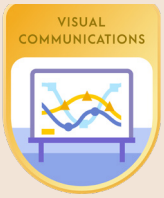
Engineered *Escherichia coli* with estA Gene Produces an Esterase to Break Ester Bonds Between Fatty Acids and 4-Nitrophenol



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Sewer systems contain a number of different solid materials that cause blockages and restrained waste water flow. Fatty obstructions, solid materials in sewer systems, are one of the major prevalent issues. These obstructions, known as 'fatbergs', are clumps of non-biodegradable solid matter held together by solidified fats. Currently, these obstructions are removed by machinery, which has a cost. In addition, the machines experience regular wear and tear, leading to increased maintenance costs. The mechanical breakdown of 'fatbergs' is a temporary solution as lipids resolidify later on during the water treatment process. To reduce the probability of fat reformation, this project uses genetically engineered *Escherichia coli* to produce an esterase enzyme which cleaves the ester bond within triglycerides present between the glycerol and fatty acids. For testing purposes, the compounds of 4-nitrophenol with two different lengths of carbon chains (4-nitrophenyl octanoate and 4-nitrophenyl palmitate) were introduced to the engineered *E. coli*. When the ester bond existing in 4-nitrophenol was broken by the esterase, the compound turned yellow. Both the visual and spectrophotometry tests revealed a more significant color change with the 4-nitrophenyl octanoate. This was due to the esterase being better suited for shorter carbon chains. For implementation into the wastewater treatment system, the plan is to incorporate the bacteria into the current fat removal process. Once the fat is isolated, it will be inserted into a bioreactor containing the *E. coli*. After the lipids have been broken into their chemical components of glycerol and fatty acids, these parts could be re-introduced into the water purification process with a reduced likelihood of reforming as the activation energy required in order to reform that ester bond is higher. In this case, the reaction of breaking the ester bond is favoured. Future directions of the project include: adjusting the promoter to be more suitable to the wastewater treatment system, possibly turning the results of lipid degradation into a biofuel and adding a lipase component better suited for longer carbon chains.

Keywords: *Escherichia coli*, esterase, fatbergs, 4-nitrophenyl, triglycerides

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Background

With the rapid surge in urban development and population growth, the availability of freshwater is becoming a major problem. Big cities have already established wastewater treatment processes; however, many individuals do not realize the impact of what they are putting into these systems. These individuals usually have limited information on what it takes to turn their waste into clean and safe to use water. One of the most extensive issues within wastewater treatment systems is the accumulation of non-biodegradable substances, which are held together by congealed fats due to the cooler temperatures within the pipe network. This problem has occurred in sewers of large cities such as Kingston, England, where a 15 ton fatberg was found in 2013 (Curran and Thomas 2015). Small towns such as High River, Alberta, Canada also experiences the issue of sewer blockages due to obstructions caused by fats, oils, and greases. Our synthetic biology team has witnessed the buildup of lipids firsthand, through visiting the High River Wastewater Treatment Plant and speaking with local wastewater representatives.

The current practice to remove fatty blockages is by using a machine with pressurized air to fragmentize the solidified fat (Craigie and Lund 2018). Once this is accomplished, the particles are inserted back into the sewage channels. However, the issue with this method is that the fat particles can still clump back together, creating a repetitive cycle of breaking down the fat and having it reform into blockages. This method is both expensive and inefficient for wastewater treatment facilities. There is a substantial costs associated with maintaining the equipment used to facilitate the manual break down of lipids. Jason Craigie and Eugene Lund of the High River wastewater treatment plant estimated that the costs to maintain removal equipment each year is between 5,000 and 10,000 dollars (Craigie and Lund 2018).

Further research on this problem revealed that bacteria *Escherichia coli* could be genetically engineered to produce an enzyme that is capable of chemically breaking down lipids. The breakdown occurs by cleavage of the ester bond between the glycerol backbone and the three fatty acids, both of which make up a fat molecule. Our synthetic biology team specifically targeted short chain triglycerides by using an *estA* gene (Dodson 2007) from the organism *Pseudomonas aeruginosa* that produces the esterase enzyme. Breaking down longer chains of fatty acids required a lipase enzyme. As a highschool synthetic biology team, the *estA* gene was a better starting point so it was decided to specifically target shorter chains of fatty acids.

When triglycerides are chemically broken down, reformation is less likely to occur. In this, if introduced when fatty obstructions are first isolated, it may reduce the efforts needed to clear fatty obstructions, and as a result, reduce the cost for operating the corresponding machinery.

In order to confirm that the genetically engineered *E.coli* was performing its designed task, it was tested on a compound similar to triglycerides. The chemical 4-nitrophenyl is similar to triglycerides, both of which contain ester bonds. However, the ester bond in 4-nitrophenyl links a single fatty acid chain, instead of the three fatty acid chains that are linked by ester bonds in triglycerides. This fatty acid is attached to a color ring that turns a yellow color when the ester bond is broken. This is useful for testing as it affirms the cleavage of the ester bond if the color were to appear. The wavelength of light (400 nm) can be measured using a spectrophotometer.

Part Design

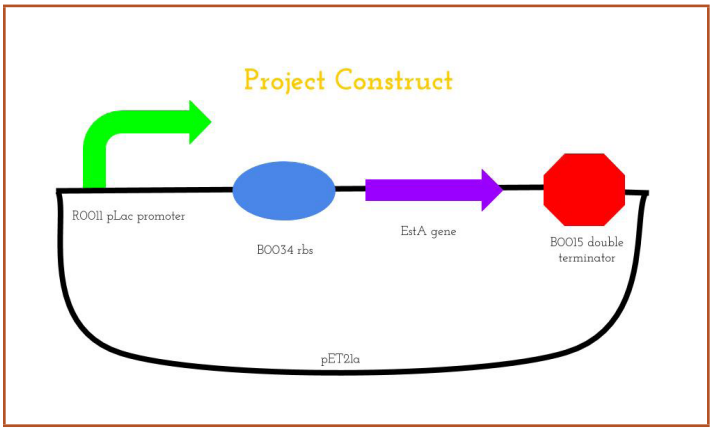

Figure 1. *The project construct, including the pLac promoter, ribosome binding site, Est A gene, and double terminator.*

For the promoter we used a pLac promoter (Part:BBa_B0011), which is Isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible and is naturally found in *E. coli*. Using an IPTG-inducible promoter allowed for the testing and comparison of differences in gene expression with and without IPTG.

We used a standard ribosome binding site, B0034 (Part:BBa_0034) and the double terminator B0015 (Part:BBa_0015). The gene we used is an esterase-producing gene called EstA, which originally comes from *pseudomonas aeruginosa* (Part:BBa_2694001). For our terminator, we chose the double terminator B0015, as it is known to be reliable. (Part:Bba_B0015).

Originally, our plasmid was synthesized on the plasmid backbone pET21a, and that was the backbone we used in our testing. However, we transformed our plasmid onto the backbone pSB1C3 to submit it to the iGEM registry. Our team has submitted two parts to the iGEM registry. One of these parts is the EstA gene (Part:BBa_

K2694001) that is a part of our construct, and the other is the composite part which includes our entire construct (Part: BBa_K2694000).

Materials and Methods

Proof of Concept Protocol with 4-Nitrophenyl Octanoate and 4-Nitrophenyl Palmitate

The purpose of the initial testing was to qualitatively observe whether the engineered bacteria was producing the *EstA* protein or not. Moreover, the produced *estA* was functional. The testing was conducted with two different types of 4-nitrophenol with carbon chains i.e. 4-nitrophenyl octanoate (Sigma-Aldrich, Oakville, ON) which has an eight carbon chain attached, and 4-nitrophenyl palmitate (Sigma-Aldrich, Oakville, ON) which has a sixteen carbon chain attached.

The first step was to grow a culture of untransformed *E. coli* DH5α, overnight. It is the same strain in which we expressed our plasmid. The second culture of engineered *E. coli* was grown overnight with the *estA* gene. These cultures were grown in 5mL of LB broth with 5 µL of ampicillin per tube. In a sterile 15 mL tube, a detergent solution of Triton X-100 (Sigma-Aldrich, Oakville, ON) was added to the phosphate buffer (with a molarity of 0.2 M and a pH of 7) in order to yield a 1% detergent phosphate solution (ratio: 1 mL Triton X-100 to 99 mL phosphate buffer). Next, 4-nitrophenyl octanoate and 4-nitrophenyl palmitate were added separately at a 1:50 dilution (Ratio: 100 µL and add it to 4.9 mL of the mixture) resulting in a 1 mM concentration of 4-nitrophenyl, 1% detergent phosphate solution (the reaction mixture).

To facilitate more accurate test results 1.5 mL of each of the reaction mixtures were put into centrifuge tubes as controls. Two different amounts of the overnight cultures, 500 µL and 1,000 µL (one containing untransformed *E. coli* DH5α, and the other containing the engineered *E. coli*) were pelleted in the D1008 EZee Mini-Centrifuge at the maximum speed of 7,000 rotations per minute (RPM) (RCF of 2,680xg) for 5 minutes. The cell pellets were resuspended with the reaction mixture, and then incubated at 37°C at 200 RPM. Every 5 minutes, centrifuge tubes were removed from the incubator to take pictures for documenting the color change.

Spectrophotometry Lab with 4-Nitrophenyl Octanoate and 4-Nitrophenyl Palmitate

To perform quantitative testing using 4-nitrophenyl esters, four cultures of *E. coli* DH5α were grown overnight. Two of the cultures contained *E. coli* DH5α that had not been transformed to contain the engineered plasmid, to be used as controls, while the other two cultures contained DH5α with the engineered plasmid. Since the plasmid contains an ampicillin resistance gene, the cultures with the plasmid-containing *E. coli* were grown with 1 µL of ampicillin per tube. This was done to eliminate any cells that did not successfully receive the plasmid. After this step, 25 µL of 50 mM IPTG was added to 50mL of the overnight cultures containing the plasmid, and the one containing no plasmid.

Next, an OD_{600} reading was taken using a spectrophotometer to determine the initial optical density of the cultures prior to centrifugation. A sample of 100 µL was extracted from each culture, which was then pipetted into a microcentrifuge tube. This step was repeated three additional times for the two cultures containing the plasmid, for a total of four tubes each, and one additional time for the two cultures that did not contain the plasmid, for a total of two tubes each. Then, all twelve tubes were spun in a microcentrifuge at 7,000 RPM for 5 minutes, in order to create pellets. In the next step, Triton X-100 detergent was added to a 100 mM phosphate buffer solution having a pH of 7, resulting in a 1% detergent phosphate solution (1 mL detergent to 99 mL phosphate buffer). A spectrophotometric reading of this mixture at 400 nm was taken to be used as a reference point. Next, the 4-nitrophenyl esters were both added separately to the detergent phosphate solution in a 1:50 dilution (100 µL of 4-nitrophenyl added to 4.9 mL of the detergent phosphate solution), resulting in a 1 mM 4-nitrophenyl, 1% detergent phosphate solution, which was the reaction mixture. At this point, another spectrophotometric reading of the new reaction mixture was taken. After this, the cell pellets were resuspended in the reaction mixture at 37°C and shaken at 200 RPM. A spectrophotometric reading was taken initially when the reaction mixture was first added to the cell pellets. The subsequent readings were noted after every 3 minutes for the next 30 minutes. This procedure was repeated for a total of 2 trials.

Laboratory Safety and Precaution

All experiments within the lab were performed with an utmost focus on proper safety procedures, such as wearing the correct safety equipment, and carefully following all lab safety procedures. Everyone involved in the experimentation wore standard lab goggles, coats, and disposable gloves when entering the workspace, and while handling any of the equipment. The risk of contamination was eliminated through the use of proper sterilization methods, such as hand washing every time before entering or leaving the lab. The area of experimentation and equipment was sterilized using a bleach-based spray cleaner. In addition to this, we regularly autoclave or wash our lab equipment, and educate each student on the proper care and maintenance of such materials. Biohazard caution signs are posted around our lab to ensure that students and faculty are aware of the necessary safety precau-

tions. All the lab safety fixtures, such as eye washing stations, are inspected on a regular basis to ensure that they are as effective as possible in case of emergencies. All lab procedures have been executed under the careful supervision of mentors and teachers.

Figure 2. *Image A and image B show centrifuge tubes containing the 4-nitrophenyl ester reaction mixture for the palmitate (A) and octanoate (B) esters with no cells added. The images all show the results after five minutes of incubation. The middle row shows the response for a negative control where DH5α cells without any plasmid were added to reaction mixtures containing 4-nitrophenyl-palmitate (C) or 4-nitrophenyl-octanoate (D). The bottom row shows the response for the BBa_K2694000 plasmid transformed into DH5α cells when added to reaction mixtures containing 4-nitrophenyl palmitate (E) or 4-nitrophenyl octanoate (F). All reaction mixtures were 1mM of the nitrophenyl ester with 1% v/v Triton X-100 in 0.1M pH 7 phosphate buffer. No color change was observed in samples depicted on images A-D. Samples shown on images E and F changed their color to yellow.*

Since both 4-nitrophenyl octanoate and 4-nitrophenyl palmitate were ordered in a powdered form, our mentors helped in converting it into the solution form and dilute it. This made it easier and safer for us to work with in a high school lab.

The engineered *E.coli* will not be directly added into the current wastewater treatment systems. Instead, either cultures or purified enzymes would be added to the fatty obstructions removed from the system by staff. There are already measures in place to kill bacteria using UV light before it exits the wastewater treatment plant, though that would not be necessary given the current integration plan.

Results

Degradation of 4-Nitrophenyl Octanoate and 4-Nitrophenyl Palmitate by Engineered *E. coli*

After the completion of initial testing, which involved introducing our engineered *E. coli* and multiple controls to both 4-nitrophenyl octanoate and 4-nitrophenyl palmitate, there was a slight change in color (Figure 2).

Quantitative Testing Results: Color Change in 4-Nitrophenyl Octanoate and 4-Nitrophenyl Palmitate After Addition of Engineered *E. coli*

As seen with the initial qualitative testing, there was a slight change in color when our engineered *E. coli* was introduced to both 4-nitrophenyl octanoate and 4-nitrophenyl palmitate (Figure 3).

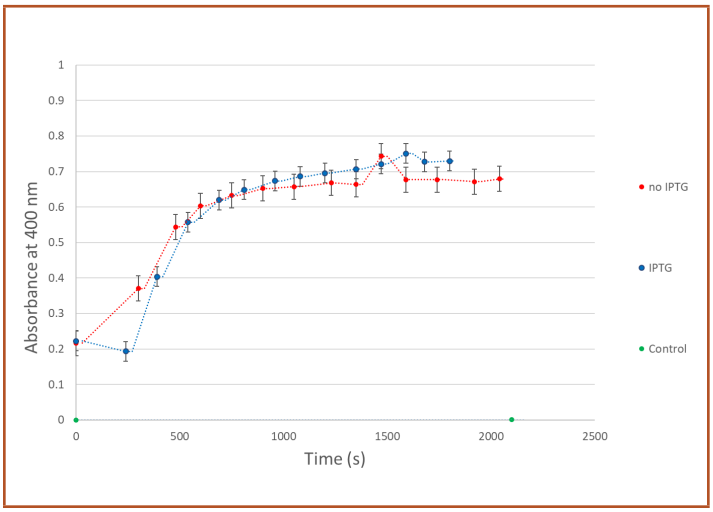

Figure 3. *The above graph displays the average absorbance rates for the reaction of 100 µL of overnight culture with 1 mM of 4-nitrophenyl octanoate. This graph shows that the engineered E. coli was producing the estA gene as the color of the solution progressively turned a deeper yellow with the introduction of the bacteria; however, the control (4-nitrophenyl octanoate without the engineered E.coli) did not depict any color change. There were minimal differences between tests done with and without IPTG.*

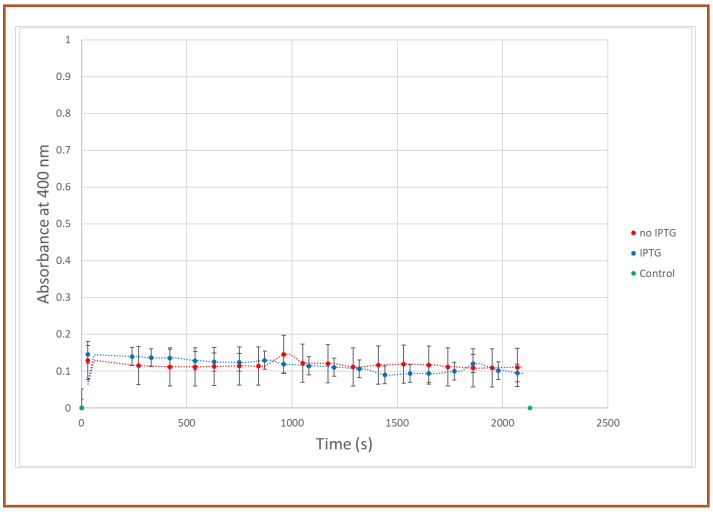

Figure 4. *The above graph displays the average absorbance rates for the reaction of 100 µL of overnight culture with 1 mM of 4-nitrophenyl palmitate. This graph shows that the engineered E. coli was producing the estA protein, as shown when the solution became yellow with the introduction of our engineered E. coli. The results were confirmed further when the control (our reaction mixture and 4-nitrophenyl palmitate), which was not introduced to the engineered E. coli, did not experience any color change.*

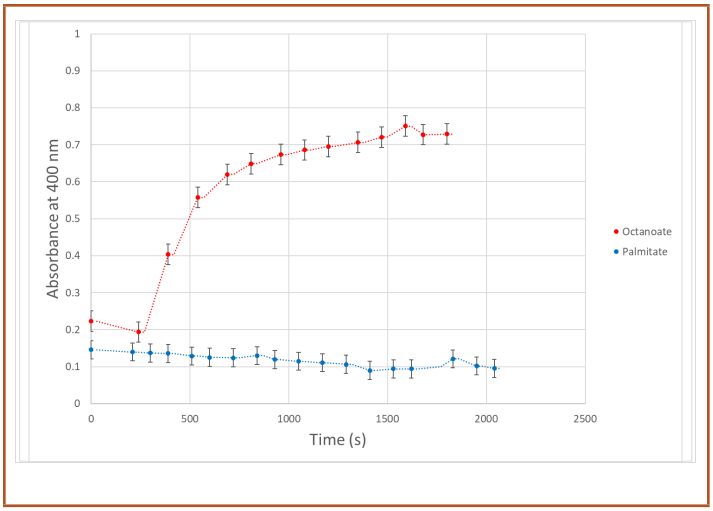

Figure 5. *The above graph displays the average absorbance rates for the reaction of 100 µL of overnight culture with a 1 mM concentration of 4-nitrophenyl octanoate (red) and 100 µL of overnight culture with a 1 mM concentration of 4-nitrophenyl palmitate (light blue). This graph shows that, our engineered E. coli was producing the EstA protein because both of the solutions became yellow, however; the color change was greater with 4-nitrophenyl octanoate.*

Discussions

The *estA* gene expressed in *E. coli* produced an enzyme which successfully cleaved the ester bond in 4-nitrophenyl esters. Color change of the samples confirmed the breakage of ester bonds (Figure 1: Images E and F). The exact color change of the samples was validated using spectrophotometry which is based on light absorbance levels in Figures 2 and 3, as well as the comparison in Figure 4. The objective of this testing was to compare the effectiveness of the esterase enzyme at breaking the ester bonds in 4-nitrophenyl octanoate and palmitate with different carbon chains attached to 4-nitrophenol. Color change indicated the *estA* protein broke the ester bond in both chain lengths, however it was more effective in breaking short chain fatty acids (4-nitrophenyl octanoate), which is depicted in the comparison between the absorbance values of 4-nitrophenyl octanoate and 4-nitrophenyl palmitate in Figure 4.

It is concluded that the engineered *E. coli* successfully produced the esterase enzyme and broke the ester bond within both 4-nitrophenyl octanoate and 4-nitrophenyl palmitate. The controls did not undergo any color change, showing that it was the engineered *E. coli* that enabled the color change due to the cleavage of the ester bond.

The addition of IPTG had no significant difference in the test results. These results may be due to the notoriously leaky pLac promoter, which allowed for the continual expression of the *estA* gene without the need of IPTG, causing the cells to passively produce the esterase enzyme.

When the ester bonds in fatty acids are broken, the pH shifts to become more acidic. As the overall goal in this project is breaking down fat, the bacteria was introduced to various fats that are found in sewer systems (though the engineered *E.coli* would be introduced in an isolated environment outside of the wastewater treatment systems). Butter, vegetable oil, and olive oil (which are found in sewers as a result of kitchen and restaurant waste) were mixed with a red cabbage pH indicator which has been used in high school classroom experiments. The expected result was for the indicator to turn pink/red, indicating an increase in acidity due to lipid breakdown. This indicator turns red for more acidic substances and blue for more basic ones. Unfortunately, during the only round of testing, the red cabbage indicator turned blue on one of the butter plates, which would indicate a basic change in pH. After consulting with mentors, the reason for this change was not discovered. Further research will be necessary to determine the cause of the pH change.

In the future, it may be beneficial to add a lipase component to the system, making the bacteria more efficient in breaking down triglycerides with long chain fatty acids. The leaky promoter (pLac) currently used in the

gene will be replaced with a more suitable part for the wastewater treatment environment. Further testing will be performed on different chain lengths of fatty acids. To expand on this project in the next year is, a team goal is to design a part that can turn the resulting fatty acids from lipid breakdown into a biofuel. Additionally, we would like to improve the efficiency of our system. Literature provided by our mentors suggested that the esterase enzyme would be more effective at a higher pH and that constant agitation could increase the reaction rate by up to 10 times (Jamie et al. 2017).

Acknowledgements

This project would not have been possible without our mentors from FREDsense, Dr. Robert Mayall and Emily Hicks; as well, we would like to thank our teachers, Jessica Larocque and Catherine Côté, who guided the direction of our project in terms of research and testing. Jason Craigie and Eugene Lund of the High River wastewater treatment plant provided valuable information about fatty obstructions in sewer systems and their management in our community. MindFuel and the GeekStarter program provided support through funding and valuable workshops. We would also like to thank the Knights of Columbus, Cargill, and the Catholic Women's League for supporting our team. The iGEM Foundation provided an educational opportunity to share our project and learn more about other topics in the field of synthetic biology through competing in the 2018 Giant Jamboree. We would also like to thank the University of Calgary iGEM team for helping us through the spectrophotometer labs.

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