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THE BIOCONVERSION OF PLASTIC MATERIALS

by

BRYAN A. STUBBLEFIELD

Under the Direction Eric S. Gilbert, Ph.D.

ABSTRACT

Plastics are highly useful economically because of their resistance to diverse types of environmental and chemical agents and their ability to be molded into many types of products. Globally, plastic production is greater than 20 million metric tons per year. However, their widespread use and often their disposable nature results in significant plastic accumulation in the environment. Plastics are made of hydrocarbons, materials that are biodegradable depending on their molecular structure and size. It is hypothesized that pre-treatment of plastic materials could enhance their bioavailability, facilitating their microbial biodegradation. In this dissertation, a process was developed to treat nylon 6,6 polymers by acid hydrolysis to produce a microbial growth medium. The chemical composition of the medium was determined by low pressure liquid chromatography-spectrophotometry and electrospray ionization mass spectrometry and

found that the medium was a mixture of molecules with molecular weight > 800 m/z and with similar chemical characteristics to polyamines. There was steady growth of *Pseudomonas putida* KT2440 in the medium with concomitant substrate biodegradation. Notably, the yeast *Yarrowia lipolytica* grew well in the medium when supplemented with yeast extract. A similar medium derived from nylon 6,6 containing nylon-derived particles supported the growth of *Beijerinckia* sp. and *Streptomyces* sp. BAS1. Confocal laser scanning microscopy and flame ionization gas chromatography were used to identify and quantify the production of polyhydroxybutyrate, a type of “bioplastic”. The aforementioned microorganisms were cultivated in a bench-scale bioreactor that was developed as part of this dissertation. The bioreactor had a novel impeller design resulting in enhanced mixing and rotation and also a modular format allowing for diverse configurations. The bioreactor was notable for its durability and low cost. A detailed description of its design is included in the appendices. In summary, plastic materials can potentially be processed into growth media for microorganisms and can be used for production of value-added products. The media described herein can be used in bioconversion processes using a bioreactor.

INDEX WORDS: Acid hydrolysis (AH), Bioconversion, Bioplastics, Bioreactor, Nylon 66, Oil process 1(OP1), Viscometer, Polyolefin,

THE BIOCONVERSION OF PLASTIC MATERIALS

by

BRYAN A. STUBBLEFIELD

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2016

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2016

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Office of Graduate Studies

College of Arts and Sciences

Georgia State University

May 2016

DEDICATION

South African proverb, Ubuntu – “I am because we are.”

I would like to dedicate this dissertation to the spirits of divinity and compassion, for these concepts are the true motivation of scientific research. I would also like to dedicate this work to those who dreamed of a higher education but never had the opportunity to obtain one, likewise to persons known and unknown who helped me in this journey of discovery. Starting with my ancestors and elders; Mrs. Anne Baugh, Mrs. Victoria Stubblefield, Mr. RT Stubblefield, Mr. Charles Baugh, Dr. William H. Woods; the whole of the Baugh, Stubblefield and Ohienmhen families for without their support this work of scientific innovation would not be possible.

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1 INTRODUCTION

1.1 Plastic: uses, value, and history

The first truly synthetic polymer, nylon 6, 6, (was prepared by Carothers (1935), at DuPont. This polymer was made to compete with silk in the world's commercial fabric markets. The first major commercial product produced using nylon 6,6 was hosiery, and after overwhelming acceptance, nylon 6,6 entered the home furnishing markets. Due to its lower price than the natural polymer of silk, it was able to replace that material in many applications. Later, nylon 6 was developed by BASF to compete with nylon 6,6 for a share of the nylon polymer market. To date, nylon 6,6 and nylon 6 are the leading products of the nylon market with a 90% combined share (Ulrich 1993). Polyolefins are the most widespread polymers in use to date, comprising 22.1 million metric tons produced annually (Hamilton and Reinert 1995, Czaplicka-Kolarz et al. 2010) and include the commercially important polymers polyethylene and polypropylene.

1.2 Plastic pollution in the environment: accumulation and biodegradation

The discovery of plastic polymers and resins during the early decades of the 20th century catalyzed a new era of manufacturing, but with great innovation, often come new challenges. In the plastic industry this challenge is environmental pollution. Many of the features about plastics that make them economically attractive result in their widespread distribution and persistence in the environment. Importantly, because of their low cost, much plastic materials that have been created are discarded after use, with many products being designed as single-use. In the environment, plastics are degraded through mechanical action attrition into small pieces that retain their polymeric character and resist degradation at the chemical level (Andrady 2011). When plastics end up in aquatic environments, their hydrophobicity and buoyancy results in

plastic particles of wide-ranging sizes suspended in the water column, eventually resulting in the “garbage patches” in several of the world’s oceans, and also collecting and persisting in other bodies of water of different scales in the world’s biomes. In the environment, plastics may interact with biota in ways that are harmful due to their physical persistence (e.g. accumulation in the gastrointestinal tracts of pelagic birds) and as sources of toxic chemicals (e.g. phthalates) (Andrady and Neal 2009, Andrady 2011). In contrast to many naturally occurring organic polymers, synthetic plastics comprise a pool of carbon that is not efficiently cycled back into the carbon cycle. A contributing factor to this problem is that most plastics are only biodegraded to a limited extent by indigenous soil bacteria. In a model system, low density polypropylene (LDPE) was biodegraded faster following inoculation with a mixed bacterial consortium derived from a landfill compared to its rate of biodegradation in inoculated soil. In general, technological solutions could potentially accelerate plastic biodegradation and re-entry of the elements into their respective biogeochemical cycles (Wiles and Scott 2006).

1.3 Plastic reuse and recycling

Some plastics can be collected and reprocessed into their chemical constituents for reuse; i.e. plastic recycling. In the USA, plastic recycling is generally limited to six types widely used in consumer products; these include, polyethylene terephthalate (PET), polypropylene (PP), low density polyethylene (LDPE), high density polyethylene (HDPE), polyvinyl chloride (PVC) and polystyrene (PS). Other types of plastics are not readily recycled. Products made from mixtures of plastics and other materials are not readily recycled and are generally treated as waste. Examples of products that are often made of mixed polymers are carpets and metal-coated films. Many of the recycled polymers find other uses as end of life products, such as construction material (Siddique et al. 2008, Yesilata et al. 2009, Kyaw et al. 2012). New approaches to for

these types of products would be beneficial to the environment and also potentially economically.

1.1.1 Related existing technologies

Several groups have developed chemical and physical processes for recycling polymers found in carpets, including nylon 6,6 and nylon 6. In most cases, the objective of these processes is to derive monomers that can be reused to synthesize new polymers, although some processes produce a blended material that can be extruded to form new products (Ashori and Nourbakhsh 2009). In general, the chemical-physical processes require conditions of high heat and pressure, and may need extensive purification steps. In Allied Signal's process for recycling nylon 6, superheated steam (350 °C) and high pressure (100 psig) is used to convert nylon 6 fibers to caprolactam (Wang et al. 2003). BASF has several patents on acid hydrolysis of nylon 6 coupled with superheated steam followed by multistep chemical purification (Wang et al. 2003). A notable challenge is the recovery of the resultant adipic acid, which must be removed by crystallization and filtration. Several processes have been developed that focus on melting and blending nylon fibers and PP carpet backing, occasionally with the addition of compounds that modify physicochemical features of the resulting polymer blend (Wang et al. 2003). Processes for recycling PE and PP may use a catalyst and require temperatures > 400 °C (Achilias et al. 2007).

There is limited research on biological processing of plastic waste. The most productive of these efforts has been developed by Kenny O'Connor in Dublin, Ireland and is based on a pyrolysis process for bioconversion of polystyrene, PET and polyethylene into PHAs ("bioplastics"). As the PHAs have greater monetary value than the starting waste plastic feedstock, the process was referred to as "up cycling" (Kenny et al. 2008). The same group has

developed a new process for PE that involves melting the plastic into a wax and dispersing it with a rhamnolipid biosurfactant, followed by growth with a PHA producer (Guzik et al. 2014). The Negoro group in Japan has investigated enzymes of select bacteria that can grow on that can grow on nylon (Negoro 2005, Sudhakar et al. 2007). Efforts are underway to engineer recombinant bacteria with enhanced capabilities applicable for plastic polymer bioconversion (Mazzoli et al. 2012).

1.2 Bio-based products

The development of microbial biotechnology has centered on the production of chemicals, food and pharmaceuticals. These bio-based products are developed from a myriad of organisms and include amino acids from *Corynebacterium glutamicum* fermentation (Rittmann et al. 2008), ethyl alcohol production from *Saccharomyces cerevisiae* (Shigechi et al. 2004) and antibiotics from various strains of bacteria and fungi. The potential for manufacturing diverse molecules has expanded greatly in recent years with the advent of the field of synthetic biology. The technology is now able to reach beyond the limitations of nature, to produce a wider diversity of chemical products (Keasling 2012), including higher biofuels yields (Lee et al. 2008) and plant metabolites expressed using bacteria (Xu et al. 2013).

1.3 Project rationale and hypotheses

The premise of this work is that waste plastics could be used as a source of nutrient for industrial microorganisms if the plastic polymers were depolymerized into small bioavailable molecules. This would allow waste plastics to be used in bioconversion processes for production of value-added compounds. To accomplish this, we have developed two processes for plastic depolymerization that yield products which can serve as growth substrates for microorganisms.

- 1) Acid hydrolysis can depolymerize polymers containing ester- and peptide-type linkages, notably nylon 6,6. The depolymerized nylon 6,6 can then be used as a growth substrate for microorganisms.
- 2) Microorganisms growing on nylon-derived growth media can produce a useful product.
- 3) Optimized growth of microorganisms in plastic-derived media can take place in a durable, benchtop bioreactor.

The proposed work in this dissertation will help reconnect carbon presently found in plastic waste back into the carbon cycle. The concept underlying the project is that microbes, as the primary decomposers of materials, act as the primary agents in the recycling of matter in the biosphere and their abilities can contribute to reducing plastic waste in the environment.

2 CHAPTER: ACID-HYDROLYZED NYLON CARPET FIBERS AS A GROWTH SUBSTRATE FOR MICROORGANISMS

2.1 Introduction

The amount of plastics that are discarded in the United States each year is on the order of 10^{10} kg (USEPA 2015), an amount that makes plastic polymers an intriguing potential feedstock for bioconversion processes. Under natural conditions, microbial biotransformation's of synthetic plastics are generally limited, although researchers have identified microorganisms, particularly fungal strains, which can accelerate the rates of biodegradation of select plastics (Kyaw et al. 2012, Cregut et al. 2013, Balasubramanian et al. 2014). As with many polymers, regardless of whether their origin is natural or synthetic, the large size of plastic polymers impedes uptake and catalysis. Additionally, the rate at which synthetic polymers are biodegraded may also be limited by their xenobiotic molecular structures (Tokiwa et al. 2009). Some polymeric materials that resist rapid biodegradation are made of otherwise labile monomeric

constituents; for example, cellulose (composed of glucose monomers) or chitin (composed of N-acetyl-D-glucosamine subunits). While not always the case for plastic polymers, several monomers of commonly used plastics are relatively labile; i.e. 6-aminohexanoic acid, caprolactam, styrene, terephthalic acid (Hara et al. 2007, Yasuhira et al. 2007, Gaszczak et al. 2012, Rajoo et al. 2013). Consequently, plastic polymers can be depolymerized into monomers or small oligomers; they serve as growth substrates for microorganisms. Moreover, microorganisms growing on plastic-derived media could function as biocatalysts for producing value-added products.

Plastic polymers of economic significance that could potentially be depolymerized to support microbial growth are nylons. The most commonly used nylons are comprised of repeating units of 6-aminohexanoic acid (nylon 6) or adipic acid and hexamethylenediamine (nylon, 6,6). Nylons are an interesting potential feedstock for a bioconversion process because of their abundance in a variety of commercial products, notably carpet fibers. We have developed a proprietary process that employs acid hydrolysis for nylon fiber depolymerization to produce a microbial growth medium. The objective of this work was to characterize the medium composition and to determine whether post-consumer nylon-containing carpet waste could be used to generate a medium that would support the growth of industrial microorganisms. Additionally, we hypothesized that carpets made of both nylon and wool fibers could be converted into a microbial growth medium by our process, as wool contains a significant amount of protein that will depolymerize by acid hydrolysis. The results of this work are presented herein.

2.2 Materials and Methods

2.2.1 Chemicals

Caprolactam, adipic acid, hexamethylenediamine (HMDA), nylon 6,6 (N66), nylon 6, ninhydrin spray reagent 2%, HCl and H₂SO₄ were purchased from Sigma-Aldrich, USA.

2.2.2 Microorganisms and culture conditions

The microorganisms used in this work are listed in Table 2. All cultures were stored at -80 °C prior to use. *P. putida* KT2440 was cultivated from stock originating in the ATCC culture collection. Inocula were grown overnight from frozen stock in 30 - 50 mL LB broth in an Erlenmeyer flask stored at 30 °C. The resulting cell suspension was washed once in M9 medium or 50 mM phosphate buffer prior to use. For *Yarrowia lipolytica*, 5 mg L⁻¹ yeast extract (YE) was added to the growth medium.

2.2.3 Growth medium

The growth medium was prepared by depolymerizing a known mass of N66 carpet face fibers in 30% hydrochloric acid to make a concentrate and then mixing the concentrate in M9 salts (Anderson 1946, Atlas 2004). The concentration of the acid hydrolyzed nylon medium, referred to as AH4, was 1 g L⁻¹, except during yeast cultivation when it was 2 g L⁻¹. A growth medium containing 80:20 percent N66-wool face fibers was prepared as described herein for the N66 medium. The protocol for the production of AH4 media is found in the appendix section C.4 of this dissertation.

2.2.4 Growth rate measurements

Specific growth rates for bacteria and yeast were calculated based on optical density readings at 600 nm. Bacteria were grown in 1 g L⁻¹ AH4 medium. Yeast was grown in 2 g L⁻¹ plus 5 mg L⁻¹ YE. Yeast was also grown in the corresponding concentration of YE with no AH4

in order to determine the contribution of the YE to biomass. The specific growth rates of *Streptomyces* sp. and *Penicillium* sp. were determined from wet weight calculations. The mass of the spores used for the inoculum was measured and added to 10 mL of AH4 medium in a 250 mL Erlenmeyer flask and grown at 30 °C and 150 rpm. After 7 (*Streptomyces* sp.) or 13 (*Penicillium* sp.) days of growth, the biomass was collected, centrifuged at $20,400 \times g$ for 5 minutes, decanted and wet weighed.

2.2.5 Bioreactor operation

A 450 mL bioreactor operating in batch mode was used for growth experiments. All components of the bioreactor were autoclaved prior to use. Growth experiments were conducted at 30 °C with a mixing speed of 150 rpm. The pH of the medium was maintained at 7.2 - 7.5. Filter sterilized sparged air was continuously added to the bioreactor at greater than 1.5 liters of air per minute. The bioreactor was sampled aseptically via a sampling port and pH and temperature data were continuously collected via a data logging device (Sper Scientific, USA).

2.2.6 LPLC fractionation for analysis of AH4 composition and biodegradation

Low pressure liquid chromatography (LPLC) was used to separate AH4 medium in order to characterize its chemical composition and also to measure its biodegradation during *P. putida* KT2440 growth. Two stationary phases were used: to examine the chemical composition of AH4, a methyl-based hydrophobic resin (Macro-prep Methyl HIC support, 156-0080; Bio-Rad, USA) was used; to measure substrate biodegradation during *P. putida* KT2440 growth, Davisil silica gel, 60-70 mesh was used (Grace, USA). Columns were made from 3 mL polypropylene plastic syringes with the plunger removed and with the needle opening plugged with glass wool. Each column was dry packed with silica gel and equilibrated with 100% 2-propanol at a flow rate of 1 mL min^{-1} . After packing the column to the 1 mL mark, a 2 - 3 mm

layer of sand was layered on top to keep the column level. Columns were allowed to equilibrate with the mobile phase. For columns packed with the hydrophobic resin, the mobile phase was 50% ethanol. For columns packed with silica gel, the mobile phase consisted of 3:1:1 2-propanol: acetic acid: ultrapure distilled deionized water (DDH₂O). Each time prior to use, columns were washed with 40 volumes of mobile phase. A Masterflex model 77521-50 peristaltic pump was used to add the sample to the column with a flow rate of 1 mL min⁻¹ (Cole-Parmer, USA). A constant flow rate was maintained over the course of experiments. A total of 1 mL of sample was loaded on the column and for each experiment, 1 mL fractions were obtained. With the exception of adipic acid, the collected fractions were complexed with ninhydrin, a dye which binds to amine groups, for analysis by spectrophotometer. 100 µl of sample was mixed with 25 µl of a 0.01% ninhydrin solution and 50 µl of DDH₂O in a 1.5 mL microfuge tube. Tubes were mixed for 15 minutes at 300 rpm at 98 - 99 °C. The samples were transferred to 96-well microtiter plates and scanned at 570 nm on a SpectraMax 190 plate reader (Molecular Devices, USA). Adipic acid samples were complexed with 20 mg L⁻¹ bromothymol blue and were imaged at 430 nm (presence of carboxyl groups) and 620 nm (absence of carboxyl groups) by spectrophotometer.

2.2.7 Electrospray injection mass spectrometry

Electrospray injection mass spectrometry (ESI-MS) was conducted by the Georgia State University Chemistry Core Facility using a Waters Q-TOF micro mass spectrometer equipped with an electrospray ionization source in negative ion or positive mode (Waters, Milford, MA). The instrument was calibrated with sodium formate and the mass range was from 100 - 1000 Da. The sample was introduced into the ion source through direct infusion at a flow rate of 5 mL min⁻¹. Data were analyzed using MassLynx 4.1 software.

2.2.8 *Viscometry*

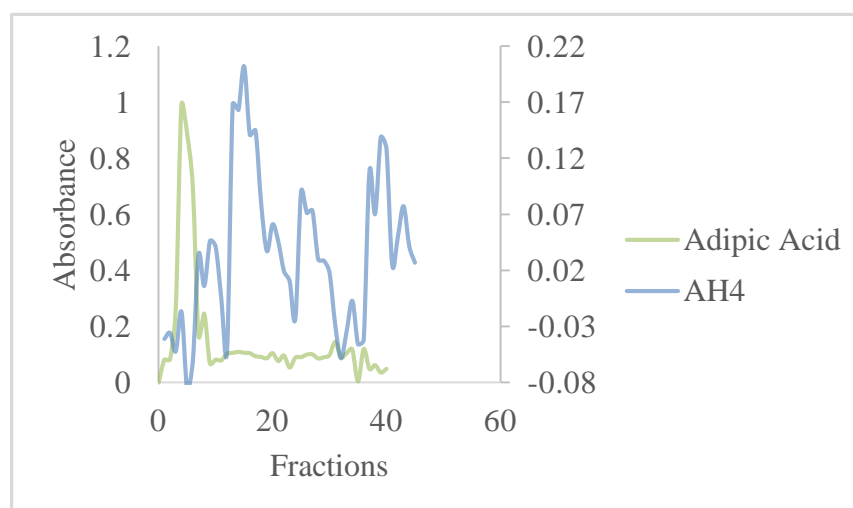
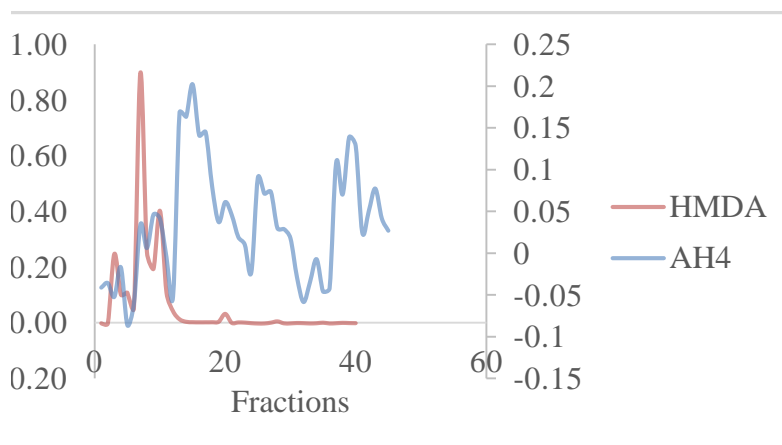
Polymer concentrations in solution can be measured by viscometry, with higher concentrations typically having greater viscosity. Changes in the viscosity of AH4 medium during growth were measured with a Cannon-Fenske tube size 200 viscometer (Sigma-Aldrich, USA). The viscometer was calibrated using DDH₂O and a minimum of 3 replicate measurements were made per time point. Samples from the bioreactor and from flasks were centrifuged at $20,400 \times g$ for 5 minutes to remove cells prior to analysis. The relative viscosity (η_r) was determined by normalizing each day's measurements to the viscosity of DDH₂O.

2.2.9 *Substrate mass measurements*

The mass of carpet fibers used to prepare each batch of AH4 medium was determined prior to processing. The mass of the resin produced from each batch of carpet fiber was also weighed. The mass of solids in the resin was determined by thermal decomposition of the resin using a Corning PC 420 hot plate (Corning, USA) set to maximum for 4 min and weighing the remaining residue.

2.3 **Results**

The composition of AH4 medium was investigated with two approaches, LPLC-spectrophotometry and ESI-MS. First, LPLC was used to fractionate AH4 medium. The absorbance of each fraction was measured spectrophotometrically at 200 nm and 280 nm (Fig. 2.1). Three standards (adipic acid, HMDA, caprolactam) prepared from authentic chemicals were fractionated and measured in an identical manner using the methods described in the section 2.2.6 of this dissertation that their spectra could be compared to the AH4 medium. Peaks in the AH4 medium with similar absorption characteristics to the standards were evident, indicating the presence of these and/or structurally similar compounds in the medium.



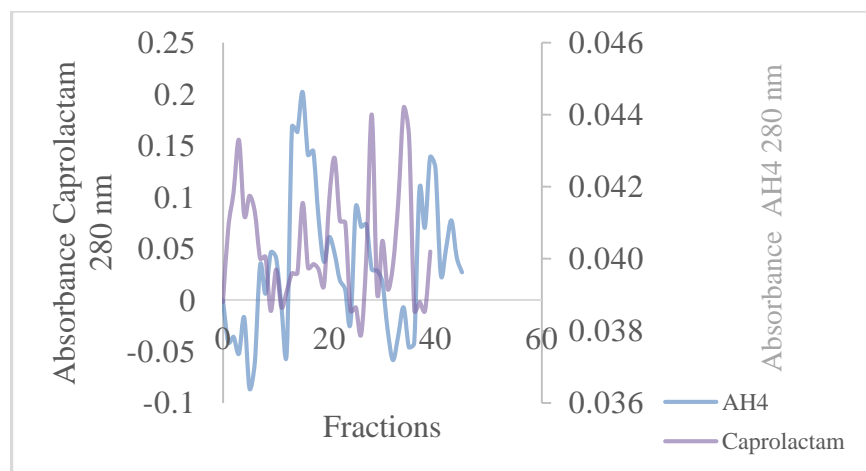
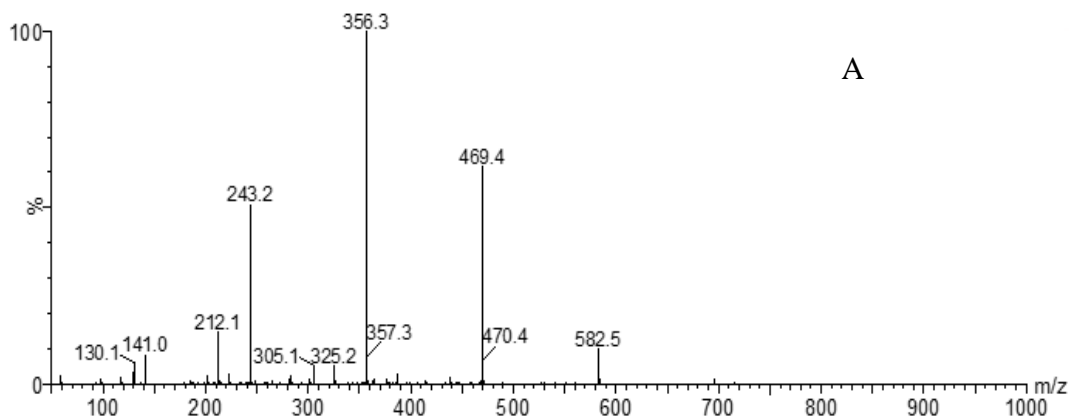


Figure 2.1 Comparison of AH4 medium with authentic standards. Analysis by LPLC-spectrophotometry, A and B, 200 nm C.280 nm.

The N66 monomer, comprised of adipic acid-HMDA ($M = 262$), was evident with an m/z ratio of 243 ($M-H_2O-H$) (Fig. 2.2A). Ion fragments separated by m/z values of 113 were evident at 356, 469 and 582, indicating molecules with varying numbers of HMDA fragments. The maximum m/z ratio of components in the mixture was 582. The data indicated that the medium was comprised of a heterogeneous mix of molecules comprised primarily of N66 monomer, HMDA-adipic acid-HMDA and N66 dimer, or structurally related molecules.



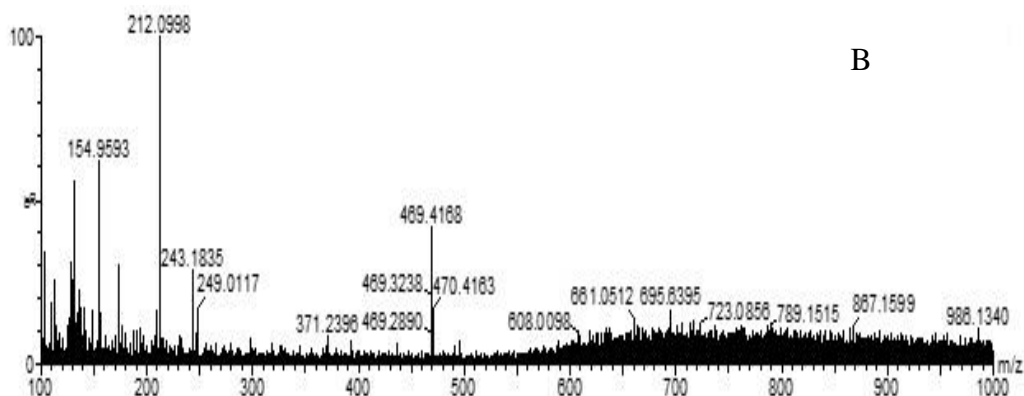


Figure 2.2 A. ESI-MS analysis of the composition of AH4, B. acid-hydrolyzed nylon-wool carpet fiber.

The mass of acid-hydrolyzed carpet face fiber contained in AH4 medium was determined. First, the mass of carpet fiber used to prepare a batch of AH4 medium with a concentration of 1 g L^{-1} The AH4 was compared to the mass of the corresponding derived resin following acid hydrolysis. In three replicate experiments, the mass of the resin was 122 ± 7 percent of the initial carpet fiber mass. Subsequently, the resin was heated to remove water. After heating, the remaining mass was 106 ± 17 percent of the initial carpet fiber mass. ESI-MS was used to determine the size range of molecules in AH4 medium. Scans from 0 – 900 m/z indicated that the largest components of AH4 medium had an m/z ratio of 800 (Fig. 2.2A). Visual inspection indicated that the resin dissolved completely in the M9 solution used for preparing AH4 medium. To determine the extent to which AH4 could serve as a growth substrate for *P. putida* KT2440, the strain was grown in a bioreactor containing AH4 medium as the sole carbon and energy source. Growth was measured turbidimetrically and demonstrated that the optical density increased over the course of 10 days (Fig. 2.3). To measure changes in substrate concentration, the optimal wavelength for analysis was first identified. Scans of AH4 medium from 190 nm to

750 nm found that the greatest absorbance was 210 nm (Fig. 2.4). Spectrophotometric analysis at 210 nm indicated a decrease in substrate concentration concomitant with growth. In order to corroborate the absorbance data, for select time points, the medium was analyzed by LPLC-spectrophotometry. The analyses indicated that the concentration of depolymerized nylon in the medium decreased 80 ± 2 percent over an 8-day period (March) or 9-day period (August) (Fig. 2.5). Viscometry analysis, used to measure polymer concentrations in solution (Shukla 2005, ASTM 2006), indicated that the relative viscosity of AH4 medium decreased from $\eta_r = 1.04 \pm 0.03$ prior to inoculation with *P. putida* KT2440 to $\eta_r = 0.98 \pm 0.01$ after the growth of strain KT2440 in flasks for 6 days. This difference was found to be significant using Student's t test ($p < 0.02$).

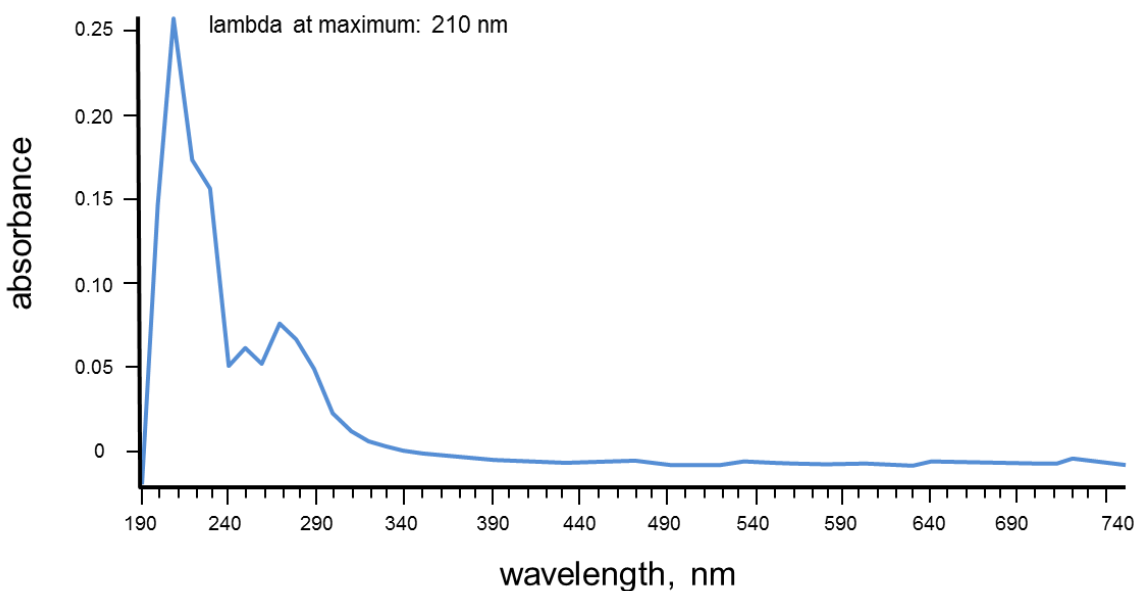


Figure 2.3 Absorbance spectrum of AH4 substrate medium. Maximum absorbance was detected at 210 nm.

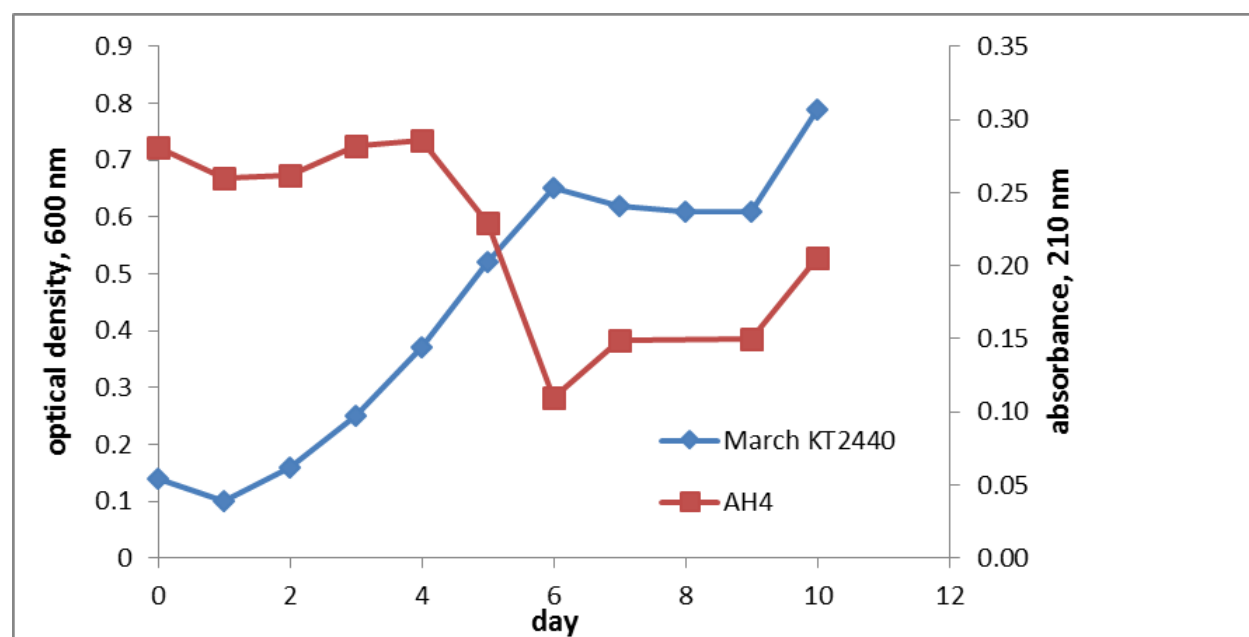
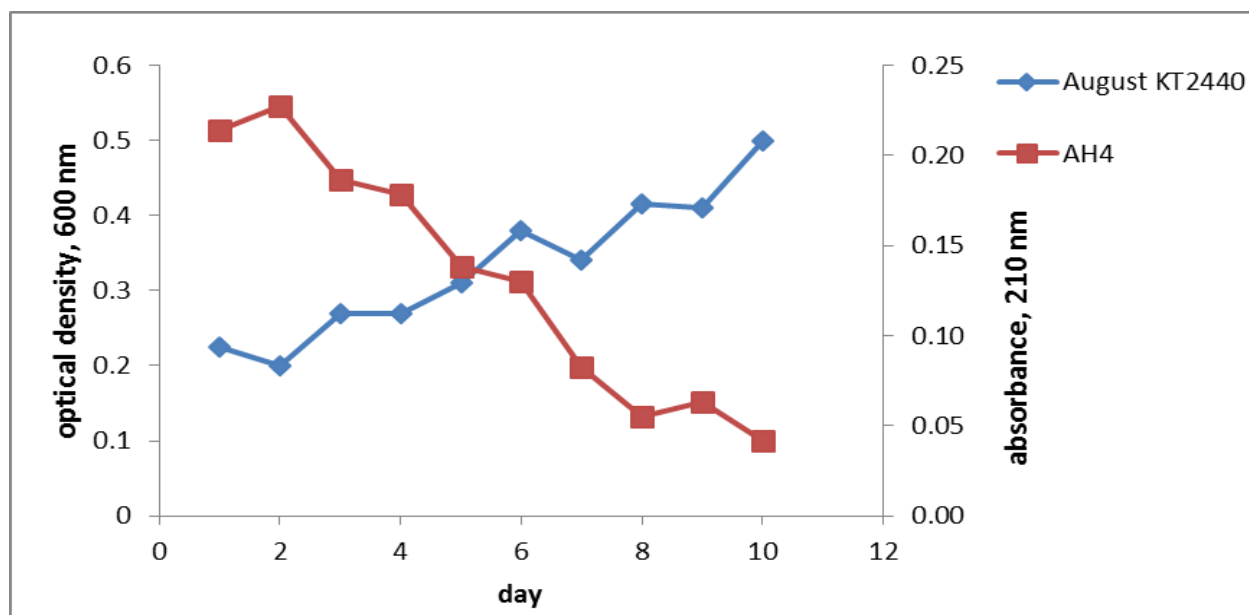


Figure 2.4 *P. putida* KT2440 growth in a bioreactor supplied with AH4 medium.

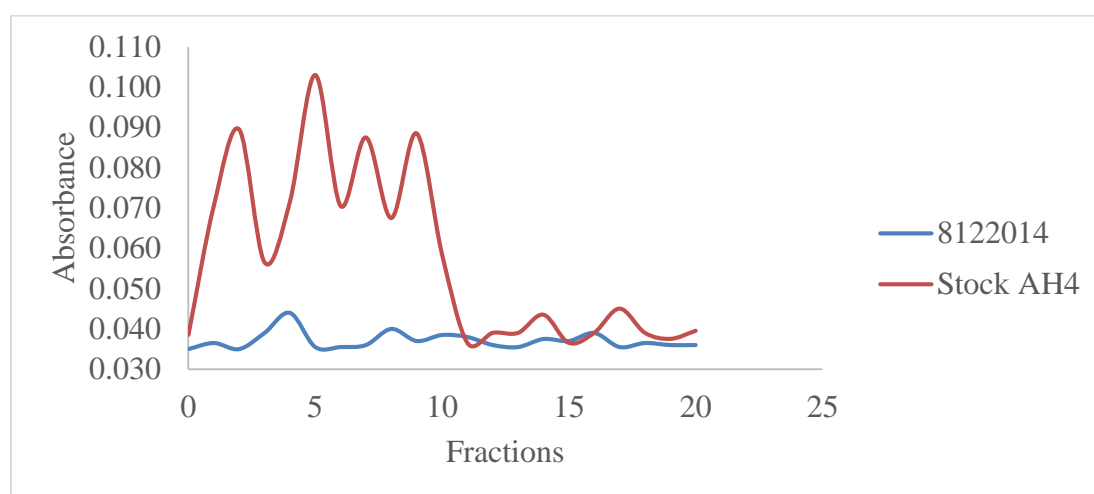
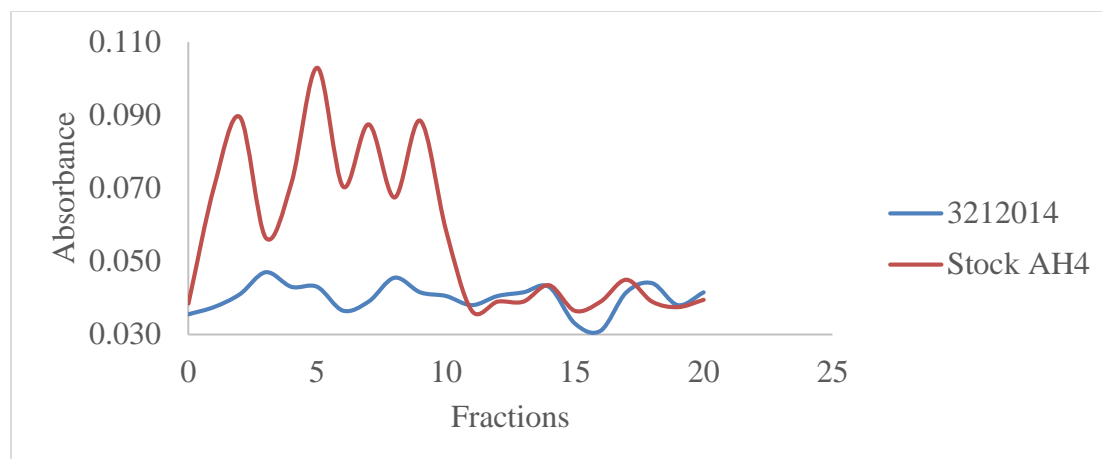


Figure 2.5 LPLC-spectrophotometric analysis of AH4 substrate utilization by *P. putida* KT2440 during bioreactor growth. Independent bioreactor experiments were conducted in August 2014.

Assessment with Student's t test determined that the reduction was significant ($p < 0.01$).

The ability of representative industrial microorganisms to use AH4 as a growth substrate was examined (Table 2). The comparison indicated that yeast supplemented with 5 mg L^{-1} yeast extract grew the most robustly. Generally, the growth of the other organisms was slow.

Experiments with *P. putida* KT2440 demonstrated that its biomass steadily increased over the course of experiments.

Table 2.1. Specific growth rates of select microorganisms using AH4 medium as a carbon and energy source

^aThe biomass of *Streptomyces* sp. and *Penicillium* sp. was measured as mg wet weight. The

Strain	maximum			
	specific growth rate, hr ⁻¹	biomass at start of experiment ^a	biomass at end of experiment	growth period, days
<i>Yarrowia lipolytica</i> (AH4 + YE) ^b	0.205	0.01	2.02	6
(<i>lipolytica</i> (YE only) ^c	0.090	0.01	0.07	6
<i>Streptomyces</i> sp. str. BAS1	0.067	50 mg	220 mg	13
<i>Bacillus subtilis</i> str. 6051	0.062	0.05	0.29	3
<i>Rhodococcus rhodochrous</i> str. DAP 96253	0.013	0.19	0.34	2
<i>Penicillium roqueforti</i>	0.009	10 mg	50 mg	7
<i>Pseudomonas putida</i> KT2440	0.005	0.15	0.80	10

biomass of all others was measured as OD₆₀₀

^bGrowth medium contained 2 g L⁻¹ nylon and 5 mg L⁻¹ yeast extract (YE)

^cGrowth medium contained 5 mg L⁻¹ YE only

Acid hydrolysis of a nylon wool blend carpet (80% nylon, 20% wool resulted in a complex mixture of molecules relative to AH4 (Fig. 2.2C). The resultant medium was used as the sole carbon and energy source to grow *P. putida* KT2440 in a bioreactor over a 4-day period. The optical density of the culture reached 1.1 ± 0.1 (600 nm) on day 3 when measured turbidity leveled off. Substrate consumption was measured by LPLC-spectrophotometry and indicated substantial biodegradation over the 4-day period (Fig. 2.6).

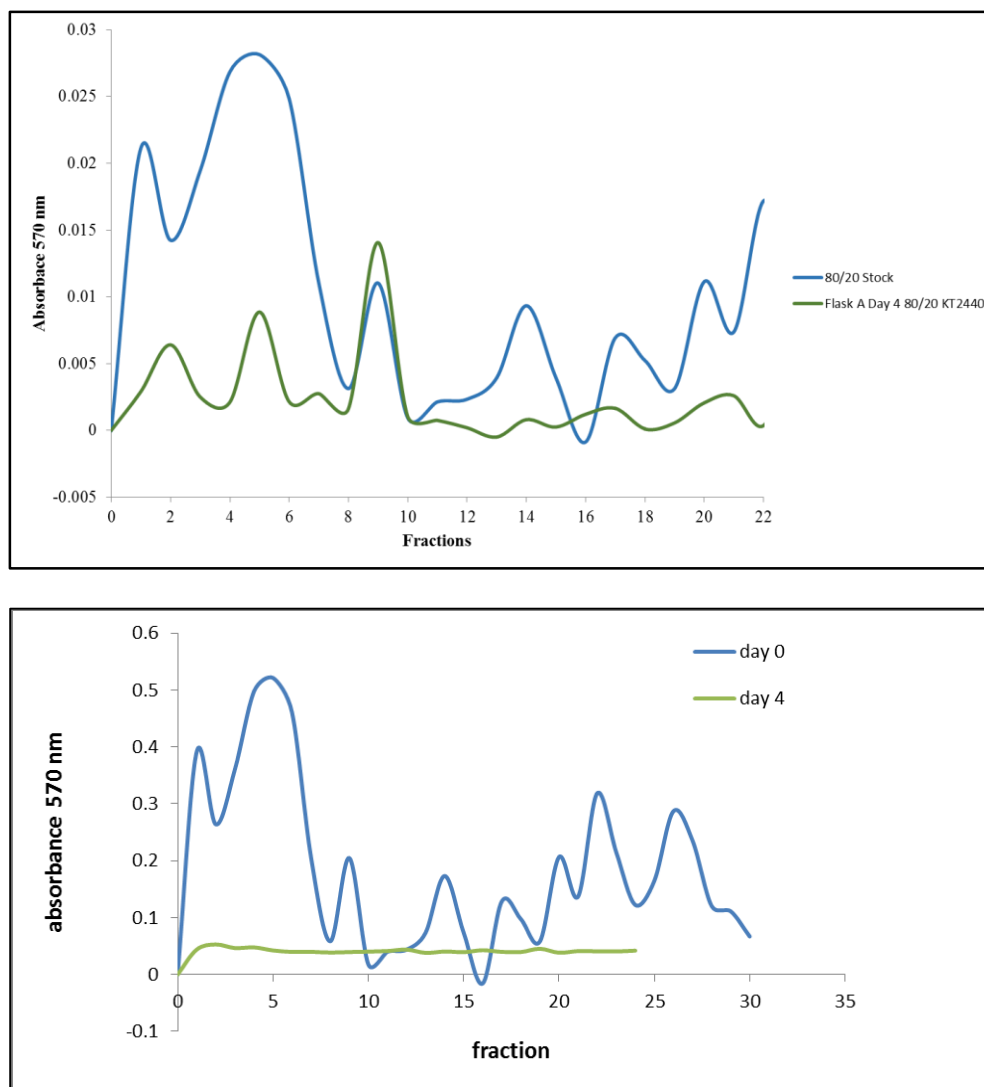


Figure 2.6 LPLC- spectrophotometric analysis of 80:20 nylon wool medium utilization by *P. putida* KT2440 during bioreactor growth. Upper panel: substantial biodegradation is evident after 4 days. Lower panel: data are plotted on separate axes to highlight similar.

2.4 Discussion

Microorganisms are increasingly used as catalysts for the production of value-added chemicals. Examples include the production of biofuels, bioplastics, biosurfactants, enzymes and drug precursor molecules (Keasling 2012). The potential for manufacturing diverse molecules has expanded greatly in recent years with the advent of the field of synthetic biology. We envision that the media described herein can serve as feedstocks for producing value-added

chemicals and simultaneously can help reduce carpet waste. A feature of the described process is that the products of acid hydrolysis do not need to be separated before they can be put to use, as long as they are labile with respect to the microorganisms employed. More broadly, the contribution of acid hydrolysis to the bioconversion process is to generate a mixture of labile molecules that is suitable for biotransformation by microbial biocatalysts.

Diverse approaches have been investigated for reclaiming carpet waste. Several groups have developed chemical and physical processes for recycling carpet polymers, including nylon 6,6 and nylon 6. In most cases, the objective of these processes was to derive monomers that could be reused for synthesizing new polymers, although some processes produced a blended material that could be extruded to form new products (Bogoczek and Pinkowska 2006, Wang 2006, Achilias et al. 2007). In general, the chemical-physical processes require conditions of high heat and pressure, and need extensive purification steps. There is a small but growing body of research on biological processing of plastic waste. One promising approach involves pyrolysis for bioconversion of polystyrene, polyethylene terephthalate (PET) and polyethylene into polyhydroxyalkanoates (PHAs; “bioplastic”). As the PHAs have greater monetary value than the starting waste plastic feedstock, the process was referred to as “upcycling” (Kenny et al. 2008). The same group has developed a new process for polyethylene that involves melting the plastic into a wax and dispersing it with a rhamnolipid biosurfactant, followed by growth with a PHA producer (Guzik et al. 2014). Other groups have identified bacteria that could grow to a limited extent on nylon and investigated nylon-degrading enzymes (Negoro 2005, Sudhakar et al. 2007). The acid hydrolysis pre-treatment presented here for facilitating nylon fiber bioconversion broadens the spectrum of plastic polymers that can be incorporated into biological treatments.

Growth in the bioreactor followed similar patterns in both bioreactor experiments, although there were some notable differences. In the March 2014 bioreactor experiment, the absorbance at 210 nm increased following substrate disappearance over the first six days. This change may have resulted from the secretion of a secondary product into the medium as the cells reached stationary phase, although the specific cause of the increase is presently unknown. Additionally, the growth of *P. putida* KT2440 in the March and August 2014 bioreactor experiments reached different maximum optical densities although the amount of substrate that was measured by LPLC-spectrophotometry to have been removed from the medium was similar. A potential explanation for this result is that substrate was taken up by *P. putida* but was not immediately metabolized. Polyamines, to which AH4 products share structural similarity, are known to bind to intracellular components including RNA (Kim 1965); a similar type of interaction may have occurred when nylon hydrolysis products were taken up by *P. putida*, affecting rates of metabolism. Alternatively, *P. putida* cells in the bioreactor may form a biofilm on the vessel. This process could contribute to differential cell densities measured in the planktonic phase. In general, the steady growth of *P. putida* KT2440 in both experiments supports the concept that acid-hydrolyzed nylon carpet can support microbial growth.

Two aspects of the results warrant specific commentary. First, the analyses of the composition of AH4 medium indicated that the processed carpet face fibers were converted to small molecules that were soluble and that it was unlikely that there were “hidden pools” of high molecular weight molecules in the medium that were not bioavailable. We think it is reasonable to assume that the LPLC-spectrophotometric profiles of the medium during *P. putida* KT2440 growth represent changes in the concentration of the total medium and not merely in a subset of

compounds that were present. Second, a comparative analysis of growth by different types of microorganisms used in industrial processes indicated that *Y. lipolytica* supplemented with 5 mg L⁻¹ yeast extract grew substantially better than the others. Additional work is required to determine whether the growth of the other organisms could be improved with nutrient supplements or whether there are requisite metabolic traits for robust growth in AH4 medium that can be identified.

Acid treatment is an effective way to transform acid hydrolyzable polymers into labile molecules that can be fed to microorganisms. We think that the approach used in this work may apply to other plastic polymers, such as polyethylene terephthalate (PET). Additionally, we hypothesize that products synthesized from blends of polymers can be converted into growth media without prior separation, as long as the component polymers form labile products following acid hydrolysis, as was the case with the 80:20 nylon-wool carpets examined in this work. In general, accelerating depolymerization of plastic polymers can facilitate their incorporation into bioconversion processes and help channel plastic waste into productive use.

3 CHAPTER: MICROBIAL PRODUCTION OF POLYHYDROXYBUTYRATE DURING GROWTH IN A NYLON-DERIVED GROWTH MEDIUM

3.1 Introduction

Waste plastics have the potential to serve as feedstocks for bioconversion processes if their bioavailability can be improved. We developed a method based on acid hydrolysis to depolymerize polymers containing ester linkages, including nylon and polyethylene terephthalate (PET), that yields mixtures of small molecules amenable to microbial uptake and metabolism. The extent of nylon depolymerization that takes place during acid hydrolysis is a function of the acid concentration, the time of exposure, the amount of mixing and the input of heat, with more

of each contributing to greater hydrolysis. This in turn means that more thorough polymer depolymerization will generally be more expensive to carry out, due to the requirement for more resources. In previous work, we examined a nylon 6,6-derived medium that was thoroughly processed and contained only soluble products, called AH4 medium. In this work, we prepared a nylon 6,6-derived medium that was less extensively processed and contained a combination of soluble products and particles, called AH2 medium. We investigated the growth in AH2 medium of *Beijerinckia* sp., a free-living aerobic, chemoheterotrophic microorganism with the ability to synthesize polyhydroxyalkanoates (PHAs; “bioplastics”) (Becking 2006) and also measured the corresponding production of Polyhydroxybutyrate (PHB). The work could shed light on the utility of nylon-derived media for bioconversion processes.

3.2 Materials and Methods

3.2.1 Culture isolation and growth

Beijerinckia sp. was isolated from a compost pile found in Piedmont Park, Atlanta Georgia. The enrichment was carried out using AH2 medium as the sole carbon and energy source. 1 g compost was added to 100 ml AH2 medium and was shaken at 200 rpm and 30 C for 8 days. Subsequently 1 ml of suspension was transferred to 100 ml AH2 medium and was incubated for 8 days. 10 µl was withdrawn from the suspension and cultured on LB agar plate. Colonies that grew were isolated and were evaluated for the ability to grow in AH2 medium, ultimately yielding a greenish fluorescent mucoid colony on LB agar. The cells when stained are gram negative, with cells that had Cluster forming morphology and typed to *Beijerinckia* sp. using Bergey’s Manual, 3rd edition fig 3.5 (Kerstens and Vancanneyt 2005).

3.2.2 *Growth medium*

AH2 and AH4 medium were prepared by depolymerizing N66 carpet face fibers in acid to make their respective concentrates and then mixing the concentrate in M9 salts (Anderson 1946, Atlas 2004). The concentration of AH2 medium was 0.5 g L^{-1} and the concentration of AH4 medium was 1 g L^{-1} . AH4 medium is prepared by more extensive acid hydrolysis of AH2 medium and results in a medium free of particle material suitable for measurements of growth by optical density Growth of *Beijerinckia* sp. in AH4 medium. The protocol to of the production of AH2 and AH4 is found in the appendix C.2, C.4 in this dissertation.

The flask experiments were done in duplicate, with each set having two treatments and one abiotic control (used for the blank in spectrophotometer). Autoclaved 250 ml Erlenmeyer flasks containing 50 ml of filter sterilized AH4 medium were prepared. Flasks were inoculated with $10 \mu\text{l}$ of *Beijerinckia* sp. and were grown over a two-day period. After two days, the mature culture was collected and was centrifuged at $20,400 \times g$ to remove the supernatant and was washed once with 50 mM phosphate buffer. After inoculation, the flasks were placed in a $30 \text{ }^\circ\text{C}$ shaker incubator at 150 RPM for 8 days. Changes in substrate concentration were analyzed by UV absorbance spectrophotometry at 210 nm.

3.2.3 *Bioreactor conditions*

A 450 ml bioreactor operating in batch mode was used for growth experiments. All components of the bioreactor were autoclaved prior to use. Growth experiments were conducted at $30 \pm 1 \text{ }^\circ\text{C}$ with a mixing speed of 150 rpm. The pH of the medium was maintained at 7.2 ± 0.2 . Filter sterilized sparged air was continuously added to the bioreactor. The bioreactor was sampled aseptically via a sampling port and pH and temperature data were continuously collected via a data logging device (Sper Scientific, USA). Samples from the bioreactor were

aseptically obtained over 10 days. Growth in the bioreactor was measured spectrophotometrically (600 nm).

3.2.4 Polyhydroxybutyrate (PHB) extraction and analysis

PHBs were extracted from cells grown in the bioreactor at the conclusion of the experiment. The total volume of cell solution in the bioreactor was removed and centrifuged at 20400 x g. The supernatant was removed and 5 g of the wet cell mass was collected for PHB extraction. 5.23 grams of wet cell mass was mixed with a 1:1 mixture of 100 percent ethanol and acetone followed by shaking at 100 rpm for 30 min. The solvent was removed and the remaining cell mass was subjected to 0.6% sodium hypochlorite at 37 °C for 1 hour with shaking at 100 rpm. The resulting mixture was centrifuged for 10 minutes at 12000 RPM. The supernatant was collected and the pellet was discarded. The supernatant was extracted with pure chloroform at a 10:1 ratio (chloroform: supernatant). The chloroform was evaporated at 45°C using a centrifuge evaporator, leaving a waxy residue at the bottom of the collection tube. (Sayyed et al. 2009) Prior to analysis by gas chromatography, PHB was dissolved in 1.0 ml chloroform. Because the PHB would precipitate during storage, before injection, the mixture was heated in a water bath at 60 °C for up to 20 minutes with vortexing to dissolve.

3.2.5 Gas chromatography

PHB production was measured by flame ionization detection gas chromatography (GC-FID). Analyses were performed with a PerkinElmer Autosystem XL gas chromatograph (PerkinElmer, Wellesley, MA), using a Supelco SPB-1 capillary column (length, 60 m, i.d. 0.32 mm; Supelco, Bellefonte, PA). The injector temperature was 210 °C, and the detector temperature was 220 °C. The carrier gas was helium (2.0 ml min⁻¹), and detector gases were

hydrogen (45 ml min⁻¹) and air (450 ml min⁻¹). PHB was analyzed with the following temperature program: initial column temperature was 160 °C, oven temperature was increased by 8 °C min⁻¹ until 200 °C; temperature was held for 10 min. (Braunegg et al. 1978)

3.2.6 Microscopy

Growth on nylon particles was visualized by bright field microscopy and confocal laser scanning microscopy (CLSM). For both types of microscopy, a cover slide chamber was used to obtain a three dimensional image of bacterial growth on the nylon particle (described below). Bright field microscopy was carried out using an AmScope B120E (AmScope, USA) microscope using a 100x oil immersion objective. Samples for bright field imaging were stained with 0.02% crystal violet. CLSM was conducted with a Zeiss LSM 510 confocal laser scanning microscope (Zeiss, Thornwood, NY) equipped with a Fluor 40X oil immersion lens. For CLSM, samples were stained using 50 µM SYTO 59 nucleic acid stain (Life Technologies, USA). Fluorescence in CLSM images resulted from excitation at 543 nm using the HeNe laser and the 488 nm line of the argon laser. Nylon particles were imaged using excitation of both lasers at a gain of 64% for the argon laser and 90-100 % for the HeNe laser, and appeared blue due to auto fluorescence. *Beijerinckia* sp. cells appeared from the SYTO 59 nucleic acid stain

3.2.7 Cover slide chamber

Microorganisms attached to particle nylon were imaged by stabilizing colonized particles using 3 percent agarose in a microscope-slide sized chamber. The chamber was constructed from two 60 x 22 mm number 1 coverslips separated by a silicone ring made from 0.89 mm i.d. silicone tubing containing a segment of 22-gauge steel wire (Fig. 3.1). To make the chamber, a 63.5 mm length of tubing was threaded with 76 mm of wire and bent to form a “donut” shape. The ring was placed in the center of the coverslip, attached with a thin coating of Barge toluene-

free contact cement (North Brookfield, MA, USA) and allowed to set overnight.

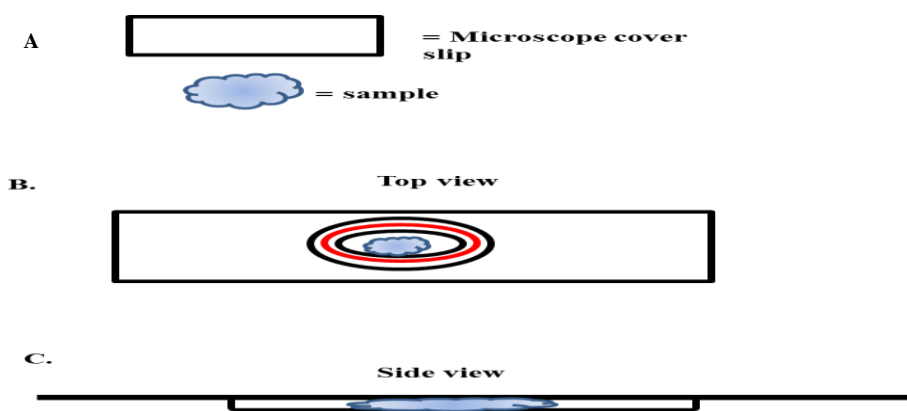


Figure 3.1 Set-up for imaging colonized nylon particles. Two glass cover slips and silicone tubing were used to make a chamber to fix nylon particles in place using 3 percent agarose. (See Methods section for details).

To image, 10 μ l of growth medium containing colonized particles were placed in a 1.5 ml microfuge tube and stained with 50 μ M SYTO 59 for 5 min. After staining, 10 μ l of sample was transferred to the chamber using a 1000 μ l pipet. 1000 μ l of melted 3 percent agarose was quickly placed over the sample and gently tilted to distribute throughout the chamber and to ensure that the sample was in contact with the cover slip. Once the agarose gelled, a thin film of contact cement was placed on the silicone ring and a second 60 x 22 mm cover slip was adhered to it for 20 min, creating a sealed, stained sample that could sit on the stage of the microscope and be manipulated for imaging. (Pittman et al. 2010)

3.3 Results

3.3.1 Growth and substrate utilization of nylon-derived media

Beijerinckia sp. grew on the soluble components of AH2 medium and on suspended nylon particles present in the medium. To evaluate growth on the soluble components of the

medium, *Beijerinckia* sp. was cultivated in a bioreactor over an 8-day period using AH2 medium as the sole carbon and energy source. After an initial decline, the optical density of the medium in the bioreactor increased exponentially after 5 days (Fig. 3.2). Growth on the particle component of AH2 was assessed by bright field microscopy and CLSM. These complementary assays indicated the presence of bacteria growing as biofilms on the surfaces of particles (Fig 3.3, Fig. 3.4). Substrate biodegradation was detected at 210 nm during the growth of *Beijerinckia* sp. on AH4 medium. The substrate could no longer be detected after 4 days.

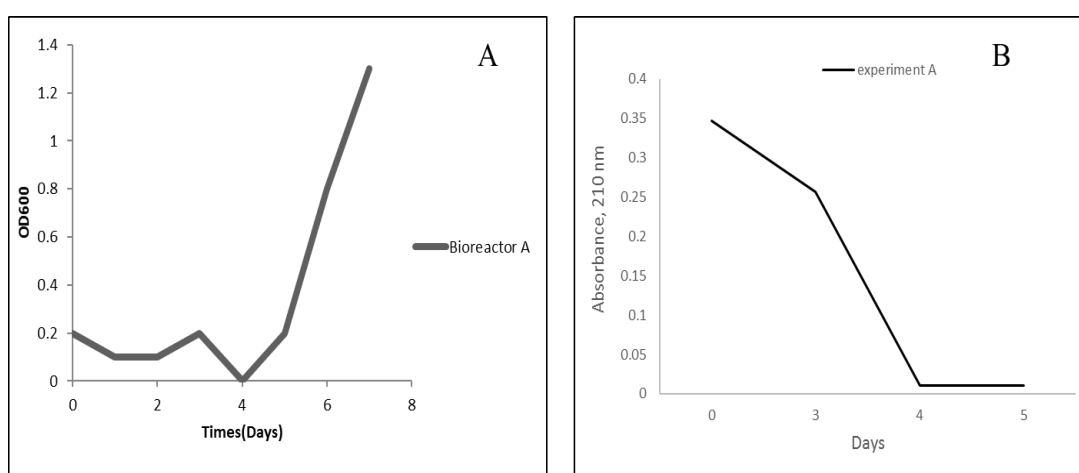


Figure 3.2 Substrate utilization during growth in flasks. A. cellular growth in the bioreactor. B. Growth of *Beijerinckia* sp. using AH2 medium in flask.

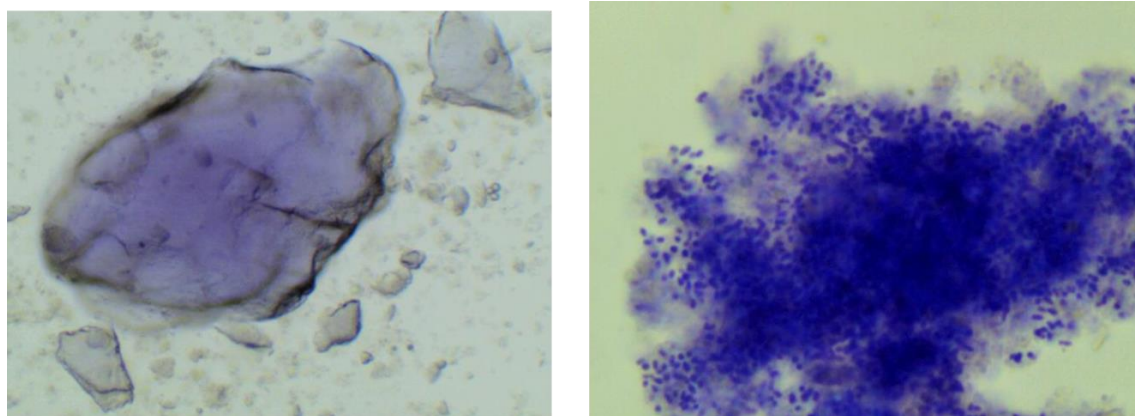


Figure 3.3 Growth of *Beijerinckia* sp. on nylon particles. Left, uncolonized nylon control. Right, colonized sample. All samples were stained with 0.2 percent crystal violet and were imaged using bright field microscopy. Magnification: 400X.

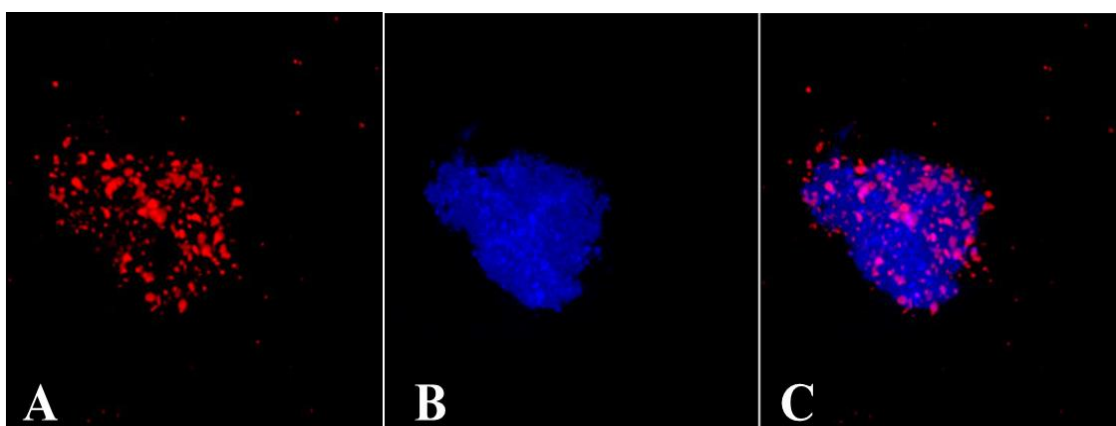


Figure 3.4 Growth of *Beijerinckia* sp. on nylon particles, confocal laser scanning microscopy. The samples were stained with the red nucleic acid stain SYTO 59. a: cellular biomass (red) B, nylon particle (blue); c, merged image showing *Beijerinckia* cells attached

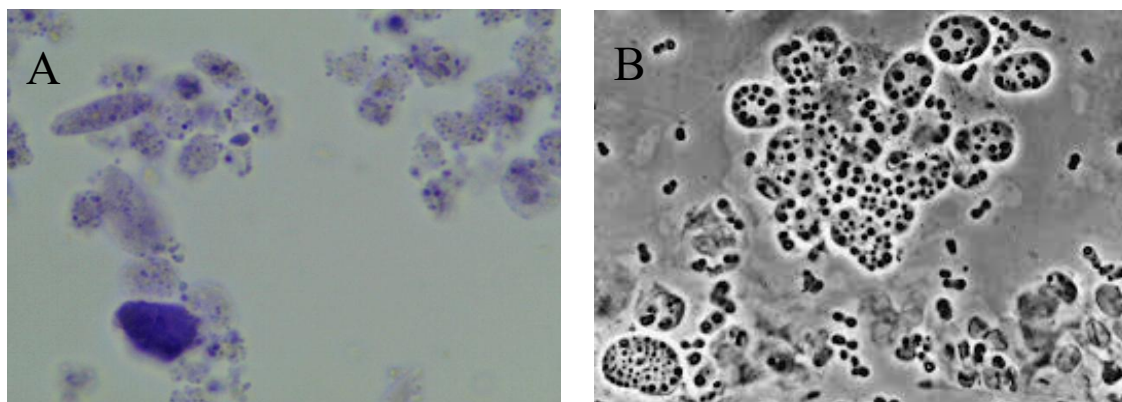


Figure 3.5 Beijerinckia growth on AH2 formation of clusters stain with crystal violet 40x, B. stock image of Beijerinckia from Bergey's Manual of Systematics Bacteriology.

3.3.2 PHB production

The presence of PHB was readily detected following Nile blue staining, visible as a bright yellow color in contrast with the red-stained bacteria (Fig. 3.5). A comparison of extracted PHB with 0.150 grams of commercial PHB standard by GC-FID indicated the presence of several recognizable peaks. Two prominent peaks that were not homologous to the PHB standard were also detected (Fig 3.6).

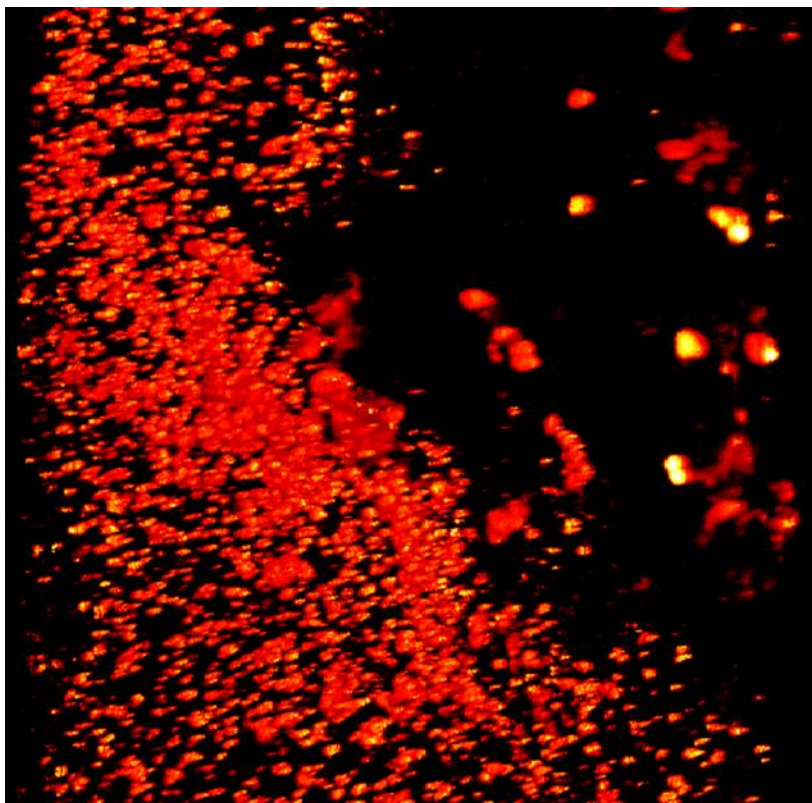


Figure 3.6 PHB production during growth of Beijerinckia sp. using AH2 medium. Red, cell biomass. Yellow, accumulated PHBs. Cells were stained with 0.2 percent Nile Blue A.

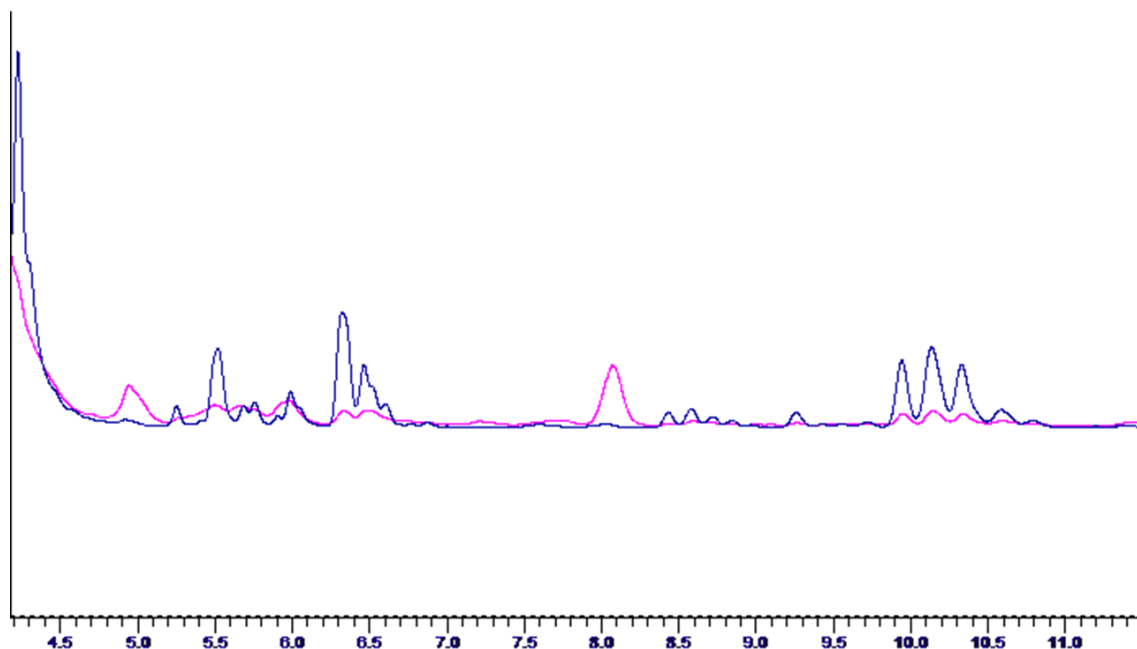


Figure 3.7 GC-FID analysis of PHB production. Blue, PHB control 0.15 g mL⁻¹. Pink, PHB extracted from *Beijerinckia* sp. grown in the bioreactor.

3.4 Discussion

Beijerinckia sp. successfully functioned as a biocatalyst, converting acid hydrolyzed nylon 6,6 into PHB. The findings support the hypothesis that a waste plastic can serve as a growth substrate for a bioconversion process if the plastic is sufficiently depolymerized to increase its bioavailability. The use of acid hydrolysis to improve plastic polymer bioavailability broadens the approaches available for bioconversion of plastic waste, which presently include pyrolysis (Kenny et al. 2008), melting and dispersal and enzymatic treatment (Negoro 2000). In the present work, we determined that PHB could be produced during the growth of *Beijerinckia* sp. on AH2 medium; we anticipate that a diversity of products can be made during growth on nylon-derived media using both wild-type and engineered strains of microorganisms. The optimization of PHB production by microorganisms and its subsequent recovery have been extensively analyzed (Suzuki et al. 1986) and future work will focus on optimizing the process presented herein.

Three points emerging from this research related to growth in AH2 medium warrant additional comment. First, surface-attached growth was evident on particles, although it is unclear whether the particle-associated bacteria metabolized their particles, only used the particles as surfaces and then metabolized soluble material in the surrounding medium, or a combination of both. Second, we think that bioprocesses based on soluble media such as AH4 medium are likely to be simpler to optimize due to the thoroughly dissolved carbon source, but that AH2 medium could function in select processes and be advantageous due to the predicted lower production costs. With the extraction process of PHB the nylon particles are unaffected and the PHB is separated from the nylon by being dissolved in the chloroform extract. Third, a challenge for evaluating bioconversion processes involving particle suspensions is assessment of microbial growth. This work introduces a novel approach for examining microbial growth on nylon 6, 6-derived particles based on agarose stabilization, CLSM and digital image analysis. The technique could likely be adapted to assess microbial growth on other polymer-derived particles. Overall, the presented work indicates that optimization of bioconversion processes based on nylon-derived media could be rewarding.

4 CHAPTER: BENCH-SCALE BIOREACTOR WITH NOVEL IMPELLER DESIGN

4.1 Introduction

Bioreactors hold a central place in the production of pharmaceuticals, solvents, and in the treatment of waste water (Bramucci and Nagarajan 2000, Rushmore et al. 2000, Wang et al. 2005, Ezeji et al. 2007). Bioreactors are typically used to enhance the control of microbial activities, particularly with regard to biotechnology. Important features that bioreactors provide are optimal temperature, pH and aeration and also can support high cell densities. The use of bioreactors in industry is increasing, creating a demand for skilled personnel.

Efforts to incorporate bioreactors into an undergraduate curriculum have been developed. Manhattan College, New York, NY modified their curriculum so that seniors worked with bioreactors in order to prepare their students for an career in (Assaf-Anid and Hollein 2002). The Karlsruhe Institute of Technology offers a one-week course in bioprocess engineering where up to 12 pairs of students each operate a Minifors bench top bioreactor and cultivate *E. coli* containing expressing a recombinant green fluorescent protein (Henkel et al. 2015). A simple design using a modified Erlenmeyer flask and a brewer's airlock has been used to introduce principles bioreactor use in fermentation (Minerick and Schulz 2005). Another bioprocessing laboratory designed for undergraduate seniors used two BioFlow 3 fomenters for the class (Shuler et al. 1994). Notably, these courses focused primarily on training students in bioengineering and chemical engineering degree programs.

In spite of their significance to industry, bioreactors are rarely part of undergraduate science curricula. We think that the experience of operating a bioreactor would greatly benefit undergraduates both as scientists and as job-seekers. It is hypothesized that most science teaching laboratories decline to include bioreactors in their curriculum because either the cost is prohibitive, the equipment is too complex or fragile, or both. Moreover, the incorporation of bioreactors into undergraduate teaching curricula could be feasible if the cost of the unit was low and if it were durable and easy to use. The following sections describe a bioreactor design that meets these criteria and that has novel design elements, including a new impeller and modular assembly, that will contribute to expanding student access to fermentation science.

The impeller is the component of a bioreactor that imparts homogenous mixing to the liquid medium in the bioreactor vessel, keeping cells and nutrients well-mixed to stimulate maximal growth (Gogate et al. 2000, Puthli et al. 2005). It is essential that the impeller rotate smoothly to avoid introducing irregular mixing in the bioreactor, which may result in foaming and suboptimal growth. Smooth rotation is also important to avoid uneven wear of the impeller parts due to mechanical friction, especially since the impeller must actively rotate uninterrupted for long lengths of time. The standard structure of a spinner flask impeller involves a pivot point for the spinning action placed on a fixed glass rod with a plastic base. The rotation action is made with a magnetic rod inserted into the plastic base. The impeller spins once the bioreactor vessel is placed on a magnetic stir plate. Two of the major producers of fermenters, Bellco and Corning, each employ designs of this type. Although spinner flasks were first design to facilitate the growth of low shear animal cells, its basic physical design can be used for microbial fermentation reactions.

In this work, a novel impeller design was developed. The impeller was comprised of two parts: a modified conical tube containing a magnetic stir bar and a steel bolt that functions as an axle around which the conical tube rotates and which attaches the impeller to the bioreactor vessel. Three steel nuts attached to the axle act as a ball bearing-like system and are responsible for the impeller's steady spin. The impeller hangs from and is secured to the cap of the bioreactor vessel. The presented design results in an impeller that spins smoothly, rapidly and for extended lengths of time. The impeller design is described in detail in Appendix A.

4.2 Materials and Methods

4.2.1 Bioreactor overview

Details regarding the design and construction of the bioreactor can be found in the accompanying supplemental document (Appendix A). Briefly, the bioreactor has four modules: 1) vessel, 2) air pump, 3) heating unit and 4) data logger (Scragg 1991, Olisti 1992). The vessel comprises a 500 ml spinner flask with a modified cap containing ports that provide access for probes to sample pH, temperature and oxidation-reduction potential and also to withdraw solution. Conditions in the vessel were continuously monitored by a data logger (Sper Scientific, USA). The vessel was stirred by an impeller driven by a magnetic stir bar and stir plate. The design of the impeller is novel and is described in the attached document. Filter-sterilized air was pumped into the bioreactor using a variable flow aquarium pump (80 gal capacity) set at 1/3 its highest rate. The temperature of the vessel was maintained by circulating hot water through a heat jacket.

4.2.2 Bacterial strains and growth media

Streptomyces BAS1 was isolated by enrichment culture from soil using acid-hydrolyzed nylon as the sole C source. Prior to inoculation, strain BAS1 was cultivated on nutrient agar overnight at 30 °C. AH1 medium was prepared by soaking nylon 6,6 (N66) pellets in a hydrochloric acid solution according to a proprietary protocol to induce partial depolymerization, and after neutralization to pH= 7.0, resulted in a particle suspension of N66. The suspension was mixed with M9 salts to make a growth medium and contained 0.5 g of N66 per liter of growth medium. *Pseudomonas putida* KT2440 was cultivated on nutrient agar at 30 °C overnight and was grown in the bioreactor in 0.2 g L⁻¹ succinate-M9 medium without glucose.

4.2.3 Analysis of bioreactor performance

Measurements of pH, temperature and redox potential were collected with a combination probe (Sper Scientific USA). Readings were taken hourly during the operation of the bioreactor and the data were stored on an SD card.

4.2.4 Analysis of growth

Streptomyces sp. strain BAS1 growth was analyzed by confocal scanning laser microscopy (CLSM). At each sampling, 100 μ l of culture were withdrawn from the bioreactor and were stained with 1 μ l of 20 μ M SYTO 59 nucleic acid dye. Image stacks of biomass attached to N66 particles were collected. The SYTO 59 dye fluoresces red and distinguishes cellular biomass from the N66 particles to which they are attached and which fluoresce blue. To quantify the biomass, the image analysis software program COMSTAT was used (Heydorn et al. 2000). CLSM was carried out using an LSM 510 instrument (Zeiss, USA), with excitation by argon laser at 543 nm wavelengths and a helium/neon laser at 488 nm. *Pseudomonas putida* KT2440 growth was measured turbidimetrically at 600 nm using an Ultrospec 2000 spectrophotometer (Pharmacia, USA). Three replicate independent bioreactor runs were conducted for *Streptomyces* strain BAS1 and two replicate independent runs were carried out for *P. putida* KT2440.

4.3 Results

4.3.1 Bioreactor performance

Streptomyces BAS1 was grown in the bioreactor for 7 days. Hourly measurements of pH, temperature and oxidation-reduction potential were collected for three independent bioreactor runs of 168 hours (Figs. 1 – 3). In two of the three runs, the pH fluctuation was less than 0.1 log units relative to the initial pH; for run C the fluctuation was less than 0.2 log units.

In terms of temperature, once the bioreactor equilibrated, the vessel remained within 1 °C of the initial setting, with the exception of run C, which had a downward spike at 140 hours. In this case, the data indicate that the bioreactor returned to its set temperature within 8 hours. In terms of redox, two of the three runs indicate a change in voltage of less than ± 5 mV relative to the initial redox measurement. In the case of run A, a steady decline in redox due to pH was measured.

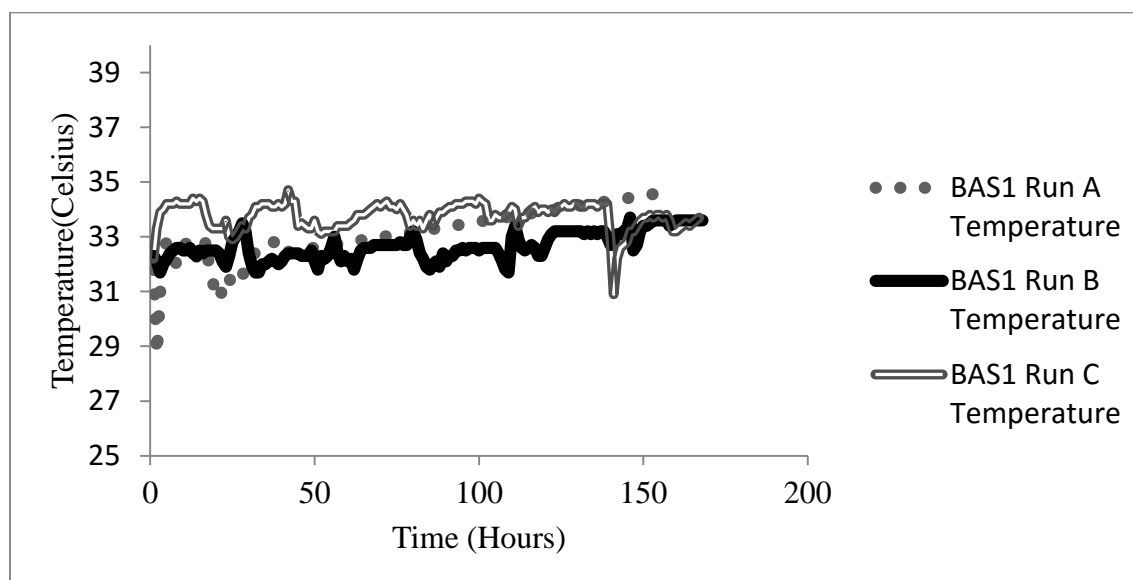


Figure 4.1 Temperature level in bioreactor vessel during growth of *Streptomyces* BAS1. Dotted line, run A. Dark line, run B, and clear line run C.

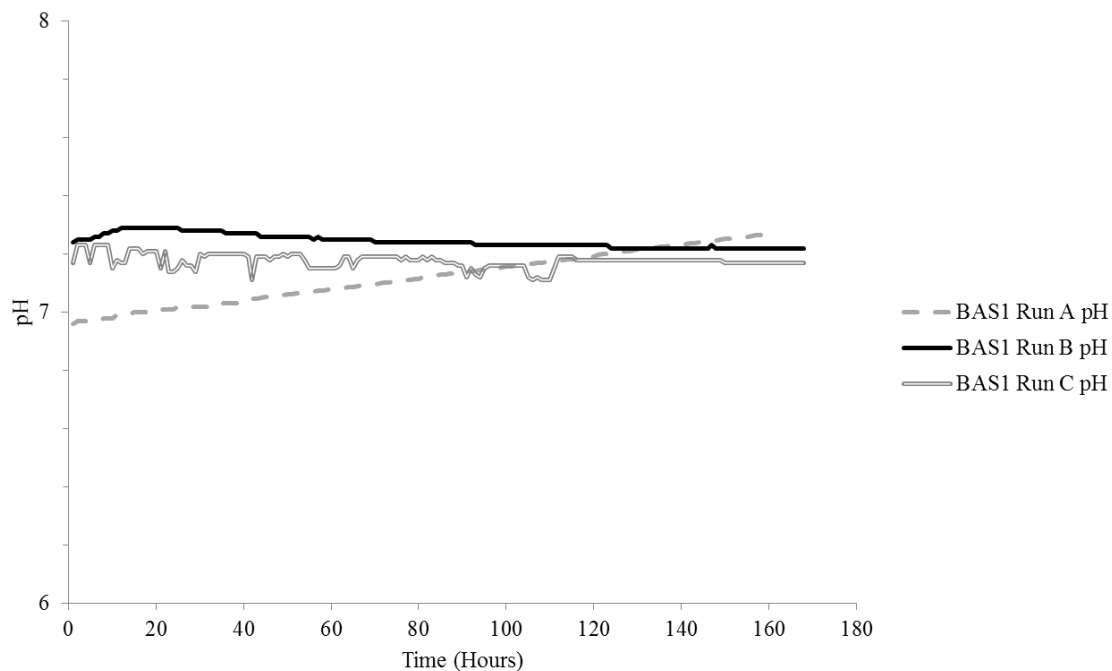


Figure 4.2 pH level in bioreactor vessel during growth of *Streptomyces BAS1*. Light dash line, run A. Solid black line, run B, Clear line run C.

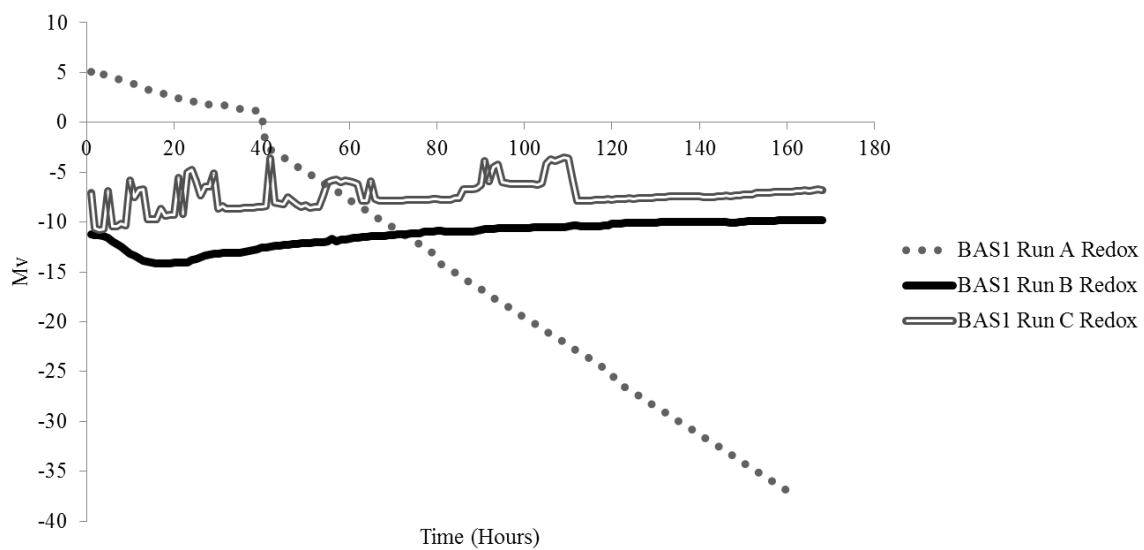


Figure 4.3 Redox level in bioreactor vessel during growth of *Streptomyces BAS1*. Dotted line, run A. Dark line, run B, and clear line run C.

P. putida KT2440 was grown in the bioreactor for 3 days. Hourly measurements of pH and temperature were collected (Figs. 4 -6). During run A, the temperature stabilized at 31 °C. An adjustment to the temperature was made which lowered the temperature to 26 °C where it remained stable for the duration of the experiment. In run B, the temperature was set at 28 °C and remained stable throughout the course of the experiment. For both experiments, the pH was maintained at 6.9 ± 0.05 .

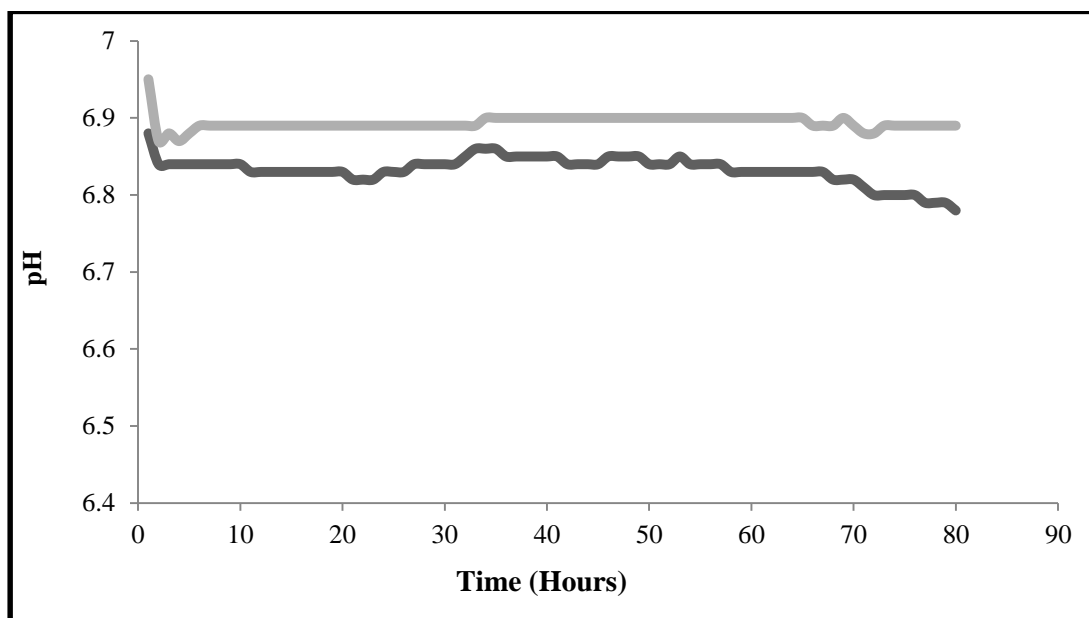


Figure 4.4 pH level in bioreactor vessel during growth of *Pseudomonas putida* KT2440. Light line, run A. Dark line, run B.

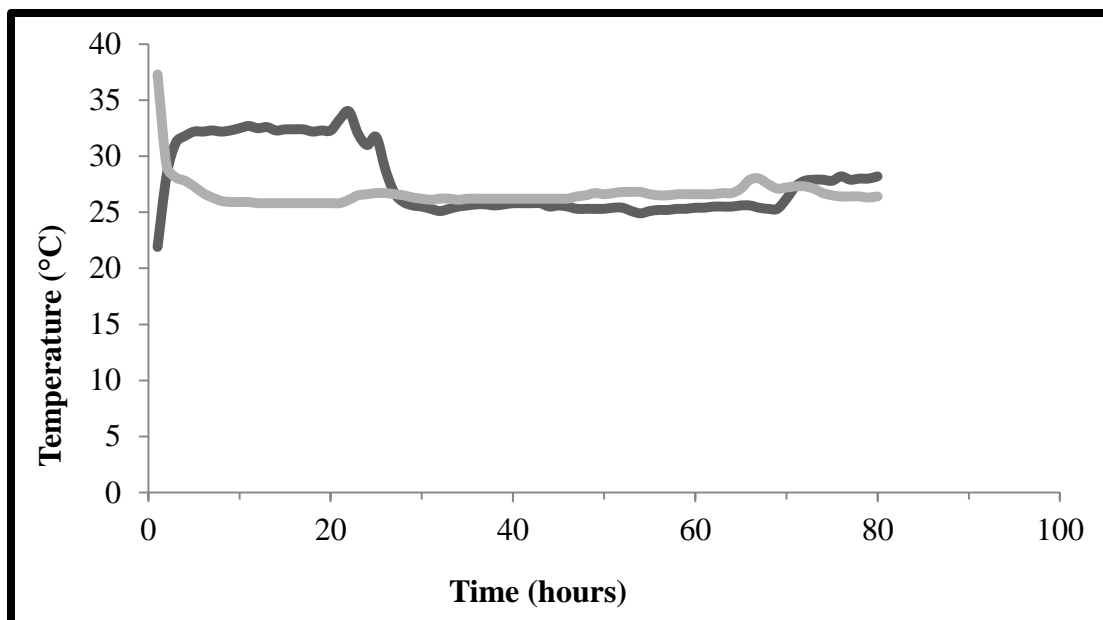


Figure 4.5 Temperature in the bioreactor vessel, *Pseudomonas putida* KT2440 growth. The difference in starting temperature was due to introduction of the temperature probe before the cooling of autoclaved media. Light line, run A. Dark line, run B.

4.3.2 Growth

Growth of *S. BAS1* on AH2 N66 was evident in each of the bioreactor runs (Fig. 4.6-4.7). The growth of *P. putida* KT2440 with succinate as the growth substrate was comparable during both runs of the bioreactor (Fig. 4.8).

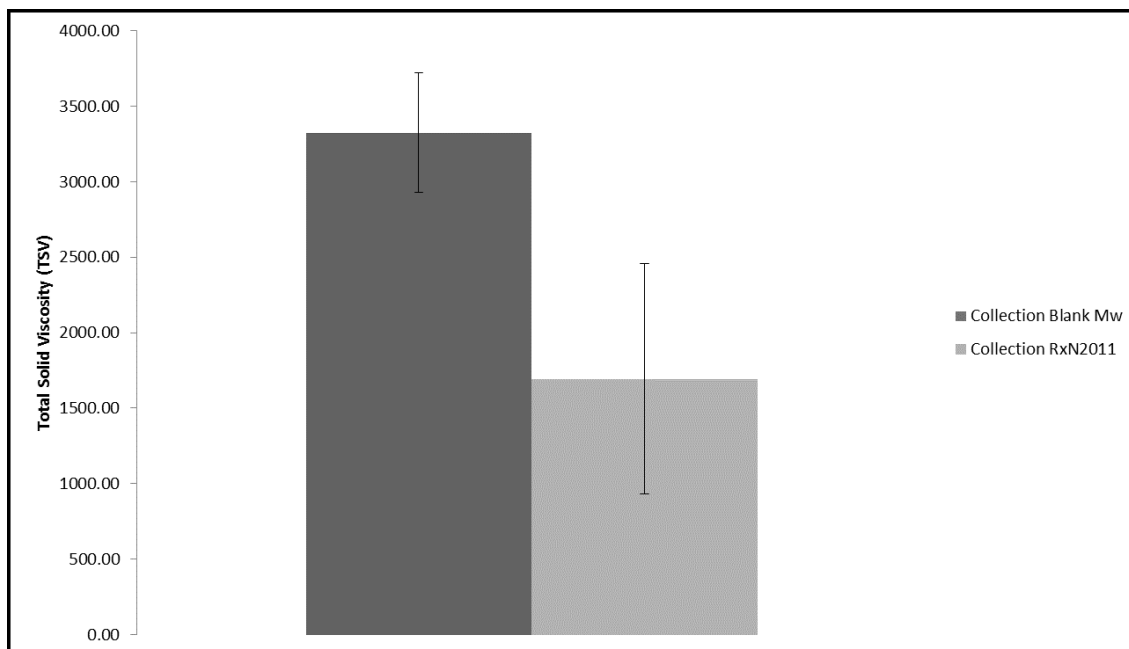


Figure 4.6 Note there is there is a significant difference between the experimental infraction group and the infraction blank in TSV. Note that when compared there is a greater than 50% reduction between the blank and sample.

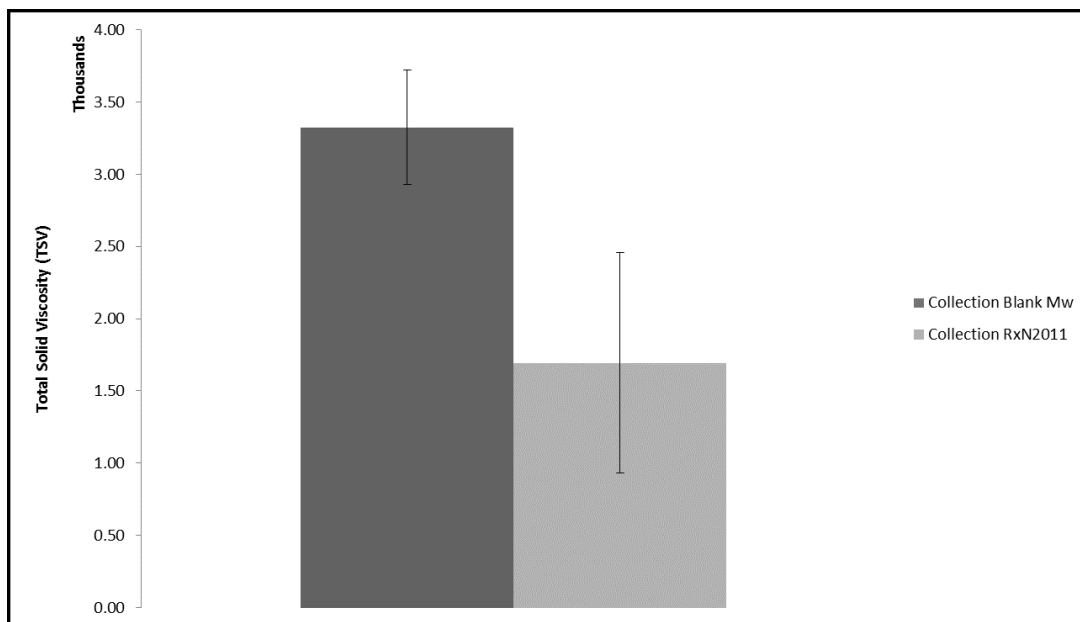


Figure 4.7 Note there is there is a significant difference between the experimental infraction group and the infraction blank in TSV. Note that when compared there is a greater than 50% reduction between the blank and sample.

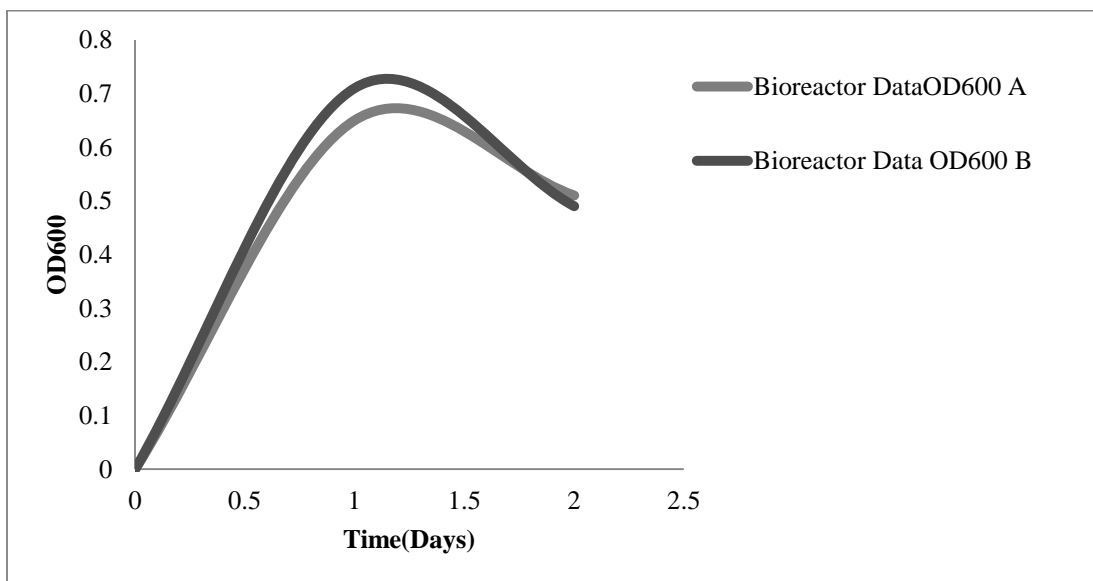


Figure 4.8 *Pseudomonas KT2440* with succinate as the growth substrate.

4.4 Discussion

The rationale for employing a bioreactor for cultivation of microorganisms is controlled conditions leading to predictable growth. The bioreactor design described in this work achieved this objective for a filamentous and a non-filamentous microorganism growing on either particle or soluble substrates for experiments ranging in length from 3 to 7 days. The results indicate that the presented bioreactor design could be broadly useful for growing diverse microorganisms under a range of conditions. Additionally, the modular design of the bioreactor enables different types of experiments. For example, if aeration is not required in the fermentation process, the air pump can be removed from the system.

The impeller employed in this work has the following features that make its use advantageous:

1. An axle extending downwards from the bioreactor vessel cap that stabilizes the impeller, preventing wobble at a wide range of speeds.
2. A set of bearings that are placed along the length of the axle which facilitate stable and smooth rotation of the spinner.
3. The ability to adjust the vertical length of the impeller to accommodate the size of different vessels.

In combination, these features result in stable mixing of the bioreactor vessel contents.

The design differs from that of Harker (US patent 3,572,651; 1971) in that the bearings are fitted along the entire length of the axle rather than being housed only underneath the cap of the spinner flask vessel. The design differs from that of Scharf (US patent 3,649,465; 1972) in

that the rotation of the spinner in the presented design takes place along the entire length of the axle rather than in a piece located at the distal end (Harker 1971, Harold 1972).

A notable inconsistency among the data was the redox values measured for *S. sp. str.* BAS1 for run A compared to the data collected for runs B and C. A likely explanation is that after run A, an air stone was added to the end of the air input tube responsible for aerating the growth medium. The air stone improved the sparging of air bubbles, which improved the availability of air as the microbial population increased over time. The change in the aeration resulted in consistent redox measurements for runs B and C.

Bioreactors currently on the market are generally intended for users in the pharmaceutical and biotechnology industries. Quality entry-level instruments such as the Sartorius Biostat Aplus bioreactor (Sartorius, Germany) typically begin at around \$10,000. This price point is likely to be prohibitive to many teaching institutions and that a device that could be offered in the area of \$1000 - \$1500 would be attractive. As tested, the bioreactor cost \$2,400 (Appendix B), with approximately half of the cost associated with the peristaltic pump used for heating the vessel. This pump was used because it was available in the laboratory; it would be reasonable to use a less expensive pump and achieve the same efficiency of fluid circulation through the water jacket. The cost of peristaltic pumps begins around \$100, indicating that there is room to lower the cost of producing the presented bioreactor.

The presented design could be effective for educating both secondary and undergraduate students on principles of bioreactor operation. It also could serve the needs of researchers who want to incorporate a fermenter into their program but are not prepared to invest the amount of money currently required to acquire a full-scale bioreactor.

5 CONCLUSIONS

5.1 A novel approach for plastic bioconversion based on acid hydrolysis

The presented data support the hypothesis that plastic waste could serve as a feedstock for production of value-added products. Many if not all waste plastics can be redirected into productive uses as biological substrates. There is cautious enthusiasm regarding the potential to use microorganisms to handle mixed polymer wastes, using single species of microorganisms in some cases, and in others, employing microbial communities to expand the metabolic potential of the system. The described growth media will be suitable for natural microbiota and for synthetic biology applications.

Research presented in this dissertation shows that acid treatment is a straightforward and effective way to transform acid-hydrolyzable polymers into labile molecules that can be fed to microorganisms. Notably, the AH4 medium described herein is suitable for diverse microorganisms and may potentially be used as a general growth medium and could potentially be used to produce microbial metabolites for commercial use. In this dissertation, a blended wool and nylon carpet fiber was used to make a growth medium. These data appear to be the first to demonstrate the biodegradation of a mixed polymer consumer product. In principle, the same type of approach could be used for nylon and cotton fiber, or other cellulose-based products.

5.2 A cost effective bench top bioreactor

Bioreactors currently on the market are generally intended for users in the pharmaceutical and biotechnology industries. Quality entry-level instruments such as the Sartorius Biostat Aplus bioreactor (Sartorius, Germany) typically begin at around \$10,000. This price point is likely to be prohibitive to many teaching institutions and that a device that could be offered in the area of \$1000 - \$1500 would be better suited for educational purposes. As tested, the bioreactor cost \$2,400 (Appendix B), with approximately half of the cost associated with the peristaltic pump used for heating the vessel. The cost of peristaltic pumps begins around \$100, indicating that there is room to lower the cost of producing the presented bioreactor. The presented design could be effective for educating both secondary and undergraduate students on principles of bioreactor operation. It also could serve the needs of researchers who want to incorporate a fermenter into their program but are not prepared to invest the amount of money currently required to acquire a full-scale bioreactor.

5.3 Summary of features and innovations related to AH4 medium

The growth media described in this dissertation and the processes to make them have the following features:

1. Diverse microorganisms can grow in AH4 medium.
2. Minimal residual materials remain after microbial growth.
3. The concentrated product can be stored and transported.
4. The process is adaptable to diverse polymers.
5. The process is suitable for mixed plastics.

6. The process is amenable to “end of life mixed plastics” contaminated with biological products like whey and slaughterhouse effluent.
7. The process will work without changing the existing formulation of plastics that exist on the market.
8. Hydrochloric acid is volatilized during processing, which simplifies adjusting the pH of the resulting growth medium.
9. For AH2 medium, the addition of acetic acid reduces re-polymerization and improves substrate solubility.

5.4 Summary of features and innovations related to the benchtop bioreactor

The impeller and system setup employed in this work has the following features that make its use advantageous:

4. An axle extending downwards from the bioreactor vessel cap that stabilizes the impeller, preventing wobble at a wide range of speeds.
5. A set of bearings that are placed along the length of the axle which facilitate stable and smooth rotation of the impeller.
6. The ability to adjust the vertical length of the impeller to accommodate the size of different vessels.
7. A modular nature, which makes the bioreactor system able to accommodate different types of experiments.

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APPENDICES

Appendix A: Bioreactor assembly

Overview:

The bioreactor is comprised of three modules. Instructions for their construction are described in the following sections.

I. Bioreactor vessel assembly

A. Cap assembly

1. Using a permanent pen, draw two lines to divide the cap into four quadrants of equal size (**Fig. 1A, 1B**). The lines will serve as guides for drilling holes for the ports and the impeller.
2. Make holes in the cap for the pH port and other ports. Find the center of each quadrant in which the brass ports fittings will be placed. Be sure that the holes are placed such that the brass fittings will not contact the bioreactor vessel once the completed cap is screwed on. Use a hand drill with a $\frac{3}{4}$ " bit to start the hole and then enlarge it such that each brass port can be inserted (**Fig. 2B**).
3. A hole for the impeller should be drilled at the center of the cap (**Fig. 2A**). The hole should be drilled with a 2.5 mm bit to accommodate the 10" bolt used to anchor the impeller.
4. When all the holes are made, use a hand wrench to insert the brass fittings into the cap.
5. The impeller should be assembled prior to insertion into the cap. Instructions for constructing the impeller are detailed in the next section.

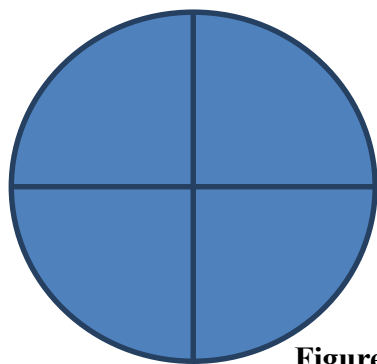


Figure 1A

Top View

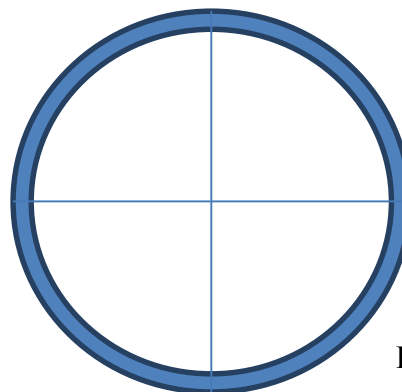


Figure 1B

Bottom View (with draw lines imposed to match the top)

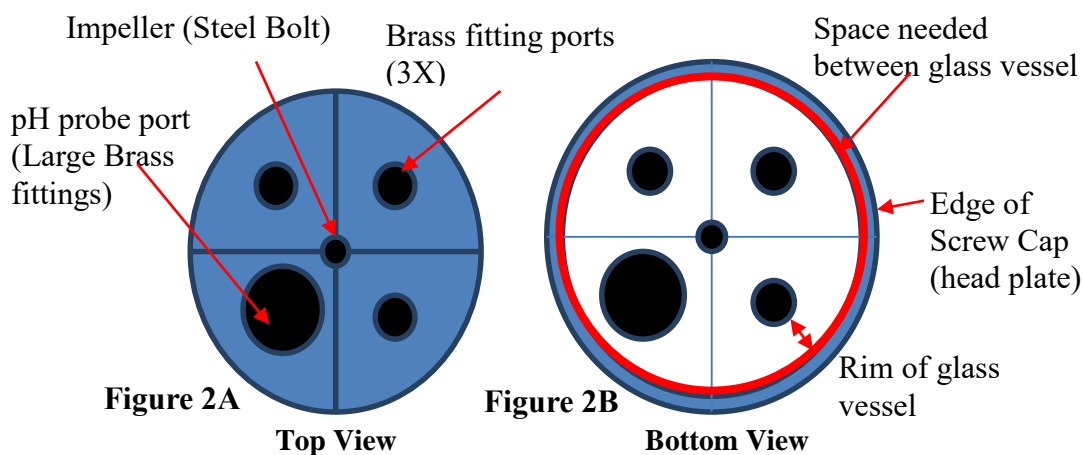


Figure 2A

Top View

Figure 2B

Bottom View

I. Impeller construction

The impeller is comprised of two parts: a modified conical tube containing a magnetic stir bar and a bolt that functions as an axle around which the spinner rotates and which attaches the impeller to the bioreactor vessel.

6. Spinner component. The impeller is located within the vessel and its principal function is mixing of the vessel contents. The impeller is constructed from a modified 15 mL conical tube. The bottom section of the conical tube was cut away using a rotary drill to make a section with a total length of 8.5 cm (**Fig. 3A, 3B**). One cm above the cut, two holes of

equal size are drilled opposite one another through the modified conical tube. A magnetic stir bar will be inserted through the two holes. This component will function as the spinning element of the impeller that will mix the medium in the bioreactor.

7. Slide the magnetic stir bar through the two drilled holes at the bottom of the modified conical tube (**Fig. 3C**). The stir bar should fit tightly. If it does not have a tight fit, place 0.5 cm sections of Pharmed BPT tubing (<http://www.plastics.saint-gobain.com/>) over the ends of the stir bar to keep it fixed in the modified conical tube
8. Axle component. Cut three pieces of BPT tubing, each 0.5 cm in length. Slide one piece onto the 10” bolt all the way to the bottom (**Fig. 3E**).
9. Slide on the first of three nuts.
10. Repeat steps 7 and 8 until there are three pieces of tubing and three nuts placed on the 10” bolt
11. Place the modified conical tube cap onto the bolt. The .5 cut BPT tubing will hold the cap to the bolt.
12. Place the modified conical tube over the 10” bolt and screw it into the conical tube cap. This will complete the impeller construction (**Fig. 3F**).
13. Insert the end of the 10” bolt of the impeller through its hole in the bioreactor cap (step 4). Anchor the impeller in place by screwing on a dome shaped nut (**Fig. 4A, 4B**)

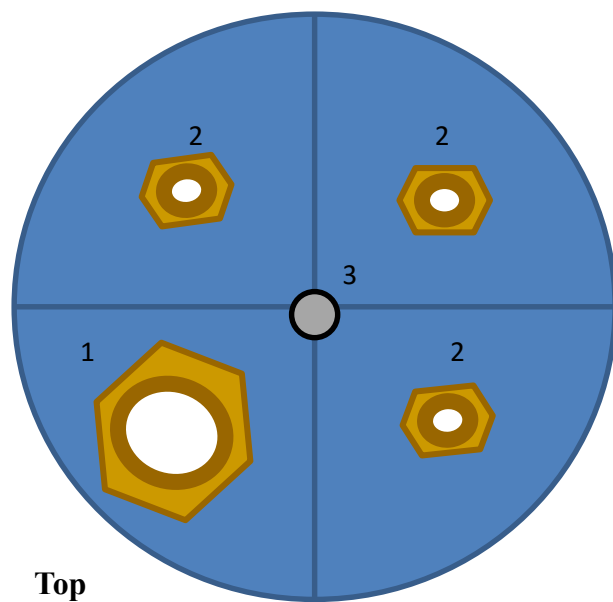
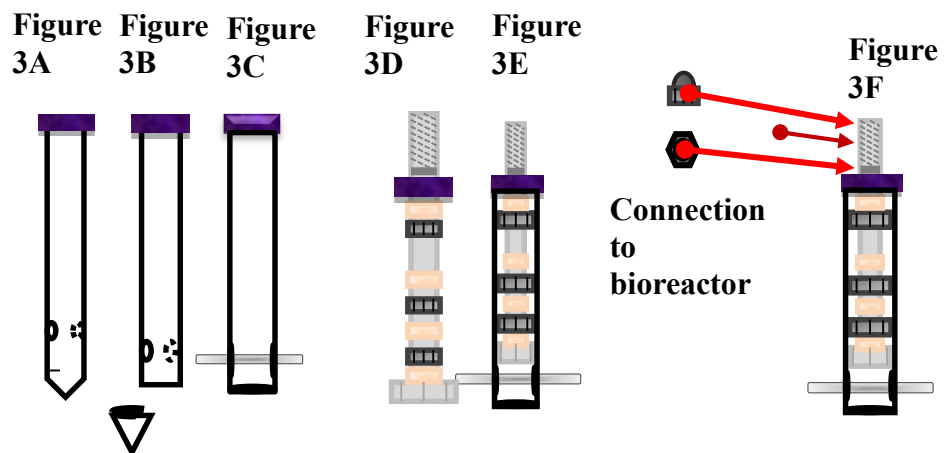
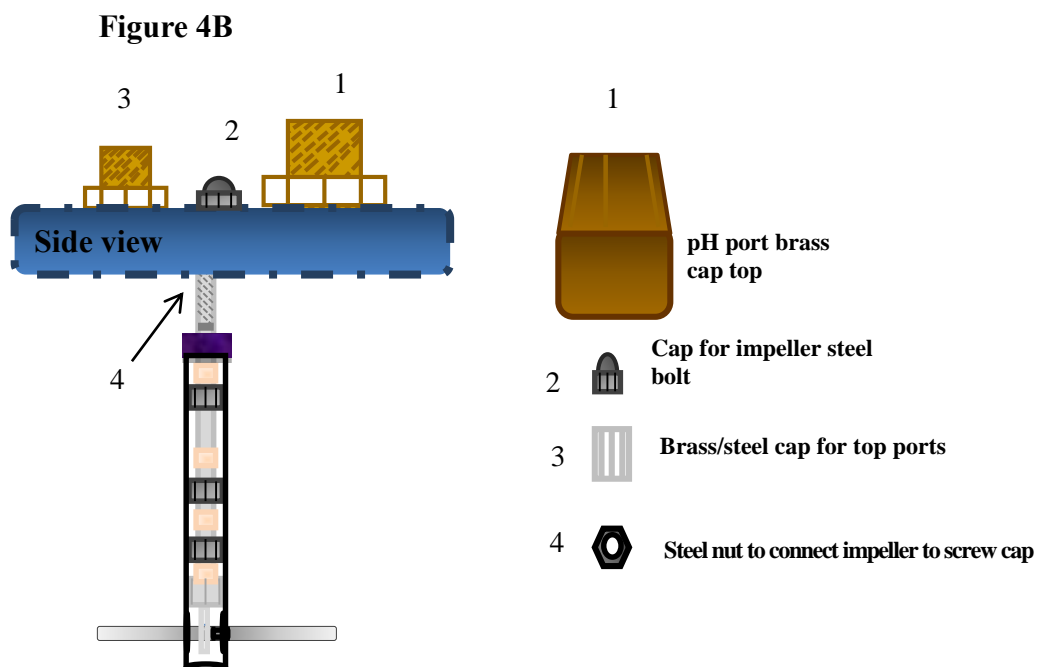


Figure 4A



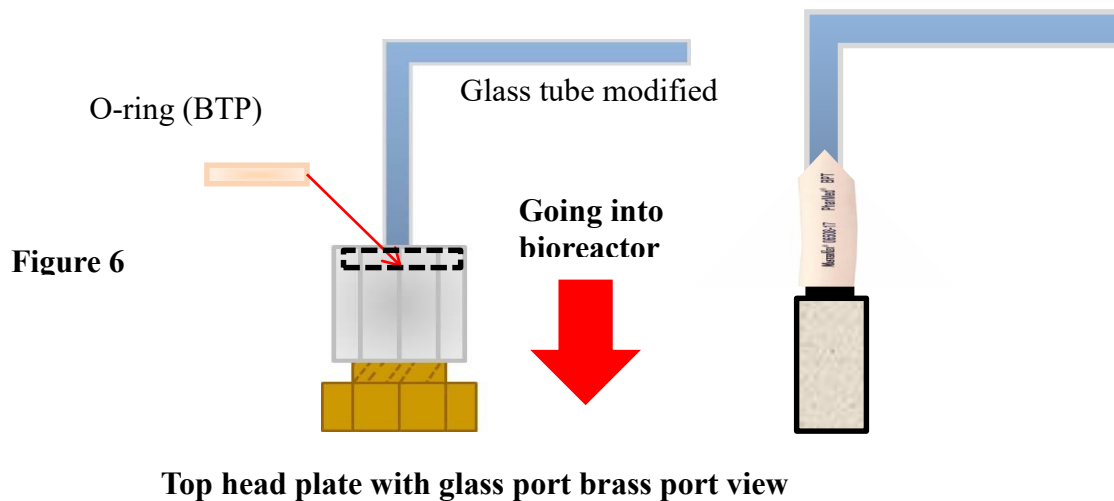
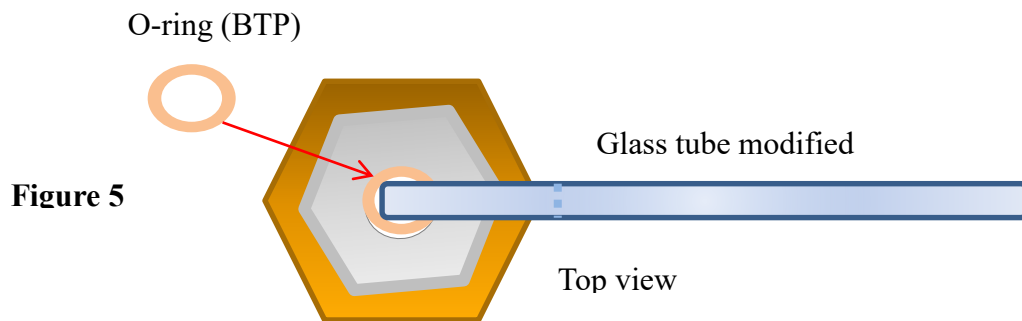


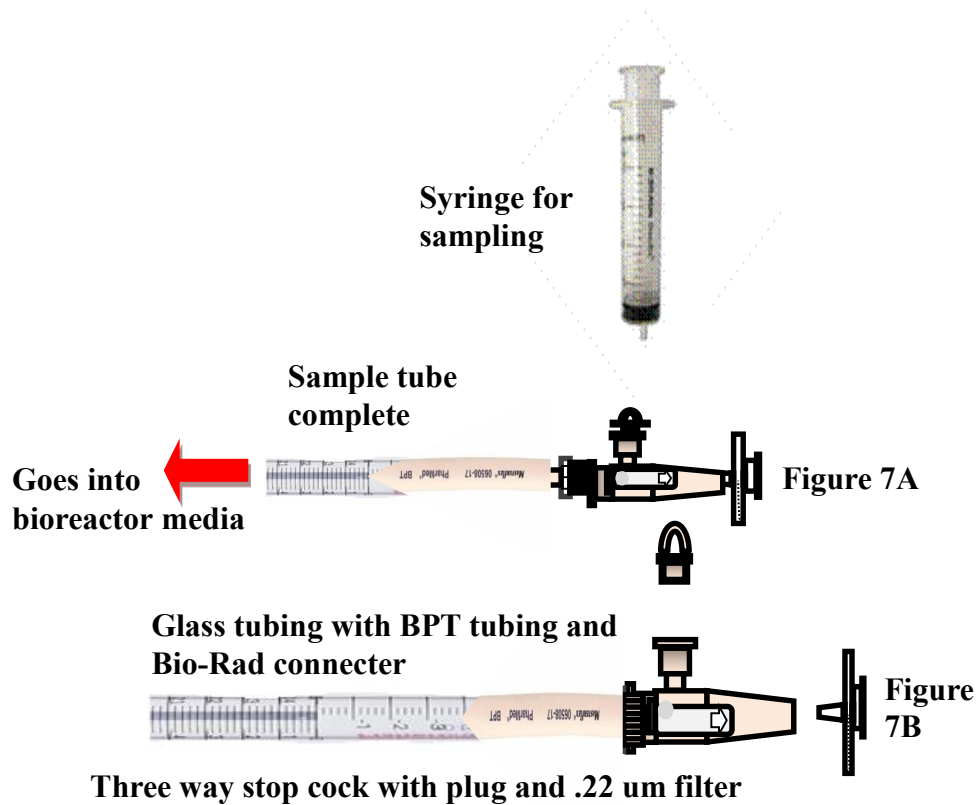
C. Sample port set-up

The sample port is used to aseptically withdraw fluids from the bioreactor vessel. It is located in the vessel cap (**Fig. 9**) and is comprised of a glass tube and associated fittings.

14. Glass tube preparation. Modify a borosilicate glass tube using two pairs of pliers and a glass flame. If using a 1 mL glass pipette as the glass tube, remove any cotton plugs prior to use. The tube should be long enough to reach to the bottom of the bioreactor vessel and extend through the vessel cap, bending outside the vessel to improve access to the sample port once all the components of the bioreactor are in place (**Fig. 5**, **Fig. 9**). To connect the glass tube to a brass port, place a 0.5 cm section of BPT tubing over the end of the tube and then connect the other end to the brass port.

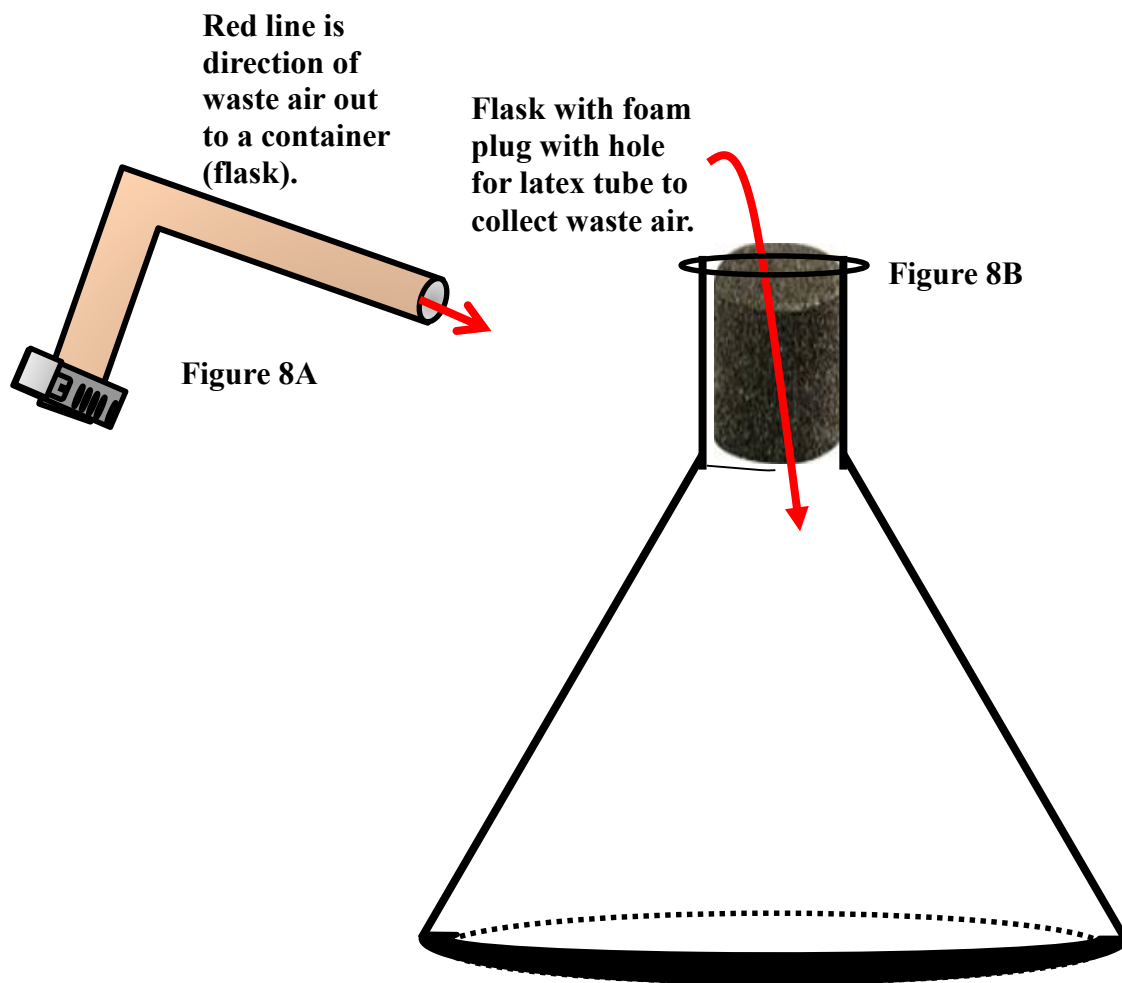
15. Use BPT tubing to connect an air stone (sparger) to the glass tube used for air transport (**Fig. 6**). Note the length of the stone when preparing tubing to hold the air stone in place within the bioreactor vessel.
16. Addition of sample port. Modify the end of the glass tube to facilitate aseptic sampling. Add components to the glass tube as illustrated in **Fig. 7A** to permit connection with a three-way stopcock.
17. Attach the stopcock as illustrated in (**Fig. 9**) and insert the modified glass tube through the brass fitting in the cap.
18. Add a 0.22 μm filter on the tip of the three way stop cock to keep the vessel aseptic during sampling (**Fig. 7B**).
19. The side port of the three-way stopcock is used for sampling with a luer-lock syringe (**Fig. 7A, 7B**). Sterilize the side port with 95% ethanol or 20% bleach. Seal the side port with a female luer plug when not in use.





D. Air exhaust set-up

20. The air exhaust port is required to prevent a buildup of air pressure inside the bioreactor vessel. To assemble, attach a 12-inch-long section of latex tubing to the air exhaust port on top of the cap. Use a hose clamp to secure it (**Fig. 8A**)
21. Insert the other end of the latex tube into a sterile Erlenmeyer flask sealed with a foam plug with a hole for latex tube to collect waste air (**Fig. 8B**)



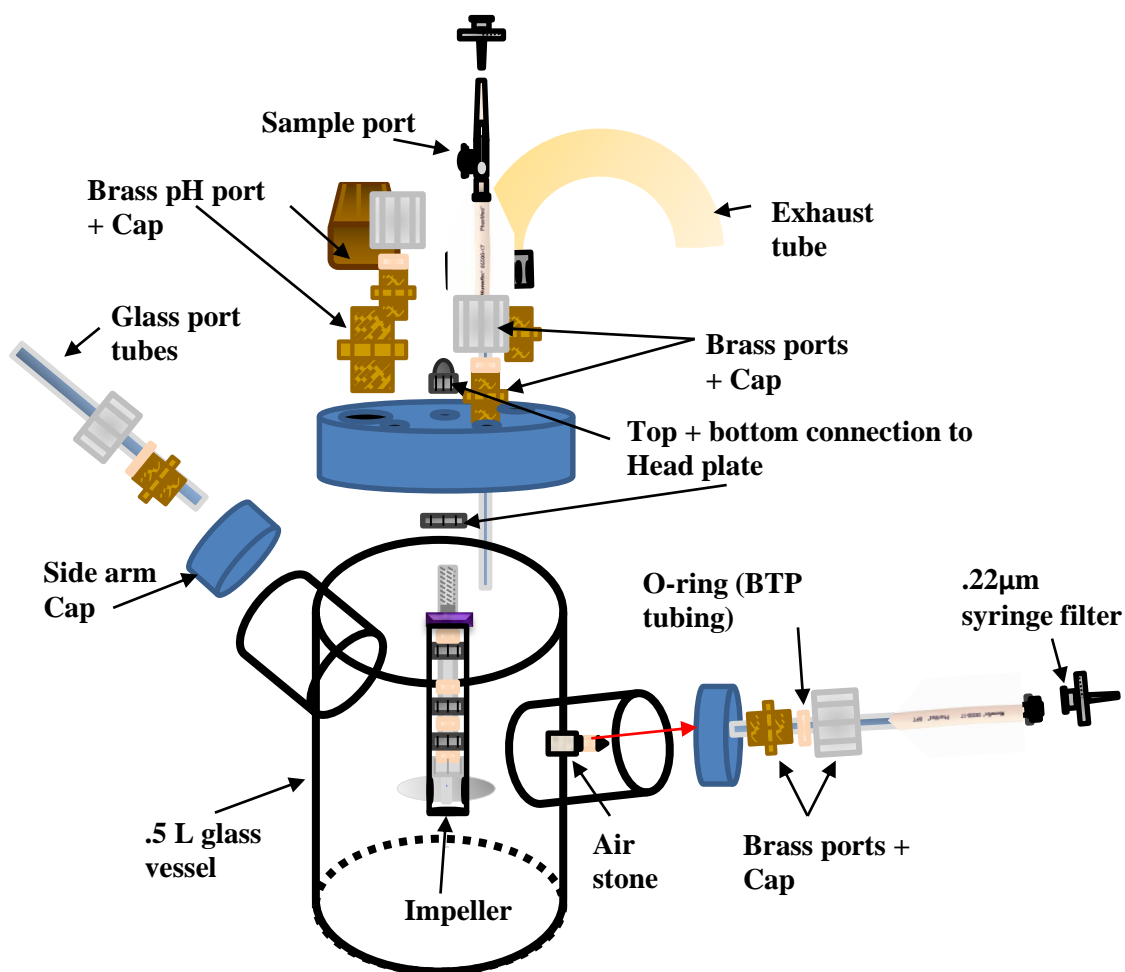


Figure 9

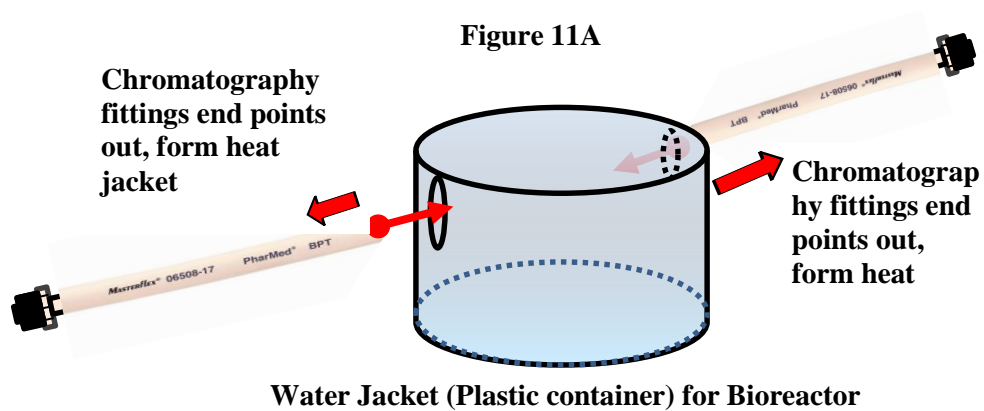
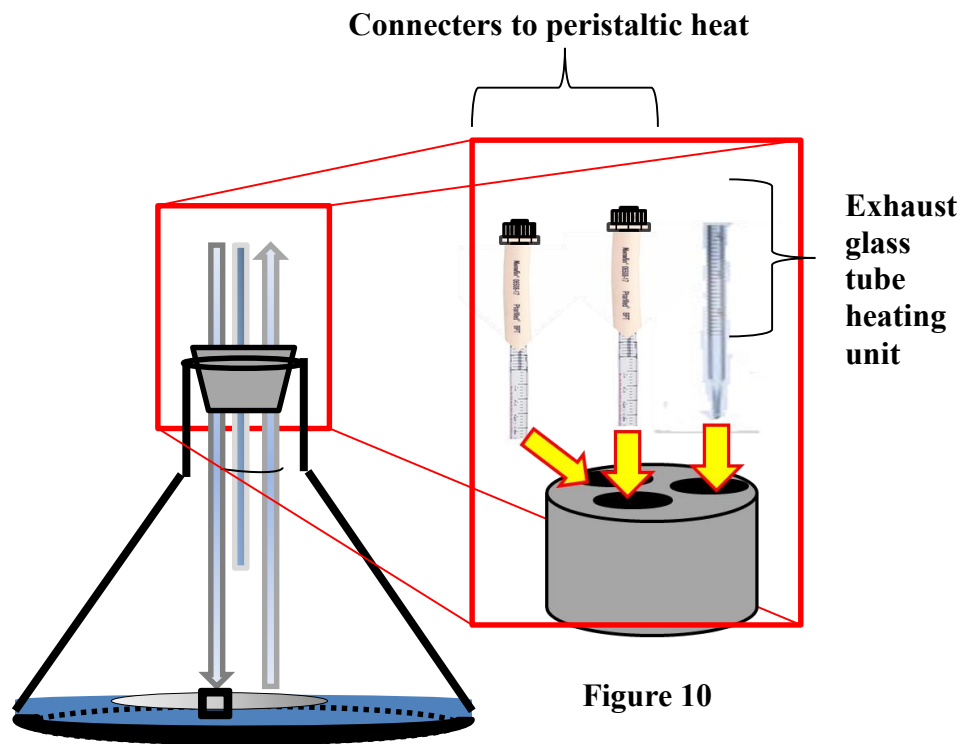
I. Heating and aeration

E. Water jacket assembly

The water jacket surrounds the bioreactor vessel and keeps the temperature inside constant. A heater warms the water to a suitable temperature and a pump is used to transfer the heated water through the water jacket. The following sections describe the steps for construction of the water jacket.

Heating flask preparation. A 250 mL Erlenmeyer flask contains the water to be heated. The flask is sealed with a modified rubber stopper containing three glass tubes (**Fig. 10**). One of the tubes leads to the water jacket. Another tube returns water from the water jacket to the heating vessel. A third tube serves as an air exhaust.

22. To make the modified rubber stopper, drill three holes as illustrated in **Fig. 10** and insert 1.5 mm diameter glass tubes, inserted so as not to touch the bottom of the flask.
23. A magnetic stir bar is placed inside the heating flask to keep the water at uniform temperature while heating. The assembled heating vessel is placed on a hot/stir plate.
24. Water jacket assembly: The water jacket is a plastic container that surrounds the bioreactor vessel. Two holes are drilled at opposite ends of the water jacket container are for tubes bringing water to and from the heating vessel (**Fig. 11A**) The volume of water surrounding the bioreactor vessel is approximately 10 mL. The water jacket should be covered to reduce heat loss.
25. Peristaltic Pump: The flow of heated water from the heating vessel to the water jacket is maintained by a peristaltic pump. 3.2 mm i.d. BPT tubing connects the heating vessel with the pump and the pump with the water jacket. The flow rate should be empirically tested for mL min⁻¹. (**Fig. 11B**)



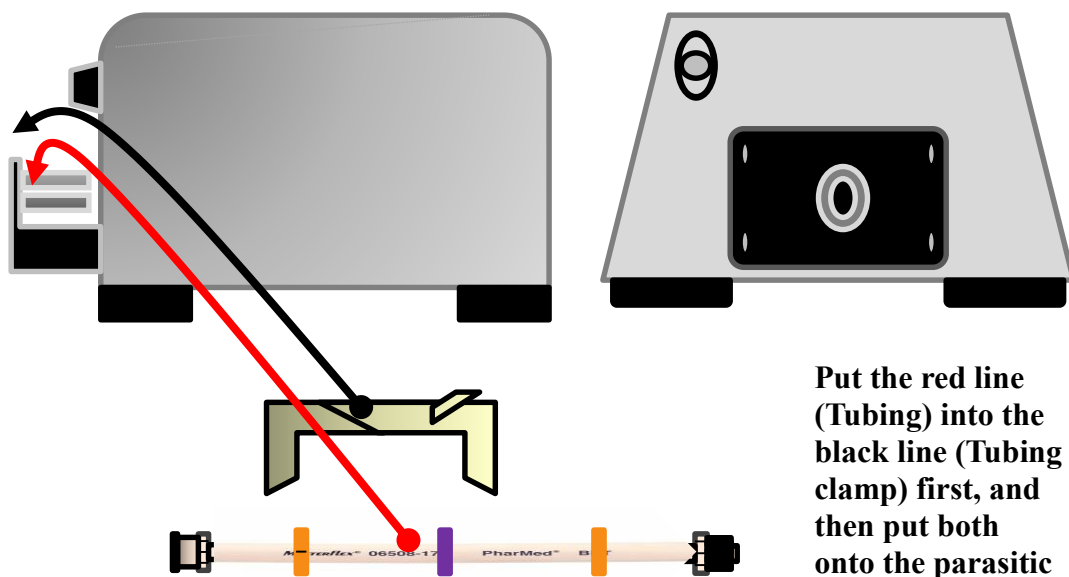


Figure 11B

F. Air pump set-up

The growth medium in the bioreactor vessel must be well aerated to maximize microbial growth. Aeration involves an air pump and a safety flask.

26. Air pump: The air pump is a Top Fin 4000 aquarium pump with BPT tubing to deliver air to the safety flask and on to the bioreactor vessel (**Fig. 12**).
27. Use BPT tubing to connect the air pump to the inlet tube in the safety flask (see below).
28. Safety flask: The air pump moves air through the system by positive pressure. A safety flask must be connected in line in case there is an obstruction or any reason that results in a build-up of air pressure in the system. In the event of a pressure build-up, an opening on the safety flask will pop, allowing pressure to be released and preventing an explosion.

Once the safety flask is connected to the air pump, use BPT tubing to connect to the air inlet of the bioreactor vessel. The filter will be between the bioreactor and tube from the safety flask. (**Fig. 13**)

29. See **Figure 13** for the safety flask set-up. Briefly, a 250 ml vacuum flask is placed in line between the air pump and the bioreactor vessel. A 0.22 μm filter must be inserted between the safety flask and the glass flask. A suitable air flow rate is empirically tested for ml min^{-1}

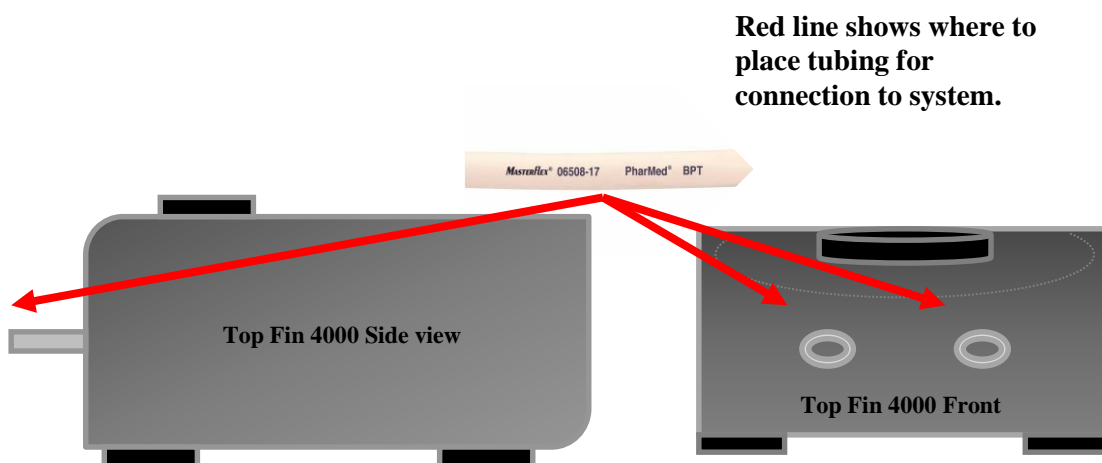
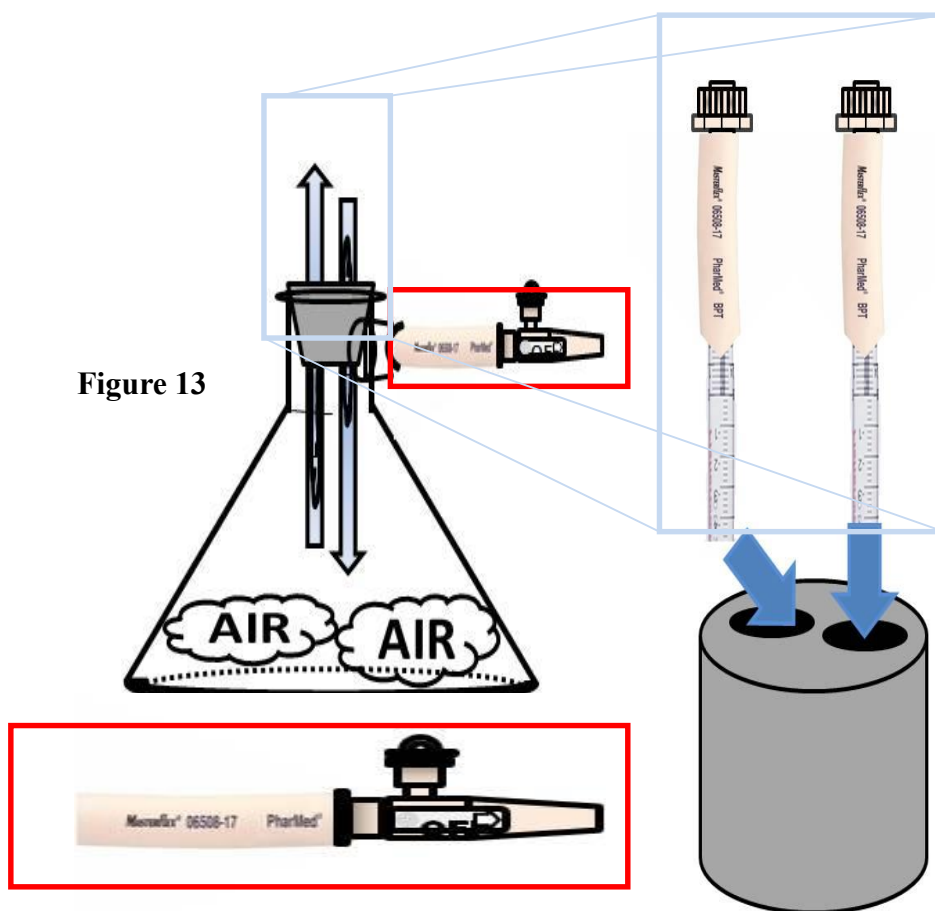


Figure 12



The blue box shows the placement of tubes in rubber cork, the red box is the placement of three-way stopcock.

G. Data collection

Data logger set-up

30. The data logger records data measured by a pH/redox/temperature inserted into the bioreactor vessel via a port in the cap. (Fig. 14)

Figure 14

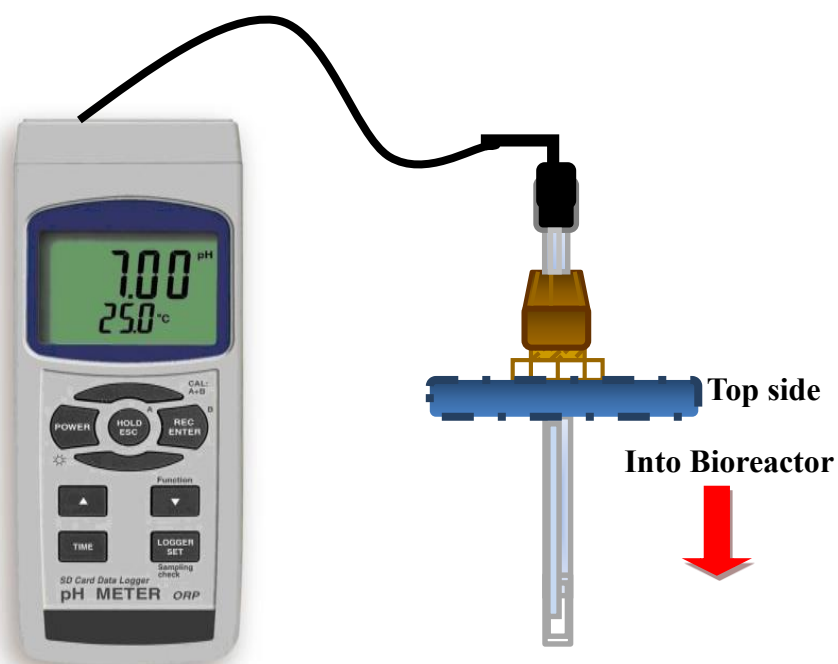
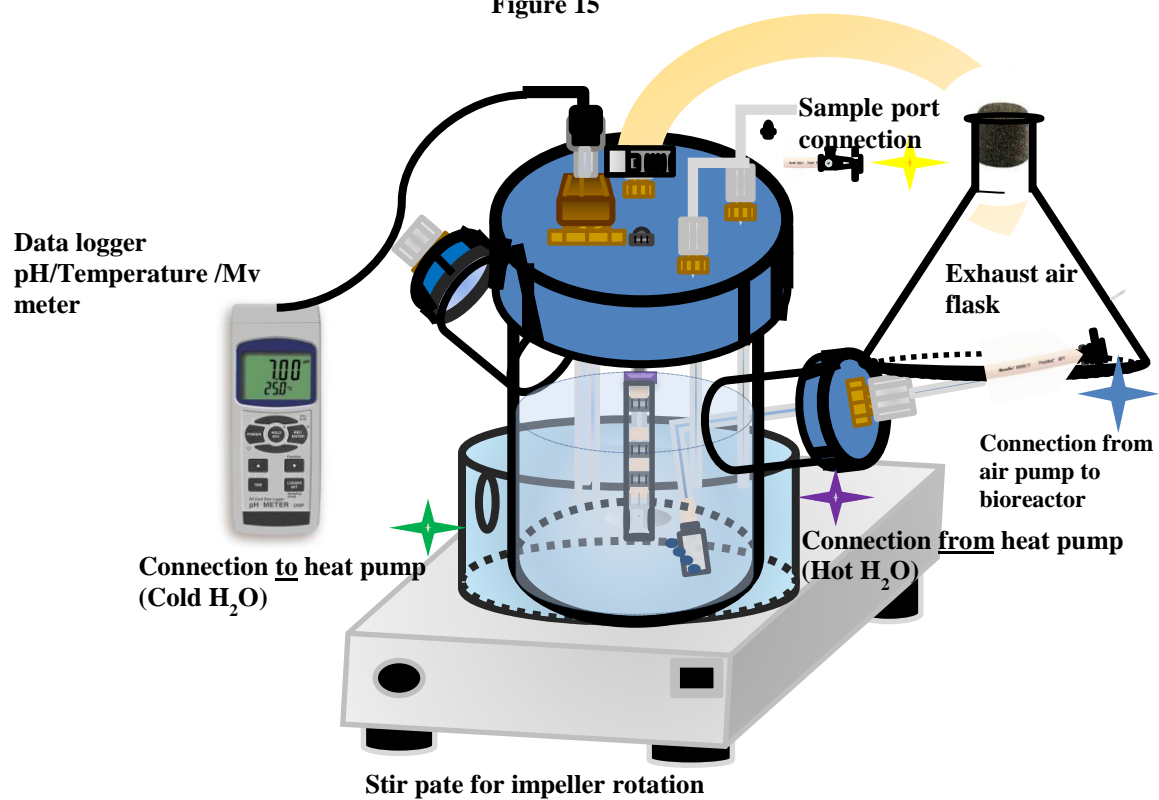


Figure 15





Connections:

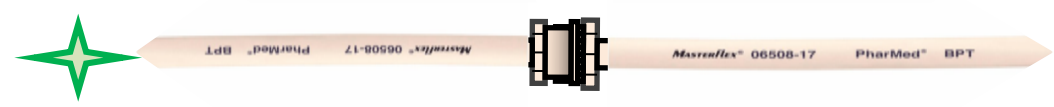
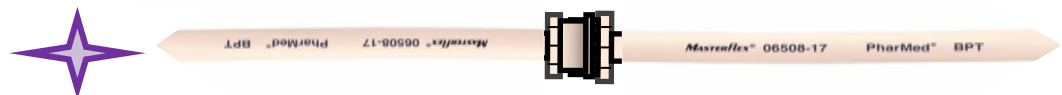
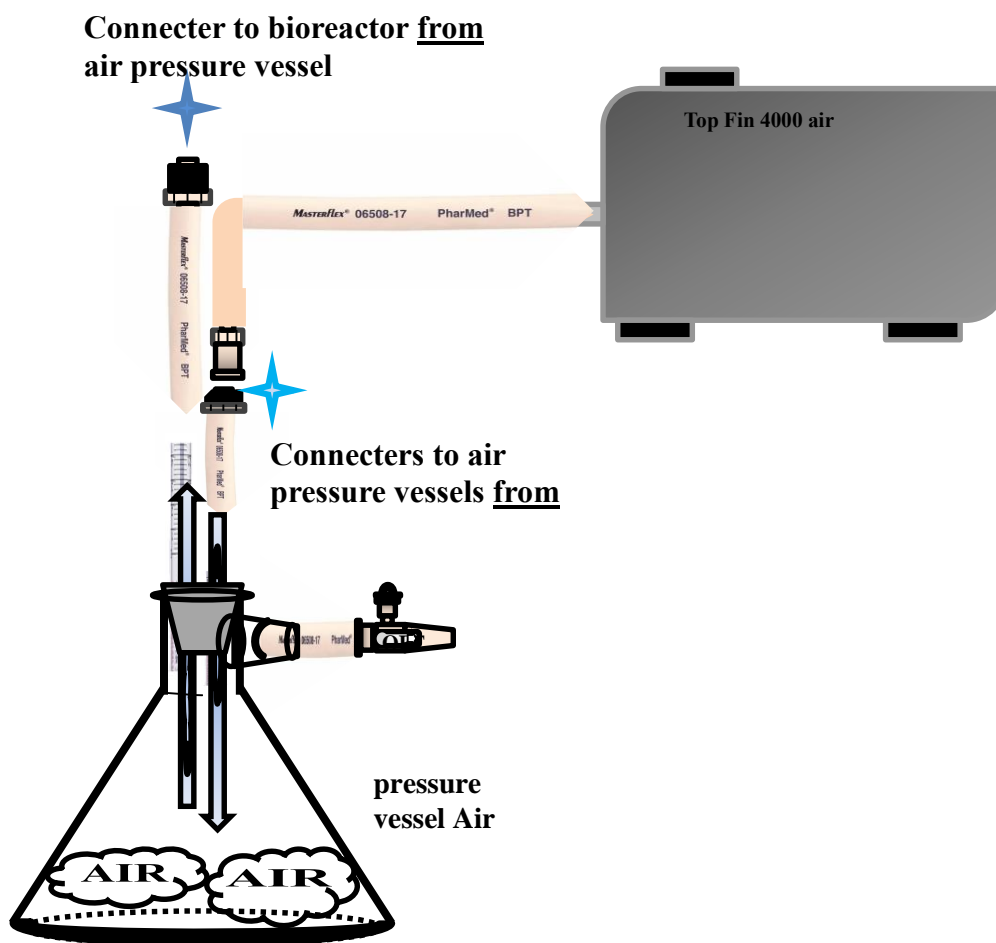


Figure 16



Connections:

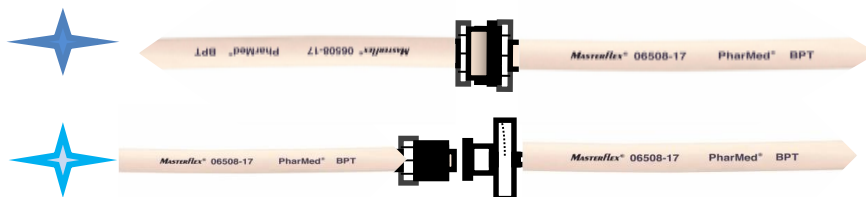
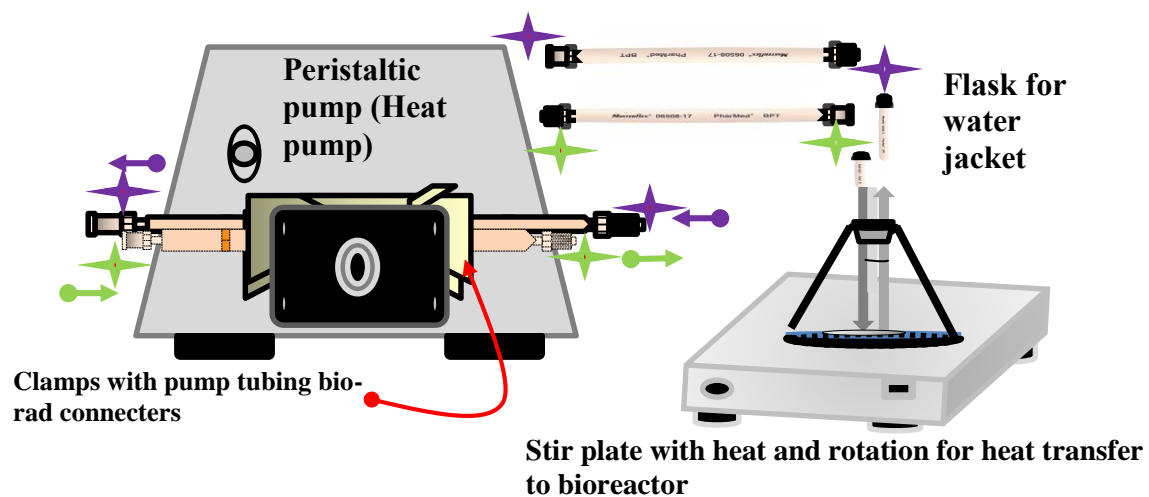
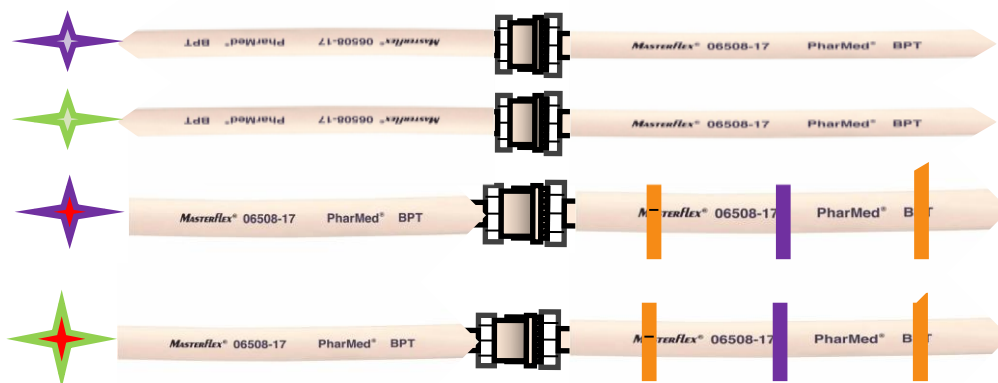


Figure 17



Connections:



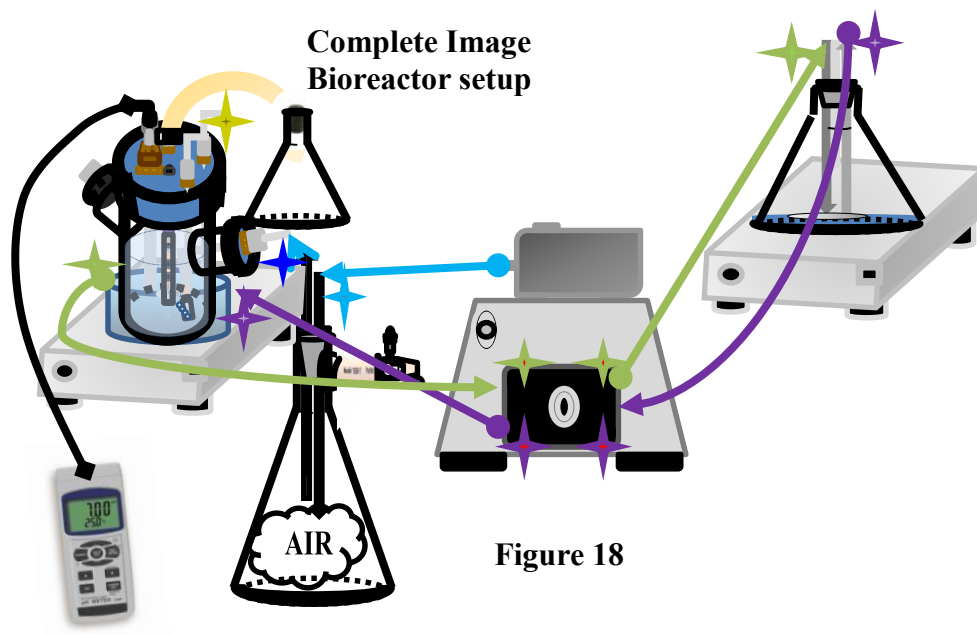
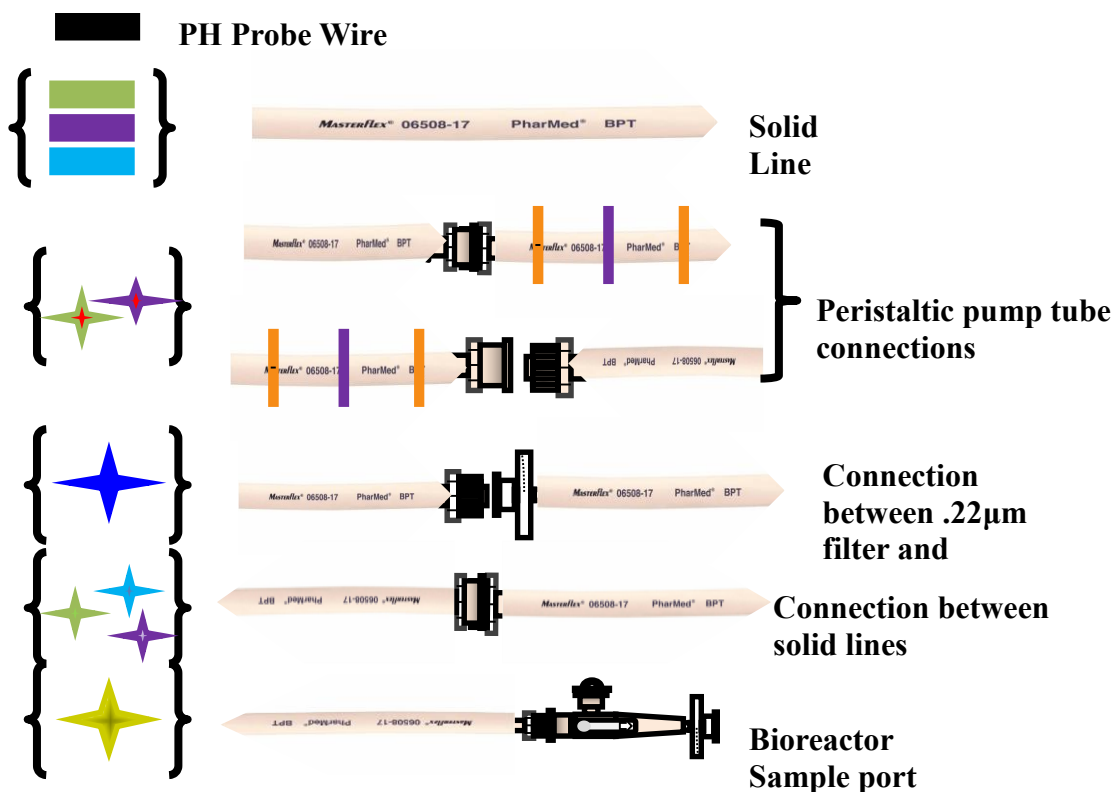


Figure 18



IV. Sterization and maintenance of bioreactor system

A. Sterilization

31. Autoclave the bioreactor vessel. Remove stopcocks and filter from the sample port. Open all ports and cover with aluminum foil. Autoclave the bioreactor vessel for 20 min at 15 psi. 2. Any tubing connected to open ports to the bioreactor are sterilized and can be autoclaved with the same procedure as in step one.
32. Bleach the other components. The pH /temperature/redox probe and any device that will go into the bioreactor should be left overnight in a 20 percent bleach solution.
33. The three way stopcock should be left overnight in a 20 percent bleach solution.
34. After the system is started, a 20 percent bleach solution should be placed into the waterjacket to inhibit the growth of microorganisms in the heating system .

B. Maintenance and starting the system

35. After each run, clean the bioreactor of all organic material using a gentle detergent.
36. Add growth media to the bioreactor vessel prior to autoclaving in order to sterilize. Alternatively, use an autoclaved funnel and pre-sterilized media through the pH probe port under aseptic conditions.

Appendix A.1 Parts Dimensions and Catalog Numbers:**Bio-Rad Luers:**

3.2 mm Barb to Male Luers **Catalog # 731-8226**,

3.2 mm Barb to Female Luers **Catalog # 731-8223**

3-Way Stopcocks **Catalog # 732-8107**

Female Luers T-Connector **Catalog# 731-8229**

Brass Fittings:

Top: 1/8" tube fitting nuts are **Catalog# 23101** for Swagelok or **21801** for Parker

Bottom: **Catalog# A-715**, WATTS brass pipe Nipple 1/8"

pH Top: **Catalog# A-335 41-s**, WATTS Short Rod Nut 5/8" Flare

pH Bottom: **Catalog# A-370 or A-350**, WATTS 5/8" Flare, 5/8" Flare x 1/2" MIP

BPT PharMed Tubing:

Catalog# 96880-06, PharMed BPT Tubing 06508-13, 7.6 m

(25 ft)/pk

Falcon Tubes (15 ml):

Catalog# 50-869-570, Tubes, Plastic Centrifuge; Falcon; Thermo Scientific; Conical; 15

mL; 125/Pk falcon tubes 15 ml (125 pcs.)

Filters (.22 µm):

Catalog # EW-81053-02 (Mfr# SLGP033RS)

Millipore Syringe Filters

Glass Flask (250 ml):

Catalog # FB-500-250; Reusable; Narrow-mouth; Erlenmeyer; Capacity: 250mL;

Graduated: 50 to 225mL; Stopper number 6; Marking area; ASTM E 1404, Type I; 12/Pk

Glass Pipettes:

Catalog# 7464D49, Pipette, and 1 ml in 1 / 10 graduated

Glass Vessel (500 ml):

Bellco Part # 1967-10500 or Bioprocess Spinner Flask, 500mL,

Dimpled Bottom **Catalog#** CLS-1430-500

Heat Pump (Peristaltic):

Master flex L/S Variable-Speed Drive w/ Remote I/O; 600 rpm

Catalog Item# HV-07528-10, Model# 77521-50 (model without remote)

Hot Plate plus Stirring:

Catalog # 11-100-49SH, Ceramic top; 7 x 7in.; 120V 60Hz

Stir hotplate 7x7 cerm 120vst

Latex Exhaust Tubing:

Catalog# 62996-564 VWR Amber Latex Rubber Tubing 5/16" I.D., 1/16" wall

Magnetic Stir Bars:

Catalog #CH-1033, Bel Art Products, No.:371100138X Magnetic Stirring Bars, 25.4mm x 9.5mm

Magnetic Stir plate:

Catalog# 14-490-200 Thermos Scientific No. S194615, 120V 60Hz 0.45A, 21w, Top plate dimensions: 4.25X4.25in (10.8X10.8 cm)

Peristaltic Pump Tubing:

Catalog# 95714-48, Purple/White 2.79mm I.D.

pH Meter SD Card Data logger – 850061 Kit:

pH/Temperature Meter with Real-Time Data Logger

Data logging pH/Temperature meter with SD card

Plastic Water Jacket:

Dimensions: Top Diameter: 4 1/2" Height: 3 1/8"

Capacity: 16 oz. **formerly known as new spring L-5016**

Pump Clamps (Peristaltic):

Catalog# EW-07519-80; Master flex L/S small cartridges for 07519-05 and -06 pump heads

Rubber Stoppers:

Catalog# 14-140H Stopper, Rubber; Fisher brand; Two hole; Size **No.: 6**; Hole size: 5mm, **Catalog#** 14-140K Stopper, Rubber; Fisher brand; Two hole; **Size No.: 8**; Hole size: 5mm

Steel Bolt:

Model# 801076 **Internet#** 204633238

1/2 in.-13 tpi x 8 in. Zinc-Plated Hex Bolt Grade 2

Steel Cap (for Head plate)

Nut size (A.K.A. diameter) is the size bolt that the nut is used with. The same is true of the thread count. Thus a 1/4"-20 nuts fits a 1/4"-20 bolts, to cover impeller bolt top

Steel Clamp for Exhaust Tube:

Home Depot **Model # 6712595 Internet # 202309385** 1/2" - 1 1/4"

Steel Nut(s):

The Hillman Group **Catalog Model# 43468 Internet# 204775232**

1/2 Stainless Steel Jam Nut hex nut for impeller rotation. 1/4 Stainless steel 18-8 Stainless steel 3169 for head plate (connection to impeller/bottom of head plate)

Vacuum (Filtration) Flask:

Catalog # K953760-0000, Capacity: 1000mL; No. 8 Stopper Joint Capacity: 1000mL;

No. 8 Stopper Joint; with side arm; No. 2 hose connection

Appendix B: Bioreactor Cost

Product type	Amount required	Source	Dollars (US)Price	Est. cost
spinner flask, 500 ml	1	various	150	150
peristaltic pump	2	various	100	200
Brass Metal Connectors	6	Home Depot	5	30
1 ml glass tubes	9	Fisher Scientific	0.5	4
Heating stir plate	2	various	75	150
250 ml Erlenmeyer Flask	3	Cole Palmer	10	30
Various size silicon Tube Air/Liquid	Various	Cole Palmer	25	25
Latex Tubing For Exhaust	Various	Cole Palmer	6	6
Top Fin 80 gallon aquarium variable Flow air Pump	1	PetSmart	20	20
Top Fin Air stone	2	PetSmart	5	10
15 ml conical tube, polypropylene	1	Fisher Scientific	1	1

Brass/ Steel Gas Chromatography Nut for top connection	5	Phenomenex	31	156
Magnetic Stir Bar	2	Fisher Scientific	6	12
0.22 µm Nylon Air filters	1	Fisher Scientific	3	3
250-500 ml round Plastic Container for Heat Jacket	1	Wal Mart	2	2
Data logging pH/Temperature Meter with Probes	1	Sper Scientific	256	256
Rubber Stoppers with 3 ports for tubing	2	Fisher Scientific	2	4
Nuts 10 to 5 mm inner Dia Note: larger than Dia of bolt	3	Home Depot	2	6
Steel or Nylon construction bolt , 8 mm in length, 5mm in Dia	1	Home Depot	2	2
Nut Cap for bolt same Dia. Of Steel/Nylon Bolt	1		4	4
Chromatography fittings		Bio-Rad	25	25
			Total cost:	1096
Comparive Cost to Store bought bioreactor				
New Brunswick 1.5 Liter Bioreactor/Fermenter M1369-1601	1	New Brunswick	9,895	9,895
Shake Flask Set Up				
Incubator Shaker	1	New Brunswick	1,895	1,895
Erlenmeyer 250ml Baffled Flask	1	Fisher Scientific	209	209
Foam Plugs	1	Finn Scientific	4.30	4.30
			2,109	2,109
		Comparison with 1.5 Kit	Comp arison with 1.5 Kit	
Totals	Total with add on lab reactor	Price Difference	Differ ence with add on To Lab made reactor	

Price of Laboratory Made Bioreactor	2,784.73	7,603	7,110	
Price of 1 extra Laboratory Made Bioreactor	0	9,402	n/a	
New Brunswick 1.5 Liter Bioreactor/Fermenter	0	n/a	n/a	
Shake Flask Set Up	0	7,786	n/a	

Cost estimate to construct a 500 mL laboratory GSTaR bioreactor

Appendix C: Protocols for Preparing Nylon-Derived Growth Media

The following protocols describe the requirements to produce nylon-derived media using acid hydrolysis. Each of the media was prepared using nylon-based carpet fibers but the outlined approach could be applicable for other sources of nylon polymers.

C.1 Protocol for preparing AH1

Begin with a 100 mL Pyrex screw top bottle. Add 5 M or higher sulfuric acid (H₂SO₄) and combine with 1 gram of N66 pellets. Allow the mixture to stand for 24 hours. After this time, it will have a viscous look. If using unstained nylon, the color of the mixture will be golden to clear. Screw the cap onto the bottle and place the mixture on a rotary shaker at 200 RPM. It will dissolve in approximately 2 hours. When the N66 pellets are completely dissolved in the sulfuric acid, add it incrementally to 900 mL of distilled deionized water (DDH₂O). The mixture should be added slowly to the water to reduce the clumping of the dissolved nylon. Use a magnetic stir bar to mix while adding the acid hydrolyzed N66 to water. When all of the acid-hydrolyzed N66 has been added to the DDH₂O, adjust the pH to 6.8 - 7.5 by adding 10 M NaOH or NaOH pellets. Continue to stir the mixture with a magnetic stir bar while adjusting the pH. Approximately 8 – 10 grams

of NaOH pellets are needed to reach the correct pH. Once the pH has been adjusted, add the M9 salts as described below and then readjust the pH back to 6.8 - 7.5. Autoclave the medium for 15 minutes at 15 psi and 121 °C. After autoclaving, allow the medium to return to room temperature. The solution should contain flocs ranging from 1 - 20 mm in diameter, with a whitish hue and various shapes.

C.2 Protocol for preparing AH2

Place 1 gram of N66 pellets or carpet fiber into a glass flask, add 20 mL DDH₂O and 20 mL glacial HCl or H₂SO₄. Let the mixture shake at 250 - 300 RPM for 25 hours minimum at room temperature. The mixture will form a clear to golden colored solution. Add the mixture to 900 mL M9 solution. Note that the solution should be stirred at a fast rate when the acid hydrolyzed N66 is added to the M9 and it should be added slowly to reduce the formation of precipitates. After the N66 has been added to the M9, adjust the pH to 7.5 - 8.0 by adding 10 M NaOH or NaOH pellets. Note: be careful adding strong base to the acidic solution. The final volume should be adjusted to 1000 mL by adding additional M9. Autoclave for 15 minutes at 121° C and 15 psi. This solution will have very fine particles, whitish in color and no bigger than 2 millimeter in diameter.

C.3 Protocol for preparing AH3

Start with a 125 mL vacuum sidearm flask containing 1 gram of N66 pellet or carpet fiber that has been allowed to soak in a solution of 10 mL DDH₂O and 10 mL glacial HCl for 25 hours (20 mL total volume). The vacuum flask must be connected to a vacuum line with trap in place. Place the flask containing the mixture on a stirring hotplate with mild heat (dial 5-7; approximately 80 °C) with the magnetic stirrer set at 3 plugged with a rubber stopper. Note: the apparatus must

be operated in a fume hood and appropriate safety precautions must be taken. When the vacuum to the sidearm flask is turned on, the solution in the flask will bubble, helping to volatilize the HCl and DDH₂O from the flask. The solution should be evaporated until the viscosity of the solution increases. The N66 solution should be heated and evaporated until the solution contains a fine resin. Turn off the vacuum, but with mild heat; add 100% acetic acid (2 mL) to the resin that has formed at the bottom of the flask. Next add DDH₂O (150 mL) slowly to the resin and mix with stirring to keep chunks from developing. If any chunks form, filter and weigh them and then reprocess them in HCl as was done at the beginning of this protocol, for AH2 and AH3. The reprocessed N66 will be added back in the next step of the process. Add the acetic acid-treated resin and any reprocessed N66 to 500 mL of M9 salts and adjust the pH to 6.8-7.5 with 10 M NaOH. Add approximately 500 mL more of M9 until the total volume is 1000 mL. Autoclave for 15 minutes 15 psi 121 °C. The solution should be clear with a few very fine flakes. After 24 hours, there will be a small amount of precipitate that forms at the bottom of the solution.

C.4 Protocol for preparing AH4

Start with a chemical reflux set up; this will keep the maximum amount of heat in the system to carry out the complete hydrolysis of the N66 material. See Fig. 1. Begin with a 200 mL glass round bottom flask, and to this connect a glass condenser with tap water flowing through it to cool the solution in the round bottom flask, which will keep any of the N66/HCl mixture from evaporating. The round bottom flask/condenser set up is placed in a 1000 mL beaker filled with tap water on a stirring hotplate set at the temperature of boiling water. The water in the beaker must cover the maximum surface area of the round bottom flask. Place inside the round bottom flask a Teflon stir bar for constant mixing of the N66/HCl mixture. Weigh out

1 gram of N66 pellets or carpet material; add this to the round bottom flask and set up the reflux apparatus. Add 100 mL of a 70% DDH₂O and 30% glacial HCl solution to the round bottom flask. Set the stir plate temperature to high and mix and heat the solution to the boiling point of water in the beaker. Make sure the condenser has water recirculating through it. Set it for 2 - 4 hours. Note: the process must be carried out in a fume hood.

After the allotted time there should be, depending on the dyes present, a colored solution of hydrolyzed N66 in the round bottom flask. At this step, the solution can be filtered (0.22 µM filter) or centrifuged (13000 RPM) to remove any residual particulates. When working with carpet fibers, some residual particulates may form from carpet backing that was present on the fibers. Pour the hydrolyzed N66 solution into a 500 mL glass beaker, and boil off the HCl and water. This step should be about 45 minutes to an hour. Continue boiling the solution until a thin resin develops at the bottom of the beaker. It will have the deep color of any dye present in the N66. Note: it is important to monitor the resin-forming step; do not let it burn. To the N66 resin add 200 mL of DDH₂O, without stirring. Add 10 M NaOH to the dissolved resin until it reaches pH 6.8 - 7.5. Filter sterilizes the 200 mL of solution using a 0.22 µm filter under sterile conditions.

During or before the filtering process, prepare 800 mL of a solution of autoclaved M9 (15 minutes at 15 psi, 121°C). When the 200 mL N66 solution is ready, add it to the M9, making a final volume of 1000 mL of sterile medium. M9 solution is prepared according to Handbook of Microbiological Media, third edition by Ronald M. Atlas. Note: The M9 can be replaced with an autoclaved solution of 0.008 grams of yeast extract dissolved in 800 mL DDH₂O. AH4 was

prepared with a modified M9 containing no magnesium salts and was supplemented with 1 mg per liter of yeast extract.

Appendix D:

INTRODUCTION

D.1 Production of a microbial growth medium based on non-acid hydrolyzable plastics

In contrast to nylon and PET, many plastics cannot be depolymerized with acid. Several commercially significant plastics fall into this category, including polyethylene (PE) and polypropylene (PP). An approach to depolymerizing this group of plastics involves heat treatment. The resultant products are hydrophobic, meaning that they will not dissolve in water. They can however be blended with biodegradable natural oils and subsequently dispersed into an aqueous solution using a biodegradable surfactant. In this way, the components of the plastic can be used as the basis of a growth medium for microorganisms.

MATERIALS AND METHODS

D.2 Inocula preparation

All bacteria were stored at -80 °C. *P. putida* KT2440 was cultivated from stock originating in the ATCC culture collection. Inocula were grown overnight from frozen stock in 30 - 50 mL LB broth in an Erlenmeyer flask at 30 °C. The resulting cell suspension was washed once in M9 or 50 mM phosphate buffer prior to use.

D.2.1 Preparation of olive oil-HDPE growth medium (OP1)

D.2.2 Chemicals

Kroger brand extra virgin olive oil was used in this work (Kroger, USA). Bags synthesized from HDPE were obtained from Kroger, USA. SDS was obtained from Amersham Biosciences AB, Sweden.

D.2.3 Protocol for preparing OP1 medium

Begin by blending the olive oil and the HDPE. There is a ratio of olive oil to HDPE that allows for optimal blending of the plastic with the olive oil; if the ratio goes below 10:1 olive oil:HDPE, the HDPE will not disperse. To make OP1, 10 parts of olive oil to 1 part HDPE is required. Under a fume hood, on a hotplate, heat 1 gram of HDPE **WITHOUT** oil in a 300 mL Pyrex beaker. Heat the plastic until it reaches its melting point and becomes liquid. Add 5 mL olive oil and mix with a metal spatula, chopping any lumps of plastic, until the oil and plastic are well mixed. Note: the temperature should be below the burning point of the olive oil, approximately 200 °C. If smoke forms inside the beaker, the temperature is nearing the flash point of the oil; allow the beaker to cool slightly so that the oil does not ignite. Add an additional 5 mL of olive oil. Continue to mix the oil-plastic blend, stirring by hand, not stir bar. The olive oil-HDPE mixture will form a smooth viscous liquid. Continue mixing for an additional 10 minutes. Remove the beaker from the hot plate and let it stand for 20 minutes. It will develop

into a wax/butter-like material. Once the wax-like material has formed, add 7 mL 10% SDS to the wax oil/HDPE and mix by hand for 20 minutes after mixing, a cottage cheese-like material will form. To this, add when cool and solid with mixing with a stir bar, 200 mL of M9 salts (no carbon source). The resulting solution will be turbid and will have a greenish color. This solution is referred to as the olefin solution. Note: the green color is derived from the color of the olive oil. Additionally, if the HDPE contains a coloring agent, this compound will also be present in the olefin solution. Use a stir bar to mix the solution for 20 minutes. Autoclave the olefin solution at 121 °C and 15 psi for 15 minutes Add it to 800 mL of autoclaved M9.

D.3 OP1 batch culture experiments

Growth in OP1 medium was investigated using batch culture. Sterile 250 mL Erlenmeyer flasks containing 50 mL of OP1 were inoculated with *P. putida* KT2440 at cell densities ranging from 5.5×10^5 to 6.6×10^6 CFU mL⁻¹. The inoculated flasks were incubated at 30 °C with 200 rpm shaking. On day 1 and day 4, 1 mL was collected from each flask and was centrifuged at 13,000 rpm for 3 min. The resultant pellet was resuspended in 100 µL of 50 mM phosphate buffer and was enumerated by serial dilution and plate count.

D.3.1 LPLC-Sudan Black assay

Culture supernatant from the OP1 flask experiments was collected and 1 mL was centrifuged for 5 minutes at 12,000 RPM. The supernatant was taken out and placed in a fresh 1.5 mL tube. A ratio of 100 µl of prepared Sudan B Black dye was added to 900 µl of centrifuged supernatant. Samples were vortexed to mix and were fractionated in the same fashion

as the ninhydrin assay (chapter 2). Fractionated samples were transferred to microtiter plates and were analyzed at 595 nm on a Victor³ plate reader.

RESULTS

D.4 Microbial growth in OP1 medium

Growth of *P. putida* KT2440 using the OP1 medium (HDPE-olive oil-SDS) as the sole carbon and energy source was measured by serial dilution and plate count. Duplicate measurements indicated an increase in CFU per mL of 8 – 25 fold that reached 1×10^8 CFU mL⁻¹ after 4 days. Growth in OP1 was examined by microscope and revealed clusters of cells aggregated around particle oil droplets in the aqueous medium (Figure D.1.). Substrate utilization was measured by fractionation of the spent medium using a hydrophobic interaction column, followed by staining of the collected fractions with Sudan Black and measuring absorbance spectrophotometrically. These data revealed extensive reduction of the substrate following 8 days of microbial growth (Figure D.2.). Interestingly, analysis of uninoculated media found that complexation of the Sudan Black with the oil-HDPE mixture resulted in slightly less absorbance than that measured for the oil-alone control. After 8 days of microbial growth, the absorbance of the spent media was at background levels for both the oil-HDPE mixture or the oil-only control, indicating that extensive metabolism had occurred.

D.5 DISCUSSION

A method for bioconversion of these hydrophobic plastics could be impactful because of their abundance in post-consumer waste and their prominence in the environment. Additional research is required to understand and to optimize the process presented in this work using HDPE, olive oil and SDS. Nonetheless, there have been few efforts to recycle these types of plastic waste and we think that the described approach could be successfully employed to reduce waste and simultaneously produce value-added products.

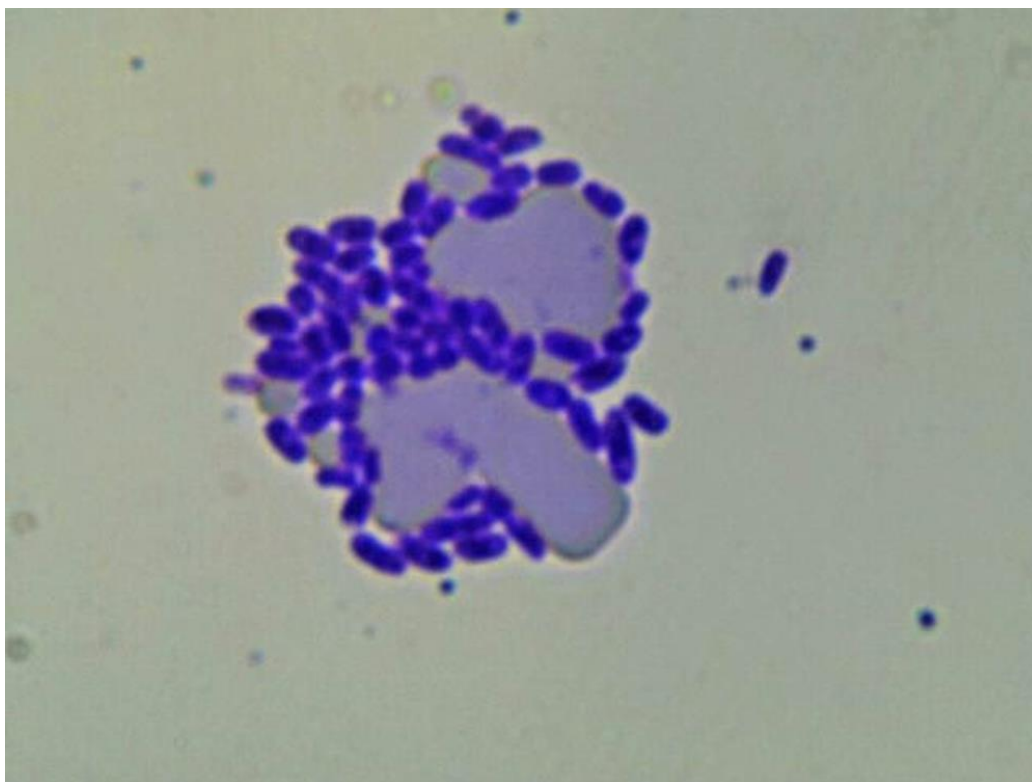


Figure D. 1. *P. putida* KT2440 growth on OP1 medium. Bright field microscopy; cells stained with crystal violet. Magnification: 40 \times .

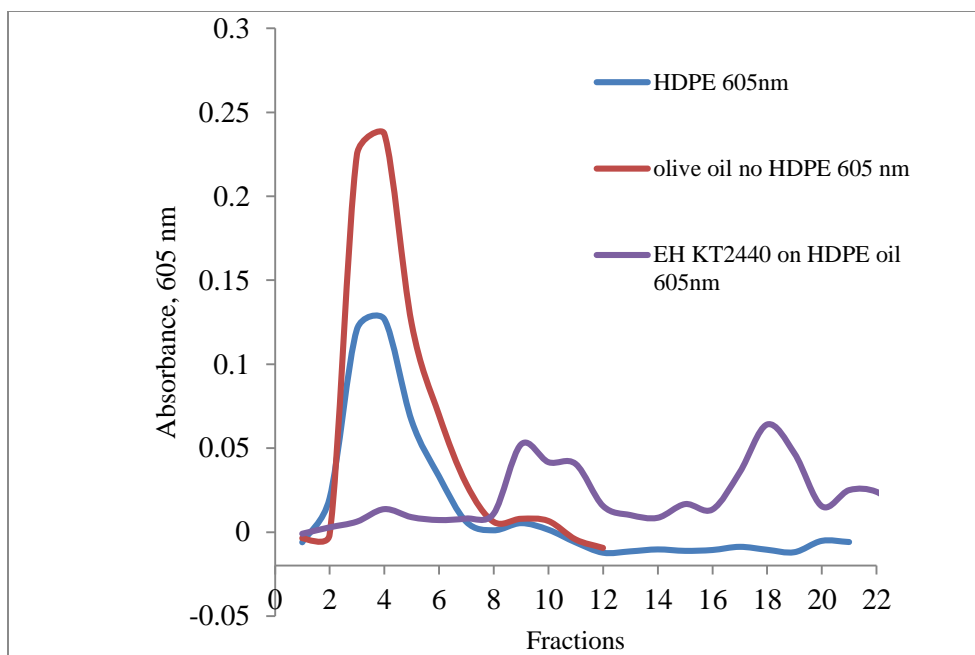


Figure D.2. LPLC-HIC analysis of OP1 substrate utilization by *P. putida* KT2440 grown in batch culture. Red, olive oil only; blue, olive oil + HDPE on day 1; purple, olive oil + HDPE on day 8. Representative data; experiments carried out in duplicate.