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# The Effects of Nutrients on the Efficiency of Motor Oil Bioremediation by *Pseudomonas putida*

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## ABSTRACT

Bioremediation is an innovative solution to reduce harmful chemicals in the environment using microorganisms and plants, making pollution removal eco-friendly and cost effective. Nutrients are often applied to enhance the process of bioremediation, allowing for larger, more active colonies of bacteria to grow and consume contaminants. The purpose of this project was to study the limits and effects of adding various amounts of nitrogen, phosphorus, and potassium to the environment of *Pseudomonas putida* as it consumed hydrocarbons in motor oil. In the experiment, test tubes of water, motor oil, bacteria, and varying amounts of nutrients were observed for two days. At the end of the two day period, the amount of oil left in the tubes was analyzed using a spectrophotometer to calculate the turbidity as absorbance numbers. It was found that there was a distinct negative trend in absorbance with each increase in nutrients, signifying that the nutrients allowed for greater growth and hydrocarbon consumption by *Pseudomonas putida*. However, there was not an observable limit to the benefits of adding nutrients. This is likely due to *Pseudomonas putida*'s strong chemotaxis towards hydrocarbons, and not towards other nutrients. Additionally, it was discovered that increasing the potassium promoted more oil consumption, due to potassium's key role for cellular transportation and moderation of turgor pressure. The results of this experiment can be used to guide which nutrients to prioritize in the bioremediation process, but more research must be done to discover the full extent of adding nutrients to a realistic environment.

## Introduction

Environmental pollution has been a growing global crisis since the first days of industrial innovations. Throughout the history of industrialization, this issue has been discussed in countless news reports, political debates, and scientific research, making it a concern of people around the world. This issue is contentious because the main source of worldwide pollution is due to anthropogenic causes such as poor agricultural practices, urban runoff, nuclear waste, or the petroleum industry (Khan & Ghouri, 2011). With this, it is clear to see that the health and wellbeing of human and wildlife populations is threatened by this anthropogenic pollution. Fortunately, many government entities and corporations are taking steps to decrease the severity of environmental pollution by applying innovative and efficient techniques to remove petroleum waste and heavy metals. One such technique is bioremediation, which is the elimination of harmful substances from the environment (Davey, 2020). Bacteria, fungi, and plants are commonly utilized for bioremediation purposes due to their ability to efficiently break down contaminants in water and the soil. Bioremediation using bacteria can be a remedy for harmful chemicals, petroleum waste, and heavy metals, and it is one of the most widely-used techniques to remove these substances from ecosystems (Davey, 2020). Furthermore, many studies have explored the benefits and possibilities of using bacteria in bioremediation, making it an innovative solution to the increasing problem of environmental contamination (Hesnawi & Adbeib, 2013; Ojuederie & Babalola, 2017; Vinothini et al., 2015).

Vinothini et al. explored the species of oil-consuming bacteria naturally found in soil (2015). Researchers collected soil samples from gas stations and automobile repair shops, and then identified the main species of bacteria in each. They found that *Pseudomonas putida* and *Bacillus cereus* were present in all samples, and that the former species was superior in removing oils from the environment. They then grew alfalfa and tomato plants in contaminated soil, and discovered a symbiotic relationship between the plant and the bacteria (Vinothini et al., 2015). In this relationship, *Pseudomonas putida* removes toxins from the soil directly around the roots of the plant, which supports the growth of both organisms.

Researchers in South Africa noted that microorganisms use a variety of processes to convert or remove soil contaminants, including coagulation of heavy metals, precipitation, oxidation, or diffusion (Ojuederie & Babalola, 2017). They explained that bacteria produce substances called siderophores that increase mobility and decrease bioavailability of contaminants to remove them from the environment. These bacteria have biosorptive capabilities that allow them to bind contaminants to their cell walls (Ojuederie & Babalola, 2017). The authors also noted that some plants and fungi also have this ability and when used in combination with bacteria, the productivity of the bioremediation process is increased.

Other methods to enhance the process of bioremediation include the use of fertilizers and organic nutrients, which allow larger, more active colonies of bacteria to grow and consume contaminants (Hesnawi & Adbeib, 2013). However, the researchers claimed that fertilizers can potentially alter the properties of water, and an alternative treatment must be found. The specific treatments the researchers used were poultry manure and chemical urea fertilizer, and the researchers concluded that the chemical fertilizer was surprisingly superior in stimulating the growth of bacteria.

## Research Question and Hypothesis

While many experiments have explored the techniques of bioremediation and the ideal species of bacteria to implement, few have directly studied the correlation between raw nutrients and the bioremediation process in order to determine how nutrients can be used more effectively (United States Environmental Protection Agency, Office of Solid Waste and Emergency Response, 1995). Bioremediation has its limitations, as it is entirely dependent on the environmental conditions and the strength of the microbial population (Azubuike et al., 2016). For this reason, the researcher was intrigued by the response of bacteria to different amounts of beneficial nutrients. Would an increase in the amount of raw nutrients *Pseudomonas putida* receives positively affect the bioremediation of hydrocarbons in motor oil? The researcher hypothesized ($H_1$) that the addition of raw macronutrients to support the growth of *Pseudomonas putida* would positively affect the bioremediation of hydrocarbons in motor oil until a certain extent, mainly due to the ability of the bacteria to utilize the nutrients as a stimulus for greater consumption of oil and an extra substrate for growth (Hesnawi & Adbeib, 2013). For a null result ($H_0$), the researcher hypothesized that the addition of raw macronutrients to support the growth of *Pseudomonas putida* would not affect the bioremediation of hydrocarbons in motor oil. In this experiment, the bacteria were grown and tested with different amounts of nutrients in a test tube with motor oil. The effectiveness of the bacteria was measured using a spectrophotometer to calculate the turbidity of the overall solution, and then the measurements were compared to determine the best amount of nutrients to add. The independent variable of this experiment was the amount and type of raw nutrient (nitrogen, phosphorus, potassium) added to the test tube, and the dependent variable was the intensity of light received by the spectrophotometer, which measured the turbidity of the solution. Some of the controlled variables included the initial amount of bacteria, the initial amount of oil, the amount of water, the experiment location and conditions, and the duration of the experiment. At the end of the study, the results could be applied/scaled to oil spills or leaks to decrease the time and environmental costs needed to successfully restore the environment. To have a wider scope of applications, this experiment could even be repeated with bacteria species that are superior in removing other harmful substances.

## Method

### Safety

The experimenter cultivated the bacteria in a Biosafety Level 1 laboratory, specifically the laboratory supervised by the teacher sponsor. Basic laboratory precautions such as the washing of hands, wearing of safety garments, and reporting of accidents to the supervisor were taken, and all of the equipment was sterilized. The supervisor of this experiment was Mrs. Pawadee Elliott, and the adult sponsor was Dr. Sara Fox. During the experiment itself, there were no accidents or injuries.

### Materials

- *Pseudomonas Putida* (living, 20 ml, Carolina Biological Supply Company)
- Test tubes (9)
- Test tube rack
- Cuvettes (10)
- Deionized Water (545 ml)
- Motor Oil (135 ml)
- Ammoniacal Nitrogen powder (6.3 g, RAW ELEMENTS- NPK Industries)
- Available Phosphate (Phosphorus) powder (6.3 g, RAW ELEMENTS- NPK Industries)
- Soluble Potash (Potassium) powder (6.3 g, RAW ELEMENTS- NPK Industries)
- Spectrophotometer
- Graduated cylinder
- Inoculation loop (3)
- Bunsen burner
- Scale
- Incubator
- Micropipette (1 ml, blue tip)
- Blue pipette tips, 1 ml (28)

### Procedures

The researcher used 9 test tubes. First, they measured 20 ml of water in a sterile graduated cylinder and poured that exact amount into each sterilized test tube. The *Pseudomonas putida* bacteria was prepared using the aseptic technique. The researcher re-suspended the bacteria in the original test tube by holding the top of the tube in one hand and flicking the bottom of the tube with the other hand until the bacteria were re-suspended.

The researcher took the inoculation loop and passed it over the bunsen burner for 3 seconds, and then waited for the loop to cool for 10 seconds. Then, they took off the cap of the bacteria test tube and sterilized the mouth of the tube by passing it over the burner three times. The experimenter then dipped the inoculation loop into the bacteria tube without touching the sides, and then withdrew it in the same manner. The experimenter inserted and removed the inoculation loop into the tubes of sterilized water. This was repeated for all tubes.

5 ml of motor oil was measured and poured into each test tube as well. Each tube was labeled with a number 1 through 9. The experimenter did not put in any nutrients for tube 1. Tubes 2, 3, and 4 received 0.6 grams of nitrogen, phosphorus, and potassium, respectively. Tube 5 received 0.1 grams of each nutrient, tube 6 received 0.2 grams of each nutrient, tube 7 received 0.3 grams of each nutrient, tube 8 received 0.4 grams of each nutrient, and tube 9 received 0.5 grams of each nutrient. A scale was used to measure the nutrients.

The tubes were placed in a test tube rack, in an incubator set to 37 degrees Celsius. The experimenter waited 2 days (48 hours) for the bacteria to grow. After that, the researcher took out the tubes and carefully transferred 5 ml of the contents of each test tube into sterilized cuvettes using the micropipette.

The experimenter powered on the spectrophotometer and waited 15 minutes for it to warm up, selecting "600 nm" as the wavelength. They then filled a cuvette with 5 ml of water and inserted the water solvent cuvette, setting the dial to 100 Percent Transmittance. After, the solvent cuvette was removed. Each of the nine cuvettes were analyzed by inserting them into the spectrophotometer. These steps were completed three times for the three trials.

## Results

Each test tube in this experiment contained *Pseudomonas putida*, oil hydrocarbons, and varying amounts of nutrients in order to determine the ideal amount of nutrients to promote hydrocarbon consumption. The amount of oil left in the tubes after two days was measured using a spectrophotometer to find the absorbance number. The absorbance number for each tube is displayed in Table 1, and the trends can easily be seen in Figure 1. Additionally, Figure 2 includes error bars for the standard deviation of each sample.

**Table 1:** *Light intensity measurements of the samples for all trials.*

| Artificial Environment Sample | Absorbance Units (Trial 1) | Absorbance Units (Trial 2) | Absorbance Units (Trial 3) | Absorbance Units (Average) |
|---|---|---|---|---|
| Control (1) | 0.170 | 0.189 | 0.143 | 0.167 |
| Nitrogen Sample (2) | 0.190 | 0.159 | 0.146 | 0.165 |
| Phosphorus Sample (3) | 0.150 | 0.084 | 0.075 | 0.103 |
| Potassium Sample (4) | 0.085 | 0.122 | 0.086 | 0.098 |
| Tube 5 | 0.097 | 0.239 | 0.232 | 0.189 |
| Tube 6 | 0.146 | 0.169 | 0.163 | 0.159 |
| Tube 7 | 0.090 | 0.174 | 0.175 | 0.146 |
| Tube 8 | 0.078 | 0.111 | 0.124 | 0.104 |
| Tube 9 | 0.088 | 0.104 | 0.090 | 0.094 |

**Figure 1**

*Absorbance number for each test tube, in all three trials*

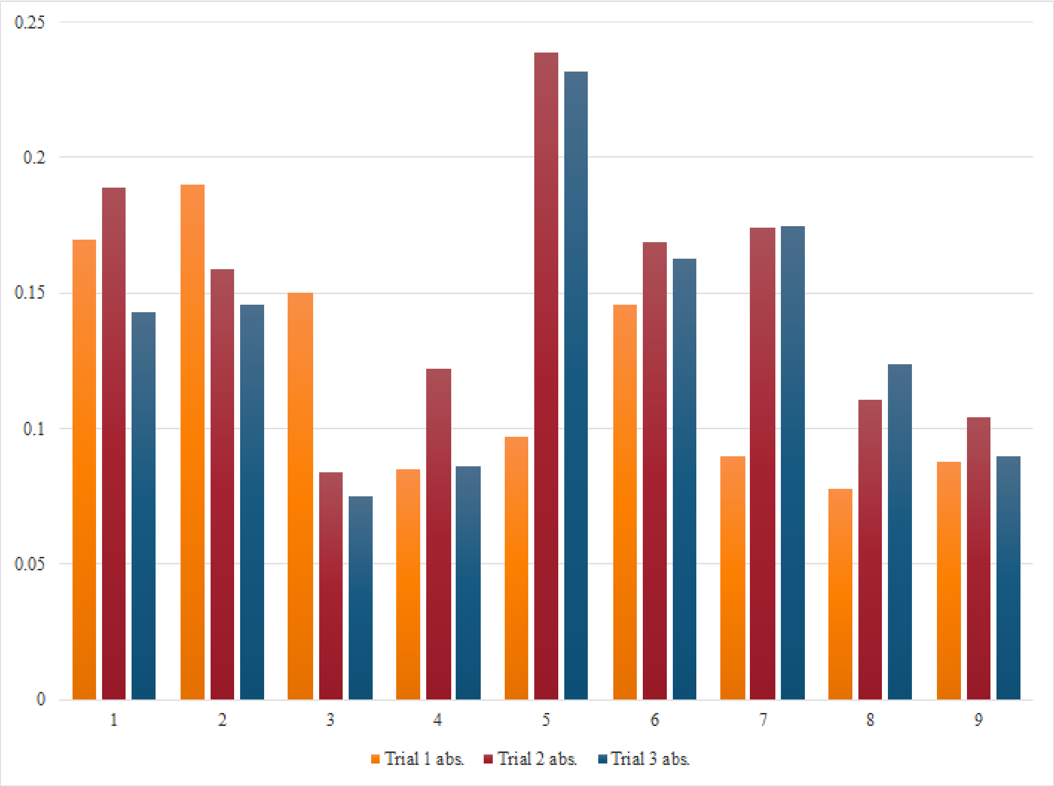
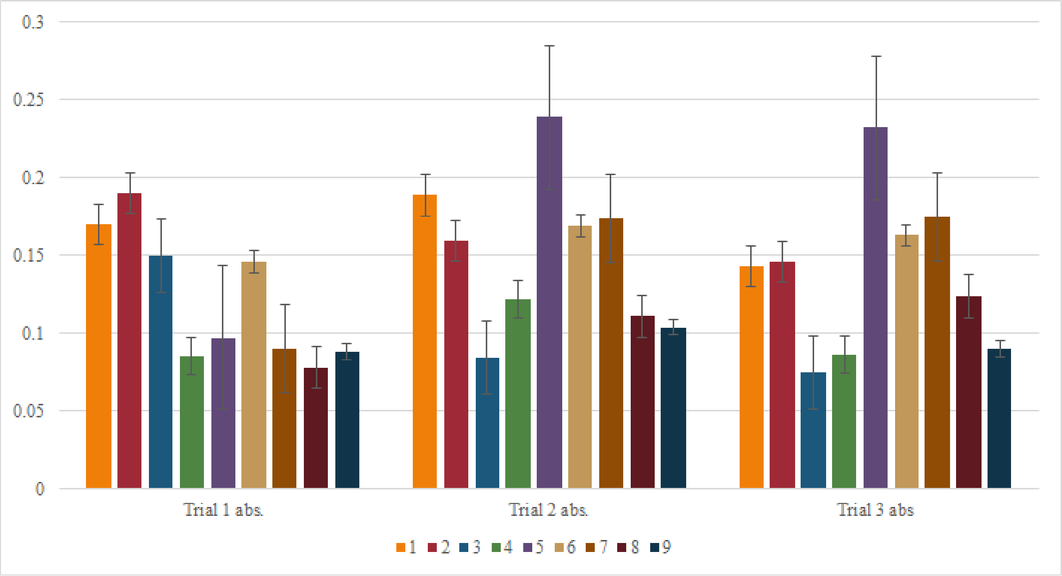


**Figure 2**

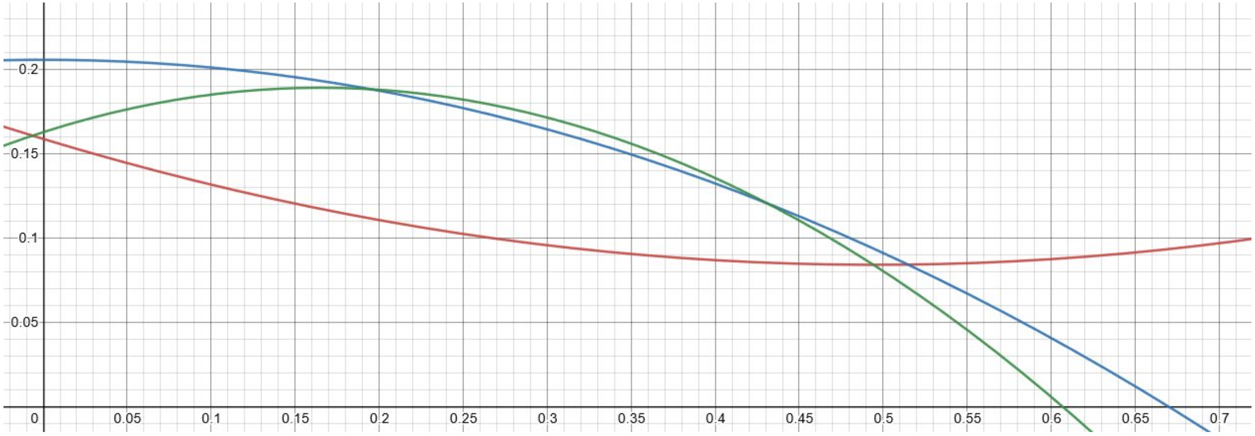
*Absorbance numbers for each trial with standard deviation*



The researcher also performed statistical analysis of this data using quadratic regression and ANOVA. To find a pattern in tubes 1 (control), 5 (0.1 g of each nutrient), 6 (0.2 g of each nutrient), 7 (0.3 g of each nutrient), 8 (0.4 g of each nutrient), and 9 (0.5 g of each nutrient), the experimenter plotted the data and used a graphing calculator to determine an ideal line of fit (See Figure 3). For the equations of Trials 1, 2, and 3, the $r^2$ values were 0.611, 0.784, and 0.705, respectively. This means that the quadratic model fits the data moderately well.

**Figure 3**

*Quadratic regression model for all 3 trials - absorbance units*



- ● Trial 1: $y = 0.305x^2 - 0.302x + 0.159$
- ● Trial 2: $y = -0.459x^2 + 0.206$
- ● Trial 3: $y = -0.97x^2 + 0.32x + 0.163$

Lastly, the researcher conducted an ANOVA test for all the data values from each trial. The data is categorized as significant or not significant with a significance level of 0.05.

Null Hypothesis: There is no significant difference between all the absorbance numbers in the experiment.

Alternate Hypothesis: There is a significant difference between all the absorbance numbers in the experiment.

After the ANOVA test was performed, the researcher got a f-statistic of 2.81929 and a p-value of 0.03229. Because the p-value is less than 0.05, the researcher can conclude that there is a significant difference between the absorbance numbers of each tube.

## Discussion

This experiment was conducted to determine the ideal amount of nitrogen, phosphorus, or potassium for *Pseudomonas putida* to consume a greater amount of hydrocarbons, based on the hypothesis that more macronutrients would promote bioremediation to an extent. The absorbance numbers of varying mixtures containing water, oil, bacteria, and nutrients were calculated by a spectrophotometer. Overall, from tubes 1 (control), 5 (0.1 g of each nutrient), 6 (0.2 g of each nutrient), 7 (0.3 g of each nutrient), 8 (0.4 g of each nutrient), and 9 (0.5 g of each nutrient), there is a significant decreasing trend of absorbance numbers, signifying that the oil and water mixture become clearer, or less turbid, with the addition of more nutrients. The tubes with the lowest absorbance numbers were tubes 4, 8, and 9, and tubes 1, 2, and 5 had the highest absorbance numbers. Of tubes 2, 3, and 4 (0.6 g of a single nutrient each), tube 4 (potassium) had the lowest absorbance number. Additionally, while all tubes appeared to have a bacteria film between the oil and water, the researcher observed that more bubbles formed in the tubes with lower numbers, an obvious sign of vigorous biological activity. Lastly, the researcher conducted various statistical tests, confirming that there was a trend and a significant difference between the tubes, rejecting the null hypothesis.

The results of this experiment adequately supported the experimenter's hypothesis that the addition of raw macronutrients to support the growth of *Pseudomonas putida* would positively affect the bioremediation of hydrocarbons in motor oil until a certain extent, mainly due to the ability of the bacteria to utilize the nutrients as a stimulus for greater consumption of oil and an extra substrate for growth (Hesnawi & Adbeib, 2013). To completely support the hypothesis, the absorbance numbers must show a significant decrease with each increase in nutrients, with a slight increase at the end. However, there only appeared to be a steady increase in absorbance number, so the hypothesis is not fully supported.

Initially, the researcher was curious if adding extra macronutrients in *Pseudomonas putida*'s environment would cause a decline in oil consumption due to the bacteria prioritizing the extra nutrient substrate, but this was not the case. In fact, a thick bacterial film was observed between the water and oil, demonstrating that the bacteria prioritized the hydrocarbons as their primary substrate. This is due to the ability of *Pseudomonas putida* and other oil-consuming bacteria to sense hydrocarbons in water, known as chemotaxis. Generally, chemotaxis is an ability of motile bacteria to react to certain chemicals in their environment, whether the reaction is to flee or approach the chemical (Lacal, Muñoz-Martínez, Reyes-Darías, Duque, Matilla, Segura, Calvo, Jímenez-Sánchez, Krell, & Ramos, 2011). In *Pseudomonas putida*, chemotactic reactions to hydrocarbons are determined by the McpT chemoreceptor, using direct ligand binding of carboxylates to the microorganism. Simply put, receptors in the bacteria bind to a crystallized form of carboxylic acid, an organic compound found in oil and petroleum products (Lacal et al., 2011). By using this receptor, *Pseudomonas putida* is attracted towards the hydrocarbon concentration in the test tube.

Furthermore, the researcher speculates that *Pseudomonas putida* does not have a strong system or receptor to the nutrients used in this experiment, unlike its attraction to hydrocarbons. This would mean that the increasing trend of oil consumption with the addition of nitrogen, phosphorus, and potassium is that the bacteria came into contact with the nutrients rather than actively seeking them out in the test tube environment. The higher the concentration of nutrients in the tube, the more often *Pseudomonas putida* would come into contact with and consume nutrients. However, more research needs to be done in order to test this idea.

Additionally, it was observed that the tubes containing 0.6 g of potassium had an overall lower absorbance than the rest of the single nutrient tubes. In bacteria such as *Pseudomonas putida*, potassium is key in cellular transportation and moderation of turgor pressure inside the cell (Epstein, 2003). The bacteria were cultivated in water for this experiment, which could have had an increasing effect of osmotic pressure on the bacterial cell wall. The researcher believes adding potassium to the sample promoted the transportation of water and nutrients through pressure-activated channels within the cell, relieving some of the pressure and allowing for greater population growth (Epstein, 2003).

Despite these positive discoveries, there were several possible errors within this experiment. When measuring out the ingredients, there was likely human or machine error regarding the exact amount of nutrient or other ingredient in the experiment. Another potential error was the accuracy of the spectrophotometer, mainly due to the fact that the spectrophotometer was an older model and has been used many times before. Three trials were conducted to minimize these errors and collect more precise results. While the results of this experiment did not identify a limit to the amount of extra nutrients beneficial to the growth of *Pseudomonas putida*, the insight gathered from this study could be a helpful guideline to the most effective raw macronutrients to use in an oil spill accident. Based on the data and observations of the researcher, adding more organic potassium compounds to a cleanup job with oil-consuming bacteria would be beneficial in the bioremediation process. Additionally, it should be noted that while bacteria like *Pseudomonas putida* thrive on an increasing abundance of nutrient substrate, this experiment did not include other factors like algae bloom or undesired bacteria growth in a real-life environment. More research must be done to provide the best possible combination of nitrogen, phosphorus, and potassium for hydrocarbon-consuming bacteria without having unintended environmental consequences. In order to gather more information about the effects of nutrients on *Pseudomonas putida*, this study could be extended to include a small imitation of an oil spill with natural water and biological conditions. This would help identify the consequences of an excess amount of nutrients on the local ecosystem. Another similar study could be done to identify the limit of nutrients on bacterial growth but measured in concentration rather than mass in order to better apply the results to a realistic clean-up job. To find the limit of nitrogen, phosphorus, and potassium's benefits on bacteria growth, the experiment must be extended for larger amounts of nutrients, and also include several different species of hydrocarbon-consuming bacteria.

## Conclusion

In conclusion, adding an increasing amount of nitrogen, phosphorus, and potassium to *Pseudomonas putida* only positively affected the consumption of hydrocarbons due to the bacteria's ability to prioritize hydrocarbons as a food source, called chemotaxis. Thus, the hypothesis was mostly supported because there was a visible negative trend in absorbance. Overall, the 0.6 g potassium sample and 0.5 g of each nutrient sample had the lowest absorbance, meaning that *Pseudomonas putida* was able to consume more oil. Even though there are a few possible errors to the experimental procedures, other variations of these trials could further support the results. The possibilities of nutrients' effectiveness on hydrocarbon-consuming bacteria must still be explored and limitations in their applications must be exceeded.

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