

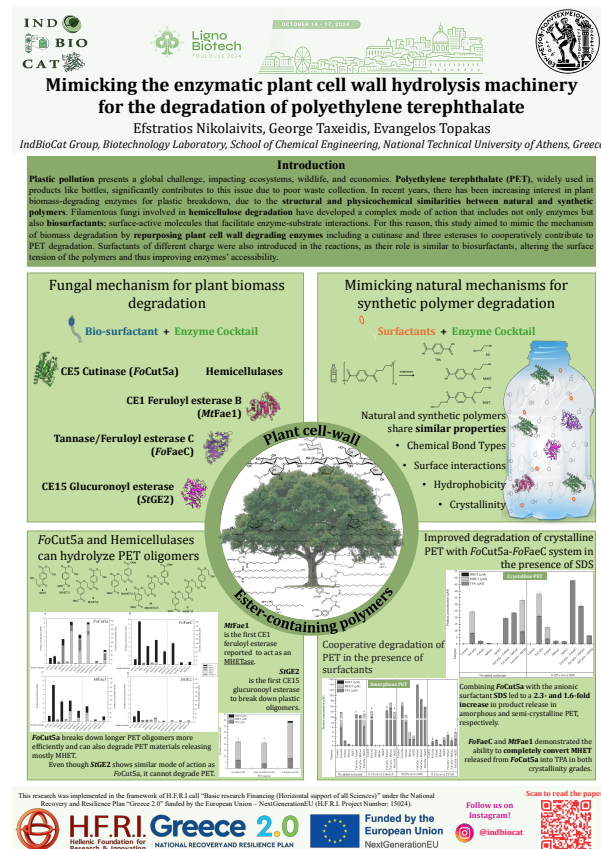
D3.4 Publication and/or presentation in International Conference on the selective degradation of PET & PLA polymers

The results from the investigation of the selective degradation of PET and PLA, which formed the core focus of the experimental activities within WP3, were disseminated at 4 international conferences through a total of 5 poster presentations, and one scientific publication at a peer-reviewed journal.

Poster presentations

- **LignoBiotech 2024**, Toulouse, France (14-17/10/2024), attended by over 250 participants.

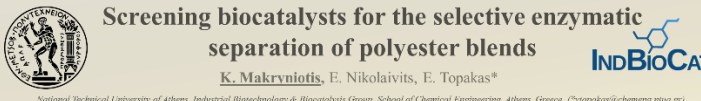
1) “Mimicking the enzymatic plant cell wall hydrolysis machinery for the degradation of polyethylene terephthalate” by Dr. Efstratios Nikolaivits.



The poster described the use of fungal enzymes in a synergistic manner for the selective and complete enzymatic depolymerization of PET, as a part of WP3.

- The 3rd International Electronic Conference on Catalysis Sciences, online (23-25/04/2025), attended by over 500 participants.


2) “Screening biocatalysts for the selective enzymatic separation of polyester blends” by Mr. Konstantinos Makryniotis (PhD candidate).



Screening biocatalysts for the selective enzymatic separation of polyester blends
 K. Makryniotis, E. Nikolaivits, E. Topakas*
National Technical University of Athens, Industrial Biotechnology & Biocatalysis Group, School of Chemical Engineering, Athens, Greece. (*topokar@chemeng.ntua.gr)

The challenge of managing complex packaging waste streams

Packaging materials, mostly utilized in food-related applications, contribute significantly to pollution. Mixed-polymer packaging poses a challenge for **mechanical recycling** due to its poor material properties. **Incineration and chemical recycling** offer partial solutions, they conflict with circular economy goals or lack efficiency for similar-type polymer mixtures.



Enzymatic recycling offers a promising alternative, through selective breakdown of polymers like PLA and PET, addressing challenges associated with complex packaging waste streams.

Experimental procedure

17 serine hydrolases (proteases & esterases)

In-house
Heterologous expression (*P. pastoris* & *E. coli*)

Commercial
Dissolution in optimal buffers

Plastics of aim

PLA
Semi-crystalline PLLA

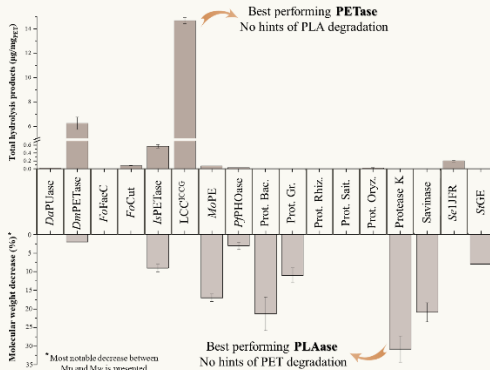
PET
Semi-crystalline PET

Degradation evaluation

Molecular weight alterations (PLA) through GPC

Hydrolysis products (PET) through HPLC

Classification of investigated enzymes



Best performing PETase
No hints of PLA degradation

Best performing PLAase
No hints of PET degradation

*Most notable decrease between Mn and Mw is presented

Enzyme	Classification
<i>DaPUase</i>	PETase
<i>DmPEase</i>	Dual-function
<i>FoFaeC</i>	Inactive
<i>FoCut</i>	PETase
<i>IsPEase</i>	Dual-function
<i>LCC^{CCG}</i>	PETase
<i>MoPE</i>	Dual-function
<i>P/PHOase</i>	PETase
Prot. Bac.	PLAase
Prot. Gr.	PLAase
Prot. Rhiz.	Inactive
Prot. Sait.	Inactive
Prot. Oryz.	PLAase
Protease K	PLAase
Savinase	PLAase
Se1JFR	Dual-function
S/GE	Dual-function

Enzymes for selective degradation in polyester blends

Selective degradation of PET and PLA using LCC^{CCG} and Protease K could purify packaging waste streams, facilitating efficient recycling and promoting sustainable waste management.

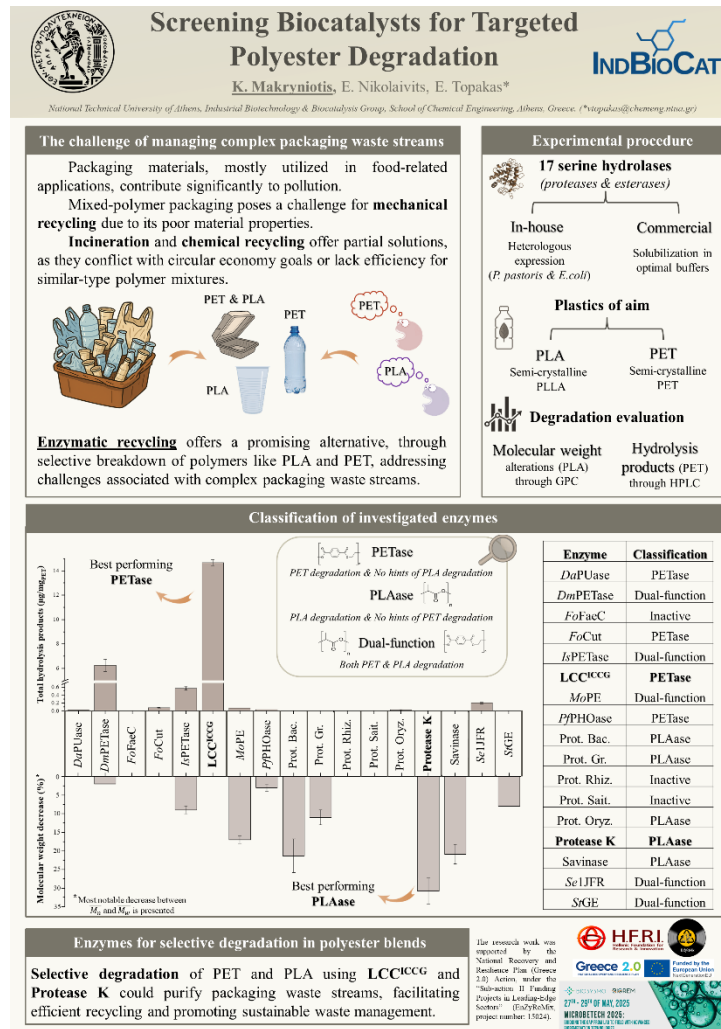
This research work was supported by the National Research and Reference Plan (Contract 2/0 Action, under the "Education 1: Training Program 12, Leader Edge Sector" (NSRF, MIS, project number: 1N254).

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ECOS 2025
The 3rd International Electronic Conference on Catalysis Sciences
23-25 April 2025 Online

3) “Comparing the secretome response of *Aspergillus* and *Fusarium* species on chemically treated plastics” by Mrs. Markella Papi (PhD candidate).


4) “Screening Biocatalysts for Targeted Polyester Degradation” by Mr. Konstantinos Makryniotis (PhD candidate).



Both posters presented results accomplished through WP3, focusing on the investigation of 17 serine hydrolases, including esterases and proteases, for their ability to selectively degrade project’s key polyesters, PET and PLA. Through heterologous expression in *E. coli* and *P. pastoris*, followed by polymer degradation screening utilizing GPC and HPLC analyses, distinct substrate preferences were observed. Esterases exhibited limited polymer selectivity, while proteases favored PLA degradation. Notably, LCC^{ICCG} exhibited the highest PET hydrolysis activity (PETase), whereas Protease K and Savinase (PLAases) efficiently degraded amorphous and semi-crystalline PLA, respectively.

- **Joint International Conference of Mikrobiokosmos & CEESME**, Thessaloniki, Greece (22-24/09/2025), attended by over 200 participants.

5) “Engineering the activity of a thermophilic esterase from *Zhizhongheella caldifontis* for MHET degradation” by Mr. Konstantinos Grigorakis (PhD candidate).




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

Konstantinos Grigorakis¹, Christina Feroouli¹, Natalia Kastana¹, Efstratios Nikolaiivits¹, Evangelos Topakas^{1*}

¹Industrial Biotechnology & Biocatalysis Group, Biotechnology Laboratory, School of Chemical Engineering, National Technical University of Athens, Athens, Greece

*topakas@central.ntua.gr



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, ZcMHEase (ZcEST_D355N) and ZcBHEase (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 ISMHEase. 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

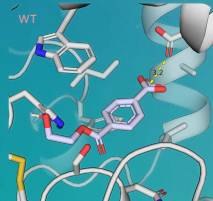


Figure 1: Interactions in the active site of ZcEST. The negatively charged MHET species clashes with D355.

- 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).

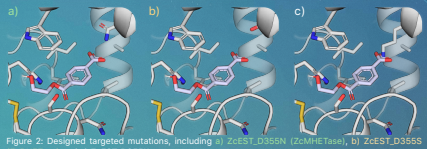


Figure 2: Designed targeted mutations, including a) ZcEST_D355N (ZcMHEase), b) ZcEST_D355S (ZcBHEase) and c) ZcEST_D355K.

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

Results

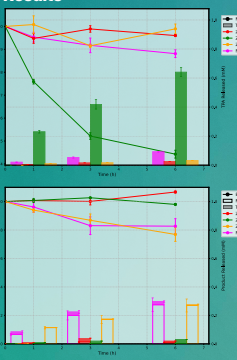


Figure 3: MHET consumption (lines) and release of TPA (bar plots) by wild-type ZcEST (Zc), ZcMHEase (ZcMHEase) and engineered ZcMHEase (ZcD355N) and ZcBHEase (ZcD355S). Error bars represent standard deviations of 3 samples.

Enzyme	TpA (%)
ZcEST	38.22±0.90
ZcMHEase	68.56±0.27
ZcBHEase	88.27±0.30
ISMHEase	54.19±0.08

Table 1: Melting temperature (DSF)

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

Here it is worth noting that F355A 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 ZcMHEase (D355N) and ZcBHEase (D355S) showed 21-fold and 51-fold higher catalytic activity than wild-type in MHET and BHET respectively.
- Both variants exhibited improved thermostability (>66 °C) compared to current benchmark ISMHEase, meeting industrial needs.
- Further work could explore introducing a positive charge in the active site, inspired by ISMHEase (Figure 5), synergistic integration with PETases and scale-up.
- These advances move enzymatic recycling closer to practical alternatives for plastic management.

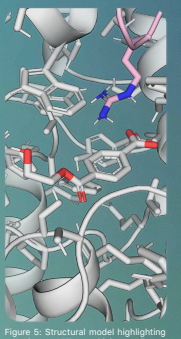



Figure 5: Structural model highlighting R411 in ISMHEase, which contributes to its high catalytic activity.

Acknowledgements



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Scientific publication

A review research paper was published on [Catalysts](#) journal in an open access format titled “Protein Engineering for Industrial Biocatalysis: Principles, Approaches, and Lessons from Engineered PETases” by K. Grigorakis, C. Ferousi and E. Topakas.

The paper explores the principles and methodologies of protein engineering, emphasizing rational design, directed evolution, semi-rational approaches, and the recent integration of machine learning. These strategies have significantly enhanced enzyme performance, even rendering engineered PETase industrially relevant. Insights from engineered PETases underscore the potential of protein engineering to tackle environmental challenges, such as advancing sustainable plastic recycling, paving the way for innovative solutions in industrial biocatalysis. More specifically, this work is correlated with making enzymes specific for specific polymers with high yields that make them industrially relevant.

Open Access Feature Paper Editor's Choice Review

Protein Engineering for Industrial Biocatalysis: Principles, Approaches, and Lessons from Engineered PETases

by Konstantinos Grigorakis , Christina Ferousi  and Evangelos Topakas * 

IndBioCat Group, Biotechnology Laboratory, School of Chemical Engineering, National Technical University of Athens, Zografou Campus, 9 Iroon Polytech Str., 15772 Athens, Greece

* Author to whom correspondence should be addressed.

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