BACTERIOPHAGE FACILITATED EXTRACTION OF POLYHYDROXYALKANOATES PRODUCED BY *PSEUDOMONAS OLEOVORANS* GROWN ON SYNTHETIC CRUDE GLYCEROL

A Thesis

by

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ABSTRACT

Polyhydroxybutyrate (PHB), a type of polyhyroxyalkanoate (PHA) bioplastic, is an attractive alternative to traditional petrochemical-derived plastics, such as polypropylene. However, PHB is costly to produce due to high feedstock and extraction costs. Crude glycerol from biodiesel production is a potential economic carbon source for PHA production, but crude glycerol often contains high levels of impurities, such as sodium salts and methanol, which might inhibit microbial growth. This study investigated the ability of *Pseudomonas oleovorans* to produce PHB when grown on glycerol and glycerol impurities, followed by the application of bacteriophage to extract PHB from PHB-bearing cells.

During growth on glycerol, *P. oleovorans* was able to accumulate up to 31.1 % of cell dry weight (CDW) as PHB. The accumulation of PHB was influenced by molar carbon:nitrogen ratios (C/N) in the growth media, with the highest accumulation of PHB occurring at a C/N ratio of 21.5. Additionally, *P. oleovorans* was found to tolerate the common glycerol impurities NaCl (0-2 g/L) and methanol (0-2 g/L) with no significant loss of PHB accumulation. A bacteriophage, Ke14, targeting *P. oleovorans* was isolated from soil samples in College Station, TX. Ke14 was found to be able to lyse PHB-bearing cells once they were allowed to return to exponential growth stage.

We have shown *P. oleovorans* is an attractive candidate for PHB production due to its ability to produce PHB in the presence of common glycerol impurities found in biodiesel by-product crude glycerol with no adverse effect on PHB accumulation.

Bacteriophage facilitated lysis of glycerol containing *P. oleovorans* was observed. This indicates that alternative methods to traditional PHB extraction methods may be feasible.

DEDICATION

This thesis is dedicated to my wife, Kelsey. Without your constant encouragement and support, I would be streets behind.

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Thanks to my parents. I have been blessed with two amazing sets of parents who have provided endless support and encouragement. Finally, thanks to my amazing wife. She has selflessly endured long laboratory hours and an unnecessarily cranky husband while never ceasing to reassure and encourage.

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1. INTRODUCTION

Polyhydroxyalkanoates (PHAs) are biopolymers synthesized and stored in intracellular granules by many microorganisms [1-5]. PHAs are attractive alternatives to traditional petroplastics such as polypropylene [6, 7] due to their biodegradability and biocompatibility. PHAs can also be widely used in medical applications [8]. However, they are expensive to produce, with common PHAs such as [R]-3-hydroxybutryic acid (PHB) costing between 3.1 and 4.4 USD/kg [9] compared to polypropylene costing less than 1 USD/kg [10, 8]. PHB is typically removed from PHB producing cells through extraction using solvents such as chloroform and dichloroethane or through hypochlorite digestion [11, 12]. Because large-scale production cost of PHB is governed by costs of feedstock (25-45% production cost) [9] and separation processes (30% production cost) [13], more cost-effective production of PHB could be attained by utilizing better separation methods and cheaper carbon sources, such as waste glycerol from biofuel production.

Biofuels such as biodiesel are increasingly sought as alternatives to traditional petroleum fuels and products due to their sustainability and decreased greenhouse gas emission [14, 15]. The production of biodiesel through transesterification generates significant levels of crude glycerol as a byproduct (1 kg of crude glycerol is produced per kg of biodiesel) [16]. Increasing biodiesel production demands are expected to increase the availability of crude glycerol; biodiesel production is expected to reach 37 billion gallons per year in 2016, resulting in almost 4 billion gallons of crude glycerol [17].

However, crude glycerol generated from the transesterification process (which requires alkyl alcohols and catalysts such NaOH) contains high levels of impurities, such as sodium and potassium, methanol, and soaps [18-20]. The toxicity and growth-inhibitory effects of common crude glycerol impurities limit the productivity of some microorganisms when used as a sole carbon source [20, 19]. Despite these limitations, there is significant interest in utilizing crude glycerol as a low-cost feedstock to generate value-added products [20, 21, 19, 22, 16] including PHB [22, 20].

Over 300 different microorganisms have been found to be capable of producing PHAs such as PHB [23, 24, 1, 5, 25]. Production of PHB can be both growth- and non-growth-associated [26, 25, 6, 27, 28], and occurs under nitrogen- limiting conditions [29, 24, 23]. While nitrogen-limiting conditions are necessary for PHB production, the carbon/nitrogen ratio (C/N, moles of carbon per mole of nitrogen) of the media in which microorganisms are cultured has been shown to affect both PHB accumulation and the chain length of PHB produced [30-32]. *Pseudomonas oleovorans* is a well-studied, Gram negative gammaproteobacteria, which is known to produce growth-associated PHB [27, 5, 33]. *P. oleovorans* has been found to tolerate high salt concentrations [34], and is highly tolerant of organic solvents [35]. Additionally, *P. oleovorans* is capable of growth with glycerol as the sole carbon source [36-39]. Because of these characteristics, *P. oleovorans* was selected as an ideal candidate for PHB production using crude glycerol as the sole carbon source.

Once the bacterium has accumulated PHB granules, disruption of the cells and release of the product poses an additional challenge. Bacteriophage lysis is an enticing

alternative extraction technique for removing PHA granules from cells without the need for additional solvents or chemicals, thereby reducing the costs of PHB extraction [40, 41, 13, 42]. Previous studies have focused on extraction of PHA via cloning phage lysis cassettes into PHB-producing strains which were cultured on rich media, or on the utilization of glycerol for PHB production. These methods often require additional chemicals or treatments to express the lysis plasmids, and must carefully balance cell stability/viability and extraction facilitator [13, 41, 42]. No study has been conducted using lysis of PHB-filled cells, which are typically in a stationary phase of growth, with exogenously supplied phage. Further, the interactions of phages with their hosts while in stationary phase is generally a well-studied area.

It has been found that some phages do not readily bind to or infect stationary phase cells [43, 44]. Accordingly, this method requires controlled culture and growth conditions in order to properly induce phage binding and cell lysis for PHB extraction. In this study, *P. oleovorans* was selected to test the effects of glycerol impurities and C/N ratio on growth and PHB accumulation, followed by phage-facilitated lysis of cells to release PHB. To our knowledge, this is the first study to investigate glycerol as a sole carbon source to cultivate PHB-accumulating bacterium, followed by phage-based PHB extraction.

The goals, specific tasks and hypotheses of this study were:

Goal 1: Determine culture conditions favorable to PHA production in *Pseudomonas oleovorans* on biodiesel waste glycerol.

Hypothesis 1: Increased C:N ratio will result in higher PHB accumulation as percent cell dry weight (% CDW)

Task 1a: Develop and validate method to quantify PHB using photospectroscopy.

Task 1b: Grow *P. oleovorans* at varying glycerol and ammonium concentrations to control C:N ratio at fixed NaCl concentrations. Extract and quantify PHB to determine PHB as % CDW

Hypothesis 2: Increased NaCl content will result in lower PHB accumulation as % CDW

Task 1c: Grow *P. oleovorans* with varying NaCl concentrations and fixed glycerol and ammonium concentrations to determine NaCl effects on PHB accumulations as % CDW.

Hypothesis 3: The presence of methanol at expected crude glycerol concentrations will not inhibit PHB production.

Task 1d: Grow *P. oleovorans* with varying methanol concentrations and fixed glycerol, ammonium, and salt concentrations to determine NaCl effects on PHB accumulations as % CDW.

Goal 2: Characterize a bacteriophage capable of lysing *P. oleovorans* for PHB extraction.

Hypothesis 4: A lytic bacteriophage capable of infecting *P. oleovorans* can be isolated from soil samples and characterized

Task 2a: Isolate *P. oleovorans* phage from soil samples collected at various locations throughout College Station.

Task 2b: Perform adsorption velocity test to determine kinetics of isolated phage adsorption.

Task 2c: Perform one-step grow test to determine phage latency period and burst size. Add phage to glycerol-grown cells for lysis. Measure phage and cell OD to see if cell lysis occurs for PHB containing cells.

Task 2d: Perform sucrose gradient ultracentrifugation to isolate extracted PHB from cell debris after lysis. This data can then be compared to unlysed control to determine efficiency of PHB release due to phage treatment.

2. LITERATURE REVIEW

2.1 Biodiesel process

Biodiesel is a fuel composed of alkyl esters obtained from a variety of organic sources of triacylglycerols including, but not limited to: vegetables oils, animal fats, or microbial and algal lipids. Triacylglycerols lipids which are composed of three fatty acids attached to a glycerol backbone (Fig. 1). To produce biodiesel triacylglycerols are reacted with an alcohol, such as methanol or ethanol, in a transesterification (Fig. 2) process which results in the formation of three alkyl esters (biodiesel) and one molecule of glycerol. This process results in the formation of 1 kg of glycerol for every 10 kg of biodiesel produced. As global biodiesel production is estimated to increase to 37 billion gallons per year by the year 2016, this will mean that almost 4 billion gallons of crude glycerol will be produced [17]. Accordingly, glycerol is increasingly being pursued as a value-added byproduct from biodiesel production [22, 20].

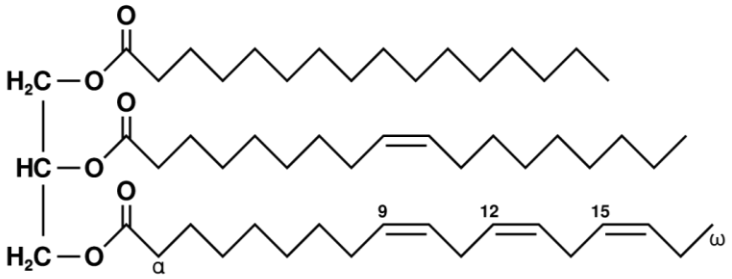


Figure 1. Typical triacylglycerol molecule structure. In this case composed of glycerol, palmitic acid, oleic acid, and alpha-linolenic acid.

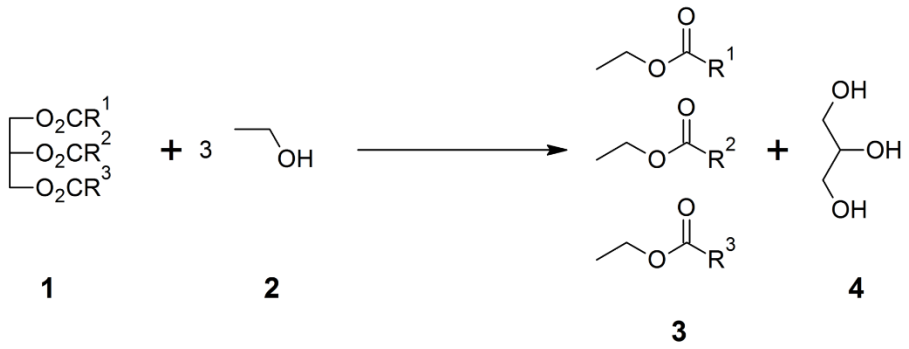


Figure 2. Typical biodiesel reaction wherein triacyglycerol (1) is reacted with an alcohol (2) to produce alkyl esters (3) and glycerol (4).

The transesterification reaction is typically catalyzed by the addition of strong base, e.g. NaOH [45, 46]. Accordingly, the resultant crude glycerol waste stream is characterized by the presence of residual methanol and salts [18, 20]. However, the exact composition of crude glycerol can vary depending on the source and catalyst. Pyle et al. [19] found that the methanol content of crude glycerol can vary from 12.8 to 28.3% (w/w). The soap content was found to range between 15.3 and 25.17% (w/w). The ranges in soap content are largely attributable to the difference in feedstock for biodiesel production. These soaps can be split into free fatty acids and salt through the addition of strong acid such as HCl [47, 19]. Pyle et al. also found the glycerol from KOH catalyzed sources to contain up to 31,250 ppm of elemental potassium. Similarly, NaOH catalyzed sources had elemental sodium levels of 12,550 ppm. Trace amounts of boron, calcium, and silicon were also found. Similarly, Wijesekara et al. [21] found that crude glycerol could contain up to 3% salts and 50% methanol (w/w). Rymowicz et al. [48] found up to 4% salt but

only 0.1% (w/w) methanol. This variation in methanol concentration is likely due to differeing levels methanol recovery in various biodiesel/glycerol producing systems. Accordingly, any system designed to use crude glycerol as a sole or primary carbon source must utilize microorganisms capable of withstanding moderate concentrations of salts and methanol.

2.2 Crude glycerol applications

An enticing option for utilization of waste crude glycerol is in the production of chemicals through biotechnology [20]. Hiremath et al. have shown that *Klebsiella pneumoniae* ATCC 15380 are capable of producing 56 g/L of 1,3-propanediol, a chemical utilized in the synthesis of various polymers, using crude glycerol [49]. André et al. were able to use crude glycerol-grown *Aspergillus niger* to produce 21.5 g/L of oxalic acid [50]. Similarly, Morgunov et al. were able use crude glycerol-grown *Yarrowia lipolytica* NG40/UV7 to produce 115 g/L of citric acid [51]. Sabourin-Provost and Hallenbeck found *Rhodopseudomonas palustris* to be capable of photochemically converting glycerol to H_2 at 75% of the theoretical efficiency (6 mole of H_2/mole of glycerol) [52]. Additionally, Pyle et al. produced 1.63 g/L of docosahexaenoic acid, an omega-3 polyunsaturated fatty acid human diet supplement, using crude-glycerol grown *Schizochytrium limacinum* [19]. Several studies have shown a variety of microorganisms are capable of converting crude glycerol into ethanol at high yields, 25-27 g/L [53, 54]. Similarly, bioplastics such as polyhyhroxyalkanoates (PHAs) are attractive alternatives to

traditional petroleum derived plastics due to their biodegradability and biocompatibility [55-57].

2.3 Bioplastics

Bioplastics are alternatives to traditional petroplastics which can be defined according to European standard EN13432 as plastics which are biodegradable and compostable. These bioplastics can be further divided into bio- or fossil based bioplastics depending on the resources utilized in their production. Hereafter, bioplastic will refer to bio-based bioplastics per EN13432. Bioplastics are a highly diverse field of desirable products which are increasingly being pursued as alternative to due to their sustainability and renewable sources [28].

Different bioplastics hold varying physical properties and applications. Common bioplastics include starch-, cellulose-, and protein-based bioplastics, polylactic acid, and PHAs.

A common starch-based bioplastic is thermoplastic starch (TPS) [28]. TPS is attractive due to its ready biodegradability and low cost and commonly used in food packaging [58, 28]. Cellulose-based bioplastics range from wood pulp cartons used in food and plant packaging to cellulose acetate membranes. Protein-based bioplastics include formaldehyde-free adhesives for wood treatments such as Soyad® adhesive [59]. Polylactic acid is a polyester with high transparency and gloss that makes it an attractive alternative food-packaging choice [28]. PHAs are another bioplastic with specific,

desirable characteristics that serves as both a biodegradable alternative to petroplastics, such as polypropylene, and biocompatible medical material.

2.3.1 Polyhydroxyalkanoates (PHAs)

PHAs (Fig. 3) are compostable polyesters first discovered by Maurice Lemoigne in 1926 [33]. PHAs accumulate in intercellular granules which functional as energy and carbon storage for bacteria [60]. PHAs are desirable products due to their biocompatibility for medical applications such as sutures, heart valves, urological stents, surgical meshes, etc. [8]. PHAs are currently sold by Metabolix, Kaneka Corporation/Proctor & Gamble, Mitisubishi Gas Chemical, and Biomer as Biopol®, Nodax®, Biogreen, and Biomer, respectively. However, typical costs of glucose produced PHAs, such as [R]-3-hydroxybutryic acid (PHB), are very high, between 3.1 and 4.4 USD/kg [9]. Because substrate/materials costs can account for up to 20% of the total cost of PHB production [9], it is crucial to utilize a low cost feedstock alternative such as crude glycerol.

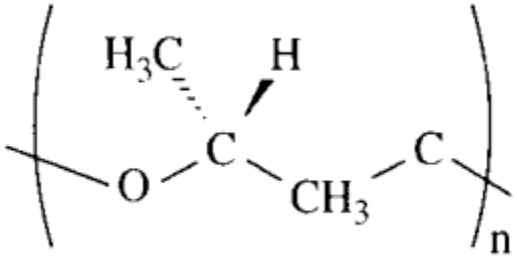


Figure 3. Structure of [R]-3-hydroxybutryic acid monomer, a typical bacterially produced PHA.

2.3.2 Bacterial production of PHAs

Several bacterial strains have been found to be capable of producing PHB at varying culture conditions and yield (Table 1) [23, 24, 1, 5]. *Pseudomonas oleovorans* is a well-studied soil isolate that was known to produce PHB in nitrogen-limiting conditions [5, 27, 33]. Lageveen et al. [5] found that nitrogen limited *P. oleovorans* grown on various alkanes accumulated PHB up 24% cell dry weight (CDW). However, cells grown with no nitrogen limitation produce approximately 5% PHB of CDW. Likewise, Brandl et al. [27] found that alkanoate-grown *P. oleovorans* accumulated up to 35.6% PHA of CDW when grown with 10 mM octanoate and 5 mM NH^+ (C-N ratio of 16/1) [27]. They found that lower C/N ratios led to decreased PHA content - 35.6% PHA and 24.2% PHA at C/N ratios of 16/1 and 8/5, respectively.

Table 1. Known PHB accumulating bacterial strains capable of growth using glycerol as sole carbon source shown with associated PHB accumulation, nutrient conditions, and glycerol impurity tolerances

Strain	PHB Accumulation (% CDW)	Carbon Concentration	Nitrogen Concentration	Cell Dry Weight	Salt Tolerance	Methanol Tolerance	Study
Pseudomonas oleovorans	31	5 g/L Glycerol	0.5 g/L $(NH_4)_2HPO_4$	0.8 g/L	2 g/L	2 g/L	This study
	25	20% (v/v) *n*-octane	3.5 g/L $NaNH_4HPO_4$	2 g/L	-	-	[5]
Zobellella denitrificans	87	20 g/L Glycerol	0.53 g/L NH_4Cl_2	4.8 g/L	50 g/L	-	[61]
Bacillus megaterium	60	20 g/L Glycerol	1 g/L $(NH_4)_2SO_4$	7.7 g/L	-	-	[9]
Ralstonia eutropha	60	5% (w/v) Glycerol	0.36-7.2 g/L NH_4Cl_2	1.8 g/L	-	-	[62]
Cupriavidus necator JMP 134	41-10	10 g/L Glycerol	0.95 g/L $(NH_4)_2SO_4$	50 g/L	3-20 g/L	-	[63]
Paracoccus denitrificans	38-13	10 g/L Glycerol	0.95 g/L $(NH_4)_2SO_4$	50 g/L	3-20 g/L	-	[63]
Cupriavidus necator DSM4058	38-13	50 g/L Glycerol	0.5 g $(NH_4)_2SO_4$	-	-	0-125 g/L	[64]

P. oleovorans has also been shown to be capable of growth with glycerol as a sole carbon source [36-38]. Freitas et al. [36] cultivated *P. oleovorans* on glycerol in a fed-batch reactor in nitrogen limiting conditions while maintaining a small nitrogen concentration in the bioreactor. They found that *P. oleovorans* could accumulate biomass up to nearly 20 g/L while producing 8.11 g/L of extracellular polysaccharide. Likewise, *P. oleovorans* has been shown to accumulate up to 1.9 g/L cell weight in when grown with glycerol in shake flasks [38]. Ashby et al. [39] were able to grow *P. oleovorans* on pure glycerol in order to synthesize mixed chain-length PHAs. They found that *P. oleovorans* could accumulate up to PHB up to 40% CDW. Additionally, *P. oleovorans* has been found to tolerate up to 20% NaCl concentrations [34], and is highly tolerant of organic solvents [35]. As discussed in Section 2.1, crude glycerol from biodiesel production is typically high in salts and methanol. Accordingly, *P. oleovorans* is an ideal candidate for production of PHB in crude glycerol.

2.4 Bacteriophage applications

Bacteriophage, or phage, are viruses that specifically infect and lyse various bacteria. Phage specificity and lytic affects are desirable characteristics in multiple agricultural, medical, and biotechnological fields [65, 66]. Accordingly, it is important to determine phage characteristics for lysis. Relevant phage characteristics include the adsorption velocity and associated constants [67]. The adsorption rate constant (often reported in units of mL/min) is a measure of the rate at which phage in media will bind to the surface of host cells. It is also known that the presence of divalent cations, such as

Ca^{2+} and Mg^{2+}, can affect the adsorption kinetics [68, 69]. Additionally, it is useful to determine the burst, the average number progeny created by a single phage, and the latent period, the time between phage infection and the release of progeny. These characteristics are crucial in determining phage application concentration, media characteristics, and efficacy for controlled lysis.

2.4.1 Agricultural applications

In agricultural applications, bacteriophage have been investigated to induce lysis in *Lactococcus lactis* bacteria for the development of cheese [70, 71]. Genes from the phages ΦUS3 [71] and Φ31 [70] were expressed in order to lyse *L. lactis* ceels to release various intracellular enzymes such as β-galactosidase which aid in the ripening and falvor formation of cheeses. Similarly, phage can be utilized to protect crops either though application or the development of transgenic plants capable of utilizing phage endolysin genes. T4 lysozyme containing transgenic potato plants were found to be capable of disrupting phytopathogenic bacteria such as *Erwinia carotovora* by Vries et al .[72]. In similar food production applications, Hughes et al. [73] isolated bacteriophage SF153b which was capable of lysing *Enterobacter agglomerans* 53b which had been isolated from a food processing factory. SF153b was able to excrete polysaccharide depolymerase to disrupt biofilm formation. Depolymerase expression and cell lysis were combined to actively disrupt strain 53b biofilms.

2.4.2 Medical applications

Phage have also been studied for medical applications in both animals and humans [74, 75]. Phage therapy, the treatment of bacterial infections via phage, has been utilized since the 1900s [75]. The high specificity of phage in host selection make them ideal candidates for targeting specific anti-biotic resistant bacterial strains. However, there is currently a lack of understanding and scientific study in the efficacy of phage therapy[75]. As a result of the lack of scientific understanding of phage efficacy, there is substantial hesitancy to utilize phage therapy in human medicine. However, there are numerous emerging studies observing the use of phage cocktails, mixtures of multiple phage exhibiting multiple host ranges [76-78].

2.4.3 Biotechnological applications

Within biotechnology, phage and phage lysis genes have been utilized to facilitate PHB extraction and cell lysis [13, 42, 41, 40]. Fidler and Dennis [40] found that recombinant *Escherichia coli* strain could be used to grown PHB to high concentrations. The cells could then by lysed with a bacteriophage to release the accumulated PHB. Due to the limitations of infecting/lysing cells during stationary phase, they also investigated an intracellular plasmid containing T4 phage lysozyme genes. They were able to successfully lyse *E. coli* cells containing high concentrations (15 g/L) of PHB. Similarly, Yu et al. [42] were able to clone λ phage lytic genes (*S*$^{-}$*RRz*) and PHB biosynthetic genes (*phb*CAB) into *E. coli* strain JM105. The resultant strain (VG1) was able to autolyse and release stored PHB. Growth in batch cultured on glucose resulted in PHB accumulation

of 8.54 g/L while batch-fed culture reached PHB yields of 26 g/L. In addition to recombinant *E. coli* strains capable of autolysis and expression of PHAs, phage-based lysis of several naturally high PHA-expressing bacteria has been studied.

Hori et al. [41] found that gram-positive *Bacillus megaterium* could be modified by inclusion of *Bacillus amyloliquefaciens* phage holing and endolysin genes. The resultant strain contained xylose-inducible autolysing features. They were able to confirm through sucrose gradient centrifugation and Sudan black B staining that the autolysis successfully separated the PHB granules from the cell debris. Similarly, Martínez et al. [13] were about to construct a strain from *Pseudomonas putida* KT2440 which was capable of controlled autolysis. Endolysin (*ejl*) and holing (*ejh*) coding genes were cloned into *P. putida* KT2440 to allow for 3-methylbenzoate induced lysis. They also were able to determine successful extraction of PHA through sucrose gradient centrifugation. Unlike the recombinant *E. coli* strains, *P. putida* expressed far lower levels of PHA, 1 g/L. However, Martínez et al. utilized a two-step fermentation process wherein cells were grown in rich media before being resuspended in minimal media for PHB accumulation as opposed to a fed-batch cultivation technique.

3. MATERIALS AND METHODS

3.1. Strains and culture conditions

P. oleovorans was purchased from America Type Culture Collection (ATCC number # 29347). All experiments were performed in a 30°C incubator while shaken at 150 rpm in 250-mL Erlenmeyer flasks. PHB accumulation occurred in modified P1 ammonium mineral salts media (5.0 g of K_2HPO_4, 0.5 g of $NaSO_4$,and 0.4 g of $MgSO_4 \cdot 7H_2O$ per 1 liter of solution) amended with varying concentrations of glycerol and $(NH_4)_2HPO_4$ and 0.1% (vol/vol) trace mineral solution (1 liter of trace mineral solution contains 2.78 g of $FeSO_4 \cdot 7H_2O$, 1.98 g of $MnCl_2 \cdot 4H_2O$, 2.81 g of $CoSO_4 \cdot 7H20$, 1.47 g of $CaCl_2 \cdot 2H_2O$, 0.17 g of $CuCl_2 \cdot 2H_2O$, and 0.29 g of $ZnSO_4 \cdot 7H_2O$) [79, 5]. *P. oleovorans* was pregrown in Reasoner's 2A (R2A) broth medium in a 30°C incubator while shaken at 150 rpm for approximately 12 hrs to provide inocula for samples. Cells were centrifuged at 10,000xg for 10 min to pellet before resuspension in modified P1 media amended with glycerol to OD_{600} = 0.9-1.0. The resuspended *P. oleovorans* was added to test samples at a ratio of 1:25 by volume. Cells were incubated for 48 hrs to reach stationary phase at OD_{600} ~ 1.2.

Experiments were performed at C/N ratios (the ratio of input molar carbon to input molar nitrogen) ranging from 10.8 to 143.3. The C/N ratio was determined by comparing the molar concentration of glycerol and ammonium-nitrogen in the minimal media. To test the effects of common glycerol impurities, NaCl was added to P1 media for PHB accumulation samples at concentrations of 0.1, 0.3, 0.5, 1, and 2 g/L. All tests were

conducted at 0.5 g/L of NaCl unless otherwise stated. Likewise, methanol was added to samples at concentrations of 0.5, 1, and 2 g/L (0-40% (w/w) of glycerol per typical crude glycerol concentration [19]) to determine its effects on PHB accumulation.

3.2 Bacteriophage methods

3.2.1 Bacteriophage isolation

Soil samples were taken from four locations in College Station, TX. Soil samples were taken from 5-10 cm below the soil surface. 50 g of each soil sample was added to 250-mL Erlenmeyer flasks containing100 mL of R2A media. Flasks were shaken at 150 rpm at 30 °C for 1 hr. The R2A/soil mixture was then centrifuged for 10 min at 10,000xg. The resulting supernatant was filter sterilized through a 0.22 µm syringe filter. *P. oleovorans* cultured in R2A media was added to the filtrate and incubated for 24 hrs at 30 °C. The enrichment was then centrifuged for 10 min at 10,000xg, and the supernatant was again filter sterilized through a 0.22 µm syringe filter. The filtrate was titered using standard methods [80] to determine phage concentration as PFU/mL. Pickates were collected from plaques and resuspended in 500 µL of fresh R2A media. The resulting suspension was then plated and titered. New pickates were collected and titered again to purify phage. Phage were stored in R2A media at 4 °C. Titer was periodically monitored and no decay was detected over 6 months.

3.2.2 Bacteriophage adsorption

All adsorption assays were performed in a 30 °C incubator while shaken at 150 rpm. *P. oleovorans* was cultured in R2A to mid-log growth (OD_{600} ~0.5) before pelleting at 10,000xg for 10 min and resuspension in fresh R2A media to OD_{600} = 1.0 (4.3×10^{10} CFU/mL). Bacteriophage Ke14 was added to resuspended *P. oleovorans* at an input multiplicity of infection (MOI) of 0.005 - MOI is the ratio of phage to host bacteria. Input MOI was determined by the final OD_{600} of the resuspensions and the final input concentration of phage as from lysate as PFU/mL. Twenty µL samples were taken at time intervals and diluted 100X in R2A media precooled on ice to 0 °C. The resulting dilution was centrifuged at 12,000xg for 2 min and the supernatant plated. Plaques were counted to determine free phage measured as plaque forming units (PFU) per mL. Because it is known that the presence of divalent cations (such as Ca^{2+} and Mg^{2+}) can affect the adsorption kinetics of some phages [68, 69], absorption kinetics were also measured at 1, 2, 5, and 10 mM each Ca^{2+} and Mg^{2+} concentrations. All adsorption velocity tests were replicated at least 3 times.

3.2.3 One-Step growth analysis

P. oleovorans cultured in either R2A media to OD_{600} ~0.5 or glycerol media to accumulated PHB at OD_{600} ~1.2 were pelleted at 10,000xg for 10 min and resuspended in fresh R2A media amended with 1 mM of Ca^{2+} and Mg^{2+} to OD_{600} ~5. Ke14 was added to the resuspended cells at MOI of 0.001. The mixture was incubated at 30 °C while shaking at 150 rpm for 10 and 20 min for R2A- and glycerol-grown cells, respectively. Samples

were then centrifuged at 5,000xg for 4 min and resuspended in fresh R2A medium amended with 1 mM of Ca^{2+} and Mg^{2+}. The resuspension was diluted by 1000X with fresh R2A media in a 250-mL Erlenmeyer flask and placed in a 30 °C incubator with shaking at 150 rpm. At each time interval, 100 µL was removed from the suspension and mixed with 100 µL of mid-log-phase *P. oleovorans* and plated. An additional 500 µL sample was taken immediately following culture dilution (0 min) and mixed with 5 µL of chloroform to determine adsorbed and free phage. The samples were vortexed for 30 seconds and the centrifuged at 5,000 x g for 4 min before 100 µL of the supernatant was removed for plating. All measurements from 60 min after dilution and onwards were also prepared on 100X dilution plates.

3.2.4 Cell lysis

Cells were cultured to accumulate PHB in modified P1 + 5% (w/v) glycerol media for 48 hrs to a final OD_{600} of ~1.2. Cells were pelleted at 10,000xg for 10 min resuspended to OD_{600} = 0.20 (~10^8 CFU/ mL) in fresh R2A media amended with 1 mM Ca^{2+} and Mg^{2+} and 0.5 g/L $(NH_4)_2HPO_4$. The growth of the resuspension was monitored via absorbance at 600 nm to determine when cells reentered exponential growth. At various time intervals, 5 mL of cells was removed from the resuspension, diluted to OD_{600} = 0.20, and amended with bacteriophage at MOI = 10. Corresponding controls received equal volumes of R2A medium instead of phage for comparison with phage-added samples. The OD_{600} of each sample was monitored for 120 min to measure cell mass. After 120 minutes, samples were centrifuged for 5 min at 5,000xg to pellet cells. The resultant supernatant

was plated to determine free phage as PFU/mL. The pellet was then resuspended in fresh R2A and plated to determine viable cell count as CFU/mL.

3.3 PHB quantification

PHB was quantified though photospectroscopy via conversion of PHB to crotonic acid as described previously [18, 81, 82]. Briefly, 25 mL of PHB containing cells were pelleted at 10,000xg for 10 min and resuspended in 1 mL of deionized water. The resuspension was vacuum concentrated and weighed to determine cell dry weight. One mL of concentrated sulfuric acid was added to hydrolyze the cells and convert the PHB to crotonic acid. Acid/cell mixture was heated at 70 °C for 4 hrs and brought to room temperature. The mixture was then neutralized by the addition of 7 mL of 4N NaOH. The mixture was then centrifuged at 13,000xG for 4 min. The supernatant was then diluted in DI to 25% and measure at A_{235}. Known concentrations of PHB (from 115 to 1250 mg/L) were treated similarly and measured to create a standard curve.

3.4 Transmission electron microscopy

Electron microscopy of bacteriophage was performed with high titer lysate (10^{10} PFU/ml). Phages were applied to 400-mesh carbon-coated Formvar grids and negatively stained with 2% (wt/vol) uranyl acetate. After being allow to air dry, specimens were observed on a JEOL 1200EX transmission electron microscope operating at an acceleration voltage of 100 kV. At least three virions were measured to determine mean

head and tail size where mentioned. Magnification was calibrated using a carbon grating replica (Electron Microscopy Sciences No. 80051).

3.5 Sucrose density-gradient centrifugation

PHB-containing granules in media after lysis were separated from cell debris through sucrose density-gradient centrifugation [41, 83, 13]. Briefly, after lysis 50% of sample was treated to determine total PHB as described above. The remaining 50% was placed on sucrose layers prepared by overloading at 20% and 15% (w/w) sucrose solutions. Samples were centrifuged at 150,000xg for 1.5 hrs. The PHB remaining in cell debris was measured by collecting the sediment after centrifugation. The PHB released from cells was determined indirectly by measuring difference in PHB reaming in cell debris and total PHB of sample after lysis [13].

4. RESULTS AND DISCUSSION

4.1 Culture effects on PHB accumulation

4.1.1 Effects of impurities on PHB accumulation

To determine the effects of common crude glycerol impurities on *P. oleovorans growth*, samples were prepared at methanol and NaCl concentrations ranging from 0-2.0 g/L in the presence of 5 g/L glycerol (equivalent to 0-40% (w/w) NaCl contamination of crude glycerol). *P. oleovorans* was capable of growth in the presence of common glycerol impurities at all observed concentrations (Table 2). The common impurities of crude glycerol observed in this study do not adversely affect PHB accumulation in *P. oleovorans* (Table 2).

Table 2. The effects of impurities on PHB accumulation and concentration.

NaCl Concentration (g/L)	Glycerol Concentration (g/L)	Methanol Concentration (g/L)	PHB Accumulation (% CDW)	PHB Concentration (mg/L)	Final Cell Dry Weight (mg/L)
0.0	5.0	0.0	32.3±3.8	243±38	752±64
0.1	5.0	0.0	31.8±3.5	242±27	762±14
0.3	5.0	0.0	33.4±0.7	240±2.2	710±5
0.5	5.0	0.0	31.1±3.5	202±27	643±59
1.0	5.0	0.0	31.7±3.6	241±44	758±60
2.0	5.0	0.0	28.0±1.8	206±16	727±41
0.5	5.0	0.5	36.1±6.4	249±43	690±36
		0.0	32.1±6.2	204±30	638±54
0.5	5.0	1.0	38.1±9.3	233±48	633±42
		0.0	30.5±3.3	203±23	665±13
0.5	5.0	2.0	31.1±6.2	222±52	712±73
		0.0	32.1±4.5	200±37	627±99

P. oleovorans is capable of accumulating up to 28% PHB as CDW when grown in the presence of 2.0 g/L of NaCl (Table 2). While cells accumulated slightly less PHB at

higher NaCl concentrations, the differences were not found to be statistically significant via two-sample *t*-tests at $p < 0.05$. The maximum tested NaCl levels fall within expected salt concentrations in crude glycerol (3-4% (w/w)) [21, 48]. This suggests that *P. oleovorans* is an ideal candidate for PHB production utilizing crude glycerol as a sole carbon source due to its tolerance for saline conditions. Other PHB accumulating strains have displayed comparable salt tolerance within the tested range (Table 1).

To determine the effects of methanol on PHB accumulation in glycerol-grown *P. oleovorans*, parallel samples were grown with glycerol only, methanol only, and glycerol/methanol mixtures in the presence of NaCl. Similarly to NaCl, *P. oleovorans* demonstrated no statistically significant decrease in PHB accumulation when grown in the presence of methanol, via paired sample *t*-tests at $p < 0.05$ (Table 1). *P. oleovorans* exhibited no growth when grown in media containing only methanol as a carbon source. This suggests that while methanol does not inhibit the growth of *P. oleovorans* on glycerol as a carbon source, methanol itself cannot be utilized by *P. oleovorans* as a sole carbon source. As methanol concentrations have been found to vary from 12.8 to 28.3% (w/w) in crude glycerol [19], it is necessary to utilize methanol tolerant bacterial strains when producing PHB. While one other PHB accumulating strain, *Cupriavidus necator* DSM4058, was found to be methanol tolerant (Table 1), this is the first study to our knowledge that has observed the effects of methanol on PHB accumulation.

4.1.2 Effects of C/N ratio on PHB accumulation

In order to determine the effects of varying C/N ratios on the accumulation of PHB by *P. oleovorans,* samples were prepared with varying input concentrations of glycerol as a carbon source and $(NH_4)_2HPO_4$ as a nitrogen source. Under all observed nutrient conditions *P. oleovorans* was able to grow to comparable maximum densities OD_{600} ~1.2 after 48 hrs (Table 3). Further, there were no significant variations in growth characteristics across applied C/N ratios (Fig. 4). However, as glycerol concentrations increased, the final pH decreased. Other studies have shown that PHB production is linked to acidity generation [61, 84]. Accordingly, acid generation at higher glycerol concentrations may limit the maximum biomass formation. While there were not significant differences between different C/N ratios on cell growth rates, differences in PHB accumulation were observed.

Table 3. The effects of C/N ratio on cell growth

$(NH_4)_2HPO_4$ Concentration (g/L)	**Glycerol Concentration (g/L)**	**C/N Ratio (mol/mol)**	**OD_{600}**	**Final pH**
0.3	10	23.9	1.3±0.04	6.63±0.09
	15	35.8	1.1±0.03	6.60±0.07
	20	47.8	1.0±0.05	6.54±0.06
0.5	5	7.2	1.2±0.05	7.05±0.07
	10	14.3	1.1±0.04	6.67±0.09
	15	21.5	1.1±0.02	6.65±0.08
	20	28.7	1.2±0.01	6.50±0.04
1.0	10	7.2	1.1±0.03	6.70±0.05
	15	10.8	1.3±0.06	6.64±0.03
	20	14.3	1.2±0.06	6.59±0.05

P. oleovorans accumulated PHB in all observed culture conditions (Fig. 5). PHB accumulation ranged from 31.1% CDW as PHB to 14.7% CDW as PHB at C/N ratios of 21.5 and 143.3, respectively (Fig. 5).

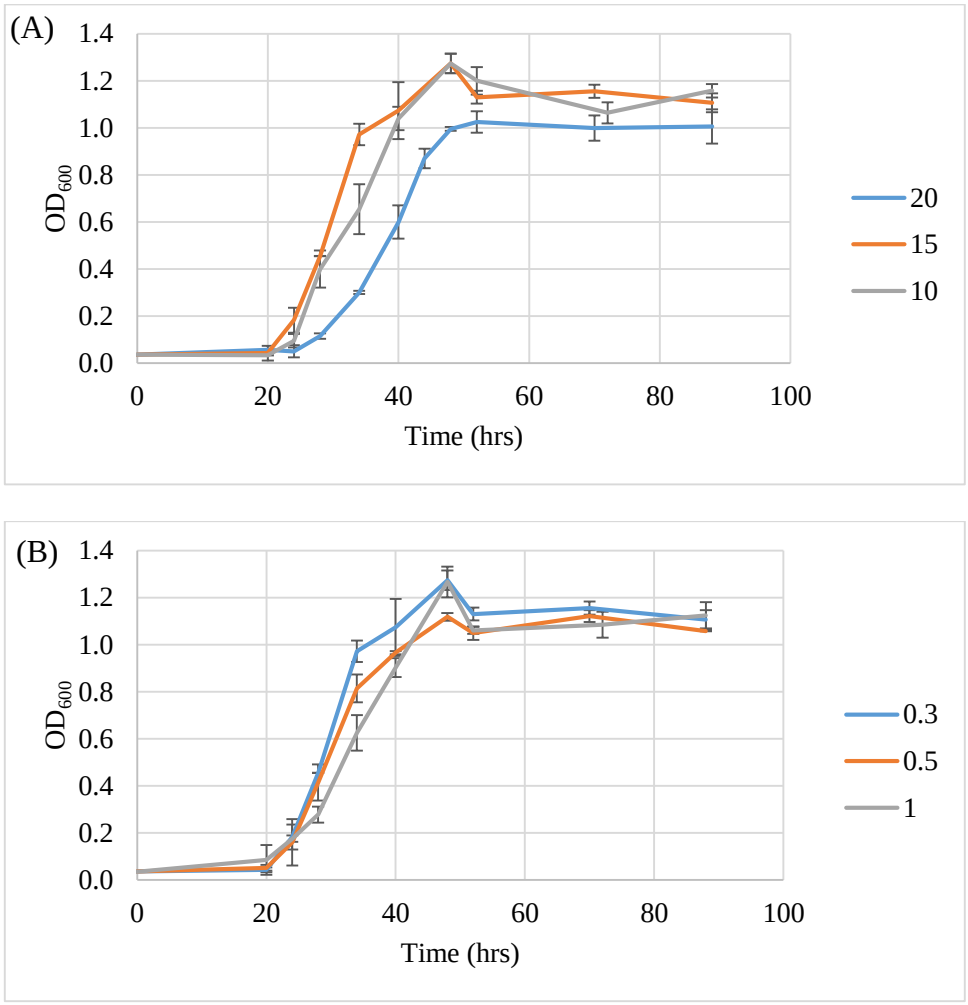


Figure 4. Effects of carbon (A) and nitrogen (B) concentrations on growth. (A) $(NH_4)_2HPO_4$ concentration fixed at 0.5 g/L. Glycerol concentrations shown in legend as g/L. (B) Glycerol concentration fixed at 15 g/L. $(NH_4)_2HPO_4$ concentrations shown in legend as g/L.

The highest observed PHB accumulation corresponds with the lowest C/N ratio under which nitrogen is growth-limiting. Generally, PHB accumulation decreases as the C/N ratio is increased under nitrogen-limiting conditions. This corresponds to previous work observing the effect of C/N ratio on PHB accumulation in other growth-associated PHB accumulating strains *Pseudomonas resinvorans* [30] and *Cupriavidus taiwanensis* [31]. Both strains similarly saw peak PHB accumulation at lower nitrogen-limited C/N ratios, and displayed similar trends of decreasing PHB accumulation as the C/N ratio increased. Additionally, the decrease in PHB accumulation as C/N ratio increases may be due to formation of different chain length PHAs.

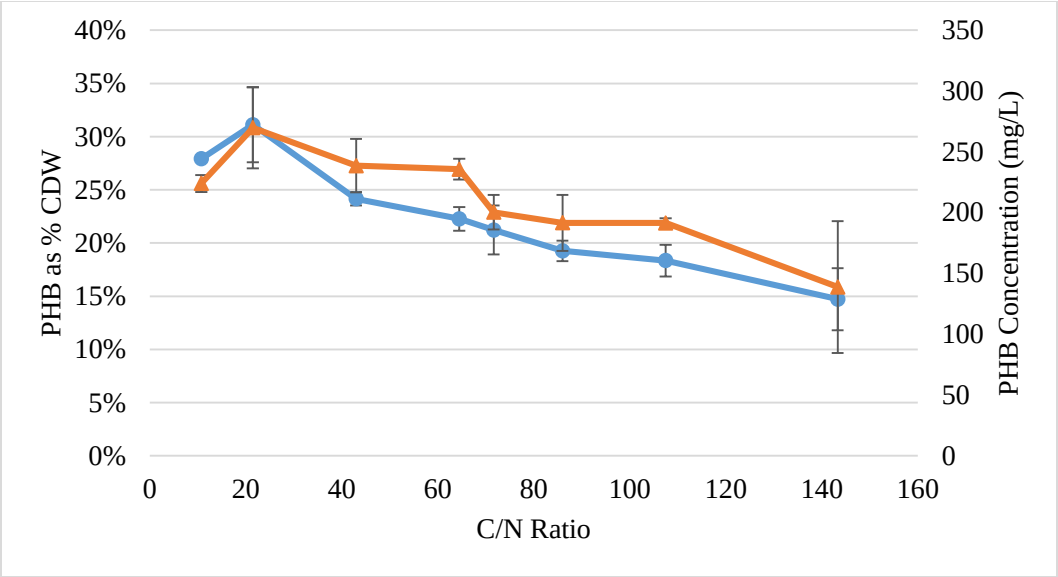


Figure 5. PHB accumulation and yield for cells cultivated at varying C/N ratios. (●) values shown as % CDW (mass of PHB/total cell cry mass), (▲) values shown as PHB Concentration (mass of PHB per volume culture). All samples grown in aerobic conditions at 30 °C while shaken at 150. All samples contain 0.5 g/L NaCl.

Kato et al. [32] found that increasing C/N ratio led to a greater accumulation of larger chain length PHAs such as 3-hydroxyoctanoate and 3-hydroxydecanoate. *P. oleovorans* is known to accumulate PHAs at chain lengths other than PHB, such as polyhydroxyvalerate (PHV) [85, 86]. Because our method measures PHB as opposed to other chain length PHAs, the reported PHB accumulation in this study might underrepresent the total PHAs accumulated by *P. oleovorans* at higher C/N ratios.

4.2 Phage isolation and adsorption kinetics

A phage capable of lysing *P. oleovorans* has been isolated – Ke14 (Fig. 6). Ke14 is a tailed phage, characteristic of a siphophage. Ke14 was isolated in June of 2014 from soil samples near railroad tracks in College Station, TX.

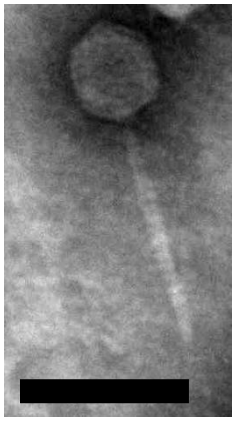


Figure 6. Bacteriophage Ke14 at 50K magnification. Scale bar 100 nm.

Ke14 was observed to bind to mid-log, R2A-grown cells at a rate of 3.6 x 10^{-10} ± 3.3 x 10^{-11} mL/min at a confidence of 95% (Fig. 7A).

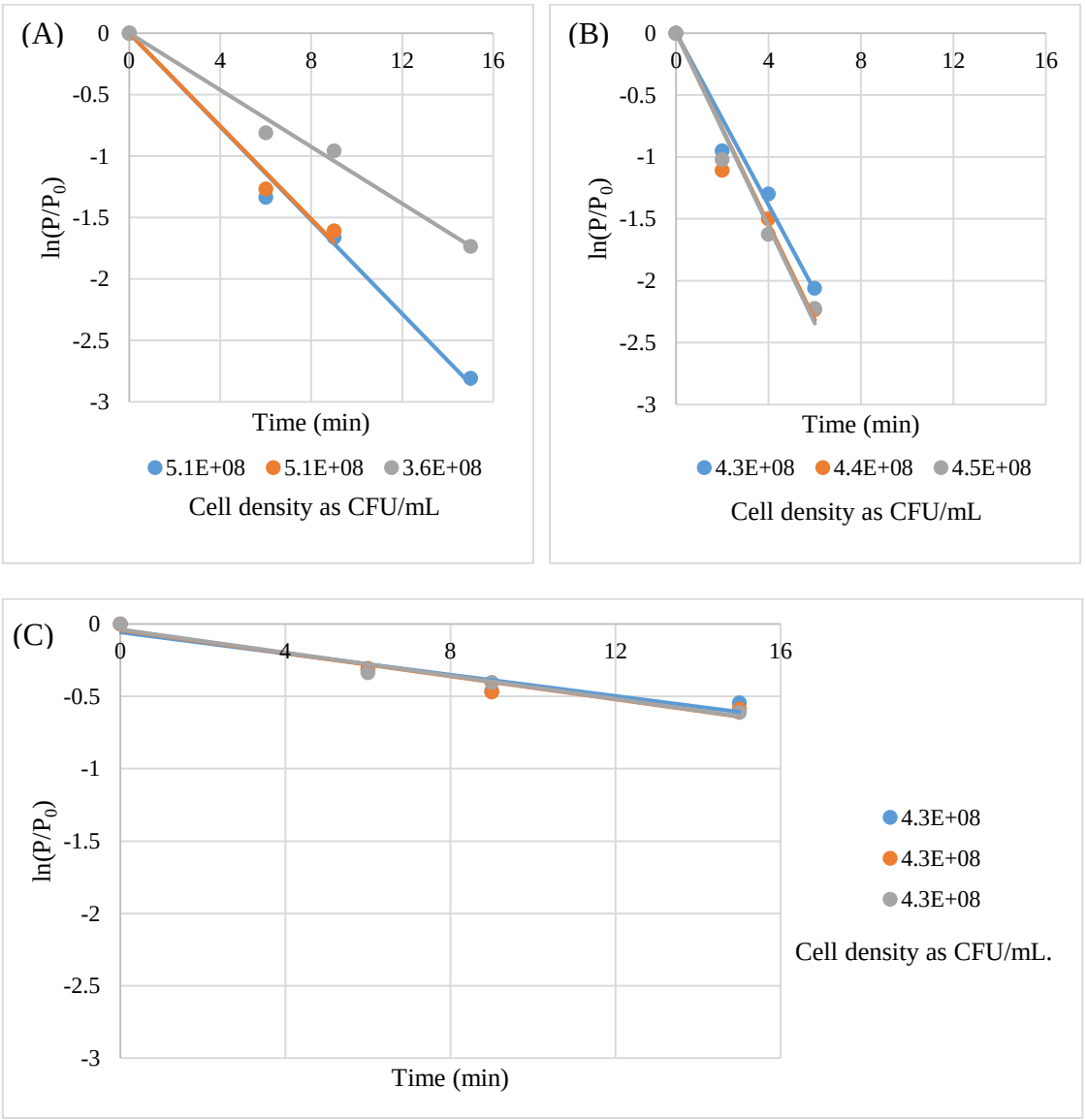


Figure 7. Ke14 adsorption kinetics. The y-axis represents natural log of the ratio of free phage at time *t* divided by the initial free phage as CFU/mL. Amount of *P. oleovorans* measured as CFU/mL at which test shown in legend. (A) Adsorption of R2A-grown cells without the presence of divalent cations. (B) Adsorption in R2A-grown cells in the presence of 1 mM of Ca2+ and Mg2+ each. (C) Adsorption in Glycerol-grown cells in the presence of 1 mM of Ca2+ and Mg2+ each.

This rate is approximately five fold slower than E. coli λ phage adsorption, which occurs at a rate of ~5 x 10^{-9} mL/min [68]. Because it is known that the presence of divalent cations (such as Ca^{2+} and Mg^{2+}) can affect phage adsorption kinetics [68, 69], absorption kinetics were also measured at varying Ca^{2+} and Mg^{2+} concentrations (Fig. 8). The fastest adsorption constants were measured in the presence of 1 mM each of Ca^{2+} and Mg^{2+}, with absorption occurring ~2.5-fold faster than without any cations, 8.5 x 10^{-10} ±4.3 x 10^{-11} mL/min for R2A-grown cells at a confidence of 95% (Fig. 7B). However, when *P. oleovorans* was grown to saturation in glycerol media (the state in which PHB accumulation is optimal) in the presence of 1 mM each of Ca^{2+} and Mg^{2+}, the adsorption rate was approximately ten times slower than that of cells cultured in R2A, 9.0 x 10^{-11} ±4.9 x 10^{-12} mL /min at a confidence of 95% (Fig. 7C).

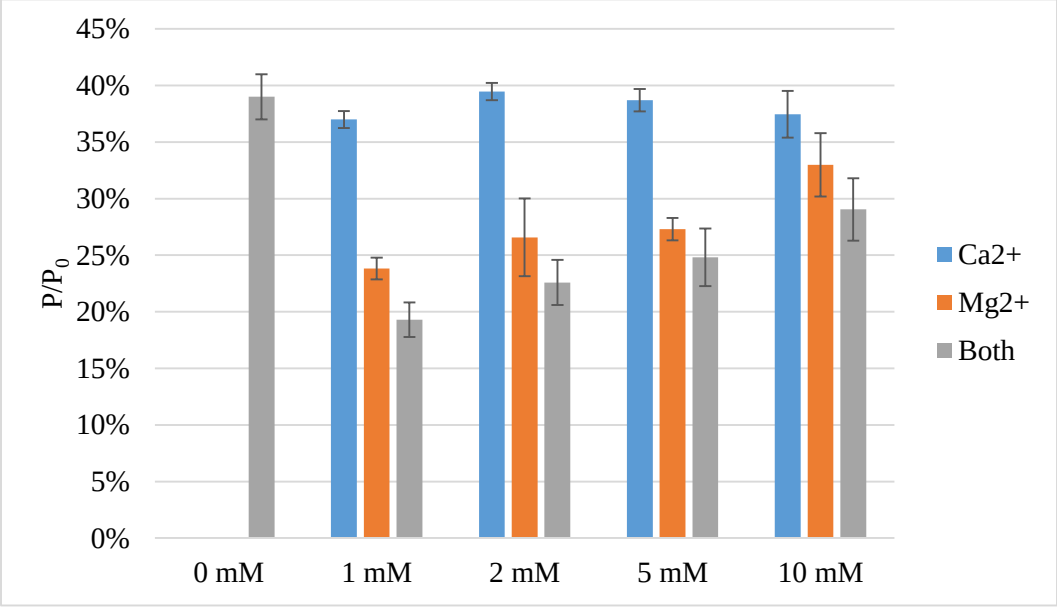


Figure 8. Effects of divalent cations Mg^{2+} and Ca^{2+} on phage adsorption. The units on the y-axis represent percent remaining free phage in solution as free phage in solution normalized to the free phage at time zero

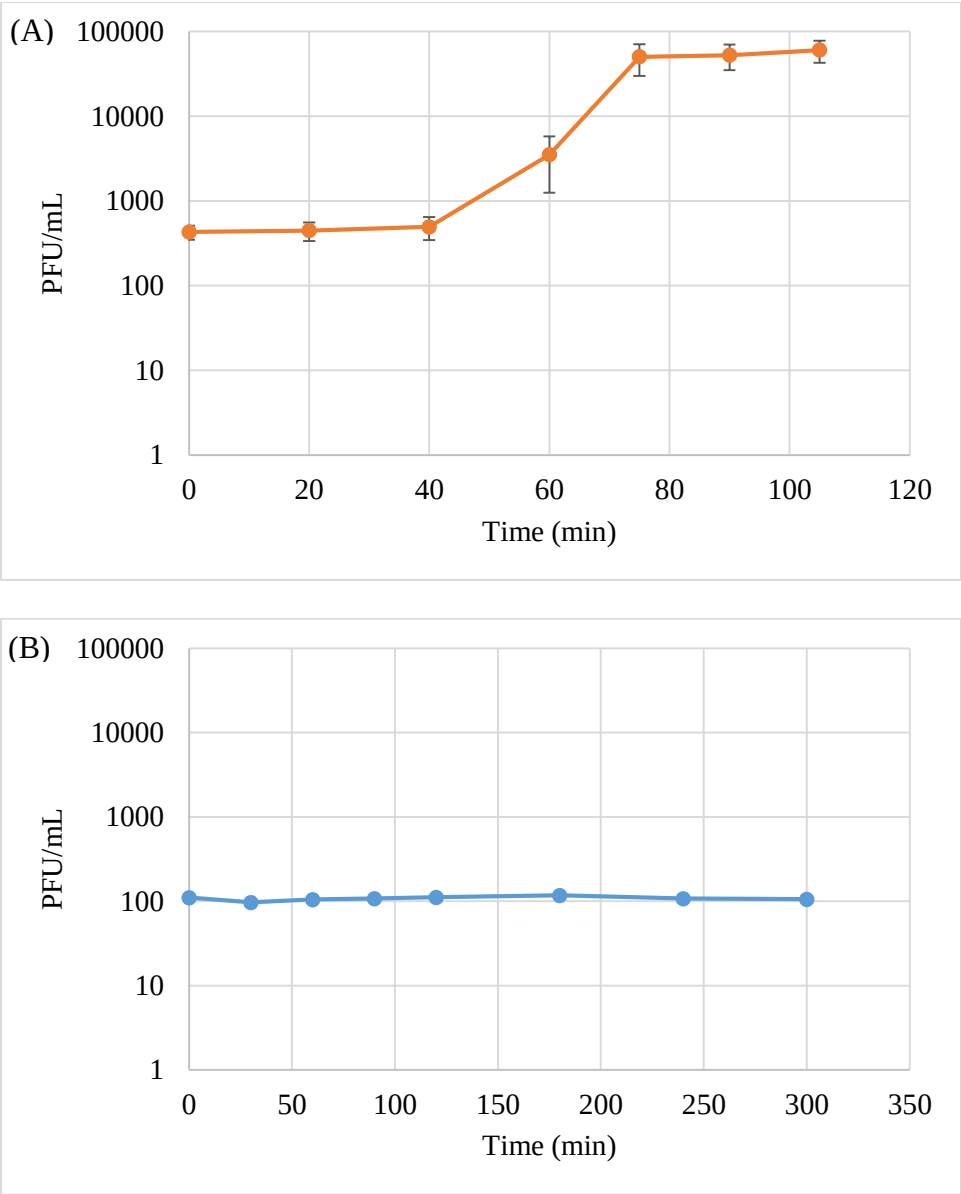


Figure 9. Ke14 one-step growth for R2A- and glycerol-grown *P. oleovorans*. Time zero is measured at start of 1000X dilution after phage adsorption at MOI of 0.001. Initial samples were treated with and without 10 µL of chloroform to differentiate free phage and bound phage. (A) One-step growth for mid log phase R2A-grown cells. (B) One-step growth for stationary phase Glycerol-grown cells performed in both glycerol and R2A media.

While the exact reason for the decreased absorption rate constants found in glycerol as opposed to R2A-grown cells was not determined, it may be due to changes in

the availability of binding sites and the configuration of the cell wall [87-89]. Hadas et al. [43] found that the adsorption of phage T4 was linked to the physiological state of its host, *E. coli*. Specifically, they found that as the growth rate (μ) decreased, absorption occurred at a lower rate. Similarly, Sillankorva et al. [44] found that phage ΦS1 absorbed to *Pseudomonas fluorescens* at a lower rate when cells were in stationary as opposed to exponential growth phase. In this study, PHB-bearing *P. oleovorans* cells were cultured in glycerol media for 48 hrs until they reached stationary phase at OD_{600} ~1.2. These previous studies correspond to our finding that the rate at which Ke14 absorbs to PHB-bearing *P. oleovorans* cells would be lower than the rate at which it absorbs to exponential growth stage cell.

Ke14 was also evaluated for burst size (the number of progeny phage per initial bound phage) and latent period (the time between infection and the release of progeny phage). Ke14 demonstrated a burst size of 110 and a latent period of 40 minutes when infecting mid-log phase cells cultured in R2A medium (Fig. 9A). This burst size is comparable to other well-studied phages such as T4 or lambda [90-92]. Similarly, the latent period is comparable to wild-type phage T4 optimal latent period when grown in LB media (19-46 min) [92]. While Ke14 was readily capable of infection and lysis of *P. oleovorans* cells grown under "optimal" conditions of R2A medium, Ke14 was not able to lyse glycerol-grown *P. oleovorans* (PHB-bearing cells cultured to stationary phase in glycerol media) immediately after resuspension in either R2A or fresh glycerol media (Fig. 9B). Hadas et al. [43] found that T4-infected *E. coli* cultured on glycerol as the sole carbon source displayed lower burst size and a greater latent period than those cell culture

in Luria-Bertani broth containing glucose (LBG). However, the infection observed by Hadas et al. occurred in log phase *E. coli* whereas our study observed infection in stationary phase. Accordingly, our expected latent period and burst size exhibit greater disparity to those observed in rich media. Similar trends regarding the effect of minimal media on lysis were observed by Sillankorva et al. when *P. fluorescens* was grown in minimal vs rich glucose-containing media [44]. Additionally, nitrogen-starved *E. coli* cells infected with phage MS2 were found to be incapable of lysis even after additional nitrogen was added to the culture [93]. Likewise, MS2-infected *E. coli* at stationary growth phase were unable to release progeny phage [94]. Culture of *P. oleovorans* on glycerol media results in hosts which are inferior to exponential growth phase host, i.e. glycerol-grown cells have differing cell morphology, cell wall surface characteristics, and metabolic processes. Accordingly, the lack of observed lysis in glycerol-grown cells might be due to poor host quality.

4.3 Lysis of PHB accumulated cells

In order to achieve lysis of PHB-bearing cells, *P. oleovorans* was cultured in glycerol-containing media until stationary phase. The PHB-containing cells were then pelleted at 10,000xg for 10 min, resuspended in fresh R2A medium to OD_{600} = 0.2, and placed in a 30 °C incubator, shaking at 150 rpm. After resuspension, an extended lag phase was observed, followed by a return to exponential growth after approximately 4 hrs (Fig. 10A). When phage was added to samples immediately after resuspension in R2A, no lysis was observed for up to 3 hrs of observation (Fig. 10). Similarly, phage added to

samples 120 min after resuspension showed no detectable lysis. However, once the cells moved into exponential growth, lysis was observed in samples taken at 240 and 300 min (Figs. 10D and 10E). All cells lysis observations were confirmed through plating samples to determine viable cells as CFU/mL. Samples were centrifuged at 5,000xg and resuspended in fresh media to remove free phage.

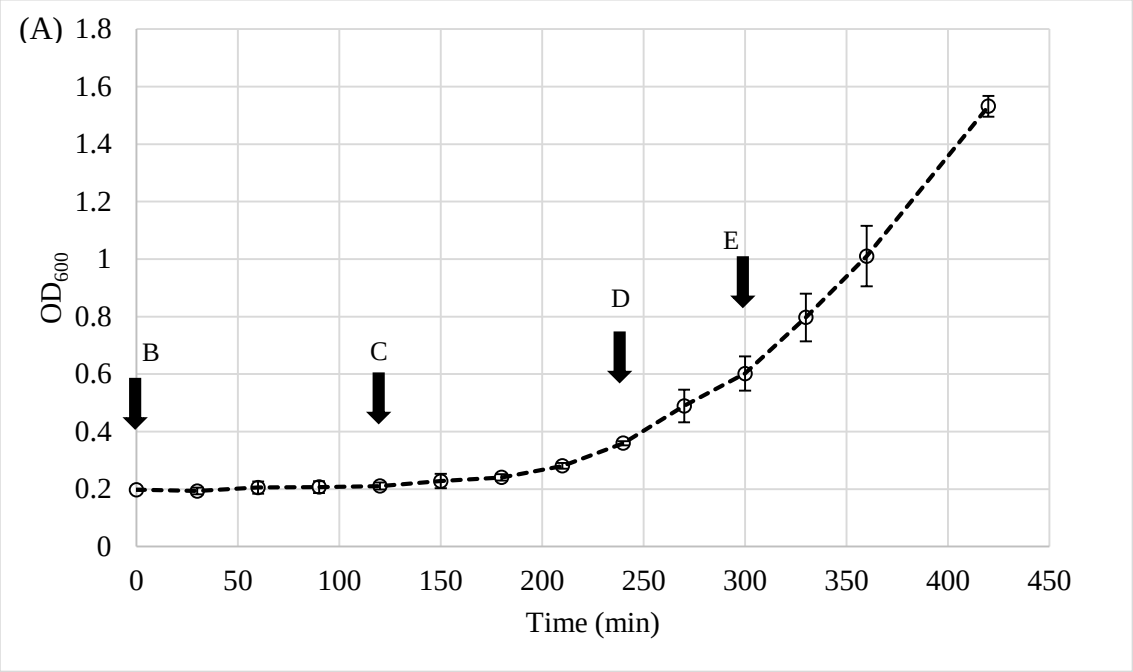


Figure 10. Lysis of PHB containing cells. x-axis corresponds to time from dilution. Test samples were removed from resuspension of PHB-containing cells to receive phage addition for lysis. Corresponding controls which received R2A media addition instead of phage. x-axis corresponds to time from dilution. Test samples receiving phage are marked with circles and solid lines. Controls receiving R2A instead of phage are marked with x and dotted line. Arrows indicate sampling point for phage addition. (A) Growth of R2A-resuspended, PHB-containing cells from which lysis samples were removed. (B) 0 min phage addition. (C) 120 min phage addition. (D) 240 min phage addition. (E) 300 min phage addition.

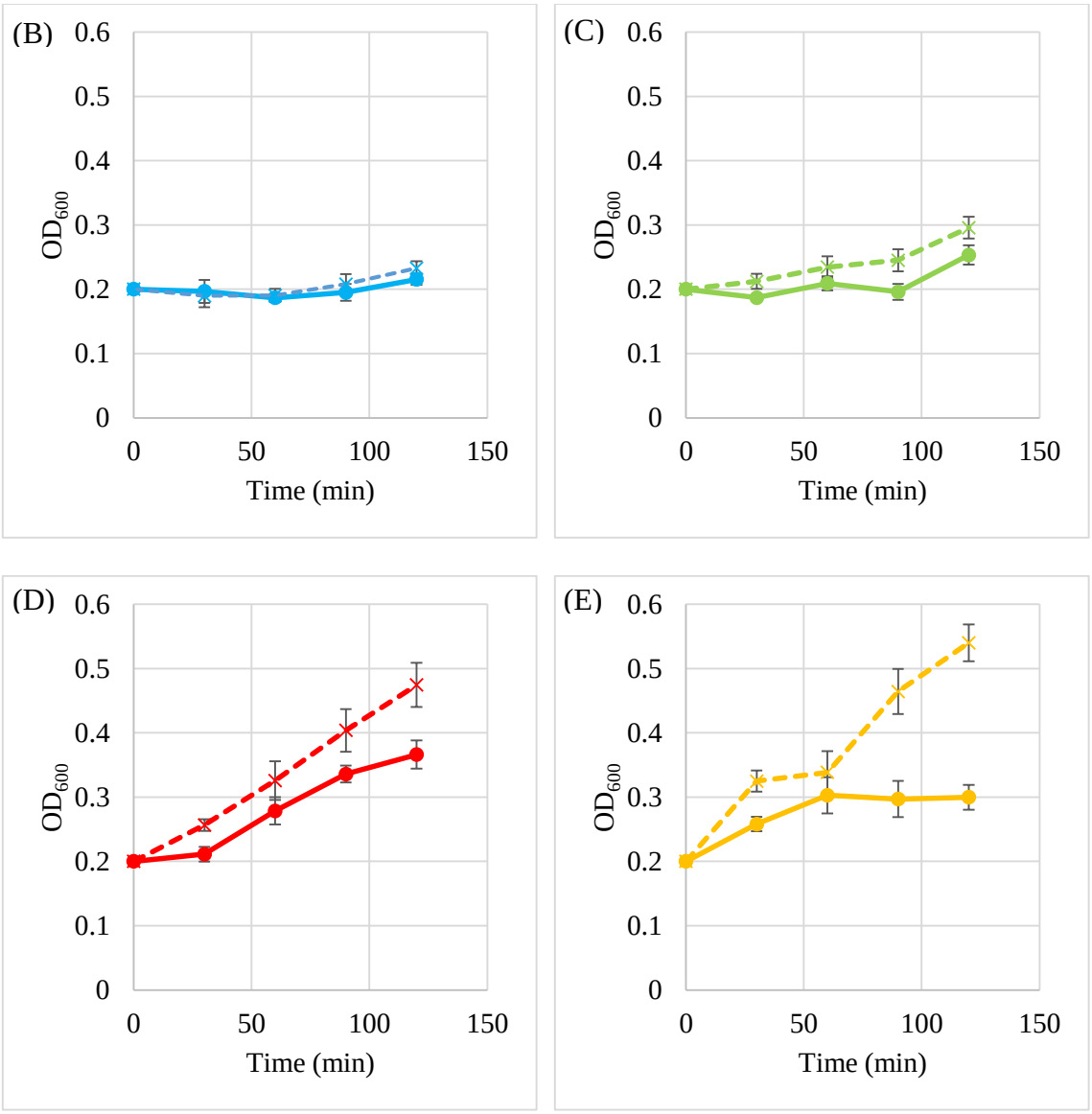
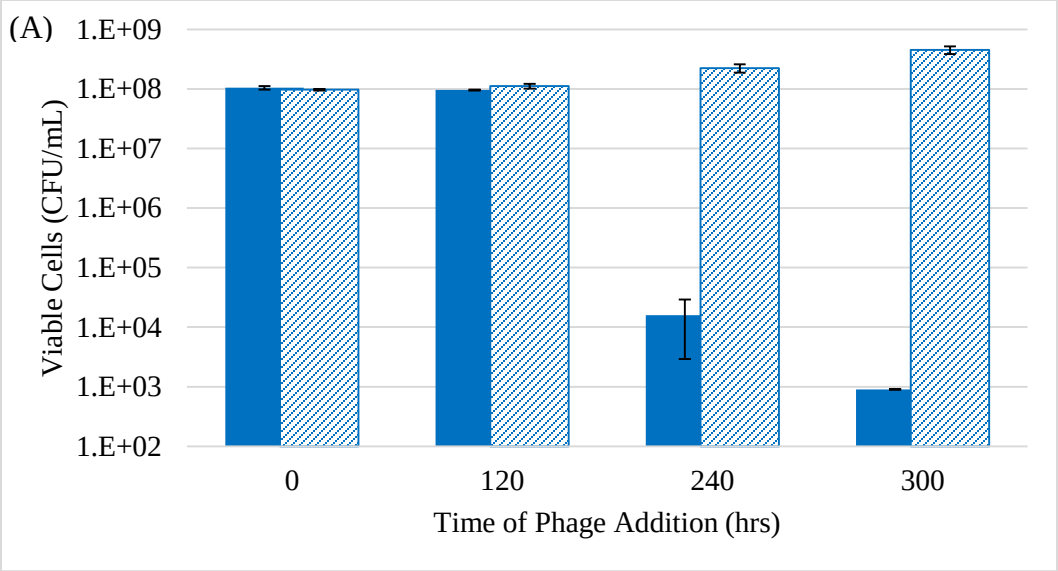


Figure 10. Continued.

In samples taken at 300 min, no CFU were detected above the measurement threshold of 10^3 CFU/mL - compared to the control samples' viable cell concentration, approximately 10^8 CFU/mL (Fig. 11).



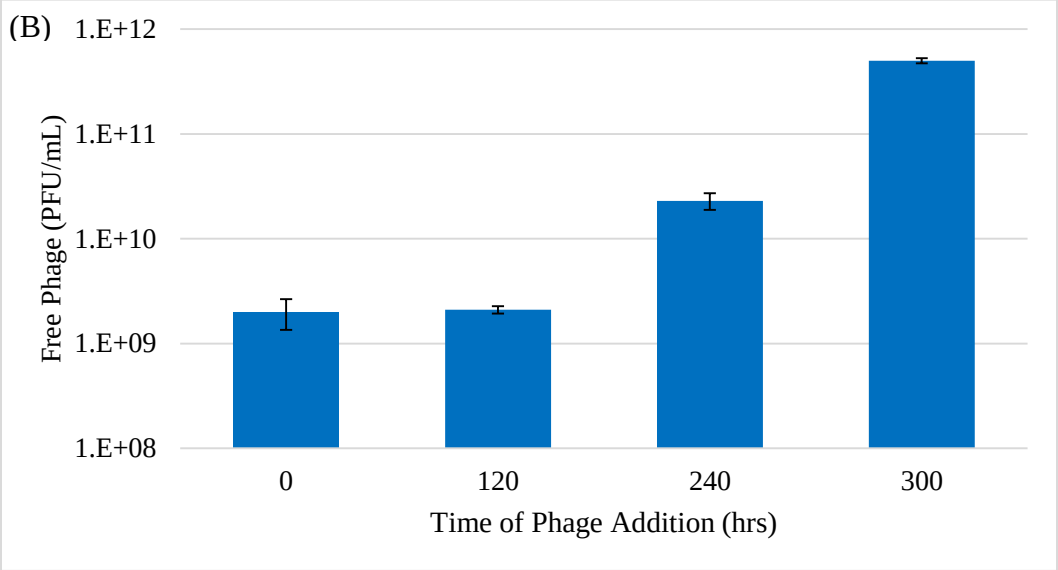


Figure 11. Viable cells (A) and free phage (B) in cultures of PHB-accumulated cells following return to rich medium and addition of phage to induce cell lysis. Solid bars indicate test samples and patterned bars indicate controls receiving no phage. Viable cells measured CFU/mL and free phage are measured as PFU/mL.

To determine the effects of returning cells to exponential growth in rich medium on the PHB accumulated by *P. oleovorans*, samples were taken to measure the PHB

concentration over time. The PHB content of cells did decrease after resuspension and dilution in R2A medium, from 31% PHB as CDW at 0 min to 22% PHB as CDW at 120 min (Fig. 12). Franz et al. [95] modeled the production and consumption of PHB in *Ralstonia eutropha* when cells were allowed to accumulate PHB before ammonium chloride was added to stimulate biomass formation. They found that PHB was consumed in order to produce biomass. Similarly, *Bacillus megaterium* has been shown to consume internally stored PHB to produce biomass [96]. It is probable that the decrease in PHB content of glycerol-grown *P. oleovorans* is likely due to consumption of internal PHB as the cells reenter exponential growth. However, the amount of PHB consumed by the cells while returning to a phage-susceptible state is only ~20% of the total PHB accumulated, suggesting that this strategy may be viable for PHB extraction.

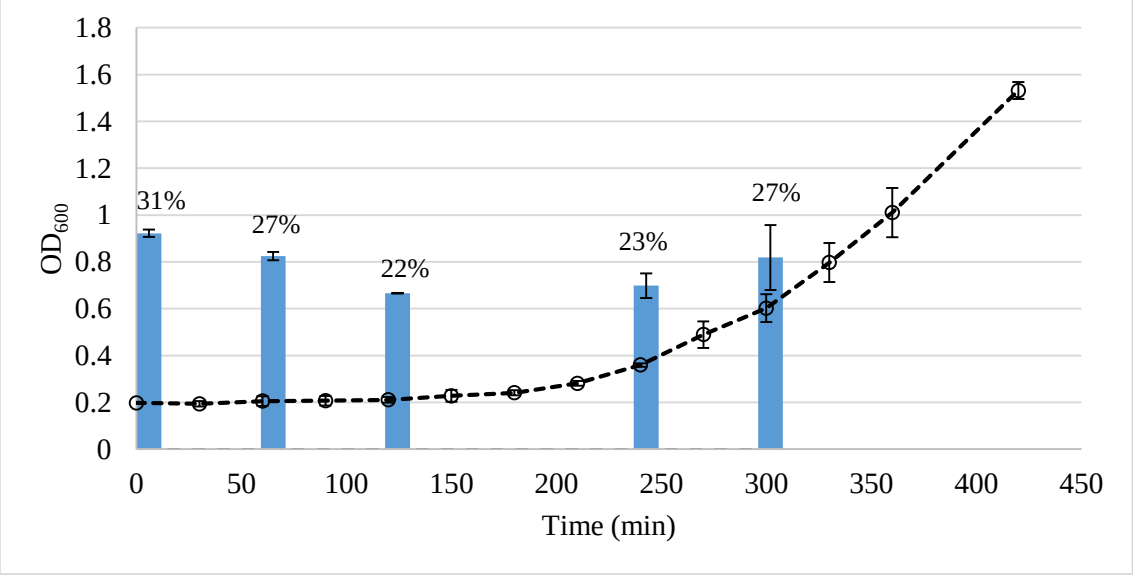


Figure 12. Changes of PHB content in glycerol-grown *P. oleovorans* after dilution and resuspension in R2A over six hours. Percent of cell dry weight as PHB is indicated by bars and corresponding cell growth shown by (o) with dotted line.

The efficacy of PHB extraction by phage was measured through sucrose-gradient density ultracentrifugation. This method separates the PHB-containing granules released into the media from those granules still contained in cell debris. After 120 min of incubation/lysis, samples receiving bacteriophage addition at 300 min (Fig. 10E) were placed on sucrose-gradients alongside corresponding controls which received no phage addition. Phage-facilitated lysis resulted in the release of approximately two thirds of accumulated PHB (Fig. 13). The non-negligible amount of PHB contained in cell debris after cell lysis may be potentially due to failure of PHB granule release despite disruption of the cell envelopes. Martínez et al. [13] found that *P. putida* cells lysed through the expression of an auto-lysing cassette released only 9% of the total PHB produced. Hori et al. [41] were also able to release about two thirds PHB accumulated in *B. megaterium* after self-disruption. While not evaluated in this study, incomplete release of PHB-containing granules may be affected by the hydrophobic interactions between PHB-containing granules and cell debris [41]. Accordingly, effective methods need to be developed to maximize PHB release in phage-facilitated extraction.

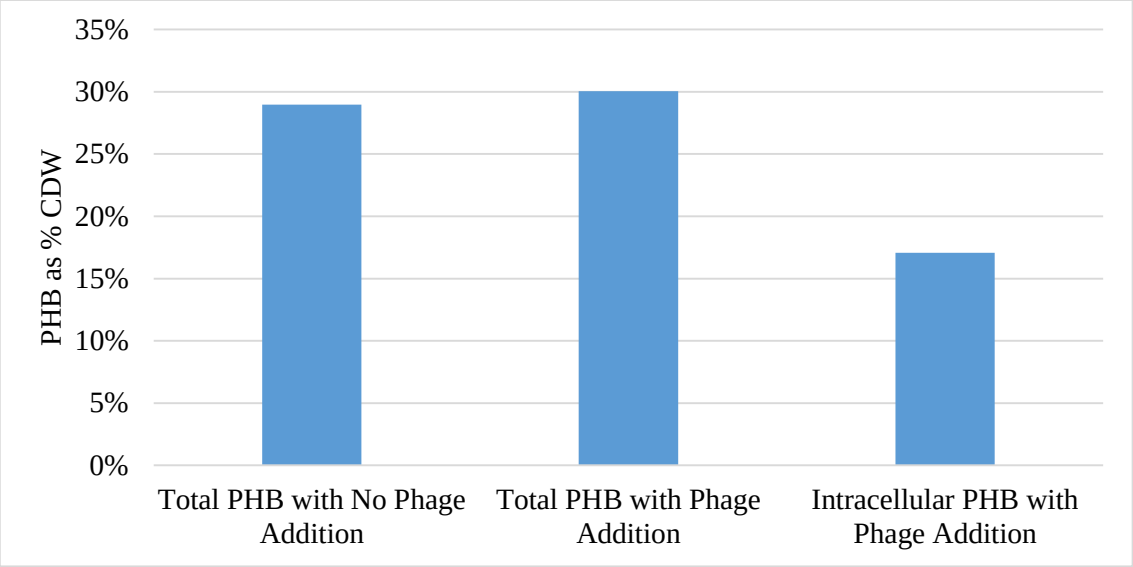


Figure 13. Release of PHB due to bacteriophage-facilitated lysis. After addition of phage to samples at 300 min, cells were placed on sucrose-gradients to separate intra- and extra-cellular PHB. Total PHB was measured in cells receiving phage treatment and controls receiving corresponding amount of R2A. After ultracentrifugation, PHB remaining in cell debris was measured to determine amount of PHB extracted through lysis.

5. CONCLUSIONS

In this study, *P. oleovorans* was found to be capable of utilizing crude glycerol as a sole carbon source while being unaffected by common crude glycerol impurities, sodium salts and methanol, in order to produce PHB. In addition, *P. oleovorans* is able to accumulate PHB at a range of C/N ratios with maximum PHB accumulation, 31.1% PHB as CDW, occurring at C/N ratio of 21.5. This study also isolated *P. oleovorans* targeting bacteriophage Ke14. Ke14 was found to be able to lyse cells cultured in rich medium and PHB-containing cells cultured on glycerol, but only after these cells were returned to a rapidly growing state in rich medium. These results provide examples of how PHB production costs can potentially be reduced through utilization of cheaper feedstocks and extraction techniques.

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