What Is the Effect of a Mixed Culture of *Pseudomonas fluorescens* and *Anabaena* on the Degradation of Polyvinyl chloride?

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ABSTRACT: As our use of plastics continues to increase, the difficulty in degrading these plastics when they reach natural waterways increases as well. The degradation of polyvinyl chloride plastic (PVC) in particular, has posed difficulties for researchers as PVC's even balance of electrical charges complicates the degradation process. The objective of this study was to expose two bacteria - *Pseudomonas fluorescens* and *Anabaena* - to each other to produce an increased supply of energy for a more effective method of degrading PVC. To test this, samples of PVC cloth were massed before and after exposure to these two samples of bacteria to determine if degradation was present or not. After undergoing these trials, the methodology demonstrated that the proposal to expose both bacterium to each other to attempt to degrade PVC was a success, as the degradation rates of this combination of bacteria surpassed previous degradation rates of PVC in the field.

KEYWORDS: Bioremediation; environment; Polyvinyl chloride; *Pseudomonas Fluorescens*; *Anabaena*; degradation; Oxygen; Carbon Dioxide.

INTRODUCTION

Globally, humans produce over 200 million tons of plastic per year.[1,2] Of this, it is estimated that 91% of this plastic isn't recycled.[3,4] Plastics that aren't recycled often end up in landfills, where the chemicals permeate the ground as a result of exposure to UV light from the sun, posing dangers to our air and soil. Additionally, much of this plastic ends up in our waterways: in oceans, lakes, and rivers. It is estimated that almost 8 million metric tons of plastic finds its way to our oceans every year.[5] When these materials enter our water, they disperse harmful chemicals that disrupt the ecosystems within the ocean. Among these plastics, polyvinyl chloride (PVC) has been identified as an extremely hazardous plastic due to the severity of its degradation effects. Upon degradation, it leaches chlorinated organic compounds such as organotin and dimethyltin into the water, which are substances known to be toxic to an organism upon ingestion or exposure.[6]

Polyvinyl chloride is created when the polymerization of initiator compounds and Vinyl Chloride Monomer (VCM) droplets occurs. This starts a chain reaction, which forms the basic monomer of PVC. After water is removed from this structure, polymerization can occur further, and the final product is formed. The even spread of hydrogen and carbon particles throughout common PVC products such as food packaging, water pipes, and medical devices makes it difficult for PVC to be degraded naturally.[7]

Researchers have investigated alternative pathways and conditions that can enhance the degradation of PVC. For example, photodegradation has been linked to the degradation of PVC, as scientists have found that the UV rays emitted by the sun can degrade the polymers in PVC. Certain experiments have even found a link between the degradation of PVC and increased heat, as thermal degradation studies have shown that PVC mass decreases under temperatures greater than 250 degrees.[8] The field of bioremediation has proved to be one of the most promising. Using bioremediation, scientists have investigated the possibility of using bacteria to break apart the chains within plastic structures. Considering the scale of this problem in oceans, this solution could be one of the most effective and feasible possibilities if properly executed.

Specifically, experiments such as the well-known trials with Anand Chakrabarty's superbug in 1971,[9] have demonstrated the possibility for a bacterial strain that could degrade different forms of plastic by simplifying the structures holding the plastics together. These experiments have advanced the possibility for a solution to the plastics within our oceans using bioremediation. In this instance, this experiment served as a steppingstone for other projects to investigate the effects of bacterium in trying to degrade the plastic that is present within the ocean waters. For example, in 2016, a great advancement within the field was made, as a group of scientists in Japan discovered the specimen *Ideonella sakaiensis* 201-F6 which was capable of degrading plastic using only the structure of plastic as its sole energy source.[10] It utilized an enzyme called PETase, which simplified the structure of the plastic and allowed for the degradation process to occur solely based on the structure of the plastic alone. Just a few months later, scientists improved upon the invention using genetic engineering, making the degradation process more effective.

Yet, even with thousands of experiments analyzing the possibilities of using bacteria to degrade plastic, an underlying problem has halted the possibility for these bacteria to be integrated into an aquatic environment: competition. When these bacteria are tested within a laboratory setting, researchers of-

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ten provide a constant supply of substrate within the agar the bacteria are cultured in, such as glucose or peptone to assist in the degradation process, which are not resources that are always available within a natural setting. Bacteria in our oceans meet an unprecedented level of competition from other bacteria. This competition from other bacteria limits their access to resources such as oxygen, an electron acceptor vital to the degradation process, which in turn halts the breaking down of the plastic.

This lack of resources has trumped the possibility of utilizing such bacteria that have evolved to possess characteristics to degrade PVC as these bacteria are not able to break down the plastic without access to these resources. Figure 1 demonstrates this by modeling the release of chloride atoms within bacterial degradation of plastic. This figure demonstrates the limitation of bacterial degradation within the natural environment by illustrating the drop phase, or the phase where release of chloride atoms (an indicator of plastic degradation) becomes stagnant.[11] This phase demonstrates the scenario where a result of a lack of natural resources, such as an energy source or an electron acceptor such as oxygen, stalls the plastic degradation process.

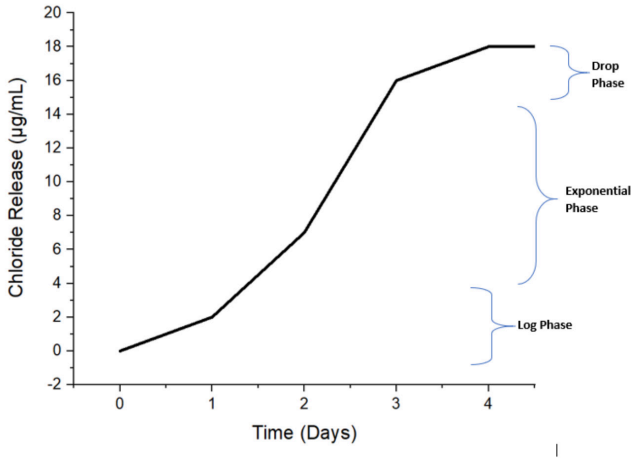

Figure 1. Shows the four phases of plastic degradation first introduced by Asha Yabannavar and Richard Bartha in 1993[12] modeling the limitations of bacterial degradation as a result of a lack of energy within the environment.

In relation, this project aims to modify the relationship that a bacterium has with its surroundings by introducing another specimen of bacteria to continuously supply oxygen, an electron acceptor vital to degradation, so that the bacteria capable of PVC degradation can overcome this drop phase even within the natural environment. *Pseudomonas fluorescens* is a bacterium that has been labeled as capable of degrading PVC plastic. However, without a presence of oxygen, it is not able to complete the process of degradation. *Anabaena* is a cyanobacteria, or a blue-green algae species, that produces oxygen as a byproduct. It requires carbon dioxide (CO_2) to produce oxygen. *Pseudomonas fluorescens* produces carbon dioxide as a byproduct of its natural processes. By coupling these bacteria together within a sample, we can effectively create a source of energy between the two that allows the *Pseudomonas fluorescens* bacteria to degrade plastic within our waters without interruption due to competition from surrounding microbial life within the water.

Therefore, we hypothesize for this experiment that if the cyanobacterium *Anabaena* and the soil bacteria *Pseudomonas fluorescens* are exposed to each other through a mixed culture, they will be able to more effectively degrade polyvinyl chloride (PVC) plastic than *Pseudomonas fluorescens* alone.

RESULTS AND DISCUSSION

In the collection of data, it was found that the total difference in weight was greater in the samples that contained both the *Pseudomonas fluorescens* and the *Anabaena* versus the *Anabaena* alone and the samples that did not contain bacteria. Differences between the mass of the PVC squares within each group *(Pseudomonas fluorescens* and *Anabaena, Pseudomonas fluorescens*, and no bacteria present) were present, but an overall trend was consistent between each group. This is demonstrated in Table 1, where the weight of the weight boat, the weight of the plastic before and after exposure, and total differences in weight of the PVC are listed.

Table 1. Demonstrates the weight of the PVC before and after exposure to bacterial strains after exposure to bacterial strains.

	Weight of Polyvinyl Chloride Cloth Squares (in grams)			
Bacterial Strain in Sample	Weight Prior to Exposure with Bacteria	Weight of Weight Boat	Weight After Exposure to Bacteria	Total Difference in Weight
Pseudomonas Fluorescens and Anabaena				
Trial 1	0.0276	2.8042	2.8308	0.0010
Trial 2	0.0301	3.235	3.2624	0.0027
Trial 3	0.0290	2.4674	2.4943	0.0021
Trial 4	0.0414	2.0835	2.1228	0.0031
Trial 5	0.0232	2.5059	2.5286	0.0005
Pseudomonas Fluorescens				
Trial 1	0.0431	2.6809	2.7227	0.0013
Trial 2	0.0301	2.4464	2.4749	0.0016
Trial 3	0.0272	2.6362	2.6622	0.0012
Trial 4	0.0284	2.7500	2.7770	0.0014
Trial 5	0.0272	2.5729	2.5988	0.0015
No Bacteria Present				
Trial 1	0.0302	2.7273	2.7567	0.0009
Trial 2	0.0281	2.9331	2.9596	0.0016
Trial 3	0.0416	2.8712	2.9122	0.0006
Trial 4	0.0283	2.9035	2.9303	0.0015
Trial 5	0.0272	2.7736	2.8001	0.0007

The first column of values demonstrates the weight of PVC prior to any exposure with bacteria. This value is then followed by the weight of the weight boat that the PVC cloth was placed within to mass it after it was exposed to the bacterium. The weight of the boat and the cloth together is also shown. The total difference in the last column shows the difference in the value of the weight of the PVC before and after exposure to bacteria. It demonstrates a change present in all samples. However, the change is much more visible in the sample containing *Pseudomonas fluorescens* and *Anabaena* versus the samples citing only the *Pseudomonas fluorescens* or no bacteria at all. For example, differences such as 0.0031 were

only present when both strains were present. Figure 3 adds to significance of these values by modeling degradation as a percentage of the initial weight.

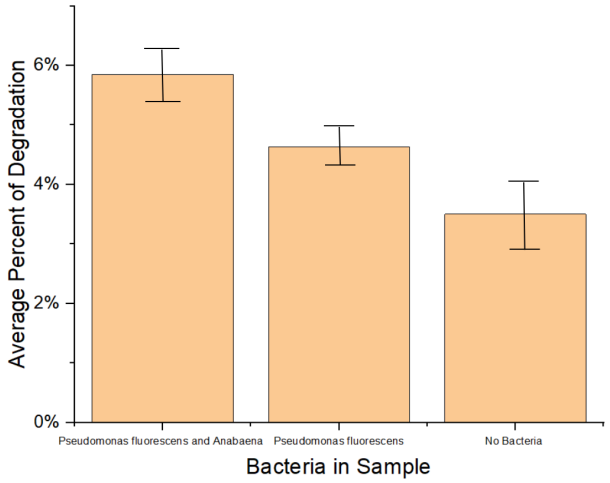

Figure 2. Demonstrates the changes in the mass of the PVC cloth during the experimental trials, calculated as the average of the 5 trials.

To truly acknowledge all possibilities of error within calculations, Figure 2 demonstrates the percent of degradation rather than just the difference in weight. Different weights of PVC could have increased or decreased rates of degradation (as PVC squares with greater surface area are more susceptible to degradation), and so the percentage of the degradation was a necessary addition. This model demonstrates that the percentage of degradation for both strains of bacterium (5.85% average rate of degradation) was constantly greater than the rate of degradation for just one strain of bacteria (4.6% average rate of degradation) and the percentage of degradation for samples without bacteria (3.5%). These differences between percentage of degradation are statistically significant. This was calculated using a two sample T-test where the p-value between a comparison of percentages of degradation between samples of the groups was calculated to by 0.00627, which was lower than the assigned significance level of 0.01. By adding this comparison, the hypothesis is supported further. Figure 3 attempts to test if possible confounding variables, such as the differing weights of the PVC squares used for each sample, could have significantly impacted these values.

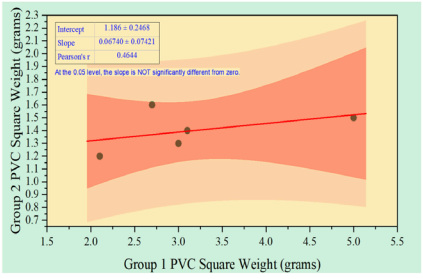

Figure 3. Models the average percent of degradation for different samples by dividing the starting weight of the PVC to the weight after exposure.

Additionally, to ensure the reliability of the results, an analysis to test how uniform the squares of PVC were, was necessary. If the weights of each square were not uniform, then the degradation rates could have been influenced by the different weight of the squares. To ensure this was not the case, a linear regression line analysis was done to demonstrate the uniformity between the weight of the PVC squares. This test aims to better understand if the differences between the weights of the PVC squares are statistically significant or not. If the differences in the weights of the squares are not statistically significant, then it demonstrates that the possibility of the differences between the weight of each PVC square being a confounding variable within the experiment is limited. However, if they are statistically significant, then the differences in the weight of the PVC squares could lead to changes in the percentage of degradation. The result of this analysis was that the differences between the weights of the different groups of PVC squares were not significant, as the differences of the weights yielded a p-value of 0.4464, which was greater than the significance level of 0.01. Therefore, the validity of measuring the difference of weight between the PVC squares remains, as the weights of the cloth itself will not cause a significant change within the results. Lastly, Figure 4 attempts to investigate if differing weights between the weight boats utilized could have significantly changed the results as well.

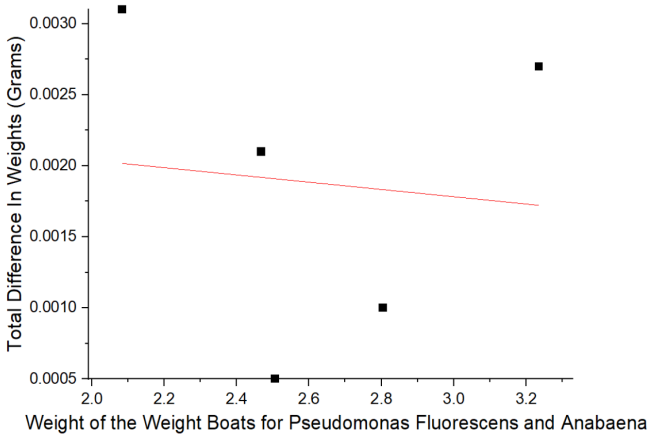

Figure 4. Demonstrates a linear regression analysis between the weight of the weight boats for the samples with *Pseudomonas fluorescens* and *Anabaena* and the total difference in weights (in grams).

A linear regression analysis between the weight of the weight boats and the total difference in weight was also conducted, as a test to see if the differences of the weight of each weight boat had any effect on the resulting difference in weight for the PVC squares. In the end, a value of r= -0.16 demonstrated that such a correlation was not present. Therefore, the weights of the weight boats did not impact the weights of the PVC squares; it was the degradation process itself that resulted in the changes in the plastic.

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DISCUSSION

Selection of Weight as a Factor to Measure Degradation

The mass of the individual PVC squares was utilized as the dependent variable to demonstrate the different rates of degradation for each sample. The weight of each sample should decrease as a result of the degradation of the phthalates that are a part of the industrial plastic structure. This degradation also releases chloride atoms as well, which furthermore supports the idea that the degradation of plastic leads to the loss of weight within each PVC square. Weight has been utilized as a measuring factor within several experiments testing degradation as well.[13] However, an addition of a quantitative way to measure the oxygen present in the tubes, such as the use of Durham Tubes, would provide a great deal if insight into the processes occurring in the test tube themselves. The study of the level of oxygen in the tube would demonstrate the activity of the *Anabaena*, which would produce information that would either better clarify the results in this experiment. Looking towards continuations of this project in the future, such a method will be utilized.

A Comparison of the Results of the Mixed Culture and the Pseudomonas fluorescens Strain Alone

Within the actual results of the experiment, it was evident that the culture that had contained each bacterium was more successful in degrading the PVC than the samples containing just *Pseudomonas fluorescens* alone or no bacteria at all. Within the actual measurements of the weight, an average difference in weight for samples containing both bacterium was 0.00188 milligrams (with a standard deviation of 0.0011 milligrams), the average difference in weight with one bacteria within the sample was 0.0014 milligrams (with a standard deviation of 0.00015 milligrams), and average difference in weight for no bacterial samples was 0.00106 milligrams (with a standard deviation of 0.0029). The differences between all three treatments were significant.

Statistical Analysis to Validate Results

First, to assess the reliability of the conditions set by the methodology of the experiment, significance values were tested between the PVC squares of each group. If the differences in weight between the PVC squares of the groups were statistically significant, then the results of the experiment would be invalidated, as the differences in mass of PVC squares across groups would interfere with the percentage of mass of degradation when compared to the original mass of the PVC square from group to group. The presence of an increased amount of PVC within samples could also lead to an increased level of degradation between certain samples. To ensure that this was not present within the experiment, a paired T-test was created to test the statistical significance of the differences. The null hypothesis stated that the difference of the two groups of PVC squares would equal zero, while the alternate hypothesis stated that the differences between the groups would be greater than zero. However, a p-value of 0.4464, which is greater than the assigned significance level of 0.01, led to the conclusion that the differences between the squares were not significantly different, and that the trials would not be impacted by the weight of the squares significantly.

Additionally, another paired T-test was created to analyze whether the results of the trials supported the hypothesis or not. The null hypothesis within the test was that the rate of degradation by the *Pseudomonas fluorescens* alone would equal the rate of degradation by the *Pseudomonas fluorescens* and the *Anabaena* together. The alternate hypothesis, therefore, was that the difference in the weight of PVC squares in the groups of both bacteria together minus the *Pseudomonas fluorescens* strain alone would be greater than 0. Since the p-value yielded, where p=0.00627, is less than the significance level of 0.01, the null hypothesis can be rejected, and the alternate hypothesis can be accepted. This proves that the rate of degradation exemplified by the mixed culture of bacteria was statistically significantly higher than the rate of degradation conducted by the *Pseudomonas fluorescens* strain alone, supporting the original hypothesis of the experiment.

To further show the validity of the methodology, a linear regression analysis between the weight of the weight boats and the outcome of the differences in weight in the samples containing both bacteria was conducted. It could be inferred that if such a correlation existed, then the weight of the weight boats could have influenced the differences in the weight to of the PVC squares due to the differing weight boats for each sample changing the mass of the measurements. Yet, within this test, it was shown that a value for r=-0.16 demonstrated that there was no correlation present between the two variables, and therefore, the differences in weight were a result of the degradation itself, not a result of the differing weights of the weight boats.

Importance of Results and Future Outlook

The results of this experiment are important to the issue of plastics ending up in our oceans because they provide evidence for the use of bacterial degradation as a possible solution. Bacterial degradation is integral in trying to rid our waterways of plastics as bacteria can use the structures of these plastic materials as energy and break them down naturally. This could lead to a massive cost reduction in our current efforts and allow us to apply this solution to all waterways, not just a specific area. The underlying problem with an approach involving bacteria has been the lack of energy that these bacteria can acquire when trying to degrade complex structures. This study shows that by coupling two strains of bacteria, we can supply a more plentiful source of oxygen for bacteria capable of degrading plastic. It allows these bacteria to degrade the plastic without nearly as much competition from the environment, which leads to a more efficient process of degradation. These findings could allow us to replicate the success that we have in laboratories with plastic degradation in our aquatic environments as well, which could be a huge step forward for the field of bioremediation.

In a wider context, this study is useful to national governmental agencies such as Environmental Protection Agency (EPA) as the agency has sponsored several different projects

to try to clean up the plastic debris within our oceans.[14] The application of research could potentially change the approach utilized within these projects, as rather than investing in large capture devices - as the EPA currently does for its local projects[15] the agency would be able to apply the projects to a larger scale and still cut costs with the application of these two bacteria. Such a fact would make the results relevant to people on a local scale as these bacteria are both biosafety level (BSL) 1, meaning with proper precaution they can be utilized by people other than just scientists and researchers. Local people could contribute to the project in a much bigger way if these solutions are implemented simply by investing in a possible product that uses the combination of these bacteria to clean up plastic waste within waterways. However, before such ideas are implemented, it is necessary to try to expand the scale of this experimentation. The use of these two bacteria to degrade plastic must be first tried within nature, as several experiments involving bioremediation to degrade plastic often demonstrate promising results within the lab but do not work within nature. It is necessary to scale up the number of samples within the trials as well as the time period that the plastic is exposed to both bacteria to better understand and interpret the results.

CONCLUSION

Using multivariate methods to analyze the results, a conclusion can be drawn stating that the hypothesis was supported by the experiment. Specifically, the statistical significance between the variables was less than 1% (0.0627% was the exact value), demonstrating that the correlation between the conjugation between both bacterial samples led to increased PVC degradation.

Additionally, beyond just the statistical significance, a substantive significance was shown in this experiment by projecting the data over time. Considering that the difference between both bacteria and just one bacterium was statistically significant in just a span of five days, the difference between the rates of degradation of both bacteria in the sample versus just one bacteria in the sample of the bacteria could continue to increase over time. This data modeled that the correlation between the variables was casual, or that the degradation level of both bacteria was a result of both working together on the PVC sample, not just the result of one bacterium. However, to further prove this, future trials will be conducted with a control group of *Anabaena* as well. This will assist in the understanding of the results within these trials, and better understand the roles that each bacterium plays in the degradation process.

Within this experiment, it was concluded that the combination of both strains of bacteria together could assist each other in overcoming the limitations of plastic degradation caused by competition in the environment. Looking towards the substantive meaning of this finding, one can state that if utilized properly in the future, the combination of both strains of bacteria together in one sample could open new doors for the bioremediation of complex plastics such as polyvinyl chloride.

Materials and Methods

Fifteen test tubes were required for this experiment, each with test tube caps to ensure that the oxygen produced is trapped within the tube. 500 ml of liquid agar broth was made and was stored in a 37-degree Celsius freezer to ensure that the agar allowed for the growth of both bacteria. Two strains of bacteria, *Pseudomonas fluorescens* and *Anabaena* were used, each of which was ordered from Carolina Biological. A PVC tablecloth was used because it was made of 100% polyvinyl chloride. An autoclave and a 70% ethanol solution were used to sterilize all substances that were touched by the bacteria. A vortex mixer was used to continuously mix the strains while they were degrading the PVC to ensure that the samples of bacteria interacted with each other. Finally, the experiment was conducted in a Biosafety level 1 laboratory under a laminar hood to prevent any possible airborne contamination.

Methodology

Using sterilized scissors, the PVC bath cloth was cut into squares of length 1 cm x 1 cm to obtain a total of 15 squares. The PVC cloth squares were then disinfected by rinsing them with ethanol and allowing them to sit for 5 minutes. Then the sheets were labeled by writing the letter "B" on the first five sheets, "O" on the next five, and then "N" on the last five. Each group of five sheets was numbered from 1-5 randomly. Then, the mass of these squares was measured on the analytical balance. After massing, the PVC sheets were placed into an airtight bag using autoclaved forceps.

Next, using a bulb pipette, 13 mL of liquid agar broth was pipetted into each tube. At the end of this procedure, 15 test tubes containing liquid agar broth were present, and the test tube caps were placed on the tubes.

The first five test tubes were labeled with the letter B (which represented the sample for "both strains") and then a number from 1-5 depending on the order that tube was picked up. The next five were marked with the letter O for "one strain" along with numbering from 1-5 and then the next five.

Next, the inoculating loop was flamed over the Bunsen burner to ensure the removal of the contaminants. The loop was placed within the bacterial vial containing *Pseudomonas fluorescens* and used to scoop a portion of bacteria from the test tube. This process was repeated until all test tubes with the letter "O" contained the *Pseudomonas fluorescens* bacterium.

Then, the metal inoculating loop was flamed once more and used to pick up bacteria from the test tube containing "*Pseudomonas fluorescens*". This bacterium was added first to the tube labeled "B1". Next, the loop was flamed once more used to pick up a small portion of bacteria from the "*Anabaena*" vial. The inoculating loop was also added to the test tube labeled B1 and stirred. This process was repeated until all test tubes with the letter B contained both samples of bacterium.

The last five test tubes were not exposed to any bacterial samples since these samples would serve as controls for the experiment.

After, the test tube caps of all fifteen test tubes were removed, and 1 PVC cloth square was added to each tube, depending on the number and letter was labeled as. For example, the square with the labels "B1" was added to the test tube labeled "B1" and so on. All the test tube caps were placed back on after the squares were added, and the test tubes were placed within a

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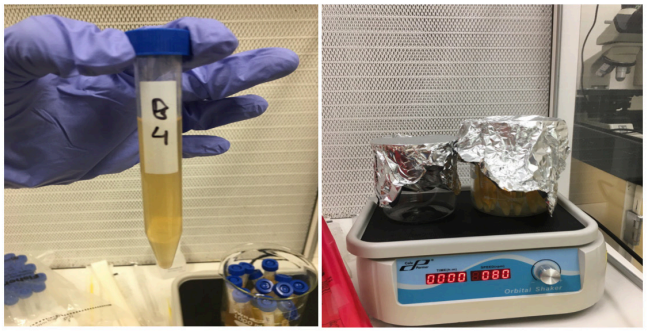
beaker and covered by an autoclaved piece of foil. The beaker was then placed on the vortex machine, and vortexed at a speed of 100 rpm-for 5 days, as demonstrated within Figure 2.



(a) (b)

Figure 2. Shows a plastic tube incubated with both strains of bacteria (a) and shows the experimental setup used to vortex the samples (b).

After 120 hours, the test tubes were removed from the beaker covers. Using another pair of autoclaved forceps, all 15 PVC cloth squares were removed from each tube and placed in a 70% ethanol solution. After washing and letting the sheets dry, each sheet was massed.

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