Engineering the activity of a thermophilic esterase from *Zhizhongheella caldifontis* for MHET degradation

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Introduction

Polyethylene terephthalate (PET) is the world's most extensively recycled polymer, the dominant material for beverage packaging, and valuable enough to drive sustained R&D into post-consumer recovery strategies.[1] Enzymatic depolymerization can fully convert PET into its monomers, terephthalic acid (TPA) and ethylene glycol (EG), enabling separation from mixed-plastic streams, upgrading to higher-value chemicals, and re-synthesis of virgin-quality PET, in contrast to the chain-degrading nature of conventional thermomechanical recycling.[2,3] PETases can recycle PET, but the buildup of the intermediate MHET slows the process.[4] We engineered a thermotolerant esterase from ***Zhizhongheella caldifontis*** (ZcEST) to improve enzymatic recycling of PET. Two tailored variants, ZcMHETase (ZcEST_D355N) and ZcBHETase (ZcEST_D355S), showed dramatic boosts in activity on the PET intermediates MHET and BHET, with up to 21-fold and 56-fold increases compared to the wild-type enzyme respectively and increased temperature stability compared to current benchmark IsMHETase. Experiments using HPLC, thermal stability assays, and kinetic analysis confirmed both the efficiency and resilience of the new variants. Together, these advances position ZcEST-derived hydrolases as strong candidates for industrial PET recycling and demonstrate how targeted protein engineering can accelerate the development of circular plastic solutions.

Methodology

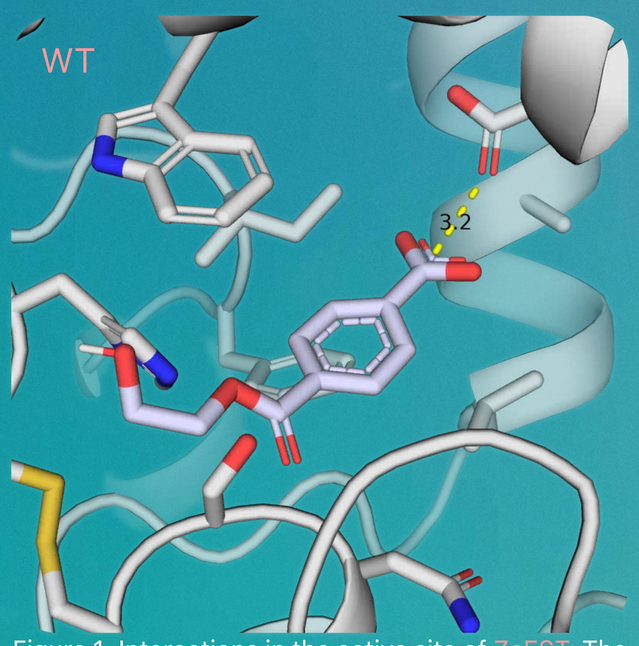

Figure 1: Interactions in the active site of ZcEST. The negatively charged MHET species clashes with D355.

- MHET has a predicted pKa of 3.77, meaning that at the common PETase working ranges of pH > 7 it is negatively charged.
- The wild-type active site of ZcEST possesses an aspartic acid residue (D355) that inhibits MHET binding at this pH range (Figure 1).

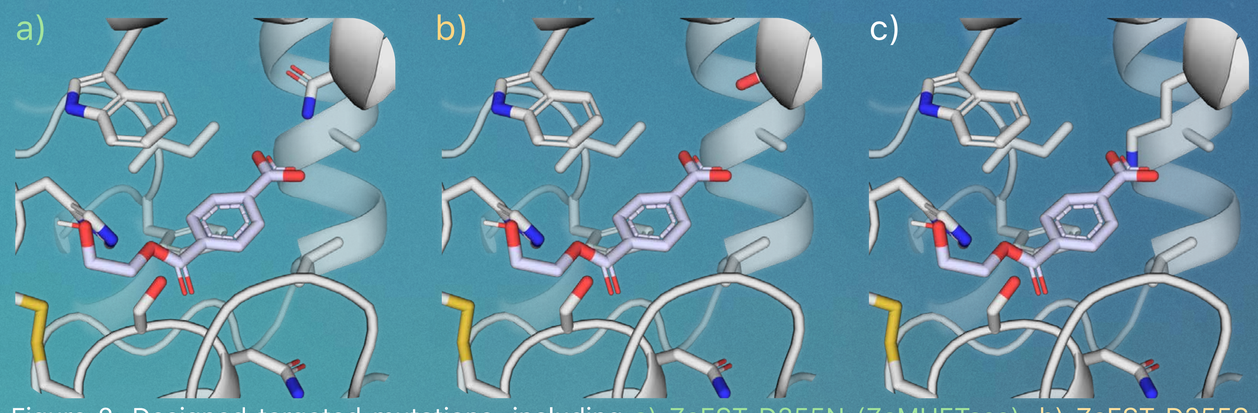

Figure 2: Designed targeted mutations, including a) ZcEST_D355N (ZcMHETase), b) ZcEST_D355S (ZcBHETase) and c) ZcEST_D355K.

- A series of targeted mutations were designed (Figure 2) to test:
  - Adding a positive charge (D355R and D355K), inspired from IsMHETase
  - Hydrogen bonding (D355N)
  - Increased space (D355S and D355A)
- Only D355N and D355S were successfully constructed with site-directed mutagenesis and subsequently assayed.

Results

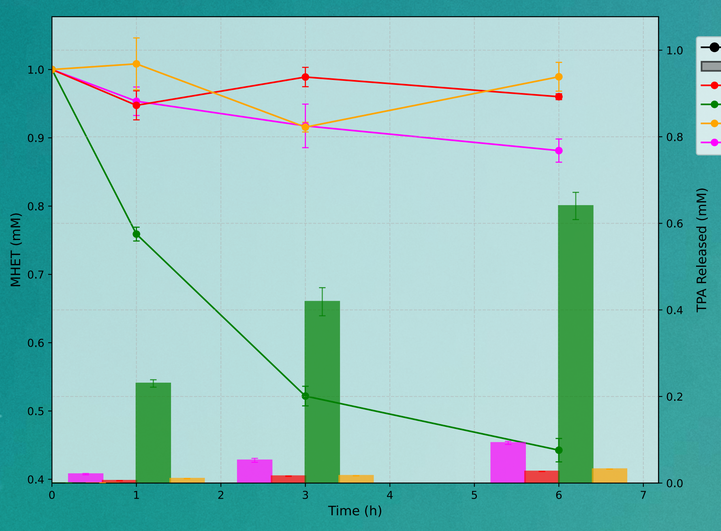

Figure 3: MHET consumption (lines) and release of TPA (bar plots) by wild-type ZcEST (Zc), feruloyl esterase FoFAE and engineered ZcMHETase (ZcD355N) and ZcBHETase (ZcD355S). Error bars represent standard deviations of 3 samples.

Table 1: Melting temperature (DSF)

Enzyme	T_m (℃)
ZcEST	58.72±0.29
ZcEST_D355N	66.56±0.27
ZcEST_D355S	66.27±0.30
IsMHETase	54.19±0.08

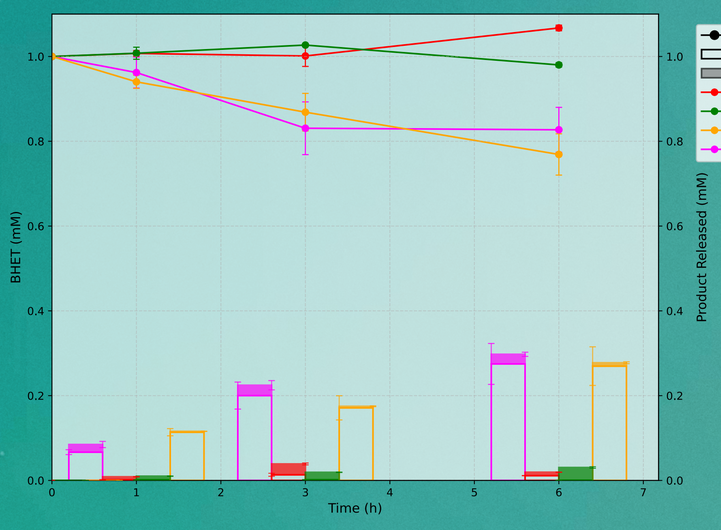

Figure 4: BHET consumption (lines) and release of TPA and MHET(bar plots) by wild-type ZcEST (Zc), feruloyl esterase FoFAE and engineered ZcMHETase (ZcD355N) and ZcBHETase (ZcD355S). Error bars represent standard deviations of 3 samples.

Here it is worth noting that FoFAE carries a serine (S) at the amino acid position point of interest.

Discussion

- Targeted engineering of ZcEST from ***Zhizhongheella caldifontis*** enhanced hydrolysis of PET intermediates.
- Substitution of D355 removed unfavorable interactions with MHET, validating the structural hypothesis.
- Variants ZcMHETase (D355N) and ZcBHETase (D355S) showed 21-fold and 51-fold higher catalytic activity than wild-type in MHET and BHET repsectively.
- Both variants exhibited improved thermostability (>66 °C) compared to current benchmark IsMHETase, meeting industrial needs.
- Further work could explore introducing a positive charge in the active site, inspired by IsMHETase (Figure 5), synergistic integration with PETases and scale-up.
- These advances move enzymatic recycling closer to practical alternatives for plastic management.

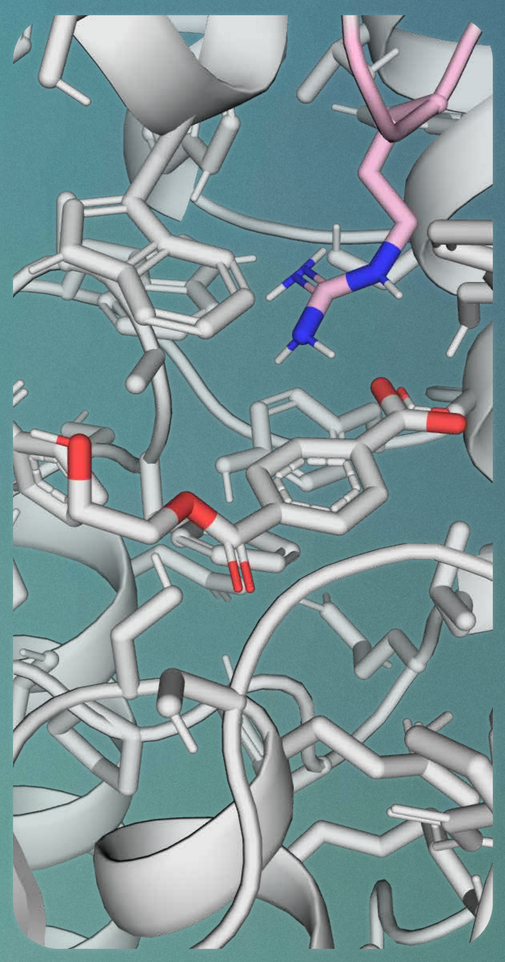

Figure 5: Structural model highlighting R411 in IsMHETase, which contributes to its high catalytic activity.

Acknowledgements

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