

Isolation and Identification of Plastic-Degrading and Oil-Degrading Bacteria In a Soil Sample

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“On my honor, I have neither given nor received unauthorized aid on this lab report”

## Introduction

With the rise of climate change and pollution as a whole comes an emerging need to clear these contaminants for the prosperity of life on Earth. Recently, researchers have wondered if they could help the environment with the environment itself. Bioremediation uses natural products or organisms like microbial life or plants (enzymes) to detoxify its environment. Science Direct describes this type of technology as potentially being able to clear the ecosystem of both man-made and natural contaminants like plastic and better public health by proxy. Although the focus of this specific research, the benefits for the environment go past just plastic degradation. For example, it would be a sustainable and cost-efficient way to treat hazardous waste or it could recover soil and enhance its quality by breaking down contaminants and other substrates in the rhizosphere by supplementing microorganisms. Off the coast, these organisms and enzymes could potentially clean up oil spills or clean up wastewater. The possibilities once the process is mastered could truly be endless and thus inspire a lot of hope.

The soil sample was collected from the Hartsville Foxes Corner Gas Station. The location was selected due to its proximity to petroleum products being so close to the gas pumps and its proximity to litter. Much of the trash beside these sides were food that contained grease or very old plastic objects. Simply because of the probabilities from known plastic and oil degraders, should all the experiments be accurate, then an oil degrader should at least be isolated.

## Purpose

The objective is to isolate and culture oil-degrading and possibly plastic-degrading bacteria from a soil sample from a gas station.

## Materials and Methods

### April 19, 2023: Nutrient Broth Enrichment Cycle One

Firstly, the bacteria needed to be enriched and selected only for one or two strains. To do so, two 100ml tubes became the containers for enrichment as seen in Figure 1. In both tubes, 25mls of Bushnell-Haas(BH) broth(1:10 dilution with water) and 2 spoonfuls of the soil was added. Then, in one tube labeled “plastic” around 50 pieces of a mix of thinly cut Walmart plastic bags and thin plastic material were added as the carbon source. In the other tube labeled “oil”, around 6mls of vegetable oil was added as their carbon source. Then, both tubes were loosely closed and left to incubate at 30°C for 7 days.

### April 27, 2023: Bushnell-Haas Broth Enrichment Cycle Two With Known Carbon Sources

April 26, 2023 the two tubes were centrifuged at 1500 rpm for 5 minutes to separate any contaminants from the first enrichment such as other soil products and leaves as seen in Figure 2 and 3. On April 27, 2023, the second enrichment process was set up but this time with PolySnow as opposed to regular plastic bags, this way we could confirm the exact carbon source down to the chemical compound that the bacteria is degrading and not just give a general term of “plastic”. Polysnow is simply just sodium polyacrylate, a polymer commonly used in baby diapers and detergents as a chelating agent. The product is commonly used and wasteful so finding a degrader to cleanse the environment from it would be extremely beneficial.

In two new 100ml tubes, the one labeled “plastic” received around 20mls of the supernatant alongside 20mls of the BH broth and 100mg of polysnow. The one labeled “oil” received around 20mls of the supernatant from the previous “oil” sample, 20 mls of the BH broth, and 4mls of vegetable oil. Then, the tubes were resealed and incubated at 30°C for 7 days.

**May 4, 2023: Bushnell Agar Plate Isolation and Tetrazolium Biochemical Test**

At this point, the bacteria were enriched and it was time to isolate them onto agar plates and make colonies out of them. As shown in Figure 4, four plates were set up. On two plates, Luria Broth(LB) agar was used and on the other two BH agar was used. On the LB plates, simply 200 $\mu$ l of the respective samples from the enrichment tube in Figures 2 and 3 were pipetted onto the plates and spread for 2 minutes. For the BH agar plates, the procedure for “oil” and “plastic” differed. For “plastic”, 0.2g of polysnow was added to the plate and the excess was tapped out. Then, 200 $\mu$ l from the Figure 3 enriched tube sample was spread onto the plate. For “oil”, 25 $\mu$ l of oil was first spread over the plate and emulsified for about 3 minutes. Then, 200 $\mu$ l from the Figure 2 enriched tube sample was spread and re-emulsified onto the plate. They were all capped and incubated at 30° for a day. May 5, 2023 the LB plate of the plastic sample was put under a UV light to confirm biofluorescence.

Additionally, a Tetrazolium biochemical test was performed to prove degradation. Eight tubes were set up, 6 controls and 2 regarding the experiment. Each “ingredient” was added in equal parts of 2ml quantities. Displayed in Figure 6, four controls were executed. From left to right: the first contained water, oil, and 0.2% tetrazolium, the second(same as the 5th from Figure 5) contained the Ridx suspension, oil, and 0.2% tetrazolium (the one positive control), the third contained water, oil, and more water, and the fourth contained the Ridx suspension, oil, and water. As shown in Figures 5 and 7, one tube contained the plastic sample, oil, and water(a control). The next tube contained the oil sample, more oil, and water (a control). Then next contained the oil sample, oil, and 0.2% tetrazolium. The next contained the plastic sample, oil, and 0.2% tetrazolium. All tubes were sealed and left at room temperature for 24 hours.

## May 8, 2023: Gram Staining and Morphology Observations

Lastly, the samples were plated onto a coverslip, Gram stained (Figure 9 and Figure 10), and observed under a microscope at oil magnification (100x). For the LB samples the following process was repeated with each plate (plastic and oil sample). Add a loopful of water onto the cover slip. Then, add a loopful of a colony that is isolated and create a smear. Let the smear air dry and then heat fix it. After allowing the slide to cool, stain with Crystal Violet for 60 seconds. Next, rinse with water. Then, apply Iodine solution for 60 seconds and rinse again. Then, use the Gram Stain Decolorizer for 3 seconds to wash the stains off and immediately rinse with water. Lastly, add Safranin to counterstain for 30 seconds and wash with water. After repeating with the desired samples, let the slides dry and observe them under oil to determine morphology.

### Data

Figure 4 and Figure 8 depict the growth of each of the plates at its plating and after letting it grow for four days. Figures 5,6, and 7 depict the results of the Tetrazolium biochemical tests the day it was performed and the next day. Figure 9 and Figure 10 depict the Gram-staining results from the LB agar grown samples of each of the degraders.

### Results and Discussion

#### Plating on LB Agar as Opposed to BH Agar

Type	Plastic Sample on LB	Plastic Sample on BH	Oil Sample on LB	Oil Sample on BH
Amount of Growth/Notes	A great lawn of biofluorescent bacteria grew	Only probably around 15 dispersed colonies could be identified	A great lawn of bacteria grew	Scraping is apparent on the agar but a solid lawn of isolated colonies grew

All plates expressed some type of growth indicating successful isolation of bacteria as shown in Figure 8. The lawns grown were significant as compared to when they were just plated as shown in Figure 4. However, at this point there was still some hesitation on the growth being actual plastic/oil degraders as within the enrichment experiments, there were other carbon sources present (the leaves in the dirt sample that could not be extracted). In future experiments, a cleaner sample should be used to begin with to insure the bacteria is actually only using the respective oil and plastic bags as carbon sources and not something else. Later, this confusion is alleviated with the following tetrazolium experiment to confirm its ability to degrade oil.

Furthermore, for the BH sample plates, less oil and Polysnow could have been used to create a more level field for bacteria to grow on. The bacteria grown on LB media clearly had more space and grew much faster. Perhaps if the surface was more level (without polysnow texture, excess un-emulsified oil, and rips in the agar), the BH media plates would have grown just as well.

### Tetrazolium Biochemical Test Results

#### Control Experiments

Tube	Water +tetrazolium	Ridx +tetrazolium	Water -tetrazolium	Ridx -tetrazolium
Result	-	+	-	-

#### Sample Experiments

Tube	Plastic Sample -tetrazolium	Oil Sample -tetrazolium	Oil Sample +tetrazolium	Plastic Sample +tetrazolium
Result	-	-	-	+

\*Positive = pink, Negative= clear

Firstly, controls were established and the results were as expected as seen in Figure 6. In the two samples without the bioindicator, negative results presented. This makes sense as nothing is causing a change in the tubes. Additionally, the plain water negative controls also came out negative as expected with no enzymes to interact with the tetrazolium. The positive control also showed expected results turning a deep pink color indicating the biochemical reaction and experiment is working. The Ridx contains enzymes for oil-degradation.

Moving on, the same test was performed on the isolated bacterium as seen in Figure 7. As expected, the tubes lacking the bioindicator lacked indication of a biochemical reaction. However, interestingly the oil sample indicated no results of active degradation/oxidation while the plastic sample did. This data would suggest that a plastic degrading bacteria was isolated but not an oil degrading one. Even so, perhaps since the plastic bags were biosynthetic and thus contain other carbon sources other than pure plastic, it is just an oil degrader. In further experiments, perhaps the bacteria can be enriched with purely polysnow to begin with to limit introducing other sources of oxidation. Still, the oil sample not giving any indication of oxidation is confusing as oil degrades are a lot more present than plastic degraders. Perhaps it just couldn't break down the amount of oil given to it since it had been in such a concentrated amount or it was just weaker (is less suited for the environment but outcompeted the oil-degraders to begin with when there were other carbon sources in the beginning). Later results showed signs of endospores which appear when conditions are not ideal for a bacteria.

### **Gram-Staining Test Results**

LB Plate Plastic Sample	LB Plate Oil Sample
Pink and Circular	Purple and Rod-Like with Endospores

Due to the limited growth of plastic degraders on the BH media agar and constraint of time, Gram staining was only performed on the BH oil sample, and the LB media agar samples. As microscopic observations were performed, only the LB media agar grown samples gave clear results as to morphology and thus are the only two listed in the figure. In future experiments, hopefully all samples can be plated and inspected and the experiments can be repeated to make sure problems like over decolorization did not occur and confirm results. Figure 9 depicts gram-positive (purple result) bacillus that seem to be caught in phases with endospores. This suggests that it isn't in ideal conditions for growth which makes sense if it is not an oil-degrader in an environment where the only carbon source is oil. Figure 10 depicts a gram-negative coccus. It doesn't seem to be over decolorized but is pink and it is pretty spherical. This suggests the plastic degrader is gram-negative, a coccus, and bioluminescent. However, there are microscopic limitations to the results as perhaps the shapes could be morphed by the heat fixation of the experiment(maybe a negative stain should be performed) and even at the oil level the bacteria were so small they were hard to see (maybe use a more powerful microscope).

After close comparison with known oil degrading bacteria presented by the NIH, Figure 9 seems to depict *Exiguobacterium sibiricum* (gram-positive bacteria shown to occasionally oil-degrade) or a common bacillus and Figure 10 seems to depict *Pseudomonas fluorescens* (biofluorescent, gram negative, contains oxidases, but is not a coccus). However, only a genetic screening can confirm these hypotheses.

### **Conclusion**

Essentially, after two rounds of enrichment (the first containing oil and plastic bags and the second containing oil and polysnow), both samples of bacteria from the soil were successfully plated onto both BH and LB agar plates. Additionally, they were confirmed for their ability to degrade oil through a Tetrazolium biochemical test (which the plastic sample tested positive for but not the negative). And finally, their morphology was studied through a simple gram-staining. Ultimately, a plastic-degrader was successfully isolated despite its odd combination of characteristics being a gram-negative cocci that is bioluminescent.

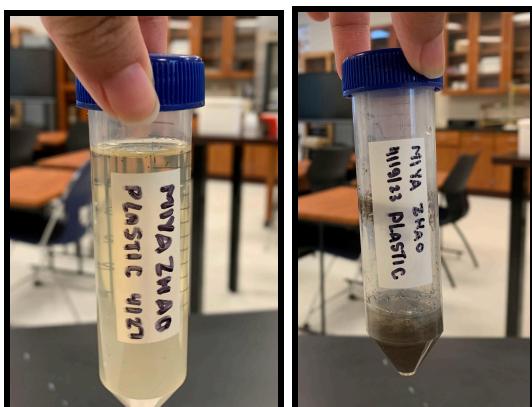
## Figures

**Figure 1** - First Enrichment Tubes



*Plastic-Degrader sample(left) and Oil-Degrader sample (right)*

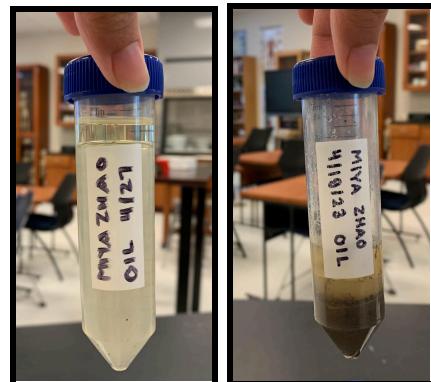
**Figure 3** - Plastic- Degrading Extracted Sample After Centrifusion



*Cleaned Sample (left) and the dirt remnants (right)*

**Figure 2** - Oil- Degrading Extracted Sample

After Centrifusion



*Cleaned Sample (left) and the dirt remnants (right)*

**Figure 4** - Isolation of Bacteria on Agar



*Left to right : Bushnell-Haas Agar oil sample, Bushnell-Haas Agar plastic sample, LB Agar oil sample, LB Agar plastic sample*

**Figure 5 - Tetrazolium Biochemical Tests**

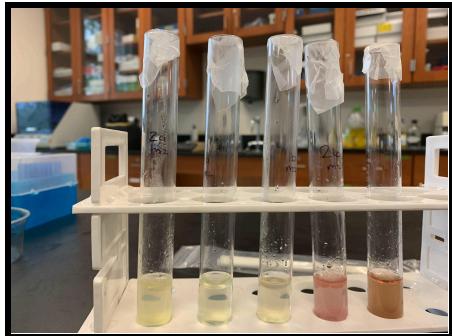
on May 4, 2023



*Left to right: plastic sample -tetrazolium, oil sample -tetrazolium, oil sample +0.2% tetrazolium, plastic sample +0.2% tetrazolium, Ridx suspension +0.2% tetrazolium*

**Figure 7 - Tetrazolium Biochemical Tests**

on May 5, 2023 (Results)



*Order same as in Figure 5*

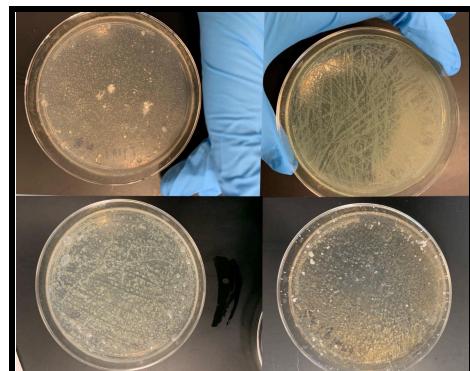
**Figure 6 - Control Tetrazolium Biochemical Tests**

on May 5, 2023



*Left to right: water +tetrazolium, Ridx suspension +0.2% tetrazolium, water -tetrazolium, Ridx suspension -tetrazolium*

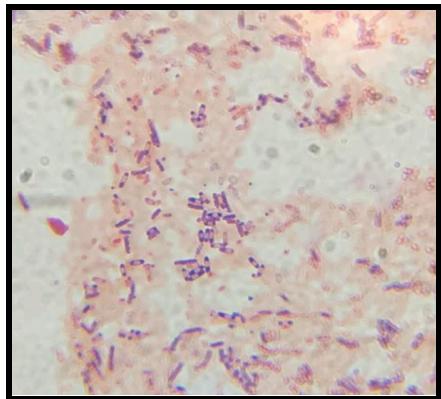
**Figure 8 - Plates on May 8, 2023**



*Left to right (top): BH oil sample plate, LB plastic sample plate*

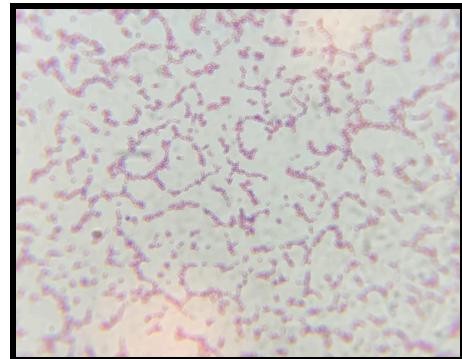
*Left to right (bottom): LB oil sample, BH plastic sample plate*

**Figure 9 -** Gram Staining of Oil Degraders  
from LB Plate



*Gram-positive bacillus with endospores at  
oil magnification*

**Figure 10 -** Gram Staining of Plastic  
Degraders from LB Plate



*Gram-negative coccus at oil magnification*

## References

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## Appendix

**Difco™ Bushnell-Haas Broth**

Approximate Formula\* Per Liter

Magnesium Sulfate .....	0.2 g
Calcium Chloride .....	0.02 g
Monopotassium Phosphate .....	1.0 g
Diammonium Hydrogen Phosphate ...	1.0 g
Potassium Nitrate .....	1.0 g
Ferric Chloride .....	0.05 g
*Agar .....	20.0 g

**Nutrient Broth**

Approximate Formula\* Per Liter

Peptone .....	15.0 g
Yeast extract .....	3.0 g
Sodium chloride .....	6.0 g
D(+) -Glucose .....	1.0 g

\*\*Distilled water was later added to create a diluted sample for both the recipes (1:10)

**Bushnell-Haas and Oil Agar**

Bushnell-Haas Solution with Agar .....	A Petri-dish
Vegetable Oil .....	~ 250 µl

**Bushnell-Haas and Plastic Agar**

Bushnell-Haas Solution with Agar .....	A Petri-dish
Polysnow™ .....	~ 0.2 g

\*Polysnow™ was added on the dish then the excess was tapped out