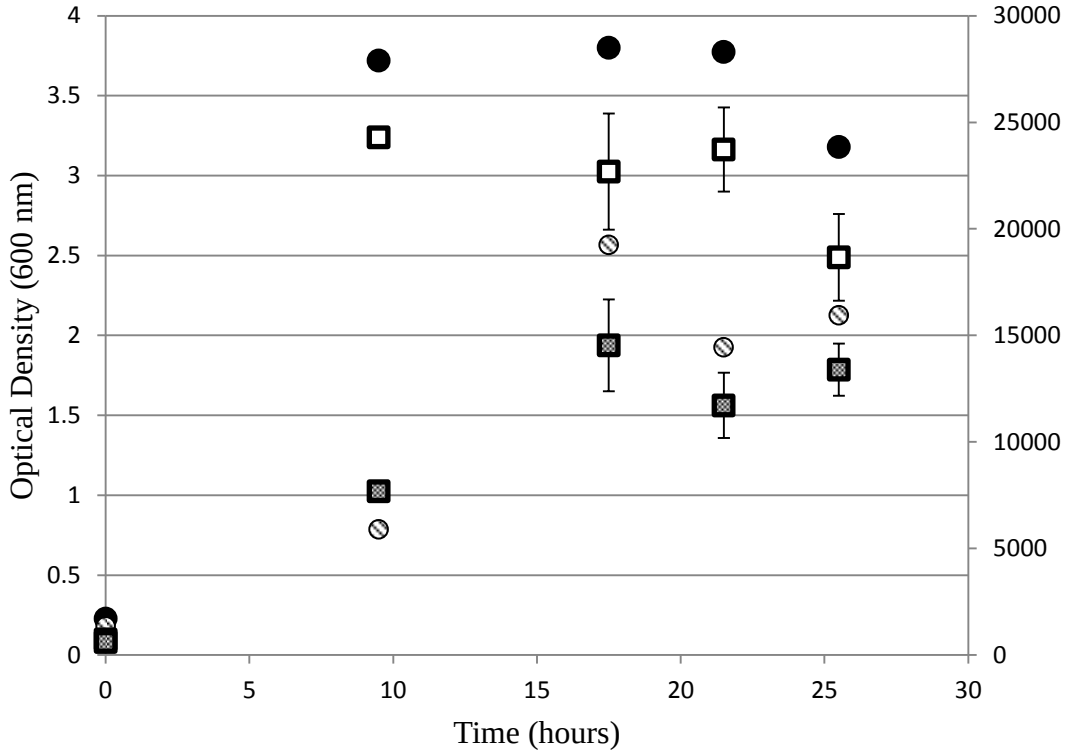
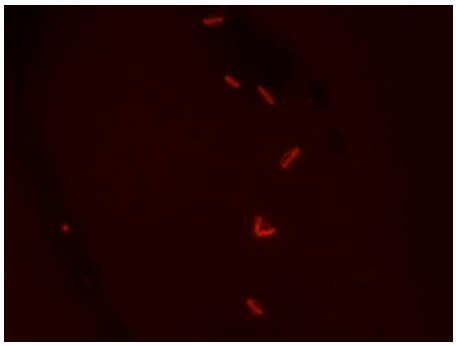
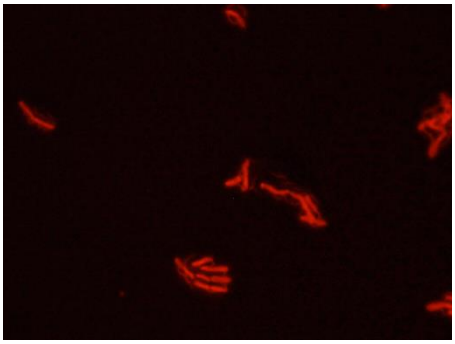
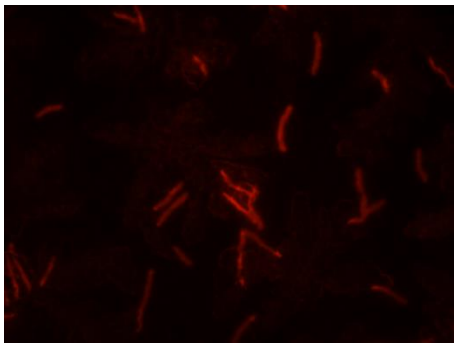
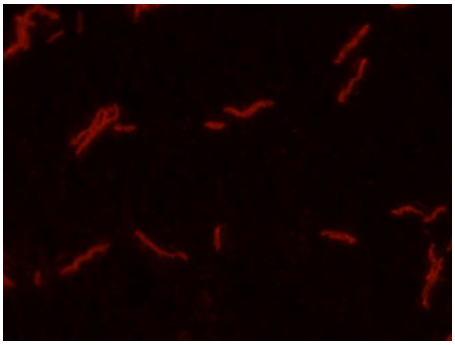
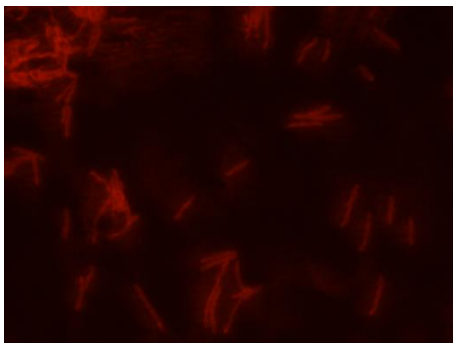
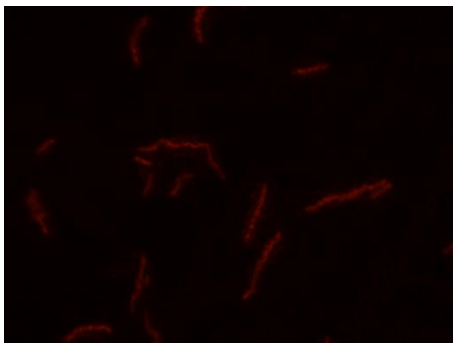
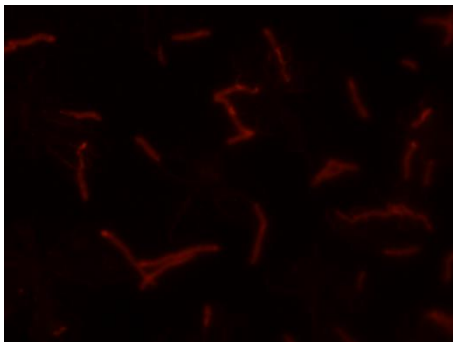
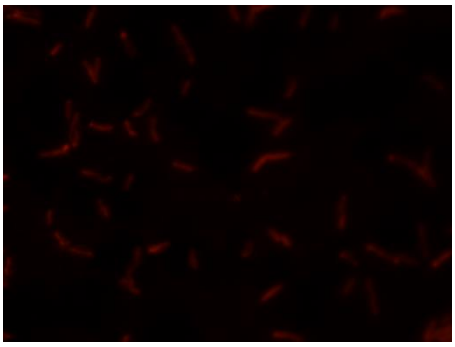


Figure 3.7. Comparison of oxygen diffusion rates on the growth and fluorescence profiles *T. thermophilus* HB8 in 697 *Thermus* medium. Culture tubes that were "standing" in the rack had lower oxygen availability then the tubes that were "tilted" since tilted tubes mix better when shaken. The fluorescence of Nile Blue stained cells was measure for the tilted (▣) tubes and for the standing (▩) tubes. Optical density was measured for tilted (●) tubes and standing (⊘) tubes, in this case the standard deviations were omitted for clarity (see Figure 3.6.). Culture tubes were incubated in a shaking water bath at 73 ^{0}C.

Incubation Time	Standing Tubes	Tilted Tubes
0 Hours		
9.5 Hours		
17.5 Hours		
21.5 Hours		

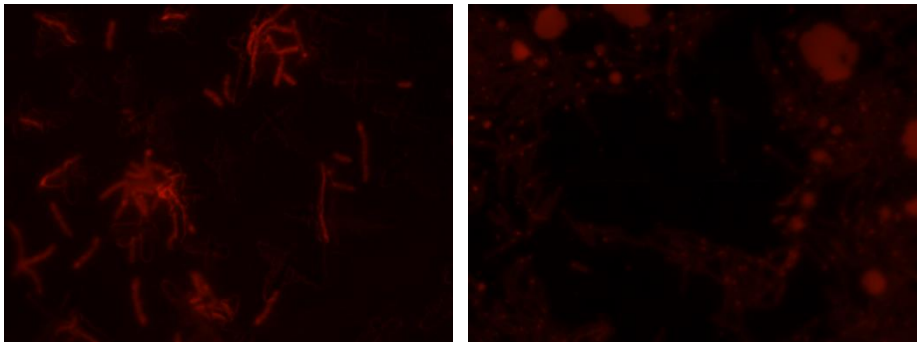
25.5 Hours 

Figure 3.8. Comparison of oxygen diffusion rates on fluorescent microscopy imaging on *T. thermophilus* HB8 in 697 *Thermus* medium. Culture tubes that were "standing" in the rack had lower oxygen availability then the tubes that were "tilted" since tilted tubes mix better when shaken. Cells were stained with Nile Blue staining solution (see section 2.4.) and heat-fixed to the microscope slide. Magnification = 600X. Culture tubes were incubated in a shaking water bath at 73^0C.

3.3.4. Measuring for PHA production by transferring viable cells into PHA-inducing medium

The first attempt with *P. putida* LS46 as a positive for PHA production provided proof of principal to test *Thermus* sp. by this method. The conditions and results which agree with previous data obtained for *P. putida* LS46 are shown in Table 3.1. (Sharma, et al., 2012). When grown on octanoic acid. *P. putida* LS46 produced medium chain length PHAs containing eight carbon (C8) 3-hydroxyacyl monomers, and PHA was accumulated in the cells to as high as 48% of the dry cell weight (dcw). *P. putida* LS46 grown on glucose produce PHAs with a ten-carbon (C10) 3-hydroxyacyl monomers. No PHA was detected when grown on 697 *Thermus* medium, which is consistent with literature detailing nutrient limitation being required for PHA synthesis initiation.

The second experiment with *P. putida* LS46 shows PHA accumulation already beginning after seven hours of incubation when transferred into medium containing octanoic acid (Figure 3.9). Octanoic acid was efficiently converted and accumulated in the cells up to 25% of the dcw by 21 hours post-inoculation. Sodium gluconate fed cells showed reduced PHA production with no PHA accumulation at seven hours and accumulation to only 5% of dcw after 21 hours. The octanoic acid results agreed with the first experiment and growth on sodium gluconate showed similar monomer composition to growth on glucose.

These conditions were repeated with *T. thermophilus* HB8 using octanoic acid and glucose. Although good cell growth was obtained, there were no peaks for any of the 3-hydroxyacyl monomers. The experiment was then repeated with *T. filiformis* WRT using octanoic acid and sodium gluconate. This particular strain was tested due to the

phaC amplicon obtained by PCR (Chapter 4.3). Good growth was obtained, but like *T. thermophilus* HB8, no PHA monomer peaks were detected. Fluorescent microscopy images were taken of Nile Blue stained cells after incubation to visualize PHA granules (Figure 3.10). As with previous fluorescent imaging attempts, granule-like fluorescence was observed in *T. thermophilus* HB8, but GC analysis failed to detect 3-hydroxy fatty acid methyl esters. Fluorescence was very clear in *P. putida* LS46, and 3-hydroxy fatty acid methyl esters were detected by GC analysis.

Table 3.1 Accumulation of PHA by *Pseudomonas putida* LS46. The optical density (OD) was measured after incubation. PHA composition is given in percent of PHA by cell dry weight (%CDW) for each carbon-length monomer.

Sample	Conditions	Final OD	% C4	% C6	% C8	% C10	% C12	% total
Glucose	Media-Exchanged. Incubated for 12 hours	6.76	0	0	1.88	5.18	0	7.08
Glucose	Media-Exchanged. Incubated for 20 hours	3.06	0	0	3.40	10.22	0	13.62
Glucose	5% inoculum. Incubated for 24 hours	3.06	0	0	1.03	5.34	0	6.37
697 medium	5% inoculum. Incubated for 24 hours	0.67	0	0	0	0	0	0
Octanoate	Media-Exchanged. Incubated for 12 hours	9.36	0	1.65	19.63	0	0	21.28
Octanoate	Media-Exchanged. Incubated for 20 hours	6.16	0	3.70	43.26	0	0	46.96
Octanoate	5% inoculum. Incubated for 24 hours	8.73	0	3.26	48.30	0	0	51.56
697 medium	5% inoculum. Incubated for 24 hours	11.36	0	0	0	0	0	0

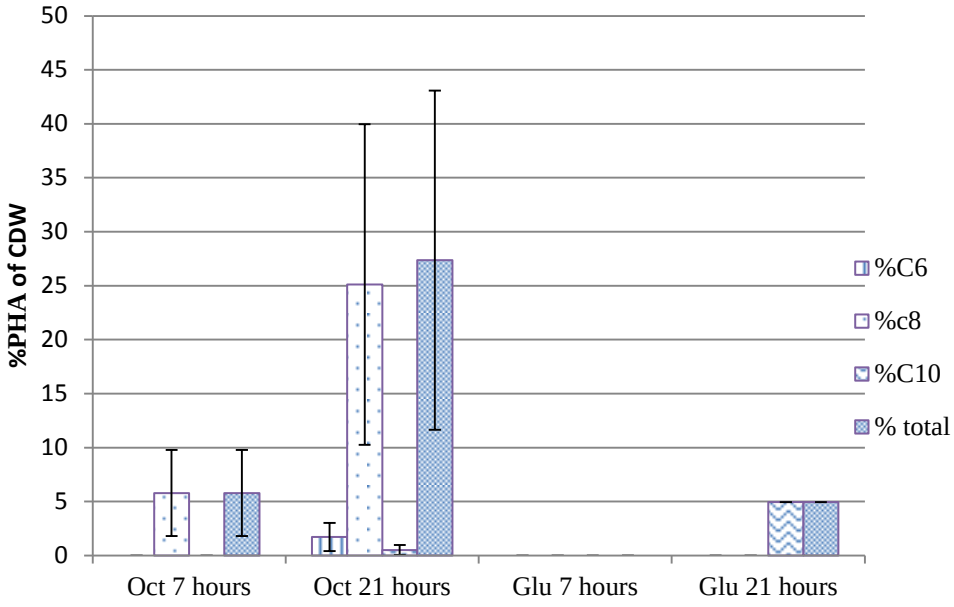


Figure 3.9. Accumulation of PHA by *Pseudomonas putida* LS46 grown on octanoic acid (Oct) and sodium gluconate (Glu). The PHA composition is given in percent of PHA by cell dry weight (% dcw) for each carbon-length monomer.

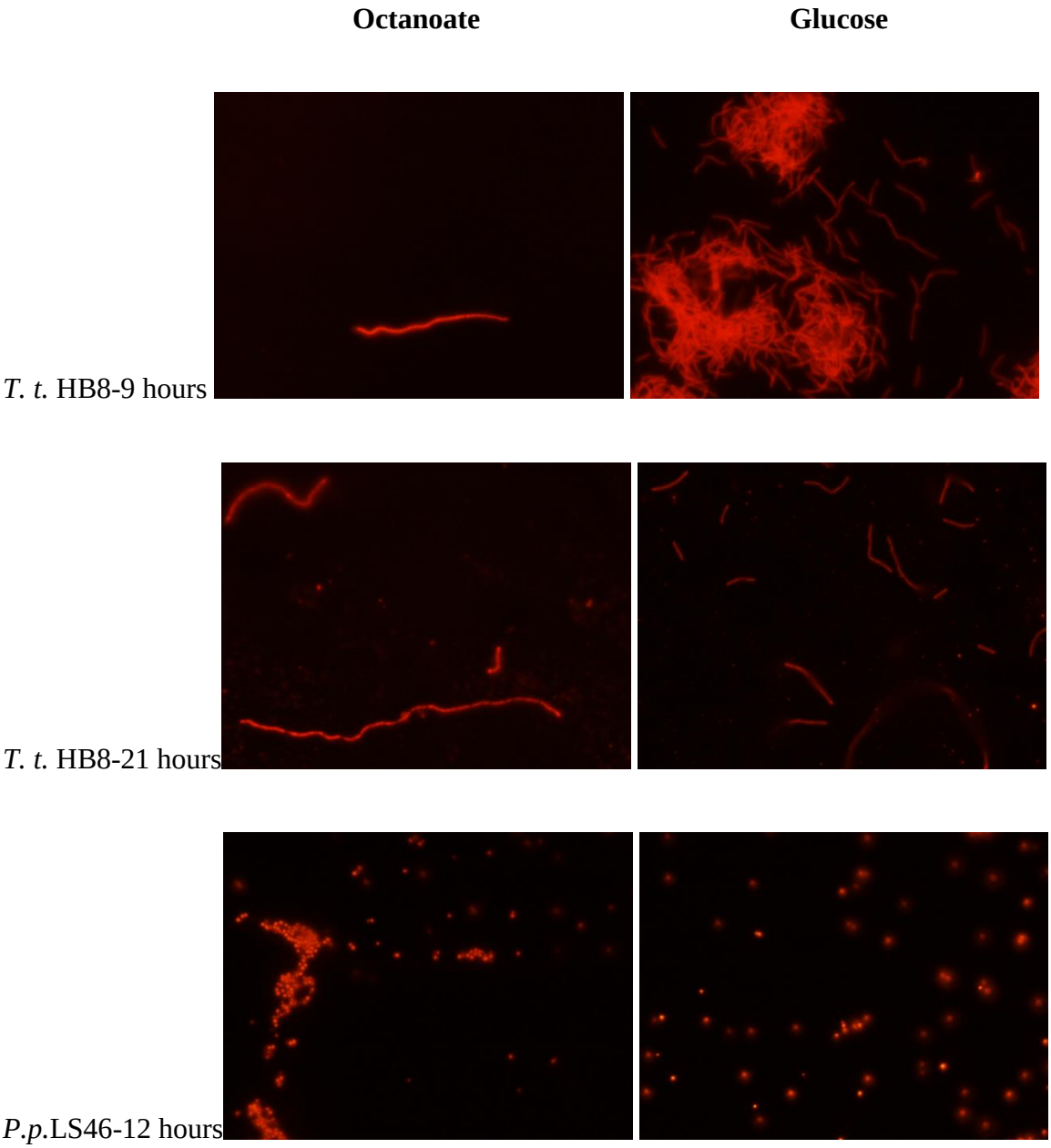


Figure 3.10. Fluorescent microscopy images of *Thermus thermophilus* HB8 and *Pseudomonas putida* LS46 transferred into media containing octanoic acid or sodium gluconate, after initial growth on 697 *Thermus* medium. Cells were stained with Nile Blue solution (see section 2.4.) and heat fixed to the microscope slide. Magnification = 600X

3.4. Conclusion

The optimum growth temperatures were determined for *T. filiformis* strains and a variety of carbon sources were initially tested in Ramsay's minimal medium. Using *T. filiformis* WRT, we determined that 0.2 g/L ammonium sulfate was a suitable nitrogen source that supported biomass growth and depletion of this nutrient could promote PHA biosynthesis. *T. thermophilus* HB8 does not appear to produce PHA in nitrogen-rich, 697 *Thermus* medium. Although brightly fluorescent granules were observed in the cells by fluorescent microscopy after staining with Nile blue, no 3-hydroxy fatty acid methyl esters was detected in the cells by gas chromatography, strongly suggesting that the granules observed by fluorescent microscopy were not made of PHAs. Substrate utilization by *T. thermophilus* HB8 was investigated using octanoic acid and sodium gluconate, but it was determined that *T. thermophilus* HB8 does not grow on these substrates. Our hypothesis that peptides from the rich inoculum were supporting growth was confirmed. These results contradict those published by Pantazaki et al. (2003), as we found no evidence for PHA production by *T. thermophilus* HB8.

Chapter 4: Genetic Analysis of PHA Synthesis by *Thermus* sp.

4.1. Introduction

The PHA synthase enzyme is present in all bacteria that synthesize PHAs. Thus, the presence of the gene encoding PHA synthase, *phaC*, can be used to indicate the potential of a species to synthesize PHAs. In this chapter the *16S* rDNA gene (rDNA) was used for phylogenetic analysis of PHA producing bacteria. *In silico* bioinformatics was used to design primers for polymerase chain reaction (PCR) analysis of *Thermus thermophilus* HB8 and three *Thermus filiformis* strain (MOK, NGM, WRT).

4.2. Experimental Design and Methods

4.2.1. Construction of *16S* rDNA phylogenetic tree of PHA accumulating microorganisms

There are four classes of PHA producing microorganisms which are characterized by their PHA synthases as previously discussed (Chapter 1.3.2). *16S* rDNA sequences were used to construct a neighbour-joining phylogenetic tree of PHA microorganisms. Neighbour-joining trees display evolutionary relatedness in members of the same class. Although not all of the members of the same PHA class are related, distinct clades were observed in which there is relatedness. In an effort to improve *in silico* analyses and *phaC* primer designs, *Thermus* sp. and members of its phylum were included in a *16S* rDNA neighbour-joining phylogenetic tree with members of all four classes of PHA producing microorganisms. The PHA producing microorganisms were selected based on their appearance in literature and availability of their *16S* rDNA sequences in the NCBI database. *16S* rDNA consensus sequences for *T. filiformis* strains were received from Dr.

Matthew Stott (a Senior Scientist, with the Extremophile Research Group, GNS Science Wairakei Research Centre, Wairakei Taupo, New Zealand) and included in the tree. Working cultures of these same *T. filiformis* strains were sequenced with *16S* primers designed from *T. thermophilus* HB8 (chapter 4.2.2) and were also included in this tree. *E. coli* K12 was included for context.

The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentages of replicated trees, in which the associated taxa clustered together in the bootstrap test (1000 replicates), are shown next to the branches. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The analysis involved 56 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 779 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

4.2.2. Sequencing *16S* rDNA from *Thermus sp.*

DNA was extracted from the four candidate *Thermus* sp. strains for PHA production. A *16S* PCR reaction was conducted to establish the presence of *Thermus* sp. DNA in the reaction. Universal primers (928F and 336R) for the *16S* rDNA were applied. The PCR reaction mixture typically consisted of 10µL of ReadyMix™REDTaq PCR

reaction with $MgCl_2$, 2.5µL of a 10µM solution of forward and reverse primers, 4µL H_2O and 1µL of the desired DNA. The concentrations of DNA and primers were varied and water could be substituted with 2µL or 4µL of Polymer-Aide PCR enhancer when attempting to optimize the PCR mixture. Denaturation occurred at 94 ^{0}C for 45 seconds, the annealing temperature was on a gradient from 48 to 60 ^{0}C for 75 seconds and the elongation phase at 72 ^{0}C for 60 seconds over 35 cycles to determine the optimum annealing temperature for each primer set.

The completed PCR reactions were run through an agarose gel to and DNA was stained with RedSafe™ nucleic acid staining solution. The gel was visualized under ultraviolet light and selected bands excised. A Zymoclean™ Gel DNA Recovery Kit was used to isolate 7µL of the amplicon. A DNA concentration between 5 and 20 ng/µL was sent with the primers for sequencing by MacrogenUSA. The *16S* PCR amplicon was sequenced to verify the identity of the *Thermus* species stain.

4.2.3 Detecting the *phaC* gene using designed degenerate primers

The first course of action was to verify the legitimacy of the designed *phaC* degenerate primers. The NCBI method for browsing a genome with primers was applied *in silico* with some success. Since degeneracies will not work using this method, their positions were randomly assigned. This had the desired effect of detecting primarily *phaC* genes in various microorganisms belonging to the respective PHA class. To check the validity of these degenerate primers on extremophiles, the Class III degenerate primer set was aligned to *Ferroglobus placidus* and *Haloferax mediterranei* ATCC 33500 (Figure 4.5). After legitimizing the designed PHA primer classes, they were all compared against the genome of *T. thermophilus* HB8 in search of its elusive *phaC* gene.

Initial attempts with the PHA primer sets resulted in promising bands in *T. filiformis* NGM. To verify these results the primer sets were optimized to produce bands on PHA positive microorganisms; in this case *Cupriavidus necator* H16 for Class I and *Pseudomonas putida* LS46 for Class II. Focus was primarily shifted to these primer classes due to initial results in which amplicons were obtained for *T. filiformis* NGM with Class I primers (Figure 4.6). After the optimization of the PCR conditions, the primers were applied to *T. thermophilus* HB8, *T. filiformis* MOK, *T. filiformis* WRT and *T. filiformis* NGM. Ultimately three Class I, a Class II, a Class III and a Class IV primer sets were tested on the four strains of *Thermus*. Any potential *phaC* bands from *Thermus* sp. were to be sequenced to ascertain the identity of this gene.

4.2.3. Bioinformatic analysis of genes associated with PHA metabolism present in *Thermus thermophilus* HB8

To date, the *phaC* gene in *Thermus thermophilus* HB8 has not been identified despite reports of PHA production by this strain and physical characterization of its PHA synthase (Pantazaki, et al., 2003). Here we describe the *in silico* search for this elusive gene in all four strains for *T. thermophilus* in the IMG-JGI database (Markowitz, et al., 2005). This consists of *T. thermophilus* HB27, *T.t.* HB8, *T.t.* JL-18 and *T.t.* SG0.5JP17-16.

To begin, the PHA synthase enzyme was searched by name in the four strains of *T. thermophilus*. Next, the *phaC* genes of representative members of PHA producing classes were used as reference genes to probe the *Thermus thermophilus* HB8 genome by nucleotide BLAST and tBLASTx analyses. The reference genes were obtained from *Pseudomonas putida* F1 (Class II), *Bacillus cereus* ATCC 10987 (Class III) and

Ralstonia eutropha JMP134 (Class I). In addition, the *phaC* genes from *Nakumurella multipartita* DSM 44233, *Haloferax mediterranei* ATCC 33500 and *Ferroglobus placidus* DSM 10642 were applied against *T. thermophilus* HB8 using a tBLASTx due to closer evolutionary relatedness or, in the case of the latter two microorganisms, their ability to grow under extremophilic conditions. Finally, the amino acid sequence of *Bacillus* sp. INT005 PHA synthase was located on Uniprot, and this sequence was compared to a translated nucleotide query of *T. thermophilus* HB8. Due to thermophilic production of PHA by *Bacillus* sp. INT005, it was hypothesized it may have increased likeness to a PHA synthase in *T. thermophilus.*

The Enzyme Commission number (EC 2.3.1.-) was used to attempt identification of the *phaC* gene in the *T. thermophilus* strains. COG 3243 refers to PHA synthase and was searched for in *Thermus* strains. (Cai, et al., 2011) Interpro numbers and TIGRfam numbers obtained from the three different classes of PHA synthases belonging to *Ralstonia eutropha* JMP134, *Pseudomonas putida* F1, and *Bacillus cereus* ATCC10987 were used to probe *T. thermophilus*. Finally pfam 00561 and pfam 07167, an abhydrolase (common in many enzymes) and N-*terminal* domain respectively, which are associated with all three classes of PHA synthases, were compared to the *Thermus* genomes.

4.3. Results and Discussion

4.3.1. Construction of *16s* phylogenetic tree of PHA accumulating microorganisms

A neighbour-joining tree was created of PHA producing microorganisms, *E. coli*, and member of the phylum *Deinococcus-Thermus* to determine the existence of any evolutionary relatedness between *Thermus* sp. and other PHA producers (Figure 4.1).

Some evolutionary relatedness was seen between members of the same class of PHA producers. However, the branch-point where *Deinococcus-Thermus* species separate from known PHA producers clustered in only 10% of the trees produced. This tree provided little insight into the Class of PHA producing microorganisms with which *Thermus thermophilus* HB8 associates, as there was no apparent phylogenetic relation to other PHA producing microorganisms.

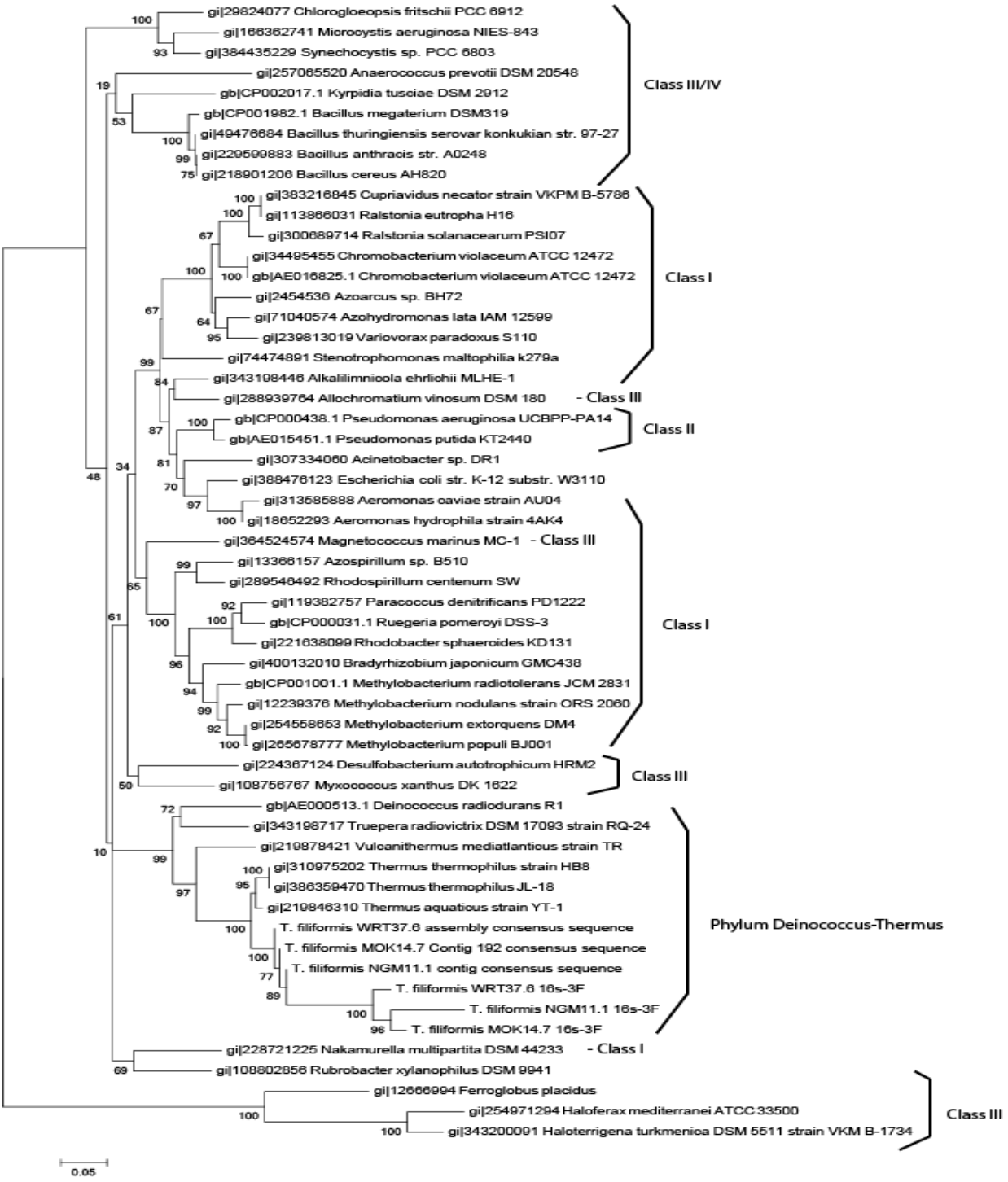


Figure 4.1. Neighbour-joining tree of *16S* rDNA amongst select PHA accumulating microorganisms and *Thermus* sp.

4.3.2. Sequencing *16S* in *Thermus sp.*

Initial attempts to attain amplification of *16S* rDNA using universal primers were not successful. New *16S* PCR primers were designed from the sequence of *T. thermophilus* HB8 and in the meantime three different DNA extraction methods were applied to ensure that PCR quality DNA was available to the universal primers. Having not obtained a PCR band with any of the extracted DNA samples, it was determined that the universal primers were unsuitable for amplification in *Thermus* sp. which was supported by *in silico* analysis.

Four new sets of primers were obtained for the amplification of *16S* rDNA in *T. thermophilus* HB8. Two sets of primers were obtained from literature; one of which was given the name Nested *16S* (Gregory, et al., 2005) which was designed for *T. thermophilus* IB-21 and the other Uni16S (Beffa, et al., 1996) designed for isolation of *Thermus* sp. from hot composts. Two other primer sets were designed to be specific for *T. thermophilus* HB8. One set of primers was selected from a compilation provided by the Primer3 engine. This set's forward sequence is 5'-GGCATTGTAGCACGTGTGTC-3' and the reverse is 5'-TTAGGAATCTTCCGCAATGG-3'. The second set of specific primers was developed using Primer BLAST. The forward sequence is 5'-TCGGGTAGAGCCCGCTCCAT-3' and the reverse sequence is 5'-GGCGGACGGGTGAGTAACGC-3'.

These primers were tested on the DNA earlier extracted from *T. thermophilus* HB8 (Figure 4.2). The UNI16S primer set unexpectedly produced a much smaller band than anticipated and efforts with this set were discontinued to avoid troubleshooting this issue. The other primer sets were tested on the three strains of *T. filiformis.* The Primer3

set showed early promise, and provided bands for *T. thermophilus* HB8 grown on 697 *Thermus* medium or glucose as earlier described (Chapter 3.3.4), along with a band for *T. filiformis* MOK (Figure 4.3). Sequencing results of *T. thermophilus* HB8 matched its genome through BLAST analysis, lending confirmation to the substrate-comparing growth experiment. The Primer3 set ultimately became the best option to attempt sequencing (Figure 4.4), with denaturation occurring at 94 ^{0}C for 45 seconds, the annealing temperature reduced to 50 ^{0}C for 75 seconds and the elongation phase occurring at 72 ^{0}C for 60 seconds over 35 cycles. Upon sequencing, the quality of the reverse strand was low but still aligned fairly well with the better quality forward strand. The sequences of the forward strands from all three strains of *T. filiformis* aligned most closely to *T. thermophilus* when input to BLAST and were included in the phylogenetic tree (Figure 4.1). These sequences also matched well to consensus *16S* rDNA sequences.

Although contamination cannot be completely ruled out since these primers don't necessarily amplify for all microorganisms, these results along with the lack of amplification with the universal primer set provide some security in the identity of the DNA in use. This preliminary *16S* DNA data was sufficient enough to begin analyzing these strains for the presence of a *phaC* gene.

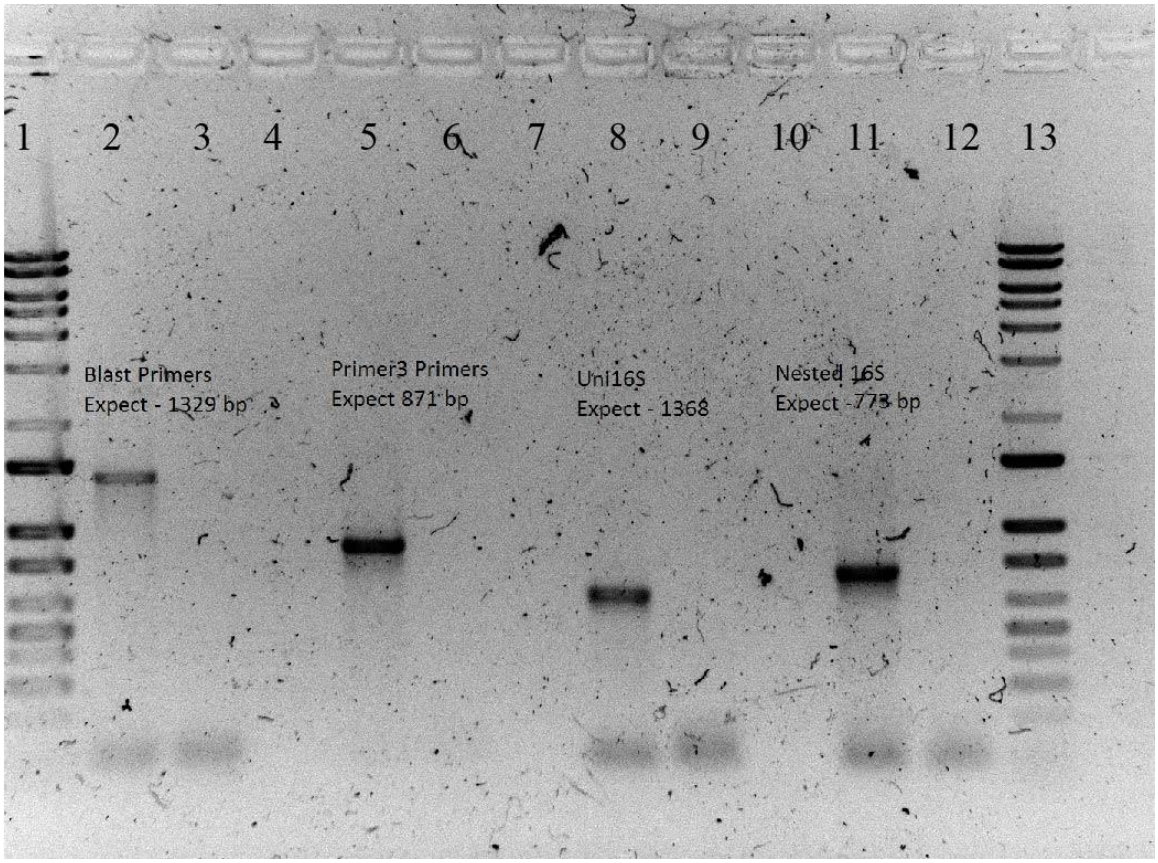


Figure 4.2. Agarose gel showing the amplification of DNA when comparing four *16S* rDNA primer sets. Wells 1 and 13 are the .1-10 kb DNA ladder. The other wells contain PCR reaction mixtures. Wells 2 and 3 contains Blast designed *16S* primers, only well 2 contains *T. thermophilus* HB8 DNA. Wells 5 and 6 contain Primer3 designed *16S* primers, only well 5 contains *T. thermophilus* HB8 DNA. Wells 8 and 9 contain the UNI*16S* primers, only well 8 contains *T. thermophilus* HB8 DNA. Wells 11 and 12 contain the Nested*16S* primers, only well 11 contains *T. thermophilus* HB8 DNA. Wells 4,7 and 10 are empty.

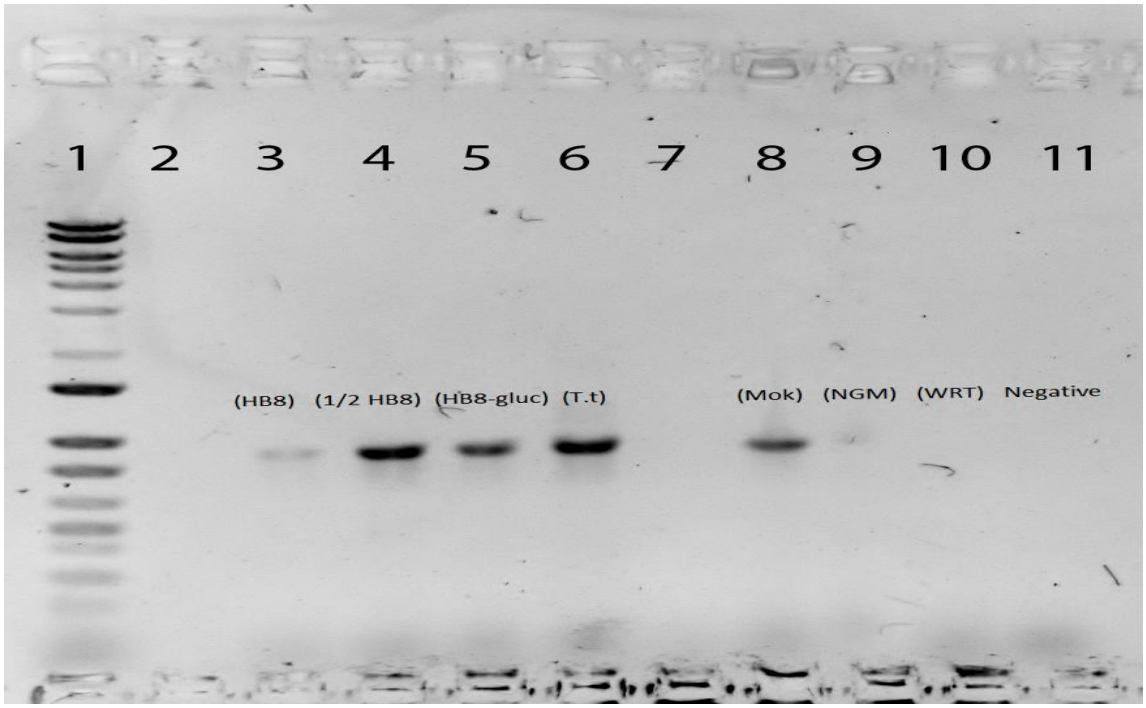


Figure 4.3. *16S* rDNA amplification of experimental *Thermus thermophilus* HB8 grown normally on 697 *Thermus* medium or in minimal medium supplemented with 1% glucose using Primer3 set of primers. First attempt to amplify *16S* DNA in *T. filiformis* strains with Primer3 set. Chris, define what is each lane. Well 1 contains the 0.1-10kb DNA ladder, wells 2 and 7 are empty. The remaining wells contain PCR reaction mixtures using the Primer3 *16S* primer set. Well 3 has *T. thermophilus* HB8 DNA extracted from 697 *Thermus* medium, of which 1µL of a 98.2 ng/µL solution of DNA was added to the reaction mixture. Well 4 contains the same DNA, diluted down to 49.1 ng/µL. Well 5 has *T. thermophilus* HB8 DNA extracted from minimal medium supplemented with 1% glucose, of which 1µL of a 108.7 ng/µL solution of DNA was added to the reaction mixture. Well 6 has a different cell line of *T. thermophilus* HB8 DNA extracted from 697 *Thermus* medium, of which 1µL of a 98.7 ng/µL solution of DNA was added to the reaction mixture. Well 8 has *T. filiformis* MOK DNA extracted from 697 *Thermus* medium, of which 1µL of a 1009.8 ng/µL solution of DNA was added to the reaction mixture. Well 9 has *T. filiformis* NGM DNA extracted from 697 *Thermus* medium, of which 1µL of a 831.3 ng/µL solution of DNA was added to the reaction mixture. Well 10 has *T. filiformis* WRT DNA extracted from 697 *Thermus* medium, of which 1µL of a 982.9 ng/µL solution of DNA was added to the reaction mixture. Well 11 is a negative control, it contains no DNA.

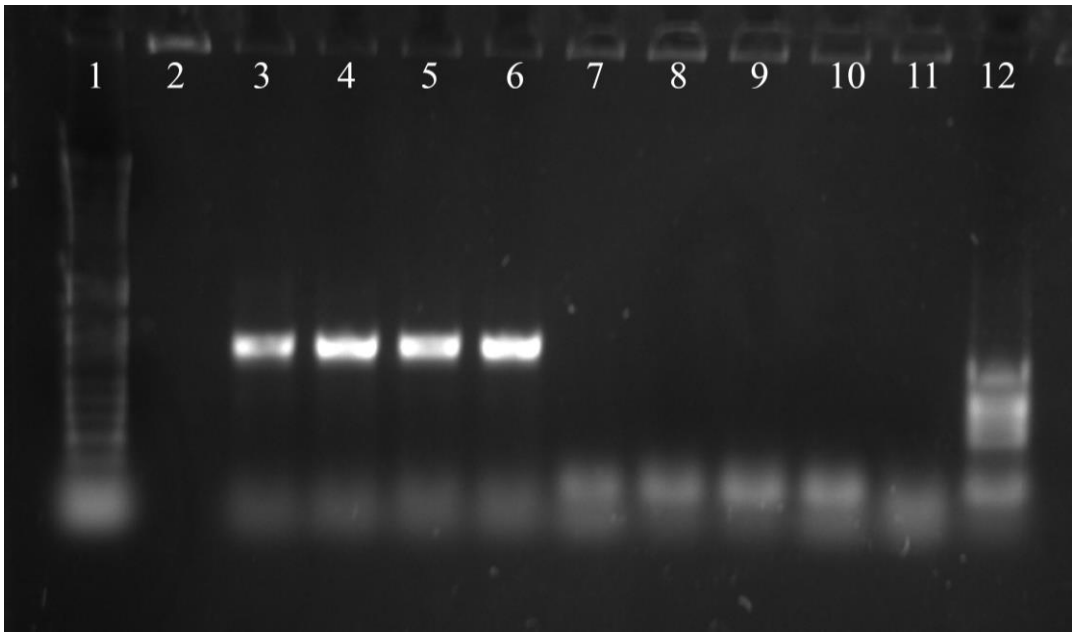


Figure 4.4. Succesful amplification using Primer3 set of primers designed to probe for *16S* rDNA as shown in this agarose gel. Also shown in the first attempt to amplify *phaC* using the Class I designed degenerate primers on *T. thermophilus* HB8 and the Class I PHA positive *Cupriavidus necator*. Well 1 contains a 0.1-10 kb ladder. Well 2 is empty. Wells 3 through 6 are PCR reaction mixtures containing Primer3 *16S* primers. Well 3 contains 1 µL of 49.1 ng/µL *T. thermophilus* HB8 DNA. Well 4 contains 1 µL of 83.1 ng/µL *T. filiformis* NGM DNA. Well 5 contains 1 µL of 101.0 ng/µL *T. filiformis* MOK DNA. Well 6 contains 1 µL of 98.3 ng/µL *T. filiformis* WRT DNA. Wells 7 through 11 are PCR reaction mixtures containing Class I degenerate *phaC* primers and 1 µL of 49.1 ng/µL *T. thermophilus* HB8 DNA. Well 7 contains the standard concentration of primers (10 µM). In well 8, 5 µM primers were added. In well 9, 5 µM primers were added and 3 µl of water was substituted with PCR enhancer. Well 10 contained no DNA and class I primers. Well 11 contained no DNA and Primer3 *16S* primers. Well 12 contained 10 µM class 1 primers and *C. necator* H16 DNA.

4.3.3. Detecting the *phaC* gene using designed degenerate primers

The degenerate primer sets designed on alignments of the four classes of PHA producing microorganisms performed satisfactorily during *in silico* analysis. Browsing the NCBI nucleotide collection database with these primers resulted in preferential identification of *phaC* genes of members of the same class. When aligned to known PHA producers, the results were agreeable. Alignment of Class III primers to *Haloferax mediterranei* ATCC 33500 showed only one mismatch, the rest matched with the specific nucleotides or the degenerate nucleotides (Figure 4.5). *Ferroglobus placidus* showed four mismatches to the Class III primers. Ideally the combination of all four classes of PHA primer sets would be able to detect all *phaC* genes, and though this is unlikely, we believe that the primers developed might be suitable for the screening of *phaC* in microorganisms. Due to the degenerate nature of the primers, there is increased probability of false positives which cannot be predicted *in silico*. The size of the band would likely vary from the theoretical length in the case of a false positive allowing these primers to be tested and any band sequenced to verify its identity.

The Class II primers set was the first tested, due to the ability of *T. thermophilus* HB8 to produce medium-chain length PHA monomers similar characteristic of Class II (Pantazaki, et al., 2003). The thermocycler settings were identical for those used in amplification of *16S* above, where denaturation occurred at 94 ^{0}C for 45 seconds, the annealing temperature was 50 ^{0}C for 75 seconds and the elongation phase at 72 ^{0}C for 60 seconds over 35 cycles. The PCR mixture was as described in the methods (Chapter 2.5) and it was shown that a tenfold dilution of the DNA provided amplification of what we assume is the *phaC* gene in *Pseudomonas putida* LS46 (Figure 4.7). The expected

product size was 457 base pairs and the Class II primers only showed two mismatches when aligned to *P. putida* ND6, which supports this assumption. Having obtained a PHA positive reaction, the Class II primer sets were applied to *Thermus* sp. with extra emphasis on *T. thermophilus* HB8. Variations of primer concentration, from 1 to 20 µM, and dilutions of DNA of 1-fold, 10-fold and 20-fold along with substitutions of water with PCR enhancer and lowering annealing temperature to 48 ^{0}C did not amplify the template. The Class II primer set appears to be functional however *Thermus* sp. does not appear to have a conventional Class II PHA synthase.

The degenerate Class I primer set was hypothesized to be most likely to amplify any present *phaC* gene in *Thermus* sp. due to the previous success with the Class I specific STI primers. These degenerate primers have five total mismatches to the template of *Cupriavidus necator* H16 and a predicted product of 803 base pairs. Use of an annealing temperature gradient determined a 47.9 ^{0}C temperature to be optimum for annealing. The reaction was carried out with a 1/10 dilution of the DNA and 10 µM primer concentrations which was suitable for amplification with the Class I primers and universal *16S* primers (Figure 4.8). The appearance of a second band on the gel from Class I primer amplification is discouraging, as it could a false positive, however is consistent with the earlier results from STI on *T. filiformis* NGM and *N. nitrogenifigens* Y88. Testing these conditions on *Thermus* sp. did not result in amplification.

Specific primers for Class I were also tested to improve the likelihood of detecting *phaC*. The primers set RSpha were designed for *Cupriavidus necator* H16 and the STI primers were designed for *N. nitrogenifigens* Y88. The RSpha primers had successful amplification of *C. necator* H16 with thermocycler program identical to that

for Class II primers but failed to amplify any of the *Thermus* strains. STI primers would be expected to produce an amplicon of approximately 500 base pairs in *C. necator* H16. The STI primers amplified under Class I thermocycler settings, but there were at least three bands which smeared together (Figure 4.4). The annealing temperature most suited for amplification with STI was 52 ^{0}C but still two distinct bands were seen. When applied to *Thermus* sp. using 1/10 the DNA and 10 µM primers, amplification was obtained on *T. filiformis* WRT but none of the other *Thermus* strains. Before trying to obtain amplification using the other *Thermus* strains, conditions were optimized so the band in *T. filiformis* WRT could be sequenced and identified as *phaC* or a false positive (Figure 4.9).

The sequencing quality of the excised STI band on *T. filiformis* WRT was below acceptable measure. This strain was then cultured from a glycerol stock and new DNA extracted. Amplification still occurred and no result on the negative reaction would indicate that the amplification was indeed due to *T. filiformis* WRT DNA. Changing cell lines, growth at 68 ^{0}C and the satisfactory sequencing of the previous cell line discourage the notion of possible contamination by another PHA producing microorganism. The amplicon was sent for sequencing and the quality was much improved from the first attempt. The forward (~400 bp) and reverse (~200bp) sequences did not overlap, despite the amplicon being just over 400 bp, although the quality of sequencing dropped near the 3'-ends, which may explain the difficulty in overlap alignment. BLAST analysis of both sequences did not reveal the possible identity of the sequenced band, and had no resemblance to any PHA synthases. This result could be explained by the expression of a divergent PHA synthase compared to mesophilic PHA producers. However, the amplicon

is 100 base pairs shorter then theoretically predicted, other Class I primers did not detect this gene, the presence of multiple bands when first probing *C. necator* H16 and the poor sequencing results strongly favour false positive reactions.

Finally Class III primers were used to probe *C. necator* H16 and the *Thermus* strains. As expected no result was obtained for *C. necator* H16, but the absence of result from *Thermus* sp. discouraged further investigation. Class IV primers were initially tested but not rigorously. Due to the similarity between Class III and Class IV PHA synthases and the fact that Class IV microorganisms are mainly *Bacillus* sp. it did not seem an appropriate course of action.

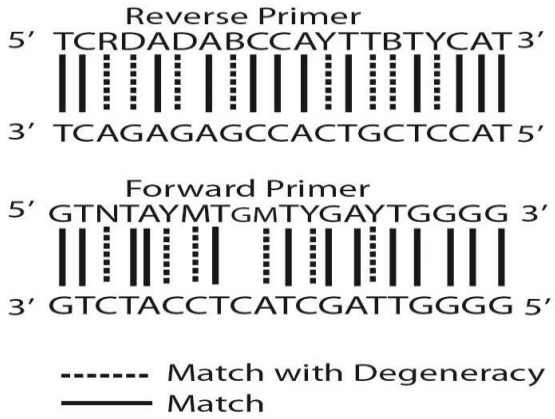


Figure 4.5. Alignment of Class III PHA primers to the *phaC* gene of *Haloferax mediterranei* ATCC 33500.

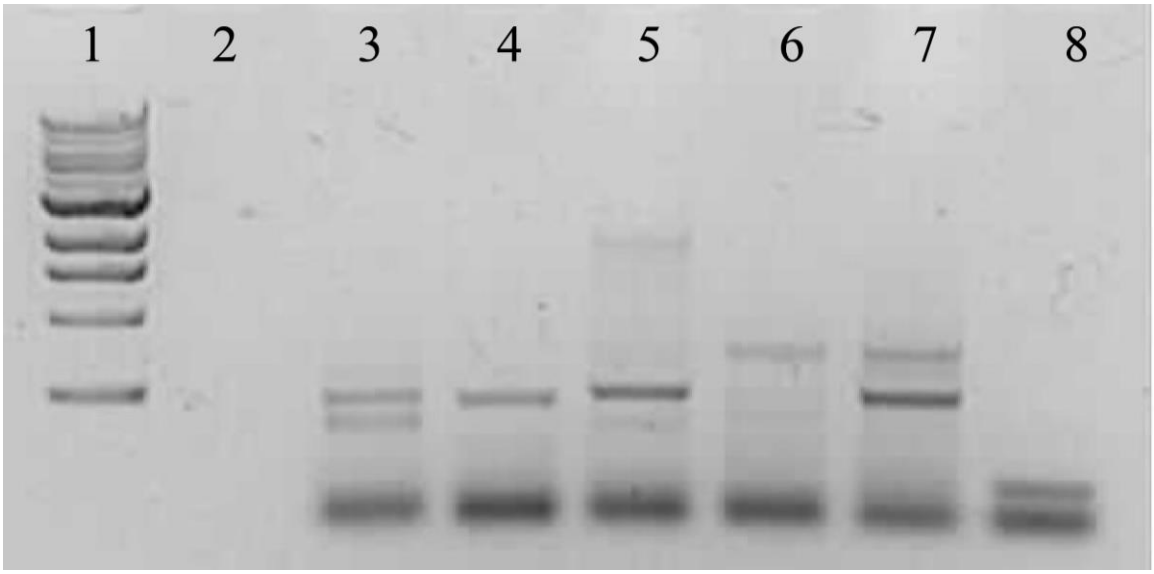


Figure 4.6. Agarose gel showing potential amplification of the *pha*C gene at around 500 base pairs using STI primers. *Novosphingobium nitrogenifigens* Y88 is used as a Class I positive. *Thermus filiformis* NGM is probed alongside three soil samples taken from thermophilic hot pools in New Zealand and *E. coli* which is used as a negative for PHA production. Well 1 is 0.5-10 kb ladder. Well 2 was empty. Wells 3 through 8 contained PCR reaction mixtures containing STI primers. Well 3 contained *N. nitrogenifigens* Y88 DNA. Well 4 contained *T. filiformis* NGM DNA. Well 5,6 and 7 contained DNA from different soil samples taken from New Zealand hot springs. Well 8 contained *E. coli*, a negative for PHA production.

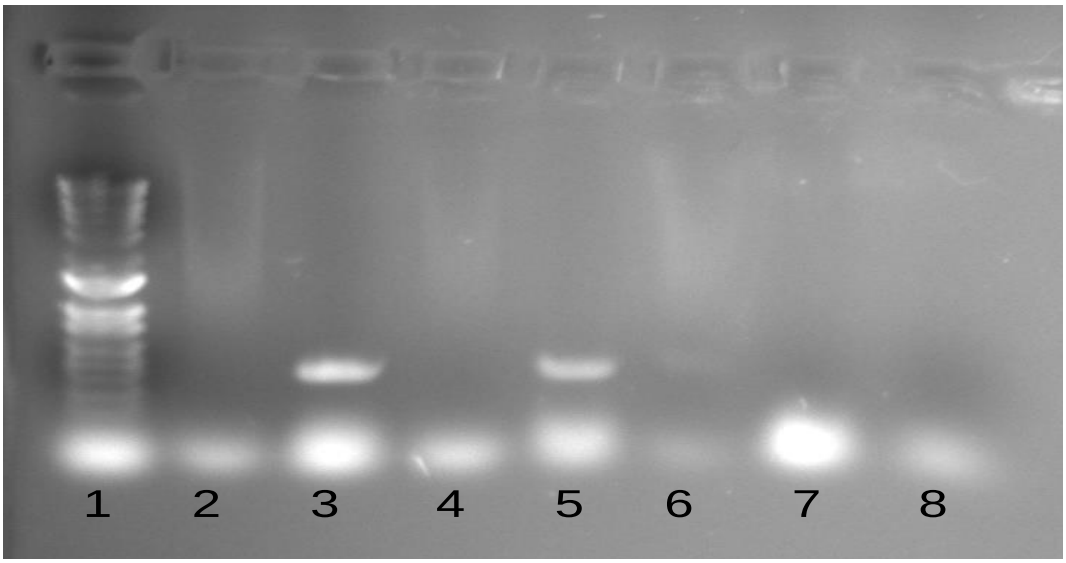


Figure 4.7. Succesful amplification using Class II primers designed to probe for the *phaC* gene in *Pseudomonas putidas* LS46 as shown in this agarose gel.. 1) 0.1-10 kb ladder; 2) Standard Reaction (2.5 µL of 10 µM forward and reverse Class II primers, 4 µL water, 10 µL RedSafe® PCR reaction mix, 1 µL of 1422 ng/µL of *P. putida* LS46) 3) Standard reaction with diluted 142.2 ng/µL DNA added; 4) Standard reaction in which 2µL of water has been exchanged with PCR enhancer; 5) Standard reaction with diluted 142.2 ng/µL DNA added and 2µL of water has been exchanged with PCR enhancer 6) Standard Reaction with diluted 1µM primers; 7) No DNA; 8) No DNA, 1 µM primers.

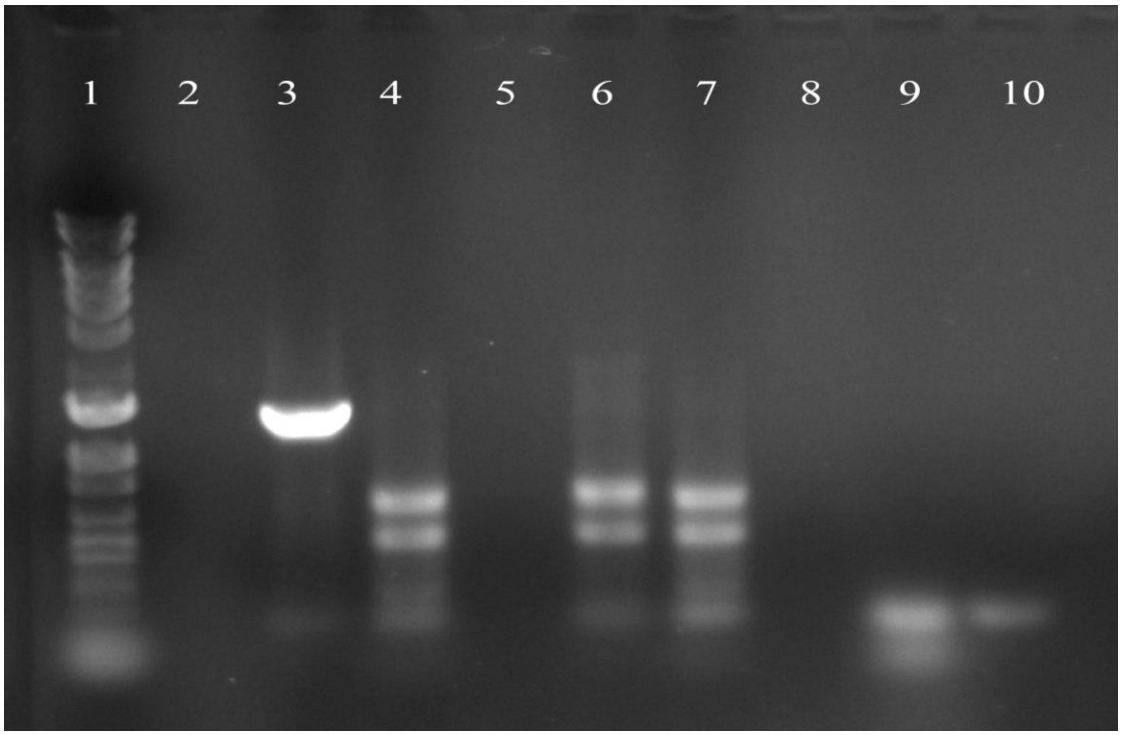


Figure 4.8. Succesful amplification using Class I primers designed to probe for the *phaC* gene in *Cupriavidus necator* H16 as shown in this agarose gel. Well 1 contains 0.1-10 kb ladder. Well 2 is empty. Well 3 (1.) contains a standard PCR reaction mix using universal *16S* primers and *C. necator* H16 DNA. Well 4 (2.) contains Class I primers and *C. necator* H16 DNA. Well 5 is empty. Well 6 (3.) is the same as well 4 only 2 µL of water is substituted for PCR enhancer. Well 7 (4.) contained nearly twice as much Class I primer as well 4, since all 4 µL of water is substituted from primers. Well 8 is empty. Well 9 is a standard PCR reaction mix with Class I primers and no DNA. Well 10 is a standard PCR reaction mixture with universal *16S* primers and no DNA.

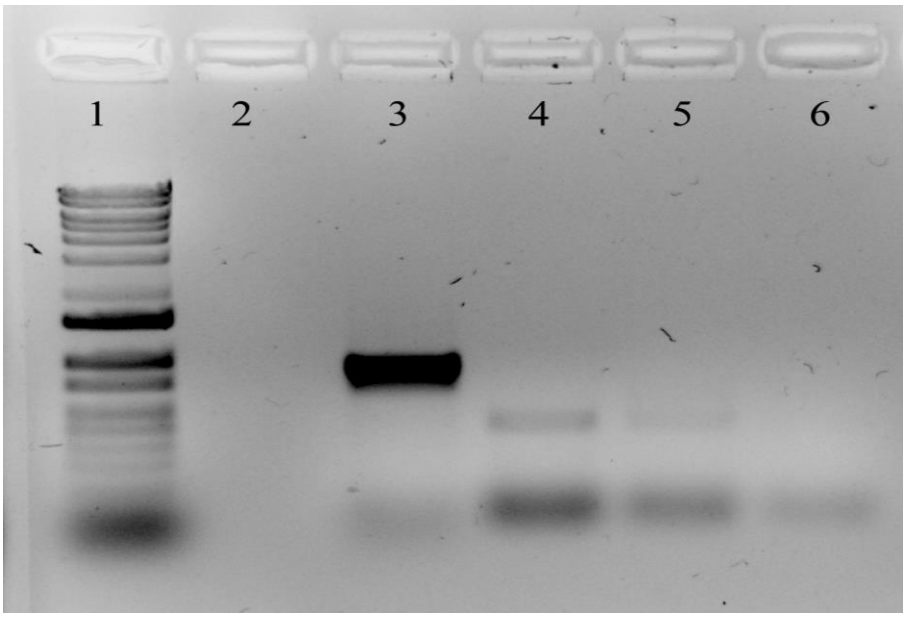


Figure 4.9. Successful amplification using STI primers, designed to probe for the *phaC* in Class I PHA producer *N. nitrogenifigens* Y88, on *T. filiformis* WRT as shown in this agarose gel. Well 1 is a 0.1-10 kb ladder. Well 2 is empty. Well 3 is *16S* amplification with designed Primer3 16S primers on *T. thermophilus* HB8 DNA. Well 4 is *phaC* amplification with 10 µM STI primers. Well 5 is *phaC* amplification with 5 µM STI primers on *T. filiformis* WRT. Well 6 is a *16S* negative.

Table 4.1. Results of probing for selected gene, *16S* or *phaC*, with designed primers on Class I positive *Cupriavidus necator*, Class II positive *Pseudomonas* putida LS46 and *Thermus* species. The results whether amplification occurred (+), no amplification occurred (-) or was not determined (ND) are represented here.

Gene Targets	***16S***		***phaC***			
Primer Set	**928F/336R**	**T.t. 16s**	**Class I**	**Class II**	**Class III**	**STI**
C. necator N-1	+	ND	+	ND	-	+
P. putida	+	ND	ND	+	ND	ND
T. thermophilus HB8	-	+	-	-	-	-
T. *filiformis* MOK	-	+	-	-	-	-
T. filiformis NGM	-	+	-	-	-	-
T. filiformis WRT	-	+	-	-	-	+

4.3.4. Bioinformatic analysis of genes associated with PHA metabolism present in *Thermus thermophilus* HB8

An early candidate for a potential PHA synthase was a putative acyl-CoA synthetase, however its occurrence in all four strains of *T. thermophilus* in the IMG-JGI database and *E. coli* suggest that this enzyme is more likely involved as a ligase for lipid biosynthesis rather than in PHA synthesis. The analysis of *T. thermophilus* HB8 by BLAST analysis using reference *phaC* genes yielded no significant result from any of the selected microorganisms. The amino acid sequence for the PHA synthase of *Bacillus sp.* INT005 provided a partial hit to a hypothetical protein on the pTT27 plasmid of *T. thermophilus* HB8. The result alone is not enough to confirm a *phaC* gene. The hypothetical protein gene of *T. thermophilus* HB8 was then used as a reference to find other similar sequences by BLAST analysis, hoping to identify a likeness to other PHA synthases. Very poor similarity was seen between this protein and all other sequenced genomes, with no *phaC* genes being identified. Therefore, the identified hypothetical protein cannot be identified as a *phaC* gene.

Searching for the enzyme EC 2.3.1.-, which corresponds to PHA synthase, resulted in zero hits in all the available *T. thermophilus* genomes. A look at the KEGG map for butanoate metabolism in *T. thermophilus* HB8 shows the absence of a PHA synthase despite the presence of preceding enzymes in the production of PHB, notably the presence of *phaA* and *phaB* (Figure 4.10) Searching using the COG tag 3243 for a PHA synthase had no improved result. Using the Interpro and TIGRfam numbers associated with all classes of PHA synthases did not provide any potential gene targets that could be a PHA synthase. The search conducted using pfam 00561 and pfam 07167,

an abhydrolase and N-terminal domain, provided hits for the abhydrolase in all four *Thermus* strains. Upon further analysis, this domain is not confined to PHA synthases and the obtained hits did not appear to be PHA synthases. No results were obtained from the N-terminal conserved domain in any of the *Thermus* strains.

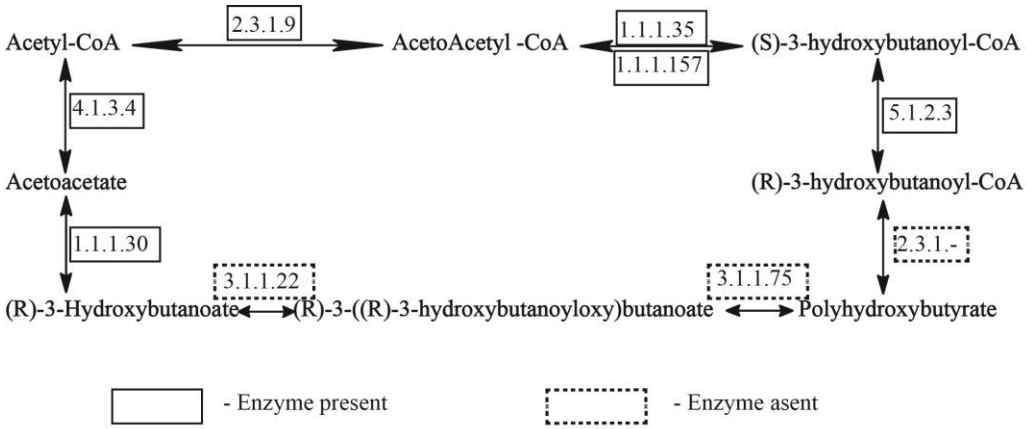


Figure 4.10. KEGG pathway for butanoate metabolism in *Thermus thermophilus* HB8 retrieved from the IMG-JGI database (Markowitz, et al., 2005). Encased is the polyhydroxybutyrate product. Enzyme commission numbers in blue or pink are present in the genome and the rest are not. Refer to Table 4.2.

Table 4.2. Enzyme commission (EC) numbers relevant to the production of PHA, their corresponding names and their appearance in *T. thermophilus* HB8.

E.C. Number	Enzyme Name	Status
2.3.1.-	Polyhydroxyalkanoate Synthase (*phaC*)	Absent
3.1.1.75	Poly(3-hydroxybutyrate) depolymerase	Absent
3.1.1.22	Hydroxybutyrate-dimer lyase	Absent
1.1.1.30	3-hydroxybutyrate dehydrogenase	Present
4.2.1.55	3-hydroxybutyryl-CoA dehydratase	Present
5.1.2.3	3-hydroxyacyl-CoA dehydrogenase	Present
1.1.1.36	Aceto-acetyl-CoA reductase	Absent
1.1.1.35	3-hydroxyacyl-CoA dehydrogenase	Present
1.1.1.157	3-hydroxybutyryl-CoA dehydrogenase	Present
4.2.1.17	Enoyl-CoA hydratase	Present
2.3.1.9	Acetyl-CoA C-Acetyltranferase (*phaA*)	Present
2.4.1-	3-hydroxyacyl-CoA-acyl carrier protein transferase (*phaG*)	Absent

4.4. Conclusion

The putative *phaC* gene in *Thermus* sp. could be genetically divergent from classical PHA synthases due to the extreme temperature at which *Thermus* species grow. Reports of a mixed medium-chain and short-chain length PHA polymer may explain the difficulty in identifying this gene (Pantazaki, et al., 2003). The objective of this study was to identify the PHA synthase gene (*phaC*) in *Thermus* sp. through *16S* phylogeny, *in silico* bioinformatic analyses, and probing with primers though polymerase chain reaction. The phylogenetic data showed that *Thermus* sp. is not closely related to other PHA producing microorganisms. The bioinformatic analyses did not reveal *phaC* in *T. thermophilus* by function, domain, or BLAST analyses. At best a protein query of the PHA synthase from thermophilic *Bacillus* sp. INT005 against *T. Thermophilus* HB8 resulted in a hypothetical protein, which did not resemble other PHA synthases through BLAST analyses. The designed primers did not amplify against the *Thermus* sp. templates. However, the STI primers did result in a band. A poor sequence was obtained from the smaller than expected band and did not align with other *phaC* genes.

The inconclusive hypothetical protein gene and the detection of an amplicon using STI primers could support the hypothesis that the PHA synthase of *Thermus* sp. is highly divergent; however the overall result of our genetic analysis favours the absence of a *phaC* gene.

Chapter 5: Concluding Remarks

This study characterizes growth and PHA production of *Thermus thermophilus* HB8 and three strains of *T. filiformis* (MOK, NGM and WRT), while simultaneously performing a genetic analysis for the presence of a *phaC* gene that encodes the PHA synthase. Due to preliminary PCR results and its relatedness to *T. thermophilus* HB8, the hypothesis was that *T. filiformis* would produce PHA and that the *phaC* gene could be elucidated by PCR. The experimental design consisted of characterizing PHA production in *T. filiformis* and probing for *phaC* using primers, while using *T. thermophilus* HB8 as a positive control for PHA production.

Chapter 3 describes the efforts to characterize growth and PHA accumulation in *Thermus* species. The difficulties in detecting PHA production in *T. thermophilus* HB8 complicated the experimental design. Attempts to reproduce the results by Pantazaki et al. (2003) did not succeed and experiments were designed to grow the *Thermus* species on 697 *Thermus* medium, followed by transfer of the cells into minimal medium, to overcome the growth difficulties in minimal medium that were occurring. While this did not produce PHA in *T. thermophilus* HB8 or *T. filiformis* WRT, the PHA positive *Pseudomonas putida* LS46 produced PHA under the same conditions.

In Chapter 4, the results of bioinformatic analyses of the *Thermus thermophilus* genome sequence were reported. A phylogenetic analysis based on *16S* rDNA sequences indicated that *T. thermophiles* did not associate with other known PHA producing bacteria. Bioinformatic analyses offered a few potential genes that could be interpreted as a *phaC* candidate, and those genes that were tentatively identified as likely to serve other purposes. Finally the PCR analyses with various primer sets designed to amplify the four

classes of PHA synthases resulted in amplification with one particular primer set in *T. filiformis* WRT. The STI primer designed for the *phaC* from *Novosphingobium nitrogenifigens* Y88 resulted in an amplicon that was sent for sequencing. Although this amplicon could represent amplification of a *phaC* from *T. filiformis* WRT, BLAST analysis determined that the gene encoded a hypothetical protein with nothing in common with PHA synthase. Thus, this amplicon was likely a false positive. Furthermore, no amplification occurred in *T. thermophilus* HB8 with this primer set.

The results from the genetic analyses, together with attempts to produce PHA in *T. thermophilus* HB8 and *T. filiformis* strains (WRT, NGM and MOK) infer an absence of PHA accumulation. Although "absence of evidence is not evidence of absence" and does not dismiss the possibility that *Thermus* sp. can produce PHA, it can be concluded that previous reports of *T. thermophilus* HB8 27634 are irreproducible and that *Thermus* sp. is not a strong candidate for the thermophilic production of PHA.

The inability to reproduce *T. thermophilus* HB8 reports of PHA production on minimal medium supplemented with sodium gluconate or octanoic acid is likely to due to a knock-out in our strain of *T. thermophilus* HB8 or an error on their behalf such as contamination (Pantazaki, et al., 2003). Although our findings suggest *Thermus* sp. are not great candidate microorganisms for PHA production, it only strengthens the need for continued research into thermophilic PHA production. Described PHA producers *Caldimonas taiwanensis*, *Pseudomonas* sp. SG4502 and *Bacillus* sp. INT005 are prime candidates for optimization of their PHA production capabilities. *Ferroglobus placidus* grows optimally at 85ºC and has been annotated with a *phaC* gene making it a strong

candidate for thermophilic PHA accumulation. Finally, the primers designed here could be applied to thermophilic isolates to screen for more potential PHA producers.

References

Amara AA, Rehm BHA. 2003. Replacement of the catalytic nucleophile cysteine-296 by serine in class II polyhydroxyalkanoate synthase from *Pseudomonas aeruginosa*-mediated synthesis of a new polyester: identification of catalytic residues. Biochem. J. **374**(2): 413-421.

Andreeßen B, Lange AB, Robenek H, Steinbüchel A. 2010. A.Conversion of Glycerol to Poly(3-Hydroxypropionate) in Recombinant *Escherichia coli.* Appl. Environ. Microbiol. **76**(2): 622-626.

Aneja KK, Ashby RD, Solaiman DKY. 2009. Altered composition of *Ralstonia eutropha* poly(hydroxyalkanoate) through expression of PHA synthase from *Allochromatium vinosum* ATCC 35206. Biotechnol. Lett. **31**(10): 1601-1612.

Beffa T, Blanc M, Lyon P-F, Vogt G, Marchiani M, Fischer JL, Aragno M. 1996. Isolation of *Thermus* strains from hot composts (60 to 80 degrees C) Appl. Environ. Microbiol. **62**(5): 1723-1727

Berlanga M, Montero MT, Hernandez-Borrell J, Guerrero R. 2006. Rapid spectrofluorometric screening of poly-hydroxy-alkanoate-producing bacteria from microbial mats. Int. Microbiol. **9**(2): 95-102.

Braunegg G, Sonnleitner B, Lafferty RM. 1978. A rapid gas chromatographic method for the determination of poly-B-hydroxybutyric acid in microbial biomass. European J. Appl. Microbiol. Biotechnol. **6**: 29-37.

Cai L, Tan D, Aibaidula G, Dong Z-R, Chen J-C, Tian W-D, Chen G-Q. 2011. Comparative genomics study of polyhydroxyalkanoates (PHA) and ectoine relevant genes from *Halomonas* sp. TD01 revealed extensive horizontal gene transfer events and co-evolutionary relationships. Microbial Cell Factories. **10**(88)

Choi J-I, Lee S-Y. 1997. Process analysis and economic evaluation for Poly(3-hydroxybutyrate) production by fermentation. Bioprocess Engineering. **17**(6): 335-342

Diamond D. 2001. Determination of ammonia (phenolate) by flow injection analysis. QuikChem® Method 10-107-06-1-I. - [s.l.]. Lachat Instruments.

Faccin DJL, Martins I, Cardoza NSM, Rech R, Ayub MAZ, Alves TLM, Gambetta R, Secchi AR. 2009. Optimization of C:N ratio and minimal initial carbon source for poly(3-hydroxybutyrate) production by *Bacillus megaterium*. J. Chem. Tech. Biotechnol. **84**(12): 1756-1761.

Fu J, Zhang XL, Sharma PK, Spicer, V, Krokhin OV, Fristensky B, Wilkins JA, Sparling R, Cicek N, Levin DB. 2012. Elucidation of bioplastic production using *Pseudomonas putida* LS46 from industrial wastes and its cellular production mechanism. Gairdner Foundation-Genome Canada "State of the Science" Event, November 27th and 28th, Ottawa, Canada.

Fukui T, Shiomi N, Doi Y. 1998. Expression and Characterization of (R)-Specific Enoyl Coenzyme A Hydratase Involved in Polyhydroxyalkanoate Biosynthesis by *Aeromonas caviae*. J. Bacteriol. **180**(3): 667-673.

Gregory ST, Carr JT, Rodriguez-Correa D, Dalhberg, AE. 2005. Mutational Analysis of 16S and 23S rRNA genes of *Thermus thermophilus*. J. Bacteriol. **187**(14): 4804-4812.

Hai T, Hein S, Steinbüchel A. 2001. Multiple evidence for widespread and general occurrence of type-III PHA synthases in cyanobacteria and molecular characterization of the PHA synthases from two thermophilic cyanobacteria: *Chlorogloeopsis fritschii* PCC 6912 and *Synechococcus* sp. strain MA19. Microbiology. **147**(11): 3047-3060.

Hai T, Lange D, Rabus R, Steinbüchel, A. 2004. Polyhydroxyalkanoate (PHA) Accumulation in Sulfate-Reducing Bacteria and identification of a Class III PHA Synthase (PHAEC) in *Desulfococcus multivorans*. Appl. Environ. Microbiol. **70**(8): 4440-4448.

Hoffmann N, Steinbüchel A, Rehm BH. 2000. The *Pseudomonas aeruginosa* phaG gene product is involved in the synthesis of polyhydroxyalkanoic acid consisting of medium-chain-length constituents from non-related carbon sources. FEMS Microbiol. Lett. **184**(2): 253-259.

Hudson JA, Morgan HW, Daniel RM. 1987. *Thermus filiformis* sp. nov. a Filamentous Caldoactive Bacterium. Int. J. of Syst. Bacteriol. **37**(4): 431-436.

Huismann GW, Wonink E, Meima R, Kazemier B, Terpstra P, Witholt B. 1991. Metabolism of Poly(3-hydroxyalkanoates)(PHAs) by *Pseudomonas oleovorans*. J. Biol. Chem. **266**(4): 2191-2198.

Ibrahim MHA, Willems A, Steinbüchel A. 2010. Isolation and characterization of new poly(3HB)-accumulating star-shaped cell-aggregates-forming thermophilic bacteria. J. Appl. Microbiol. **109**(5): 1579-1590.

Jendrossek D, Handrick R. 2002. Microbial degradation of polyhydroxyalkanoates. Annu. Rev. Microbiol. **56**: 403-432.

Jia Y, Kappock J, Frick T, Sinskey AJ, Stubbe J. 2000. Lipases Provide a New Mechanistic Model for Polyhydroxybutyrate (PHB) Synthases: Characterization of the

Functional Residues in *Chromatium vinosum* PHB synthase. Biochemistry. **39**(14): 3927-3936.

Jia Y, Yuan W, Wodzinska J, Park C, Sinskey AJ, Stubbe J. Mechanistic Studies on Class I Polyhydroxybutyrate (PHB) Synthase from *Ralstonia eutropha*: Class I and III Synthases Share a Similar Catalytic Mechanism. Biochemistry. **40**(4): 1011-1019.

Kadouri D, Jurkevitch E, Okon Y. 2005. Ecological and Agricultural Significance of Bacterial Polyhydroxyalkanoates. Crit. Rev. Microbiol. **31**(2): 55-67.

Kitamura S, Doi Y. 1994. Staining Method of Poly(3-hydroxyalkanoic acids) producing Bacteria by Nile Blue. Biotechnol Tech. **8**(5): 345-350.

Klinke S, Roo GD, Witholt B, Kessler B. 2000. Role of phaD in Accumulation of Medium-Chain-Length Poly(3-Hydroxyalkanoates) in *Pseudomonas oleovorans*. Appl. Environ. Microbiol. **66**(9): 3705-3710.

Kranz RG, Gabbert KK, Madigan MT. 1997. Positive Selection Systems for Discovery of Novel Polyester Biosynthesis Genes Based on Fatty Avid Detoxification. Appl. Environ. Microbiol. **63**(8): 3010-3013.

Langenbach S, Rehm BHA, Steinbüchel A. 1997. Functional expression of the PHA synthase gene phaC1 from *Pseudomonas aeruginosa* in *Escherichia coli* results in poly(3-hydroxyalkanoate) synthesis. FEMS Microbiol. Lett. **150**(2). 303-309.

Lee SY. 1996. Bacterial Polyhydroxyalkanoates. Biotechnol. Bioeng. **49**(1): 1-14.

Lee T-R, Lin JS, Wang S-S, Shaw G-C. 2004. PhaQ, a New Class of Poly-B-Hydroxybutyrate (PHB)-Responsive Repressor, Regulates phaQ and phaP (Phasin) Expression in *Bacillus megaterium* through Interaction with PHB. J. Bacteriol. **186**(10): 3015-3021.

Nelson DL, Cox MM. 1993. Principles in Biochemistry. Fourth edition.Lehninger. W.H. Freeman and company. New York.

LeMeur S, Zinn M, Egli T, Thöny-Meyer L, Ren Q. 2012. Production of medium-chain-length polyhydroxyalkanoates by sequential feeding of xylose and octanoic acid in engineered *Pseudomonas putida* KT2440. BMC Biotechnol. **12**(53)

Lowry O, Rosebrough N, Farr A, Randall R. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**: 265-275.

Madison LL, Huisman Gjalt W. 1999. Metabolic Engineering of Poly(3-hydroxyalkanoates):From DNA to Plastic. Microbiol. Mol. Biol. Rev. **63**(1): 21-53.

Markowitz VM, Korzeniewski F, Palaniappan K, Szeto E, Werner G, Padki A, Zhao X, Dubchak I, Hugenholtz P, Anderson I, Lykidis A, Mavromatis K, Ivanova N, Kyrpides NC. 2005. The integrated microbial genomes (IMG) system. Nucleic Acids Res. **34**(1):D344-348.

Matsusaki H, Abe H, Taguchi K, Fukui T, Doi Y. 2000. Biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxyalkanoates) by recombinant bacteria expressing the PHA synthase gene phaC1 from *Pseudomonas* sp. 61-3. Appl. Microbiol. Biotechnol. **53**(4): 401-409.

Matsusaki H, Manji S, Taguchi K, Kato M, Fukui T, Doi, Y. 1998. Cloning and Molecular Analysis of the Poly(3-hydroxybutyrate) and Poly(3-hydroxybutyrate-co-hydroxyalkanoate) Biosynthesis Genes in *Pseudomonas* sp. Strain 61-3. J. Bacteriol. **180**(24): 6459-6467.

McCool GJ, Cannon MC. 2001. PhaC and PhaR Are Required for Polyhydroxyalkanoic Acid Synthase Activity in *Bacillus megaterium*. J. Bacteriol. **183**(14): 4235-4243.

Mittendorf V, Robertson EJ, Leech RM, Krüger N, Steinbüchel A, Poirier Y. 1998. Synthesis of medium-chain-length polyhydroxyalkanoates in *Arabidopsis thaliana* using intermediates of peroxisomal fatty acid β-oxidation. PNAS. **95**(23): 13397-13402.

Müh U, Sinskey AJ, Kirby DP, Lane WS, Stubbe J. 1999. PHA Synthase from *Chromatium vinosum*: Cysteine 149 Is Involved in Covalent Catalysis. Biochemistry. **38**(2): 826-837.

Nagamani P, Mahmood S. 2011. Synthesis of SCL-LCL-PHA Co-Polymer from Starch by *Pseudomonas*. Pharmacologyonline. **3**: 935-943

Ojumu TV, Yu J, Solomon BO. 2004. Production of Polyhydroxyalkanoates, a bacterial biodegradable polymer. Afr. J. Biotech. **3**(1): 18-24.

Oshima T, Imahori K. 1974. Description of *Thermus thermophilus* (Yoshida and Oshima) comb. nov., a Nonsporulating Thermophilic Bacterium from a Japanese Thermal Spa. Int. J. Syst. and Evol. Microbiol. **24**(1): 102-112.

Pantazaki AA, Tambaka MG, Langlois V, Guerin P, Kyriakidis DA. 2003. Polyhydroxyalkanoate (PHA) biosynthesis in *Thermus thermophilus*: Purification and biochemical properties of PHA synthase. Mol. Cell. Biochem. **254**(1-2):173-183.

Pantazaki AA, Ioannou AK, Kyriakidis DA. 2005. A thermostable B-ketothiolase of polyhydroxyalkanoates (PHAs) in *Thermus thermophilus*: Purification and biochemical properties. Mol. Cell. Biochem. **269**(1): 27-36.

Pantazaki AA, Papaneophytou CP, Lambropoulou DA. 2011. Simultaneous polyhydroxyalkanoates and rhamnolipids production by *Thermus thermophilus* HB8. AMB express. **1**(1): 17.

Papi RM, Mimikakou GE, Pantazaki AA, Kyriakidis D. 2008. Identification of PHA loci in *Thermus thermophilus* HB8 genome. The FEBS Journal. **275**(1): 422-422.

Park SJ, Lee SY. 2003. Identification and Characterization of a New Enoyl Coenzyme A Hydratase Involved in Biosynthesis of Medium-Chain-Length Polyhydroxyalkanoates in Recombinant *Escherichia coli*. J. Bacteriol. **185**(18): 5391-5397.

Peoples OP, Sinskey AJ. 1989. Poly-B-hydroxybutyrate (PHB) Biosynthesis in *Alcaligenes eutrophus*. J. Biol. Chem. **264**(26): 15289-15303.

Peoples OP, Sinskey AJ. 1989. Fine structural analysis of the *Zoogloea ramigera* phbA-phbB locus encoding beta-ketothiolase and acetoacetyl-CoA reductase:nucleotide sequence on phbB. Mol. Microbiol. **3**(3):349-357.

Plackett D, Vasquez A. 2005. Natural polymer sources. Green Composites: Polymer Composites and The Environment. Baillie Caroline. – Cambridge. Woodhead Publishing Limited.

"Retention in Landfill." *Canadian Plastics Industry Association*. http://www.plastics.ca/EnvironmentalSustainability/RetentionLandfill/index.php

Poirier Y, Dennis DE, Klomparens K, Sommerville, C. 1992. Polyhydroxybutyrate, a biodegradable thermoplastic, produced in transgenic plants. Science. **256**(5056): 520-523.

Qi Q, Rehm BHA, Steinbüchel A. 1997. Synthesis of poly(3-hydroxyalkanoates) in *Escherichia coli* expressing the PHA synthase gene phaC2 from *pseudomonas aeruginosa*: comparison of PhaC1 and PhaC2. FEMS Microbiol. 157(1): 155-162.

Qi Q, Rehm BHA. 2001. Polyhydroxybutyrate biosynthesis in *Caulobacter crescentus*: molecular characterization of the polyhydroxybutyrate synthase. Microbiology. **147**(12): 3353-3358.

Ramsay B, Saracovan I, Ramsay J, Marchessault R. 1991. Effect of nitrogen limitation on long-side-chain poly-B-hydroxyalkanoate synthesis by *Pseudomonas resinovorans*. Appl. Environ. Microbiol. **58**(2): 744-746.

Rehm BH. 2003. Polyester Synthases: natural catalysts for plastics. The Biochemical Journal. **376**(1): 15-33.

Rehm BHA, Steinbüchel A. 2002. PHA Synthases: The Key Enzymes of PHA Synthesis. Biopolymers Online. Steinbüchel A. Jon Wiley and Sons.

Rehm BHA, Krüger N, Steinbüchel A. 1998. A New Metabolic Link between Fatty Acid de NovoSynthesis and Polyhydroxyalkanoic Acid Synthesis. J. Biol. Chem. **273**(37): 24044-24051.

Ren Q, Sierro N, Witholt B, Kessler, B. 2000. FabG, an NADPH-Dependent 3-Ketoacyl Reductase of *Pseudomonas aeruginosa*, Provides Precursors for Medium-Chain-Length Poly-3-Hydroxyalkanoate Biosynthesis in *Escherichia coli*. J. Bacteriol. **182**(10): 2978-2981.

Romo DM, Grosso MV, Solano NC, Castano DM. 2007. A most effective method for selecting a broad range of short and medium chain-length polyhydroxyalkanoate producing microorganisms. J. Biotechnol (Electronic). **10**(3)

Satoh Y, Tajima K, Nakamoto S, Xuerong H, Matsushima T, Ohshima T, Kawano S, Erata T, Dairi T, Munekata M. 2011. Isolation of a thermotolerant bacterium producing medium-chain-length polyhydroxyalkanoate. J. Appl. Microbiol. **111**(4): 811-817.

Satoh Y, Minamoto N, Tajima K, Munekata M. 2002. Polyhydroxyalkanoate Synthase from *Bacillus* sp. INT005 Is Composed of PhaC and PhaR. J. Biosci Bioeng. 94(4): 343-350.

Sharma PK, Fu J, Cicek N, Sparling R, Levin DB. 2012. Kinetics of medium-chain-length polyhydroxyalkanoate production by a novel isolate of *Pseudomonas putida* LS46. Can. J. Microbiol. **58**(8): 982-989.

Sheu D-S, Chen W-M, Yang J-Y, Chang R-C. 2009. Thermophilic bacterium *Caldimonas taiwanensis* produces poly(3-hydroxybutyrate-co-3-hydroxyvalerate) from starch and valerate as carbon sources. Enz. Microbial Technol. **44**(5): 289-294.

Sheu D-S, Chen W-M, Lai Y-W, Chang R-C. 2012. Mutations Derived from the Thermophilic Polyhydroxyalkanoate Synthase PhaC Enhance the Thermostability and Activity of PhaC from *Cupriavidus necator* H16. J. Bacteriol. **194**(10): 2620-2629.

Sheu D-S, Wang Y-T, Lee C-Y. 2000. Rapid Detection of polyhydroxyalkanoate-accumulating bacteria isolated from the environment by colony PCR. Microbiology. **146**(8): 2019-2025.

Solaiman DKY, Ashby RD. 2005. Genetic Characterization of the Poly(hydroxyalkanoate) Synthases of Various *Pseudomonas oleovorans* Strains. Current Microbiology. **50**: 329-333.

Spiekermann P, Rehm BH, Kalscheuer R, Baumeister D, Steinbüchel. 1999. A sensitive, viable-colony staining method using Nile red for direct screening of bacteria that

accumulate polyhydroxyalkanoic acids and other lipid storage compounds. Arch. Microbiol. **171**(12): 73-80.

Steinbüchel A, Hustede E, Liebergesell M, Pieper U, Timm A, Valentin H. 1992. Molecular basis for biosynthesis and accumulation of polyhydroxyalkanoic acids in bacteria. FEMS Microbiol. Rev.**9**(2-4): 217-230.

Steinbüchel A, Füchtenbusch B. 1998. Bacterial and other biological systems for polyester production. Trends in Biotechnol. **16**(10): 419-427.

Sudesh K, Kunasundari B. 2011. Isolation and recovery of microbial polyhydroxyalkanoates. eXPRESS Polymer Letters. **5**(7): 620-634.

Tajima K, Igari T, Nishimura D, Nakamura M, Satoh Y, Munekata M. 2003. Isolation and characterization of *Bacillus* sp. INT005 Accumulating Polyhydroxyalkanoate (PHA) from Gas Field Soil. J. Biosci. Bioeng. **95**(1): 77-81/

Timm A, Steinbüchel A. 1990. Formation of Polyesters Consisting of Medium-Chain-Length 3-Hydroxyalkanoic Acids from Gluconate by *Pseudomonas aeruginosa* and Other Fluorescent Pseudomonads. Appl. Environ. Microbiol. **56**(11): 3360-3367.

Tokiwa Y, Calabia BP, Ugwu CU, Aiba S. 2009. Biodegradability of Plastics. Int. J. Mol. Sci. **10**(9): 3722-3742

Valentin HE, Dennis D. 1997. Production of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) in recombinant *Escherichia coli* grown on glucose. J. Biotechnol. **58**(1): 33-38.

Valentin HE, Steinbüchel A. 1994. Application of enzymatically synthesized short-chain-length hydroxy fatty acid coenzyme A thioesters for assay of polyhydroxyalkanoic acid synthases. Appl. Microbiol. Biotechnol. **40**: 699-709.

Vo MT, Lee KW, Jung YM, Lee Y-H. 2008. Comparative effect of overexpressed phaJ and fabG genes supplementing (R)-3-hydroxyalkanoate monomer units on biosynthesis of mcl-polyhydroxyalkanoate in *Pseudomonas putida* KCTC1639 J. Biosci. and Bioeng. **106**(1): 95-98.

Yew S, Jau M, Yong K, Abed R, Sudesh K. 2005. Morphological studies of *Synechococcus* sp. UNIWG under polyhydroxyalkanoate accumulating conditions. Malaysian J. Biotechnol. **1**(1): 48-52.

Yuan W, Jia Y, Tian J, Snell KD, Muh U, Synskey AJ, Lambalot RH, Walsh CT, Stubbe J. 2001. Class I and III Polyhydroxyalkanoate Synthases from *Ralsltonia eutropha* and *Allochromatium vinosum*: Characterization and Substrate Specificity Studies. Arch. Biochem. Biophys. **394**(1): 87-98.

Appendix: Sample Calculations

Sample calculation for gas chromatography data

To calculate the percent dry cell weight of each carbon monomer, their peak areas were compared to that of our internal standard (benzoic acid). The ratios were calculated by comparing peak areas of equal concentrations of 3-HA monomer standards to benzoic acid.

The equation is:

$$\frac{\text{mL of Benzoic acid solution X Carbon monomer peak area}}{\text{sample weight (mg) X Internal standard peak area}} \text{ X R X 100\%}$$

R = 1.9 for C4, 1.27 for C6 and C8, 1.04 for C10 and 1.44 for C12