

# Hydrolysis of PET by fusing modified hydrophobins to cutinase

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Polyethylene terephthalate (PET) is a widely used and highly recyclable plastic polymer common in food packaging, plastic water bottles, and fabrics such as polyester. Despite recent efforts, most enzymes researched for hydrolysis of the polymer have proven to be moderately inefficient in hydrolytic capabilities for high-crystallinity PET. However, the recently discovered leaf-branch compost cutinase (LCC) is particularly suited toward these ends given its relatively high PET hydrolysis activity and thermostability, capable of reaching a depolymerization rate of 93.2 mgTAEq/ (h\* mgenzyme) at 65°C, around 30 times higher than most other enzymes tested so far. In addition, fusing an enzyme with another protein exhibiting hydrophobic properties, such as a hydrophobin, can significantly increase the levels of PET hydrolysis, potentially improving the hydrolysis 16-fold over the level with the free enzyme. Despite the improvement, one of the major downsides to this method is the formation of inclusion bodies, or accumulated clusters of the fusion proteins, as hydrophobins form disulfide bridges between cysteine residues amongst themselves when expressed. The effects among the different types of hydrophobins fused to the enzyme vary, as removing the disulfide bridges of a class I hydrophobin via substitution of cysteine with another non-compromising amino acid was significantly less detrimental to the structural integrity of the protein when compared to class II hydrophobins and has a negligible effect on the self-assembly of the protein. We plan to express a fusion protein of LCC with the HYD1 class I hydrophobin in *E. Coli* through transformation using a plasmid prepared with general recombinant DNA techniques, followed by purification of the enzyme to use in PET hydrolysis to test how much the efficiency of the PET depolymerization improves for potential commercial use in other systems.

**Keywords** Polyethylene terephthalate, Hydrolysis, Fusion protein, Leaf-branch compost cutinase, Hydrophobins

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Watch a video introduction by the authors at <https://youtu.be/spNz3J8ARQQ>

## Background

Polyethylene terephthalate (PET) is the world's packaging choice for many foods and beverages because it is hygienic, strong, lightweight, shatterproof, and retains freshness. It is most commonly used for packaging carbonated soft drinks and water. PET is a very inert material that is resistant to attack by microorganisms and does not react with food products, which is why it is widely preferred for packaging foods, beverages, and pharmaceuticals. PET is also recyclable and highly sustainable: it is the most recycled plastic in the United States and worldwide. PET can be recycled repeatedly under ideal conditions, back into containers for foods, beverages and personal care products, or into the carpet, clothing, automotive parts, construction materials, industrial strapping, and scores of other products (Tournier et al., 2020).

It is estimated that of the 359 million tons of plastics produced annually worldwide, 150–200 million tons accumulate in landfills or in the natural environment, despite the high recyclability of the polymer (Tournier et al., 2020). In addition, recycling of PET and modification of its properties for different applications by traditional procedures involve harsh chemical and physicochemical treatments. Enzymatic modification, particularly by cutinases such as Thc\_Cut1 (Figure 1), has been recognized as a powerful alternative in the past decade and, besides offering new avenues for PET recycling, has the additional advantage of creating a modified PET with increased dyeing efficacy and improved binding to polyvinyl chloride without altering the polymer's bulk properties.

## Systems Level

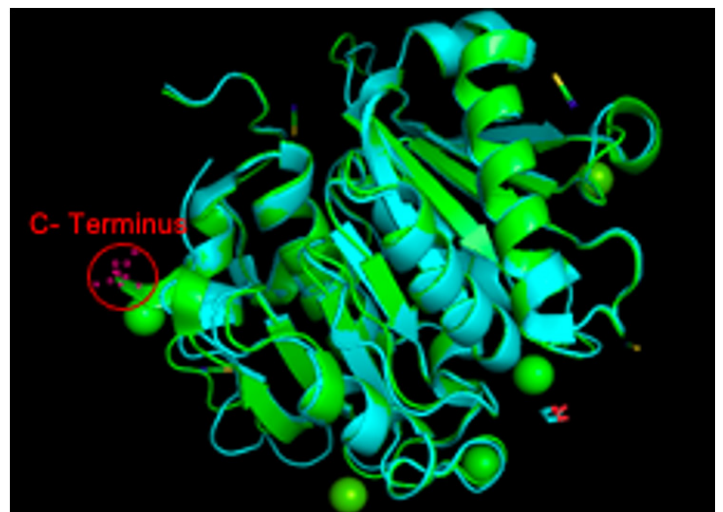
Enzymatic hydrolysis of PET has the inherent disadvantage of occurring at a very low rate for reasons that aren't entirely clear. One specific potential limiting factor is the access of the epitopes (active site of the enzyme) to the substrate. Many enzymes that act on insoluble substrates would usually contain protein

domains for substrate binding.

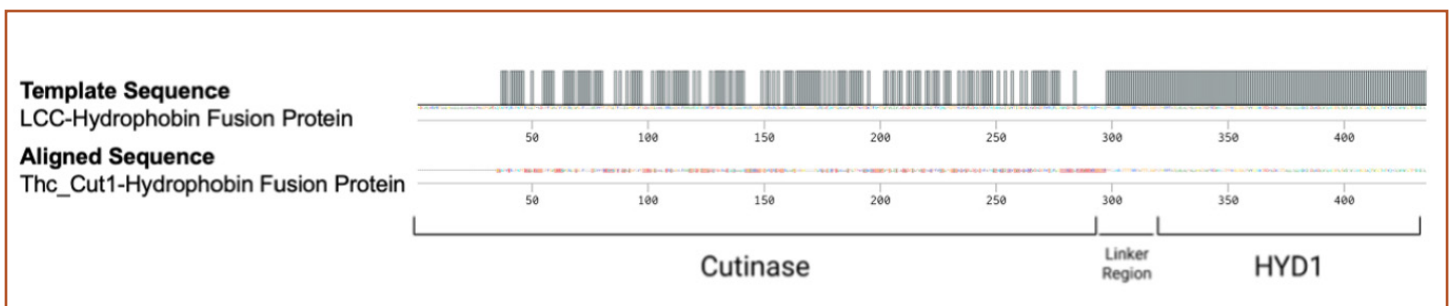
However, with our design, we propose instead the fusion of hydrophobins, small cysteine-rich proteins of exclusively fungal origin that can naturally adsorb to hydrophobic surfaces and interfaces between hydrophobic (air, oil, and wax) and hydrophilic (water and cell wall) phases. The mechanism by which the hydrophobins stimulate the enzymatic activity of cutinases on PET is essentially unknown, however, and could involve the creation of a more hydrophilic surface, the binding and targeting of the cutinases to PET, or even the direct modulation of their activity (Ribitsch et al., 2015).

## Device Level

Cutinases, also known as cutin hydrolases, are enzymes discovered from phytopathogenic fungi that grow on cutin as the sole carbon source. These enzymes share catalytic properties of lipases and esterases, presenting a unique feature of being active regardless of the presence of an oil-water interface, making them a potential biocatalyst



**Figure 2:** A model alignment of LCC (Green) and a different yet similar cutinase taken from *Thermobifida Cellulosilytica*, Thc\_Cut1 (Blue) that has previously been fused to hydrophobins (Ribitsch et al., 2015).



**Figure 1.** Amino acid sequence alignment between a fusion protein for LCC (UniProt accession no. G9BY57) and an alternate fusion for Thc\_Cut 1 (GenBank accession no. ADV92526.1), both with the HYD1 class I hydrophobin (UniProt accession no. D8QCG9). The grey bars at the top indicate where the protein sequences align.



**Figure 3.** Removing the four-disulfide bridges would remove the amino acid sequences labeled S1, S2, S3, and S4 from both hydrophobins DewA and HYDI and would result in the absence of disulfide bridges. The process of hydrophobins connecting with other hydrophobins via disulfide bridges would be nulled, and no inclusion bodies would form (Cheng et al., 2020).

in several industrial processes involving hydrolysis, which, combined with their high stability in organic solvents, further solidifies their potential for use in PET depolymerization (Pio et al, 2009).

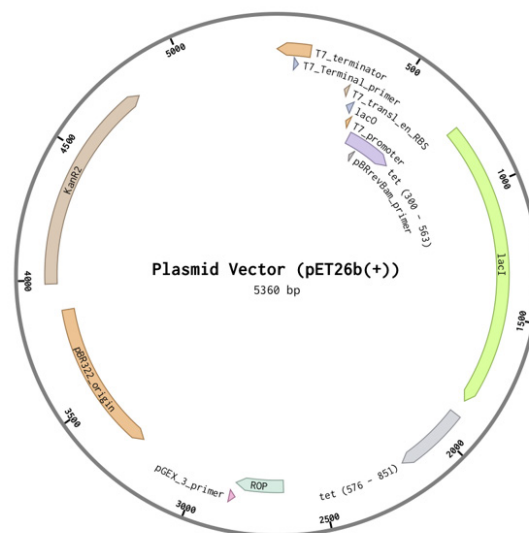
Of the variety of cutinases, however, Leaf-Branch Compost Cutinase (LCC) has been found to be approximately 33 times more efficient than most other enzymes tested so far, capable of reaching an initial PET-specific depolymerization rate of 93.2 mgTAEq/ (h \* mgenzyme) at 65°C. They can also maintain a relatively high thermostability at a melting temperature of 84.7°C (Tournier et al., 2020).

Fused by a linker at the C-terminus of the LCC protein is the hydrophobin. (Figure 2). Hydrophobins are small, cysteine-rich proteins produced by fungi. They can naturally adsorb to hydrophobic surfaces and form elastic films by self-assembling at interfaces, which could possibly create challenges for large scale production. They have also been found to facilitate or accelerate the enzymatic activity of cutinase hydrolysis of PET. However, due to their high cysteine content, hydrophobins tend to form inclusion bodies, leading to aggregation and thus hindering effectiveness.

There are two main identifying distinctions in labeling hydrophobins between Class I and Class II hydrophobins. Class I hydrophobins tend to have a diverse amino acid sequence between different types of hydrophobins. They can only dissolve in strong acids and have only one, varied inter-cysteine spacing. Examples include hydrophobins DewA and HYD1 (Figure 3), which do not form covalent bonds via disulfide bridges; and, after removing the four-disulfide bridges by replacing the cysteine residues with serine, do not express inclusion bodies (Cheng et al., 2020).

Compared to Class I, Class II hydrophobins tend to have a conserved amino acid sequence, can be easily dissolved within detergents, and have short, regular inter-cysteine spacing. Examples include hydrophobins HFB4 and HFB7.

Both hydrophobins are natural hydrophobins that do not form inclusion bodies when expressed with cutinase, but only when expressed at the suboptimal temperature of 25°C for no more than 5 hours. However, when these



**Figure 4:** A plasmid model showing the region taken up by our fusion protein (Green) within the pET26b(+) vector

hydrophobins, expressed within an optimal environment, are shown to have enhanced PET hydrolysis by up to 16-fold when fused with the Thc\_Cut1 cutinase (Ribitsch et al., 2015).

Although hydrophobins are primarily a result of protein production by fungi, it is shown that there can also be natural soluble bacterial hydrophobins as well, specifically BSIA. This bacterial hydrophobin lacks cysteine, which eliminates the formation of disulfide inclusion bodies (Hobley et al., 2013). However, we chose to design the protein with the HYD1 class I hydrophobin, in the effort of continuing with a hydrophobin of fungal origin to follow previous experiments best, allowing the cysteine residues

to be removed without compromising the self-assembly of the protein (Cheng et al., 2020).

## Parts Level

In order to express the fusion protein in transformed *E. Coli*, a plasmid is created by inserting the genes for each of the proteins into the vector pET26b (+) through gibson assembly connected by the linker region from cellobiohydrolase I in *T. reesei* (UniProt accession no. P62694), allowing expression of the enzymes without the pelB signal peptide (Figure 4). The genes coding for LCC, HYD1, and the linker region were codon-optimized for expression in *E. Coli*.

## Safety

In our case, it would be safest to conduct the actual experiment in our high school biotechnology laboratory. When working with the bacteria, we plan to consult our mentors with expertise for the general precautions of handling *E. Coli*, including the proper personal protective equipment (Gloves, Lab Coats, etc.) and safely disposing of materials and reagents.

In addition, we would need to consider the limitations imposed due to the current pandemic. If we were all to gather for the experiment, it would be best to do so after all members have been vaccinated, as well as continuing to follow whatever guidelines and protocols put in place by the lab or school.

## Discussions

Since our immediate goals are to conduct proof of concept experiments, we will not release these products into the environment. Additionally, we recognize that the exact effects of our bacteria on the ecosystem cannot be precisely predicted, and thus we plan to conduct an environmental impact analysis regarding the potential effects of our design on the ocean and marine life regarding the byproducts that may be released by the enzymatic processes of our protein.

## Next Steps

To further advance with this project, we would really need to be able to get together in a lab and fully test our experiment, as outlined by our design. If anyone were to pick up this project with the intent of further developing the design, we would hope that they incorporate elements of circular permutation to further improve the catalytic activity of the protein.

## Author Contributions

All authors contributed to the design idea. E.R. and J.E.

assisted with visuals and graphics. J.E. and S.H. took the lead in writing the manuscript. S.H. recorded the project's video.

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