UNIVERSITY OF CALIFORNIA SAN DIEGO

Biodegradation of a renewable polyester polyurethane

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in

Chemistry

by

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2020

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LIST OF ABBREVIATIONS

PU - Polyurethane

GCMS - Gas Chromatography Mass Spectrometry

LCMS - Liquid Chromatography Mass Spectrometry

MDA - 4,4'-methylenedianiline

MDI - 4,4'-methylene diisocyanate

M9 - Minimal salts

PBS - Phosphate Buffered Saline

LB - Lysogeny Broth

PDA - Potato Dextrose Agar

OD - Optical Density at 600nm

SEM - Scanning Electron Microscopy

PCR - Polymerase Chain Reaction

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PREFACE

This work was done in collaboration with Algenesis Materials, who are working to develop renewable and biodegradable polyurethane plastics. With the growing issue of plastic pollution and the exponential production of plastics, a solution is to reshape our current production system in a more sustainable manner. This research was done with the contribution of many individuals from the UCSD Department of Chemistry & Biochemistry, UCSD Biological Sciences Division, and Algenesis Materials.

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I would like to acknowledge Professor Mayfield for his guidance throughout my master's research and for giving me the opportunity to be a part of such an important project. I would also like to acknowledge Professor Burkart and Professor Pomeroy for their support of the project and their valuable insight. Thank you to Professor Jerry Yang for giving feedback on the work.

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Chapters 1 through 4, in part, have been submitted for publication of the material under the title, "Rapid biodegradation of renewable polyurethanes with identification of associated microorganisms and breakdown products," as it may appear in Bioresource Technology Reports 2020. Natasha R. Gunawan*, Marissa Tessman*, Ariel C. Schreiman, Ryan Simkovsky, Anton A. Samoylov, Nitin K. Neelakantan, Michael D. Burkart, Robert Pomeroy, and Stephen P. Mayfield. The thesis author was a coauthor of this paper.

ABSTRACT OF THE THESIS

Biodegradation of a renewable polyester polyurethane

by

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Professor Stephen P. Mayfield, Chair
Professor Michael D. Burkart, Co-Chair

In recent years, there has been a surge of interest in replacements for typical petroleum-based products due to the environmental strain of fossil fuels. Renewably-sourced and readily-biodegradable plastics have the potential to make plastic production a more sustainable process. The following study analyzes the biodegradation process of a 52% bio-based polyurethane through the identification of organisms associated with biodegradation, an analysis of the chemical breakdown into starting monomers, and physical analysis of degradation. Experiments involved incubating polyurethane in compost and soil environments, adapting organisms to utilize the polyurethane as a carbon source, as well as employing

enzymes to break down the polyurethane structure. Several organisms appeared to successfully use the polyurethane as a carbon source. When reacted with commercial esterases, the starting chemical precursors were seen through GCMS and LCMS, indicating breakdown of the polyurethane structure into starting monomers. Through understanding the biodegradation process of the polyurethane, there is the potential to develop a more renewable plastic production system through the recycling of monomers.

Chapter 1: Introduction

It has become apparent that plastic pollution poses a great problem to the planet, and that the current production system cannot be maintained indefinitely. By the end of 2017, humans had generated over 7 billion metric tons of plastic waste, with the majority persisting in the environment or landfills at the end of its use.[1] A challenge is to develop plastics that can be degraded while still maintaining industry standards of durability. The desired trait of plastics' resistance to natural degradation is now a concern as plastic production has increased exponentially over time. In fact, it is predicted that if current plastic production growth trends are to continue, we will produce roughly 1.1 billion tonnes of plastic in 2050.[1] With this in mind, there is a need for more sustainable plastic production practices. While limiting global plastic consumption is difficult to pursue, developing plastic products from renewable feedstocks that are able to undergo biodegradation at the end of its use provides a powerful solution.

Several bio-based alternatives to fossil fuels have been developed over the years. A potential source for plastic precursors is algae, which have fast growth rates under photosynthetic conditions and can be grown with non-potable water and non-arable land.[2] Additionally, algae possess unique metabolic pathways for producing hydrocarbons that can be converted into valuable chemicals, including plastic precursors and polyurethanes.[3,4] In the modern bio-manufacturing sector, efficient molecular biology tools for several strains of green algae have been developed, making it feasible to use these organisms as platforms for generating high-value products.[5] These advances make algae a more renewable alternative for making plastic products than petroleum. However, it is important to note that the term 'bio-based' is not interchangeable with 'biodegradable', as it is often marketed. Rather, biodegradability is another element that should be considered.

Among the different types of plastics, PUs have the potential to be renewably sourced and subsequently biodegraded.[6] Unlike many other polymers, PUs can be synthesized from a diverse set of monomers. A typical PU contains repeating urethane bonds produced by linking a polyol—a hydrocarbon with at least two hydroxyl groups—and a diisocyanate, but the identity of the polyol or the molecule carrying the isocyanate can vary widely in composition and size, making PUs an ideal canvas for innovation in renewable and biodegradable plastics (Fig. 1.1). Here, the diisocyanate used is MDI, which

is widely used for synthesizing PUs. Previous studies have demonstrated that polyester PUs, in which the polyol contains repeating ester linkages, can degrade through chemical hydrolysis, as well as biological hydrolysis by various enzymes that are naturally possessed by microorganisms, such as urethanases, proteases, lipases, and esterases - most of which fall under the class of hydrolases.[7,8]

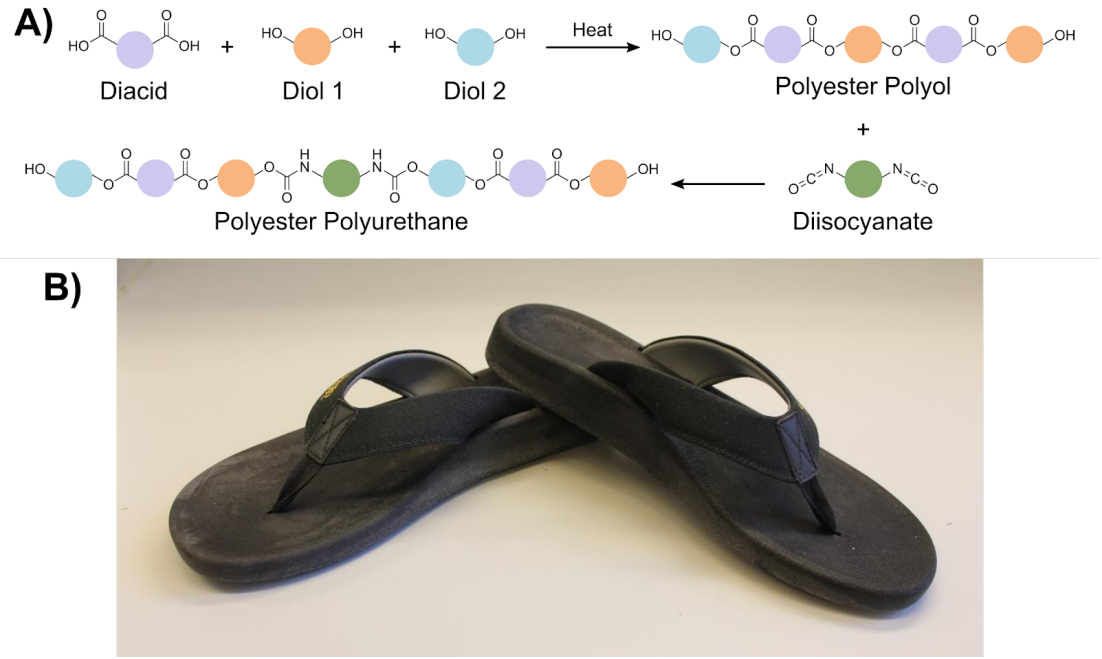

Figure 1.1: A) General polyester polyol and polyurethane (PU) syntheses and structure. B) Algenesis bio-based PU flip flop prototype.

Within the class of PUs, there exists polyester and polyether PUs (Fig. 1.2). Typically, the synthesis of a polyether PU occurs by reaction of an epoxide, rather than a diacid, with diols and an isocyanate.[9] In this study, we focus on a polyester PU, as it is known to be more susceptible to microbial degradation due to the repeated ester bonds that are readily hydrolyzed by esterases.[7] Due to the stability of ether bonds, cleavage of ethers is uncommon without additional reagents or extreme conditions.[10] In a study that looked at various laboratory-synthesized polyurethanes, all polyester PUs studied were susceptible to fungal attack, whereas polyethers were relatively resistant to fungal growth.[11] In a separate study, it was found that when polyether PUs biodegraded, it occurred at a slower rate than polyester PUs.[12] It is important to note that the urethane linkages within the PU structure are also susceptible to

enzymatic cleavage, although it is unclear whether they can be hydrolyzed directly, like ester bonds, or following breakdown into lower molecular mass structures.[7]

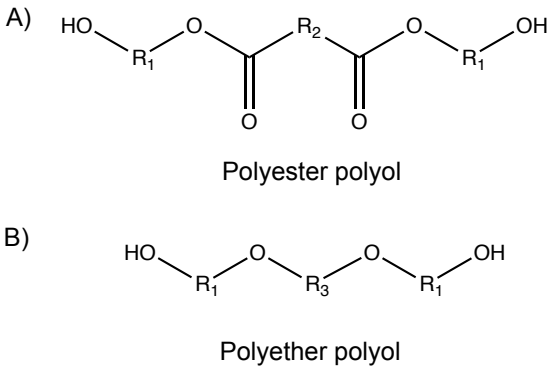

Figure 1.2: General structure of A) polyester polyol and B) polyether polyol. The two polyols make up the backbone of the two types of polyurethanes. Typically, R_1 comes from diols, R_2 comes from a diacid, R_3 comes from an epoxide.

Several chemical methods have been shown to cleave polyesters, demonstrating a mechanism that enables degradation of polyester PU into monomers, rather than into smaller plastics, also known as microplastics. Heating pre-ground polyester PU with glycols at atmospheric pressure was shown to depolymerize the PU into urethane and polyol products through ester bond cleavage.[13] Although this chemical method enables degradation of PU into monomers, it is not a naturally occurring process, making it less useful for plastic pollution in the environment, where plastic waste typically ends up. However, knowing the products of chemical hydrolysis can give way to understanding the expected products from biological hydrolysis of PUs through enzymes. Generally, hydrolysis of ester bonds produces a carboxylic acid and an alcohol, while hydrolysis of urethane bonds produces an amine, an alcohol, and carbon dioxide (Fig. 1.3). We can expect hydrolysis of the ester and urethane linkages within the PU to produce diols, a diacid, and a diamine, with a byproduct of carbon dioxide (Fig. 1.4). The diamine in this case is MDA — a byproduct of the urethane hydrolysis of MDI.

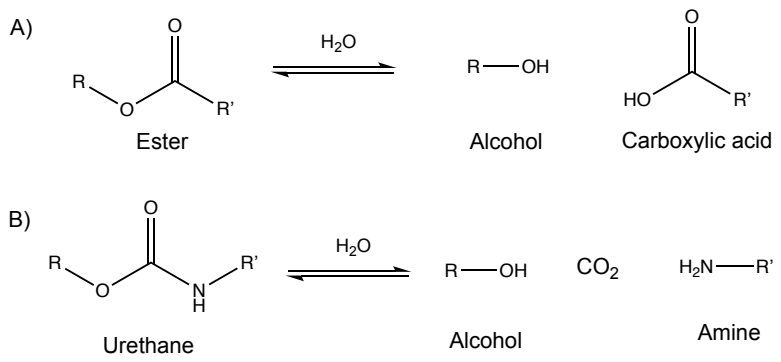

Figure 1.3: General products of A) ester hydrolysis and B) urethane hydrolysis.

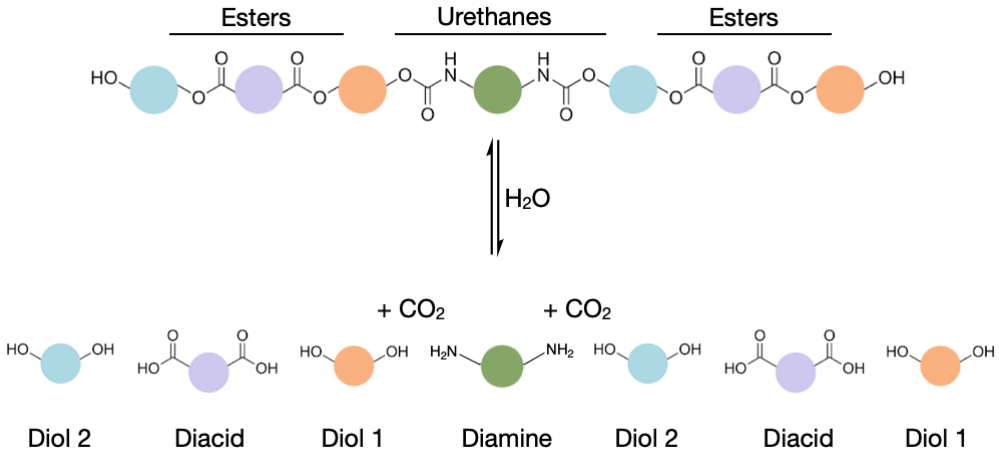

Figure 1.4: Expected products of hydrolysis of ester bonds and urethane bonds within the polyester PU structure. Diagram is simplified with assumption of complete hydrolysis of each bond.

The prospect of depolymerizing plastics is valuable because it provides a solution to a growing issue with plastic pollution: the accumulation of smaller plastic particles, also known as microplastics. Many non-biodegradable plastics tend to break apart into smaller pieces when exposed to the natural environment, often because they can only be partially depolymerized, if at all. This poses an ecotoxicological effect ranging from disturbances to reproductivity, metabolism and liver physiology.[14] Additionally, the bioaccumulation potential of microplastics is negatively correlated to its size, which highlights concerns about plastics in marine environments. In the past, several bacteria and fungi have been identified that are able to depolymerize different types of polyester plastics through esterase enzymes.[6] Biodegradation of polyester PUs is largely controlled by the activity of secreted or surface-

bound enzymes and their ability to access ester and urethane bonds. Knowing this, there is a potential for plastic to be redesigned into a more naturally renewable product in both pre- and post-production stages. The depolymerization of plastics opens up the exciting potential for monomer extraction for subsequent regeneration of the polymer.

This thesis highlights the development of a renewable polyester PU flexible foam by Algenesis Materials. It uses a multidisciplinary approach to understand the post-production biodegradation of a renewable, commercially-relevant PU product by microorganisms. Previously, the method for synthesizing biologically-sourced polyester polyols had been developed for various applications.[15] Here they were formulated into PU flexible foams to meet specifications for real-world commercial products, specifically flip-flop footbeds and shoe midsoles. This PU foam had a 52% biological content by mass, as the isocyanate used was derived from petroleum sources. Degradation of the PU was examined under two different conditions: through exposure to natural compost and soil environments, and through *in vitro* enzymatic hydrolysis. We were able to demonstrate rapid PU biodegradation via change in physical properties, and isolate a number of organisms associated with PU biodegradation from the two environments. We were also able to identify the primary molecular breakdown products of our PU foams, including intact starting monomers of the polyols. SEM imaging was used to further visualize degradation of the PU and interactions with relevant microorganisms. These data clearly demonstrate the potential to use PU to create commercial products that are sustainably-sourced, biodegradable, and potentially recyclable.

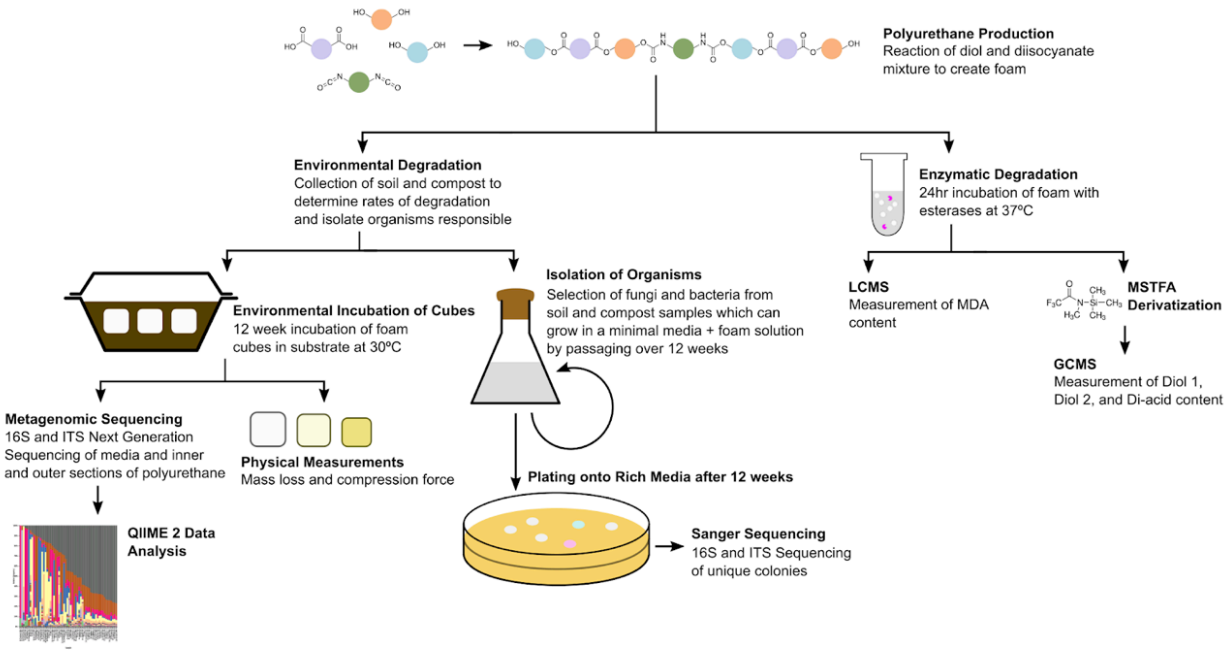

Figure 1.5: Experimental workflow of methods used within this study to analyze biodegradation processes of Algenesis Materials bio-based PU.

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Chapter 2: Biodegradation in natural environments

2.1 Background

In previous literature, several bacteria and fungi from environmental sources have been found to demonstrate biodegradation properties, attributed to enzymes typically used for surviving in natural environments. Many fungi utilize enzymes to degrade natural biopolymers, such as xylan and cellulose.[16] In order to study relevant biodegradation processes, it was important to mimic plastic waste in the environment. To do so, the PU was exposed to a surplus of microorganisms from compost and soil environments. Although this was done in a lab setting, we attempted to mimic the natural environment through modifications in temperature and humidity. Changes in physical degradation as well as biological growth were monitored.

2.2 Experimental

Two environmental samples were collected. Compost was collected from Roger's Community Garden at UCSD. Soil was collected from Solis Hall at UCSD. PU was made in a 2cm thick mold and cut into 2cm cubes. PU cubes were then covered with the environmental material in two separate containers at 30°C with high humidity.

Samples were weighed and compression-tested according to ASTM D3574 C- 50% Compression Force Deflection using an AFG 2500N compression tester (MecMesin) equipped with a MultiTest-dV sample stage at a rate of 100mm/min for 10 cycles.[17] Data was reported for the 10th cycle for all cubes. Initial, pre-degradation mass and force deflection measurements were taken prior to incubation and marked accordingly to monitor specific cubes and account for minor variations between cubes. Mass and compression force deflection measurements were taken in triplicates at 4, 8 and 12 weeks. The mass loss and maximum force were normalized as a percentage of initial, pre-degradation values.

PU cubes were removed from the incubation environments at 4, 8 and 12 weeks of environmental incubation and cut into sections. Sections from the exterior and interior surfaces of the cube were used for further analysis. Environmental media samples were collected at the same time points and analyzed in the same manner. DNA was extracted in triplicate and Next-Generation Sequencing was performed using the

Earth Microbiome Project protocols for 16S (bacteria) and ITS (fungi) analysis.[18] Next-Generation Sequencing was performed on the Illumina MiSeq for 2x250bp reads. Sequencing was conducted at the Institute for Genomic Medicine at UCSD. Sequencing reads were then processed using the QIIME 2 pipeline.[19] Sequence quality was analyzed and sequences were trimmed to 220bp and 230bp for 16S and ITS samples, respectively. Sequences were also quality-filtered using the 'deblur' feature. Taxonomy databases SILVA and UNITE were then used to analyze filtered sequences.[20,21] Raw sequencing reads can be found through the BioProject Accession numbers: PRJNA625105 and PRJNA625106.

2.3 Results and Discussion

2.3.1 Physical degradation

The bio-based PU showed marked degradation after incubation in compost and soil, compared to controls (Fig. 2.1). The top and bottom of all pre-incubation cubes had a smooth and closed-cell exterior skin, while the four sides of the cubes were cut to expose the porous interior. Over the course of the 12 weeks all six sides became noticeably porous, to the point where there was little to differentiate the sides from the top and bottom. The color also varied between the controls and incubated samples. It is important to note that while yellowing is a typical sign of PU degradation, some of the color difference may have come from the organisms or substances in the environments themselves.[29]

Cubes were washed thoroughly and dried overnight to remove any debris. In addition to visible observations, mass loss and reduction in compression force deflection (CFD) were measured at four-week intervals. Triplicate measurements normalized against pre-degradation values for each cube are pictured in Fig. X. After 12 weeks, samples in compost lost 30±3% mass and 41±3% CFD. Samples in soil showed greater degradation with 71±9% mass loss and similar 71.5±0.8% CFD decrease. The decrease in maximum force over time can be partially explained by the loss in mass through a change in surface area where the force is applied and a change in surface density, resulting in more stress per molecule and therefore a lower force. Decreased chain length, physical orientation of the remaining polymer chains, and degraded PU cell structure within the cubes may also be a factor in the CFD decrease over time.

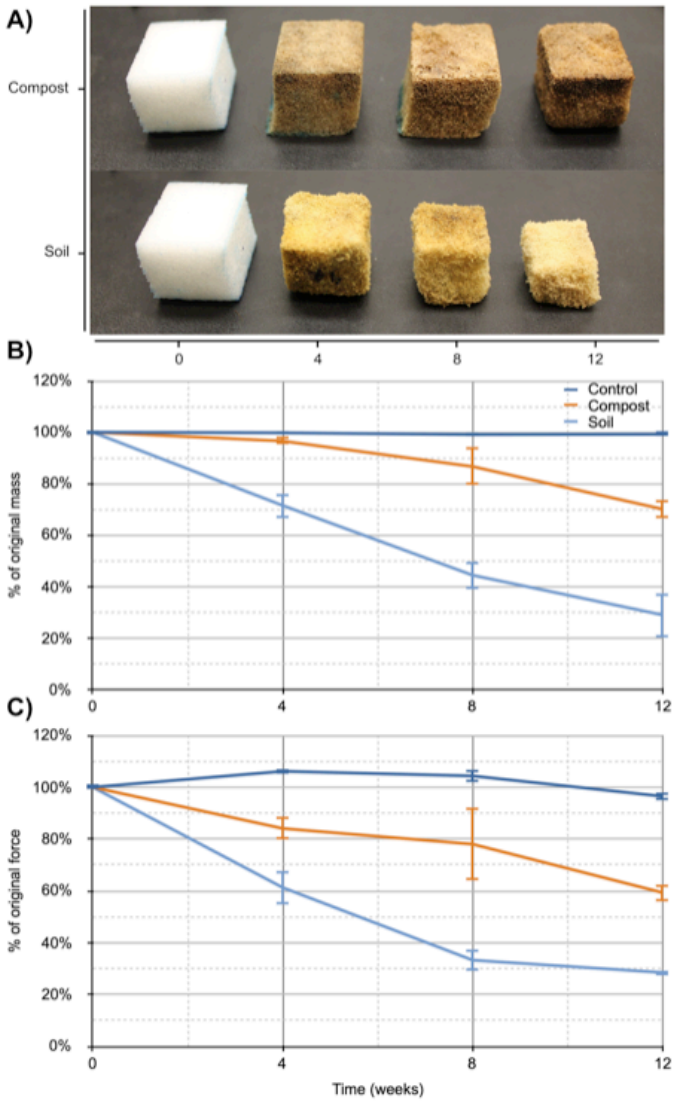

Figure 2.1: Biodegradation of PU cubes over 12 weeks. Degradation was analyzed through **A)** change in appearance, **B)** cube mass, and **C)** maximum force at 50% compression force deflection (CFD). Error bars indicate sample standard deviations of the triplicate measurements. For compost and soil mass loss, $p<0.5\%$, and for compost and soil CFD, $p<0.1\%$ (Table S2).

2.3.2 Identification of organisms associated with biodegradation of the PU

To identify the consortia of bacteria and fungi present on the environmental-incubated PU cubes, 16S and ITS metagenomic sequencing was employed. Analysis of the compost, soil and seawater media showed the background microbial community present in each sampling, compared to the community in the PU interior and PU exterior.

Here, the top 5 organisms refer to the 5 most abundant organisms found in the interior of the PU cubes at 12 weeks. From compost, the top 5 bacteria were *Pseudomonas, Pigmentiphaga, Roseomonas, Bordetella and Phenylobacterium,* and the top 5 fungi were were *Arthrographis, Thermomyces, Apiotrichum, Mortierella* and an uncharacterized fungus. From soil, the top 5 bacteria were *Pigmentiphaga, Roseomonas, Phenylobacterium, Xanthobacter, Aridibacter* and the top 5 fungi were *Arthrographis, Thermomyces, Apiotrichum, Aspergillus* and an uncharacterized fungus. In both environments, bacteria was enriched significantly (Fig. 2.2). Several fungi were enriched slightly although they were already abundant in the background media. An uncharacterized fungus, which was heavily present in both compost and soil, was most similar to a soil fungus with GenBank accession number JX489840.1.[30]

Table 2.1 highlights which of the top 5 organisms from each sample were found in greater relative abundance in the interior PU than in the media at 12 weeks using the ratio, I:B, for each genus.

I:B = (relative abundance in PU interior %) / (relative abundance in background environment %)

It was found that the majority of bacteria were enriched on the interior PU, while several fungi were not, including *Thermomyces* and *Mortierella* in compost, and *Thermomyces* and *Aspergillus* in soil.

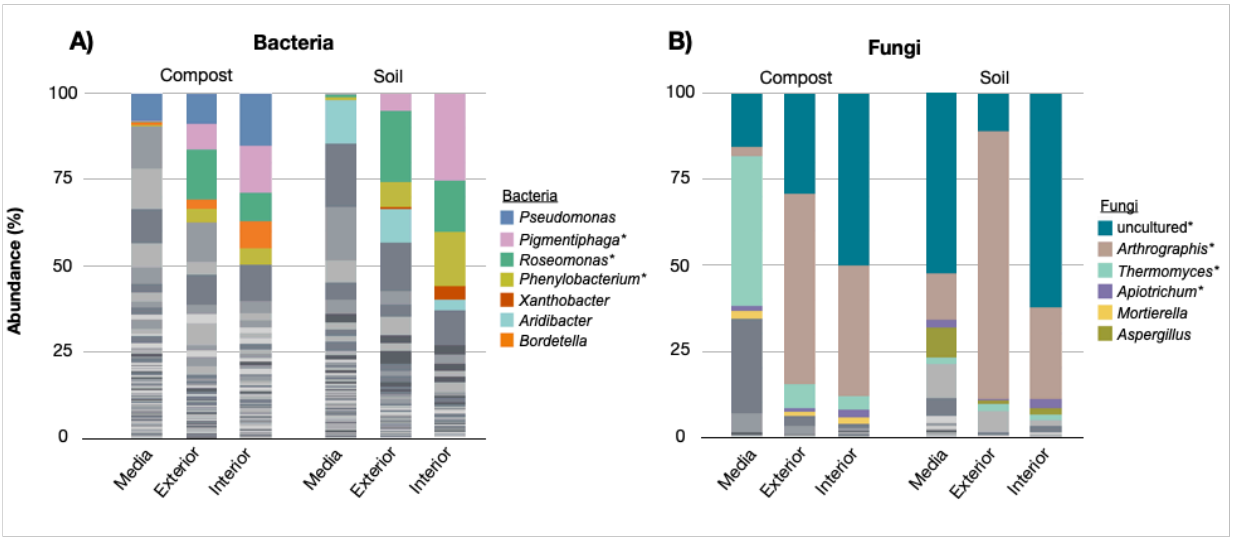

Figure 2.2: Biodiversity associated with biodegradation of PU in compost and soil by **A)** bacteria and **B)** fungi at 12 weeks. Genus-level analysis of the media, the exterior surface of PU and the interior section of PU were compared. The 5 most abundant genera found on the PU interior of each sample are colored and labeled. *Found in both compost and soil samples.

Table 2.1: Relative abundances of top 5 bacteria and fungi genera associated with biodegradation in compost and soil and the ratio of relative abundance in interior PU to relative abundance in media at 12 weeks (I:B). Blue corresponds to organisms with at least 50% greater abundance in interior PU than in media (I:B $\geq$ 1.5), red corresponds to organisms that didn't have 50% greater abundance in interior PU (I:B < 1.5). **Xanthobacter* showed no abundance in the media and 3.84% abundance in interior PU at 12 weeks.

	Top 5 Bacteria		Top 5 Fungi	
Environment	Genus	I:B		I:B
Compost	*Pseudomonas*	1.95	*uncultured*	3.24
	Pigmentiphaga	211.08	*Arthrographis*	13.06
	Roseomonas	19.88	*Thermomyces*	0.09
	Bordetella	9.36	*Apiotrichum*	1.49
	Phenylobacterium	21.68	*Mortierella*	0.72
Soil	*Pigmentiphaga*	504.60	*uncultured*	1.19
	Roseomonas	18.11	*Arthrographis*	2.00
	Phenylobacterium	29.11	*Apiotrichum*	1.39
	Xanthobacter	n/a *	*Aspergillus*	0.22
	Aridibacter	0.25	*Thermomyces*	1.00

The top 5 bacterial genera from both compost and soil were generally found to increase in abundance in the PU interior over the course of 12 weeks, with pronounced growth from week 8 to 12, compared to relatively unchanging abundance in media. For fungi, this was less apparent. Species population analysis of the top 10 species was also conducted, comparing abundance in the PU interior to abundance in the media, where the top 10 species are defined as those found in highest abundance in the PU interior at 12 weeks. The majority of bacteria from compost and soil were found in greater abundance in the PU compared to the media, especially by 12 weeks, demonstrating preferential selection over time (Fig. 2.3). For fungi, the trend was less clear.

Several of the organisms from compost and soil that had the highest abundance in the PU interior have previously been associated with the biodegradation of petroleum-based materials and plastics. Notably, *Pseudomonas* species have been found to degrade polyester PUs, *Pigmentiphaga daeguensis* AN-4a has been shown to degrade aniline at low concentrations, and *Phenylobacterium* has been used to bioremediate petroleum-contaminated soil.[22,23,24,25] Additionally, *Arthrographis kalrae*, *Thermomyces lanuginosus,* and *Aspergillus fumigatus* have been found to degrade polyester PUs.[26]

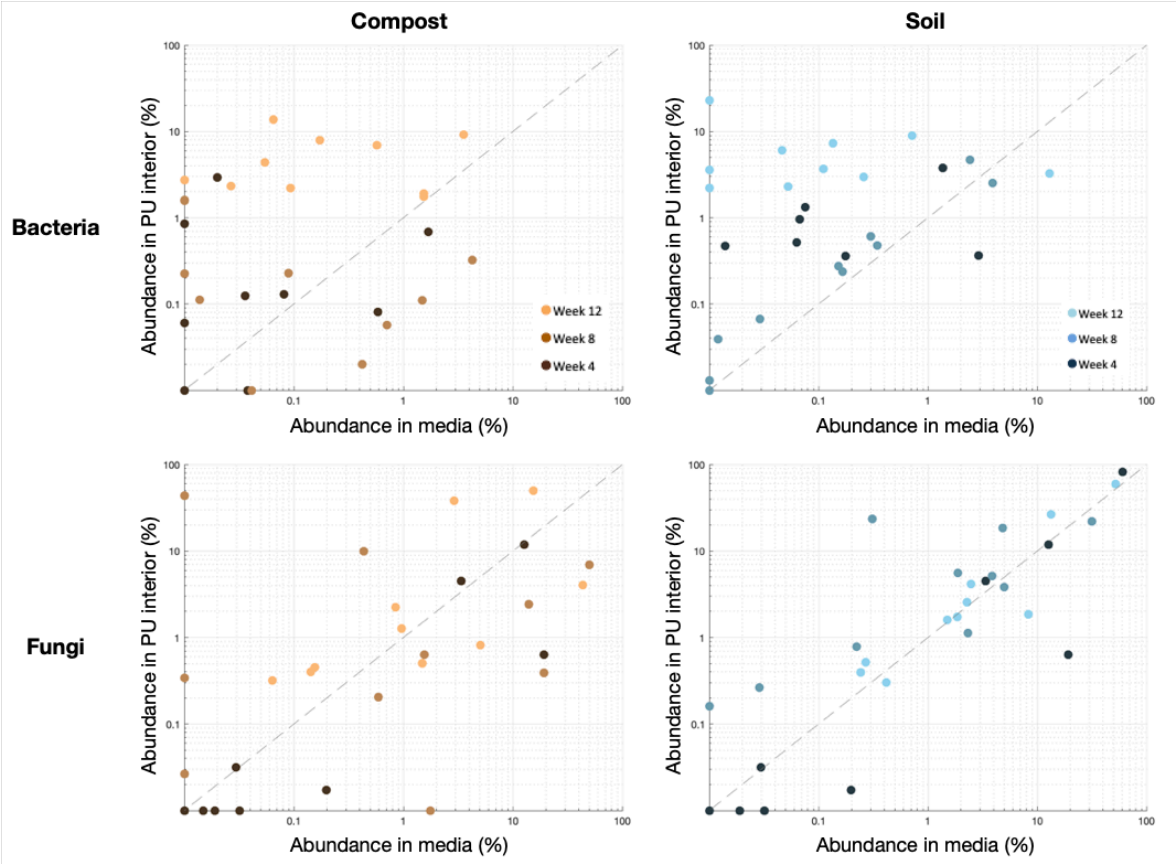

Figure 2.3: Scatter plots of abundance of top 10 species in PU interior and in media at 4, 8 and 12 weeks. Top 10 species are defined as the most abundant species at 12 weeks in the PU interior. Dashed line is x = y; organisms enriched in the PU interior lie above this line and those more populous in the media lie below. Abundances less than 0.01% were rounded up to 0.01% for convenience in plotting. Top graphs represent bacteria in compost and soil. Bottom graphs represent fungi in compost and soil.

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Chapter 3: Isolation of PU-degrading microorganisms from natural environments

3.1 Background

Although Chapter 1 provides an idea of what organisms are highly present on the PU from natural environments, the presence of those organisms does not necessarily verify biodegradation. To enrich and isolate microorganisms responsible for degradation of the foams, we serially passaged microbial samples derived from soil or compost through 10 passages in M9 media, where PU was the sole carbon source. This process allowed us to identify organisms able to utilize the PU to survive, indicating biodegradation of the PU. This experiment provided a powerful way to allow us to screen for key organisms. In previous literature, similar experiments had been conducted where organisms were able to grow in the presence of monomers as a sole energy source.[27,28]

3.2 Experimental

To identify organisms from compost and soil that were capable of utilizing the PU to grow, organisms were inoculated into M9 minimal media with PU as the sole carbon source [appendix 1]. PU foam was frozen in dry ice and pulverized with a high-speed blender to create fine particulates and then autoclaved to ensure sterility. In 125mL Erlenmeyer flasks, 25mL of minimal media, 0.5g PU particulate, and 1g of inoculum from compost or soil was added, along with control flasks with inoculum but no PU, and a control flask with PU M9 media but no inoculum. Flasks were shaken at 100rpm at room temperature. 1mL of the liquid in the flask was then used to inoculate a fresh minimal media flask for the subsequent incubation period. Fresh flasks were inoculated weekly for 8 weeks and then bi-weekly at weeks 10 and 12. At the end of each passage, day 7 or day 14 from inoculation, 1:1000 and 1:10,000 dilutions were prepared and 50µL was plated onto LB and PDA media and grown at room temperature for 48 hours. Individual colonies with unique morphology were picked from each plate from the 10th passage. ThermoFisher Phire Plant Direct PCR Master Mix (cat# F160S) with appropriate primers for 16S (515F, 806R) and ITS1 (ITS1-F, ITS2) were used to PCR amplify each selected colony.[29,30,31,32] The samples were sent to Eton Biosciences for Sanger sequencing.

To analyze individual organisms for their growth in the PU M9 media, each individually picked colony was grown up in sterile LB overnight. 1mL of each culture was then used as individual inoculants into fresh PU M9 media flasks, prepared in the same way as the first part of this experiment. The samples were then measured for initial OD values at 600nm absorbance using a UV-Vis spectrophotometer. OD values were then taken every 24 hours for each sample and monitored for 7 days. At the end of the 7 day period, growth cycles were graphed and used to determine the highest-growing organisms.

SEM imaging was used to get a visual understanding of the interactions between organisms on the surface of the PU. PU cubes were cut with a sterile razor into 1–2mm thick slices, approximately 4x4mm in size, and autoclaved. For each inoculant, one slice from the open-celled interior and one slice from the closed-cell exterior surface was prepared. Each set of slices was placed in 15mL culture tubes with 5mL of M9 media and inoculated with 500μL of liquid culture. The samples were incubated shaking at room temperature for 72 hours, then fixed in an ethanol gradient.[33] After attaching the foam slices to aluminum stubs using carbon tape, an Emitech K575X Sputter Coater was used to deposit an iridium layer on the foam slices, excess of which was dusted off with compressed air prior to imaging. All samples were imaged at high vacuum using an FEI Quanta FEG 250 scanning electron microscope, at magnifications ranging from 600x–20,000x. Each micrograph, including controls, was visually inspected for the presence of fungi and bacterial colonies, as well as changes in topography of the foam.

3.3 Results and Discussion

3.3.1 Adaptation of organisms to use PU as sole carbon source

Throughout the course of the experiment, visual changes in the cultures indicated to us that degradation was occurring in some form, allowing organisms to survive. It was speculated that the organisms were using the PU monomers as an energy source. Therefore, those able to survive were most likely able to degrade the PU on its own or survive in the consortia with the other organisms. Visual changes were seen throughout each serial passaging, including increased turbidity, color change, and sinkage of PU. In particular, the culture derived from soil appeared to become turbid rapidly with each passage, as well as develop a pink color all over the surface of the PU material. This indicated that there

were perhaps few organisms that were thriving and able to readily degrade the PU, causing the color change.

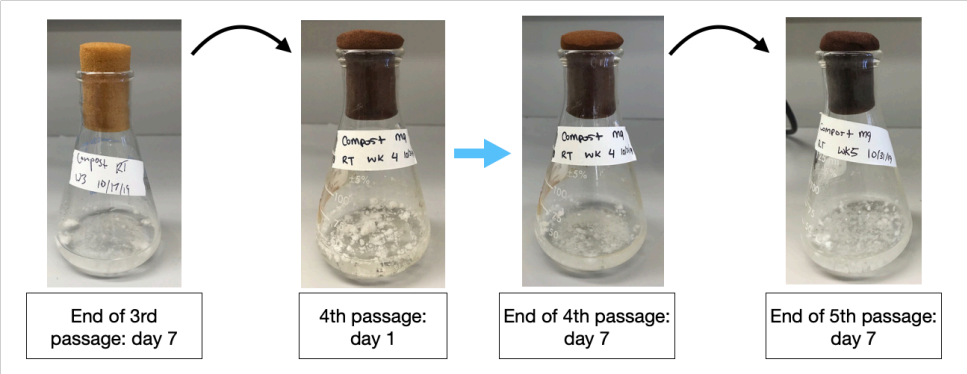

Figure 3.1: Serial passaging of organisms from compost in the PU M9 media from the 3rd passage to the 5th passage

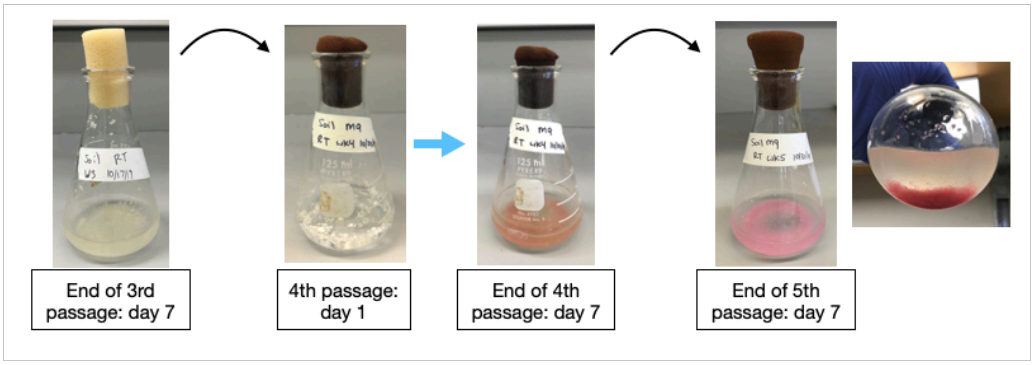

Figure 3.2: Serial passaging of organisms from soil in the PU M9 media from the 3rd passage to the 5th passage.

By plating the cultures over multiple passages, it became evident that a consortium of surviving organisms was stabilizing in both compost and soil samples over time, while minimal media control flasks lacking PU had few surviving organisms, indicating that organisms from compost and soil were utilizing the PU to survive (Fig. 3.3). No fungi were observed on plates by the 10th passage. Since we did not observe significant enrichment of fungi over time in the environmental incubation samples, it is possible the same occurred here; however, it is also possible that the setup of the consortia in a shaker flask culture could be more ideal for bacterial growth than fungi, which may need other nutrients than those provided in the media to compete with the surrounding bacteria.

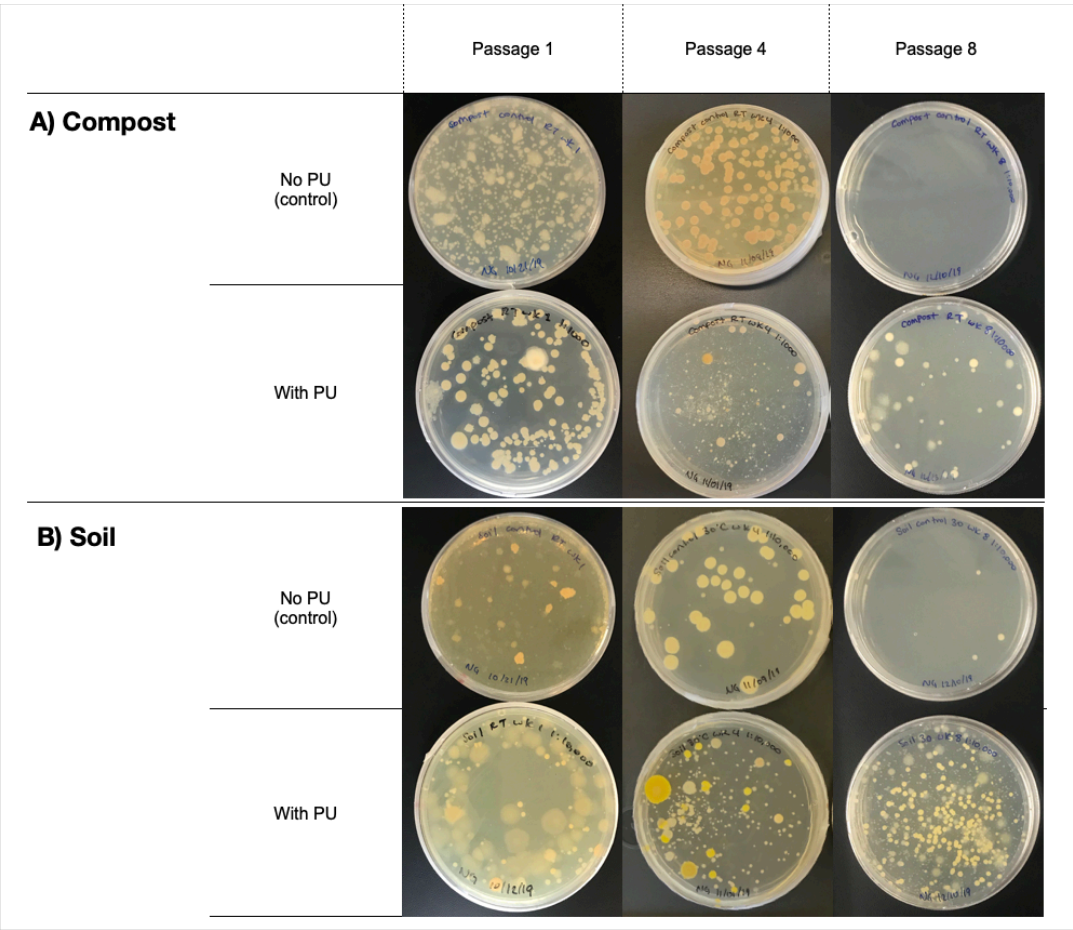

Figure 3.3: Visual analysis of samples plated on LB from A) compost and B) soil isolation experiment to identify organisms surviving on a minimal media with PU with weekly passages.

Table 3.1 displays the organisms enriched from the 10th passage, noted through their ability to survive aerobically using the PU as a sole carbon source. Notably, the consortia of organisms found here are not all of the most abundant organisms found in the PU cubes previously incubated in the environmental samples, suggesting that the top organisms from those experiments may not be the primary PU consumers, but organisms that can thrive as part of consortia living on the PU. This may also be because there is a greater abundance of nutrients in the natural environment than in the defined PU M9 media.

Table 3.1: List of remaining organisms surviving after 10 rounds of serial passaging in PU M9 media.

	Family	Species
Compost	*Burkholderiaceae*	*Achromobacter*
	Brucellaceae	*Brucella*
	Pseudomonadaceae	*Pseudomonas*
	Rhizobiaceae	*Rhizobium*
	Xanthomonadaceae	*Stenotrophomonas*
Soil	*Flavobacteriaceae*	*Chryseobacterium*
	Oxalobacteraceae	*Herbaspirillum*
	Brucellaceae	*Ochrobactrum*
	Nocardiaceae	*Rhodococcus*
	Xanthomonadaceae	*Stenotrophomonas*

Several of these bacteria genera have been cited to degrade plastic products and precursors. In particular, several *Rhodococcus* species have been found to degrade diesel-oil and hydrocarbons.[34,35] *Ochrobactrum* has been found to degrade engine oil in contaminated soil.[36] *Stenotrophomonas* has been shown to degrade polycyclic aromatic hydrocarbons and a *Chryseobacterium* has been isolated that was capable of degrading polyethylene.[37,38] A *Rhizobium* sp. has also been found that degrades phthalate esters.[39] The *Pseudomonas* genus, which has been shown to be involved in biodegradation processes, appeared in compost in the environmental incubation results as well as in this selection experiment.

Additionally, a fungi, *Cladosporium sp.*, was tested on its own as it appeared to easily grow in the PU M9 media, as a contamination in several samples. Although fungi did not grow abundantly in the restricted media, compared to bacteria, it was clear that *Cladosporium sp*. was able to survive over time and grow rapidly. This corroborated previous studies on biodegradation of PUs by *Cladosporium* fungi.[40]

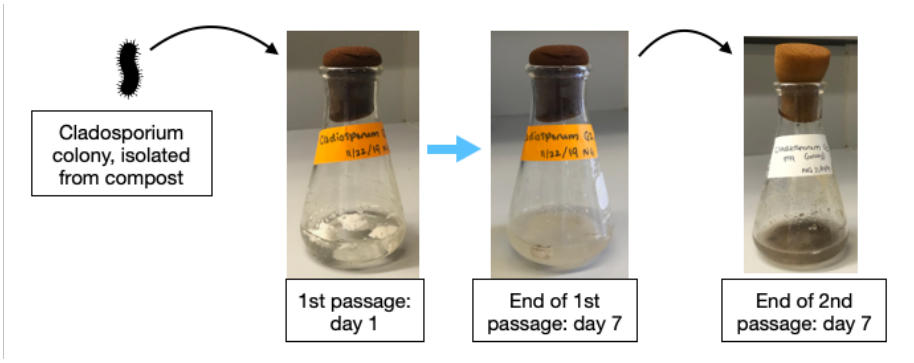

Figure 3.4: Serial passaging of *Cladosporium* fungi in the PU M9 media from the 1st passage to the 2nd passage.

3.3.2 Downselection of high-performing organisms

Once consortia of organisms were selected for biodegradation properties, it was useful to determine which organisms were primarily involved in the degradation process rather than just living on the consortium. To do this, organisms from the adaptation experiment were tested individually against the PU material. Growth was then monitored by plating samples and measuring OD over time to down-select for high-performing organisms. High growth could be an indication of high production of proteins, such as enzymes that are useful for biodegradation. In the future, sampling at different intervals during the growth period would be needed to confirm production of enzymes using proteomics analysis.

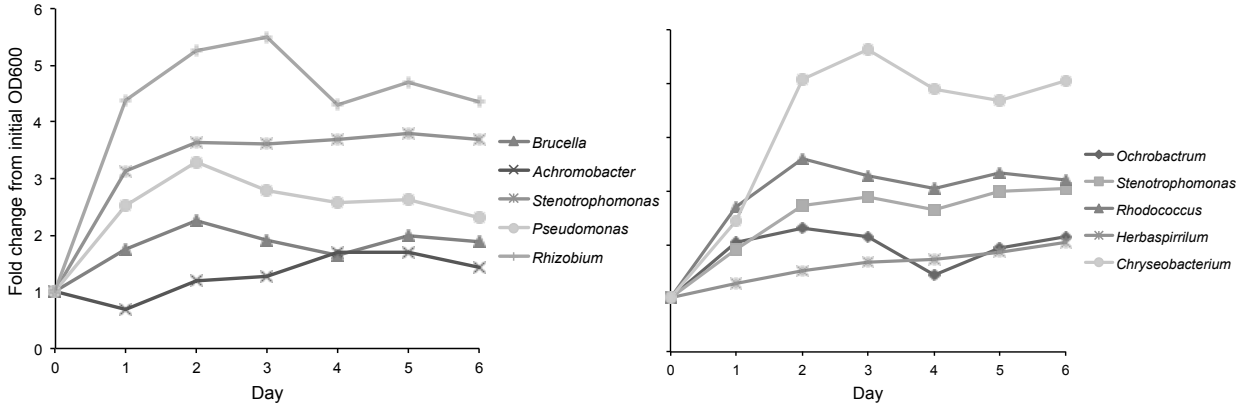

Figure 3.5: Fold change in OD values of individual isolates from compost (left) and soil (right) in minimal media with PU as sole carbon source.

Through assessing growth rates of the organisms in the PU M9 media, its was found that *Rhizobium, Stenotrophomonas* from compost, *Chryseobacterium*, and *Rhodococcus* had the highest growth rates in the PU M9 media. Interestingly, the *Rhodococcus* from soil resulted in a bright pink coloration of the PU, which could be due to interactions with the breakdown products of the PU or the nutrients in the media. Once the culture is taken off the shaker, the sample changes to a yellow color. It is speculated that the red coloration occurs when the cells are growing with active degradation processes and generous aeration. Previously, a *Rhodococcus* has been found to utilize picric acid as a nitrogen source using a metabolite, resulting in a color change of orange to red, which suggests that there may be similar interactions with this PU.[41]

High growth rates indicated that the organisms, on their own, are able to readily utilize the PU to grow. Additionally, this experiment provided information on how each organism grows on the PU material, providing insight into how these properties may be manipulated for post-production biodegradation. In the future, these downselected organisms can be studied for their enzymatic activity in the presence of PU.

3.3.3 SEM imaging of microorganisms

SEM imaging provided a qualitative look at the organisms' interactions on the surface of the PU foam. Inoculants from soil passaging experiment was imaged on the PU surface. Observations of the soil-isolated bacteria on the closed-cell skin surface of the PU showed an abundance of open-celled areas with bacteria, suggesting the ability for bacteria to degrade and penetrate the skin of the PU (Fig. 3.6B). We also observed the presence of biofilms, which could potentially be a mechanism of bacterial attachment to the PU surface [appendix 2]. Several studies have concluded that bacterial growth on plastic tends to be related to the formation of biofilms.[42] Furthermore, it has been found that there is a positive correlation between starvation of plastic-degrading bacteria and hydrophobicity, which could explain the formation of biofilms in conditions where plastic materials were the sole carbon source.[43] To understand interactions of fungi with the PU, samples were also inoculated with an isolate of *Cladosporium sp.*, which we found to utilize polyester PUs as its sole carbon source. Comparison between the PU control and PU inoculated with *Cladosporium sp.* shows extensive topographical changes (Fig. 3.6D). Significant surface

degradation and extensive binding of *Cladosporium sp.* with the foam indicates degradation of the PU for utilization by the fungus for growth.

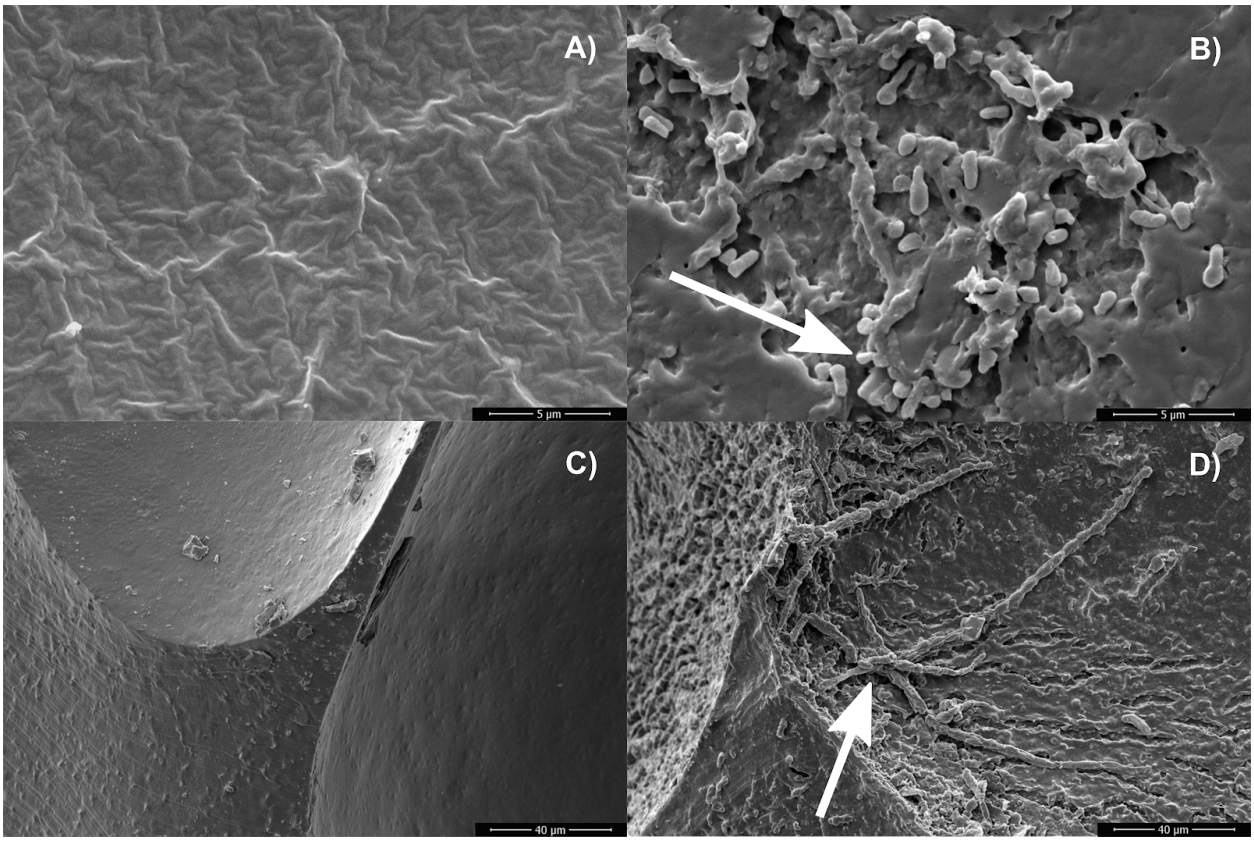

Figure 3.6: SEM micrographs of PU foam. A) Control sample of the exterior of PU foam, taken at 8000x magnification. B) Closed-cell surface of the PU with bacteria enriched in soil-PU adaptation experiments at 8000x magnification. C) Interior of PU control sample at 1000x magnification. D) Interior of the PU with *Cladosporium sp.*, taken at 1000x magnification

Chapter 3, in part, has been submitted for publication of the material under the title, "Rapid biodegradation of renewable polyurethanes with identification of associated microorganisms and breakdown products," as it may appear in Bioresource Technology Reports 2020. Natasha R. Gunawan*, Marissa Tessman*, Ariel C. Schreiman, Ryan Simkovsky, Anton A. Samoylov, Nitin K. Neelakantan, Michael D. Burkart, Robert Pomeroy, and Stephen P. Mayfield. The thesis author was a coauthor of this paper.

Chapter 4: *In vitro* enzymatic breakdown of PU into starting monomers

4.1 Background

To test the ability of known polyester degrading enzymes (hydrolase esterases) to break down our PU foam into monomers, commercially-available enzymes were assayed for their ability to depolymerize our PU foams. Esterase and lipase enzymes from four organisms were tested that were previously found to degrade PUs in the literature: *Aspergillus niger*, *Candida rugosa*, *Bacillus subtilis*, and various *Pseudomonas* species.[44,45,46] As these enzymes hydrolyze ester bonds found in lipids and sterol esters, it was hypothesized that the ester and urethane bonds found in our PU foams would hydrolyze to produce the starting polyol monomers—diol 1, diol 2, and diacid—and MDA, the diamine that originates from cleavage of the urethane bond formed by the isocyanate MDI used to make our PU.[47] This *in vitro* method served to model the degradation products expected in the environmental samples. Further degradation product analysis, such as the sampling of monomers in the complex environments or the generation of CO_2 or methane gas, was outside the scope of this study.

4.2 Experimental

PU foam was frozen using liquid nitrogen and crushed with a Qiagen TissueLyser (cat# 85300). Foam particulate was washed with MilliQ water to remove any soluble contaminants and then dried overnight in a desiccator. To screen for enzyme activity, four enzymes were tested at a concentration of 400ug/mL in PBS with 1mg of PU [appendix 3]. All samples were prepared in triplicate. In addition, tubes containing enzymes without foam and foam without enzymes were prepared as controls. Samples were shaken for 24 hours at 37°C, then frozen immediately to prevent further enzyme activity. Initial tests involved analysis of the two diols to determine which enzymes were able to degrade the PU more efficiently. The commercial enzymes included lipase from *Aspergillus niger* (cat# 62301), lipase from *Candida rugosa* (cat# L1754), esterase from *Bacillus subtilis* (cat# 96667), and cholesterol esterase from *Pseudomonas* species (cat# IC10543991).

For GCMS analysis, samples were acidified and the products were extracted with EtOAc, derivatized with MSTFA, and run on a GCMS as described in the Supplementary Methods.

Chromatograms were created by subtracting the no-substrate control from an average of the triplicate sample chromatograms. 375ppm standards containing diol 1, diol 2, and diacid 1 diluted in PBS media, as well as a series of calibration standards, were treated to the same extraction and GCMS method. Each peak was integrated using the instrument's integration tool. The mass percent of each compound in the proprietary PU foam was known. From these values, the expected concentration of each compound at 100% PU foam degradation and the percent degradation of the PU foam to each product was calculated according to formulas 1 and 2. Values are reported to a 95% confidence interval.

1. $C_{100\% \text{ degradation}} = (\%\ m/m_{\text{compound}}) \times m_{\text{foam}} / m_{\text{sample}} \times 10^6$
2. $\%\ m/m_{\text{product}} = C_{\text{product}} / C_{100\% \text{ degradation}} \times 100\%$

For LCMS analysis, a 500ppm MDA standard was prepared by heating MDA in PBS media for several hours until complete dissolution. The MDA standard and one of the *Pseudomonas* sp. enzyme and foam samples were filtered with a 0.22µm syringe filter. 10µL was injected into a Waters Acquity SQD LCMS system with a 2.1x150mm 3µm ACE C18-PFP column. The buffers were 0.1% formic acid buffer at pH 3.35 (A) and 100% ACN (B). The run started with 0.5min of 99% A at 0.2 mL/min, then ramped to 100% B by 0.7min and held until 15min before returning to the original eluent conditions at 20min. The MS had an ESI probe set at 3.5kV and 350°C. Positive mode scans were taken from 150–1000m/z. A 140-element moving-average of the raw chromatograms for standard and sample was applied.

4.3 Results and Discussion

Initial screenings were conducted for four enzymes: *Aspergillus niger* lipase, *Candida rugosa* lipase, *Bacillus subtilis* lipase, and *Pseudomonas* cholesterol esterase. Two of the enzymes demonstrated demonstrated high polyol production during degradation of PU: lipase from *Bacillus subtilis* and cholesterol esterase from *Pseudomonas* species. Cholesterol esterase from a *Pseudomonas* species demonstrated highest diol peaks, indicating that it had the greatest efficiency at cleaving the ester bonds within the PU structure (Fig. 4.1). The samples reacted with the lipase from *Bacillus subtilis* also had pronounced production of diols.

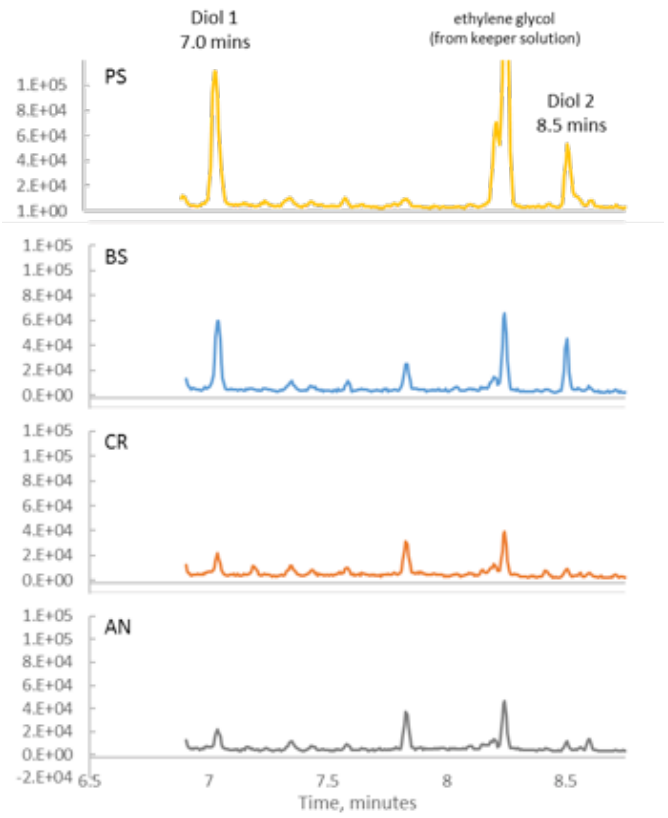

Figure 4.1: GCMS chromatograms showing diols present from degradation of foam after 24 hours from four esterases/lipases. Triplicates of no-enzyme controls were averaged and subtracted from sample triplicates

The cholesterol esterase also became of interest because the *Pseudomonas* genus is common in soil and compost, has been found to bioremediate petroleum-contaminated environments, and is a species shown by metagenomics sequencing to be abundant in our compost sample over the 12-week environmental degradation.[48] This enzyme is a sterol esterase, involved in metabolic transformations of cholesterol and its esters within biological systems and has previously been purified from *Pseudomonas fluorescens*, labelled 'CE1'.[49] Thus, the cholesterol esterase was further analyzed for the presence of diols and diacids, the expected depolymerization products of the polyester polyols, by GCMS, and for MDA by LCMS. Standards in the PBS matrix were run in parallel to compare retention times and to account for other peaks present solely in the matrix. Each peak identity was also confirmed by its mass spectra in both the GCMS and LCMS standards and samples. The PU foam chromatogram (Fig. 4.2) shows all three breakdown product peaks with the same retention times as the standards. Integration of the peaks

corresponded to 7±2%, 3±2%, and 1±0.04% complete degradation into diol 1, diol 2, and diacid 1, respectively. appendix 4 Four prominent peaks appeared later in the chromatogram at 9.8, 10.1, 11.27, and 11.47 min, which were identified by their unique mass spectra as partial degradation products: dimers and trimers of diols and diacids. To further validate the identity of the four peaks, one of the degraded samples was treated with strong base to completely hydrolyze the remaining water-soluble polymer fragments and analyzed again by GCMS (Fig. 4.3). Notable in the base-hydrolyzed GCMS chromatogram was the complete disappearance of the partial PU fragment peaks and an increase of all three final product peaks, corresponding to 39±2%, 43±4%, and 32±2% PU degradation into diol 1, diol 2, and diacid 1, respectively. These data indicate that the cholesterol esterase degraded 38±6% of the PU foam into a combination of polyol monomers and water-soluble dimers and trimers in 24 hours.

The presence of MDA in the enzyme-degraded PU foam sample was detected by the LCMS mass trace at 199.2m/z, corresponding to the $MDA+H^+$ ion as confirmed by MDA standard in PBS matrix run under the same conditions. These results indicate that the esterase targeted the ester bonds present in the polyol and, to a lesser extent, the urethane bond between the polyol and the MDI, showing evidence for PU degradation into the polyol monomers and MDA.

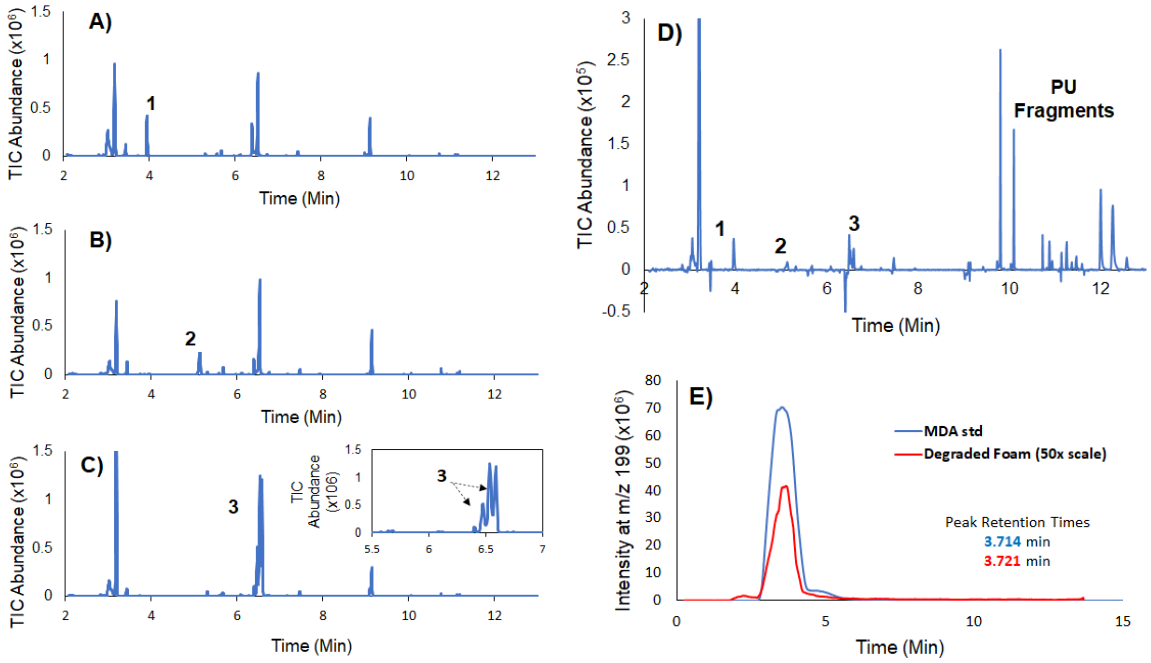

Figure 4.2: GCMS chromatograms of **A)** diol 1, **B)** diol 2, and **C)** diacid 1 standards with corresponding peaks labeled 1, 2, and 3, respectively, in PBS media. Unlabeled peaks, present in all chromatograms, are PBS media traces. **D)** GCMS chromatogram of PU foam degraded by *Pseudomonas sp.* cholesterol esterase for 24 hours, with biodegradation product peaks labeled. **E)** LCMS chromatogram of the same sample. Mass trace is MDA at 199.2m/z. Blue is the standard in PBS media, and red is the enzyme degraded PU foam sample.

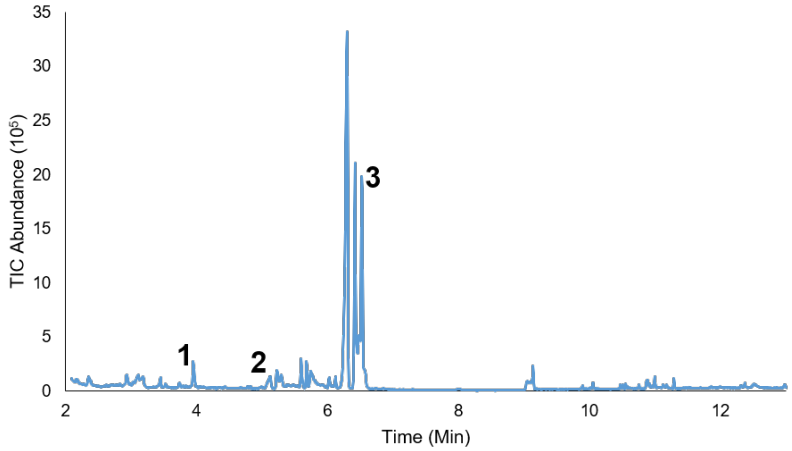

Figure 4.3: GCMS chromatogram of PU foam degraded by *Pseudomonas sp.* cholesterol esterase for 24 hours, then hydrolyzed with base to identify the PU fragments, labelled 1-3. Note the disappearance of the peaks around 10 and 12 min, and the increase in abundance of peaks 1, 2, and 3 to ~10x that of the original sample.

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Chapter 5: Key enzymes associated with biodegradation

5.1 Background

It has been well-studied that biodegradation of PU by microorganisms occurs through various enzymatic processes. Chapter 4 provides a look at commercial enzymes that are able to cleave ester bonds within the PU structure. In future research, analyzing the proteomics of organisms that degrade the PU can allow us to isolate more enzymes that may be key for biodegradation processes. In the past, a wide variety of lipase enzymes from bacteria have been found to degrade polyesters into soluble oligomers.[50] More recently, an enzyme, named a PET hydrolase, was engineered to depolymerize PET into monomers at a rapid rate.[51] Previously other PET-biodegrading enzymes were engineered.[52] Although PET is a different type of plastic, this provides evidence that enzymes can be used as a powerful tool to regenerate plastics. In previous literature, several organisms have been isolated that employ enzymes to degrade PUs specifically. An enzyme, known as a "polyurethanase-lipase" has been purified from *Bacillus subtilis*, isolated from a soil environment, labelled 'lipA'.[53] Esterases from a *Pseudomonas aeruginosa* strain were found to degrade PU into monomers through hydrolysis of the ester-bonds.[39] In this study, enzymes of interest were isolated from bacterial cultures and expressed in *Escheria coli*. This serves as a potential methodology for developing key PU-degrading enzymes for potential use in post-production applications. Two enzymes were of interest that had previously been cited as having PU biodegradation properties: a cholesterol esterase (CE1) from *Pseudomonas aeruginosa* and a lipase (lipA) from *Bacillus subtilis*.[49,54]

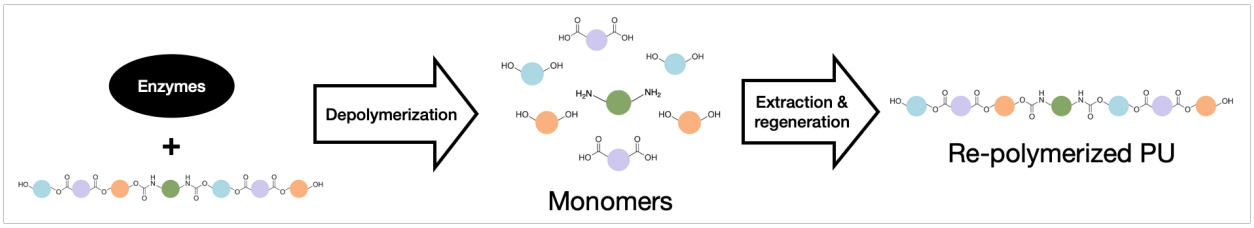


Figure 5.1: Diagram of the potential process of biorecycling.

5.2 Experimental

DNA was extracted from bacteria, *Pseudomonas aeruginosa*, isolated from soil, and *Bacillus subtilis*, isolated from a culture of strain 168 (from the Süel lab at UCSD) using the QIAGEN DNeasy UltraClean Microbial Kit (cat# 12224-50). Primers were designed to amplify genes of interest. The primers were used to PCR amplify the respective enzymes from the DNA extract with NEB Q5 Hot Start Hi-Fi DNA Polymerase (cat# NEB #M0515). Amplicons were ran on a 1% agarose gel and cleaned using the Wizard SV Gel Cleanup kit (cat# A9281). Sequence-confirmed genes were then inserted into a pET28a vector with a His-tag and chemically transformed into DNA-encoding *E. coli* DH5alpha cells, using the NEBuilder Hi-Fi DNA Assembly Cloning Kit (cat# NEB #E5520) and Chemical Transformation Protocol. pET28-SUMO-KaiA was a gift from Susan Golden (Addgene plasmid # 68049).[55] Transformants were screened and successful transformants were then transformed into protein-expressing *E. coli* BL21 cells. Successful transformants were then grown in 2xYT media and IPTG-induced (500uM) for 16 hours at room temperature. Western blot analysis with Mouse-Antibody (primary) and His-Antibody (secondary) was done to confirm successful protein induction. lipA was purified using Qiagen Ni-NTA agarose (cat# 30210) and lipase activity was analyzed using Sigma Aldrich Lipase Activity Assay kit (cat# MAK046).

5.3 Results and Discussion

Successful PCR amplification of the region of interest was confirmed through separation by electrophoresis on a 1% agarose gel and sequencing by Sanger Sequencing (Figure 12). It was confirmed that the active sites were intact. A conserved serine residue holds the active site that is typically involved in catalysis for esterases. In *Bacillus subtilis* lipase the serine residue appears in the pentapeptide Ala-Xaa-Ser-Xaa-Gly.[54] Usually for lipases the unique pentapeptide has glycine instead of the first alanine residue. For CE1, the unique pentapeptide within CE1 that holds the conserved serine residue is Gly-Xaa-Ser-Xaa-Gly.

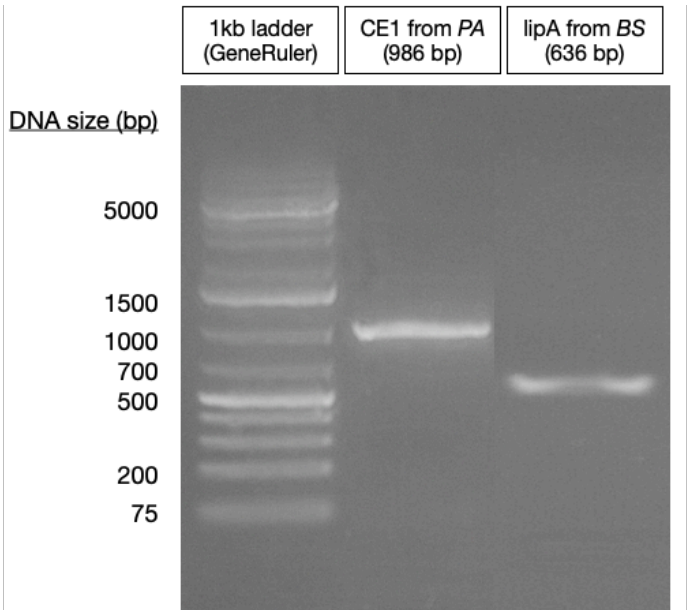


Figure 5.2: Successful PCR amplification of genes for enzymes, Cholesterol Esterase (CE1) from *Pseudomonas aeruginosa*, and lipase (lipA) from *Bacillus subtilis*.

Once enzymes of interest were successfully transformed into *E. coli* BL21 cells, samples were IPTG-induced. Confirmation of protein induction was done through Western blot analysis with clear bands at the expected protein size in the induced sample and no band in the non-induced sample (negative control). The sample was then purified using high imidazole concentration to elute the protein. The purified protein was eluted from 250-500mM of imidazole and elution fractions were combined. The lipase activity of the purified protein was found to be 0.33umol glycerol produced/min, compared to -0.03umol glycerol produced/min for uninduced BL21 cells.

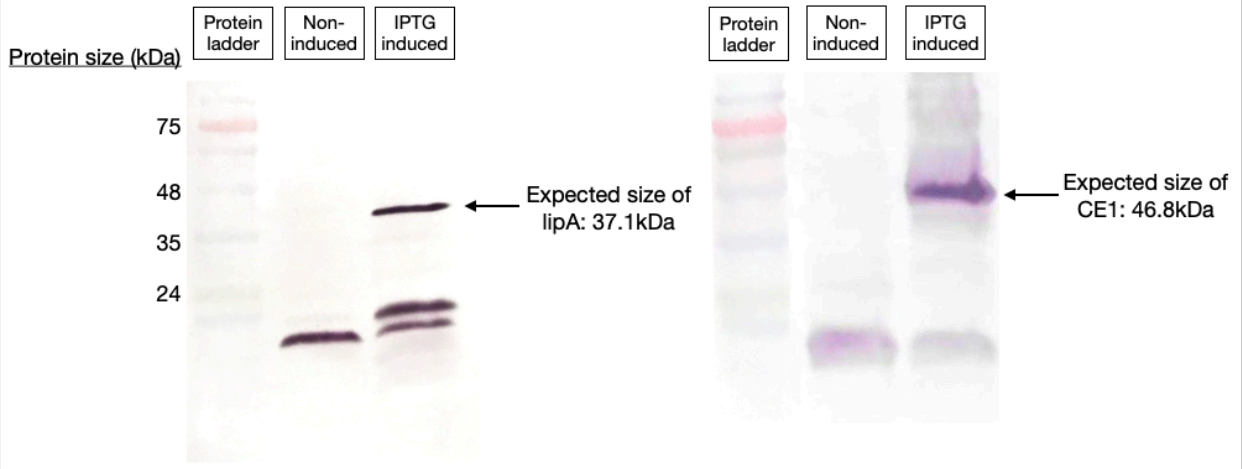


Figure 5.3: Western blot analysis of IPTG-induced enzymes expressed in *E. coli* BL21 cells. lipA (left) from *Bacillus subtilis* is seen in IPTG-induced samples with expected size of 37.1kDa. CE1 (left) from *Pseudomonas aeruginosa* is seen in IPTG-induced samples with expected size of 46.8 kDa. His-antibody was used to analyze the tagged proteins.

Conclusions

The development of more sustainable plastics provides an opportunity to reshape the current plastic production system. Here, the post-production degradation processes of a renewable polyester polyurethane were studied to better understand what a more sustainable plastic life cycle could look like where environmental costs in pre- and post-production are considered. We were able to demonstrate clear physical degradation of the PU in compost and soil and identify several organisms that grew in abundance on the PU. Downselection experiments were then conducted to isolate organisms that were able to survive utilizing the PU as a sole carbon source. This enabled us to screen for organisms that were biodegrading the PU. Through SEM imaging, we were able to capture the close-up interactions of organisms on the surface of the PU and noticed biofilm formation and topographical changes on the PU. Identifying the chemical breakdown products of enzymatic degradation was also an important tool to confirm the expected products of biodegradation of the PU structure. Through hydrolysis of the ester and urethane bonds it was found that the resulting breakdown products were two diols, a diacid, and a diamine.

Along with developing solutions to tackle the growing problem of plastic pollution, it is important to be clear about what is classified as true biodegradation. Often when marketing sustainable products, the term ‘biodegradable’ is used interchangeably with terms such as ‘renewable’ and ‘plant-based’ or used even though the product may require additional reagents or extreme conditions to depolymerize. Often, plastic waste ends up accumulating in the natural environment, where manipulating conditions as we see fit is no longer relevant. Rather, understanding how natural biodegradation processes occur can give way to a complete redesign of how plastic is designed and discarded in the future.

Together these data demonstrate that it is possible to create commercially viable PU products that have an end-of-life biodegradation option and would be much more environmentally-friendly than current PU products. This study also suggests it is possible that PU products could be recovered at the end of their useful life to be depolymerized and the depolymerization products isolated and used as feedstocks to resynthesize new PUs, potentially over many rounds of recycling. Biorecycling is a novel and important

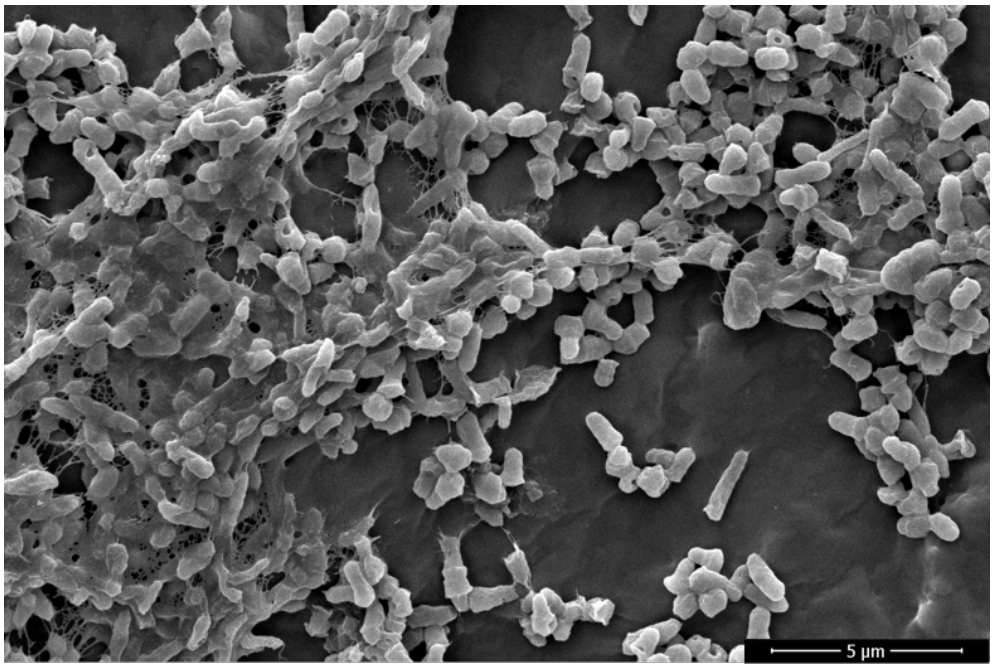
field that could provide a solution to the growing plastic pollution issue. This would represent a new paradigm in plastic recycling and a great advance for both the PU industry and the environment.

APPENDIX

1. Composition of M9 minimal salt media

Compound	**Components (g/L)**
Na_2HPO_4	6.78
KH_2PO_4	3.00
NH_4Cl	1.00
NaCl	0.50
$MgSO_4•7H_2O$	0.0250
$Na_2EDTA•2H_2O$	0.0108
$FeCl_3•6H_2O$	0.00270
$FeSO_4•7H_2O$	0.00150
NaOH	0.00138
Na_2CO_3	0.00115
$CaCl_2•2H_2O$	$8.00x10^{-4}$
$MnCl_2•4H_2O$	$5.95x10^{-4}$
$ZnSO_4•7H_2O$	$3.60x10^{-4}$
$CuCl_2•2H_2O$	$1.70x10^{-4}$
$(NH_4)_6Mo_7O_{24}•4H_2O$	$1.75x10^{-5}$
Na_2SeO_3	$8.50x10^{-6}$

2.

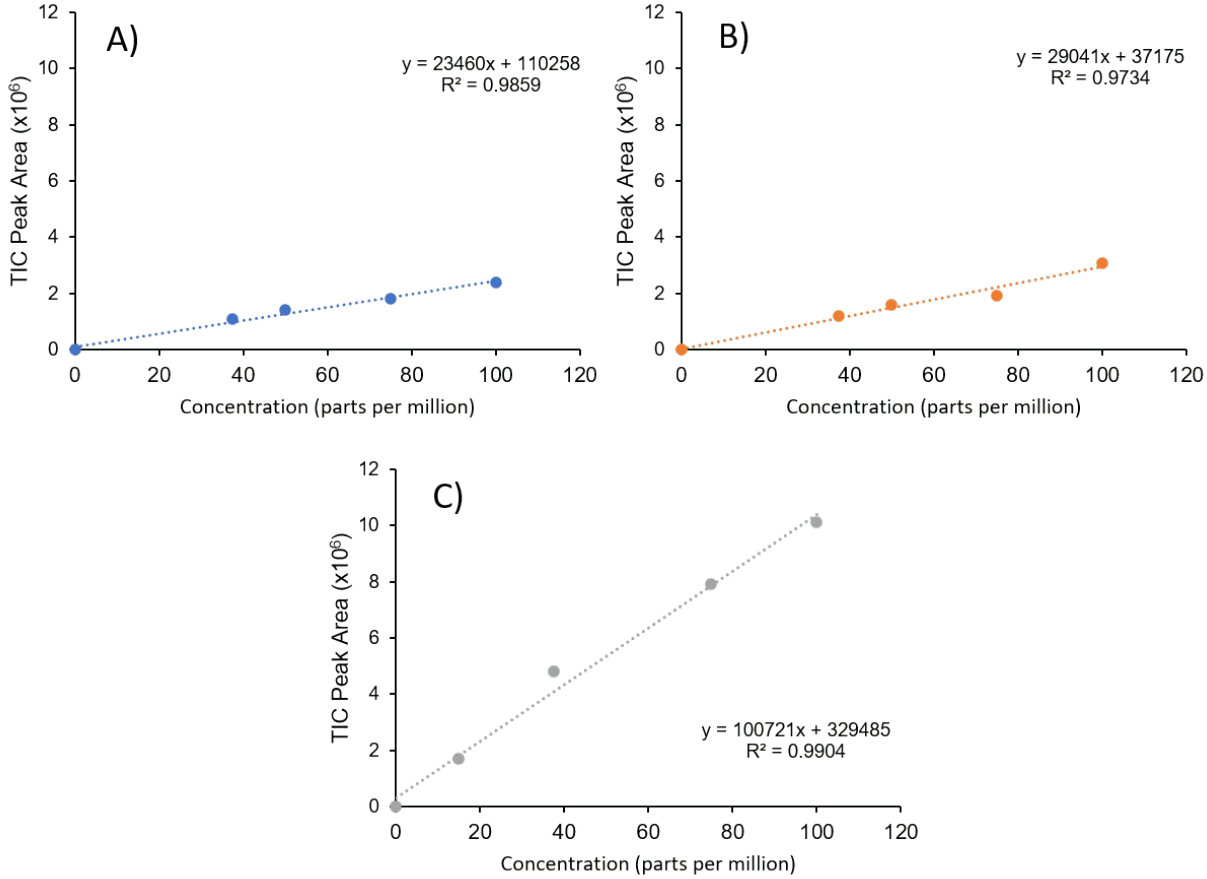


Bacteria enriched in the soil-PU adaptation experiment of this study (Table 1) 72 hours after inoculation on the PU foam (8000x magnification). Arrow indicates bacterial colonies producing biofilm on the surface of the foam.

3. Composition of Phosphate-Buffered Saline media.

Compound	**Components (g/L)**
NaCl	137
KCl	2.7
Na_2HPO_4	10
KH_2PO_4	1.8

4.



Calibration curves of **A)** diol 1, **B)** diol 2, and **C)** diacid 1 used to determine the concentration of products in the *Pseudomonas sp.* cholesterol esterase - degraded PU foam particulates. Standards were prepared by dissolving the diols and diacid in PBS media and performing the EtOAc extraction and GCMS analysis described in the Supplemental Methods. All standards and samples were integrated using the ChemStation software integration tool.

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