Biodegradation of Solid Polyurethane by *Pestalotiopsis microspora*

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Abstract

The increased overproduction and use of plastic in the past decades has caused many environmental issues. *Pestalotiopsis microspora* (*P. microspora*), an endophytic fungus, has been found to be able to break down polyurethane, a polymer found in some plastics. This study aims to explore the capability of the functionality of *P. microspora* for biodegrading polyurethane (PUR), in aerobic and anaerobic conditions. *P. microspora* was grown in in the presence of solid PUR for two weeks. Samples were taken every two days and the absorbance of each sample was measured at 540 nm to detect a decrease in PUR content. A decrease in PUR content was not observed based on the spectrophotometer readings but observations of the fungal growth patterns show an association between *P. microspora* growth and the PUR. These observations may suggest PUR degradation or consumption by *P. microspora*. Observations of the fungal growth also showed the ability of *P. microspora* to grow in low oxygen conditions. These findings suggest the potential of *P. microspora* application in future plastic waste clean up systems.

Biodegradation of Solid Polyurethane by *Pestalotiopsis microspora*

Introduction

The production and use of plastics has increased dramatically over the past decades. Global plastic demand has grown almost 200 times since the 1950s with the production of 1.5 million tons in 1950 increasing to 299 million tons in 2013 (WorldWatch, 2015). A study published in the *American Association for the Advancement of Science (AAAS)* found that 4.8 to 12.7 million tons of the 300 million tons of plastic generated in 2010 was dumped into the ocean, and the annual averages in the years following the study were estimated to increase (Jambeck, J.R., 2015). Further, the dumping of millions of tons of plastic into landfills not only causes space to be taken up but also causes known and yet to be discovered health problems by polluting the groundwater nearby with chemicals that the plastics may be made of or may hold (Magda, M., 2013). Plastic accumulation in the environment has caused increasing problems such as disrupted animal patterns and disturbed ecosystems, causing billions of dollars in damage (PlasticsEurope, 2010).

There have been efforts to address our plastic problem, the most notable one being recycling. Recycling addresses the production aspect of the problem by allowing us to produce less plastic. However, despite the numerous recycling awareness programs taught in the American schools and the many new waste sorting technologies developed, only 25 percent of the plastics produced in the United States are recycled each year (WorldWatch, 2015). The percentage of plastic recycled annually has been stagnant in recent years but is expected to increase nevertheless (Gourmelon, G., 2015). While recycling addresses the production aspect of our plastic problem, it doesn't address the existing plastic that pollutes our many environments.

Researchers studying plastics have turned to bioremediation, the use of biological processes to breakdown unnatural pollutants, as a possible solution to the plastics polluting our environment. The use of biological processes to address plastic accumulation in the environment would prevent more synthetic chemicals and systems from being introduced to the environment.

PUR is an industrial polymer found most commonly as the foams in furniture or in paints and adhesives. PUR monomers are formed from the condensation reaction of a polyisocyanate and a polyol (Fig. 1). Variations of the urethane R groups give the polymer different properties.

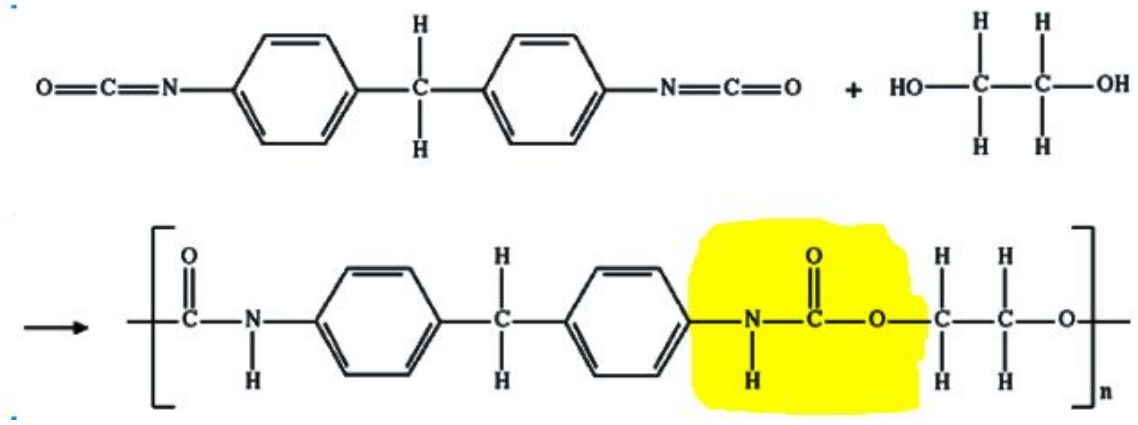

Fig. 1 Diphenylmethane diisocyanate and ethane diol bond together to form a PUR monomer. *P. microspora* cleaves the ester bond that links the isocyanate and polyol together. The ester group is seen highlighted in the figure.

Several bacteria and fungi have already been discovered that can break down PUR (Russell, et al., 2011). Most fungi that display PUR biodegradability are soil fungi such as fungi in the genus *Alternaria*, *Plectosphaerella*, *Geomyces*, *Nectria*, and *Neonectria* (Cosgrove, L., 2007). In most studies conducted to test the applicability of these fungi in bioremediation, such as the one conducted by Long and Cravatt on strains of fungi in the genus *Alternaria*, a landfill

environment was simulated by burying the organism with PUR at high temperatures under low oxygen conditions (Long, Z., & Cravatt, F., 2011). These studies have limitations that often include the inability of the organism to survive or grow at normal rates in low oxygen conditions (Cosgrove, L., 2007). The inability to grow anaerobically or under low oxygen conditions is a problem because landfills conditions often entail low oxygen availability (Crabbe J. R., 1994). If the organism cannot survive in low oxygen conditions its potential for application in bioremediation in landfills is decreased. There are also other factors to consider when simulating landfill environments including certain greenhouse gasses such as methane that are commonly produced in landfills and may inhibit organism growth (Pathirana, R. A., 1984). Another limitation is the rate at which the organisms break down the plastics. In many of the studies searching for bioremediation techniques with certain organisms, the rate of plastic breakdown observed was not significant enough that efficient application could be possible (Long, Z., & Cravatt, F., 2011). This is observed in these studies because most of the organisms discovered to be able to break down plastics have only been observed to use plastics as alternative food sources causing their breakdown rates to be much slower than needed.

Several isolates of the endophytic fungus *P. microspora* have been identified to be able to break down PUR and use it as a sole food source (Russell, et al., 2011). This is significant because it is one of the few fungi that have been found to be able to use a plastic as its sole food source. Another fungi *Aspergillus niger* was also found with this characteristic but its PUR breakdown rates were mild compared to that of *P. microspora* (Cosgrove, L., 2007). *P. microspora* breaks down PUR polymer chains by cleaving the ester bond that forms between the isocyanate and polyol that bond to form a PUR monomer. Previous research conducted with *P.*

microspora demonstrated its ability to degrade PUR in a liquid suspension (Russell, et al., 2011). No research has been conducted that tests the ability of *P. microspora* to break down solid PUR. Liquid PUR is used during manufacturing and construction but solidifies after use. PUR exists most commonly as a solid in disposal form so it is not applicable for the study to test the breakdown of liquid PUR. This project seeks to determine whether *P. microspora* can break down solid PUR and whether it can survive in low oxygen conditions in order to explore its role in future plastic waste removal systems.

Research Questions

Can *P. microspora* break down solid PUR?

Can *P. microspora* survive in a low oxygen environment?

Objective

The objective of this project is to determine if *P. microspora* can breakdown solid PUR and survive in an low oxygen environment in order to explore its application in the bioremediation of landfills.

Hypothesis

It is hypothesized that *P. microspora* will be able to break down solid PUR.

It is hypothesized that *P. microspora* will be able to survive in a low oxygen environment.

Null Hypothesis

P. microspora will not be able to break down solid PUR.

P. microspora will not be able to survive in a low oxygen environment.

Variables

For the first research question, there are two negative controls, one positive control, and one experimental group. The first negative control had just potato dextrose broth (PDB) broth. The second negative control had PDB broth and fungus. The positive control had PDB broth and PUR. The experimental group had PDB broth, PUR, and fungus.

For the second research question, there is a positive control and an experimental group. The positive control was the set of flasks with vented caps that allowed for aeration. The experimental group was the second set of flasks with closed caps that prevented aeration.

Materials

- *P. microspora* (16 mL, Vesp.co, Inc)
- PDB (1 L)
    - Dextrose sugar (10 g, VitaCost)
    - Yeast extract (1.5 g)
    - Potato cubes (250 mg)
    - Deionized water (dI H_2O) (200 mL)
- Distilled Water
- HD 2024 (3.12 mL, Hauthaway Corp.)
- Hot Stirrer/Plate (Cornino)
- 1.5 mL Eppendorf tubes

- V-5000 Visible Spectrophotometer
- Microscope slides (Baxter)
- T-75 flasks
- 1 mL Rectangular cuvettes
- Micropipette (P-1000 and P-200)
- Titer plate shaker
- Autoclave (Bio-Clave)
- Eppendorf Centrifuge 5418
- Vortex Genie 2
- Task Wipers (Kimwipes)
- Microscope
- Methylene blue stain
- Incubator (Boekel Scientific)
- Fume Hood
- Analytical Balance (Sargent Welch)

Methods

PDB preparation and *P. microspora* storage

Potatoes were scrubbed clean and bad spots were cut out. They were then cut up into 1 inch cubes and rinsed twice with tap water and once more with dIH_2O. dIH_2O was used to rinse two 800 mL flasks and then each flask filled with 500 mL of dIH_2O. The potato cubes were then weighed and 125 mg of the potato cubes were placed in each flask and then boiled in the flasks

until they were soft. The potato broth was filtered into a 1 L flask with cheesecloth and the potato cubes were discarded. The broth was then heated and 1 g of yeast extract and 10 g of dextrose sugar was added. The broth was autoclaved at 121°C then stored at 4°C.

P. microspora was obtained in the form of liquid culture and stored at 4°C. A standing culture was prepared in a flask with 50 mL of PDB and 5 mL of liquid culture. The standing culture was then grown at room temperature, 25°. 10 mL of the standing culture was transferred to a new flask every week and 40 mL of PDB was added.

P. microspora **inoculation and control preparation**

Biodegradability of PUR by *P. microspora* was tested by growing the fungus with HD 2024, a soft, co-solvent free, aliphatic, waterborne, polyurethane dispersion. The PUR liquid media (PUR-L) was a 1 L mixture of PDB and 10 mL of HD 2024 (Russell, et al., 2011). The PUR was added after autoclaving the broth. HD 2024 came in a liquid form and later solidified to serve as the solid PUR in the experiment. It was added to the broth after heating it to ensure that it mix thoroughly and did not solidify early as it solidifies at cooler temperatures.

P. microspora was grown at two different concentrations, one low, 2.5%, and one high, 7.5%. These concentrations indicate that the liquid culture of *P. microspora* was inoculated in broth with these percentages of culture to broth. These two concentrations were chosen based on both availability of the liquid culture and the unknown rate at which *P. microspora* would grow. Having two concentrations of the fungus would also show whether a difference in the amount of fungus present would significantly affect the rate at which the PUR would be broken down at.

Each concentration was treated to two different conditions in addition to two more controls. First a negative control was made for each concentration that consisted of just broth. This was prepared by adding 40 mL of PDB broth to two T-75 flasks. Second, a positive control was made for each concentration that consisted of broth and PUR, this was the PUR-L mentioned before. This was prepared by adding 40 mL of PUR-L to two T-75 flasks. Third, another negative control was made for each concentration that consisted of broth and fungus. This was prepared by inoculating 1 mL and 3 mL of the liquid culture with 39 mL and 37 mL of PDB broth, respectively. The two 40 mL mixtures were then put in two T-75 flasks. The different volumes accounted for the different fungal concentrations. Last, the experimental group was prepared for each concentration that consisted of broth, fungus, and PUR. This was prepared by inoculating 1 mL and 3 mL of the liquid culture with 39 mL and 37 mL of PUR-L, respectively. The different volumes accounted for the different fungal concentrations. The two 40 mL mixtures were then put in two T-75 flasks.

The four conditions, shown diagrammatically in Figure 2 with one flask for each concentration, totaled to eight T-75 flasks. This entire set of flasks was duplicated. The first set of flasks were fitted with vented caps to allow aeration. The second set of flasks were fitted with closed caps that prevented oxygen flow from the outside, the only oxygen in the flasks was the oxygen that was in them during the initial inoculation. Both sets of flasks were grown on a shaker in an incubator at 25^{O} for a period of two weeks. The second set of flasks which had closed caps were left untouched until the end of the two week period to prevent aeration.

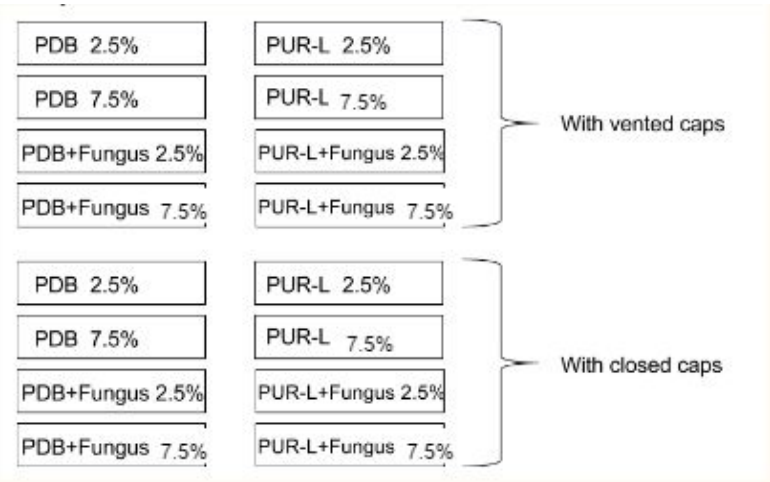

Figure 2. Visual representation of flask setup

Spectrophotometric analysis of PUR content

The wavelength of maximum absorbance of HD 2024 was determined by reading the bell curve graph plotted from the absorbance readings of a 2% HD 2024 solution for each wavelength from 400 nm to 800 nm with 10 nm intervals. The wavelength with the highest absorbance of HD 2024 was 540 nm.

A standard curve for HD 2024 was then made using a V-5000 Visible spectrophotometer at 540 nm with a HD 2024 concentration range from .05% to 2% (Fig. 4). The concentrations were made using serial dilutions to ensure continuity despite any possible errors. Dilutions were made with PDB and PDB was used as the blank.

1 mL samples were taken from each flask from the first, vented set every two days for two weeks and stored in 1.5 mL Eppendorf tubes. The samples were homogenized with a vortexer then centrifuged for 1 min at 14000 rpm in an Eppendorf centrifuge to pellet the fungal matter (Russell, et al., 2011). The supernatant was diluted to a 12.5% concentration with PDB

broth the absorbance was taken with a V-5000 Visible spectrophotometer at 540 nm to determine a relative rate of clearance of the broth. The actual absorbance of the samples were calculated by multiplying by 8 to get a reading for a 100% sample. Samples from the duplicate were tested at the end of the two weeks. All samples were taken either in a fume hood or under extreme sterile caution as to prevent contamination of the flasks. The samples taken were stored at 4° for further spectrophotometric analysis if needed. The absorbance readings were used to extrapolate the PUR concentration from the standard curve for HD 2024.

Results

The absorbance of the samples taken from the flasks during the two week period were run through the spectrophotometer at 540 nm and the readings were graphed. The general trend observed from the absorbance readings showed an increase as opposed to the expected decrease. There is some error observed in the first three of samples taken for some of the flasks because there was growth in the samples when they were stored at 4°C.

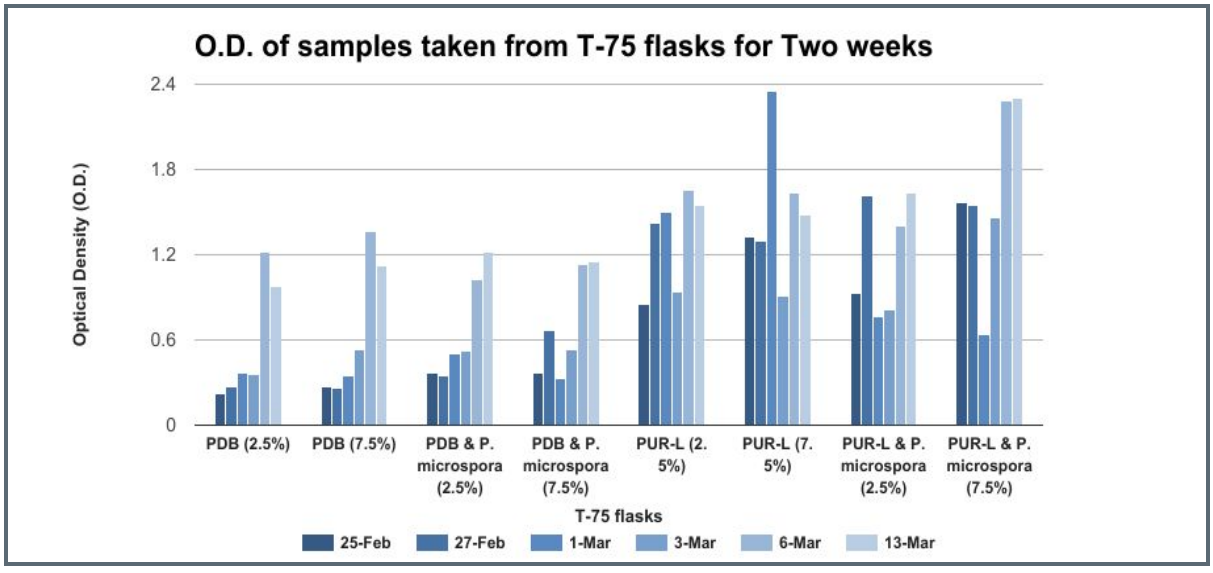

Figure 3. Samples were taken from each flask six times during the 2 week period and run through the spectrophotometer at 540 nm.

The standard curve for HD 2024 was not able to be used because the absorbance readings taken from the samples did not represent the PUR content in the flasks.

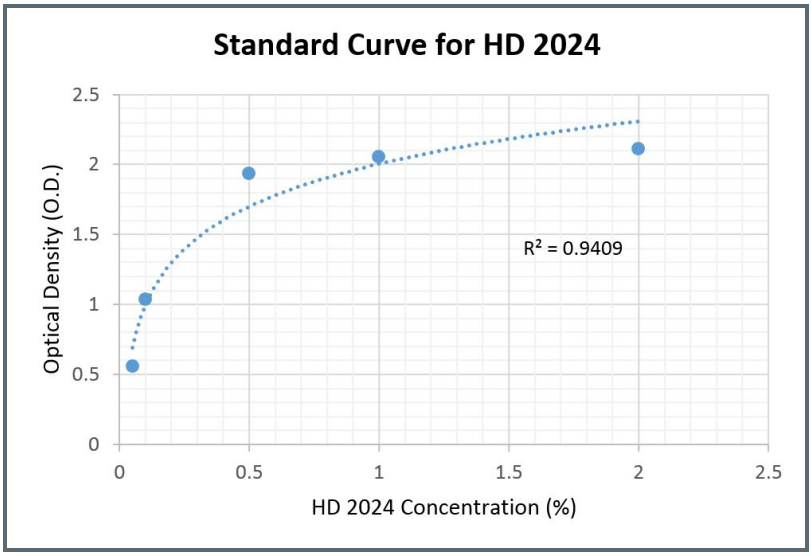

Figure 4. A standard curve was made from which HD 2024 concentration could be extrapolated from OD readings of the positive control and experimental group samples.

P. microspora growth and reproductive development was confirmed by looking at samples under 400x magnification (Fig. 5a and Fig. 5b). The presence of spores indicate reproductive growth (Fig. 5a).

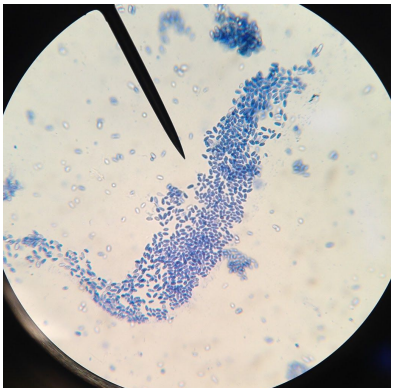

Figure 5a. *P. microspora* spores stained with methylene blue at 400x magnification. This showed the reproductive growth of *P. microspora* over the two week period.

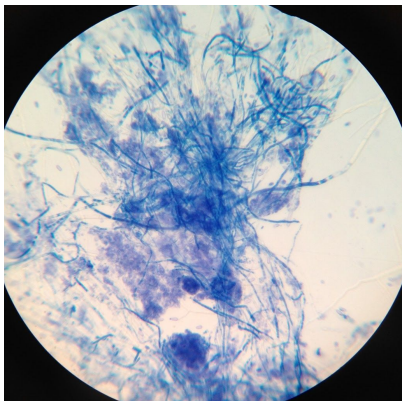

Figure 5b. *P. microspora* mycelia stained with methylene blue at 400x magnification. The mycelia hyphae are observed in this image to be wrapped in a mass.

Discussion

Data analysis

P. microspora was observed growing in both the closed and vented flasks showing that it could grow aerobically and anaerobically. This means that *P. microspora* can be effective in landfill applications as landfills have low oxygen levels and most fungi and bacteria that can break down plastics can grow without oxygen.

The spectrophotometric method proved to be ineffective in detecting a decrease in PUR content. This occurred for two reasons. One, the PUR sank to the bottom of the flasks so the samples that were taken from the flasks did not contain any PUR. This was most likely due to the fact that the PUR in the PUR-L mixture is much denser than the broth so over time, the PUR sank to the bottom. Because of this, the absorbance readings were not an accurate representation of the PUR of the flasks. The other reason that the spectrophotometric method was unsuccessful in determining the PUR content was the fact that the growth of the fungus interfering with the absorbance was not taken into account when initially developing and adopting the method used. The concurrent fungal growth is the reason for the increase in absorbance over the two week period which counters the original expectation that the absorbance would decrease due to decreased PUR levels over time (Fig. 3). Because of these errors, the PUR content decrease could not be quantified.

Although PUR content decrease was unable to be quantified, it was observed over the two week period. Observations of the way the fungus grew in the flasks showed a clear relationship between *P. microspora* and PUR. In the flasks that didn't have any PUR, the fungus grew as free floating clumps that grew only in the broth. In the flasks that had PUR, the fungus grew only where the PUR had deposited, on the sides and bottom of the flask. At first, I speculated that the fungal growth on the deposited PUR was due to it ability to grip onto the rugged surface as opposed to the smooth surface in the flasks without PUR. However, after researching fungal growth patterns, I found that the way the fungus was growing showed that it was using the solid PUR as a food source. Fungi growing in broth will wrap their mycelia around small bits of their food, in this case the bits of solid PUR, and break it down this way (Darby,

R.T., 1968). Close observation of the growth pattern as seen in Figure 6a and Figure 6b shows that the mycelia hyphae gripped onto the solidified PUR and wrapped around it until individual pieces of PUR were pulled off the container wall and were surrounded inside mycelial masses. These observations indicate that *P. microspora* is using the solid PUR as a food source.

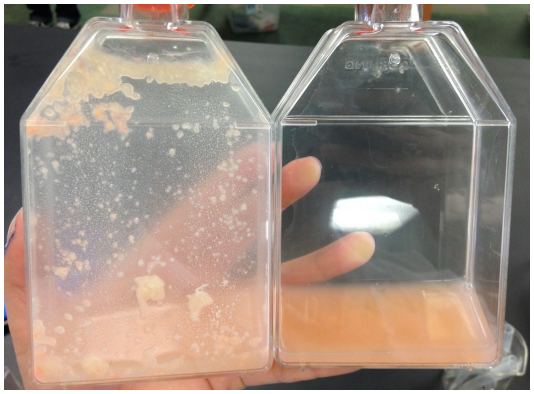

Figure 6a. *P. microspora* is observed growing on the sides and bottom of the flasks where a white layer, the solidified PUR, has formed. This is an upright display of the T-75 flask.

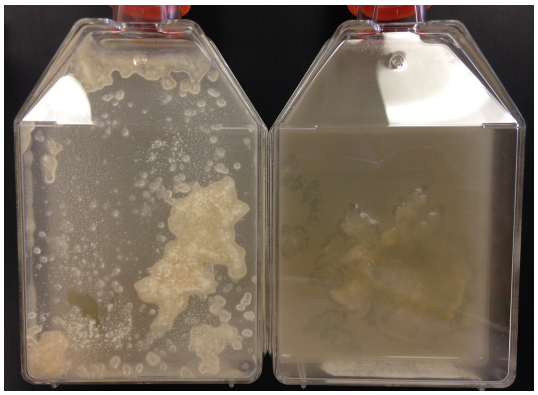

Figure 6b. *P. microspora* is observed growing on the sides and bottom of the flasks where a white layer, the solidified PUR, has formed. This is shows the T-75 flask laying on its back.

Method ~~Choosing~~ Selection

It should be noted that since the first set of flasks was duplicated, with the only difference being closed and vented caps, the second positive controls and the experimental groups for both sets were prepared at the same time. This ensured that any error that may have occurred during the preparation would not affect the comparison of the sets as both sets would have the same error.

In calculating the actual absorbance readings from the diluted samples that were run through the spectrophotometer, several problems occurred. One error was that while the samples were diluted to 12.5% dilutions, the blank was not diluted to the same factor. Because of this, the absorbance readings came out negative and therefore could be used to calculate the actual absorbance reading backwards. To account for this, I decided to recalculate certain values in the situation. By adding the absorbance reading from a sample that had an undiluted blank to the absorbance reading of the undiluted blank, I got the value X which represents the absorbance of the sample prior to the automatic subtraction of the absorbance of the blank by the spectrophotometer machine. Then by taking the value X and subtracting the absorbance of reading of a diluted blank, the accurate reading was produced. These calculations can be seen in Figure 7.

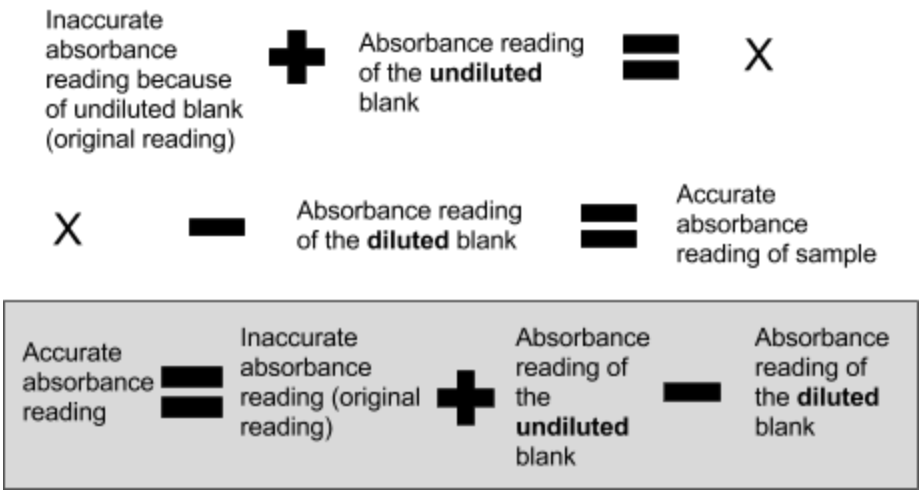

Figure 7. The formulas that were derived to account for not diluting the blank during the spectrophotometric analysis of PUR

Limitations and Sources of Error

Certain limitations exist due to the use of the spectrophotometric method as a way to analyze PUR content. The degradability of PUR by *P. microspora* was unable to be quantified because the method used did not fully address all aspects of the experiment. The sinking of the solidified PUR bits was not anticipated and neither was the interference caused by the concurrent fungal growth. This method was originally chosen because it was expected that the contents of the flasks, including the broth and the PUR, would remain a homologous solution. This however was not true and the results were affected because of that.

One thing that should be added to the experiment is another positive control that would include just *P. microspora* and PUR to confirm that *P. microspora* can in fact survive on just PUR.

Conclusion

In conclusion, the methods used were not able to address whether *P. microspora* could break down solid PUR. Nevertheless, an association between PUR deposition and *P. microspora* observed from the growth pattern of *P. microspora*. It could be speculated that the association between *P. microspora* and the PUR is due to the fungus breaking down the solid PUR. This assumption can be further explored and the results could reveal the potential of *P. microspora* in future plastic waste systems. The ability of *P. microspora* to grow in low oxygen conditions should also be noted as this makes *P. microspora* an even more valuable potential player in addressing landfills.

Future Work

Future research should be conducted to quantify the rate of PUR breakdown by *P. microspora*. This can be done with two different methods. The first step would to to identify the polyol that makes up the pUR polymer in HS 2024. This could be done by analysis of the datasheet given with the sample or by contacting Hauthaway Corp., the company that donated the HD 2024. Then a pure sample would have to be obtained of the polyol. Then using the spectrophotometer, a standard curve would be made with the polyol at a determined wavelength and then PUR breakdown could be extrapolated from the standard curve. The High Performance Liquid Chromatography (HPLC) could also be used here. The samples could be run through the HPLC with the pure sample of the polyol and the amount of the polyol in the samples could be used to determine the rate of PUR breakdown over time. Either of these two methods would have to be further researched but they can be used to quantify PUR breakdown.

It would be even more beneficial to test how efficiently *P. microspora* can break down a

sheet of PUR foam because foam is the most common form the PUR exists as. Surface area would have to taken into account and the HPLC or spectrophotometric method described can be used to determine the rate of breakdown of the PUR foam.

Lastly, because the breakdown of PUR by *P. microspora* is enzymatic and enzyme functionality can be manipulated through factors such as temperature and pH, it would be important to manipulate these factors to determine the optimal conditions in which *P. microspora* could survive and break down PUR at. It is also important to note that landfill conditions including temperature, pH, and pressure, may vary over a wide range of possibilities so it would also be important to test whether *P. microspora* can survive and breakdown PUR at the conditions that a landfill could experience.

Acknowledgements

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