

ARTICLE OPEN ACCESS

High-Efficiency PET Degradation With a Duo-Enzyme System Immobilized on Magnetic Nanoparticles

Siddhi Kotnis  | Siddhant Gulati | Qing Sun

Department of Chemical Engineering, Texas A&M University, College Station, Texas, USA

Correspondence: Qing Sun (sunqing@tamu.edu)

Received: 7 November 2024 | **Revised:** 20 February 2025 | **Accepted:** 23 February 2025

Funding: This work was supported by funds provided by a National Science Foundation (NSF) grant to Q. S. (2203715), USA; NSF Emerging Frontiers in Research and Innovation (EFRI) to Q. S. (2132156), USA; Texas A&M Excellence Fund X-grants to Q. S., Texas A&M, USA; Texas A&M Targeted Proposal Teams (TPT) grant to Q. S., Texas A&M, USA; NIH NIAID R01 to Q. S. (R01AI165433), Texas A&M, USA. This work was partially supported by the National Science Foundation (NSF) under Award No. 2330245, which funds the Engineering Research Center for Carbon Utilization Redesign through Biomanufacturing-Empowered Decarbonization (CURB).

Keywords: bioconjugate formation | co-immobilization | nanoparticle enzyme assembly | PET degradation

ABSTRACT

The widespread consumption of PET worldwide has necessitated the search for environment-friendly methods for PET degradation and recycling. Among these methods, biodegradation stands out as a promising approach for recycling PET. The discovery of duo enzyme system PETase and MHETase in 2016, along with their engineered variants, has demonstrated significant potential in breaking down PET. Previous studies have also demonstrated that the activity of the enzyme PETase increases when it is immobilized on nanoparticles. To achieve highly efficient and complete PET depolymerization, we immobilized both FAST-PETase and MHETase at a specific ratio on magnetic nanoparticles. This immobilization resulted in a 2.5-fold increase in product release compared with free enzymes. Additionally, we achieved reusability and enhanced stability of the enzyme bioconjugates.

1 | Introduction

Polyethylene terephthalate (PET) is a widely used thermoplastic for packaging, constituting up to 67% of the total packaging plastic in the US (Benyathiar et al. 2022). Due to its resistance to chemical and mechanical changes, it is a significant challenge to manage PET waste efficiently (Malik et al. 2017). The two major ways of recycling and upscaling PET are through chemical and mechanical recycling (Smith et al. 2022). Chemical recycling is achieved through solvolysis or pyrolysis where PET is degraded to its monomers of ethylene glycol (EG) and terephthalic acid (TPA) (Cao et al. 2022). These processes are economically unfeasible and involve harsh reaction conditions, leading to the release of harmful toxins into the environment (Rahimi and García 2017; Sinha et al. 2010). On the other hand, mechanical recycling reshapes PET into other desirable shapes, but this can only be done for a limited number of cycles before

the polymer loses mechanical strength and cannot be used further (Schyns and Shaver 2021).

An environmentally friendly and cost-effective alternative is the biodegradation of PET using enzymes as biological catalysts to degrade PET into its monomers (Taniguchi et al. 2019; Wei et al. 2022). Among these enzymes, PETase exhibits high activity at room temperature, making it ideal for PET degradation at mild reaction conditions (Yoshida et al. 2016). Following the discovery of PETase in 2016, there have been many mutations of PETase showing better activities and thermostabilities, (Lu et al. 2022; Son et al. 2019; Cui et al. 2021) within which FAST-PETase exhibited the best performance with 10 times better activity than wildtype PETase at 30°C. Moreover, PETase, along with MHETase, shows a synergistic effect in degradation of PET to monomers TPA and EG (Knott et al. 2020). The presence of MHETase, even small amount, is

This is an open access article under the terms of the [Creative Commons Attribution-NonCommercial-NoDerivs](#) License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

© 2025 The Author(s). *Biotechnology and Bioengineering* published by Wiley Periodicals LLC.

crucial for efficient complete depolymerization of PET (Knott et al. 2020).

One of the major obstacles to commercialize the enzyme system is the enzyme aggregation and instability at higher concentration (Shirke et al. 2018). Reports indicate that at concentrations exceeding 150 nM, PETase begins to aggregate, resulting in reduced enzymatic efficiency (Gercke et al. 2021). To overcome these challenges, enzyme immobilization has emerged as an attractive alternative to solve the enzyme aggregation and instability problem (Maghraby et al. 2023; Roth et al. 2016; Zanuso et al. 2021; Tsai et al. 2013). Magnetic nanoparticles, within all different supporting platforms for enzyme immobilization (Maghraby et al. 2023), have advantages such as being biofriendly and easy separation (Bilal et al. 2018; Schwaminger et al. 2019a; Leong et al. 2020). Immobilization of single enzyme PETase on nanoparticles including cobalt phosphate nanoflowers and iron oxide nanoparticles has shown increased PET degradation efficiency (Schwaminger et al. 2021; Jia et al. 2021). However, these system has not achieved complete PET degradation due to the limitations of the single-enzyme approach.

In this work, we propose a novel approach of immobilizing both FAST-PETase and MHETase on iron oxide nanoparticles in appropriate ratio to maximize PET degradation and achieve complete depolymerization of PET to TPA and EG. These bioconjugates, formed through the electrostatic interactions between his tag on the enzymes and the nanoparticle, (Schwaminger et al. 2019b) enhances the efficiency of the PET degradation system. Additionally, the bioconjugates exhibit improved stability and reusability, demonstrating the potential of the immobilization system for plastic waste management.

2 | Experimental Section

2.1 | Expression and Purification of Proteins

pBbE8k-FAST PETase was constructed and transformed on *E. coli* NEB5 α and then on *E. coli* SHuffleT7 for expression. pET21a-MHETase was constructed and transformed on *E. coli* NEB5 α and then *E. coli* C41(DE3) for expression. The enzymes were expressed and then purified using the hexa-histidine tag with Ni-NTA column.

2.2 | Immobilize MHETase and Fast PETase on MNPs

Varying concentrations of the enzyme mixture(0.1–0.3 g/L) were added to 1 g/L magnetic nanoparticles (MNPs, iron oxide nanoparticles, 15 nm, Sigma Aldrich) and incubated at 25°C with shaking at 250 rpm for 2 h. There are two approaches to addition of enzymes on the MNPs, simultaneous and sequential. The simultaneous approach involves addition of both enzymes at the same time, while the sequential approach involves addition of MHETase at $t = 0$ h and then addition of FAST-PETase at $t = 1$ h. The MNP-enzyme nano-bioconjugates are separated using a Magnet. The supernatant was analyzed for quantifying the protein through DC Protein Assay (Biorad). The DC protein assay is a colorimetric assay for protein concentration measurement based on the Lowry assay.

This assay primarily consists of two steps leading to color development, the reaction between protein and copper in an alkaline medium, and the subsequent reduction of Folin reagent by the copper-treated protein. This results in the formation of a characteristic blue color, which can be recorded using absorbance at 750 nm. A 10% SDS-PAGE Gel is also run with the supernatant to visualize the enzyme immobilized on the MNPs. The nano-bioconjugates were washed thrice with PBS and stored in PBS at 4°C.

2.3 | Enzyme Activity on PET Film

PET film (Goodfellow ES301445) was washed with 70% Ethanol. 1 μ M (0.029 g/L) total enzyme was incubated with the PET film to a final volume of 300 μ L reaction medium (pH 7.5 50 mM NaH₂PO₄, 100 mM NaCl, 10% (v/v) DMSO) for 96 h at 30°C and 250 RPM. Samples were collected at 96 h, and heat treated at 85°C for 20 min to denature and inactivate enzymes in the solution. The samples were passed through 0.22 μ m spin filters before HPLC analysis. The TPA concentration was then determined through HPLC analysis at 240 nm. Same steps are followed for bioconjugates as well, where nano-bioconjugates (NBCs) are added to achieve a final enzyme concentration of 1 μ M.

2.4 | Reusability and Stability

The MNPs were separated from the reaction medium using a magnet. They were washed thrice with PBS (pH 7.4) and stored at 4°C. pNPA (*para*-Nitrophenyl acetate) was used as a substrate for the determination of activity of the bioconjugates and free enzyme for reusability and stability. For the assay, a final concentration of 2 mM pNPA and 5% (v/v) DMSO was used with a 200 μ L final reaction volume of PBS (pH 7.4) (Zhu et al. 2022). The assay is conducted in a 96 well plate and the absorbance is monitored at 405 nm for 10 min with 30 s intervals.

3 | Results and Discussion

3.1 | Immobilization of Enzymes on MNPs

PETase and MHETase have shown a synergistic effect in the complete depolymerization of PET to its monomers of TPA and EG (Knott et al. 2020). The presence of MHETase, even small amount, is crucial for efficient complete depolymerization of PET. However, the concentration of MHETase has minimal impact on the depolymerization performance of the two enzyme system (Knott et al. 2020). With the mutant variant FAST-PETase, we observed a similar behavior, achieving optimal product yield at a molar ratio of 20:1 between FAST-PETase and MHETase. At MHETase concentrations lower than this, the product release began to decline (Supplementary Fig. 1).

Aiming to maximize PET degradation, we sought to immobilize the enzymes on nanoparticles at the optimal ratio of 20:1. Given the relatively minor ratio of MHETase, we employed two immobilization strategies to maximize enzyme loading at this ratio: a simultaneous method, where both enzymes were added

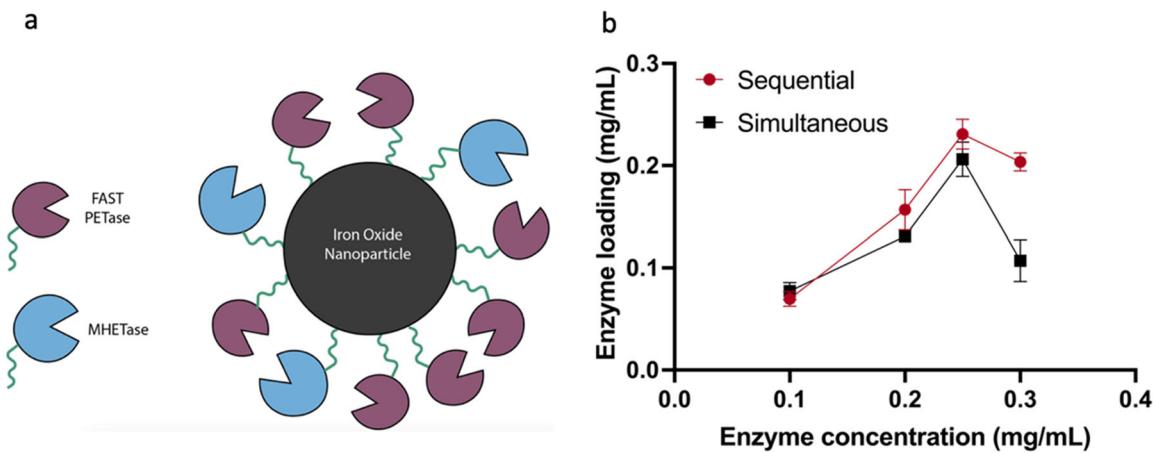


FIGURE 1 | Overall scheme of the paper and total enzyme immobilization on magnetic nanoparticles. (a) FAST-PETase and Mhetase immobilizing on the iron oxide nanoparticle through a histag. (b) The comparison in enzyme loading for sequential (black) and simultaneous (pink) addition of enzymes.

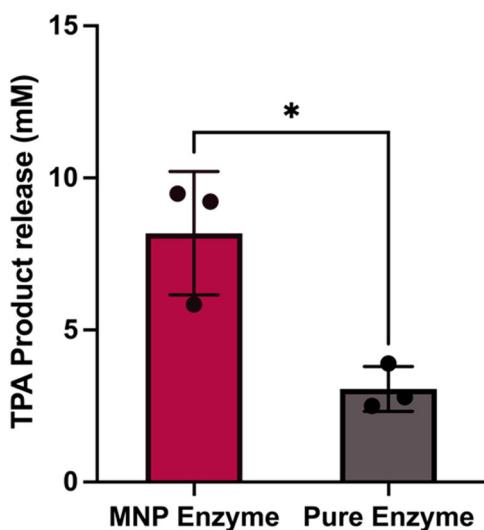


FIGURE 2 | Comparison of TPA released (mM) for a 1 μ M concentration of enzyme for both, bioconjugates and free enzyme samples ($p = 0.018$).

together, and a sequential method, where Mhetase was added before FAST-PETase.

The immobilization of FAST-PETase and Mhetase onto nanoparticles was verified qualitatively using SDS-PAGE analysis (Supplementary Fig. 2) and quantitatively using protein concentration DC Assay. The analysis of the supernatant before and after magnetic beads immobilization indicated the successful enzyme binding onto the nanoparticles Figure (1b). We explored enzyme concentrations ranging from 0.1 to 0.3 g/L for both immobilization methods. While sequential addition showed no advantages at the lower concentration of 0.1 g/L, it displayed increased enzyme loading at 0.3 g/L, likely due to reduced competitive binding, steric hindrance of enzymes, or aggregation that happens at higher enzyme concentration as compared to simultaneous addition. Mhetase is two-fold higher in molecular weight as well as 1.8-fold larger in dimensions when compared to FAST-PETase, which is one of the factors resulting into steric hindrance (Knott et al. 2020). At an initial concentration of 0.3 g/L, enzyme loading for sequential

addition (~0.2 g/L) was approximately double that of simultaneous addition (~0.1 g/L). Notably, sequential addition resulted in a higher proportion of Mhetase on the nanoparticles, as evidenced by SDS-PAGE analysis of the supernatants (Supplementary Fig. 2). Since highest enzyme loading was observed for the initial total enzyme concentration of 0.25 g/L and sequential addition was favored for higher percentage of Mhetase immobilization, we used these conditions for the subsequent activity assays.

3.2 | Activity on PET Film

Bioconjugates, formed using sequential addition with an enzyme concentration of 0.25 g/L, were tested for enzyme activity on PET films by high-performance liquid chromatography (HPLC) analysis of depolymerization products. TPA were analyzed using HPLC traces at a wavelength of 240 nm (UV) (Supplementary Fig. 3). The peak areas were quantified against a TPA calibration curve (Supplementary Fig. 4). Comparing bioconjugates to an equivalent free enzyme system, the bioconjugates released approximately 8 mM of TPA, significantly higher than the 3 mM observed from the free enzyme system (Figure 2). This enhanced activity is likely due to the increased stability of enzymes, which prevents aggregation above a concentration of 150 nM, as well as the synergy between FAST-PETase and Mhetase from close proximity, which has shown to be beneficial (Knott et al. 2020).

Notably, since the bioconjugates have both FAST-PETase and Mhetase, we observed complete depolymerization of PET to TPA without detecting reaction intermediates like BHET or Mhet in the chromatogram of HPLC traces (Supplementary Fig. 3). Furthermore, we observed a product release of 8 mM TPA after a 96 h incubation of PET film at 30°C.

3.3 | Recyclability

We further tested the recyclability of the bioconjugates through magnetic separation. Instead of PET depolymerization, we used *para*-nitrophenyl acetate (*p*NPA), an alternative substrate for

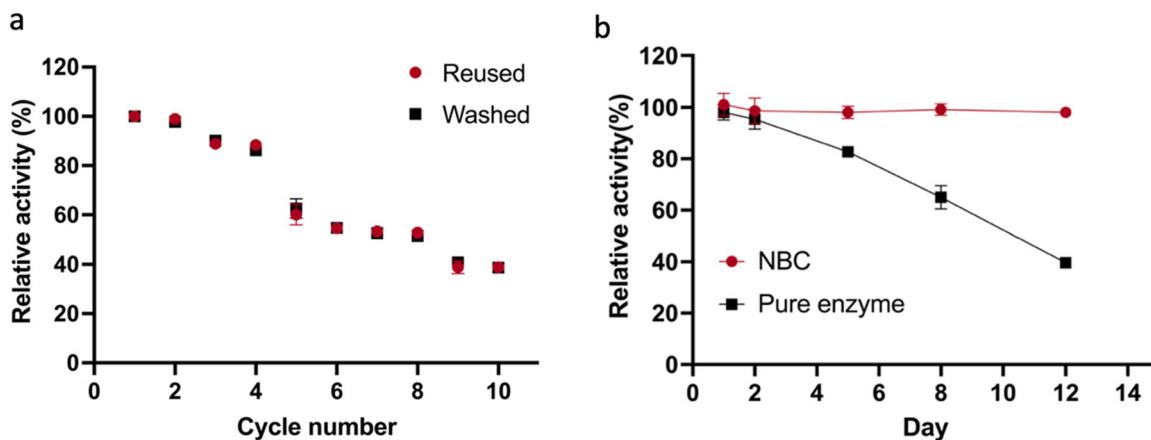


FIGURE 3 | Reusability and stability of bioconjugates. (a) Reusability of enzymes on pNPA substrate for reused samples and control samples with equivalent number of wash samples. (b) Stability of both, bioconjugates and free enzyme samples on pNPA substrate.

esterase, which upon hydrolysis, yields *para*-nitrophenol – a compound detectable by its yellowish-green color and quantifiable at 405 nm absorbance. Through successive cycles, we found that the bioconjugates retain over 80% of their initial activity for the first four cycles, with a subsequent decline to 50% activity after cycle 4, remaining active up to cycle 10 (Figure 3a). Control samples washed with PBS (pH 7.4) showed the same decline trend in activity, suggesting that the activity loss was not due to enzyme deactivation or substrate inhibition, but potentially from agglomeration and deagglomeration of the bioconjugates during magnetic separation (Figure 3a) (Schwaminger et al. 2021). Despite some loss of activity, the bioconjugates demonstrated significant reusability for up to ten cycles, with minimal effort required for magnetic separation.

3.4 | Stability of Bioconjugates

The stability of bioconjugates was assessed by comparing the activity of enzymes between bioconjugates and equivalent free enzymes, both stored at 4°C. To measure enzyme activity post-storage, *p*NPA assay was used. The bioconjugates containing both FAST-PETase and MHETase showed stability for more than a week without any significant loss (Figure 3b). Conversely, the free enzymes, stored at the same temperature, showed a gradual decrease to approximately 40% of their initial activity after a 12-day period.

4 | Conclusion

The immobilization of enzymes onto inorganic scaffolds has proven effective to enhance enzyme activity. In our study focusing on degrading PET using dual-enzyme system, we opted to immobilize FAST-PETase and MHETase onto iron oxide nanoparticles. These two enzymes work in synergy to completely depolymerize PET to its monomers. Through sequential addition, we successfully immobilized these enzymes on the iron oxide nanoparticles, ensuring an excess of FAST-PETase enzyme. This sequential addition approach not only facilitated efficient immobilization but also improved the total enzyme loading on the nanoparticles compared with simultaneous addition. This two-enzyme system on nanoparticles exhibited a

2.5-fold increase in TPA formation compared with free enzyme system. Furthermore, we demonstrated the reusability of the enzyme bioconjugates using simple magnetic separation. Additionally, the enzyme bioconjugates showed enhanced stability when stored at 4°C compared to the free enzyme. Overall, we achieved complete depolymerization of PET using our enzyme bioconjugates, along with additional advantages of reusability and stability through immobilization on nanoparticles. This work could apply to other multi-enzyme systems with non-equimolar requirements to enhance activity through immobilization.

Author Contributions

Qing Sun did the conceptualization. Siddhi Kotnis designed and conducted the experiments. Siddhant Gulati helped with earlier constructs and experiment assay. Siddhi Kotnis drafted the manuscript. Qing Sun revised the manuscript and did the funding acquisition.

Acknowledgments

This work was supported by funds provided by a National Science Foundation (NSF) grant to Q. S. (2203715), USA; NSF Emerging Frontiers in Research and Innovation (EFRI) to Q. S. (2132156), USA; Texas A&M Excellence Fund X-grants to Q. S., Texas A&M, USA; Texas A&M Targeted Proposal Teams (TPT) grant to Q. S., Texas A&M, USA; NIH NIAID R01 to Q. S. (R01AI165433), Texas A&M, USA. This work was partially supported by the National Science Foundation (NSF) under Award No. 2330245, which funds the Engineering Research Center for Carbon Utilization Redesign through Biomanufacturing-Empowered Decarbonization (CURB).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that supports the findings of this study are available in the supporting material of this article.

References

- Benyathiar, P., P. Kumar, G. Carpenter, J. Brace, and D. K. Mishra. 2022. "Polyethylene Terephthalate (Pet) Bottle-To-Bottle Recycling for the Beverage Industry: A Review." *Polymers* 14, no. 12: 2366. <https://doi.org/10.3390/polym14122366>.

- Bilal, M., Y. Zhao, T. Rasheed, and H. M. N. Iqbal. 2018. "Magnetic Nanoparticles as Versatile Carriers for Enzymes Immobilization: A Review." *International Journal of Biological Macromolecules* 120: 2530–2544. <https://doi.org/10.1016/j.ijbiomac.2018.09.025>.
- Cao, F., L. Wang, R. Zheng, L. Guo, Y. Chen, and X. Qian. 2022. "Research and Progress of Chemical Depolymerization of Waste Pet and High-Value Application of Its Depolymerization Products." *RSC Advances* 12, no. 49: 31564–31576. <https://doi.org/10.1039/D2RA06499E>.
- Cui, Y., Y. Chen, X. Liu, et al. 2021. "Computational Redesign of a Petase for Plastic Biodegradation Under Ambient Condition by the Grape Strategy." *ACS Catalysis* 11, no. 3: 1340–1350. <https://doi.org/10.1021/acscatal.0c05126>.
- Gercke, D., C. Furtmann, I. E. P. Tozakidis, and J. Jose. 2021. "Highly Crystalline Post-Consumer Pet Waste Hydrolysis by Surface Displayed Petase Using a Bacterial Whole-Cell Biocatalyst." *ChemCatChem* 13, no. 15: 3479–3489. <https://doi.org/10.1002/cctc.202100443>.
- Jia, Y., N. A. Samak, X. Hao, et al. 2021. "Nano-Immobilization of Petase Enzyme for Enhanced Polyethylene Terephthalate Biodegradation." *Biochemical Engineering Journal* 176: 108205. <https://doi.org/10.1016/j.bej.2021.108205>.
- Knott, B. C., E. Erickson, M. D. Allen, et al. 2020. "Characterization and Engineering of a Two-Enzyme System for Plastics Depolymerization." *Proceedings of the National Academy of Sciences* 117, no. 41: 25476–25485. <https://doi.org/10.1073/pnas.2006753117>.
- Leong, S. S., Z. Ahmad, S. C. Low, J. Camacho, J. Faraudo, and J. Lim. 2020. "Unified View of Magnetic Nanoparticle Separation Under Magnetophoresis." *Langmuir* 36, no. 28: 8033–8055. <https://doi.org/10.1021/acs.langmuir.0c00839>.
- Lu, H., D. J. Diaz, N. J. Czarnecki, et al. 2022. "Machine Learning-Aided Engineering of Hydrolases for Pet Depolymerization." *Nature* 604, no. 7907: 662–667. <https://doi.org/10.1038/s41586-022-04599-z>.
- Maghraby, Y. R., R. M. El-Shabasy, A. H. Ibrahim, and H. M. E.-S. Azzazy. 2023. "Enzyme Immobilization Technologies and Industrial Applications." *ACS Omega* 8, no. 6: 5184–5196. <https://doi.org/10.1021/acsomega.2c07560>.
- Malik, N., P. Kumar, S. Shrivastava, and S. B. Ghosh. 2017. "An Overview on Pet Waste Recycling for Application in Packaging." *International Journal of Plastics Technology* 21, no. 1: 1–24. <https://doi.org/10.1007/s12588-016-9164-1>.
- Rahimi, A., and J. M. García. 2017. "Chemical Recycling of Waste Plastics for New Materials Production." *Nature Reviews Chemistry* 1, no. 6: 0046. <https://doi.org/10.1038/s41570-017-0046>.
- Roth, H. C., S. P. Schwaminger, F. Peng, and S. Berensmeier. 2016. "Immobilization of Cellulase on Magnetic Nanocarriers." *ChemistryOpen* 5, no. 3: 183–187. <https://doi.org/10.1002/open.201600028>.
- Schwaminger, S. P., S. A. Blank-Shim, I. Scheifele, V. Pipich, P. Fraga-García, and S. Berensmeier. 2019a. "Design of Interactions Between Nanomaterials and Proteins: A Highly Affine Peptide Tag to Bare Iron Oxide Nanoparticles for Magnetic Protein Separation." *Biotechnology Journal* 14, no. 3: 1800055. <https://doi.org/10.1002/biot.201800055>.
- Schwaminger, S. P., S. Fehn, T. Steegmüller, et al. 2021. "Immobilization of Petase Enzymes on Magnetic Iron Oxide Nanoparticles for the Decomposition of Microplastic Pet." *Nanoscale Advances* 3, no. 15: 4395–4399. <https://doi.org/10.1039/D1NA00243K>.
- Schwaminger, S. P., P. Fraga-García, S. A. Blank-Shim, et al. 2019b. "Magnetic One-Step Purification of His-Tagged Protein by Bare Iron Oxide Nanoparticles." *ACS Omega* 4, no. 2: 3790–3799. <https://doi.org/10.1021/acsomega.8b03348>.
- Schyns, Z. O. G., and M. P. Shaver. 2021. "Mechanical Recycling of Packaging Plastics: A Review." *Macromolecular Rapid Communications* 42, no. 3: 2000415. <https://doi.org/10.1002/marc.202000415>.
- Shirke, A. N., C. White, J. A. Englaender, et al. 2018. "Stabilizing Leaf and Branch Compost Cutinase (Lcc) With Glycosylation: Mechanism and Effect on Pet Hydrolysis." *Biochemistry* 57, no. 7: 1190–1200. <https://doi.org/10.1021/acs.biochem.7b01189>.
- Sinha, V., M. R. Patel, and J. V. Patel. 2010. "Pet Waste Management by Chemical Recycling: A Review." *Journal of Polymers and the Environment* 18, no. 1: 8–25. <https://doi.org/10.1007/s10924-008-0106-7>.
- Smith, R. L., S. Takkellapati, and R. C. Riegerix. 2022. "Recycling of Plastics in the United States: Plastic Material Flows and Polyethylene Terephthalate (Pet) Recycling Processes." *ACS Sustainable Chemistry & Engineering* 10, no. 6: 2084–2096. <https://doi.org/10.1021/acssuschemeng.1c06845>.
- Son, H. F., I. J. Cho, S. Joo, et al. 2019. "Rational Protein Engineering of Thermo-Stable Petase From *Ideonella Sakaiensis* for Highly Efficient Pet Degradation." *ACS Catalysis* 9, no. 4: 3519–3526. <https://doi.org/10.1021/acscatal.9b00568>.
- Taniguchi, I., S. Yoshida, K. Hiraga, K. Miyamoto, Y. Kimura, and K. Oda. 2019. "Biodegradation of Pet: Current Status and Application Aspects." *ACS Catalysis* 9, no. 5: 4089–4105. <https://doi.org/10.1021/acscatal.8b05171>.
- Tsai, S. L., M. Park, and W. Chen. 2013. "Size-Modulated Synergy of Cellulase Clustering for Enhanced Cellulose Hydrolysis." *Biotechnology Journal* 8, no. 2: 257–261. <https://doi.org/10.1002/biot.201100503>.
- Wei, R., G. Von Haugwitz, L. Pfaff, et al. 2022. "Mechanism-Based Design of Efficient Pet Hydrolases." *ACS Catalysis* 12, no. 6: 3382–3396. <https://doi.org/10.1021/acscatal.1c05856>.
- Yoshida, S., K. Hiraga, T. Takehana, et al. 2016. "A Bacterium That Degrades and Assimilates Poly(Ethylene Terephthalate)." *Science* 351, no. 6278: 1196–1199. <https://doi.org/10.1126/science.aad6359>.
- Zanuso, E., D. G. Gomes, H. A. Ruiz, J. A. Teixeira, and L. Domingues. 2021. "Enzyme Immobilization as a Strategy Towards Efficient and Sustainable Lignocellulosic Biomass Conversion Into Chemicals and Biofuels: Current Status and Perspectives." *Sustainable Energy & Fuels* 5, no. 17: 4233–4247. <https://doi.org/10.1039/D1SE00747E>.
- Zhu, B., Q. Ye, Y. Seo, and N. Wei. 2022. "Enzymatic Degradation of Polyethylene Terephthalate Plastics by Bacterial Curli Display Petase." *Environmental Science & Technology Letters* 9, no. 7: 650–657. <https://doi.org/10.1021/acs.estlett.2c00332>.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.