UNIVERSITY OF CALIFORNIA SAN DIEGO

Host Strain Selection for Thermoplastic Polyurethane Degradation

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Bioengineering

by

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2023

The Thesis of Ethan Shane Smiggs is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2023

DEDICATION

In recognition of their continuous support during my academic journey, I would like to dedicate this thesis to my grandparents, mother, and sister, who all provided me with the constant love and encouragement needed for me to continue my higher education in graduate school. It is with their support that I am able to complete my graduate program and become the first person in the family to complete graduate school.

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ACKNOWLEDGEMENTS

I would like to acknowledge Dr. Paul Jensen as well as his graduate students Douglas Sweeney and Johanna Gutleben for allowing access to his renowned marine *Bacillus* collection and providing advice on marine microorganism growth.

Also, I would like to acknowledge Dr. Jon Pokorski and Dr. Han Sol Kim, who provided me with the thermoplastic polyurethane powders, and offered advice and recommendations about my experimental results.

I would also like to acknowledge BASF for providing the TPU powders as well. I would like to acknowledge Dr. Bernhard O. Palsson and Dr. Jeff Hasty for serving on my thesis committee as well.

I would like to acknowledge Dr. Adam Feist and Dr. Myung Hyun Noh for supervising me during my thesis and assisting me with my experiments. It was a great opportunity for me to be involved in laboratory research that seeks to solve an immediate need of reducing TPU waste.

Finally, I would like to acknowledge my fellow lab members Richard Szubin, Ying Hutchison, Ciara De Venecia, Thomas Harris, and Kevin Valenzuela. Their support and advice were invaluable in assisting me with my experimental screenings.

ABSTRACT OF THE THESIS

Host-Strain Selection for Thermoplastic Polyurethane Degradation

by

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Master of Science in Bioengineering

University of California San Diego, 2023

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Thermoplastic polyurethanes (TPUs) are a widely-used synthetic polymer that are involved in the production of many items. Due to its widespread applications, the ensuing industrial production of TPUs has led to a worldwide increase of TPU waste. However, there is currently no available commercialized recycling process for TPU, resulting in TPU waste either being incinerated or accumulating in landfills. These main methods of dealing with TPUs pose environmental and health issues, necessitating a novel approach to safely remove

TPU waste. A promising solution is the biodegradation of TPU via microorganisms, however the mechanisms of TPU biodegradation and TPU-degrading microorganisms are not well-known. The focus of this thesis is the screening and characterization of TPU-degrading microorganisms, in particular the *Bacillus* species, as they are known to have TPU-degrading capabilities. Growth in compost, capability of TPU degradation, and genetic tractability were assessed from over 140 *Bacillus* species. In this thesis, it was determined that i). most screening approaches in evaluating characteristics of potential host strain candidates were unable to produce consistent data to verify the strain growth phenotypes in terms of growth rates and final cellular densities, and ii). experiments screening growth on TPUs indicate that strains have preferences in degrading certain TPUs, requiring approaches like adaptive laboratory evolution (ALE) to optimize degradation.

In addition, characterization of screened candidates will be utilized as starting platform strains for further TPU degradation research and optimization of the degradation pathway. Ultimately, these evolved strains will be utilized as an environmentally-friendly way of removing TPU waste.

INTRODUCTION

Since the late 1990s and early 2000s, there has been growing concern over the accumulation of plastic wastes in landfills, specifically thermoplastic polyurethane (TPU), and its potential environmental hazards [1]. Thermoplastic polyurethane is a synthetic polymer utilized in a wide range of industries for a variety of applications, such as electronics, medical devices, footwear, and textiles [2, 3]. Thermoplastic polyurethanes are an increasing environmental and health concern due to its accumulation; it is part of the polyurethane class - the 6th most commonly utilized plastic worldwide - in which 10 million metric tons of polyurethanes are produced yearly [1, 3, 4]. In addition, there is no current commercialized recycling process for this type of material due to its inherently high resistance to abiotic and biotic degradation [1, 3]. As a result of the heterogeneity of TPUs due to the combinations of different polyols and diisocyanate, it is very difficult to develop a commercial recycling process that is able to process all types of TPU waste [1, 4].

Thermoplastic polyurethanes are composed of alternating hard and soft segments, which are formed from three different structures called a polyol, diisocyanate, and a chain extender [5]. The amorphous soft segments, known as polyol, are responsible for the elasticity of the TPU [5, 6]. The crystalline hard segments consist of the diisocyanate and chain extenders, which infers properties relating to hardness and tensile strength to the overall TPU structure [6].

Although its heterogeneity and inherent resistance to degradation serve as major obstacles to recycling TPUs, an alternative method to remove TPU waste is via biodegradation with microorganisms, transforming the waste into nutrients and building blocks for bacterial colony growth [1, 5, 7]. Biodegradation of plastics typically occurs when microorganisms attach and form colonies on the surface of plastics [1, 6]. The colonies excrete enzymes that bind to the

polymers to catalyze cleavage reactions, eventually degrading the plastic into low molecular weight monomers, such as CO_2 and H_2O, which are then taken up by the colonies for further growth [1, 6]. This allows for an environmentally friendly method of removing plastic waste from the environment with minimal human intervention.

Previous research has shown that TPU is degraded by a wide variety of fungi and microorganisms, such as *Bacillus*, albeit at a slow rate [6]. Therefore, adaptive laboratory evolution (ALE) may be used on the potential *Bacillus* strain candidates to enhance their TPU degradation efficiency and increase their survivability in landfill environments. Through the usage of ALE, bacterial phenotypes can be optimized via natural selection for specific utilizations, such as increasing growth rates, tolerances to certain stresses, and most relevant to this thesis, the utilization of new substrates as a carbon source [8].

ALE is a biotechnological tool that enhances and evolves phenotypes of a population of organisms that are cultured under a certain set of parameters [8]. These standardized stresses cause the population to develop mutations to overcome the growth limitations caused by the stresses, thus allowing natural selection to boost the fitness of the population over time [9]. As ALE relies on natural selection to proliferate beneficial mutations and remove deleterious phenotypes from the population, organisms that have short generation times, such as bacteria, and propagate enormously are highly utilized to allow the rapid increase of mutations to occur throughout the population [9, 10]. ALE shows a direct correlation between certain stress and phenotypic changes that occur over time, and the mutated strains with the highest increased fitness will form a larger part of the overall population [8]. A benefit of ALE experiments is that due to its reliance on natural selection to select beneficial mutations for strain optimization, it does not require the knowledge of the genetic changes needed to cause these changes [8]. Since

the scientific community currently lacks the knowledge of specific gene-phenotype correlation due to the complexity of biological systems, evolving these strains to promote beneficial mutations bypasses this issue [8]. Therefore, ALE is extremely useful in optimizing strain capabilities in conjunction or absence of genetic engineering, which may cause undesirable changes that negatively impact the desired phenotype [8].

ALE experiments consist of a population growing under specific conditions or stress for a certain period of time, usually right before they reach stationary phase [9]. Before they reach the stationary phase, a portion of the culture is propagated to a new system under the same conditions with fresh media and is thus repeated [9]. The process of repeated exposure to the same stresses during the exponential growth phase causes the accumulation of beneficial mutations to occur randomly and spread throughout the population to increase overall fitness [9]. During the beginning of the ALE experiments, several phenotypes will appear in the population as a response to the present stresses; these mutations will compete with each other for dominance among the total population, with natural selection causing the best mutations to remain while those that provide less benefits or negatively impact the phenotype, will be removed from the population [10]. As the general population fitness increases, the growth rate of the population will increase with every propagation, indicating that the population is becoming acclimated to the stresses compared to the original population [9, 10].

Bacteria are the preferred type of organism for ALE experiments as they have numerous inherent advantages, including: they usually only require simple nutrient cultures, rapid and easy growth under laboratory settings, particularly the ability to generate hundreds of generations within weeks or months and thus increasing the rate of mutations occurring in the population [10]. One type of bacteria of interest to the biodegradation field is the *Bacillus* species,

specifically *Bacillus subtilis*, for its role in TPU degradation. Previous studies have shown that *Bacillus* species have been isolated from the surface of TPU wastes in soil and have indicated that it utilizes polyurethane lipase activity as a way to degrade the urethane bonds in TPU into carbon and nitrogen sources [6, 11]. However, isolating and identifying the genes encoding for TPU degradation via polyurethanase in *Bacillus* has not been successful due to several possibilities. Some possibilities include that the gene responsible for TPU degradation may be in a plasmid - which may explain why only some samples were able to degrade TPU - or that the gene's corresponding protein may require post-translational edits to it to function properly, making it difficult to characterize the TPU degrading gene [12]. Due to the lack of precise knowledge of the genome on TPU degradation, ALE is the preferred way of characterizing and improving TPU degradation efficiency among the *Bacillus* collection. Therefore, in this thesis, a library of *Bacillus* strains was screened for characteristics that enable for TPU degradation and survivability in the landfill environment, potentially identifying host-strain candidates with best characteristics among the collections. These strains will eventually undergo long-term ALEs to enhance their TPU degradation for future strain integration within TPU products, so they will begin degrading TPU at the end-of-life cycle of the product.

In this thesis, a *Bacillus* collection is screened for a variety of characteristics, most importantly TPU-degradation capabilities, with it serving as a platform for optimizing the TPU degradation pathway via ALE. From the screening experiments, it was shown that i). most screening approaches in evaluating preferred characteristics of potential host strain candidates, most importantly TPU degradation, were unable to produce consistent data to verify the strain growth phenotypes in terms of growth rates and final cellular densities, and ii). experiments screening growth on various TPU subtypes indicate that strains have preferences in degrading

certain TPUs, requiring approaches like ALE to optimize degradation. Ultimately, the best-performing strains from the collection - mentioned as host-strain candidates - will be evolved as a superior, biodegradable way of removing TPU wastes and its harmful effects on the environment.

CHAPTER 1: METHODOLOGY

1.1 – **TPU Preparation:**

In this thesis, both grounded and cryomilled TPU powders known as 785A, BCF35, and BCF45 were utilized as a sole carbon source to measure TPU degradation via cellular growth of the potential host-strains. These TPUs were obtained from BASF for this thesis.

To prepare the various TPU subtypes and powders for eventual usage as the strains' carbon source, 15mL tubes of corresponding TPUs were sterilized via a 70°C water bath overnight and immediately heated in a 47°C conventional oven for another day. Autoclaving TPU is not a possible solution to sterilize it, as they are prone to melt at high temperatures, causing the TPU powders to fuse together, making it difficult to aliquot at specified amounts for experiments. Before being allotted into test tubes for growth experiments, spatulas were sterilized via a Bunsen burner to prevent possible contamination between the stock TPU tubes and the test tubes to keep the stock TPUs sterile for future usage in other experiments.

1.1.1 – *Grounded TPU:*

There are two ways that the TPU pellets were prepared into a powder form: via grounding or cryomilling. Grounded TPU powder is formed when TPU pellets are physically grounded into a powder form. Due to the elastic and tough properties of the TPU pellets, they were crushed via a twin-screw extender at a temperature lower than the TPU's glass transition temperature to make the pellets easily crushed by the twin screw extender while preventing the TPU from melting. Afterwards, the crushed pellets were then grounded into a powdered form via a commercial food blender. Finally, this powder was filtered using a 1-mm microsieve, resulting in TPU powder having less than 1 mm in diameter.

1.1.2 – *Cryomilled TPU:*

Due to the elastic properties and the effective energy dissipation of the TPU pellets, it is very difficult to grind the TPU into powder. An alternative method of making TPU powder from the pellets is utilizing a cryomill. During the grinding process, the cryomill keeps the temperature at -196°C via supplying liquid nitrogen to the system. This extremely low temperature makes the TPU polymer chains immobile, thus making the TPU pellets easier to grind into powder. This causes the cryomilled TPU powder to be formed into larger pieces with more exposed surface areas compared to the grounded TPU powders.

1.2 – *Bacillus* Library Collection:

In this thesis, all experimental *Bacillus* strains were obtained from the marine microorganism library collection of Dr. Paul Jensen at the Scripps Institute of Oceanography (SIO). From this, the chosen experimental strains were limited to samples that were *Bacillus* or *Bacillus*-like. These samples were then stored in cryovials for future screening experiments. The isolation media, depending on the sample, was either 25% A1 media, seawater agar, or marine agar. The samples were stored in the freezing media of A1 media or marine broth at -70°C for future usage. For comparing the *Bacillus* collection's screening data, two control strains were also included to provide a benchmark to determine the effectiveness of each strain under each screening condition. These control strains were BS168 and BS6633. BS168 is a widely studied *Bacillus* strain that is very important in the field of bioprocessing due to its ability to secrete enzymes directly into the extracellular media [13]. The BS168 strain can also be engineered to produce a variety of enzymes and chemicals, and it is regarded as a safe organism to produce natural and engineered products [13]. Previous experiments on the innate degradation capabilities of *Bacillus* strains obtained from the American Type Culture Collection (ATCC)

showed that BS6633 had the most promising growth on TPUs; therefore, this strain was utilized as a control strain for comparison in the screening criterias.

1.3 – Initial Host Strain Screenings:

To determine the effectiveness of the preliminary screening criteria and analyze the possibility of finding alternative host-strain candidates that perform better than the control strains, initial screenings were conducted on 12 random *Bacillus* species from the SIO *Bacillus* collection, along with the control strains BS168 and BS6633. These initial screenings on the 14 strains were performed to analyze the capabilities of each strain for TPU degradation, non-salt growth, sporulation and heat-shock tolerance, and genetic tractability, as depicted in Figure 1. All strains were grown on modified M9 media with glucose (200g/L or 80mL for 4 L).

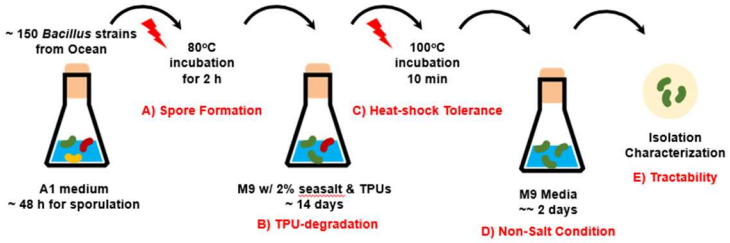

Figure 1: Initial Host-Strain Selection Screening Plan

1.3.1 – *TPU Degradation Capabilities:*

To prepare for growth on TPU, the 14 strains were initially inoculated from their cryostocks into rich A1 media containing 25% starch and 2.2% sea salt overnight at 37°C in a shaker to obtain a high initial cellular concentration. The next day, a stock solution containing 300mL of M9 media with 2.2% of sea salt and 6mL of glucose was made; this intermediate step between the initial strain inoculation and the strain exposure to TPU was utilized to gradually transition the strains from a rich media to a minimal media to avoid shock. About 150μL of the cell cultures and 15mL of the M9 solution were allocated into their respective tubes, and were left overnight in a heat block at 37°C and 1100rpm. Afterwards, the ODs of each strain from the

M9 media precultures were measured via a spectrometer. Then they were centrifuged, the supernatant discarded, and the pellets resuspended in the remaining supernatant.

The TPU degradation experiment consisted of two replicates for each of the 14 strains, resulting in 28 test tubes involved in this screening. Each test tube contained 40mg of grounded 785A TPU and 9mL of M9 media without glucose, as the 785A would serve as the sole carbon source. About 4mL of the resuspended pellets from the preculture were allocated in their corresponding test tubes, and left in the 30°C enclosed shaker overnight for 2-3 weeks. The ODs of the remaining resuspended pellets were measured every other day to determine the initial ODs of the TPU cultures. The OD measurements were taken every other day.

1.3.2 – *Non-Salt Condition Growth:*

Although the experimental strains were obtained from sediment in seawater, and thus assumed to have adapted to growth in a rich salt environment, it is practical for these strains to demonstrate survivability in the absence of salt, as landfill environments vary by location, and it is impractical to assume that most landfills will have a high salt concentration. Therefore, the 12 experimental strains and 2 control strains were grown in test tubes containing M9 media and 4g/L of glucose without sea salt, over the course of two days at 37°C, with OD measurements taken.

1.3.3 – *Sporulation and Heat-Shock Tolerance:*

The sporulation and heat-shock tolerance screenings are a necessary characteristic of potential host strains; eventually, these strains will be integrated within TPU products so they will start degrading the product as it is no longer usable. As the creation of TPU products involves high temperatures, potential host strain candidates must be able to form spores and survive the melting and extruding process of developing TPU products.

To determine the growth of strains undergoing the sporulation and heat-shock tolerance tests, 168 LB petri dishes with 1.1% of sea salt were prepared, as there are 14 *Bacillus* strains, with 4 different conditions, and 3 replicates per strain per condition.

In the first part of the sporulation purification process, the 14 strains were inoculated in M9 media in PCR tubes for 48 hours to encourage the formation of spores. Afterwards, the ODs of each strain were measured, and the tubes were centrifuged and the supernatant discarded. Then 1mL of lysozyme and PBS buffer (to maintain constant pH) were added to each strain to destroy the bacterial cell membranes, leaving intact spores. For the strain that had high ODs, and thus requiring more PCR tubes having the same OD value, the 1mL of solution was added to the first PCR tube, and then fully aliquoted to the next PCR tube to obtain as many spores as possible. All PCR tubes were incubated at 37°C for 1 hour.

Afterwards, the spore cultures were centrifuged to remove the lysozyme and PBS buffer. The pellets were resuspended in 1mL of MilliQ water and pipetted into glass bottles. The bottles were then placed in a 70°C water bath for 1 hour, and subsequently placed in a room-temperature water bath for an additional 20 minutes, and finally removed from water to obtain the heat-untreated samples.

The heat-shock tolerance had 4 conditions to determine the spores' tolerances to heat-shock. The two heat-untreated conditions were diluted 10,000X and 100,000X samples, and the two heat-treated conditions were diluted 1X and 10X samples. To create the sample dilutions, autoclaved DDW water was utilized.

- For 10X: 900μL of DDW and 100μL of sample
- For 10,000X: 990μL of DDW and 10μL of 10X
- For 100,000X: 900μL of DDW and 100μL of 10,000X

To start the process of heat-treating the samples after the room-temperature water-bath, the glass bottles containing the heat-shock condition samples were placed in a beaker of boiling water for 3 minutes. Then 100μL of the corresponding dilution conditions were added into each petri dish and labeled accordingly. The samples were streaked via beads and left in the shaker overnight for two days. The 168 petri dishes were removed from the incubator and the BioRad ChemiDoc XRS+ System was used to take images of the petri dishes and their colonies. The software programs reaConverter Standard and OpenCFU were used to count the colonies on each plate to calculate germination efficiency.

To determine if the strains have the capabilities to grow on the sea salt LB plates in the absence of heat-shock treatment, the strains were inoculated from the cryogenic stock into 3mL A1 media and 1mL of glucose overnight in a 37°C shaker. Then, the precultures were streaked on the plates after the initial incubation and left in the incubator overnight. Initially, most strains were able to grow on the sea salt LB plates, but strains No. 7- 11 did not show any visual growth, so they were restreaked from the A1 media to verify if these strains could grow on the sea salt plates.

1.3.4 – *Genetic Tractability:*

Potential host strain candidates should be able to pass the genetic tractability screening to showcase the ability to edit the genome via genetic engineering; this will allow the genome to be edited to provide the strains with more characteristics that it normally would not have.

A stock solution of 49mL of A1 media and 1mL of glucose was made, and 3mL of this solution was pipetted into 14 different 15mL tubes. The media was then inoculated with the spores from the control sea salt LB plates from the heat-shock tolerance screening, and left in the shaker overnight. Afterwards, 500μL of the A1 precultures were inoculated into 14 test tubes

containing 5mL of M9 media and glucose, and left in the shaker overnight to grow on the new media at 37°C. As only strains No. 1-6 and No. 12-13 showed growth on the minimal media and glucose, the genetic tractability experiment proceeded with only 8 strains out of the 14 strains. As the desired OD for the initial cultures was 0.1, the desired volume of the precultures was calculated to obtain this OD value in 5mL of M9 media without glucose. The OD was manually measured for each strain at the 0-, 2-, 4-, 6-, 8-, 12-, and 24- hour marks. After the second hour mark, 6mL of starvation media was added into each culture in accordance with the heat-shock treatment [14].

Ampicillin LB plates were made with 400μL of ampicillin and 400mL of agar. After 8 hours, 500μL of the heat-shocked treated samples and 100μL of the ampicillin plasmid were aliquoted into new 15mL test tubes, and left in the shaker for 1 hour at 37°C for the competent cells to uptake the plasmids [15, 16, 17]. Then 500μL of LB media was added and left in the shaker for an additional hour. Afterwards, the cultures were centrifuged, and most of the supernatant was removed. After resuspending the cell pellets in about 100μL of the remaining supernatant, the cultures were streaked on 8 ampicillin plates and left in the shaker overnight for 2 days to see if they develop colonies, and if they are able to uptake the ampicillin plasmid.

1.4 – **Entire *Bacillus* Library Screenings:**

1.4.1 – *Compost Media Condition:*

The results from the initial screenings assisted in further refining and developing a new set of screening criteria that is better suited to discovering and analyzing the most important characteristics of potential host-strain candidates, as it is expected that these strains will ultimately be utilized in landfills to degrade TPU waste. The modified screening plan is visually

shown in Figure 2, in which the two most important criteria are compost media growth and TPU degradation.

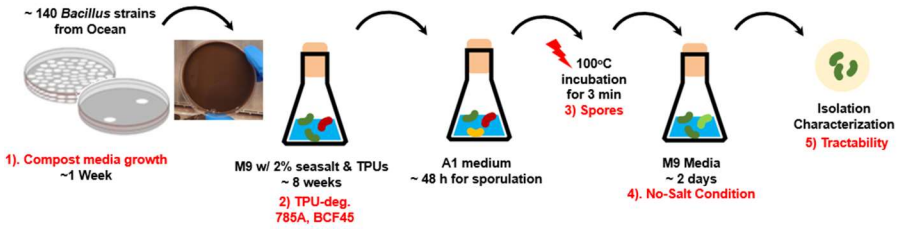

Figure 2: Modified Screening Criteria for the Entire *Bacillus* Library

As part of the newly modified screening criteria, it was highly important to determine if the library strains were able to grow in a landfill environment. Soil was obtained from a nearby landfill to create a modified compost-LB plate assay to screen strains for availability in a landfill environment.

The compost LB plates were made from a solution consisting of 800 mL/L of MilliQ water, 120g/L of the compost soil, and 12g/L of agar. As the compost soil was directly from the landfill, it had a lot of trash and microorganisms growing within it, requiring the soil to be sterilized before usage. To prepare the soil for its inclusion in the assay plates, it was filtered to remove large pieces of carbon sources and miscellaneous residue. Afterwards, it was summarily grinding into smaller pieces for it to be easily dissolved in the LB solution. The compost soil was mixed with water and autoclaved to remove the presence of other living microorganisms that may grow on the LB plates otherwise. After sterilization, agar was added to the sterilized solution, and 15mL of the mixture was distributed into petri dishes.

In preparation for the compost screening, the entire *Bacillus* library was inoculated in A1 media from their cryostocks overnight. Afterwards, 130 bacterial strains - 128 *Bacillus* strains from the SIO collection and the 2 control strains BS168 and BS6633 - were streaked on the

compost LB plates and left in the 37°C shaker to grow over a course of a few days. Each petri dish was divided into 8 sections, with each section containing a different strain. After two days of incubation at 37°C, 75 strains showed growth on the compost plates, while 54 strains did not indicate any visible growth. An additional incubation day resulted in an additional 4 strains forming colonies on the compost media. The original 54 strains that did not grow were inoculated in A1 media from the cryostock and incubated for an additional two days at 37°C. These strains were re-streaked on new compost plates, resulting in 94 out of the 130 strains growing on the soil compost. This was done to ensure if these strains had the capabilities to grow on the compost plates.

1.4.2 – *TPU Degradation Group Assessment:*

Due to the large number of strains that passed the compost media screening, it was impractical to individually grow every strain on 785A TPU to test their degradation capabilities. Therefore, the experimental strains were divided into 18 groups containing 5 different experimental strains, with Group 19 containing 2 strains. The control strains BS168 and BS6633 grew in their own separate test tubes. As the desired starting OD was 0.2, each individual strain had an OD of 0.04 in each group. To test for TPU degradation, the initial screening consisted of growing each group in 10mL of M9 media with 10g/L of grounded 785A as the sole carbon source. These test groups were incubated in a heat block at 37°C and 550 rpm for 14 days with OD600 measurements taken every other day, including Day 0 and Day 1. This experiment was done again under the same conditions to verify the results.

The second round of TPU degradation focused on the groups that showed higher growth rates compared to the rest of the groups, specifically Groups 1, 7, 10, and 11 along with isolates from Group 7 and 11, to test for consistency and reproducibility. In this round of screenings, the

same conditions were applied, however the carbon sources were grounded 785A, BCF35, and BCF45, and the group strains were propagated from A1 media precultures overnight. There was an additional 785A screening in which the strain groups were propagated from the initial group screening; as the initial screening did not have isolated from Groups 7 or 11, and included the two compost strains, the same conditions were applied. All screenings were conducted over 14 days with OD600 measurements taken every other day; however, the screenings for 785A and BCF45, which were propagated from A1 media culture, lasted for 11 days.

1.4.3 – *Featured Strains Growth Profiling:*

After the TPU-group degradation screening was concluded, the individual strains from Group 7 and 11, their respective isolates, the two compost isolates, and BS6633 were tested for their growth on all three different grounded TPUs - 785A, BCF35, and BCF45. The strains were grown on 10mL of M9 media and 10g/L of grounded 785A at 37°C and 550 rpm for 8 days. There were three replicates per strain per TPU type. From the individual strains' OD measurements, three strains - #C42, #I7, and #63 - were selected for further analysis due to their high growth on the TPUs in comparison to the rest of the strains.

These three strains underwent a short-term 2-passage ALE to verify their growth profiles on the three different grounded TPU subtypes. In this experiment, all strains were grown in 10mL of A1 media overnight to obtain an initial high growth rate, and were passed into tubes containing 10mL of M9 media with 10g/L of grounded 785A, BCF35, and BCF45; these tubes had an initial OD of 0.06-0.09, with the strains being grown at 37°C and 550 rpm. On the seventh day, a portion of the cultures with an OD of 0.06-0.09 were passed into new test tubes containing fresh media and TPUs. It was decided that strains #I7 and #63 would undergo more growth profiling to see the overall growth rate. These two featured strains were grown on 10mL

of M9 media and 10g/L of either grounded 785A, BCF35, or BCF45 at 37°C and 550 for 8 days, with two replicates per strain and TPU type.

1.4.4 – *Bradford Assay to OD600 Conversion:*

To measure the growth values of strains undergoing the screenings, a BioMate 3S UV-Visible Spectrophotometer was used to measure the optical density (OD) of strains at a wavelength of 600nm (OD600), as previous research has shown that OD600 directly correlates to cell biomass and these OD values are reliable and reproducible [18, 19, 20]. Optical density was the primary measurement utilized as the more growth a strain is undergoing, the more light will scatter, thus increasing the optical density measurement [18, 21]. For these measurements, 900μL of MilliQ water and 100μL of the strain culture are allocated into a cuvette and placed in the spectrometer to measure the optical density.

However, a negative characteristic of optical density measurements is that these measured values can be affected by the presence of other material in the culture, such as produced byproducts of the bacterial cells, and the internal sub-wavelength parts of the bacteria, which may cause additional light scattering, inflating the actual OD measurements [18]. Likewise, the size of the bacterial cells also influences OD measurements; due to their inherent size, bacteria smaller than 600 nm will be poor scatters of light, reducing the OD measurements, while the clumping of cells, due to their non-uniform large size, will result in an incorrectly large OD value [18].

As an alternative measurement for strain growth that is not affected by the presence of particles in a culture, Bradford assays were performed to provide confidence in the spectrometer data. Bradford assays are utilized to measure the total protein concentration of a given sample by having a dye bind to proteins, causing a shift of the dye's maximum absorption towards 595 nm

[22, 23]. By measuring the absorption of the dye at 595 nm, it is possible to determine the total protein concentration by measuring the amount of dye binding to the proteins [22, 23].

To compare measurements between the Bradford assays and spectrometer data, a standard curve was created between the two measurements to provide a mathematical equation that relates the two values together, as shown in Figure 3. In microtubes, 500µL of the strain culture is aliquoted and a small number of microbeads are allocated inside. An Omni Bead Ruptor 12 bead mill homogenizer is then utilized to quickly shake the microtubes to lysate the cells via physical destruction of the membranes by the beads. Afterwards, in a 96 well plate, 30µL of the lysated culture and 170µL of the Bradford reagent are placed into the well plate; for each strain/culture, there are two replicates performed in the well plate. Afterwards, the Tecan plate reader shakes the thing for 10 seconds and measures the optical density of the solution in every well. Afterwards, the Bradford measurements are converted to OD600 values via the equation derived from the Bradford standard curve for comparison.

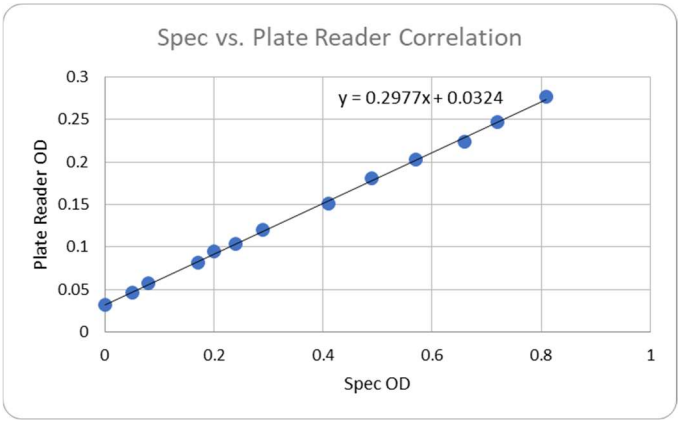

Figure 3: Correlation Between Spectrometer OD and Plate Reader OD for Bradford Assay

1.4.5 – *Effects of Processing Methods of TPU Powders:*

To compare the effectiveness of cellular growth on grounded and cryomilled TPU, BS6633 and three strains were grown on M9 media containing either 10g/L of cryomilled 785A,

BCF35, or BCF45, with 2 replicates per TPU type and strain. These strains were then cultured over 7 days at 550 rpm at 37°C on a heat block, and OD was measured daily via a spectrometer and on Day 7 via a Bradford assay. These measurements were then compared to previous grounded TPU results to compare growth rates.

1.4.6 – *Utilization of Helper Substrates:*

To test the effects of an initial limited helper substrate on the overall growth ODs of potential host strains, a strain candidate - C42 - was inoculated into 10mL of A1 media with glucose overnight at 37°C on a heat block. For this experiment, there are 20 tubes, with 2 replicates per condition. The first 10 tubes contained 10mL of M9 media along with 0%, 0.5%, 1%, 1.5%, and 2% of yeast extract; meanwhile the other 10 tubes have the same conditions except with the inclusion of 10g/L of the grounded 785A TPU. The starting OD for the cultures is 0.05 and the OD was measured via spectrometer every day for 11 days.

CHAPTER 2: RESULTS

2.1 – Initial Host Strain Screenings:

2.1.1 – *Preliminary Screenings Indicate Potential Host-Strain Candidates:*

A preliminary screening was conducted on 12 randomized strains from the SIO *Bacillus* library to test and modify the initial screening criteria, along with determining if any of the strains would exhibit better characteristics than the control strains. The presence of these initial potential host-strain candidates would indicate that it would be a worthy endeavor to screen the entire *Bacillus* library as there is a good chance of finding even more strain candidates that may exceed the capabilities of the current ones.

As exhibited in Table 1, all candidates seemed to be able to sporulate, along with the ability to germinate after 3 minutes of heat-shock treatment at 100°C. Sporulation is a necessary ability for survivability in landfills, as gradual and immediate temperature changes happen throughout the landfill cycle. By forming spores, the bacteria will be able to survive normally-intolerable conditions and only germinate after specific conditions are met, such as finding TPU waste as a carbon-source to degrade.

The most important screening criteria is the ability of strains to degrade TPU, with host-strain candidates preferably being able to degrade TPU at the same or higher rate compared to the two host strains. After growing for about 20 days, many of the experimental strains - with the exception of 7 strains and the 2 controls - were unable to adequately grow on the grounded 785A TPU as a sole carbon source, shown in Table 1. Only four experimental strains were able to reach a higher change in optical density compared to the control strains, but one strain - the CUA 1749 - was not considered for future screenings as it visually appeared as fungi in the test tubes during the overall growth process, forming extremely large colonies within the media unlike the

rest of the strains. As depicted in Figure 4, the 4 strains with the highest ODs were compared to the control strains, with one strain (CUA 1790) being able to grow higher than one of the control strains, while the other two strains (CUA 810 and CUA 1792) exhibited higher OD values than both control strains. From the initial screenings of a sample size of 14 strains, 3 different strains were identified as exhibiting higher ODs than the controls, indicating that there is a high likelihood that the *Bacillus* collection may contain other potential host-strain candidates that may exceed the controls. Therefore, a total screening of the *Bacillus* library collection was performed.

Table 1: Preliminary TPU Degradation Screening Results for ~20 Days of Culturing

No.	Strain	TPU growth	Max ΔOD_{600} (- 20 days)	Note
1	BS168	O	**0.15 ± 0.05**	Control strain
2	BS6633	O	**0.13 ± 0.05**	Control strain
3	CNT 977	X	0.09 ± 0.03	
4	CNZ 1317	X	0.10 ± 0.00	
5	CNZ 082	X	0.06 ± 0.01	
6	CNZ 058	X	0.08 ± 0.01	
7	CUA 881	Δ	0.08 ± 0.02	
8	CUA 8171	Δ	0.12 ± 0.02	
9	CUA 810	O	**0.25 ± 0.01**	
10	CUA 1796	Δ	0.07 ± 0.01	
11	CUA 1790	O	**0.18 ± 0.05**	
12	CUA 1782	O	0.10 ± 0.04	
13	CUA 1749	O	0.23 ± 0.02	Seems like fungi
14	CUA 1792	O	**0.32 ± 0.11**	

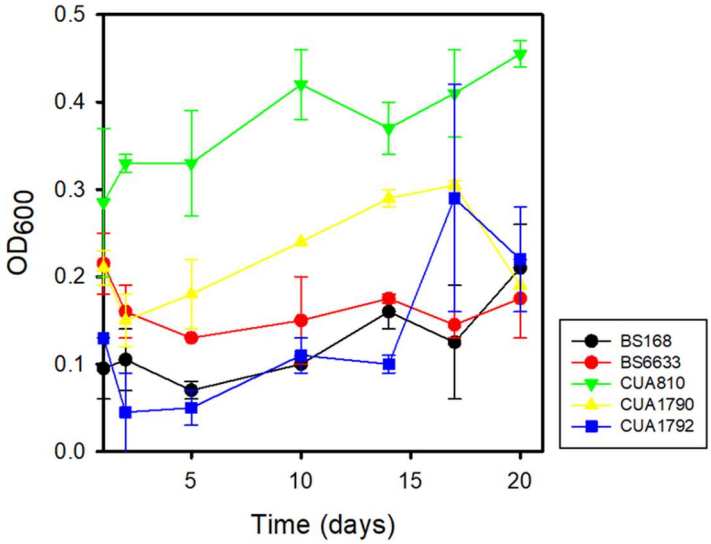

Figure 4: OD600 Measurements of the Control and Most-Promising Experimental Strains on TPU

2.1.2 – *Criteria Changes and the Usage of ALE is Mandatory for Future Strain Candidates:*

Table 2: Non-Salt Condition Growth Rates for the Initial 14 Strains

No.	Strain	Specific growth rate (h^{-1})	Note
1	BS168	**0.40 ± 0.04**	Control strain
2	BS6633	**0.31 ± 0.04**	Control strain
3	CNT 977	0.08 ± 0.09	
4	CNZ 1317	< 0.02	
5	CNZ 082	0.09 ± 0.00	
6	CNZ 058	0.49 ± 0.01	
7	CUA 881	< 0.02	
8	CUA 8171	< 0.02	
9	CUA 810	X	No growth
10	CUA 1796	< 0.02	
11	CUA 1790	< 0.02	
12	CUA 1782	< 0.02	
13	CUA 1749	0.09 ± 0.01	Seems like fungi
14	CUA 1792	0.03 ± 0.00	

Although there were some promising host-strain candidates from the initial 12 experimental strains due to their TPU degradation capabilities and heat-shock tolerance, the lackluster results in the other screenings indicate the need to alter the screening criteria and

heavily implies that ALE is mandatory for host-strain candidates to succeed in parts of the screening experiments.

In the non-salt condition experiment, most strain candidates showed minimal growth at a very slow rate, meaning that ALE is necessary to increase the strains' growth capabilities so that they may be able to grow at a rate similar to the two control strains (Table 2). The experimental strains, due to being marine microorganisms, may have slow growth rates due to their reliance on sea salt in their natural environments. Of the 12 experimental strains, 1 strain - the CNZ 058 - was the only experimental strain that was able to grow well enough to be compared and eventually succeed the growth rates of the two control strains. Most importantly, strains CUA 810, CUA 1782, and CUA 1792 which were able to degrade TPU comparatively to the control strains, were unable to have comparable growth in the non-salt condition in relation to the control strains. This necessitates the utilization of ALE to enhance the growth rate of these strains on the non-salt condition. As ALE is mandatory to enhance the growth capabilities on this screening, this screening criteria is moved to being near the bottom of the screening criteria list due to the ability of optimizing future host-strain candidates with different abilities in future ALEs.

Likewise, the genetic tractability experiment also needs some changes to increase the transformation efficiency among the experimental strains. Previously, the transformation efficient with natural competency was validated in the two control strains, and were assessed in 8 strains: the 2 control strains (Strains No. 1 and 2) and 6 experimental strains (Strains No. 3-6, 12-13), as they were the only inoculated strains to grow in 5mL of minimal media and glucose overnight in the shaker. However, the genetic tractability experiment with ampicillin plasmid only indicates that the control strains developed transformed colonies, indicating that the genetic

engineering method must be optimized for future experiments with the rest of the *Bacillus* library.

2.1.3 – *Overall Screening Results:*

Table 3: Overall Results of the Initial Host Strain Screenings

No.	Strain	TPU growth	Non-salt growth	Sporulation & germination	Genetic tractability
1	BS168	O	**0.40 ± 0.04**	O	O
2	BS6633	O	**0.31 ± 0.04**	O	O
3	CNT 977	X	0.08 ± 0.09	O	X
4	CNZ 1317	X	< 0.02		
5	CNZ 082	X	0.09 ± 0.00		
6	CNZ 058	X	0.49 ± 0.01		
7	CUA 881	Δ	< 0.02		
8	CUA 8171	Δ	< 0.02		
9	CUA 810	O	X		
10	CUA 1796	Δ	< 0.02		
11	CUA 1790	O	< 0.02		
12	CUA 1782	O	< 0.02		
13	CUA 1749	O	0.09 ± 0.01		
14	CUA 1792	O	0.03 ± 0.00		

Overall, four different screening experiments were conducted to determine if the 12 experiment *Bacillus* strains - along with the rest of the SIO *Bacillus* strain library - may be viable as host-strain candidates for TPU degradation: TPU growth, non-salt growth, sporulation and germination, and genetic tractability. As summarized in Table 3, most candidates were able to grow, albeit slowly, on the non-salt condition, but were able to successfully sporulate and germinate after heat-shock experiments. The genetic tractability experiment would need to be optimized and enhanced to increase the chances of the plasmid to be taken up by the strains. Out of the 12 experimental strains, 3 candidates were able to grow and degrade TPU as a sole carbon source, showing promise that the rest of the *Bacillus* library may offer strains with even higher TPU degradation capabilities. To enhance the TPU degradation abilities along with the other

desirable characteristics of the host-strain, it would be mandatory to utilize ALE to optimize the eventual host-strain.

2.2 – **Entire Bacillus Library Screenings:**

Although some strains were identified as having characteristics exceeding those of the control strains, there was a need to modify the current screening criteria to better fit the identification of the host-strain candidates, mainly choosing screening criteria that accurately reflects its ultimate usage in landfills to degrade TPUs. From the previous initial screenings, it was decided that the screening criteria will be changed to emphasize the most important characteristics of a host-strain in the following order: compost media condition, TPU degradation, sporulation and heat-shock and germination, no-salt condition, and finally genetic tractability. The compost media screening was decided to be the first screening as it was important for the host-strain candidates to be viable in a landfill environment; if strains were unable to grow on in an environment reminiscent of a landfill, then they would be unfit for benign host-strain candidates to optimize for future degradation of TPU wastes in landfills. The second screening involved TPU degradation across a variety of TPU compositions and testing for consistency; as TPU wastes can be composed from a variety of different chemical building blocks, it is important to screen for growth on different subtypes to identify strains that are able to degrade TPU at high rates, and also those strains that are able to degrade a variety of different TPU types.

The heat-shock tolerance, germination, non-salt condition, and genetic tractability testings are expected to be performed after the in-depth TPU tests as although these characteristics are important for host-strain candidates to perform well in degrading TPU in

landfills, they are less mandatory capabilities compared to TPU degradation, and as such can be further improved via ALE processes.

The new screening criteria was conducted for the additional ~128 spore-forming marine *Bacillus* collections from SIO, along with the previous 12 randomly-selected experimental strains in the preliminary screenings.

2.2.1 – *TPU Degradation Group Assessments Showed Higher Rates Compared to Controls:*

Of the 140 experimental *Bacillus* strains, only 94 strains were able to form colonies on the compost-LB plate. As there were too many strains to screen for TPU degradation, the strains were split into groups, with each group containing about 5 strains. These strains were grown in a test tube containing 10mL of M9 media and 10g/L of TPU. The first group screening utilized grounded 785A as the test TPU, and the cultures were grown for 14 days. Out of the 19 test groups, 4 groups and the two compost isolates showed remarkable growth compared to the BS6633 control group, as shown in Figure 5. To validate and determine consistency from the first round of group screenings, a second round of screenings with the same conditions was performed, except the second screenings include separate experiments utilizing grounded 785A, BCF35, and BCF45 to see if any strains showed preference to certain types of TPUs and determine if they can reliably degrade all three types of TPUs.

In the first group screening, Groups 7, 10, and 11 showed exceptionally higher measurements in comparison to the BS6633 control group, while the two compost isolate strains grew at about the same rate as the control, as shown in Figure 5. To validate and show consistency and reproducibility between the group strain cultures, a second set of TPU group assessments was conducted with BS6633, and Groups 1, 7, 10, and 11. This second group assessment consisted of four different screenings, with 3 screenings being utilized to test the

growth rates of the groups on 785A, BCF35, and BCF45 when the strains were propagated from initial preculture in A1 media. The fourth screening consisted of the groups in the first screening being propagated from the initial 785A screening. In Figure 5, Group 11 showed extremely high OD measurements, and thus growth rate, with Groups 7 and 10 also experiencing high measurements as well. The compost isolates seemed to follow the trajectory of the BS6633, which was the slowest and lowest growth measurements before the first screening. However, in the second screening in which the groups were propagated from the first screening culture, all strains experienced a significant decrease in achievable OD measurements, with the highest strain measurements being the two compost isolates and Group 7 (Figure 6). However, even the highest ODs from the second screening failed to even reach the measurements of Group 1 in the first screening, which was the slowest experimental group strain.

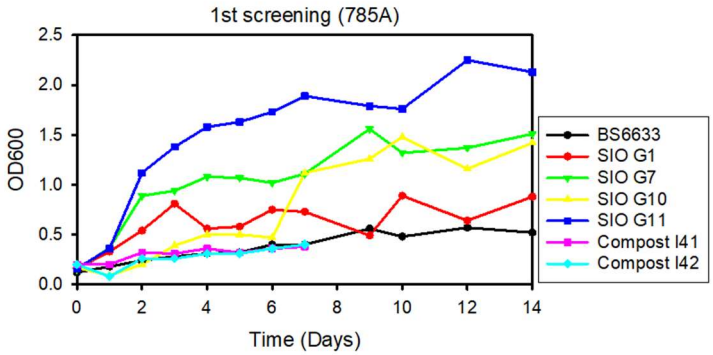

Figure 5: Plot of the Most Promising Strain Groups in the First TPU Degradation Assessment

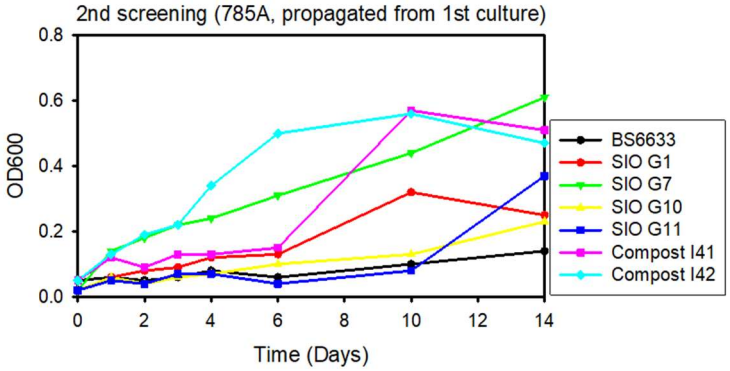

Figure 6: Plot of the Most Promising Strain Groups for the Second TPU Assessment; these strain groups were inoculated from the cultures in the first TPU assessment.

In comparison to the two previous 785A screenings, the screening of 785A when the strains were propagated from A1 media seemed to show measurements that are in-between the range of the two screenings. Surprisingly, the BS6633 control was growing almost exponentially during the screening, with it showing higher measurements compared to Group 11, an isolate from Group 7, Group 1, and Group 10 (Figure 7). The strains growing higher than BS6633 were the isolates from Group 11 and Group 7. However, even though in all three 785 screenings, Group 7 showed high OD values, the OD measurements between groups degrading the same 785A TPU were inconsistent and lacked reproducibility to verify the strain growth phenotypes.

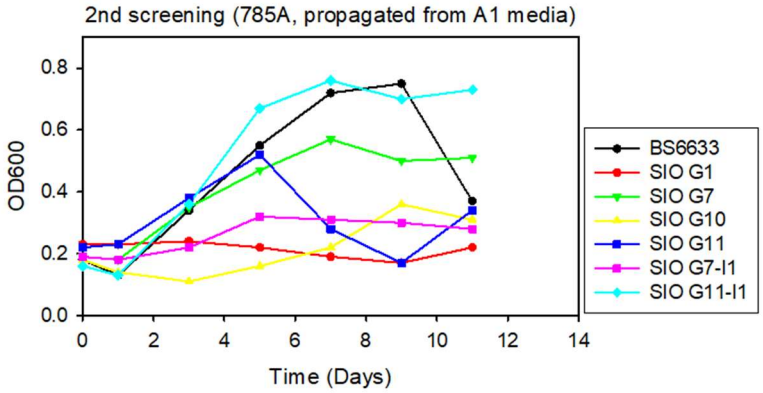

Figure 7: Plot of the Strain Groups in the Second TPU Assessment; these strain groups were separately inoculated in A1 media before being propagated into the M9 w/ 785A media

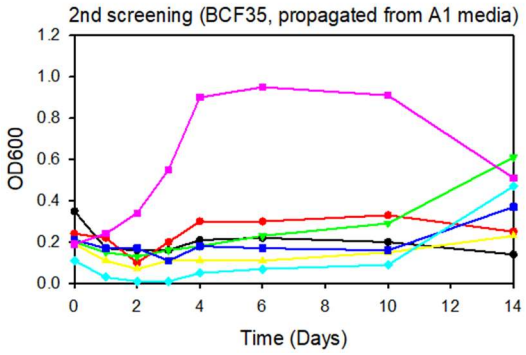

Figure 8: Plot of the Strain Groups in the Second TPU Assessment; these strain groups were separately inoculated in A1 media before being propagated into the M9 w/ BCF35 media

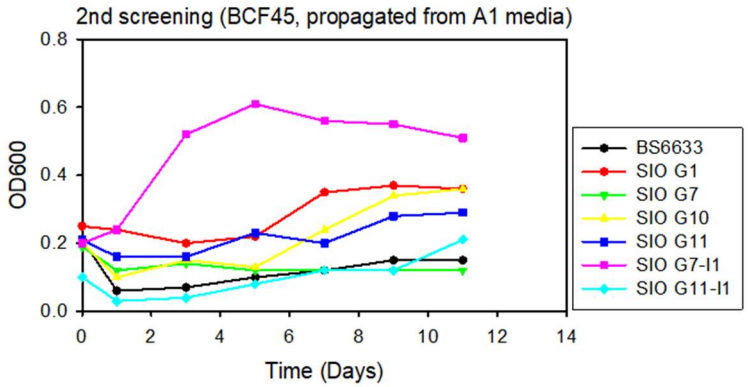

Figure 9: Plot of the Strain Groups in the Second TPU Assessment; these strain groups were separately inoculated in A1 media before being propagated into the M9 w/ BCF45 media

In the BCF35 screening, almost all the strains had difficulty growing on the TPU throughout the experiment except for the Group 7 isolate, which only showed a marked decrease in growth at the end of the experiment (Figure 8). The rest of the strains slowly grew, but seemed to start growing well after the Day 10 mark. At the end of this screening, strain groups 7 and 11, along with their respective isolates, showed higher growth. However, they showed less growth on average compared to being grown on the 785A TPU.

Likewise in the BCF35 screening, the same pattern of growth appeared in the BCF45 screening, in which the isolate from Group 7 experienced higher growth compared to the other strain groups (Figure 9). At the end of the experiment, there was a difference between the growth of the isolate from Group 7 and the rest of the strains.

Table 4: Results of the TPU Degradation Assessments of the Most Promising Strain Groups

Strains / groups	1st screening (785A, A1)	2nd screening (785A, pass)	2nd screening (785A, A1)	2nd (BCF35)	2nd (BCF45)	**15 candidates**
Group 1	++	+		-	+++	
Group 7	+++	+++	+++	++	-	**5 strains**
Group 7 – I1			+	++++	++++	**1 strain**
Group 10	+++	+	+	-	+++	
Group 11	++++	++	++	+	++	**5 strains**
Group 11 – I1			++++	+	++	**1 strain**
Compost isolate 41	+	+++				**1 strain**
Compost isolate 42	+	+++				**1 strain**
BS6633 (control)	+	+	++++	-	+	**1 strain**

From the strain grouping screenings, it seems there are a total of 15 strains from both the compost and SIO library that may be able to continue on for the next screenings.

2.2.2 – *Variation Among Featured Strain Replicates Require Alternative Growth Measurements:*

As the previous TPU assessments were conducted within groups, it was necessary to independently grow the strains from the most-promising groups to isolate and identify the specific strains that were responsible for the TPU degradation in the TPU group assessment. Therefore, the strains in Groups 7 and 11, along with their respective isolates, the two compost isolates, and BS6633 were grown individually in 10mL of M9 media with 10g/L of the TPU subtypes for 8 days. The measured OD values are shown in Figure 10. From the plot, it seems that the strains showing the highest OD values - and thus having the highest TPU degradation rate - were strains #63, #I7, and #C42. However, the replicates showed large derivations, leading to large error bars, necessitating an alternative measurement of strain growth. To provide more confidence in the OD measurements, Bradford assays were performed to measure total protein concentration as an indirect way to measure cellular growth, with the results depicted in Figure 11. Compared to the spectrometer, the Bradford assay measured lower cell concentrations. In the

growth curves, the strains growing in BCF35 showed little cell biomass compared to BCF45; in fact, BCF45 showed similar cell biomass with high accuracy.

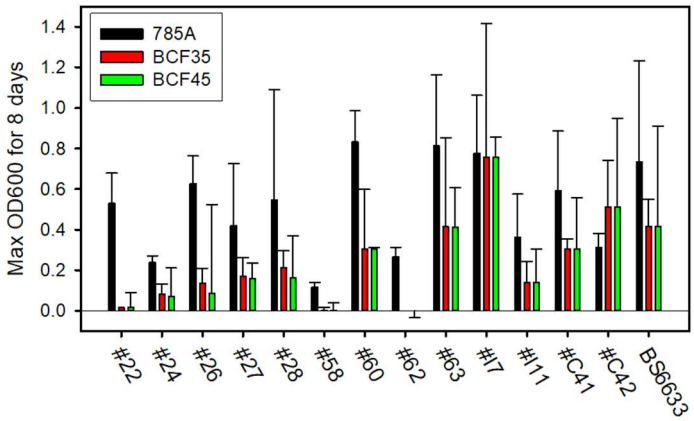

Figure 10: Spectrometer OD values for Strains in the Most Promising Groups for TPU Degradation

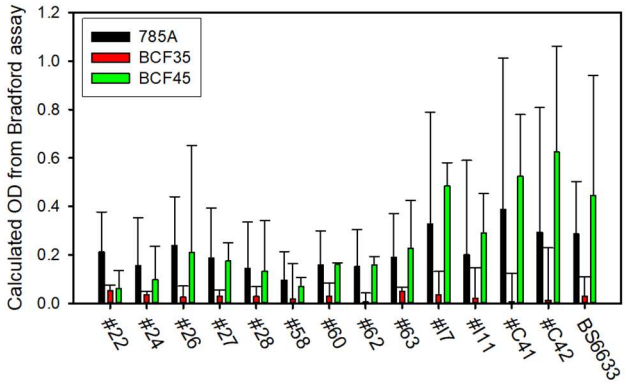

Figure 11: Calculated OD values via Bradford Assay for Strains in the Most Promising Groups for TPU Degradation

As the #C42 strain is one of the two isolates obtained from the compost soil pre-sterilization in the compost media screening, the individual growth rates of strains BS6633, #63, and #I7 on the grounded TPU subtypes were plotted, as these strains were originally from the *Bacillus* library collection, to test for growth consistency from the previous OD and Bradford measurements. In all experiments, there were large standard deviations among the replicates,

leading to error bars overlapping among the strain measurements, making it difficult to accurately and confidently determine results about the growth profiles. This indicates that it is necessary to perform alternative growth assays, such as the Bradford assay, in future screenings to provide confidence in the OD values. Figure 12 showcased the OD measurements when grown on 785A TPU; due to the overlap of error bars in all growth curves, it makes it difficult to accurately and confidently determine the growth rates of the strains, but it seems that on average, #63 showed slightly higher growth than the BS6633 strain, with #I7 exhibiting the same growth as the control strain.

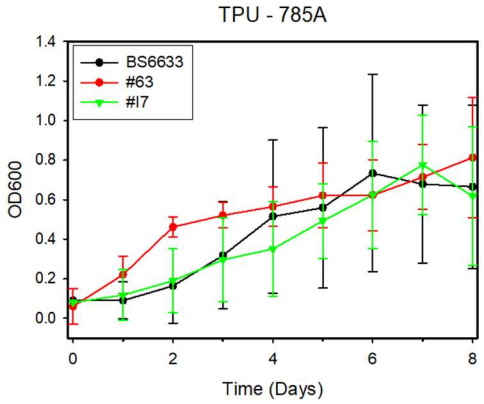

Figure 12: Growth of 2 host-strain candidates on 785A

Figure 13 depicted the OD measurements when the strains were consuming BCF35 as the sole carbon source. In this screening, it seems that strain #I7 showed substantially higher growth on average compared to the other two strains; in fact, in opposite to the 785A screening, the strains #63 and BS6633 had similar growth curves when grown on the BCF35. The final screening involved growth on the BCF45 as the carbon source, shown in Figure 14. Unfortunately, the two experimental strains failed to surpass the control strain in consuming the TPU. The differences between the growth profiles on different TPUs indicates that, besides requiring new methods to measure cell biomass, perhaps strains exhibit different degradation rates based on the specific chemical composition of the TPU.

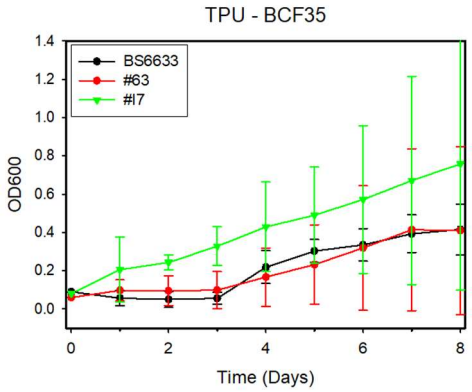

Figure 13: Growth of 2 host-strain candidates on BCF35

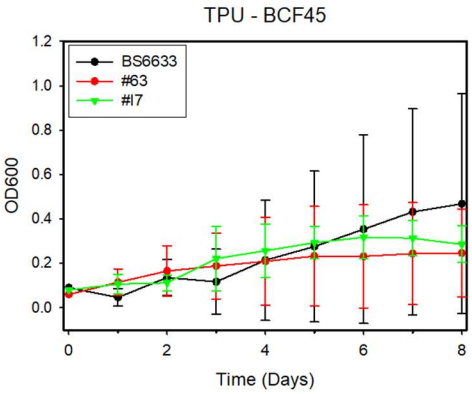

Figure 14: Growth of 2 host-strain candidates on BCF45

2.2.3 – *Short-Term ALE on Strains Indicates that Degradation Depends on TPU Composition:*

Based on the growth measurements via OD600 and total protein concentration in Figures 10 and 11, three different strains were considered to be the best host-strain candidates from the entire SIO bacterial library. These strains were #C42, #63, and #I7. Based on both the Bradford assay and OD measurements, strain #I7 showed high growth on 785A and BCF45 under both measurements, while strain #63 showed growth on both 785A and BCF45, but at much lower values compared to the other two strain candidates.

To further test strains #63, #I7, #C42, and BS6633 for growth consistency, a 2-passaged ALE was performed, with two replicates per strain and per grounded TPU subtype. Each passage

lasted for 7 days with manual OD measurements taken on Day 0, and every other day via a plate reader. On Day 7, the culture is moved to a new passage, in which a certain amount of the culture that has the same initial OD value was aliquoted into fresh media and TPU.

To showcase the growth of these strains over a prolonged exposure to TPUs, a short-term ALE was conducted, with the strain candidates and BS6633 grown on grounded 785A, BCF35, and BCF45 for two passages, in which each passage lasted for about a week. As confirmed from the previous growth data, both #C42 and #I7 rapidly grew on both 785A and BCF35 substrates during the short-term ALE, shown in Figures 15 and 16. In fact, it seemed that #I7 seemed to grow relatively well on all three TPU substrates, even though it was surpassed by strain #C42 in the BCF35 condition, as depicted in Figure 17. Meanwhile, strain #63 was the second highest-growing strain on the 785A substrate, but was one of the slowest strains on the other two TPUs; in fact, it performed even worse than the control strain when grown with BCF45.

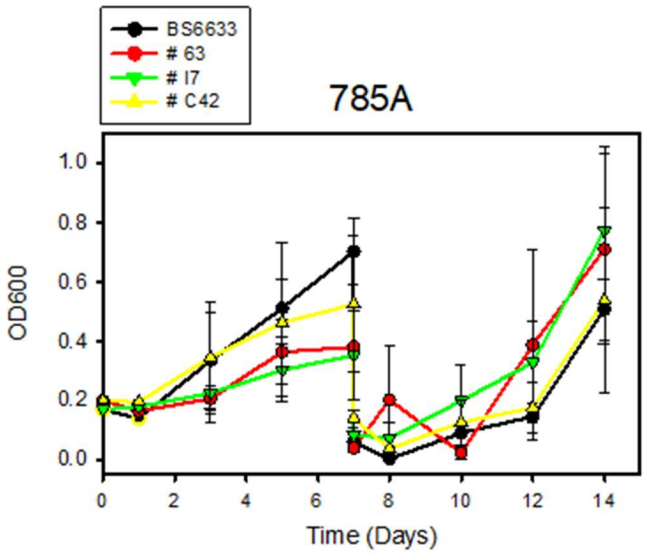

Figure 15: OD measurements of 2-passage ALE on 785A TPU

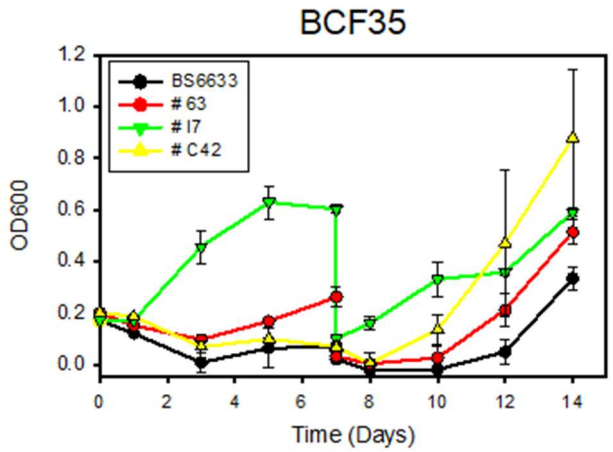

Figure 16: OD measurements of 2-passage ALE on BCF35 TPU

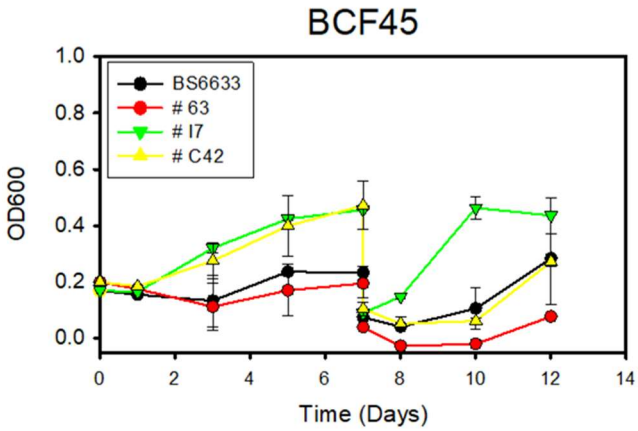

Figure 17: OD measurements of 2-passage ALE on BCF45 TPU

2.2.4 – *Grounded TPU Indicate Higher Growth Than Cryomilled TPU Due to Higher Surface Area:*

As the short-term ALE experiment indicated that strains degrade different TPUs at different rates, it was important to determine if the processing methods of these TPU powders would affect the biodegradation rates as well. Therefore, all three cryomilled TPU subtypes were grown on BS6633 and strain candidates #63, #I7, and #C42 for 7 days, and the data was compared to the previous growth rates on the grounded TPU. The cryomilled TPU, due to their

singular processing method, seems to have a higher cross-sectional area compared to grounded TPU powder, indicating that it may lead to faster TPU degradation as there would be a higher amount of surface area available for bacteria to attach and start degrading. However, calculations of cellular density via a Bradford assay implied that there was less growth compared to the grounded TPU powder, as depicted in Figure 18. The inconsistency between the grounded and cryomilled TPUs might be caused by the preparation methods of the different subtypes. As the grounded TPUs experienced three forms of deformation processes - being crushed, grinded, and sieved - compared to the cryomilled TPUs, which were just grinded, it is expected that the grounded TPU particles would be smaller and have a rougher surface compared to the cryomilled TPUs. This rough, cragged surface would confer a higher exposed surface area to the grounded TPUs, possibly allowing the bacterial strains to form more colonies on its surface as part of the biodegradation process. As more colonies form, it leads to faster degradation of the plastic, which can explain the discrepancy between grounded and cryomilled TPUs.

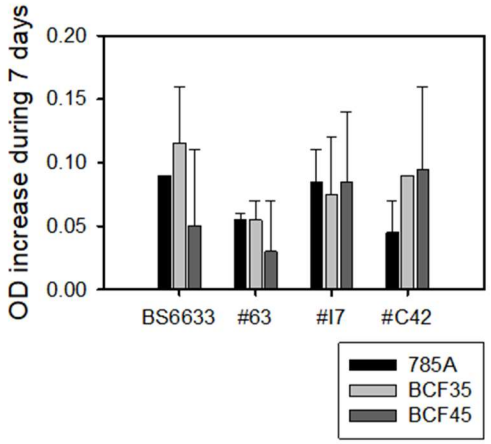

Figure 18: OD Growth on Cryomilled TPUs over 7 days

2.2.5 – *Utilization of Helper Substrates May Be Necessary for Future ALEs:*

Overall, the previous TPU screenings showed low cellular growth and TPU degradation; to assist in the biodegradation of these polymers, it was proposed to utilize a helper substrate that the strains may initially grow on, allowing them to grow at a high OD while gradually converting to use the TPU as a carbon source. This is a necessary step for future ALEs; as ALE relies on natural selection for the emergence of beneficial mutations from the population, it is necessary for a large population so there are more chances for mutations to occur throughout the population. As previous experiments measured low OD values, it indicates there is low cellular growth, meaning there are less cells and thus less chances for mutations to occur. This low population count increases the possibility that potential beneficial TPU-degradation mutations might not arise in the initial population due to its size. To solve the issues of mutation rates and population size, the addition of a limited carbon-source helper substrate may be added to initial cultures to increase the population size. The inclusion of a helper substrate will serve as the initial preferred carbon source of the inoculated strain, resulting in a large initial population after a few days, serving as the platform population for mutations to occur for ALE experiments.

A previous study grew samples of *Bacillus pumilus*, obtained from polyurethane-contaminated water, on yeast extract salts with minimal broth medium and a water-dispersible polyurethane as the carbon source [24]. The inclusion of the yeast extract salts shortened the lag phase and caused it to occur earlier compared to growth without the presence of yeast extract salts, indicating that it may assist with polyurethane degradation, as indicated with the higher bacterial count [24].

Initial testing utilized yeast extract and performed a titration calibration of yeast extract from 0-2g/L to determine the most optimal yeast extract concentration for TPU degradation. The inclusion of the yeast extract will boost the initial cell growth, thus shortening the time for it to

reach maximum OD. The presence of yeast extract does not affect the measured cell biomass from the Bradford assays. It was shown in Figure 19 that above a concentration of 1g/L, the yeast extract does not improve the initial ODs of the strains. Therefore, in future ALE experiments, it will be recommended to include 0.5g/L of yeast extract for the initial population growth, and then it is likely that mutations aiding in the consumption of TPU will emerge as the yeast extract is eventually depleted. Also, a concentration of 0.5g/L of yeast extract is preferred over 1 g/L due to there not being a large difference between the two OD values, and that the less initial amount of yeast extract included, the sooner the strains will consume the entire helper substrate and switch to TPU consumption.

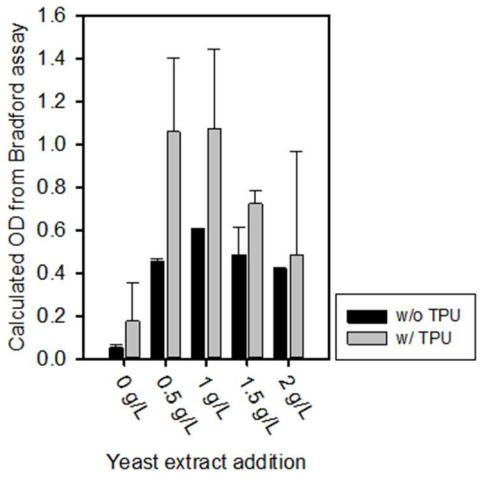

Figure 19: Yeast extract titration experiment showing calculated OD values of strains grown in the presence and absence of TPU

CHAPTER 3: DISCUSSION AND FUTURE WORK

Thermoplastic polyurethane is a synthetic plastic that is unable to be commercially recyclable, with TPU wastes ultimately accumulating in landfills or burned, causing environmental and health hazards for both humans and wildlife. An alternative way of getting rid of TPU waste in a biologically-friendly manner is via biodegradation, in which microorganisms use enzymes to break down polymers, and utilize the resulting monomers as carbon sources for cellular growth. Previous research into TPU biodegradation showed that many fungi and other bacteria, such as *Bacillus*, have the capacity to degrade TPU over a course of several months. However, due to the slow nature of biodegradation, along with the presence of other, more desirable carbon sources located in landfills, there is a need to develop bacterial strains that are specialized for quick TPU degradation and preference for TPU. By optimizing the TPU degradation capabilities of *Bacillus* strains, along with other beneficial characteristics for growth in landfill environments, these strains could be utilized to get rid of TPU waste efficiently and effectively, limiting the environmental hazards associated with prolonged TPU waste exposure.

In this thesis, the screenings of such characteristics for a *Bacillus* host-strain candidate were performed, emphasizing the abilities to degrade TPU as a food source, surviving in landfill environments, sporulating, tolerating heat-shock, and testing for genetic tractability. In both the preliminary and main screenings, there were a few strains that seemed to be likely host-strain candidates, due to their marked performance over the control strains. However, although these strains did show higher performance, the deviations among intra-replicates hampered the overall results of the screenings and served as an obstacle for comparing results. To accommodate for these inconsistencies, Bradford assays were performed and converted to OD600 measurements to compare OD600 and Bradford values. However, even the Braford values faced issues in

producing consistent data to verify the strain growth phenotypes. For future experiments, more replicates per condition and strain should be included to combat the issues of replicating the results and reducing the standard deviation between samples.

From the *Bacillus* library, we found three potential host-strain candidates that may serve as a platform for future ALE experiments to optimize their capabilities so they may be utilized in landfills to reduce TPU waste. It seems that the three strains showed different growth capabilities depending on the type of TPU being used as the sole carbon source; this hints towards the idea that more than one strain may be needed to degrade specific TPU wastes, and possibly all strains would need to undergo ALE to increase their degradation efficiencies of their less preferred TPU types. In the future, we hope that these strains may be ultimately utilized to biodegrade TPU waste, solving the health and environmental issues associated with the accumulation of this polymer in landfills.

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