

LAB REPORT

Enhancing experimental conditions for increased PETase and LCC activity and thermal stability in the enzymatic hydrolysis of PET

Ella Hu, Siri Jayaprakash, Tomas Kruecker-Green, and Keerthi Shome ■ Andover High School, Andover, Massachusetts, United States

Reviewed on 9 May 2026; Accepted on 22 June 2026; Published on 26 October 2026

Of the 400 million tons of plastic produced annually, polyethylene terephthalate alone is responsible for roughly 10% (Organisation for Economic Co-operation and Development [OECD], 2022). Only 5% of the PET is recycled, 15% is incinerated, and 75% is landfilled. Enzymes such as PETase, from *Ideonella sakaiensis*, and leaf compost cutinase (LCC), found in compost piles, have shown potential in addressing this long-standing issue. In addition to PETase, two different forms of LCC were tested: the original sequence and another engineered, more thermostable version. The efficiency of PETase and both LCCs on PET film (0.25 mm thickness) and PET powder (300 μ m) was evaluated after expression in a cell-free system (BioBits®). Insoluble components were analyzed using a scanning electron microscope (SEM), while soluble monomers were analyzed through high-performance liquid chromatography (HPLC). In 2025, PET powder showed degradation in both LCC enzymes via SEM, while PET film did not; however, some samples dried out during the process. For 2026, buffer and mineral oil were added to stabilize enzymes and prevent evaporation. With these changes, PET film exhibited degradation with PETase and both LCC enzymes, while PET powder did not. These inconsistencies indicated the need for further experimentation, and HPLC analysis was ultimately inconclusive.

Keywords: polyethylene terephthalate, leaf-branch compost cutinase, high-performance liquid chromatography, scanning electron microscope, degradation of plastic, environmental, monomers, ethylene glycol, terephthalic acid, ImageJ

The authors were mentored by Lindsey L'Ecuier, Andover High School, Andover, Massachusetts, United States. Please direct correspondence to: Lindsey L'Ecuier, Andover High School, Andover, Massachusetts, United States; lindsey.lecuier@andoverma.us. This is an Open Access article, which was copyrighted by the authors and published by BioTreks in 2026. It is distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by-nc/4.0/>), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited.

OVER 400 million tons of plastic are produced globally each year, yet only 9% of the plastic waste is being recycled (Organisation for Economic Co-operation and Development [OECD], 2022). Among the most widely used plastics is polyethylene terephthalate (PET), a thermoplastic polymer commonly found in plastic bottles. PET alone is responsible for roughly 10.2% of global plastic production. 15% of PET is incinerated in facilities, releasing a handful of toxic pollutants into the atmosphere, while 75% of all PET is dumped into landfills or the ocean, harming marine life and causing long-term ecological harm.

PETase (from *Ideonella sakaiensis*) breaks down PET by hydrolyzing the ester bonds in the polymer, converting it into intermediate products: Bis (2-Hydroxyethyl) terephthalate (BHET) and mono (2-hydroxyethyl) terephthalate (MHET). PETase is a member of the α/β -hydrolase fold family and contains a catalytic triad (Ser-His-Asp) in its active site, which

facilitates this hydrolysis reaction. The process occurs in two steps; first, the enzyme forms an acyl-enzyme intermediate with the PET substrate; second, this intermediate is hydrolyzed, releasing MHET and other products. To further degrade MHET into environmentally benign compounds, a secondary enzyme MHETase is required.

Leaf-branch compost cutinase (LCC) is a thermostable cutinase discovered in hot compost. Unlike PETase, LCC can depolymerize PET almost completely in one step. *In vitro*, LCC treatment of PET film yielded essentially only terephthalic acid (TPA, major) and ethylene glycol (EG), with only trace MHET and no detectable BHET (Sulaiman et al., 2012). This indicates that LCC hydrolyzes PET directly to its monomeric components (TPA and EG) without accumulating BHET intermediaries, thus “bypassing” the typical MHET bottleneck. Instead, LCC is able to cleave PET hydrolyzed products quickly so that the final monomers accumu-

late. Crucially, LCC and its engineered variants are highly thermostable and more likely to withstand the high temperatures of recycling facilities. For example, optimized LCC variants have the most activity around 70-80°C and retain roughly half their activity after hours at 90°C.

Materials and methods

Designing and obtaining PETase and LCC (original) and LCC (modified) vectors

Three plasmid vectors were designed in Benchling and ordered from Twist Biosciences, each containing the gene sequence for PETase, LCC (original), or LCC (modified), linked to green fluorescent protein (GFP) and integrated into the pJL1 backbone (Figures 1-3). LCC (modified) was known to be more thermostable. All sequences were codon optimized for expression in *Escherichia coli* using a ribosome binding site (RBS) calculator and parameters defined by Twist Biosciences. These vectors were designed for expression in a cell-free protein synthesis system called BioBits® (miniPCR, 2019; Stark et al., 2023). The constructs contain a T7 promoter terminator chosen to function specifically with the BioBits® cell-free expression system, which requires strong genetic parts for high affinity to the cell-free ingredients. They also contain strong RBS and a His-tag on the GFP. The vectors were ordered in the TE buffer. Twist Biosciences could not normalize the concentrations, so the resulting DNA arrived at different concentrations (PETase 471.42 ng/μL, LCC 371 ng/μL).

Evaluating enzymatic activity of PETase and LCC through GFP expression in BioBits®

BioBits®, a freeze-dried cell-free protein expression system (Figure 4), was obtained from miniPCR.

The PETase and LCC (original) plasmids were added to BioBits® reactions at concentrations of 100 ng/μL to assess fluorescence visualization. Following DNA addition, fluorescence images were captured at hourly intervals over a 9-hour period to monitor GFP expression as an indicator of protein synthesis. A plasmid encoding red fluorescent protein (provided in the BioBits® kit) served as a positive control, while nuclease-free water was used as a negative control. Following the experiment, differences in GFP expression were assessed between each experimental vector and the negative control to evaluate relative protein production. Protein production shown by GFP fluorescence was not qualitatively visible. Although research suggests that concentrations of 100 ng/μL are optimal for

LCC/PETase, we hypothesize that expression failure could be due to a number of reasons, such as excess DNA inhibiting translation, or competing for cell-free resources, thus hindering the production of GFP. This remains consistent with previous year's experiment in which multiple concentrations were tested to determine an optimal concentration; DNA at 100 ng/μL did not result in visible fluorescence.

Enzymes were placed in 105 μL of either nuclease-free water or a 1 M potassium phosphate buffer to determine which solution promoted the optimal environment for enzymatic activity and protein synthesis.

Applying PETase and LCC to PET films and powder substrates to determine the ability of the enzymes to break down plastic

Experiments were conducted using PETase and LCC (original) in addition to a modified version of LCC engineered for enhanced thermostability. DNA from the three vectors was added to separate BioBits® reactions at 100 ng/μL and allowed to incubate to allow time for expression of the enzymes. The enzymes were applied to the biaxially oriented PET films at 0.5 mm in thickness and semi-crystalline (crystallinity>40%) powder substrates with a maximum size of 300 μm. The film and powder were obtained from Dr. Nathan Crook and Dr. Tianyu Li's lab at North Carolina State University (NCSU). The enzymes with film or powder were then incubated for five days. Samples containing PETase were incubated at room temperature (22°C), while those containing the original and modified LCC were incubated at 72°C to match their optimal operating temperatures. In previous experiments, samples that were stored at 72°C dried up before the end of the incubation period. To prevent this, about 20 μL of mineral oil was added to each sample. The solution was frozen for 30 minutes, leaving the mineral oil layer to be pipetted out.

To evaluate the degradation, a scanning electron microscope (SEM) and High Performance Liquid Chromatography (HPLC) were used. Samples were prepared for SEM imaging by drying overnight in a desiccator and followed by a gold coating using a sputter coater (Denton Vacuum Desk IV). The sputtering process lasted for two minutes, resulting in a 10 μm coating. The PET films and powder substrates were imaged at 50X, 250X, and 1000X magnification. The solution that remained after the incubation period was used to evaluate the concentration of PET monomers using high performance liquid chromatography. HPLC machines separate compounds by pushing them through a column, and varying polarity of the compounds result in polar molecules exiting the column faster. A potas-

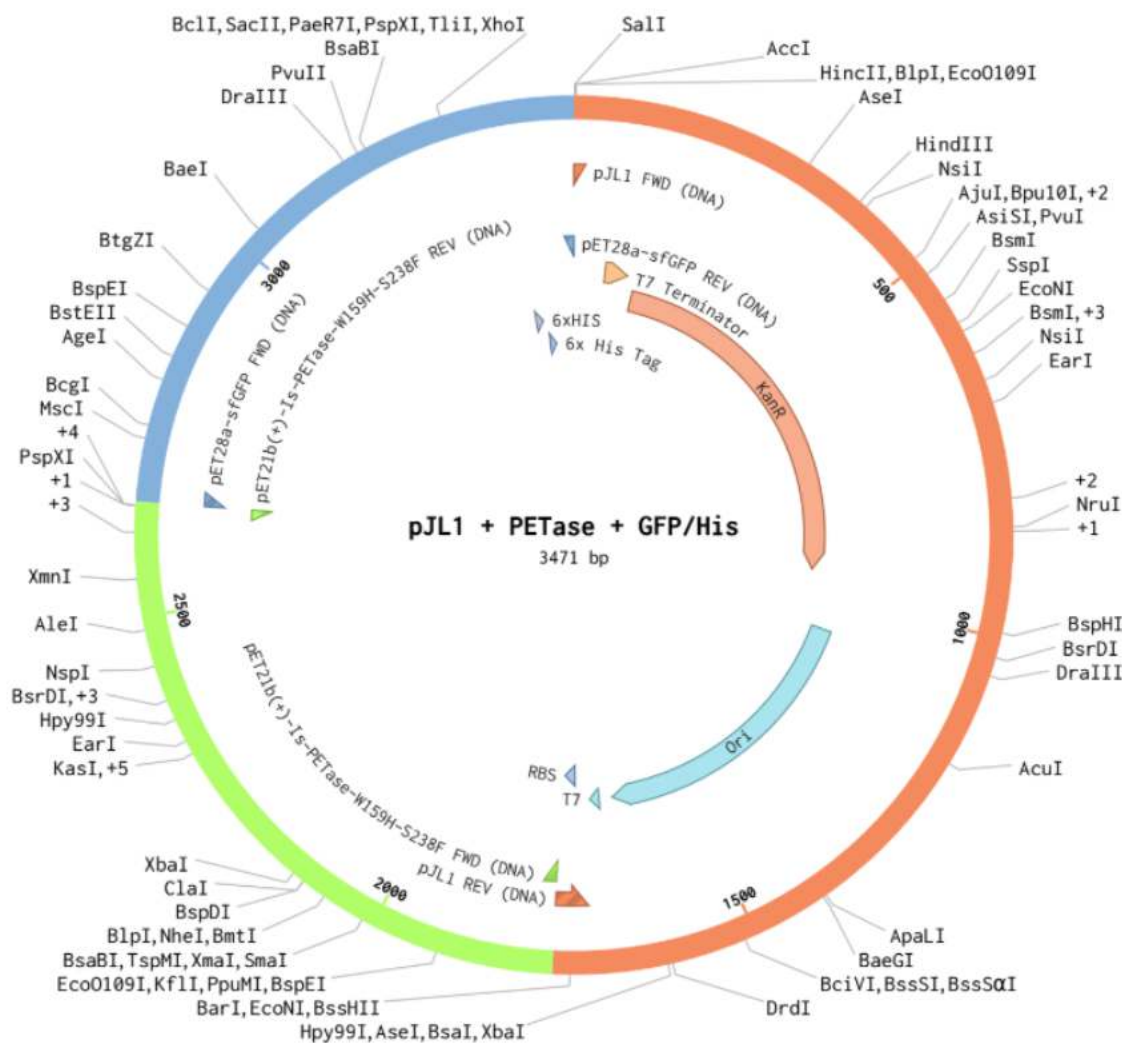


Figure 1. pJL1 + PETase + GFP Plasmid

sium phosphate buffer was used with a pH of 8. Because TPA is more polar than MHET, it has a lower retention time on the chromatogram. HPLC has a reader that detects UV absorption as molecules pass through the column. A higher concentration of molecules at any given time results in greater light absorption, producing a higher peak on the chromatogram.

Results

SEM scans showed prominent lesions on PET film following enzyme exposure indicating degradation. Lesions were visible in samples of PET film exposed to LCC (both thermostable and original) and PETase. SEM scans of PET powder, however, were difficult to quantify using ImageJ due to issues regarding particle clumping in which the software was unable to distin-

guish individual particles accurately.

HPLC was inconclusive

Trace amounts of TPA were detected at roughly 7.317 minutes and 7.618 minutes. The dominant peak was MHET, which occurred in substantial amounts at 8.413 minutes. BHET, an intermediate step in the conversion of PET to MHET, was not detected, possibly due to low concentration.

Discussions

The successful use of PETase and LCC provides a strategy for reducing the growing amount of plastic waste entering ecosystems each year. By enabling enzymatic degradation of PET, this project contributes to the goal of developing environmentally friendly methods for

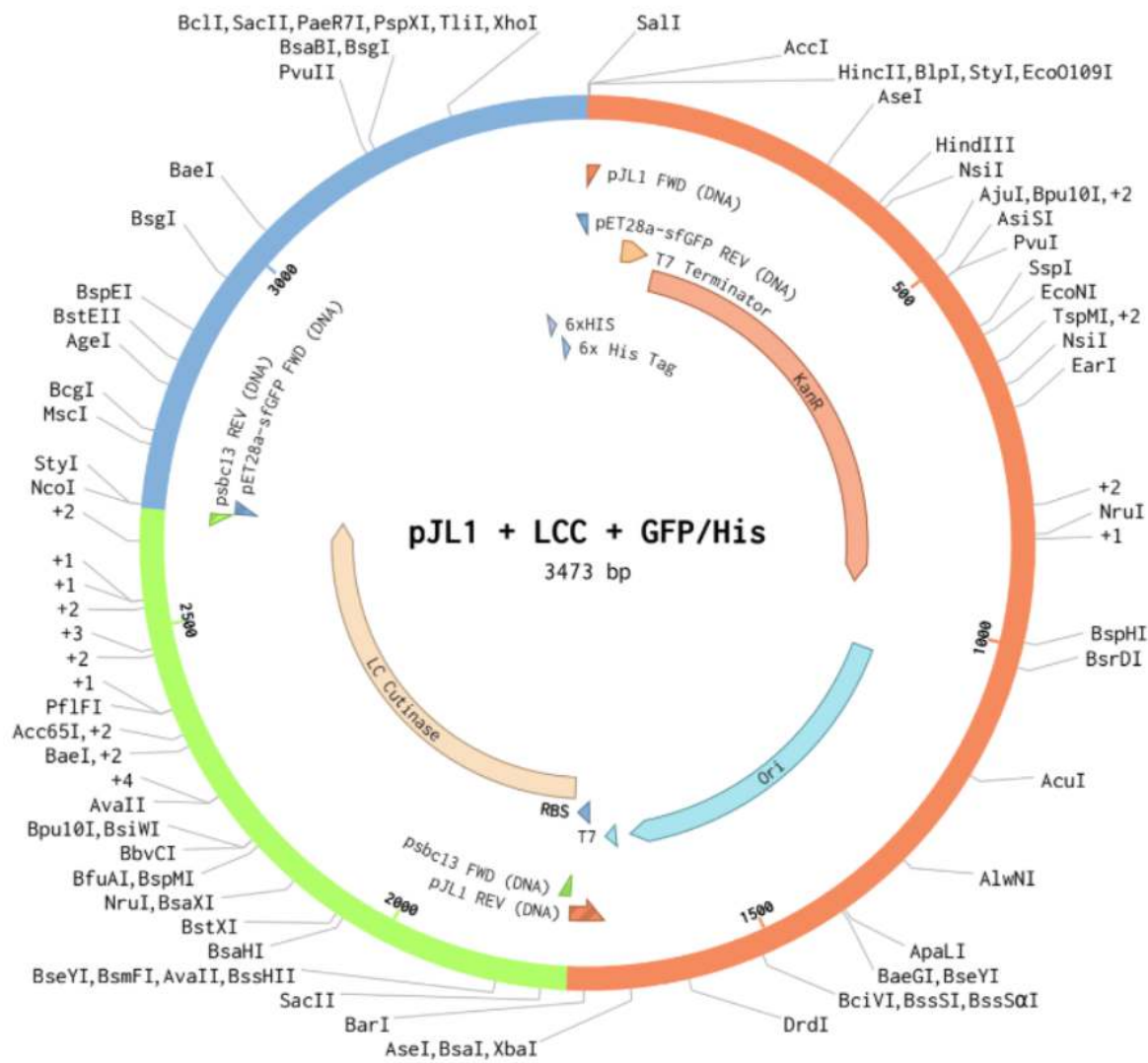


Figure 2. pJL1 + LCC (original) + GFP Plasmid

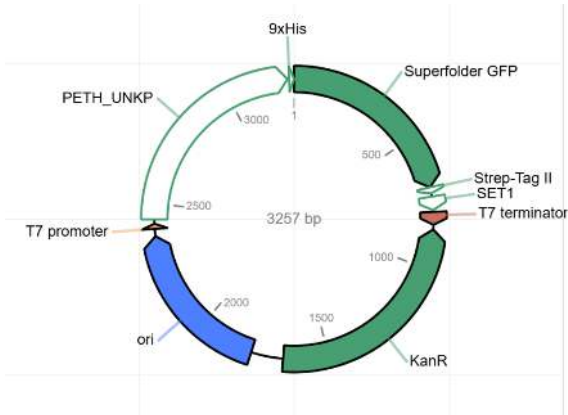


Figure 3. pJL1 + LCC (modified) + GFP Plasmid

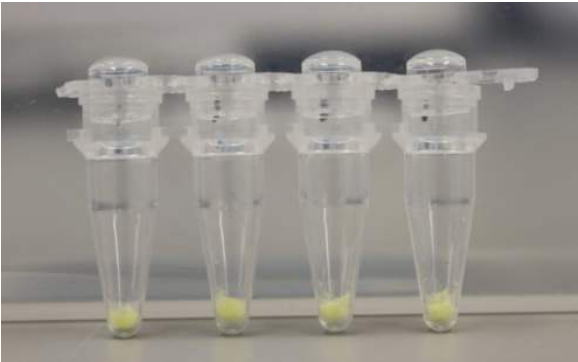


Figure 4. BioBits® (miniPCR, 2019).

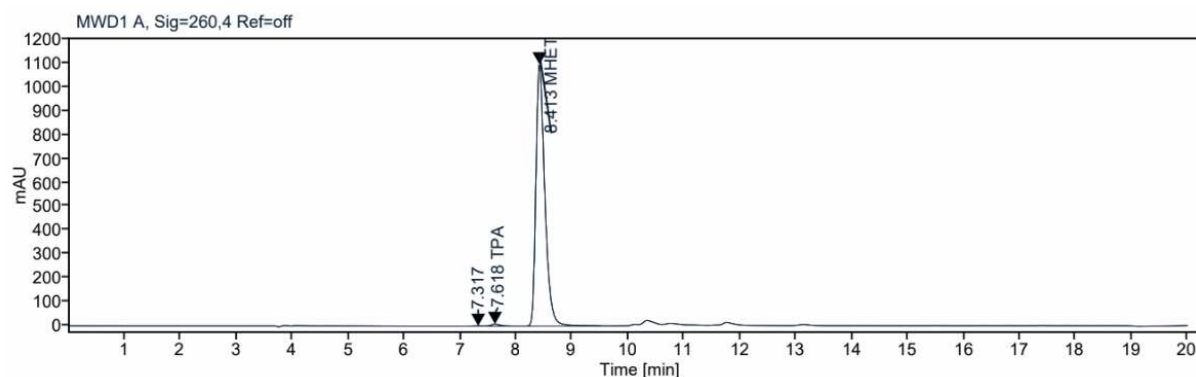


Figure 5. HPLC TPA + MHET Report

plastic recycling and waste reduction. However, the experiments encountered several challenges that may have affected the results and data collected.

Protein purification

Determining exact enzyme concentration was hindered by lack of enzyme purification, resulting in uncertainty regarding the quantity and functionality of the proteins during plastic degradation. Chemically filtered proteins in future experiments will help determine the effect of protein purification on PET degradation. A His-tag on the GFP within the plasmid constructs would separate the enzymes from the complex mixture of BioBits cellular components (Figures 1-3).

Cell-free systems don't guarantee correct protein folding

Without verification methods such as Western blotting, there was no confirmation that functional PETase or LCC enzymes were synthesized. As a result, the presence of GFP fluorescence could not be directly linked to active enzyme production, and it remains unclear whether the intended proteins were actually properly formed. Improper folding and standard cell-free expression may result in GFP expression but do not necessarily indicate enzyme activity. With proper characterization, enzyme structure can be better understood.

Removing impurities from PET powders and films

While trying to remove the plastic powders and films from PETase and LCC by rinsing in nuclease-free water, there were likely contaminants on the PET film and powders, such as DNA or BioBits® ingredients. A number of SEM scans featured extraneous substances on the surface of PET films, interfering with our observations.

Inability to remove background in SEM scans of PET powders

Extensive clumping of PET powder inhibited separation of individual particles. Without proper particle isolation, circularity was immeasurable.

Presence of non-quantified unknown substances

While quantifying the concentrations of PET monomers left behind in solution, there were innumerable amounts of additional substances that could have been tested for.

Plasmid constructs with T7 promoter and T7 terminator

The T7 promoter and T7 terminator in the plasmid constructs (Figures 1-3) were needed for BioBits® to work. They are highly efficient transcriptional components that originated in bacteriophage viruses and are needed for high affinity to the cell-free ingredients, such as RNA polymerase. However, it should be noted that these constructs would be unable to hypothetically be used in cells as they are, because the T7 promoter is so strong that it would attract a cell's RNA polymerase and other molecules away from any of the cell's own transcription and translation. Therefore, if these specific constructs were attempted to be used in cells, the cells would likely not survive.

Next steps

In future experiments, it will be crucial to address the issues highlighted in the discussion section, such as maintaining consistency with DNA and BioBits® and efficiently using equipment to measure PET degradation. Moreover, further research should be performed to explore more effective methods to determine differences in before and after PET films and powders from

SEM scans. Experiments incorporating these strategies should be conducted to confirm or enhance the data collected.

Acknowledgements

The authors would like to acknowledge Dr. Lindsey L'Ecuyer for her support in our research and her guidance with lab experiments. We also wish to thank the Andover Coalition for Education (ACE) for providing the necessary funding to carry out this project. We are very grateful for our mentors, Nathan Crook and Tianyu Li at NCSU, for guiding us with research and providing us with information and lab techniques necessary. Lastly, the authors thank all the original students, Puloma Bishnu and Krishnaveni Dole.

References

Organisation for Economic Co-operation and Development. (2022, February 22). *Plastic pollution is growing relentlessly as waste management and recycling fall short, says*

OECD. Retrieved April 9, 2025, from <https://www.oecd.org/en/about/news/press-releases/2022/02/plastic-pollution-is-growing-relentlessly-as-waste-management-and-recycling-fall-short.html>

Sulaiman, Sintawee et al. "Isolation of a novel cutinase homolog with polyethylene terephthalate-degrading activity from leaf-branch compost by using a metagenomic approach." *Applied and environmental microbiology* vol. 78,5 (2012): 1556-62. doi:10.1128/AEM.06725-11

Meet a scientist: Jessica Stark unleashes the potential of cell-free systems. (2023, August 18). miniPCR bio. Retrieved October 12, 2026, from <https://www.minipcr.com/meet-a-scientist-jessica-stark/>

miniPCR bio™ launches BioBits™ system for synthetic biology and STEM education. (2019, September 12). miniPCR bio. Retrieved October 12, 2026, from <https://www.minipcr.com/minipcr-bio-launches-biobits-system-for-synthetic-biology-and-stem-education/>