Introduction

Big industrial companies produce large amounts of biological waste, these wastes include dyes, agricultural waste, metals, and plastics such as polyethylene. Polyethylene is a major contributor to efficants and one of the most common plastics found in waters. To combat effluents, photoautotrophs, in this case, cyanobacteria (blue green algae) have been introduced into freshwater ecosystems where the wastes are found. Although Cyanobacteria have been successful in breaking down products such as Polyethylene and agricultural runoffs by turning them into digestible biomasses through the use of hydrolase, they have been known to disturb the ecosystem through overpopulation.

In order to solve this issue, we decided to create an edible PETase producing photoautotroph. *Pseudohongiella spirulinae,* can provide the mostly same function as cyanobacteria while being edible to fish. However, PET ase, a type of hydrolase that breaks down polyethylene through hydrolysis, is not found in spirulina. In order to solve this issue, we plan on inserting the hydrolase gene into the edible spirulina for natural regulation of the bacteria. On top of using an edible strain, we decided to add another feature that stimulated natural predators. Fish tend to be attracted to light due to light fragmentation in the water and will explore such objects orally. In order to promote such interaction with the bacteria, we added two proteins known as luciferin and luciferase. Luciferin breaks down to produce light in the presence of oxygen and luciferase, they are found in algae known as Dinoflagellates. Due to spirulina's ability to photosynthesize, an overpopulation of spirulina will oxygenate the water allowing for the edited spirulina to produce stronger light and cause greater amounts of predator interaction. When we ran BLAST, we found that the PETase gene found in some Cyanobacteria is similar to the Streptomyces griseoviridis strain K61 and the luciferin and luciferase genes seem similar to Chromosome 2 on humans.

Vectors

Selective marker: We chose ampicillin resistance as our selective marker. This marker is commonly found on most plasmids and ampicillin is easily obtainable so we can test to find our plasmid quickly and very easily.

Ori:We chose the ColE1 ori sequence which is a high copy number Ori, we want as much of our plasmid being created so we can create as much PETase as possible. We also aim to spread the machinery of the bacteria thinly, it will be busy with high production of our PETase gene so we hope this will in turn slow down the general growth of the bacteria.

MCS:The MCS we used was partially taken from a previously created MCS we found but we edited it so it would have more restriction sites that could match up with some sites found in the base pairs near our insert.

Special Features

Promotor: Phage Lambda PH promoter:The phage Lambda PH promoter is a promoter with a mutation that allows it to create more copies of its gene. This promoter was selected because as mentioned previously, we want as much PETase being produced as possible so we can facilitate the degradation of lots of polyethylene and overexert the bacteria.

Localization signal: We chose the N-Terminal sequence because it is the localization signal for the sec pathway. The Sec export pathway is found in nearly all bacteria including *Pseudohongiella spirulinae.* The N-Terminal sequence produces a protein that tags other proteins meant for export. In our plasmid, the N-Terminal sequence will produce a signaling protein that will tag the PETase which, in turn, enables the export of the enzyme.

Terminators: T7 RNAP terminator-The T7 RNAP is a standard terminator, it doesn't have any special features but it is an efficient RHO-independent terminator sequence which is the main feature we looked for in a terminator.

Ribosomal Binding Motif: Shine dalgarno sequence-The shine Dalgarno sequence is a ribosomal recognition sequence which will allow the bacteria's ribosomes to bind to our plasmid and transcribe our desired insert.

Insert

Luciferin and Luciferase:These two are proteins which together create a fluorescent effect. By adding this into our desired bacteria, fish will be more attracted to the spirulina and will eat more of it than they would otherwise.

PETase gene:The sequence encoding for the PETase gene will allow the Spirulina to breakdown polyethylene which it otherwise wouldn't be able to do.

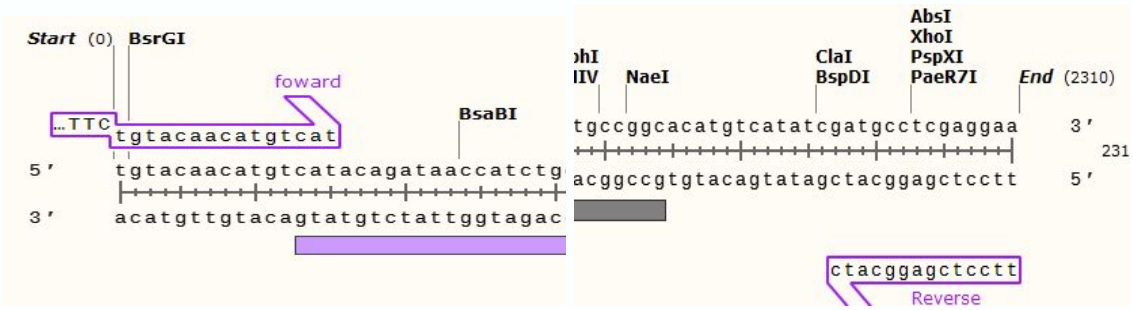
Primer Design:We added 2 priers to our initial insert. One primer was at the very end of our insert, after the terminator sequence, this primer was meant to allow us to PCR our product. This primer was made up of 14 bases with a 57% GC content to ensure that it would be effective. Our second primer which was located at the very beginning of our insert and was made up of 15 annealed bases and a 33% GC content which is lower than we would have liked but was still effective.

Restriction enzymes: For our insert and plasmid we cut with EcoR1 AND Xho1.

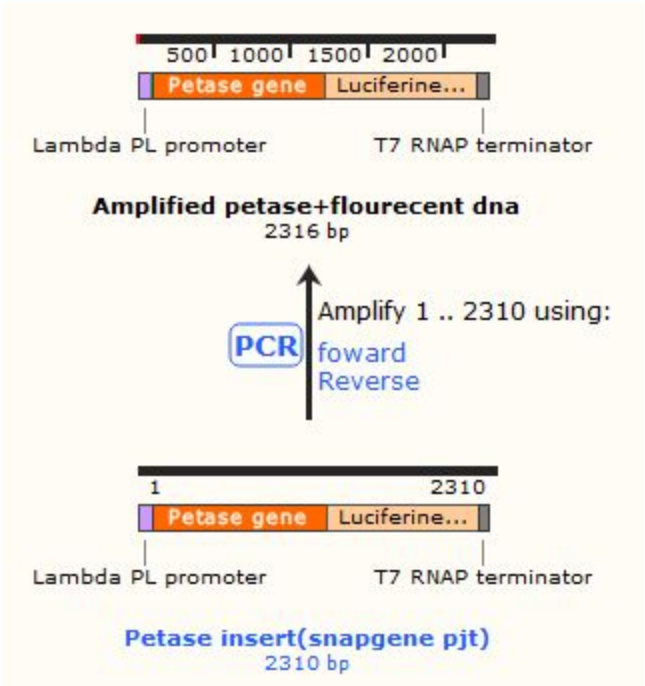
Restriction sites: At first, our forward primer did not have an EcoR1 restriction site so we added an overhang sequence which could be cut by the enzyme and when we PCRd our insert it was present.

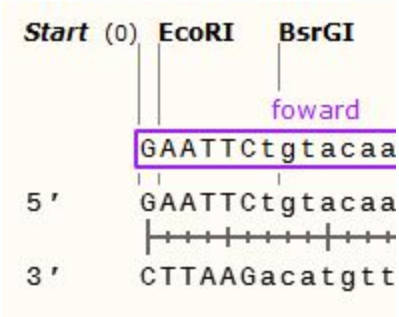
Procedure

1. Add the primers into our original insert and add in a restriction site for EcoR1 in our forward insert.



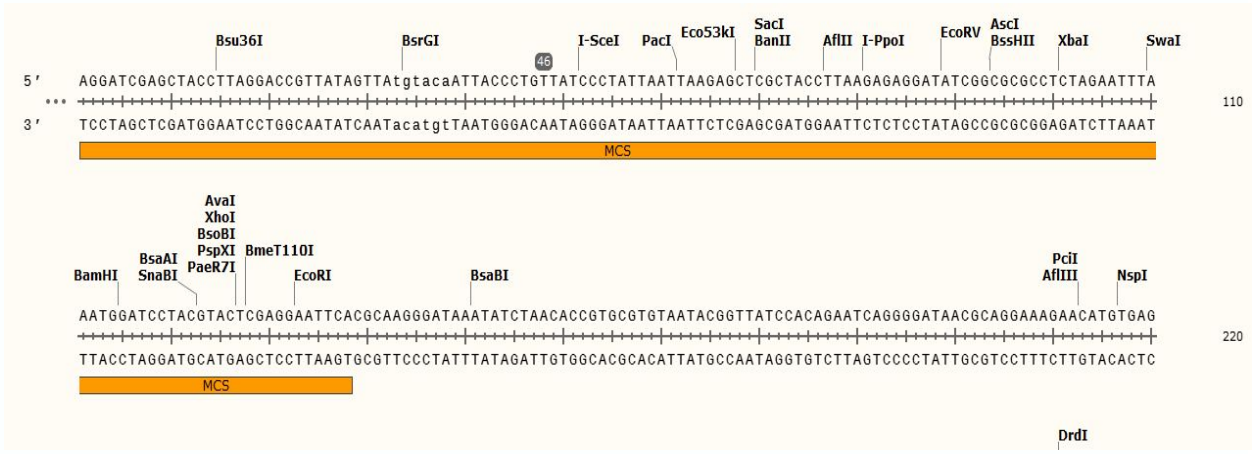
2. We then PCRd our insert and were left with an insert with our gene, our necessary sequences, and restriction sites for EcoR1 and Xho1.



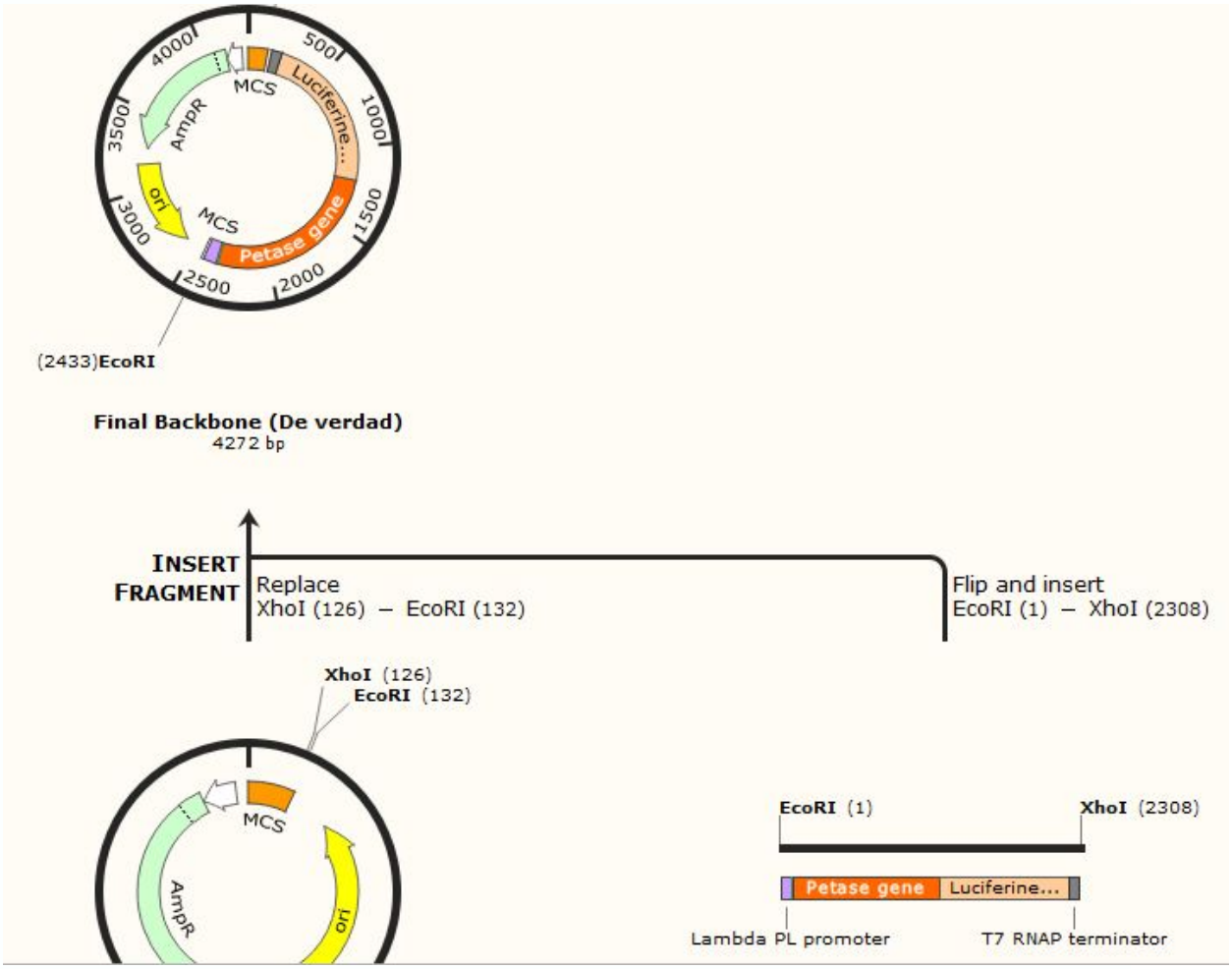


3. Once we had our insert ready, we began to modify our Plasmid. We used a plasmid that already had an ampicillin resistance gene and a high copy number Ori but we needed to insert an MCS so we wouldn't disturb the plasmids genes and so we could have the restriction sites we needed.

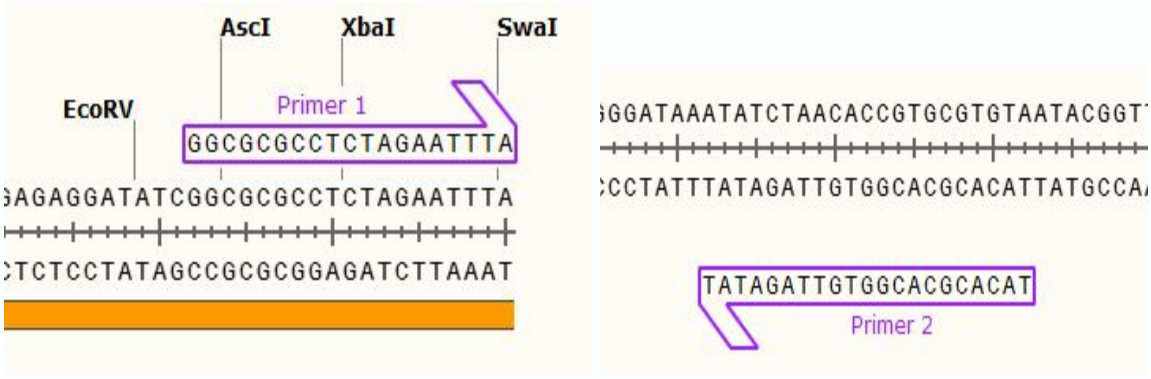
Sidenote: to modify our MCS we deleted and added bases to fit our needs.



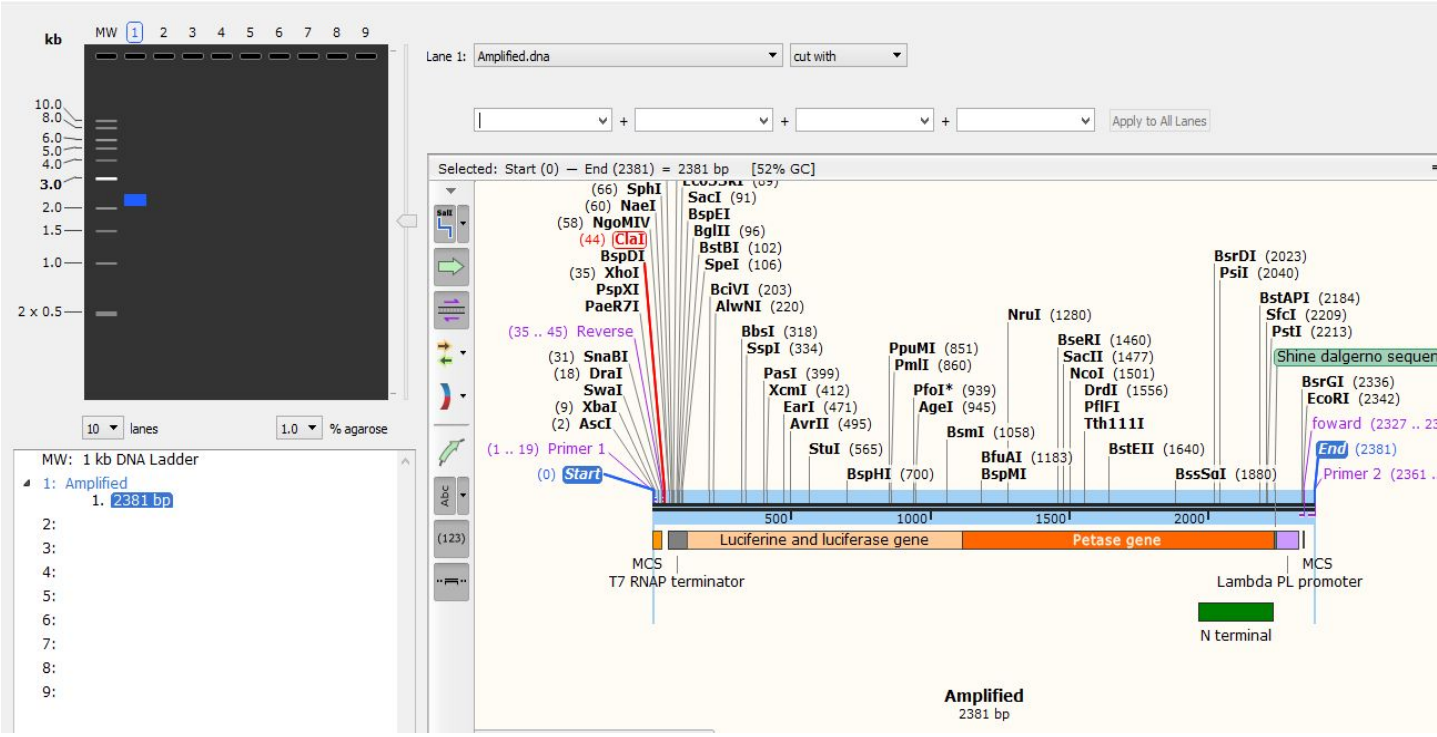
4. Once both our Insert and our plasmid were prepared, we inserted our gene into the plasmid by cutting both with the two restriction enzymes we had selected. We inserted our gene into the MCS of the plasmid through restriction and insert cloning.



5. To confirm the insertion into our plasmid backbone, we added additional primers onto our backbone and PCRd out a segment of the plasmid containing our insert.



6. Once we had our PCR fragment, we ran a mock gel electrophoresis to confirm the base pair numbers and found that the plasmid had indeed taken the insert.



Proposed Experimental Methods

To test if the insert is viable, we will collect the following data points:

1. PE levels in the water over time

2. Colonies of cyanobacteria VS. Spirulina over time

In order to do so, we can take a culture of cyanobacteria (positive control group), unaltered spirulina (Negative control), and the edited spirulina (experimental group) and add each to a sample of wastewater then measure the data points stated above. If our insert was viable, the experimental group should break down polyethylene while reproducing less than the negative control.

	Fluorescent	Colonies	PE level over time
Cyanobacteria	No	n_1 colonies	Decreasing
Spirulina (unaltered)	No	n_2 colonies	Constant
Spirulina w/ insert	Yes	<cyanobacteria and unaltered spirulina	Decreasing

Conclusion

The issue of cyanobacteria and ecosystem disruption due to overpopulation during treatment of industrial effluent can most likely be solved by using an organism that can be regulated naturally within the ecosystem such as spirulina which can become a food source for plankton, fish and other organisms in the ecosystem. However, further studies must be done in order to understand the interaction between the strain of spirulina and different ecosystems. Although spirulina is technically edible, if introduced into unfamiliar ecosystems, native organisms may not recognize it as a food source causing unregulated reproduction. Although this solution is incomplete without additional field study, other researchers should still look into modifying the genes of organisms either already existing in the ecosystem or one that can be regulated naturally within the ecosystem that is being treated for more eco-friendly bioremediation purposes.

References and Resources:

*https://www.sciencedirect.com/science/article/pii/B978012819311200022X
*https://www.omicsonline.org/open-access/biodegradation-of-polyethylene-by-green-photosynthetic-microalgae-2155-6199-1000381.php?aid=85448
*https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3430566/
*Plasmid Burden:https://www.addgene.org/mol-bio-reference/
*Fish attraction to light:https://www.underwaterfishlight.com/best-light-color-attract-fish/