Enzymes for Plastic Degradation

Krishnaveni Dole, Puloma Bishnu

Abstract

(Poly)ethylene terephthalate (PET), a plastic used in disposable water bottles, significantly contributes to ocean pollution. An engineered strain of *Bacillus tianshenii*, a gram-positive, spore-forming, halotolerant bacteria, which would contain the gene for plastic-degrading enzyme PETase, can be utilized for the bioremediation of PET. The bacteria, in their inactive spore form, would be placed alongside dried nutrients underneath a full-length water bottle label. Upon exposure to the high salinity conditions of the ocean, the bacteria would germinate and produce PETase, degrading the bottle and label. Due to limitations of a high school laboratory, proof of concept experiments will be conducted without transforming *Bacillus*. Producing PETase and Leaf Compost Cutinase, another plastic degrading enzyme, via a cell-free system or expression in *E. coli* would allow for their use in functional assays to measure the degradation ability of each enzyme on various plastic substrates under differing conditions of temperature and salinity.

Problem

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, plastic products are 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, n.d.). 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.

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

Plastic has become a modern challenge; industry pressures and consumer needs for inexpensive products have made it difficult to adopt alternatives. Thus, it is necessary to develop an environmentally responsible solution while maintaining the convenience of plastic.

Background

A solution to decrease plastics entering our oceans is bioplastics, which are made from sustainable materials and are biodegradable. However, their production process is more harmful to the environment than traditional plastics, as it releases more pollutants into the environment,

contributes to ozone depletion, and requires large amounts of land that could be better purposed for food production. Furthermore, to biodegrade, these plastics require certain conditions, often of high heat and composting facilities. The infrastructure for this process does not exist in most countries and cities. Therefore, bioplastics often end up in the recycling stream, where they contaminate batches of recyclable plastics. Lastly, bioplastics can be 20 to 50% more expensive than traditional plastics. This means that they are costly for companies and unappealing to consumers because of the cost and environmental damage (Cho, 2017).

The increasing trend of environmental consciousness has led to significant strides in removing plastic from the ocean. Innovations such as Mr. Trash Wheel, a solar-powered machine that prevents trash from entering Chesapeake Bay in Baltimore, Maryland, USA (Snow, 2017), and volunteer-based cleanup organizations such as 4Ocean, have received public support and succeeded in reducing plastic in our oceans. However, the removed trash often ends up in landfills. Therefore, the pollution is being relocated, not eliminated.

Design

In our design, we plan to engineer the gene for PETase, a PET-degrading enzyme, into *Bacillus tianshenii*, a marine isolate of the *Bacillus* genus. PETase degrades PET into organic, environmentally benign compounds ethylene glycol and terephthalic acid, both of which are naturally occuring in the ocean. We have designed a plasmid with the gene for PETase and plan to conduct a transformation experiment with *B. tianshenii*. Our design consists 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% NaCl. It can withstand pH 6.0–9.0, 10°C–50°C, and growth can occur in the presence of 0–7% NaCl. Additionally, it produces endospores in inhospitable environments, rendering itself inactive (Jiang, et al., 2014). Therefore, ocean conditions match *B. tianshenii*'s optimal conditions for growth, and outside these conditions, the bacteria would form endospores—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 be insufficient 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.

By maintaining the pre-existing design of a plastic water bottle, our solution would be cheaper to implement than bioplastics. It would not significantly increase the price of plastic bottles for families, as bacteria are cheap to culture and would result in an economy of scale. Furthermore, bacteria can be cultured in crude medium, and in contrast to bioplastics, their production does not require pollutants or high use of land.

Plastic manufacturers receive significant negative publicity for their role in producing disposable bottles. With this solution, companies will be retaining a high selling product while also partaking in a positive effort to decrease plastic pollution and improving their public image.

We surveyed 200 people asking if they would purchase a bottle with our engineered bacteria considering the benefits to the environment, estimated cost, and safety features—81.5% of respondents said they would buy our bottle. Additionally, when asked for their greatest

incentive for buying water from a particular company, almost half of surveyed respondents selected the option, “It would mean helping people or the environment through [their] purchase.” Consumers feel that they are making a change in buying a bottle with our technology, and would see direct benefits, such as when they are relaxing at beaches without viewing shored-up trash.

Our idea can also eliminate pre-existing plastic by repurposing our bacteria to be released on trash gyres or in landfills. Therefore, implementing the technology of our engineered bacteria would be beneficial and more feasible than current solutions to plastic pollution.

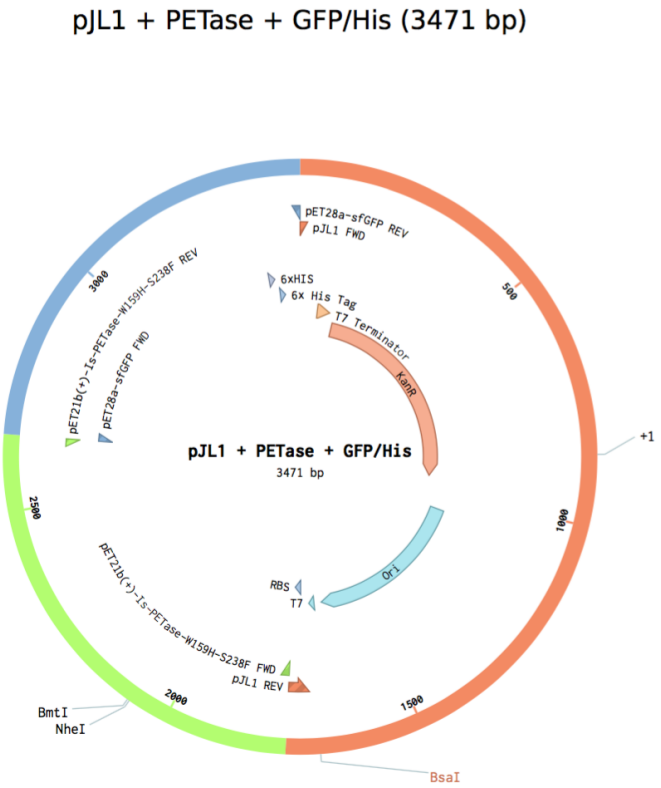
Discussions

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, using a model of the ocean microenvironment.

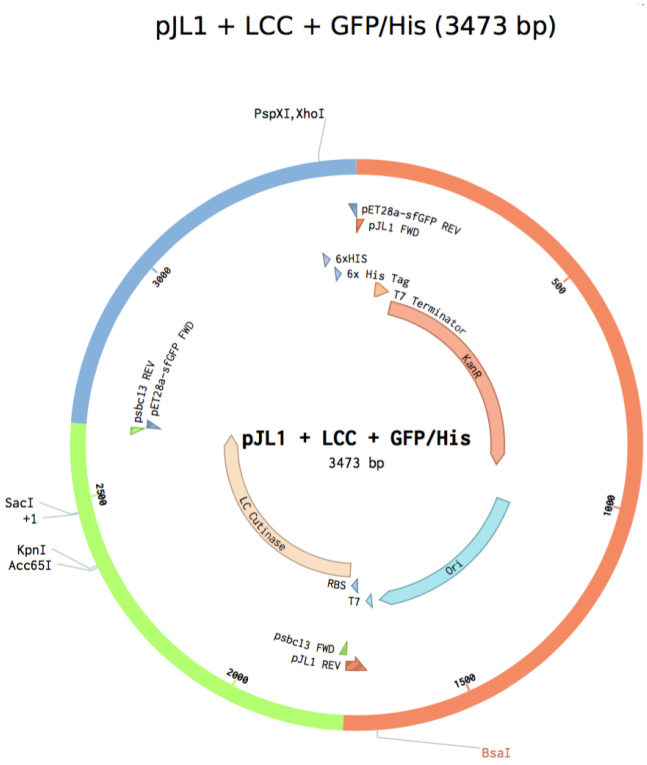
To engineer the bacteria for our design, we intend to use *Bacillus tianshenii* as our chassis. However, *Bacillus* is difficult to work with without years of experience and a designated *Bacillus* laboratory. As high school students, we have neither the skills nor the resources to engineer *B. tianshenii*. 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. We would instead like to conduct proof of concept experiments on the enzymes PETase and LCC to determine their ability to degrade plastics under various conditions, including marine environments. We will use either *Escherichia coli* or a cell-free system to express the genes for PETase and LCC.

Moreover, the gene for PETase, originally from *Ideonella sakaiensis,* is not easily compatible with *Bacillus* species, as *I. sakaiensis* is gram-negative and *Bacillus* is gram-positive. If transformed into *B. tianshenii*, it is unlikely that PETase would be expressed and fold correctly. Our plans to use *E. coli* or a cell-free system would eliminate these issues.

We will express the genes using the BioBits cell-free system, using plasmids we have created by inserting the genes for each of the enzymes into the vector pJL1 (Jewett, n.d.), which contains the T7 promoter and terminator necessary for compatibility with the BioBits system. We have integrated a PETase double-mutant gene, optimized for expression in *E. coli* (Beckham, n.d.), as well as a superfolder Green Fluorescent Protein (sfGFP) with a his tag (Mehl, n.d.) into the pJL1 backbone (Fig. 1). We have also created a plasmid with the pJL1 backbone, LCC gene, and sfGFP with a his tag (Fig. 2). By adding 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) Our designed plasmid, including the pJL1 vector and the double-mutant PETase gene with sfGFP and his tag.



(Figure 2) Our designed plasmid, including the pJL1 vector and the LCC gene with sfGFP and his tag.

It is plausible that using 5-20mL of the BioBits system, which is the standard amount provided in a kit to express GFP, will not produce enough enzyme to perform a functional assay.

Alternatively, we may express these enzymes in BL21 (DE3) competent *E. coli* (Biolabs, n.d.). This strain is commonly used to express genes with the T7 promoter and terminator, which are in our design. Also, the PETase gene and pJL1 vector are codon optimized for expression in *E. coli* (Beckham, n.d.). A transformation experiment with *E. coli* is also more likely to be successful than one using *B. tianshenii* given our skills and resources in a high school setting.

We plan to conduct functional assays to analyze PETase and LCC's efficiency in 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. We plan to measure the mass of the assay in order to assess its degradation.

Since our immediate goals are to conduct proof of concept experiments, we will not be commercially releasing these products into the environment or market. In a high school setting, 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 other scientists, engineers, and industry leaders. We hope to build on our proof of concept experiments and eventually integrate our idea into the real world, helping to eliminate plastic and saving our oceans.

Bibliography

Beckham G. Sequence Analyzer: pET21b(+)-Is-PETase-W159H-S238F Sequencing Result [Internet]. Addgene [cited 2020 April 24]. Available from: https://www.addgene.org/browse/sequence/215852/.

Biolabs NE. BL21(DE3) Competent E. coli [Internet]. New England Biolabs [cited 2020 April 25]. Available from: https://www.neb.com/products/c2527-bl21de3-competent-e-coli#Product.

Cho, Renee. The Truth About Bioplastics [Internet]. Earth Institute, Columbia University; 2017 December 17 [cited 2020 June 29]. Available from: https://blogs.ei.columbia.edu/2017/12/13/the-truth-about-bioplastics/.

Coghlan A. Bacteria found to eat PET plastics could help do the recycling [Internet]. New Scientist; 2016 March 10 [cited 2019 May 3]. Available from: http://bit.ly/2OZJ27B.

Csicsery N. Part:BBa_K936000 [Internet]. Registry of Standard Biological Parts; 2012 July 17 [cited 2020 April 24]. Available from: http://parts.igem.org/Part:BBa_K93600.

Jackson A. Every Piece of Plastic Ever Made Still Exists Today [Internet]. Planting Peace; 2015 May 21 [cited 2020 April 24]. Available from: https://www.plantingpeace.org/2015/05/plastic-footprint/.

Jewett M. pJL1 (Plasmid #69496) [Internet]. Addgene. [cited 2020 May 29]. Available from: https://www.addgene.org/69496/.

Jiang Z, Zhang D-F, Khieu T-N, Son CK, Zhang X-M. Bacillus tianshenii sp. nov., isolated from a marine sediment sample. International Journal of Systematic and Evolutionary Microbiology [Internet]. 2014 Jun 1; 64. Available from: http://bit.ly/2KSrBAA.

Reddy S. Plastic Pollution Affects Sea Life Throughout the Ocean [Internet]. PEW; 2018 September 24 [cited 2020 April 24]. Available from: https://www.pewtrusts.org/en/research-and-analysis/articles/2018/09/24/plastic-pollution-affects-sea-life-throughout-the-ocean.

Mehl R. Sequence Analyzer: pET28a-sfGFP Sequencing Result [Internet]. Addgene pET28a-sfGFP Sequencing Result - Sequence Analyzer [cited 2020 May 29]. Available from: https://www.addgene.org/browse/sequence/169849/.

Snow, Jackie. Googly-Eyed Trash Eaters May Clean a Harbor Near You [Internet]. National Geographic; 2017 February 17 [cited 2020 June 29]. Available from: https://www.nationalgeographic.com/news/2017/02/mr-trash-wheels-professor-trash-wheels-baltimore-harbor-ocean-trash-pickup/.

Surfers Against Sewage. Plastic Pollution – Facts and Figures [Internet]. Cornwall: Surfers Against Sewage; 2019 [cited 2019 May 3]. Available from: http://bit.ly/2TGrdsX.

Yoshida S, Hiraga K, Takehana T, Taniguchi I, Yamaji H, Maeda Y, Toyohara K, Miyamoto K, Kimura Y, Oda K. A bacterium that degrades and assimilates poly(ethylene terephthalate). [Internet] New York Avenue (WA): American Association for the Advancement of Science; 2016 March 11 [cited 2019 May 3]; 351(6278): 1196-1199. Available from: http://bit.ly/2Nb4gwS.

Acknowledgments

We would like to thank and acknowledge the following people who have helped us create and advance this project:

I. 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.

II. 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.

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

IV. 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.

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

VI. 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.

VII. 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.

VIII. 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.

IX. 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.

X. 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. BioBuilder Club 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.