Identification of Organophosphate Degrading Bacteria in High-Input Peanut Production Fields

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Introduction

As the global population continues to grow, pressure is put on the food supply chain to produce more calories from less land. In response, farmers and ranchers have increased their usage of chemical pesticides, fertilizers, and herbicides. In 2020, there was an estimated global use of 3 billion kg of pesticides. [1] While the use of these chemical inputs often increases crop yield, it also can lead to adverse environmental effects and health effects including groundwater contamination, high levels of pollinator death, many cancers. The active ingredients in many pesticides used today and in the recent past are organophosphate containing molecules and are known for their high toxicity which can be achieved by inhalation, ingestion, and dermal exposure. Organophosphate pesticides have also gained historic fame from use in wartime as nerve agents and achieved their peak usage in agriculture shortly after World War II. [2]

With such widespread and continuous usage of these compounds, there is a need for novel methods of their remediation from our water and soils. The method of interest of this research is bioremediation, the use of microbes to degrade chemical contaminates into more inert forms. This work looked to identify soil bacteria, collected from agricultural land regularly treated with organophosphates, with the ability to metabolize organophosphate groups for use in the treatment of contaminated soils.

Materials & Methods

The soil used in the experiment described herein was obtained from an active peanut production plot maintained by the Stephenville, Texas A&M AgriLife Research and Extension center. The plot is a known high-input area which receives consistent applications of chemical fertilizers, antifungals, and glyphosate (also known as Roundup). Soil was sampled in five sets all within less than a five-meter radius of one another and mixed to obtain a representative sample. Each soil core was taken at approximately one foot below the surface of the soil. The geographic coordinates for the collected sample were near 32.2491592 N, 98.1971305 W. It should be noted that an accompanying sample was taken by a peer along a nearby fence line which also receives consistent glyphosate applications. This accompanying sample was processed in an identical manner as the one described in this work and the results of which are discussed further in its associated work by Pennington, A.

Figure 1: A satellite image of the collection sites for the peanut field soil (red) and the associated fence line soil (blue).

Following sample collection, the soil was stored at ~ 40 ^{o}F for nearly a week prior to the inoculation of 1g of sample in LB broth at 37 ^{o}C for one week. The inoculate was then diluted in a 1:5 ratio into sets of sulfur selective media (SSM) and carbon selective media (CSM) with two solutions each containing either paraoxon or methyl parathion and further cultured for another week.

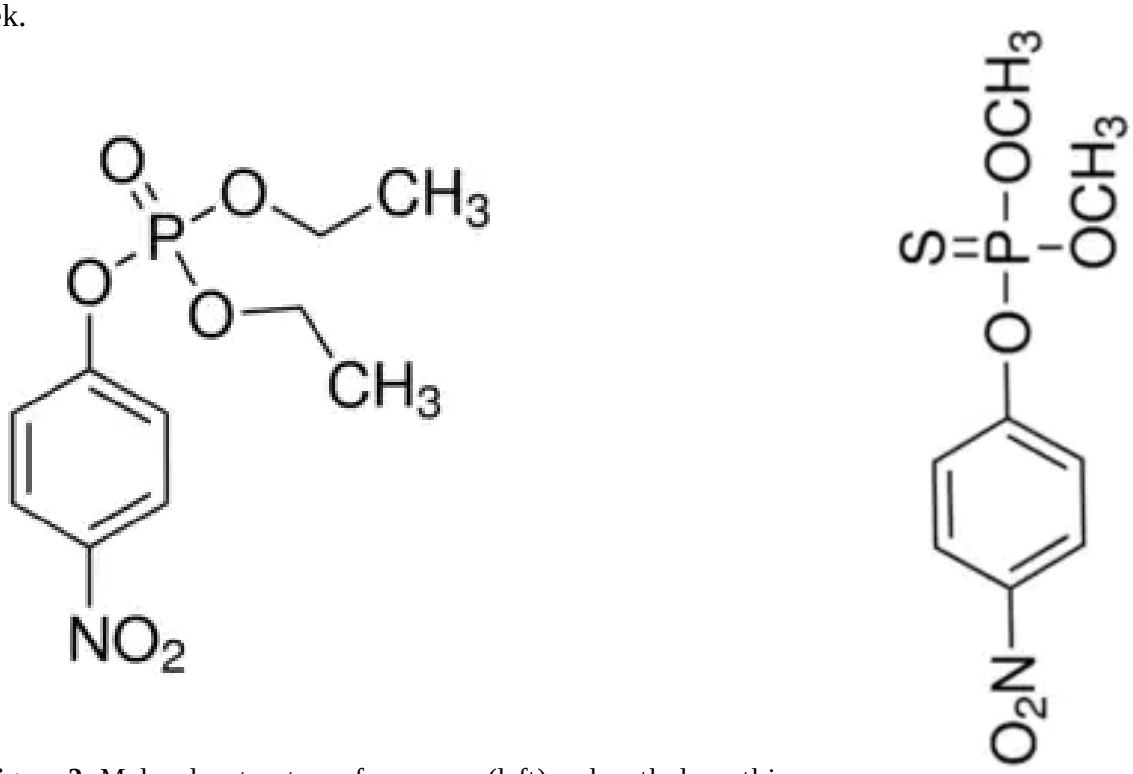

Figure 2: Molecular structure of paraoxon (left) and methyl parathion (right) and [3]

Then, 0.2 ml of each solution was removed and added to a 5 ml solution containing the same selective media and organophosphate containing compound as the previous vial along with p-nitrophenol as an indicator of organophosphate cleavage. Note that p-nitrophenol changes from colorless to yellow at a pH of 7.5. Again, these new samples were incubated for a week before the transfer of 0.2 ml of media to a new 5 ml tube was repeated for a total of four weeks and pictures obtained for each incubated batch of samples.

The following week, 1 ml was taken from final samples with obvious yellow colors to be added to 4 ml of LB revitalization broth. These samples were then incubated before being plated onto minimal media plates and colony counts obtained using a hemocytometer. Following the data collection, statistical analysis was used to determine the relationship between successful growth in SSM versus CSM medias and the results of students in different disciplines.

With regards to lab safety, caution was taken to avoid contact with either of the organophosphates and the p-nitrophenol indicator due to their known health hazards. Paraoxon and methyl parathion are both GHS classified as acutely toxic and environmentally hazardous compounds. Methyl parathion is also classified as flammable and a health hazard with GHS hazard statements H330 and H300 stating that it is fatal is swallowed or if inhaled. P-nitrophenol is also classified as an irritant and a health hazard. [4] It also is known to cause a delayed reaction in the blood with potential health effects such as cyanosis, brain fog, cataracts, and unconsciousness. [5] All compounds were handled under a biosafety cabinet and with gloves always worn.

Results & Discussion

Following the fifth week of incubation in the SSM and CSM medias, the CSM paraoxon sample was identified as having a strong yellow color and was revitalized, plated, and counted. The SSM paraoxon and both the SSM and CSM methyl parathion tubes were not selected for further testing as they were viewed as not having sufficient yellow color to indicate successful bacterial culture. The revitalized inoculate of CSM paraoxon was cultured and the colony forming units measured to be 3.50×10^7.

Figure 3: LB broth containing 1g of soil collected from the peanut field.

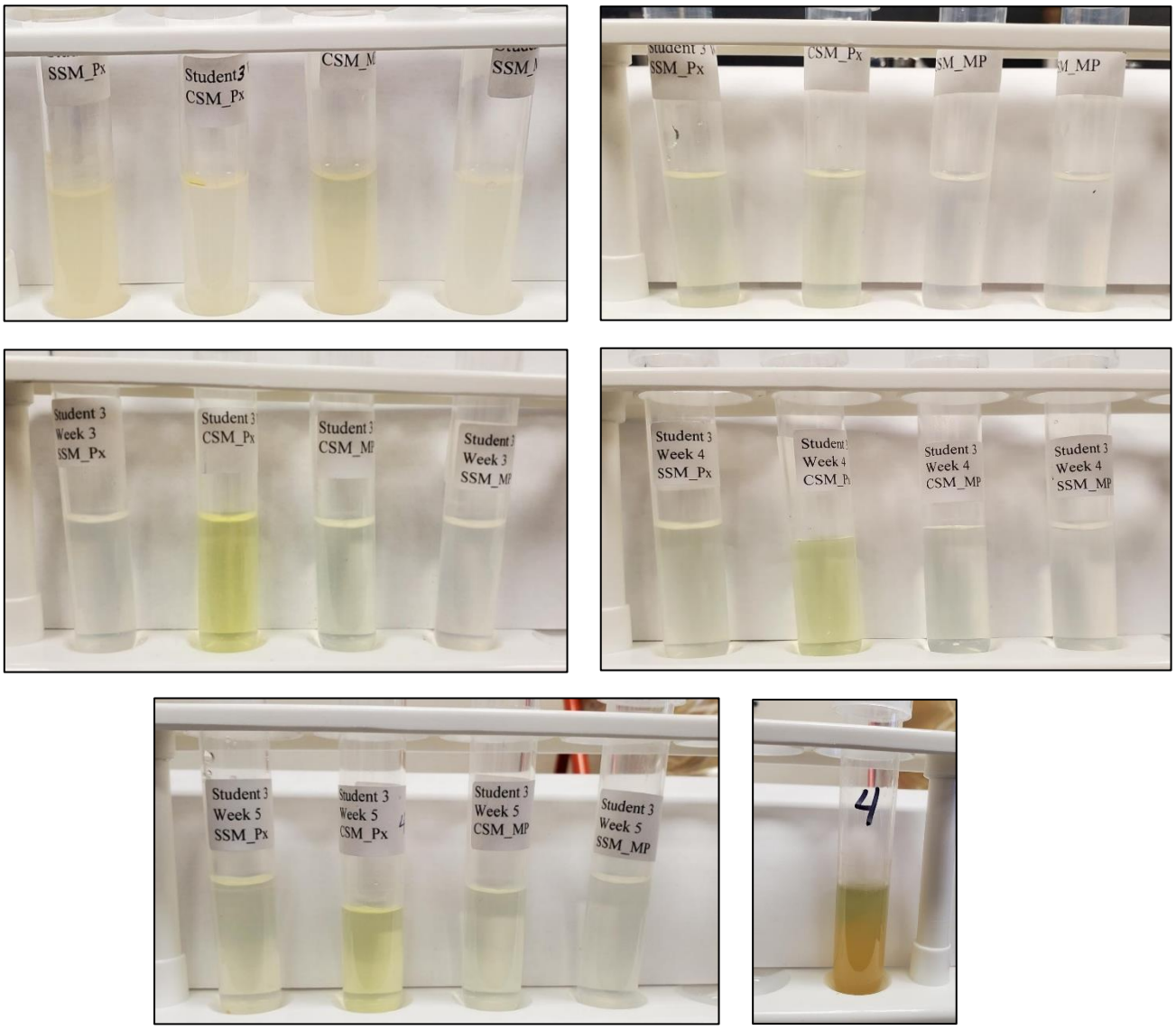

Figure 4: Selective media inoculations with organophosphates. Week 1 (top left), week 2 (top right), week 3 (mid left), week 4 (mid right), and week 5 (bottom left). Revitalization of CSM paraoxon sample after incubation (bottom right)

	SSM_Px	**CSM_Px**	**CSM_MP**	**SSM_MP**
Week 1	Med. yellow	Light yellow	Med. yellow	Light yellow
Week 2	Light yellow	Light yellow	Clear	Clear
Week 3	Clear	Dark yellow	Clear	Light yellow
Week 4	Light yellow	Med. yellow	Clear	Clear
Week 5	Light yellow	Med. yellow	Clear	Light yellow

Figure 5: Results of solution color observations for all five weeks of selective media inoculations

Statical analysis was done with the compiled classroom data using Microsoft Excel. A comparison was done between the data collected by biology and biomedical science majors and students with majors in other fields. A students t-test was done to determine if there was a significant statical difference between the two groups. The results of the t-test yielded a value of about 0.519, over ten times that of the limiting value for statical significance. Because 0.519 >> 0.05, the t-test suggests non-distinct sample groups meaning that the results gained by the BIMS and other majors are not skewed and can be considered statically undifferentiable. A second t-test was calculated to compare results between the SSM and CSM solutions. This test yielded a value of about 0.011. This result, being less than the limit of 0.05, suggests that the two data groups of SSM and CSM medias are statically distinct. There is uncertainty in the validity of these results due to the relatively small data set for SSM cultures compared to the CSM results.

SAMPLE #	STUDENT #	MP OR PX	CSM OR SSM	GROWTH IN LB	CFU COUNT	BIMS V OTHER
1	1	Px	CSM	YES	6.20E+07	BIMS
2	1	Px	SSM	YES	4.00E+04	BIMS
3	2	Px	SSM	YES		
4	3	Px	CSM	YES	3.50E+07	Other
5	5	Px	CSM	YES	1.22E+08	Other
6	6	Px	CSM	YES	3.40E+08	BIMS
7	6	Px	SSM	NO		
8	8	Px	CSM	YES	2.86E+06	BIMS
9	8	Px	SSM	YES		
10	9	Px	CSM	YES	2.25E+04	BIMS
11	10	Px	CSM	YES	7.20E+07	BIMS
12	10	Px	SSM	NO		
13	11	Px	SSM	YES	2.80E+02	Other
14	11	Px	CSM	YES	3.20E+08	Other
15	12	Px	CSM	YES	1.22E+08	Other
16	13	Px	CSM	YES	6.00E+06	BIMS
17	13	Px	SSM	NO		
18	14	Px	CSM	YES		
19	15	Px	CSM	YES	1.76E+08	Other
20	16	Px	CSM	YES	3.00E+06	Other

Table 1: Compiled data from students with successful, dark yellow colored, inoculation samples

BIMS	OTHER
2.25E+04	3.50E+07
4.00E+04	2.80E+02
2.86E+06	3.00E+06
6.00E+06	1.22E+08
6.20E+07	1.22E+08
7.20E+07	1.76E+08
3.40E+08	3.20E+08
t-test	**0.519312**

CSM	SSM
6.20E+07	4.00E+04
3.50E+07	2.80E+02
1.22E+08	
3.40E+08	
2.86E+06	
2.25E+04	
.20E+07	
3.20E+08	
1.22E+08	
6.00E+06	
1.76E+08	
3.00E+06	
t-test	**0.011041**

Table 2: Compiled data and results of the t-test calculations

In support of the t-test results, however, is the lack of sulfur atoms present in the molecule paraoxon. Because of this, a microbe should not be able to obtain essential sulfur from decomposing paraoxon and therefore be unable to survive and reproduce in the SSM media. This suggests that the SSM Px samples grew bacteria by means other than those in the scope of this research and are considered outliers or errors. Genetic identification was not completed for this dataset and therefore, the species of the surviving bacteria are unknown as well as their status of discovery.

Conclusions

It is reasonable to conclude that the CSM paraoxon sample had successfully cultured some paraoxon degrading bacteria after the five week process of successive inoculations. This is not only due to its visible yellow color, but also the bacterial count, 3.50×10^7, many magnitudes above that of the SSM sample growths which can reasonably be considered errors. This error classification is supported by the results of a t-test comparing the SSM and CSM sample groups, which yielded a value of 0.011, and the lack of obtainable sulfur in the paraoxon molecule.

The lack of methyl parathion samples with successful bacterial growth was seen throughout the class-compiled data. One possible explanation for this is derived from the higher aqueous instability of paraoxon vs. methyl parathion [6]. Because paraoxon breaks down more readily in aqueous solutions, it would be reasonable to hypothesize that rather than breaking down the organophosphate group to obtain carbon molecules, the bacteria instead metabolized the already degraded form in solution. In comparison, methyl parathion has higher stability in aqueous solutions and would be less available in its already degraded forms for bacteria to consume. This hypothesis is somewhat likely in that the broth used to culture the bacteria is

designed to grow gram-negative bacteria specifically of the Enterobacteriaceae family which are only a small part of the soil microenvironment and only encompass a small part of the metabolic niches found in the soil ecosystem. Given this, it should be concluded that until greater evidence for the successful degradation of organophosphate groups by the bacteria cultured in the CSM paraoxon samples is obtained, that there is no significant reason to believe that the experiment produced any bacteria with the unique ability to degrade organophosphate containing molecules.

References

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[3] "Sigma Aldrich", *Millipore Sigma,* Merck KGaA, Germany, 2022.

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[5] *Toxicological Profile for Nitrophenols*, Agency for Toxic Substances and Disease Registry, U.S. Department of Health and Human Services, 2022.

[6] Bharate, S., et. al., *Thionate versus Oxon: A Comparison of Stability, Uptake, and Cell Toxicity of* $(^{14}CH_3O)_2$*-Labeled Methyl Parathion and Methyl Paraoxon with SH-SY5Y Cells*, J Agric Food Chem, 2010.