Enzymes for PET degradation

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(Poly)ethylene terephthalate (PET), a plastic used in disposable water bottles, is not biodegradable and accumulates in our world's oceans, causing pollution and environmental damage. Our project proposes an engineered strain of *Bacillus tianshenii*, a gram-positive, spore-forming, halotolerant bacteria for bioremediation of these plastics. This strain would contain the gene for plastic-degrading enzyme PETase, originating from gram-negative bacteria *Ideonella sakaiensis*. The bacteria, in their inactive spore form, would be placed alongside dried nutrients underneath a full-length water bottle label. When the bacteria are exposed to the high salinity conditions of the ocean, the bacteria would germinate and produce PETase, degrading the bottle and the label. However, *B. tianshenii* is gram-positive; thus, when using parts from the gram-negative bacteria *I. sakaiensis*, the genes are unlikely to be expressed correctly in *B. tianshenii*. Also, due to limited resources in a high school environment, we would like to conduct proof of concept experiments with PETase without transforming Bacillus. The gene for PETase can be expressed via the BioBits cell-free system by inserting the gene into a backbone compatible with BioBits, designing primers, and using Gibson Assembly. Another alternative to transforming Bacillus is to conduct transformation experiments with Escherichia coli, which is more feasible given our laboratory equipment and experience in a high school setting. We also will test Leaf Compost Cutinase (LCC), another plastic-degrading enzyme, which is commonly found in plants and bacteria. In conducting proof of concept experiments with PETase and LCC, we aim to determine their ideal temperature and salinity conditions for degrading plastic substrates. To degrade PET, we plan to use a plasmid with the vector backbone pJL1 and insert the gene that produces PETase. The gene for LCC would also be inserted into a pJL1 backbone. Both plasmids include a GFP and his tag in order to determine if the plasmid is being expressed and to be used for protein purification.

Keywords: PET, (poly)ethylene terephthalate, PET degradation, plastic, bacteria

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*Watch a video introduction by the authors at*https://youtu.be/-tPquI3BtQE

Background

Due to its convenience, (poly)ethylene terephthalate (PET) is a prominent plastic used in wrappers, bottles, and more. However, plastic takes an average of 1,000 years to decompose, meaning every piece of plastic ever produced is still on Earth (Jackson 2015). Therefore, the use of plastic products is not environmentally responsible—an estimated 5.25 trillion pieces of plastic float in our oceans, and make up 60 to 90% of all marine debris studied (Surfers Against Sewage 2019). Sea turtles and seabirds often mistake floating plastic bags for food and can suffocate or starve (Reddy 2018). Plastic debris can also obstruct fishing boat motors and result in the damage or loss of fishing equipment, causing fishing and tourism industries to suffer. Due to the ocean's declining health, less fish are being caught, and those that are reeled in are of poorer quality.

These problems have pushed scientists to research eliminating plastic waste. Two enzymes, PETase and Leaf Compost Cutinase (LCC), have shown plastic degradation abilities. The bacteria *Ideonella sakaiensis* 201-F6 has a gene for the enzyme PETase, which degrades PET (Yoshida, Hiraga, Takehana, et al. 2016). *I. sakaiensis* 201-F6 hydrolyzed a thin layer of PET film in an average of 6 weeks (Coghlan 2016). LCC has also been shown to degrade PET (Csicsery 2012).

We have developed a design to remove existing and future plastic from oceans: degrading (poly)ethylene terephthalate into organic compounds using marine bacteria engineered to express the genes for PETase or LCC. We plan to conduct proof of concept experiments on these enzymes to determine their ability to degrade PET, as well as two other plastic substrates: (poly) butylene-ß-ketoadipate-co-terephthalate (PßKAT) and pNBa.

Plastic has become a modern challenge; industry pressures and consumer needs for inexpensive products have made it difficult to adopt alternatives. Our proposed method can provide an environmentally responsible solution while maintaining the convenience of plastic.

Systems level

In our original design, we planned to engineer the gene for PETase, a PET-degrading enzyme, into *Bacillus tianshenii*, a marine isolate of the Bacillus genus. We designed a plasmid with the gene for PETase and planned to conduct a transformation experiment with *B. tianshenii*. Our design consisted of a standard PET water bottle, a full-length PET label, and our engineered strain of *B. tianshenii* to be placed between the label and the bottle, along with dried nutrients.

B. tianshenii grows optimally at 30°C and pH 7.0 in the presence of 2–4% sodium chloride. It can withstand a pH range of 6.0-9.0, a temperature range of 10 to 50°C, and growth can occur in the presence of 0-7% NaCl. It produces endospores in inhospitable environments, rendering itself inactive (Jiang, Zhang, Khieu, et al. 2014). Therefore, ocean conditions match *B. tianshenii's* optimal conditions for growth, and would form endospores when outside of these conditions—including when the water bottle is in use. The label would act as a barrier between the bottle's user and *B. tianshenii*. If the user were to come in contact with the bacteria, it is likely that no harm would be done, as *B. tianshenii* is nonpathogenic and would be inactive in its spore form. Exposure to low salinity water, such as condensation or sweat, would not be sufficiently optimal conditions for *B. tianshenii* to germinate. Additionally, *B. tianshenii* can form a biofilm with the bottle as a substrate, making it unlikely that the bacteria would wash off the bottle in the ocean before the bottle is degraded.

Device level

In our original design, we intended to use *B. tianshenii* as our chassis. However, the *Bacillus* genus is difficult to work with without years of experience and a designated Bacillus laboratory. As high school students, we have neither the skills nor the resources to engineer *B. tianshenii*. We would instead like to begin experimentation with proof of concept experiments on the enzymes PETase and LCC in order to determine their ability to degrade plastics under various conditions, including marine environments. Therefore, we have opted to conduct proof of concept experiments using either *Escherichia coli* or a cell-free system to express the genes for PETase and LCC, two plastic degrading enzymes. BioBits, a freeze dried cell-free system, can be used for this, and we have designed plasmids with the T7 promoter and terminator for compatibility with the BioBits system. *E. coli* is also a feasible alternative to *B. tianshenii* for transformation experiments because in our high school setting, we have the resources to grow it, and in our proof of concept experiments, we aim to experiment with the conditions for the enzymes not for *B. tianshenii*. These alternative experiments are also beneficial because the gene for PETase, originally from *I. sakaiensis*, is not easily compatible with *Bacillus* species, as *I. sakaiensis* is gram-negative and Bacillus species are gram-positive. If transformed into *B. tianshenii*, it is unlikely that the gene for PETase would be expressed correctly and that the protein would fold correctly. Our plans to use *E. coli* or a cell-free system could eliminate these issues. We hope to produce sufficient amounts of each enzyme to conduct functional assays with plastic substrates.

Parts level

In order to degrade PET, we plan to conduct proof of concept experiments on the enzymes PETase and LCC to analyze their efficiency on breaking down three substrates under varying conditions of temperature and salinity. Two of the substrates are plastics: PET and (poly)butylene-ß-ketoadipate-co-terephthalate (PßKAT). The third substrate is pNPa, an acceptable replacement substrate for PET in assays, useful because the crystalline structure of pNPa may be easier to degrade than that of PET. It is appropriate to use multiple different substrates because the crystalline structure of each may affect the enzymes' ability to degrade it. We plan to measure the mass of the assay in order to assess its degradation.

We will express the enzymes using the BioBits cell-free system. In order to express genes in a cell-free system, we plan to create plasmids by inserting the genes for each of the enzymes into the vector pJL1 which contains the T7 promoter and terminator necessary for compatibility with the BioBits system (Jewett n.d.). The PETase gene we are using is a double mutant, optimized for expression in *E. coli* (Beckham n.d.). Also, we plan to express the gene for LCC, an enzyme used to break PET down into ethylene glycol and terephthalic acid, via a separate plasmid (Csicsery 2012). We have integrated the PETase double mutant gene, as well as a superfolded GFP (sfGFP) with his tag (Mehl n.d.) into the pJL1 backbone (Figure 1). We have also created a plasmid with the pJL1 backbone, LCC gene, and sfGFP with his tag (Figure 2). By adding the sfGFP, we can determine if the plasmid is being expressed, and using the his tag we can quantify the amount of enzyme produced using a western blot.

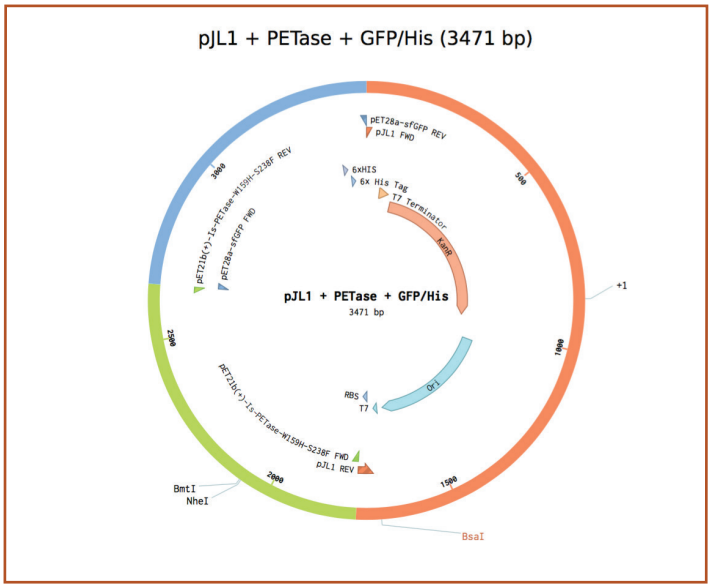

Figure 1. *The plasmid we designed using the pJL1 vector and double mutant PETase gene with a superfolded GFP and his tag.*

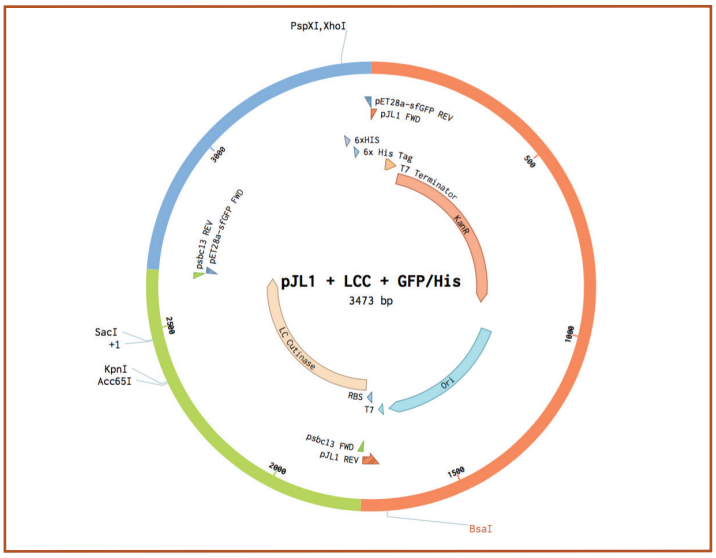

Figure 2. *The plasmid we designed using the pJL1 vector and LCC gene with a superfolded GFP and his tag.*

The limits of our high school environment render a cell-free system to be an easier method of expressing LCC and PETase. If we were to conduct transformation experiments to express the enzymes in *B. tianshenii*, cell culture and protein purification would be challenging with our limited resources and lack of experience with Bacillus.

An additional variable introduced by using a cell-free system is the amount of the system used. It is plausible that using 5-20 mL of the BioBits system, which is the standard amount provided in a kit to express GFP, will not produce enough enzyme for us to perform a functional assay. By inserting a sfGFP and his tag into our plasmids for the cell-free system, we could quantify the ratio of BioBits system reagents to enzymes produced and improve our experimental design.

Alternatively, we may express these enzymes in BL21 (DE3) Competent *E. coli* (Biolabs 2020). This strain is widely used to express genes with the T7 promoter and T7 terminator, and therefore would be fitting for our plasmid designs. Furthermore, the gene for PETase that we have inserted into the pJL1 vector is codon optimized for expression in *E. coli* (Beckham n.d.). A transformation experiment with *E. coli* is also much more likely to be successful than one using *B. tianshenii* given our skills and resources in a high school setting.

Safety

When working with cells or a cell-free system, we plan to consult our mentors with various areas of expertise. We plan to conduct our experiments in our high school laboratory or in a synthetic biology laboratory in Cambridge, MA.

Additionally, if we were to transform bacteria to produce PETase or LCC, in order to safely test our design we plan to model the ocean water microenvironment. We will place the bacteria under the label of a standard plastic water bottle, and place the label on the bottle with a dried medium of nutrients underneath. The bottle will then be placed in the simulated ocean environment. Over the course of 6 weeks, this simulated environment will be monitored and recorded. Should lab results show promising degradation without detrimental effects on the environment, we will pilot the design in a location with minimal to some occurrence of human activity, and in a medium-sized body of water with a tested salinity of at least 2.5%. A tracking tag will be placed on the bottle.

We recognize that the exact effects of our bacteria on the ecosystem cannot be predicted, and for this reason, we plan to conduct an environmental impact analysis regarding the potential effects of our design on the ocean and marine life. Since our immediate goals are to conduct proof of concept experiments, we will not be commercially releasing these products into the environment or market.

Discussions

Our original design used *B. tianshenii*, a gram-positive, halotolerant bacteria. However, genes from gram-negative bacteria do not express well in Bacillus, a predominantly gram-positive species. We plan to take the PETase gene from *I. sakaiensis* (Yoshida, Hiraga, Takehana, et al, 2016), a gram-negative bacteria, meaning the gene for PETase can not easily be integrated into *B. tianshenii* and is unlikely to express and fold correctly.Additionally, PET's crystalline structure is difficult to degrade, making the polymer nonoptimal for use in a functional assay. Thus, we have chosen two substrates in addition to PET to measure PETase and LCC's efficiency: PßKAT, and pNPa.

In a high school environment, we recognize that there are limitations to how far we can investigate, and hope to perform experimentation to the best of our ability with the help of our mentors.We hope to build on our proof of concept experiments and eventually integrate our idea into the real world, helping eliminate plastic from our oceans and saving marine life.

Acknowledgements

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1. Lindsey L'Ecuyer, our teacher, mentor, and leader of the BioBuilderClub at our school. She has encouraged and guided us from the first day we came up with the idea for this project.

2. Dr. Richard Losick, a leading Bacillus researcher from Harvard University, talked with us about transforming into Bacillus. We realized that we would not be able to accomplish this in a high school setting; Bacillus species are difficult to transform into, and since our enzyme comes from gram-negative bacteria, those coding sequences generally do not work easily in gram-positive Bacillus species. We continued to research other chassis, and realized we may run into problems purifying the protein, especially if we used a chassis that did not secrete well. This led us to consider going cell-free.

3. Patrick Holec, our BioBuilder mentor from MIT, reiterated the impressions of Dr. Losick.

4. Dr. Ally Huang, the co-creator of BioBits (freeze dried, cell-free extract), talked with us about how to express our enzymes using her system. We talked with her about the possibility of using reporters, both as separate constructs and as tags added onto the end of the enzyme sequences. Dr. Huang has also guided us through the benefits and requirements to using a cell-free system and BioBits. The vector she finds expresses best in her freeze dried, cell-free system is pJL1. We would like to demonstrate the utility of BioBits as a cell-free expression system in a high school setting. While other cell-free systems exist, such as myTxTl from Arbor Biosciences, we are concerned about storage as we do not have a -80°C freezer. Dr. Huang has guided us through the modification of parts: to use a cell free system, we must use the vector, promoter, and terminator that work best in the BioBits system. Therefore, we would like to express with the pJL1 vector, T7 promoter, and T7 terminator rather than our original salt water promoter.

5. Kosuke Seke, from Dr. Michael Jewett's lab at Northwestern, confirmed the impressions of Dr. Huang.

6. Dr. Gregg Beckham, from the NREL (National Renewable Energy Laboratory), is a leading researcher on PETase and has plasmids available on Addgene. However, they would require transformation, cell culture, and protein purification, which are challenging in our high school setting. Dr. Beckham talked with us about weaknesses in PETase, and suggested testing alternative enzymes such as LCC. He also described the PET in water bottles as amorphous and therefore inaccessible to PETase.

Thus, his lab is working on water bottle alternatives such as PßKAT, which he offered to provide to us as an alternative substrate.

7. Dr. Joyce Yang is the Sustainability Coordinator in our town, and used to work at the NREL with Dr. Beckham. She talked to us about challenges with recycling, reporting that most plastic is actually incinerated rather than recycled. It is important to find a way to recycle PET, as it is otherwise created from divergent petroleum. Both she and Dr. Beckham also suggested finding ways to upcycle PET degradation products into something useful. She suggested adding a his-tag in lieu of a large tag like GFP, noting that it's useful because it's small size. Dr. Ally Huang confirmed this should work in the BioBits system and reiterated its usefulness in protein detection and purification.

8. Michael Edgar is a part of the science department faculty at Milton Academy. He helped us locate potential sources for LCC, and advised us on how we may integrate our genes into the pJL1 backbone and assemble our DNA on Benchling.

9. Stephen Chinosi, the Director of Strategic Innovation of Andover Public Schools, and Alex Zeng, a 3D Printing Intern, worked with us to create a visual model of our design using TinkerCAD and the Andover High School Innovation Lab's 3D printers. They generously provided us with the materials and expertise we needed. The models we created are made of a water soluble polymer called PVA, and served as a visual for us to demonstrate how our final product would act.

10. Dr. Natalie Kuldell, the Founder and Executive Director of the BioBuilder Educational Foundation, has supported us and our project through presentations, media coverage, and research. This project was accomplished through participation in the BioBuilderClub, an after-school program organized by the BioBuilder Educational Foundation. BioBuilderClub engages high school teams around the world to combine engineering approaches and scientific know-how to design, build, and test their own project ideas using synthetic biology.

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