

**Induction of actinobacterial enzymes for enhanced biological depolymerization of
polyethylene terephthalate plastics**

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by

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Abstract

Polyethylene terephthalate (PET), a widely used plastic, has been accumulating in the environment at an alarming rate, posing significant ecological challenges. This research addresses PET pollution through a biological depolymerization-based approach, involving three integrated strategies: (1) physicochemical modification of PET to enhance degradability, (2) optimization of carbon sources and inducer sources for cutinase enzyme production by *Thermobifida fusca* YX, and (3) an *in silico* investigation to identify and characterize potential PET-hydrolyzing enzymes (PHEs) in the bacterium.

The first phase of the study focused on improving PET biodegradability through pretreatment techniques, including ultraviolet (UV) photooxidation, thermal oxidation, and size reduction of PET films. These treated samples were subjected to enzymatic depolymerization using both commercial immobilized enzymes and free (non-immobilized) enzymes derived from *T. fusca* YX. Analytical techniques such as Fourier-transform infrared (FTIR) spectroscopy and scanning electron microscopy (SEM) revealed that higher temperatures (above 80 °C) favored whole-cell biological depolymerization, while lower temperatures were more suitable for free enzyme-mediated depolymerization. Reduced particle size was also found to significantly improve biological depolymerization efficiency.

Following these findings, cutinase production by *T. fusca* YX was optimized to increase enzyme yield. Various carbon sources (including sodium acetate, sodium butyrate, and sodium lactate) and natural cutin inducers (extracted from Roma tomatoes, Royal Gala apples, and watermelon peels) were tested. Using Response Surface Methodology (RSM), enzyme production was enhanced by 42%, demonstrating a viable strategy for scalable and cost-effective cutinase generation for industrial applications.

In the final phase, an *in silico* analysis was conducted to explore the genomic potential of *T. fusca* YX for PET depolymerization. Six candidate cutinase enzymes were identified: three extracellular, two membrane-associated, and one intracellular. Molecular docking studies were performed to analyze their binding affinities with PET and cutin ligands, representing the first structural investigation of such interactions in *T. fusca* YX.

Overall, this study offers a comprehensive approach to improving PET biological depolymerization under environmentally benign conditions, while reducing reliance on harsh chemical depolymerization processes. The use of a naturally occurring microbial strain highlights the potential for further biotechnological advancements in sustainable plastic waste management.

Keywords

Polyethylene terephthalate, physicochemical pretreatments, chemical hydrolysis, biological depolymerization, cutinase, *Thermobifida fusca* YX, response surface methodology, central composite rotatable design, bioinformatics, PET-hydrolyzing enzymes

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This thesis is dedicated to my dad & mom,
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List of publications

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Review

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2. **Ruth Amanna,** Mahdiah Samavi, Sudip Kumar Rakshit. (2023). Biological depolymerization of microplastics and nanoplastics in water and wastewater. In *Current Developments in Biotechnology and Bioengineering* (pp. 293-314). Elsevier, ISBN: 9780323999083. <https://doi.org/10.1016/B978-0-323-99908-3.00002-6>

Abbreviations

AI	Aliphatic index
ATR-FTIR	Attenuated Total Reflectance – Fourier Transform Infrared Spectroscopy
BHET	Bis (2-hydroxyethyl) terephthalic acid
CBM	Carbohydrate-Binding Module
CCD	Central Composite Design
CDD	Conserved Domain Database
DMT	Dimethyl terephthalic acid
DSC	Differential Scanning Calorimetry
EG	Ethylene glycol
ESBRI	Evaluating Salt Bridges
GRAVY	Grand average of hydropathy
HDPE	High-Density polyethylene
HFIP	1,1,1,3,3,3-hexafluoro-2-propanol
HiC	Immobilised cutinase from <i>Humicola insolens</i>
HPLC	High-performance liquid chromatography
II	Instability index
LDPE	Low-Density Polyethylene
MHET	Mono (2-hydroxyethyl) terephthalic acid
NMR	Nuclear Magnetic Resonance
PCL	Polycaprolactone
PDB	Protein Data Bank
PET	Polyethylene terephthalate
PHA	Polyhydroxyalkanoate
PHE	PET-hydrolyzing enzyme
pNPB	Para-nitrophenyl butyric acid
pNPP	Para-nitrophenyl palmitic acid
PP	Polypropylene
PS	Polystyrene
PVC	Polyvinyl chloride

rPET	Recycled Polyethylene terephthalic acid
RSM	Response Surface Methodology
SEM	Scanning Electron Microscopy
TPA	Terephthalic acid
UV	Ultraviolet radiation
WL	Weight Loss
XPS	X-ray Photoelectron Spectroscopy

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Chapter 1

General Overview, Rationale & Objectives

1.1 Introduction

Over the past decades, plastic consumption in our society has skyrocketed. Despite individuals being cognizant of the environmental issues related to this product, plastic demand and its consumption has increased in recent years and continues to rise (Statista, 2020). Polyethylene terephthalic acid (PET or PETE) is the most common type of plastic used in several applications. PET, an aliphatic-aromatic polyester, that has applications in a wide spectrum of products such as the textile industry for fabrics, as packaging material in the food and beverage industry, etc. This polymer is lightweight, transparent, has high tensile strength and can act as a gas barrier. However, the inert properties and its resistance to break down make PET difficult to breakdown and leads to its accumulation in nature.

The annual consumption of PET has been predicted to increase by 4.1% based on a compound annual growth rate predicted for the period 2023-2030 (AgileIntel Research, 2023). In 2022 alone, 25.47 million metric tonnes of PET were consumed globally (AgileIntel Research, 2023). Although there is an increase in the use of recycled PET, the amount involved is still incomparable to the consumption of virgin PET (Eurasia Group & Suntory, 2021).

Waste PET is handled either by recycling or pyrolysis depending on the quality of the waste product. Pyrolysis has been adopted in certain countries to treat this waste and generate energy (Walker, 2021). However, pyrolysis is not a clean process as it generates multiple toxic gases and fumes containing furans, dioxins, carbon monoxide and other gases depending on the additives present in the plastics. These gases have been previously demonstrated to be harmful to all living creatures and have been classified as carcinogens (Koshti et al., 2018). Different methods of recycling of PET have been adopted by different countries. However, the quality of the waste PET collected, the amount of PET that cannot be recycled due to segregation and other issues make it a focus of considerable research. Recycling a PET bottle requires multiple steps that factor in

multiple variables like quality of waste PET, contamination, additives, and quality of recycled pellets (Lange, 2021).

Mechanical and chemical recycling are two widely used recycling methods. In mechanical recycling, PET is sorted, separated, size reduced to flakes and melted to form pellets. Recycled PET (rPET) produced by mechanical recycling generally yields lower quality polyester due to the potential migration of intentional or non-intentional compounds (Brouwer et al., 2020; Thoden van Velzen et al., 2020). In chemical recycling, PET is depolymerized to its monomers or partially depolymerized to oligomers. This includes glycolysis, methanolysis, hydrolysis, aminolysis, and ammonolysis. Chemical recycling yields comparatively higher quality rPET as it generates the monomers or oligomers during the recycling process. However, chemical recycling uses large quantities of caustic chemicals (Achilias & Karayannidis, 2004) which poses an environmental risk. The best option for clean depolymerization of PET is by biological depolymerization. However, the low yields and conversion rates associated with biological depolymerization, combined with the time it takes for the process to occur, make it less economically viable compared to chemical recycling or pyrolysis (Hiraga et al., 2019).

In the recent past, there has been tremendous increase in the efficiency of the PET hydrolases (or PETases) some of which have reported 100% biological depolymerization (Tournier et al., 2020). These enzymes obtained from mutants that are a result of genetic engineering by site directed mutagenesis. Maintaining these enzymes in carrier chassis are not economically feasible due to genetic instability of the carrier genome, potential loss of chassis, need for antibiotic resistance markers, and the loss of the mutant gene itself (Gero et al., 1997). These factors can also prove detrimental if released into natural habitats. Containment of the mutant protein or carrier chassis is one of the main reasons of mutant proteins not being used in many applications (Berry, 2007).

Despite the advancement of the efficiency of PETases, it will take tremendous effort to overcome the challenges listed above.

The best alternative to genetically modified mutants is induction of the naturally occurring bacterium with the gene of interest. Induction has always been a traditional method to enhance production of enzymes that are constitutively expressed by a microorganism (Donovan et al., 1996). Studies on induction have only focused on the best choice of inducer sources and their concentration. There are other factors that ensure maximum induction of the gene of interest including the substrate carbon source used and its concentration, presence of desirable ions and other types of inducers (Briand et al., 2016). The inducers used for induction of PET-hydrolyzing enzymes (PHEs) are cutins, lipids and other esters. Cutin is the key inducer as the chemical bonds present in natural cutin are similar to that of PET (Amanna & Rakshit, 2023). Cutin induces the enzyme cutinase which is known to have high PET-hydrolyzing activity (Fett et al., 1999). However, cutin is a polymer and it itself consists of multiple monomers depending on its natural origin is fruits and vegetables (Fich et al., 2016a). Cutin is an aliphatic polyester that is structurally analogous to PET. While using cutin as inducers, the choice of cutin origin and its concentration is key to determining the most suitable medium composition for maximum cutinase expression. Cutin consists of various long chain fatty acids that are bound to each other via ester linkages (Kolattukudy, 2003). There are usually more than three types of monomers in any given cutin. Due to the presence and concentration of various fatty acids as its monomers, induction with cutin is not expected to be a one-to-one gene expression. Most genomes of cutinase-containing microorganisms do not have most their genome annotated. *Thermobifida fusca* is one such example that has been extensively studied for its hydrolytic enzymes. However, its genome (GenBank Accession ID: CP000088.1) is still not completely annotated and is far from being

completely validated. Use of bioinformatics is key to understand the potential gene expression or change in gene expression.

All PHEs are part of the α/β fold superfamily and usually have a nucleophilic serine at the active site, which is usually part of the catalytic triad. With bioinformatics, determining non-cutinase PHEs would be possible using software like BLASTs and UniProt to mine the gene(s) of interest (Mican et al., 2024). Previous reports mention the presence of multiples cutinases in *Thermobifida fusca*, this can be confirmed using bioinformatics (S. Chen et al., 2010). Additionally, PHEs can be structurally analysed and further docked with ligands like PET, BHET and MHET. Ligands can be created in Avogadro v3.0 and docked with the mined PHEs (Flores-Castañón et al., 2022). Bioinformatics would help shed light on the interactions occurring at the microenvironmental and molecular level, thereby increasing not only our knowledge of PHEs but also adding to the PHE catalogue.

1.2 Rationale for work

The extensive and ongoing use of polyethylene terephthalate (PET) plastics over recent decades has created significant challenges for recycling systems worldwide. As traditional recycling efforts fall short of managing the sheer volume of PET waste, the development of sustainable, environmentally friendly alternatives is increasingly urgent. Biological depolymerization presents a promising and cleaner route for the disposal of persistent polymers. However, unlike other anthropogenic plastics such as LDPE, HDPE, and PLA, PET's chemically inert nature makes it particularly resistant to microbial breakdown.

A critical innovation explored in this study is the enhancement of PET's biodegradability through targeted physicochemical pretreatments. These methods—such as thermal oxidation (Panowicz et

al., 2021), size reduction (Farzi et al., 2019), ultraviolet (UV) irradiation (Day & Wiles, 1972; Falkenstein et al., 2020), and acid hydrolysis (Rajandas et al., 2012), modify the functional groups of PET and increase its carbonyl index, a key indicator of biological depolymerization. By introducing reactive oxo-functional groups and elevating the carbonyl index, the polymer becomes more susceptible to enzymatic hydrolysis, thereby facilitating biological depolymerization (Beltrán-Sanahuja et al., 2020),.

While pretreatment alters the substrate to enhance biodegradability, a second, often overlooked challenge is the availability and efficiency of the enzymes required for PET breakdown. Rather than relying on genetically modified organisms, which pose regulatory, scalability, and safety concerns. This work takes a novel approach by optimizing the factors influencing the induction of cutinase in *Thermobifida fusca* YX, an enzyme which has previously demonstrated PET-hydrolyzing activity. Traditional methods such as One-Factor-at-a-Time (OFAT) are time-consuming and fail to account for interactions between variables. This study implements Response Surface Methodology (RSM) to optimize enzyme production by efficient optimization of the concentration of multiple carbon sources and concentration of various cutins. This method has been underutilized for *T. fusca* YX, making this study one of the first to rigorously apply RSM for enzyme induction in this organism.

Induction of cutinase using naturally occurring polymers like cutin could result in a variation in other biomolecules like cAMP, enzymes for carbon consumption and transporter proteins. Additionally, the presence of a multiple monomers within cutin could lead to the expression potential cutin modifying enzymes and other cutinases. All the mentioned variables can directly or indirectly affect gene expression. The next part of the study focuses on the identifying and characterizing potential PET-hydrolyzing enzymes in the genome of *T. fusca* YX. Using in silico

modeling, the interactions between these enzymes and PET along with its monomeric forms, BHET and MHET were explored. This dual approach of experimental optimization and computational analysis offers new insights into the enzymatic depolymerization pathways of PET.

The novelty of this work lies in the optimization of cutinase induction using non-specific polymeric inducers such as cutin and also in its integrative strategy: combining pretreatment of PET, optimization of cutinase induction, and in silico study to learn of the potential PET-hydrolyzing enzymes within the genome of *T. fusca* YX. By identifying the specific cutinases expressed upon cutin induction and understanding their interactions with PET and its derivatives, this study contributes significantly to understanding the biological depolymerization of PET.

1.3 Research objectives

Based on a study of literature and identifying the research gaps, the overall objective of this work was to understand and increase the biological depolymerization of PET using PHEs from *Thermobifida fusca* YX (see Fig. 1.1). The specific objectives are as follows:

1. Pretreatment of polyethylene terephthalate (PET) using physicochemical methods and determine the increase in biological depolymerization
2. Response surface methodology for optimization of maximum cutinase production from *Thermobifida fusca* YX by modification of environmental factors
3. Genomic study of *Thermobifida fusca* YX for novels PET-hydrolyzing enzymes and their subsequent molecular docking with polyethylene terephthalate and cutin ligands

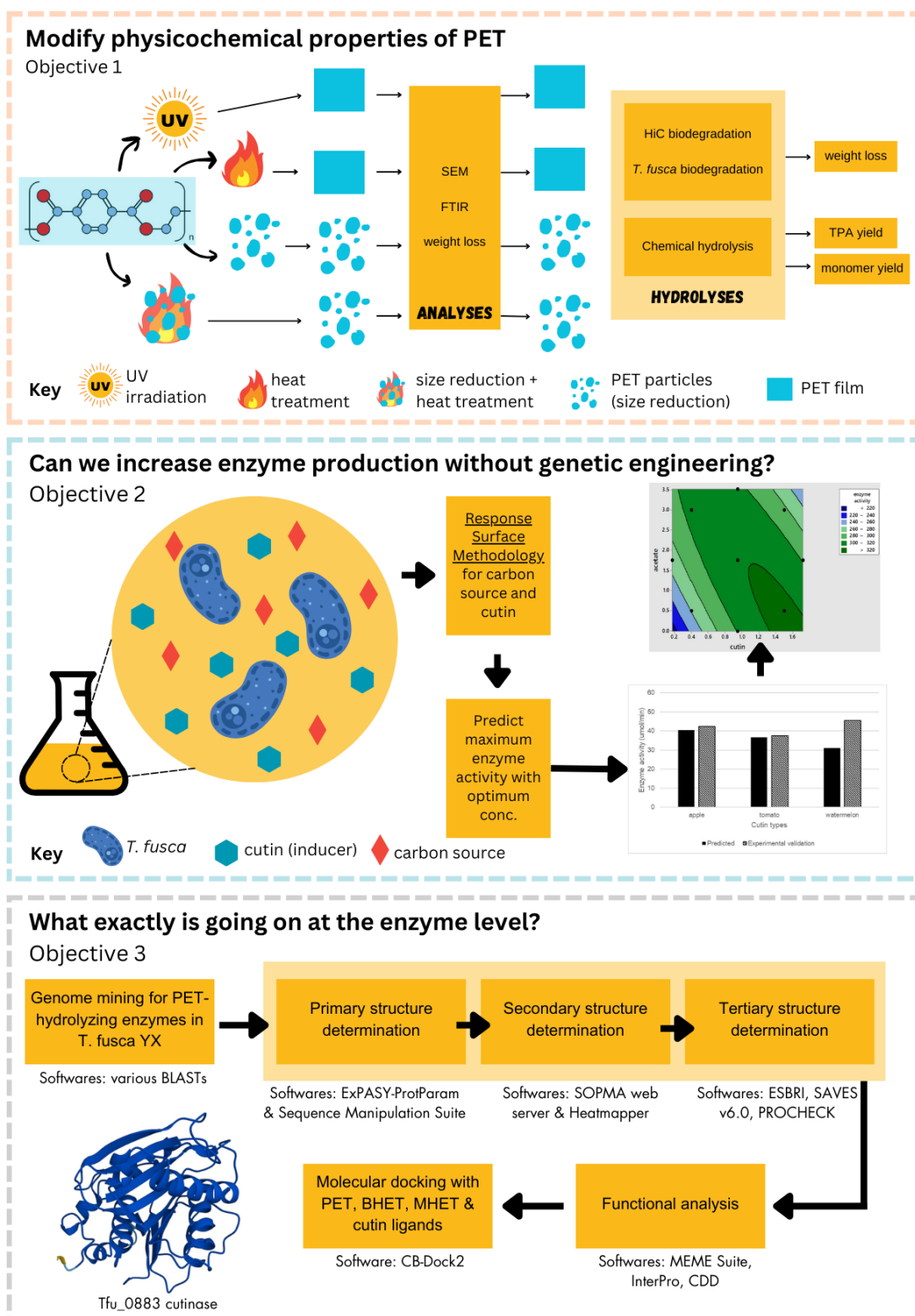


Figure 1.1 Illustration of the rationale, research objectives and the overview of the methodology followed in each research objective

1.4 Novelty of Work

The present study is novel in its comprehensive and integrative approach to enhancing enzymatic hydrolysis of PET under environmentally friendly conditions, offering a sustainable alternative to conventional chemical recycling, where the separation and reuse of caustic substances like acids and bases are difficult.

While PET's recalcitrance to biological depolymerization is well known, there has been little systematic effort to couple targeted pretreatments with enzyme optimization strategies. By examining physicochemical pretreatments that improve polymer susceptibility to hydrolysis, this work introduces a practical means of increasing PET's accessibility to enzymes—an aspect not yet fully explored in synergy with microbial enzyme induction.

A key novelty lies in the systematic optimization of cutinase production in *Thermobifida fusca* YX using Response Surface Methodology (RSM). Unlike traditional One-Factor-At-A-Time (OFAT) approaches, RSM enables rigorous evaluation of factor interactions, thereby providing higher yields of enzymes without relying on genetic modification. This study is among the first to apply RSM in the context of PET-hydrolyzing cutinase induction, particularly with natural, non-specific inducers such as cutin. The use of cutin is especially innovative, as it may co-induce a suite of enzymes and associated biomolecules, thereby broadening the potential for PET depolymerization.

Another distinctive element of this work is the exploration of *T. fusca* YX's native genomic potential through bioinformatic tools. Rather than focusing solely on engineered strains, this research investigates the inherent genetic repertoire of wild-type organisms to uncover novel PET-hydrolyzing enzymes. This systematic in silico identification and molecular docking of candidate

enzymes with PET and its monomers (BHET, MHET) has not been widely undertaken, opening new avenues for biocatalyst discovery.

Overall, the novelty of this work lies in its integrative strategy: (i) employing pretreatments to enhance PET hydrolysis using biocatalysts (ii) applying systematic induction optimization to increase enzyme yields under eco-friendly conditions, and (iii) leveraging bioinformatics to expand the “society of enzymes” encoded within the genome of *T. fusca* YX. This combination not only advances fundamental understanding of PET biological depolymerization but also charts a sustainable path toward enzyme-based solutions for plastic waste

References

- Achilias, D. S., & Karayannidis, G. P. (2004). The Chemical Recycling of PET In The Framework of Sustainable Development. *Water, Air and Soil Pollution: Focus*, 4, 385–396.
- AgileIntel Research. (2023, July 5). *Market volume of polyethylene terephthalate worldwide from 2015 to 2022, with a forecast for 2023 to 2030 (in million metric tons)*. AgileIntel Research (ChemIntel360).
- Amanna, R., & Rakshit, S. K. (2023). Review of nomenclature and methods of analysis of polyethylene terephthalic acid hydrolyzing enzymes activity. In *Biological depolymerization*. Springer Science and Business Media B.V. <https://doi.org/10.1007/s10532-023-10048-z>
- Beltrán-Sanahuja, A., Casado-Coy, N., Simó-Cabrera, L., & Sanz-Lázaro, C. (2020). Monitoring polymer depolymerization under different conditions in the marine environment. *Environmental Pollution*, 259, 113836. <https://doi.org/10.1016/J.ENVPOL.2019.113836>
- Berry, R. M. (2007). *The Ethics of Genetic Engineering*. Routledge. https://books.google.ca/books?hl=en&lr=&id=AW-qzJiqQPIC&oi=fnd&pg=PP1&dq=genetic+engineering+problems&ots=_o570sF5X2&sig=AwSw28Lst33MB3PmPejdovoJGcg#v=onepage&q=genetic%20engineering%20problems&f=false
- Bhardwaj, K. K., Kumar, R., Bhagta, S., & Gupta, R. (2021). Optimization of culture conditions by response surface methodology for production of extracellular esterase from *Serratia* sp. EST-4. *Journal of King Saud University - Science*, 33(8), 101603. <https://doi.org/10.1016/J.JKSUS.2021.101603>
- Brouwer, M. T., Alvarado Chacon, F., & Thoden van Velzen, E. U. (2020). Effect of recycled content and rPET quality on the properties of PET bottles, part III: Modelling of repetitive recycling. *Packaging Technology and Science*, 33(9), 373–383. <https://doi.org/10.1002/PTS.2489>
- Chen, S., Su, L., Billig, S., Zimmermann, W., Chen, J., & Wu, J. (2010). Biochemical characterization of the cutinases from *Thermobifida fusca*. *Journal of Molecular Catalysis B: Enzymatic*, 63(3–4), 121–127. <https://doi.org/10.1016/j.molcatb.2010.01.001>
- Day, 31, & Wiles, D. M. (1972). Photochemical Depolymerization of Poly(ethylene Terephthalate). 111. Determination of Decomposition Products and Reaction Mechanism. In *IED POJYMER SCIENCE* (Vol. 16).
- Dutta, K., Dasu, V. V., & Hegde, K. (2013). Development of Medium and Kinetic Modeling for Enhanced Production of Cutinase from *Pseudomonas cepacia* NRRL B-2320. *Advances in Microbiology*, 2013(06), 479–489. <https://doi.org/10.4236/AIM.2013.36064>
- Eurasia Group & Suntory. (2021, October). *Asia: rPET demand growth 2018-2023* | Statista. <https://www-statista-com.ezproxy.lakeheadu.ca/statistics/1310244/asia-rpet-demand-growth/>
- Falkenstein, P., Gräsing, D., Bielytskyi, P., Zimmermann, W., Matysik, J., Wei, R., & Song, C. (2020). UV Pretreatment Impairs the Enzymatic Depolymerization of Polyethylene Terephthalate. *Frontiers in Microbiology*, 11. <https://doi.org/10.3389/fmicb.2020.00689>
- Farzi, A., Dehnad, A., & Fotouhi, A. F. (2019). Biological depolymerization of polyethylene terephthalate waste using *Streptomyces* species and kinetic modeling of the process. *Biocatalysis and Agricultural Biotechnology*, 17, 25–31. <https://doi.org/10.1016/j.bcab.2018.11.002>
- Fett, W. F., Wijey, C., Moreau, R. A., & Osman, S. F. (1999). Production of cutinase by *Thermomonospora fusca* ATCC 27730. In *Journal of Applied Microbiology* (Vol. 86).
- Fich, E. A., Segerson, N. A., & Rose, J. K. C. (2016). *The Plant Polyester Cutin: Biosynthesis, Structure, and Biological Roles*. <https://doi.org/10.1146/annurev-arplant-043015-111929>
- Flores-Castañón, N., Sarkar, S., & Banerjee, A. (2022). Structural, functional, and molecular docking analyses of microbial cutinase enzymes against polyurethane monomers. *Journal of Hazardous Materials Letters*, 3, 100063. <https://doi.org/10.1016/J.HAZL.2022.100063>
- Gero, J. S., Kazakov, V. A., & Schnier, T. (1997). Genetic Engineering and Design Problems. *Evolutionary Algorithms in Engineering Applications*, 47–68. https://doi.org/10.1007/978-3-662-03423-1_3
- Hiraga, K., Taniguchi, I., Yoshida, S., Kimura, Y., & Oda, K. (2019). Biological depolymerization of waste PET. *EMBO Reports*, 20(11). <https://doi.org/10.15252/EMBR.201949365>
- Hong, H., Ki, D., Seo, H., Park, J., Jang, J., & Kim, K.-J. (2023). Discovery and rational engineering of PET hydrolase with both mesophilic and thermophilic PET hydrolase properties. *Nature Communications*, 14(4556). <https://doi.org/10.1038/s41467-023-40233-w>
- Khoramnia, A., Lai, O. M., Ebrahimpour, A., Tanduba, C. J., Voon, T. S., & Mukhlis, S. (2010). Thermostable lipase from a newly isolated *Staphylococcus xylosus* strain; process optimization and characterization using RSM and ANN. *Electronic Journal of Biotechnology*, 13(5). <https://doi.org/10.2225/VOL13-ISSUE5-FULLTEXT-22>

- Kolattukudy, P. E. (2003). Structure, Biosynthesis, and Biological depolymerization of Cutin and Suberin. *https://doi.org/10.1146/Annurev.Pp.32.060181.002543*, 32(1), 539–567. <https://doi.org/10.1146/ANNUREV.PP.32.060181.002543>
- Koshti, R., Mehta, L., & Samarth, N. (2018). Biological Recycling of Polyethylene Terephthalate: A Mini-Review. In *Journal of Polymers and the Environment* (Vol. 26, Issue 8, pp. 3520–3529). Springer New York LLC. <https://doi.org/10.1007/s10924-018-1214-7>
- Kumari, V., Kumar, V., Chauhan, R., Asif, • Mohammad, Tek, •, & Bhalla, C. (2016). Optimization of medium parameters by response surface methodology (RSM) for enhanced production of cutinase from *Aspergillus* sp. *RL2Ct. 3 Biotech*, 6(149). <https://doi.org/10.1007/s13205-016-0460-4>
- Lange, J. P. (2021). Managing Plastic Waste-Sorting, Recycling, Disposal, and Product Redesign. *ACS Sustainable Chemistry and Engineering*, 9(47), 15722–15738. <https://doi.org/10.1021/ACSSUSCHEMENG.1C05013>
- Mican, J., Jaradat, D. M. M., Liu, W., Weber, G., Mazurenko, S., Bornscheuer, U. T., Damborsky, J., Wei, R., & Bednar, D. (2024). Exploring new galaxies: Perspectives on the discovery of novel PET-degrading enzymes. *Applied Catalysis B: Environmental*, 342, 123404. <https://doi.org/10.1016/J.APCATB.2023.123404>
- Panowicz, R., Konarzewski, M., Durejko, T., Szala, M., Łazińska, M., Czerwińska, M., & Prasula, P. (2021). Properties of Polyethylene Terephthalate (PET) after Thermo-Oxidative Aging. *Materials 2021*, Vol. 14, Page 3833, 14(14), 3833. <https://doi.org/10.3390/MA14143833>
- Parmar, A. S., & Rahi, D. K. (2023). Cutinase production from *Fusarium verticillioides* using response surface methodology and its application as potential insecticide degrader. *Environmental Science and Pollution Research*, 30(36), 86484–86497. <https://doi.org/10.1007/S11356-023-28635-1/FIGURES/7>
- Rajandas, H., Parimannan, S., Sathasivam, K., Ravichandran, M., & Su Yin, L. (2012). A novel FTIR-ATR spectroscopy based technique for the estimation of low-density polyethylene biological depolymerization. *Polymer Testing*, 31(8), 1094–1099. <https://doi.org/10.1016/j.polymertesting.2012.07.015>
- Ronkvist, Å. M., Xie, W., Lu, W., & Gross, R. A. (2009). Cutinase-Catalyzed hydrolysis of poly(ethylene terephthalate). *Macromolecules*, 42(14), 5128–5138. <https://doi.org/10.1021/ma9005318>
- Statista. (2020). *Polyethylene terephthalate market size in the United States from 2014 to 2025 (in million U.S. dollars) [Graph]*. <https://www-statista-com.ezproxy.lakeheadu.ca/statistics/1098186/us-polyethylene-terephthalate-market-size/>
- Sulaiman, S., Yamato, S., Kanaya, E., Kim, J. J., Koga, Y., Takano, K., & Kanaya, S. (2012). Isolation of a novel cutinase homolog with polyethylene terephthalate-degrading activity from leaf-branch compost by using a metagenomic approach. *Applied and Environmental Microbiology*, 78(5), 1556–1562. <https://doi.org/10.1128/AEM.06725-11>
- Thoden van Velzen, E. U., Brouwer, M. T., Stärker, C., & Welle, F. (2020). Effect of recycled content and rPET quality on the properties of PET bottles, part II: Migration. *Packaging Technology and Science*, 33(9), 359–371. <https://doi.org/10.1002/PTS.2528>
- Tournier, V., Topham, C. M., Gilles, A., David, B., Folgoas, C., Moya-Leclair, E., Kamionka, E., Desrousseaux, M. L., Texier, H., Gavalda, S., Cot, M., Guémard, E., Dalibey, M., Nomme, J., Cioci, G., Barbe, S., Chateau, M., André, I., Duquesne, S., & Marty, A. (2020). An engineered PET depolymerase to break down and recycle plastic bottles. *Nature*, 580(7802), 216–219. <https://doi.org/10.1038/s41586-020-2149-4>
- Walker, T. R. (2021). (Micro)plastics and the UN Sustainable Development Goals. In *Current Opinion in Green and Sustainable Chemistry* (Vol. 30). Elsevier B.V. <https://doi.org/10.1016/j.cogsc.2021.100497>

Chapter 2

Literature Review

Part of this work has been published in **Biological depolymerization (Springer Nature)**

Amanna, R., & Rakshit, S. K. (2023). Review of nomenclature and methods of analysis of polyethylene terephthalic acid hydrolyzing enzymes activity. *Biological depolymerization*, 1-20.

2.1 Introduction

It is incomprehensible to imagine human life and advancements without plastics. In 2021 alone, 390.7 million metric tons of plastics were produced (PlasticsEurope (PEMRG), 2022) of which 80.9 million metric tons were polyethylene terephthalic acid (PET) resin (BlueWeave Consulting & Research Pvt Ltd, 2022). PET is a chemically inert polymer manufactured with varying crystallinities, plasticizers, UV-resistant additives, slip agents, and acetaldehyde quenchers depending on the final use of the polymer. The additional components further increase the inertness of the polymer. Inertness, one of its most significant attributes, is also the reason for its vast accumulation after its end-use. This has facilitated its entry and persistence in water bodies, air, and the food chain, which has dire consequences in these environments. This, in turn, has led to global concern about growing plastic and PET accumulation. Over the past decade, several countries have adopted new policies to tackle ever-increasing plastic pollution (Walker, 2021). Despite such active efforts, global plastic waste is still environmentally unsustainable. Unfortunately, the rate at which we consume PET (or any other plastic) is significantly higher than our current remediation processes (Geyer et al., 2017).

PET polymers are rigid and have increased molecular mobility around their glass transition temperature (67 °C for amorphous PET and 81 °C for crystalline PET) (Kawai et al., 2014). These temperatures are higher than average environmental temperatures. The anthropogenic nature, inertness, and high glass transition temperatures of PET are not conducive to biological depolymerization naturally in the environment. The estimated specific substrate depolymerization rate for single-use PET water bottles buried in the soil is insignificant and immeasurable (Chamas et al., 2020). This has fueled research to discover novel microorganisms and enzymes with high PET-hydrolyzing ability. There are reports of several PET-hydrolyzing microorganisms and

enzymes that do not have sufficiently high PET-hydrolyzing rates for practical applications (Koshti et al., 2018; Miri et al., 2022; Qi et al., 2022).

However, the isolation of a novel PET-hydrolyzing microorganism does not necessarily equate to a high depolymerization rate of these materials. Increasing the overall efficiency of biological depolymerization requires understanding the mechanism of action of the enzyme, the function of the tertiary structure, and its regulation and expression in the cell. The mechanism of action and the function of the tertiary structure were deduced with genetic engineering and high-definition spectroscopy (Falkenstein et al., 2020; Joo et al., 2018; Pinto et al., 2021; Yoshida et al., 2016). This has paved the way for the development of PET-hydrolyzing enzymes with exceptionally high hydrolyzing activities (Tournier et al., 2020).

With the recent focus on the discovery and characterization of several PET-hydrolyzing enzymes, reported literature on the nomenclature of these enzymes and enzyme activities are often inconsistent. This has raised confusion regarding the enzyme's catalytic function and difficulty in comparing the results of different research groups (Roth et al., 2014). This review discusses PET and the enzymes that can hydrolyze PET. This includes various PET depolymerization techniques, recent developments in PET-hydrolyzing enzymes, their classification and nomenclature, and the techniques and procedures used to characterize these enzymes. The development of new enzymatic assays that use substrates structurally and chemically similar to PET will also be discussed. This review also discusses induction of cutinase, and the factors involved in induction. Lastly, we discuss the potential use of bioinformatic tools for the discovery of new PET-hydrolyzing enzymes from sequenced genomes.

2.2 General overview of PET

Polyethylene terephthalate is a polyester that is widely used in the packaging and textile industries. This polyester is widely used due its physical and chemical attributes like transparency, thermal stability, oxygen barrier, mechanical properties, chemical inertness and rigidity (A. K. Singh et al., 2021). It can be formed into crystalline or amorphous form based on its processing conditions. As described earlier, PET (CAS: 25038-59-9) is a thermoplastic polymer of mono-(2-hydroxyethyl) terephthalic acid (MHET) linked via ester bonds. Each MHET consists of one ethylene glycol and one terephthalic acid. PET is produced through a polymerization process involving the reaction of ethylene glycol (EG) and terephthalic acid. This process typically occurs in two stages: the esterification or transesterification stage, and the polycondensation stage. In the esterification stage, terephthalic acid reacts with EG to form bis(2-hydroxyethyl) terephthalate (BHET) and water. In the transesterification stage, dimethyl terephthalate (DMT) is used instead of terephthalic acid to react with ethylene glycol, producing methanol and BHET. The resulting BHET undergoes polycondensation, where it is heated and polymerized to form long chains of PET while releasing water or methanol as by-products. The final PET resin can then be cooled, solidified, and processed into various forms (Webb et al., 2012). The reaction is illustrated in Fig. 2.1.

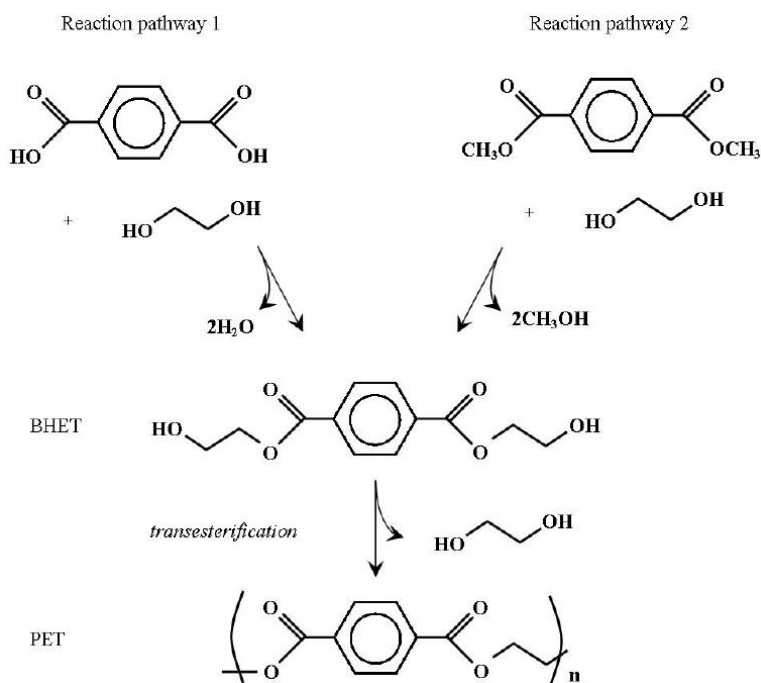


Figure 2.1 Illustration of the chemical reactions involved in the production of polyethylene terephthalate via transesterification and polycondensation (Webb et al., 2012)

2.3 Depolymerization of PET

Due to its intrinsic physical and chemical properties, the breakdown of PET is challenging. Despite a conscious effort on an individual level, the global plastic consumption has increased, thereby, increasing environmental pollution (Muringayil Joseph et al., 2024). To help mitigate this problem, our knowledge on the physicochemical effects on PET depolymerization is necessary. The methods of PET depolymerization and the factors affecting it are described below.

2.3.1 Factors influencing the depolymerization of PET

2.3.1.1 Exposure to UV radiation

Ultraviolet radiation is a short wavelength, high energy radiation source that has the ability to oxidize PET via the process of photo-oxidation. Absorption of UV radiation leads to the formation

of the initial radical by cleavage of the C-C and C-H bonds. This is followed by the propagation reaction of auto-oxidation. The prevalent cleavage mechanism for polymer backbone cleavage is the Norrish type I reaction followed by β -scission route. In semi-crystalline polyesters, chain scission usually occurs in the amorphous regions (B. Singh & Sharma, 2008). This can often lead to an increase in crystallinity and can sometimes hamper its breakdown (Falkenstein et al., 2020).

2.3.1.2 Temperature

Temperature plays a key role in the depolymerization of PET via, modification via thermal oxidation or complete depolymerization via pyrolysis (see section 2.4.3). Temperatures around the glass transition temperatures, 60-80 °C (depending on the crystallinity of PET) renders the polymer soft and begins to lose its rigidity which is caused due to increased mobility. In some cases, it can cause restructuring of the polymer chains (Thomsen et al., 2023). Prolonged exposure can result in decrease in structural integrity, change in topography and increase permeability, thus making the polymer more susceptible to depolymerization. At varying temperatures, there is varying levels of thermal oxidation via random chain scissions at C-H and C-C. PET depolymerization higher than 400 °C results in pyrolysis of PET (B. Singh & Sharma, 2008).

2.3.1.3 Particle size

Particle size plays a significant role in the depolymerization of PET. Smaller particles have a higher surface to volume ratio compared to larger particles. This increased surface area provides more sites for chemical reactions and microbial depolymerization. Smaller particle size also allows a more efficient transfer of heat, permeability of chemical and radiation. Additionally, there is a decrease in the size of polymer chains (Mehrabzadeh et al., 2000).

2.3.2 Methods for depolymerization of PET

Breakdown of PET can be followed by four different routes, viz., mechanical depolymerization, chemical depolymerization, thermal depolymerization (pyrolysis) and biological depolymerization.

2.3.2.1 Mechanical depolymerization

With the efficiency of current recycling technology, waste PET either reaches recycling facilities or ends up in landfills. Recycling facilities typically employ mechanical recycling to regenerate PET and extend its market life (Muringayil Joseph et al., 2024). The steps of mechanical recycling include separation, processing, decontamination, extrusion and pelletization, and reforming (see Fig. 2.2). The process begins with sorting to remove contaminants like PVC, as even minor contamination can significantly reduce the quality and alter the characteristics of recycled PET. After the first separation, PET is further sorted by its colour. Once the colours are sorted, the waste PET is shredded into small flakes which allows easy processing of the waste PET. Next, in the decontamination step, these flakes are thoroughly washed to remove residual contaminants such as glue and food particles. The cleaned PET flakes are then converted into pellets, which undergo extrusion and blowing, and are finally reformed into the desired shape of the product, such as bottles (Muringayil Joseph et al., 2024).

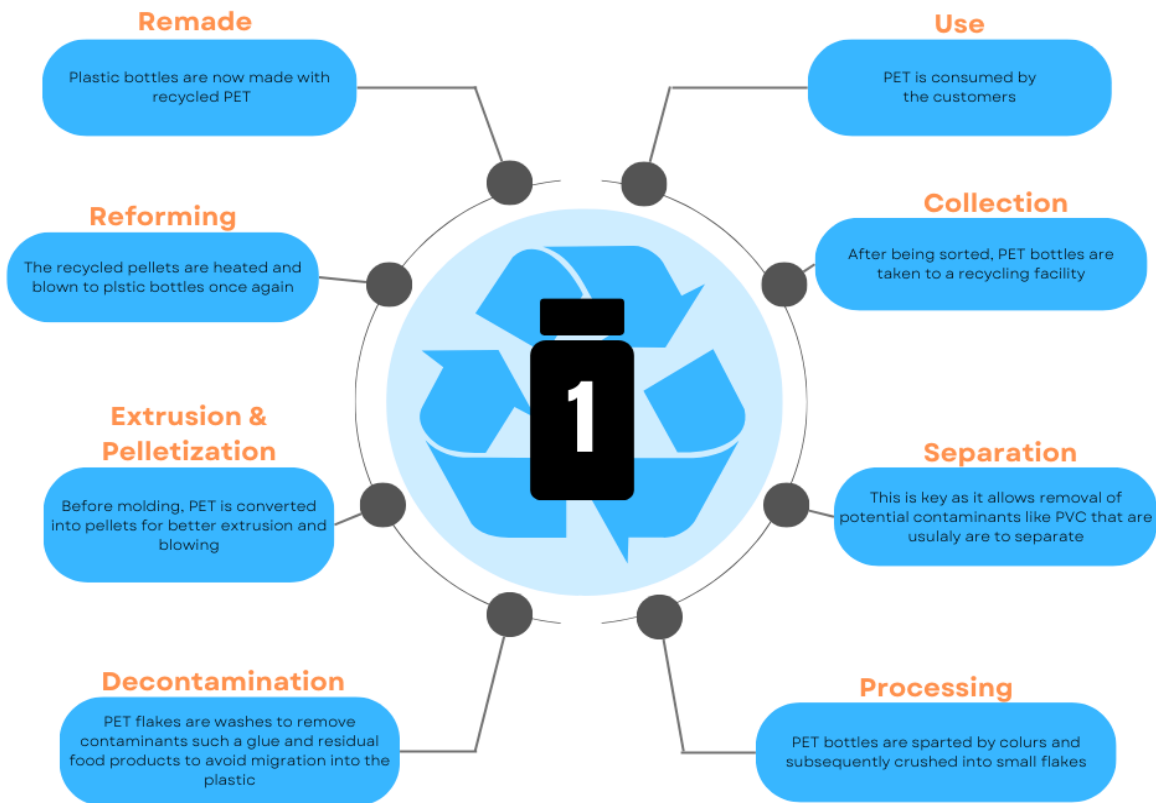


Figure 2.2. Illustration of the process flow of PET recycling

Despite being our currently used technology, mechanical recycling recycles only 9% of the plastic waste generated (Canada Environment & Climate Change, 2019). Additionally, with every cycle of mechanical recycling there is a further reduction in the quality of recycled PET generated. Despite the rigorous separation for the removal of contaminants, a few contaminants seep into the polymer itself. Studies have demonstrated that PET can be recycled a maximum of seven times before losing its marketable qualities and characteristics. Thus, mechanical recycling of PET results in PET of lower quality. Such quality PET are used for end-of-life products that do not require high quality PET. However, despite these disadvantages, life-cycle assessment of mechanical recycling showed a lower CO₂ generation compared to its chemical depolymerization counterpart (Xin et al., 2021).

2.3.2.2 Chemical depolymerization

Chemical depolymerization of PET is the complete or partial depolymerization of PET using various solvents. This approach is relatively sustainable as the generated products can be repolymerized to produce PET without a significant loss in quality depending on the breakdown products generated. Due to its ability to regenerate the monomers, and subsequent reconstruction of the polymer, chemical depolymerization is a preferred option depending on the final use of the recycled polyester (Mehrabzadeh et al., 2000). PET can be chemically degraded using alcoholysis, hydrolysis, ammonolysis, aminolysis, glycolysis and methanolysis (see Fig. 2.3). Certain recycling facilities use chemical hydrolysis to recycle waste PET, of which glycolysis is the most used technique. So far, chemical recycling is the currently used technology that follow the principles of sustainability and circular economy (Achilias et al., 2004). The chemical depolymerization types listed either use high temperature, high pressures, caustic chemicals, result in depolymerization products with limited value with the exception of glycolysis that is extensively used to regenerate PET.

Methanolysis is a depolymerization method that uses methanol as a solvent to decompose PET to dimethyl terephthalate (DMT) and ethylene glycol (EG). This can be done by three methods, viz., liquid methanolysis, vapour methanolysis and supercritical methanolysis (M. Han, 2019). Liquid methanolysis is performed at 180-280 °C at 20-40 atm pressure. The high pressure allows the methanol to stay in its liquid form despite the high temperatures. Vapour methanolysis uses superheated methanol vapours for the transesterification reaction (M. Han, 2019).

Aminolysis is a method of chemical depolymerization that uses primary amines that target the functional groups of polyesters. The primary product generated is bis(2-hydroxyethylene) terephthalamide (BHETA) (Shukla & Harad, 2006). The time of the aminolytic reaction varies

depending on the solvent ratio, presence of a catalyst, and temperature. The catalysts normally used are potassium sulphate, sodium acetate, although sodium acetate generally yields better quantities of BHETA. Aminolysis of PET can be enhanced by microwave radiation, ultrasound, UV radiation and sunlight (Chan et al., 2023).

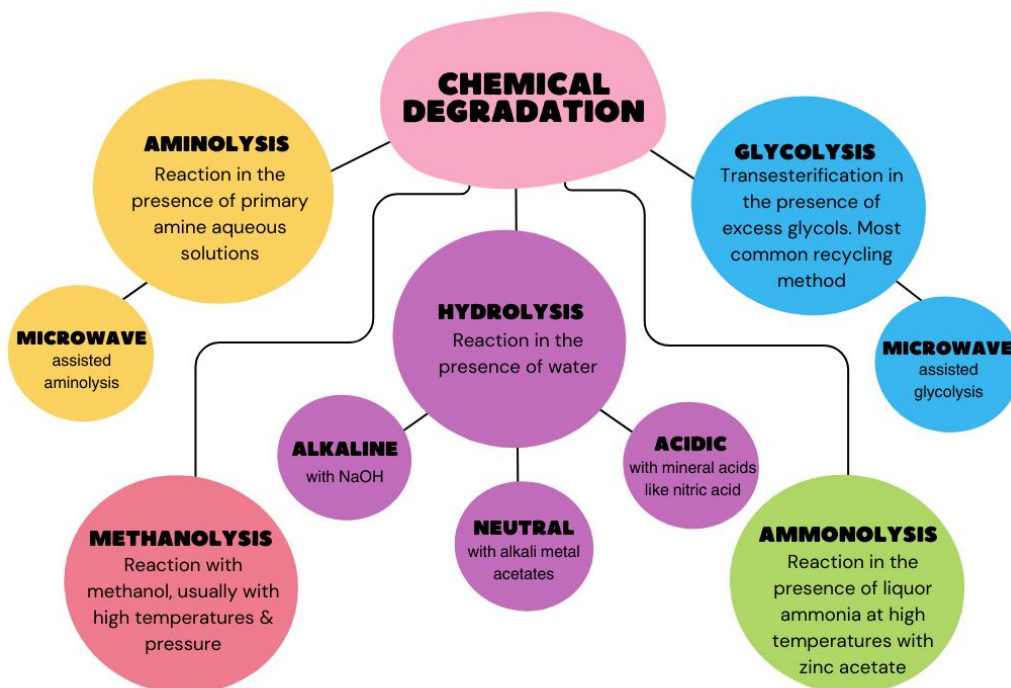


Figure 2.3. Types of chemical hydrolysis used for the breakdown of PET.

Hydrolysis of PET, as the name suggests, uses water as the solvent for the breakdown. PET hydrolysis can be further categorized into acidic, neutral and alkaline hydrolysis. This process is generally carried out under high temperatures ($>250\text{ }^{\circ}\text{C}$) and pressures (1-4 MPa) (Pereira et al., 2023). The primary products generated because of PET hydrolysis are ethylene glycol and terephthalic acid which is resultant of the scission of the ester bond in the backbone (Mcmahon et al., 1959; Stanica-Ezeanu & Matei, 2021).

Glycolysis of PET involves breaking down PET into its monomer components using glycol, typically ethylene glycol. This depolymerization reaction occurs under relatively low temperatures and atmospheric pressure, often in the presence of a catalyst such as zinc acetate or cobalt acetate. Glycolysis of PET consists of heterogeneous and homogeneous depolymerization resulting in, breakdown products like BHET and other oligomers, which can be further purified and repolymerized to produce new PET (Xin et al., 2021).

Ammonolysis of PET is a chemical recycling process in which PET is depolymerized by reacting it with liquor ammonia. This process occurs at elevated temperatures and pressures, often in the presence of a catalyst like zinc oxide or sodium methoxide. The reaction targets the ester bonds in PET, resulting in the formation of terephthalamide and ethylene glycol (Gupta & Bhandari, 2019).

2.3.2.3 Pyrolysis

PET can be depolymerised or converted to energy at temperatures between 200 to 600 °C. Pyrolysis at lower temperatures, 200-300 °C yield hydrocarbons of length C5 to C10 as breakdown products. With increase in temperature the length of the decomposed products decreases (Hujuri et al., 2013). The primary products of pyrolysis of PET are TPA, EG, hydrocarbons and gases. Pyrolytic depolymerization is typically carried out in the absence of oxygen, which breaks down the polymer chain into TPA and EG. Using pyrolysis, waste PET can be completely converted to oil and gas in 30 minutes at 375-425 °C (Olam & Karaca, 2022). Catalysts such as zeolites and mesoporous catalysts are used to increase the uniformity of the products generated (Dai et al., 2022). Although, pyrolysis is able to produce liquid hydrocarbons that can be recycled to plastic, it still produces harmful gases that can be carcinogenic and uses extremely high temperatures.

2.3.2.4 Biological depolymerization

Biological depolymerization or the biological hydrolysis of PET involves the use of a bioagent like microorganisms or an enzyme to breakdown PET. Few enzymes like cutinases, esterases, lipases, PETases and MHETases have demonstrated their PET hydrolyzing abilities and have been categorized as PET-hydrolyzing enzymes (PHEs) (see Section 2.7). The products of biological depolymerization of PET are primarily TPA and EG, however, they can vary depending on the enzyme used for biological depolymerization. Biological depolymerization is a slow process, so, to address the issue of increased PET waste, scientists have taken to engineering a select few enzymes for increased efficiency (Sulaiman et al., 2012; Tournier et al., 2020). This further complicates its translation to an environmental application due to the techniques involved in genetic engineering. Understanding the biological depolymerization of PET not only requires knowledge about the enzymes or microorganisms but also the factors influencing biological depolymerization, which are discussed below.

2.4 Factors affecting biological depolymerization

The advantages of the discovered bioagents for PET biodegraded are still limited by certain physicochemical factors of the polymer. These factors, viz., crystallinity, hydrophobicity, and surface topology of PET are described below.

2.4.1 Crystallinity

Crystallinity is the structural order of any given polymeric material. Highly ordered polymer chains result in increased crystallinity in plastics. Most PET in circulation have some level of crystallinity. For example, a single-use PET plastic bottle has different degrees of crystallinity for the three regions of the bottle, the neck is 1.2%, the body is 33.8% and the base is 11.7% (Thomsen et al., 2023). PHEs are able to cleave the amorphous regions of PET by random chain scissoring.

As the biological depolymerization progresses, the amorphous region of PET is reduced, thereby increasing the overall crystallinity of the unhydrolyzed PET (Thomsen et al., 2023). This in turn reduces the rate of biological depolymerization. Few enzymes like *IsPETase* and *LCC_{ICCG}* are able to hydrolyze the crystalline regions of PET (Tournier et al., 2020; Yoshida et al., 2016).

2.4.2 Hydrophobicity

One of the desirable qualities of PET is its hydrophobicity. This property helps impede the biological depolymerization of the polymer as the biological depolymerization reactions normally occur in aqueous environments. Hence, hydrophobicity is another factor that plays a crucial role in the biological depolymerization of PET. Increased hydrophobicity has been shown to hinder biological depolymerization and limits the hydrolysis by slowing down the reaction. Additionally, microbial colonization which is important step for biological depolymerization is dependent on the hydrophilicity of the substratum. Surface modification can be used to increase the hydrophilicity of PET, making PET more susceptible to enzymatic and microbial attack, which in turn could improve the biological depolymerization rate.

2.4.3 Surface topology

Most PET is molded such that the surface is smooth. A smooth, hydrophobic structure hinders the adherence of microorganisms. Biological depolymerization commences with adherence via pili-like protrusions followed by secretion of PHEs. The surface topology determines the amenability of adherence of microorganisms. Surfaces that are rough, have cracks, crevices and protrusions allow for pockets that allow microorganisms to adhere.

2.5 Biocatalysts used for PET biological depolymerization

The discovery and development of PET-hydrolyzing biocatalysts have been going on for decades now. Previously, PET-hydrolyzing microorganisms and enzymes were studied in either compost or soil loosely littered with PET or other plastics. Recent efforts have been focused on understanding the microbiome in areas with substantial amounts of plastics in wastewater treatment plants (Kelly et al., 2021), marine waters (Molitor et al., 2020) and outside recycling facilities (Yoshida et al., 2016). The two main types of biocatalysts studied are whole-cell microorganisms and enzymes. Depending on the downstream products desired, the appropriate biocatalyst is used. Microorganisms have a battery of enzymes that can modify and hydrolyze the polymer resulting in a combination of monomers and oligomers that the microorganism can use as a nutrient. Employing a single enzyme or a combination of enzymes would result in known hydrolysis products (Mueller, 2006; R. J. Müller et al., 2005; R.-J. Müller et al., 2001). Such products can then be harvested to regenerate PET (Tournier et al., 2020) or used as a substrate for other polymers, for example, to produce polyhydroxyalkanoate (Kenny et al., 2008).

All the PET-hydrolyzing enzymes discovered have the same catalytic triad (Asp-His-Ser), indicating that they possess the exact catalytic mechanism of PET cleavage, a serine nucleophilic attack. After a bioinformatic analysis of 69 PET-degrading enzymes, they were categorized into type I, type IIa and type IIb (see Table 2.1 for more information). Their categorization is focused on the amino acid composition and does not reflect the substrate-binding and regulatory sites of the enzymes, thereby resulting in varying enzyme activities and substrate specificities within each group. Our current understanding regarding the types of PET-hydrolyzing enzymes is limited to their structural differences and bond hydrolyzing mechanisms. PET-hydrolyzing enzymes, like restriction enzymes, could have different recognition and cleavage sites (Joo et al., 2018).

Table 2.1 Comparison between types I, IIa and IIb PET-hydrolyzing enzymes

Characteristic	Type I	Type IIa	Type IIb
Additional disulphide bond	None	Cys203-Cys239	Cys203-Cys239
Subsite I	Amino acids identical to type II also contains the catalytic site	Amino acids identical to type I, also contains the catalytic site	Amino acids identical to type I, also contains the catalytic site
Subsite II	Different residues compared to IIa and IIb	Slight variation in amino acid residues compared to IIb	Slight variation in amino acid residues compared to IIa
238 th residue of subsite II	Phe238	Phe238 or Tyr238	Ser238 (non-catalytic serine)
Extended loop	No extended loop	Highly variable residues in the extended loop	Relatively conserved extended loop
PET-degrading activity	Lower PET-degrading activity than type II	Higher PET-degrading activity than type I	Higher PET-degrading activity than type I
Bacteria examples	<i>Thermomonospora curvata</i> , <i>Thermobifida fusca</i>	<i>Pseudomonas alcaligenes</i> NBRC 14159, <i>P. litoralis</i>	<i>Ideonella sakaiensis</i> 201-F6, <i>Acidovorax delafieldii</i>

Despite the gaps and limitations in knowledge, numerous studies have described the factors affecting hydrolysis and genetic engineering for increased activity and thermostability. Studies focusing on understanding the structure and function of enzymes employ a common strategy of disrupting their function by replacing the amino acid with alanine. This results in widened substrate-binding grooves, reduction in post-substrate-binding conformational changes, or deletion of loops or lids that hinder substrate binding. These features generate enzymes with increased substrate promiscuity (Palm et al., 2019).

2.6 Nomenclature used for reporting PET depolymerization enzymes in literature

PET-hydrolyzing enzymes include cutinases, lipases, esterases, carboxylesterases, and hydrolases.

Cutinases have shown tremendous promise as opposed to other PET-hydrolyzing enzymes. However, its nomenclature has not remained uniform throughout the research literature. Cutinases have occasionally been categorized either as esterases or lipases. Cutinase behaves as an intermediate between lipase and esterase (Kleeberg et al., 2005). In addition, it has also been described as a hydrolase possessing PET-hydrolyzing activity (R. J. Müller et al., 2005).

All the enzymes ever discovered can be segregated into six classes and are assigned an Enzyme Commission (EC) number. Class 3 hydrolases catalyze the reaction by adding a water molecule across the cleaved bond. The sub-classification helps us understand the kind of bond being cleaved, an ester bond. This is the primary bond between PET monomers. According to most studies on the hydrolysis mechanism of PET-hydrolysis, ester bonds are cleaved in such systems. These fall under the esterase class of hydrolase enzymes annotated as EC 3.1. An esterase is an enzyme that cleaves ester bonds to generate acid and alcohol moieties via hydrolysis. Since there are several types of ester bonds, there are further sub-classes of esterases. PET-hydrolyzing ester hydrolases cleave the carboxylic ester bond and fall under the EC 3.1.1 class i.e., carboxylic ester hydrolase. Carboxylester hydrolases belong to EC 3.1.1.1 class. Lipases (triacylglycerol hydrolase) (EC 3.1.1.3) and cutinases (EC 3.1.1.74) cleave carboxylic bonds, Fig. 2.4 further illustrates this functional categorization. The subtle differences between the classes are further mentioned in Table 2.2 (see below). Hence, lipases and cutinases are subtypes of esterases (Roth et al., 2014).

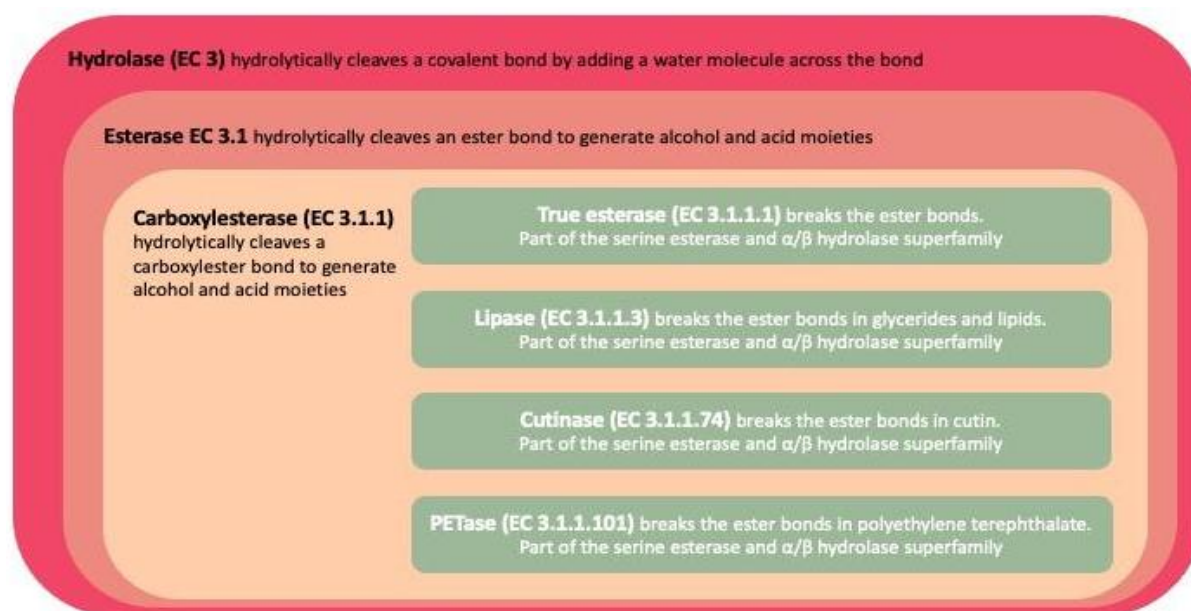


Figure 2.4 Schematic representation of the nomenclature hierarchy of PET-hydrolyzing enzymes (in green) according to EC numbers with the defining characters of each class

PET-hydrolyzing enzymes are also referred to as α/β -hydrolases because of the arrangement of the α -helices and β -sheets (S. Chen et al., 2010). This nomenclature style focuses on the enzyme's structure, not the functional groups targeted for hydrolysis. The α/β hydrolase superfamily includes lipases, esterases, proteases, dehalogenases, peroxidases, and epoxide hydrolases. The core of the tertiary structure of α/β hydrolase superfamilies canonically consists of parallel β -sheets surrounded by α -helices (Nardini & Dijkstra, 1999). PET-hydrolyzing enzymes consist of a central twisted β -sheet which is made up of 9 β -strands and surrounded by 7-9 α -helices (Erickson et al., 2022; Joo et al., 2018).

Another structural classification focuses on the active amino acid involved in the hydrolysis reaction. Lipases, esterases and cutinases with PET-hydrolyzing activity contain serine as a crucial part of their catalytic triad. Hence, these enzymes are also referred to as serine esterases (Dimarogona et al., 2015; Zhang et al., 2013) and are a sub-section of the α/β hydrolase

superfamily. This catalytic serine is present within the conserved GXSXG motif. This motif has been used as an identification marker for carboxylic ester hydrolase activity (Dherbécourt et al., 2008). The serine in serine esterases nucleophilically attacks the ester bond. The nucleophilic serine is accompanied by an acidic amino acid (usually aspartic acid or glutamic acid) and a basic amino acid (usually histidine). The most common catalytic triad present in PET-hydrolyzing enzymes are Ser-Asp-His with almost identical triad geometries (Erickson et al., 2022). In addition, the substrate binding groove is considerably hydrophobic and spans across multiple subsites. Since all serine hydrolases hydrolytically cleave ester bonds, they fall under EC 3.1. PET hydrolysis is an additional attribute of the various enzymes mentioned above and hence had no unique EC number annotated. Due to this, most PET-hydrolyzing enzymes rely on class-specific assays to determine their niche class. The novel category for PET-hydrolyzing enzymes is 3.1.1.101 was defined after the discovery of *IsPETase* from *Ideonella sakaiensis* that has a specificity towards PET as a substrate.

Table 2.2 Comparison between typical characteristics of true lipases, esterases and cutinases

Lipase (EC 3.1.1.3) (Lopes et al., 2011)	Esterase (EC 3.1.1.1) (Lopes et al., 2011)	Cutinase (EC 3.1.1.74) (Sulaiman et al., 2012)
A type of hydrolase & subclass of esterase	A type of hydrolase	A type of hydrolase & subclass of esterase
Mainly active against water-insoluble substrates, e.g., long-chain fatty acids	Preferentially hydrolyses 'simple' esters & shorter than C6 fatty acids	Preferentially hydrolyses 'simple' esters & shorter than C6 fatty acids
Can hydrolyze p-nitrophenyl palmitate better	Can hydrolyze p-nitrophenyl butyrate better	Can hydrolyze p-nitrophenyl butyrate better
More stable in organic solvents	Stable in organic solvents	Stable in organic solvents
Cutinase is sometimes	Cutinase is first considered an	Cutinase is considered an

considered a lipase.	esterase.	esterase first and then a lipase.
Requires lipid-water interface activation	Active regardless of lipid-water interface	Active regardless of lipid-water interface
Lipases have lids which take part in activation. Exceptions found.	Does not have a lid	Does not have a lid

2.7 Classes of PET-hydrolyzing enzymes

2.7.1 Hydrolases (EC 3)

A hydrolase is an enzyme that catalyzes the cleavage of a covalent bond using a water molecule. All known PET-hydrolyzing enzymes are hydrolases. Most PET-hydrolyzing enzymes are further classified to fit one of the sub-classes viz., esterases, lipases, cutinases or PETases. The few hydrolases that haven't been further classified were isolated with a non-PET substrate, a metagenomic analysis of unculturable microorganisms or a combination of both.

Metagenomic analysis of uncultured thermophiles led to the discovery of a thermostable hydrolase (Kato et al., 2018) which was later classified as a BhrPETase (Xi et al., 2021). This enzyme has a melting temperature of 101 °C, possibly the most thermostable bacterial PETase reported. The expression of BhrPETase was optimized by shifting the expression host to *Bacillus subtilis* CBS2 due to its excellent secretion system. Another such study discovered a novel polyester hydrolase, PHL7, that was able to degrade amorphous PET and post-consumer PET thermoform packaging with high efficiency. The thermostable enzyme yielded 90% weight loss of PET films at 70 °C after 24 hours with a depolymerization rate of 6.7 $\mu\text{m}\cdot\text{h}^{-1}$ at the 16-hour mark of the reaction. Inching closer to circular economy, the same group, used the TPA generated as a result of biological depolymerization to produce virgin PET films (Sonnendecker et al., 2022). The first

crystal structure of a hydrolase derived with PET-hydrolyzing activity was a type IIa hydrolase from *Pseudomonas aestusnigri* (Bollinger et al., 2020). Its primary breakdown product is mono-(2-hydroxyethyl) terephthalic acid (MHET). Dimethyl terephthalic acid (DMT) is a commonly used substrate in the commercial production of PET but not in biological depolymerization studies. Using this as a substrate, a novel enzyme, DMT hydrolase, DmtH, was discovered (Cheng et al., 2020).

2.7.2 Carboxylesterase (EC 3.1.1.1)

A carboxylesterase is a hydrolase that cleaves an ester bond to acid and alcohol functional groups, commonly referred to as esterases. The efforts to discover novel microorganisms and enzymes with esterase activity are constant. There are reports on esterases with high hydrolyzing capacity (Brueckner et al., 2008; Cheng et al., 2020; Ghodke & Punekar, 2022; Kawai et al., 2014; Qiu et al., 2020; Ribitsch et al., 2012; Sharon & Sharon, 2012) and modifying capacity (Alisch et al., 2004; Maurya et al., 2020). Some esterases require activation, like the esterase from *Clostridium botulinum* (Cbotu_EstA) (Perz et al., 2016). In addition to the isolated enzymes, Cbotu_EstA was engineered to reduce interfacial activation resulting in an increase in the enzyme binding on hydrophobic surfaces of polyesters. The engineered Cbotu_EstA showed up to a 7-fold increase in activity towards PET (Biundo et al., 2018).

Structurally, BHET is a terephthalic acid linked to two ethylene glycols by an ester bond. Using BHET as a sole carbon source, esterase containing *Enterobacter* sp. HY1 (gene: estB) was isolated (Qiu et al., 2020). Using PCL as a substrate and a metagenomic approach, a novel polyesterase, SM14est, was discovered from *Streptomyces* sp. SM14 (Carr et al., 2023). The most commonly used substrate to determine esterase activity is para-nitrophenyl butyric acid (pNPB).

2.7.3 Lipase (EC 3.1.1.3)

Lipase, as previously described, is a subclass of esterase that prefers hydrophobic substrates like glycerides. Some lipases are able to cleave structurally analogous substrates due to their attribute of substrate promiscuity, a feature observed for PET hydrolysis. Lipase expression is induced in the presence of lipids and subsequently requires interfacial activation to catalyze hydrolysis. BHET proved to be the best inducer for lipolytic *Aspergillus oryzae* (X. Wang et al., 2008). In another study, the expression of both lipase and esterase in *Yarrowia lipolytica* was increased by the addition of 5% w/w watermelon peels (Sales et al., 2020). The cutin in these peels have lipid molecules that function as inducers (Chaudhari & Singhal, 2015). In addition, the growth conditions were optimized for *Y. lipolytica* for an increased expression (da Costa et al., 2020). The combination of lipase B from *Candida antarctica* (CALB) and cutinase from *Humicola insolens* (HiC) worked synergistically to break down PET. HiC resulted in MHET accumulation which was subsequently degraded to TPA by CALB (Carniel et al., 2017).

2.7.4 Cutinase (EC 3.1.1.74)

Cutin is the insoluble, waxy polymer part of the cuticle, a plant structural component. It consists of ω -hydroxy fatty acids, dihydroxy palmitic acids, saturated and 12-monounsaturated 18-hydroxy 9,10-epoxy C18 acids, and saturated and 12-monounsaturated 9,10,18-trihydroxy C18 acids (Alves Macedo & Fontes Pio, 2005). It has fewer phenolic groups than its counterpart, suberin, in potatoes. Cutinases are enzymes that can hydrolytically cleave the ester bonds in cutin. These enzymes are inducible and are secreted to the extracellular environment. Plant pathogens produce them to disturb the structural integrity to elicit a disease. Cutinases were first isolated from pathogenic fungi, *Fusarium solani* pisi (Dantzig et al., 1986; Dickman & Patil, 1986; Maiti &

Kolattukudy, 1979) and *Aspergillus fumigatus* (Stutzenberger & Kahler, 1986). The key differences between the two are described in Table 2.3 (Ping et al., 2017).

Due to this structural similarity between cutin and PET, cutinases can effectively cleave the ester bonds in PET compared to other PET-hydrolyzing enzymes. Cutinases have higher affinities for short-chain to medium-chain fatty acid esters (Nikolaivits et al., 2018). However, a higher affinity towards the substrate does not equate to a higher hydrolysis rate (Ronkvist et al., 2009). Cutinases and lipases are the most widely studied classes of enzymes for PET hydrolysis.

Along with the classical isolation techniques, metagenomic analysis has proven helpful in screening through multiple PET-hydrolyzing enzymes (Purohit et al., 2020; Sulaiman et al., 2012). Cutinases from *Thermobifida fusca* (TfCut2), *Fusarium solani* (FsC) and *Humicola insolens* (HiC) have been of particular interest. Moreover, the most studied genus is a thermophilic actinomycete, *Thermobifida* spp. (previously known as *Thermomonospora* spp.). Their cutinases hydrolyze ester bonds of triacylglycerides in the sn-1,3- and sn-2 positions and have broad substrate specificity (S. Chen et al., 2008). The Tfu_0883 was initially reported as a hydrolase capable of degrading aliphatic-aromatic copolyesters (Kleeberg et al., 2005).

Structures can be constructed *in silico* using a combination of tertiary predicting software (Dimarogona et al., 2015) which circumvents the challenges faced using X-ray crystallography or NMR. Based on molecular docking and enzyme contact surface analysis, it was noticed that the polymer binds to the enzyme in an elongated hydrophobic groove (Tournier et al., 2020). The structures of numerous cutinases and their respective mutants are readily available on protein databanks (examples: TfCut2 UniProt ID: Q47RJ6, Cut190 UniProt ID: W0TJ64, HiC UniProt ID: A0A075B5G4).

Metal ions regulate a few cutinases. For example, Tfu_0883 is inhibited by Cr(III) (S. Chen et al., 2008). The function and stability of Cut190* from *Saccharomonospora viridis* AHK190 are regulated by Ca^{+2} ions. The Ca^{+2} ions are a co-factor that, upon binding to the enzyme, results in conformational change such that the active site is open for substrate binding (Numoto et al., 2018). The predominant breakdown products vary for each enzyme. For example, the predominant breakdown product of FsC is MHET with some TPA and traces of BHET, while HiC-catalyzed breakdown produces only TPA (Ronkvist et al., 2009).

Table 2.3 Difference between cutinase characteristics from two fungal species, *Fusarium solani* and *Aspergillus fumigatus* (Ping et al., 2017)

Characteristics	<i>Fusarium solani</i> cutinase	<i>Aspergillus fumigatus</i> cutinase
pNP-aliphatic substrates	Hydrolyzed predominantly para-nitrophenyl decanoate	Hydrolyzed predominantly para-nitrophenyl butyrate
Substrate specificity	Prefers longer substrates	Preferred shorter substrates
Size	Size: ~20kDa	Size: ~20kDa
Thermostability	Optimal temperature: 40 °C. Less thermostable.	Optimal temperature: 60 °C. More thermostable.
Polycaprolactone depolymerization	Degraded 44.3% at 40 °C	Completely degraded at 40 °C
Biosynthesis potential	Could polymerize up to 2300 MW of ω -hydroxy-hexadecanoic acid	Could polymerize up to 25,000 MW of ω -hydroxy-hexadecanoic acid

The common strategies to increase catalytic efficiency are overexpression (Araújo et al., 2007; S. Chen et al., 2010; Ping et al., 2017) and site-directed mutagenesis (Araújo et al., 2007; Zhang et al., 2013). Dong et al. (2020) focused on increasing cutinase activity towards its natural cutin substrate by reducing the steric hindrance caused by bulkier amino acids (mutations: L90A and

I213A), which allowed more flexibility in the substrate-binding site. L90A mutant exhibited 5-fold increased hydrolysis activity toward cutin. Zhang et al. (2010) evaluated a unique approach to creating a fusion protein followed by site-directed mutagenesis. The new enzyme was a combination of cutinase Tfu_0883 and the carbohydrate-binding module of the cellulase CenA from *Cellulomonas fimi*, named TfCut-CBM_{CenA}. This modification increased catalytic efficiency, specific activity, and binding (Zhang et al., 2013). The catalytic efficiency of *Fusarium solani* cutinase (FspC) ($3.2 \text{ s}^{-1} \cdot \mu\text{M}^{-1}$) was found to be higher than the cutinases from *T. fusca* (S. Chen et al., 2010). With the metagenomic approach, the leaf-compost cutinase (LCC) with a depolymerization rate of 12 mg/h/mg of the enzyme with PET at 50 °C was isolated (Sulaiman et al., 2012). Since then, several groups have mutated the enzyme to increase its efficiency and thermostability. As mentioned before, thermostability is an attractive feature. The most recent and successful development in enzymatic biological depolymerization of PET is with an engineered LCC (two mutants: ICCG & WCCG) that can withstand 94.5 °C. With a ratio of 3 mg of enzyme per 1 g of PET, these mutants biodegrade 90% of PET with yields of 16.7 g of TPA from the initial 200 g of PET in approximately 10 hours. The extracted TPA was used to blow PET bottles, which could be a step toward a circular economy (Tournier et al., 2020). An even improved variant of LCC was able to hydrolyze 1.3 g of untreated post-consumer PET with 1.25 mg of the Δ LCC variant in less than 3 days at 55 °C. This double mutant variant lacked the secretion signal and had two amino acid substitutions by site-directed mutagenesis, S101N and F243T (Pirillo et al., 2023).

2.7.5 PETase (EC 3.1.1.101)

Subclass 3.1.1.101 was formed after the discovery of one enzyme and its engineered variants. In 2016, a novel PET-hydrolyzing bacterium was isolated from a PET bottle recycling factory's yard

in Sakai City, Japan. *Ideonella sakaiensis* demonstrated potential by extensively damaging amorphous PET film and high-crystalline commercial PET bottles. Biological depolymerization commences with adherence via pili-like protrusions followed by secretion of PETase (previously classified as lipase). The primary depolymerization product is MHET, followed by terephthalic acid (TPA) and bis(2-hydroxyethyl) terephthalic acid (BHET). These products are assimilated into cellular metabolism via enzymes like MHETase and protocatechuic acid (PCA) dioxygenase (Yoshida et al., 2016).

The specificity of *Is*PETase has been hypothesized to be because of the evolution towards aromatic-aliphatic polyesters (Yoshida et al., 2016). Several studies report the crystal structure, the inactive version of *Is*PETase (X. Han et al., 2017), wild type with ligands (Joo et al., 2018), at an increased resolution to determine its active site flexibility at ambient temperatures (Fecker et al., 2018), and *Is*PETase with substrate and product analogs (C. C. Chen et al., 2018). Amino acid substitution is a standard method to study the role of predicted essential amino acids. A byproduct of this method is the generation of mutants with increased breakdown and product generated (R240A substitution near the substrate-binding site) (Joo et al., 2018) and an increase in its k_{cat} (Ma et al., 2012a). Austin et al. (2018) focused on engineering the substrate-binding site of *Is*PETase to achieve some semblance of the substrate-binding site of TfCut2. The engineered enzyme explicitly degrades polyesters containing aromatic components like polyethylene furanoate (PEF). Thermostability is an attractive feature in PET biological depolymerization due to the polymer's glass transition temperature, hence, site-directed mutagenesis (Brott et al., 2022), and rational protein engineering (Cui et al., 2021; Son et al., 2019) has been adopted to achieve the same.

Pretreating PET films with hydrophobin A (from *Aspergillus oryzae*) was a unique approach that boosted the overall hydrophilicity of the enzyme, which in turn increased hydrolysis (Puspitasari et al., 2021).

Another common strategy is the non-homologous overexpression of the enzyme in a standard microbial chassis. Using this strategy, a strain of *Pichia pastoris* was created that displayed a fully functional IsPETase on its cell surface via an anchor protein. The whole-cell biocatalyst had a 36-fold increase in PET-hydrolysis compared to the purified enzyme and showed a 50% reduction in turnover rate after seven repeated uses. The chief breakdown product was MHET, followed by BHET and TPA (Z. Chen et al., 2020). This strategy is cost-efficient, has high PET-hydrolysis capacity and has high reusability compared to purified enzymes and engineered microorganisms.

PET biological depolymerization is far more complicated than the four types of enzymes described above. These enzymes act directly on PET as a substrate. Meanwhile, metagenomes are known to have an array of enzymes in multiple metabolic pathways that are responsible for not only the complete breakdown of the polymer backbone but also, its integration into cellular (Giangeri et al., 2022) .

There are numerous reports of the specific activities of cutinases, weight loss, etc., under different conditions. Table 2.4 lists key examples of specific activities with their respective substrates and test conditions of PHEs.

Apart from the usually studied enzymes like hydrolases, cutinases, esterases, and lipases, Inderthal et al. (2021) suggests exploiting alkane hydroxylases for depolymerizations that use a single substrate where certain desired products are sought.

Table 2.4. Enzyme, origin, reaction system, and specific activity of lipases, esterases, cutinases, hydrolases, and enzyme combinations.

LIPASES				
Enzyme	Origin	Reaction system	Specific Activity	Reference
Lipozyme TL IM LIPASE	Novozyme	<i>Different protocols are used for each substrate and enzyme combination.</i>	<u>Esterase activity:</u> with olive oil: 187.3 U/mg with pNPB: 1.8 U/mg <u>Lipase activity:</u> with olive oil: 297.4 U/mg with pNPP: 45.8 U/mg	Lopes et al., 2011
PsL	Sigma, Germany	0.1M phosphate buffer pH 7.0, temperature: 40 °C, 50 rpm, 3 weeks	Negligible weight loss with PET	R. J. Müller et al., 2005
CaL	Fluka, Germany	0.1M phosphate buffer pH 7.0, temperature: 55 °C, 50 rpm, 3 weeks	Negligible weight loss with PET	R. J. Müller et al., 2005
CUTINASES				
LC Cutinase	Expressed: <i>E. coli</i> BL21-CodonPlus (DE3)	pH 8.5, temperature: 50 °C, 24 hrs	with pNPB: 4.5 ± 0.52 μ kat/mg with cutin: 6 μ mol/hr/ mg of enzyme with PET: 12 mg/h/mg of enzyme	Sulaiman et al., 2012
LC Cutinase (codon optimized)	Expressed: <i>E. coli</i> BL21-DE3	pH 8.0, temperature: 65 °C	with PET 93.2 mg of TPA equivalents/h/mg of enzyme	Tournier et al., 2020
Cutinase	<i>Fusarium solani</i> PBU-RU-5B	pH 7.0, temperature: 25 °C	with pNPB: 11.4 μ g/h/ μ g of enzyme	Nimchua et al., 2008
Cutinase (FsC)	<i>F. solani</i> f. sp. <i>psi</i>	pH 7.0, temperature: 30 °C	with pNPB: 2.8 μ g/h/ μ g of enzyme	Nimchua et

				al., 2007
FsC	Expressed: <i>Pichia pastoris</i> X33.	pH 7.0, temperature: 65 °C	Activity with pNPB: 456.5 U/mg Degraded 44.3% of PCL at 40°C	Ping et al., 2017
FsC	Expressed: <i>Pichia pastoris</i> X33.	14.5 mM Tris-HCl, 0.75% glycerol, pH 7.5, temperature: 25 °C	Catalytic efficiency with pNPB (k_{cat}/K_m): $0.32 \pm 0.03 \text{ min}^{-1} \cdot \mu\text{M}^{-1}$	Baker et al., 2012
FsC	Expressed: <i>E. coli</i> BL21-DE3	PET: 0.1 M phosphate buffer pH 7.5, 90 rpm, temperature: 37 °C, 24 hrs.	with pNPB: 210 U/mg TPA formed: 0.024 mM	Araújo et al., 2007
FsC mutant (L182A)	Expressed: <i>E. coli</i> BL21-DE3	PET: 0.1M phosphate buffer pH 7.5, 90 rpm, temperature: 37 °C, 24 hrs.	with pNPB: 976.5 U/mg TPA formed: 0.127 mM	Araújo et al., 2007
FsC mutant (V184A)	Expressed: <i>E. coli</i> BL21-DE3	PET: 0.1 M phosphate buffer pH 7.5, 90 rpm, temperature: 37 °C, 24 hrs.	with pNPB: 606.9 U/mg TPA formed: 0.049 mM	Araújo et al., 2007
FsC mutant (L81A)	Expressed: <i>E. coli</i> BL21-DE3	PET: 0.1 M phosphate buffer pH 7.5, 90 rpm, temperature: 37 °C, 24 hrs.	with pNPB: 262.5 U/mg TPA formed: 0.096 mM	Araújo et al., 2007
Cutinase (FspC)	Expressed: <i>E. coli</i> BL21-DE3	pH 7.0, temperature: 30 °C. 20 mM Tris-HCl + 10mM NaOH, pH 8.0, temp 40 °C	with pNPB: 5 µg/h/µg of enzyme Catalytic efficiency (k_{cat}/K_m): $3.2 \text{ s}^{-1} \cdot \mu\text{M}^{-1}$	S. Chen et al., 2010; Nimchua et al., 2007
Cutinase (FoCut5a)	Expressed: <i>E. coli</i> BL21-DE3	pH 6.0, temperature: 40 °C, 0.1 M citrate phosphate buffer, 10 mins.	Catalytic efficiency with pNPB (k_{cat}/K_m): $152.5 \text{ s}^{-1} \cdot \text{mM}^{-1}$	Dimarogona et al., 2015

Cutinase (<i>A. oryzae</i>)	Expressed: <i>Pichia pastoris</i> X33.	14.5 mM Tris-HCl, 0.75% glycerol, pH 7.5, temperature: 25 °C	Catalytic efficiency (k_{cat}/K_m): $3.4 \pm 0.41 \text{ min}^{-1} \cdot \mu\text{M}^{-1}$	Baker et al., 2012
Cutinase (<i>A. fumigatus</i>)	Expressed: <i>Pichia pastoris</i> X33.	14.5 mM Tris-HCl, 0.75% glycerol, pH 7.5, temperature: 25 °C	Catalytic efficiency (k_{cat}/K_m): $0.56 \pm 0.01 \text{ min}^{-1} \cdot \mu\text{M}^{-1}$	Baker et al., 2012
Cutinase (<i>A. fumigatus</i>)	Expressed: <i>Pichia pastoris</i> X33.	pH 7.0, temperature: 65 °C	Activity with pNPB: 1236.3 U/mg Thoroughly degraded PCL at 40°C	Ping et al., 2017
<i>T. fusca</i> cutinase	<i>Thermobifida fusca</i> DSM43793	pH 8.0, temperature: 60°C, with PET: 55 °C, pH 7.0	with pNPB: 7.6 $\mu\text{kat}/\text{mg}$ with cutin: 4 $\mu\text{mol}/\text{hr}/\text{mg}$ of enzyme Depolymerization rate with PET: 0.05 mg/h/mg of enzyme	S. Chen et al., 2008; R. J. Müller et al., 2005
Cutinase (Tfu_0882)	Expressed: <i>E. coli</i> BL21-DE3	pH 8.0, temperature: 60 °C	Catalytic efficiency (k_{cat}/K_m) with pNPB: $0.7 \text{ s}^{-1} \cdot \mu\text{M}^{-1}$	S. Chen et al., 2010
Cutinase (Tfu_0883)	Expressed: <i>E. coli</i> BL21-DE3	pH 8.0, temperature: 60 °C	Catalytic efficiency (k_{cat}/K_m) with pNPB: $1.5 \text{ s}^{-1} \cdot \mu\text{M}^{-1}$	S. Chen et al., 2010
TfCut-CBM _{CenA}	Expressed: <i>E. coli</i> BL21-DE3	pH 8.0, temperature: 50 °C	with pNPB: 20,000 U/ μmol protein	Zhang et al., 2013
Cutinase 1 (<i>T. cellulosilytica</i>)	Expressed: <i>Pichia pastoris</i> KM71H	PET: 1 M potassium phosphate buffer, 100 rpm, pH 8.0, temperature: 65 °C	with pNPB: 210 U/mg with PET: ~55mM TPA, negligible MHET	Gamerith et al., 2017
ThC_Cut1_koAsn (glycosylation KO)	Expressed: <i>Pichia pastoris</i> KM71H	PET: 1 M potassium phosphate buffer, 100 rpm, pH 8.0, temperature: 65 °C	with pNPB: 210 U/mg with PET: ~61mM TPA, ~2mM MHET	Gamerith et al., 2017

ThC_Cut1_koST (glycosylation KO)	Expressed: <i>Pichia pastoris</i> KM71H	PET: 1 M potassium phosphate buffer, 100 rpm, pH 8.0, temperature: 65 °C	with pNPB: 210 U/mg with PET: 62.5mM TPA, ~3mM MHET (=24%)	Gamerith et al., 2017
HiC (<i>H. insolens</i> cutinase)	Expressed: <i>Pichia pastoris</i> X33.	14.5 mM Tris-HCl, 0.75% glycerol, pH 7.5, temperature: 25 °C	Catalytic efficiency (k_{cat}/K_m): $1.76 \pm 0.43 \text{ min}^{-1} \cdot \mu\text{M}^{-1}$	Baker et al., 2012
Cutinase (<i>A. brassicicola</i>)	Expressed: <i>Pichia pastoris</i> X33.	14.5 mM Tris-HCl, 0.75% glycerol, pH 7.5, temperature: 25 °C	Catalytic efficiency (k_{cat}/K_m): $0.97 \pm 0.07 \text{ min}^{-1} \cdot \mu\text{M}^{-1}$	Baker et al., 2012
Engineered cutinase (<i>S. viridis</i> AHK190)	Expressed: <i>E. coli</i> Rosetta-gami B (DE3)	pH 8.2, temperature: 63 °C, 0.1 M Tris-HCl, 3 days.	Catalytic efficiency (k_{cat}/K_m) with pNPB: $10.4 \text{ s}^{-1} \cdot \text{mM}^{-1}$ & $2.7 \pm 0.1 \text{ mg}$ weight loss of PET	Kawai et al., 2014
Engineered cutinase (<i>S. viridis</i> Q138A)	Expressed: <i>E. coli</i> Rosetta-gami B (DE3)	pH 8.2, temperature: 37 °C, 50 mM Tris-HCl, 30 mins.	Catalytic efficiency (k_{cat}/K_m) with PBSA: $1360 \pm 2.0 \text{ s}^{-1} \cdot \text{mM}^{-1}$	Kawabata et al., 2017
ESTERASE				
Esterase	Induced <i>T. fusca</i> KW3b with suberin	PET: 50 mM phosphate buffer pH 7.2, temperature: 55 °C	Modification of PET surface observed & increase in TPA released	Alisch et al., 2004
HYDROLASES				
TfH	<i>T. fusca</i> induced for increased production	0.1 M phosphate buffer pH 7.0, temperature: 55 °C, 50 rpm, 3 weeks	Weight loss with PET-G: $49.7 \pm 1\%$ and PET-B: $22.4 \pm 1.3\%$	R. J. Müller et al., 2005
rTfH (no mutation)	Expressed: <i>E. coli</i> BL21 (DE3)	0.1 M phosphate buffer pH 7.0, temperature: 55 °C, 50 rpm, 3 weeks	Weight loss with PET-G: $54.2 \pm \text{n.d}\%$ and PET-B: $43.1 \pm 1.6\%$	R. J. Müller et al., 2005
ENZYME COMBINATIONS				

Immobilized TfCa + LCC (free)	Expressed: <i>E. coli</i> BL21-DE3	1 M potassium phosphate buffer pH 8.0, temperature: 60 °C, 120 rpm, 24 hrs	Depolymerization products (TPA, BHET, MHET): 10.42 ± 1.85 mM. Weight loss: 47.9%	Barth et al., 2016
Immobilised TfCa + TfCut2 (mobilised)	Expressed: <i>E. coli</i> BL21-DE3	1 M potassium phosphate buffer pH 8.0, temperature: 60 °C, 120 rpm, 24 hrs	Depolymerization products (TPA, BHET, MHET): 4.44 ± 0.57 mM. Weight loss: 20.4%	Barth et al., 2016

Footnotes:

a pNPB: p-nitrophenylbutyrate

b pNPP: para-nitrophenylpalmitate

c PET-G: Granini fruit juice PET bottle

d PET-B: BASF provided PET pellets

e TPA: terephthalic acid

f MHET: mono-(2-hydroxyethyl) terephthalic acid

g KO: knockout (gene)

2.8 Methods used to quantify PET hydrolysis

PET-hydrolyzing enzymes like cutinases and lipases have standard protocols to determine their primary enzyme activity, which are cutin hydrolysis and lipid hydrolysis, respectively. However, there is no standard protocol for their PET-hydrolyzing ability. The commonly used test is to assess its general esterase activity with non-analogous substrates like pNPB or (para-nitrophenyl acetic acid) pNPA which neither gives us sufficient information about PET-hydrolyzing activity nor the polymer breakdown itself. Recently, PET biological depolymerization assays using PET as a substrate are being developed and used. These along with other qualitative and quantitative methods, will be described below (for illustration see Fig. 2.5).

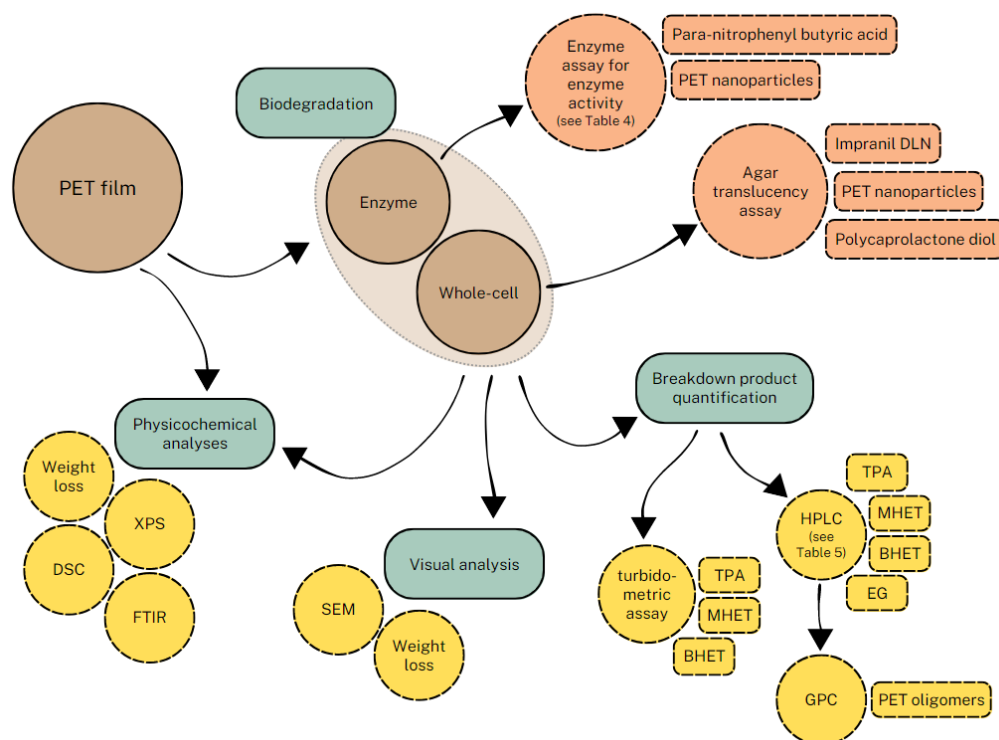


Figure 2.5 Illustration of methodologies currently in use to analyze biological depolymerization of PET.

2.8.1 Qualitative methods

A qualitative, non-specific method of determining PET-hydrolyzing ability is to observe a zone of discoloration or transparency on an agar plate, depending on the substrate used. Uniform PET nanoparticles can be prepared by dissolving PET in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) followed by a dropwise addition into an ice-cooled beaker filled with double distilled water with continuous stirring at 8000 rpm (Pütz, 2006).

Numerous substitutes, as well as their combinations, have been tested. A few examples are polycaprolactone (PCL) nanoparticles (prepared by solvent precipitation or evaporation techniques) (Jarrett et al., 1984; Nishida & Tokiwa, 1993; Wei et al., 2014), coconut oil and tributyrin (Molitor et al., 2020). Studies have also used water-emulsifiable polyesters, like Impranil® DLN emulsion (anionic aliphatic polyester polyurethane) and polycaprolactone diol (PCD_{Mn530}) (lower molecular weight PCL diol derivative). Furthermore, tributyrin was used for metagenomic library screenings (Peña-García et al., 2016).

Some studies report lipase activity using other substrates like olive oil, para-nitrophenyl long-chain aliphatic esters (usually para-nitrophenyl palmitic acid) and triacylglycerides (Lopes et al., 2011). Apart from the PET-hydrolyzing activity, lipases have also been well-studied in food, textile, and detergent industries (Contesini et al., 2010; Mehta et al., 2017).

The substrates mentioned above do not contain an aromatic component; they are not structurally analogous. Hence, these assays may not work as well as a substitute for an aromatic-aliphatic polyester like PET. With each new study, newer substrates were used to study the enzymatic function. While these studies shed light on the mechanism of enzyme action and substrate specificity, the choice of a standard substrate and protocol for determining PET-hydrolyzing activity would significantly help in comparing results and choosing the most efficient enzyme for the breakdown of PET.

2.8.1.1 Microscopy

PET hydrolysis is a surface phenomenon, and visual observation is the easiest way to observe biological depolymerization. Microscopy with regard to enzymatic hydrolysis has been observed by surface modifications on the polymer. Hydrolysis results in cracks, pits, and undulations of all shapes, sizes, and forms on the polymer surface (Malafatti-Picca et al., 2023; N. Wang et al., 2020). Esterases modified the microtopography of PET fibers as opposed to lipases which were observed with scanning electron microscopy and atomic force microscopy (Kardas et al., 2011).

Visual observations have also assisted with understanding the physical interaction between the microorganism and the polymer. For example, it was observed that *Ideonella sakaiensis* use cilia-like extensions to adhere to PET films (Yoshida et al., 2016). Other groups observed protrusions like fimbriae (Rosenberg et al., 1982), pili (Giltner et al., 2006; Martino et al., 2003), and flagella (Haiko & Westerlund-Wikström, 2013) that enable adherence to polymer films. Time-lapse fluorescence microscopy can be used to study early biofilm formation on polymers. This could help shed light on the community dynamics and preferences of microorganisms with plastics in natural environments. Microscopy provides invaluable insights; however, it cannot be quantified.

2.8.1.2 Fourier-Transform Infrared Spectroscopy (FTIR) & X-ray Photoelectron Spectroscopy (XPS)

Different treatments (physical, chemical, and biological) result in changes in the chemical structure of PET. This may not always be evident in substantial weight loss or with imaging techniques. These changes in the bonds can be observed spectroscopically by FTIR and XPS. In addition, XPS analyses determine the percentage of observed functional groups (Hurley & Leggett, 2009). Biological depolymerization results in a fluctuation of the carbonyl index of PET and is sometimes used as an indicator of depolymerization. A ratio of the intensities at the band 1710 cm^{-1} to that of

871 cm^{-1} can be used as an indicator of the carbonyl index (Torena et al., 2021a). Using a similar approach, the ratios of peak height at 1410 cm^{-1} and the peak heights at 725 cm^{-1} (C-H aromatic), 1090 cm^{-1} (C-O ester), 1240 cm^{-1} (C-O), and 1711 cm^{-1} (C=O carboxylic acid) can be indicative of PET biological depolymerization. These wavenumbers are key oxidation peaks, and an increase in their absorbances is indicative of PET polymer chain hydrolysis (Wright et al., 2021). In addition, the crystallinity peak can be calculated as well by the ratio of the peak areas at the bands 1341 cm^{-1} and 1410 cm^{-1} , and an increase in crystallinity can be indicative of PET biological depolymerization (Torena et al., 2021a). Taking this further, the lower 1340-1370 cm^{-1} ratio the more amorphous the PET polymer, making it relatively biodegradable. Similarly, higher absorption ratios of 1340-1370 cm^{-1} correspond to a higher crystalline structure (Mecozzi & Nisini, 2019).

These changes in functional groups can serve as a measure of biological depolymerization by observing the increase of crystallinity and increase in carbonyl content. Rajandas et al. (2012) developed a strategy to quantify the biological depolymerization of low-density polyethylene biological depolymerization (LDPE) using FTIR-ATR. Currently, there is no standard quantitative protocol for PET biological depolymerization using FTIR or XPS.

2.8.1.3 Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry is a thermoanalytical tool for the identification of physical and thermal transitions in polymers. These transitions give insights into the glass transition temperatures, which increase as a function of crystallinity. Crystallinity is a crucial factor in hydrolysis reactions. Amorphous or low crystalline plastics are easily hydrolyzed compared to high crystalline plastics. Commercially available plastics have varying crystallinity depending on

their function. Most naturally occurring enzymes and microorganisms prefer amorphous or low crystalline compared to high crystalline plastics.

Furthermore, as biological depolymerization progresses, the overall crystallinity increases as enzymes target the amorphous regions of the plastic (Ronkvist et al., 2009). DSC can measure this change in crystallinity. However, the increasing crystallinity does not quantify biological depolymerization but indicates that biological depolymerization could have potentially occurred.

2.8.2 Quantitative methods

In recent years with the increasing understanding of the types and mechanisms of PET-hydrolyzing enzymes, the scientific community has managed to understand the intricate details pertaining to ester bond cleavage within the polyester backbone for certain enzymes (Joo et al., 2018). Quantitative methods help us understand the extent of the breakdown.

2.8.2.1 Weight loss

A commonly used method to determine PET hydrolysis is weight loss. This is a simple quantitative method, as the name suggests, where the weight loss of a PET sample following a breakdown process is measured. As the depolymerization progresses, the PET polymer is slowly broken down into oligomeric, dimer and monomer forms that are soluble in the liquid phase. This can be quantified with weight loss. With this method, the hydrolysis products cannot be determined. More sophisticated methods like spectroscopy and HPLC are required to quantify the breakdown products.

2.8.2.2 Spectrophotometric analysis

The most common quantitative spectrophotometric analysis method to determine esterase activity is the ester hydrolysis of p-nitrophenyl acyl esters, of which pNPA and pNPB are widely used. The esterase activity of cutinase is determined by the extent of para-nitrophenol (pNP) generation, which can be spectrophotometrically quantified at 410 nm (Jabloune et al., 2020). At pH 6.0 and below, pNP predominantly absorbs at 317 nm, due to the abundance of its protonated form. While, at pH 10.0 or above, the majority of absorption occurs at 401 nm because the equilibrium is shifted to its anionic form. The absorption of pNP at neutral pH is more temperature dependent at 410 nm (Syedd-León et al., 2020). In a study, after engineering Cbotu_EstA by reducing the interfacial activation, there was a 7-fold increase in activity towards PET. There was no notable change in the reaction rate, turnover number, and catalytic efficiency towards pNPB (Biundo et al., 2018). This proved that a direct correlation could not be made between the activities towards pNPB and PET since both substrates have different intrinsic properties. This activity incongruence proves that the pNPB-hydrolyzing assay should not be the standard for gauging PET-hydrolyzing activity. PET hydrolysis should ideally result in two monomers, TPA and EG. TPA has its absorption maxima at 240 nm. Using this principle, the fluorometric estimation of TPA can correlate to the extent of biological depolymerization (Alisch et al., 2004; Wei et al., 2012). In most hydrolysis reactions, breakdown products other than TPA are generated contain the aromatic moiety as well. Hence, this method is not specific to TPA (Zhong-Johnson et al., 2021) but rather the total breakdown products containing aromatic rings.

In addition to colorimetric detection, Wei et al. (2012), developed a fluorimetric assay for polyester hydrolysis by detection of oxidized TPA. Fe(II)-EDTA complex in a basic buffer oxidizes TPA to 2-hydroxyterephthalic acid which gets excited at 328 nm and emits at 421 nm. This method specifically detects TPA among the various breakdown products generated during biological

depolymerization. Using the same principle of TPA oxidation, Nimchua et al. (2007), developed a method that generated hydroxyterephthalic acid in the presence of hydrogen peroxide at 90 °C. The fluorescence was measured at 425 nm. A slight variation of this protocol uses an excitation wavelength of 315 nm and an emission wavelength of 425 nm (O'Neill & Cavaco-Paulo, 2004; Silva et al., 2011). A thorough protocol-oriented flow-through of this assay has been elaborated by the same group (Pfaff et al., 2021). Liu et al. (2023), devised a novel high-throughput screening technique using PET as the substrate. Here, a combination of fluorescein dilaurate embedded in PET and fluorescent-protein label was used to screen *Is*PETase and its generated mutants for increase PETase activity. The reaction was a co-hydrolysis of PET and fluorescein dilaurate. This was the first proof-of-concept assay that uses an aliphatic-aromatic polymer of petrochemical origin like PET as a substrate for an enzyme assay.

The turbid nature of PET nanoparticles in an aqueous solvent was exploited for a turbidimetric analysis. The reaction system consisted of PET nanoparticles suspended in buffer with 2% agarose and the enzyme added to commence the reaction. A solidifying agent like agarose held the PET nanoparticles suspended throughout the buffer and not settle down. The decrease in turbidity (observed at 600nm) was proportional to the incubation time with the enzyme (Wei et al., 2014). Another gelling agent, like polyacrylamide was used for this method (Belisário-Ferrari et al., 2019).

The performance of polyester hydrolases was assessed by impedance spectroscopy (Frank et al., 2022). Impedance is the measure of opposition to electric flow (measured in ohms). A change in the dielectric potential of PET was used to evaluate PETase performances. In this non-invasive assessment method, a change in the dielectric potential of PET was used. This approach has the potential to be used to evaluate other plastic hydrolases as well.

2.8.2.3 High-Performance Liquid Chromatography (HPLC)

One of the most useful methods for quantifying PET hydrolysis products is HPLC. It is generally used to determine the PET hydrolysis activity by determining and quantifying the breakdown products. This method depends on several factors like the extraction protocol, the efficiency of the extraction protocol, and the sensitivity of the column and detector. The column that is usually used for the analysis of PET hydrolysis products is a C18 column with a UV detector. The same principle of aromatic absorbance in the UV range (see Section 5.2.2) is used to detect the monomers TPA, MHET, and BHET. Due to the absence of an aromatic ring in ethylene glycol, it cannot be detected with the same protocol. Some of the HPLC columns and detectors reported in PET depolymerization literature are given in Table 2.5. Even the initial rate of PET hydrolysis can be assessed with a combination of a plate-reader assay and RP-HPLC (Arnling Bååth et al., 2020). Ronkvist et al. (2009) demonstrated the correlation between the TPA detected by HPLC and the weight loss of lcPET when treated with HiC, confirming the HPLC protocol used.

Table 2.5 HPLC columns and detectors used in analysis of breakdown products in supernatant post PET biological depolymerization

PET type	Column & Detector	Products observed	Ref.
lcPET ^a , hcPET ^b , BHET ^c , MHET ^d	Cosmosil 5C18-AR-II guard column (4.6 × 10 mm) and a Cosmosil 5C18-AR-II analytical column (4.6 × 250 mm) and detected at 254 nm	TPA ^e , MHET, BHET	Yoshida et al. 2016
Commercial PET film (UBIGEO, Korea) & BHET	Eclipse Plus-C18 column and detected at 260 nm	TPA, MHET, BHET	Joo et al. 2018
BHET	Diamonsil-C18 column with a UV detector (254 nm)	TPA, MHET, BHET	Qiu et al. 2020
Cyclic PET trimer	Chromsep Microsorb 300-5 C18 column and detected at 241 nm	TPA, MHET, BHET	Billig et al. 2010
PET fibres	RP-C18 column and detected at 240 nm	TPA, BHET	Zhang et al. 2013

Footnotes:

^a lcPET: Low crystalline polyethylene terephthalic acid

^b hcPET: High crystalline polyethylene terephthalic acid

^c BHET: Bis(2-hydroxyethyl) terephthalic acid

^d MHET: Mono-(2-hydroxyethyl) terephthalic acid

^e TPA: Terephthalic acid

2.8.2.4 Gel Permeation Chromatography (GPC)

GPC is a type of size exclusion chromatography that separates sample components by size. The elution time of the standards of the expected products of breakdown can be used to separate the components in the hydrolyzed sample. Quantification of these purer components will be done by the HPLC method. Yoshida et al. (2016) dissolved PET in HFIP and used the HFIP-803 gel column to study the elution profiles of *Ideonella sakaiensis*-mediated biological depolymerization.

2.9 Induction of cutinase

Cutinase production can be enhanced through induction mechanisms as described below. Cutin is the insoluble, waxy polymer part of the cuticle, a plant structural component. The cuticle acts as a protective layer that protects the plant from pathogenic infections. Cutin consists of ω -hydroxy fatty acids, dihydroxy palmitic acids, saturated and unsaturated fatty acids (Alves Macedo & Fontes Pio, 2005). These monomers are linked to each other via ester bonds, which are similar to the bonds present between PET monomers. It has fewer phenolic groups than suberin, its structural and functional counterpart in potatoes (Nawrath, 2002). Cutinases are enzymes that can hydrolytically cleave the ester bonds in cutin. Hence these enzymes are considered for hydrolysis of PET to its monomeric components. Plant pathogens produce cutinases to disturb the structural integrity in order to elicit a disease (Köller et al., 2011). The following sections describe the molecular mechanism of cutinase induction and the factors affecting its gene expression.

2.9.1 Molecular mechanism of cutinase induction

Induction of cutinase begins when the plant pathogen encounters cutin in the cuticle. Most inducible enzymes have a basal-level expression (Köller et al., 2011). This allows some cleavage of cutin, which results in a few free monomers. These monomers are recognized and transported across the membrane via active transporters. This in turn triggers the production of cAMP and protein kinases which are responsible for the activation of gene expression for inducible enzymes like cutinases (Carvalho et al., 1999; Woloshuk & Kolattukudy, 1986). Cutinase induction utilizes transcription factors like zinc-fingers and helix-turn-helix (Podila et al., 1988). Most cutinase encoding genes contain signal peptides that allow the enzyme to be transported across the membrane into the extracellular environment. This process creates a positive feedback loop that

ultimately leads to the increased production of cutinase (Köller et al., 2011) which has been illustrated in a schematic in Figure 2.6.

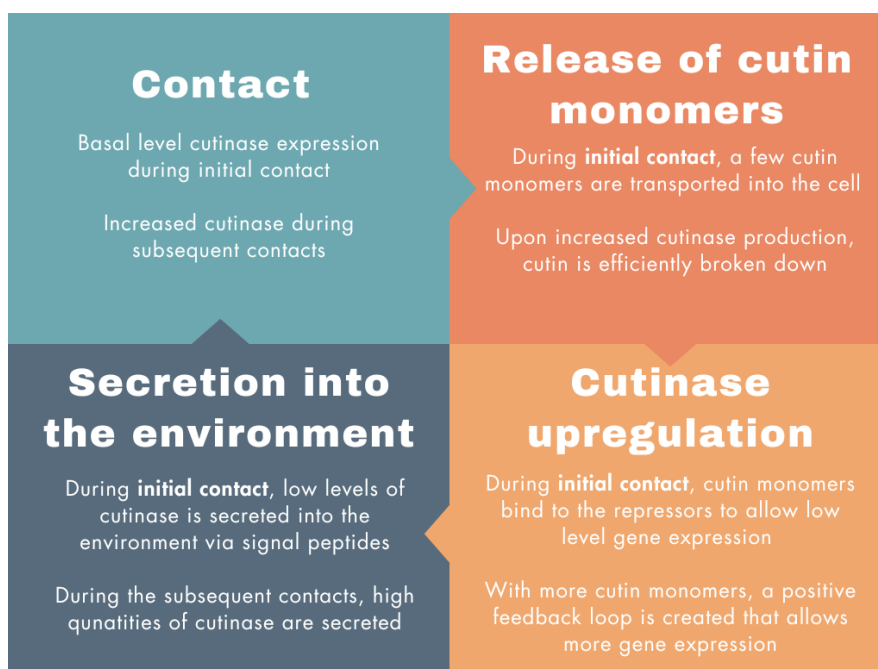


Figure 2.6 Schematic of the positive feedback loop involved in cutinase induction during the initial and subsequent contacts with cutin

2.9.2 Factors affecting cutinase induction

Most inducible enzymes are affected by inducers and carbon sources. As described earlier, cutin is a naturally occurring polymer that consists of various fatty acids of varying saturation. This means that cutin composition varies between genera and can sometimes even vary between cultivars. Studies have reported variations in monomeric constituents between species and cultivars of certain fruits like apples (Arrieta-Baez et al., 2020). The monomers, 10,16-dihydroxyhexadecanoic acid and 9,10,18-trihydroxyoctadecanoic acid, occur in nature only as components of cutin (Podila et al., 1988). These monomers widely present in apple and tomato cutin and are important in inducers of cutinase in *Thermobifida* spp (Arrieta-Baez et al., 2020;

Osman et al., 1999). Similarly, watermelon cutin has been demonstrated to induce cutinase production in *Streptomyces* spp (Chaudhari & Singhal, 2015; Sales et al., 2020).

In addition to the monomers, carbon sources have been shown to have an effect cutinase production. Most inducible enzymes are repressed in the presence of glucose due to the limited availability of cyclic AMP (cAMP), which is necessary in the induction process (Brückner & Titgemeyer, 2002). Trace amounts of glucose can cause catabolite repression. Meanwhile, an increased production of cutinase was observed under batch formation conditions, using sodium acetate and a combination of sodium lactate and sodium butyrate (He et al., 2009). The optimization of the factors affecting cutinase induction for enhanced production of cutinase or PET-hydrolyzing enzyme is specific for the microorganism.

2.10 Mining more cutinases using bioinformatic tools

Extensive bioinformatic efforts have led to the identification of numerous cutinase genes across diverse species. For instance, a study utilizing BLAST analysis with the *Fusarium solani* cutinase as a query identified 15,848 non-redundant amino acid sequences. After filtering for sequences of appropriate length, 1,243 sequences were subjected to phylogenetic analysis, resulting in 211 distinct clusters. From these, 43 cutinases were selected for recombinant expression and further molecular studies (Bhunja et al., 2018). *T. fusca* YX has two previously identified cutinases, tfcut1 and tfcut2 (tfu_1823), of which TfCut2 has been extensively studied. A similar approach to studying cutinases like in Bhunia et al., could benefit the scientific community in understanding the workings of the cutinases as PHEs. Additionally, bioinformatic analyses of 69 cutinases have revealed different types of cutinases, viz., type I, IIa and IIb (Joo et al., 2018). *Thermobifida fusca* YX was originally isolated as a plant pathogen and since then has been studied for its series of lytic enzymes. The actinomycete has been extensively studied for its cellulases. Reports include

from simple characterization to metabolic modelling using metabolic flux analysis and proteomic analysis (Adav et al., 2011; Lykidis et al., 2007; Stutzenberger & Kahler, 1986). Most previous reports determined the presence of these lytic enzymes by means of isolation. Lykidis et al. sequenced the entire genome of *Thermobifida fusca* YX and studied the genes upregulated and downregulated in cellulose depolymerization (Lykidis et al., 2007). However, with the genome sequenced, the presence of more lytic enzymes, regulatory factors and potential interacting proteins can be determined using bioinformatic tools.

In addition to the discovery of novel PHEs, the development of advanced bioinformatics tools has made it easy to predict de novo structures (Hegedűs et al., 2021; Kim et al., 2004), understand the interactions within the secondary structures (Donald et al., 2011; Kumar & Bansal, 1998), potential ion interactions, and molecular docking with relevant substrates (Y. Liu et al., 2022). Tools like the Robetta web server (Kim et al., 2004) (<https://robetta.bakerlab.org/>), and AlphaFold2 (Hegedűs et al., 2021) are crucial in de novo tertiary structure prediction which use already predicted structures of similar cutinases. The whole genome sequence of *T. fusca* YX has not been used for any other analyses yet. Although, partially annotated, the data provided with the sequence of the whole genome could provide insight into the presence of more PHEs or PET-modifying enzymes, which has been explored in Chapter 5.

2.11 Summary

Polyethylene terephthalic acid (PET) is a water-insoluble, chemically inert polymer that has been polluting the environment for decades. Despite our current recycling technologies and the future plans for recycling of PET, there will be a still vast amount of PET littered in the environment. Biological depolymerization has been catching up to the other degradative methods currently in

use. However, the increase in the efficiency of PET-hydrolyzing enzymes has only been through its engineering. As mentioned earlier, this results in challenges downstream for environmental applications. This study aims to focus on understanding all the aspects included in PET biological depolymerization, viz. effects of physicochemical factors on PET, optimizing the bacterial growth medium for the maximum production of PET-hydrolyzing enzymes and finally taking a deeper dive into the genome by mining potential enzymes present within the bacterial chassis, *Thermobifida fusca* YX.

References

- Adav SS, Ng CS, Sze SK (2011) iTRAQ-based quantitative proteomic analysis of *Thermobifida fusca* reveals metabolic pathways of cellulose utilization. *J Proteomics* 74:2112–2122. <https://doi.org/10.1016/J.JPROT.2011.05.038>
- Alisch M, Feuerhack A, Müller H, et al (2004) Biocatalytic modification of polyethylene terephthalate fibres by esterases from actinomycete isolates. *Biocatal Biotransformation* 22:347–351. <https://doi.org/10.1080/10242420400025877>
- Alves Macedo G, Fontes Pio T (2005) A Rapid Screening Method For Cutinase Producing Microorganisms. *Brazilian Journal of Microbiology* 36:388–394
- Araújo R, Silva C, O'Neill A, et al (2007) Tailoring cutinase activity towards polyethylene terephthalate and polyamide 6,6 fibers. *J Biotechnol* 128:849–857. <https://doi.org/10.1016/j.jbiotec.2006.12.028>
- Arnling Bååth J, Borch K, Westh P (2020) A suspension-based assay and comparative detection methods for characterization of polyethylene terephthalate hydrolases. *Anal Biochem* 607:. <https://doi.org/10.1016/j.ab.2020.113873>
- Arrieta-Baez D, Flores M de JP, Méndez-Méndez JV, et al (2020) Structural Studies of the Cutin from Two Apple Varieties: Golden Delicious and Red Delicious (*Malus domestica*). *Molecules* 2020, Vol 25, Page 5955 25:5955. <https://doi.org/10.3390/MOLECULES25245955>
- Austin HP, Allen MD, Donohoe BS, et al (2018) Characterization and engineering of a plastic-degrading aromatic polyesterase. *Proc Natl Acad Sci U S A* 115:E4350–E4357. <https://doi.org/10.1073/pnas.1718804115>
- Baker PJ, Poultney C, Liu Z, et al (2012) Identification and comparison of cutinases for synthetic polyester depolymerization. *Appl Microbiol Biotechnol* 93:229–240. <https://doi.org/10.1007/s00253-011-3402-4>
- Barth M, Honak A, Oeser T, et al (2016) A dual enzyme system composed of a polyester hydrolase and a carboxylesterase enhances the biocatalytic depolymerization of polyethylene terephthalate films. *Biotechnol J* 11:1082–1087. <https://doi.org/10.1002/biot.201600008>
- Belisário-Ferrari MR, Wei R, Schneider T, et al (2019) Fast Turbidimetric Assay for Analyzing the Enzymatic Hydrolysis of Polyethylene Terephthalate Model Substrates. *Biotechnol J* 14:. <https://doi.org/10.1002/biot.201800272>
- Bhunia RK, Showman LJ, Jose A, Nikolau BJ (2018) Combined use of cutinase and high-resolution mass-spectrometry to query the molecular architecture of cutin. *Plant Methods* 14:117. <https://doi.org/10.1186/S13007-018-0384-6>
- Billig S, Oeser T, Birkemeyer C, Zimmermann W (2010) Hydrolysis of cyclic poly(ethylene terephthalate) trimers by a carboxylesterase from *Thermobifida fusca* KW3. *Appl Microbiol Biotechnol* 87:1753–1764. <https://doi.org/10.1007/s00253-010-2635-y>
- Biundo A, Reich J, Ribitsch D, Guebitz GM (2018) Synergistic effect of mutagenesis and truncation to improve a polyesterase from *Clostridium botulinum* for polyester hydrolysis. *Sci Rep* 8:. <https://doi.org/10.1038/s41598-018-21825-9>
- BlueWeave Consulting & Research Pvt Ltd (2022) Global Polyethylene Terephthalate (PET) Resin Market to Boost in Coming Years – Projected to Reach Worth 114.7 Million Tons in 2028 | BlueWeave Consulting
- Bollinger A, Thies S, Knieps-Grünhagen E, et al (2020) A Novel Polyester Hydrolase From the Marine Bacterium *Pseudomonas aestusnigri* – Structural and Functional Insights. *Front Microbiol* 11:. <https://doi.org/10.3389/fmicb.2020.00114>
- Brott S, Pfaff L, Schuricht J, et al (2022) Engineering and evaluation of thermostable IsPETase variants for PET depolymerization. *Eng Life Sci* 22:192–203. <https://doi.org/10.1002/ELSC.202100105>
- Brückner R, Titgemeyer F (2002) Carbon catabolite repression in bacteria: choice of the carbon source and autoregulatory limitation of sugar utilization. *FEMS Microbiol Lett* 209:141–148. <https://doi.org/10.1111/J.1574-6968.2002.TB11123.X>
- Brueckner T, Eberl A, Heumann S, et al (2008) Enzymatic and chemical hydrolysis of polyethylene terephthalate fabrics. *J Polym Sci A Polym Chem* 46:6435–6443. <https://doi.org/10.1002/pola.22952>
- Carniel A, Valoni É, Nicomedes J, et al (2017) Lipase from *Candida antarctica* (CALB) and cutinase from *Humicola insolens* act synergistically for PET hydrolysis to terephthalic acid. *Process Biochemistry* 59:84–90. <https://doi.org/10.1016/j.procbio.2016.07.023>
- Carr CM, Keller MB, Paul B, et al (2023) Purification and biochemical characterization of SM14est, a PET-hydrolyzing enzyme from the marine sponge-derived *Streptomyces* sp. SM14. *Front Microbiol* 14:1445. <https://doi.org/10.3389/FMICB.2023.1170880/BIBTEX>

- Carvalho CML, Aires-Barros MR, Cabral JMS (1999) Cutinase: From Molecular Level to Bioprocess Development
- Chamas A, Moon H, Zheng J, et al (2020) Depolymerization Rates of Plastics in the Environment. *ACS Sustain Chem Eng* 8:3494–3511. <https://doi.org/10.1021/acssuschemeng.9b06635>
- Chan K, Kawai M, Yamake M, Zinchenko A (2023) Copper Ion Removal Using a Waste-Plastic-Derived Hydrogel Adsorbent Prepared via Microwave-Assisted PET Aminolysis. *Gels* 9:874. <https://doi.org/10.3390/GELS9110874/S1>
- Chaudhari SA, Singhal RS (2015) Cutin from watermelon peels: A novel inducer for cutinase production and its physicochemical characterization. *Int J Biol Macromol* 79:398–404. <https://doi.org/10.1016/j.ijbiomac.2015.05.006>
- Chen CC, Han X, Ko TP, et al (2018) Structural studies reveal the molecular mechanism of PETase. *FEBS Journal* 285:3717–3723
- Chen S, Su L, Billig S, et al (2010) Biochemical characterization of the cutinases from *Thermobifida fusca*. *J Mol Catal B Enzym* 63:121–127. <https://doi.org/10.1016/j.molcatb.2010.01.001>
- Chen S, Tong X, Woodard RW, et al (2008) Identification and characterization of bacterial cutinase. *Journal of Biological Chemistry* 283:25854–25862. <https://doi.org/10.1074/jbc.M800848200>
- Chen Z, Wang Y, Cheng Y, et al (2020) Efficient biological depolymerization of highly crystallized polyethylene terephthalate through cell surface display of bacterial PETase. *Science of the Total Environment* 709:. <https://doi.org/10.1016/j.scitotenv.2019.136138>
- Cheng X, Dong S, Chen D, et al (2020) Potential of esterase DmtH in transforming plastic additive dimethyl terephthalate to less toxic mono-methyl terephthalate. *Ecotoxicol Environ Saf* 187:. <https://doi.org/10.1016/j.ecoenv.2019.109848>
- Contesini FJ, Lopes DB, MacEdo GA, et al (2010) *Aspergillus* sp. lipase: Potential biocatalyst for industrial use. *J Mol Catal B Enzym* 67:163–171
- Cui Y, Chen Y, Liu X, et al (2021) Computational Redesign of a PETase for Plastic Biological depolymerization under Ambient Condition by the GRAPE Strategy. *ACS Catal* 11:1340–1350. <https://doi.org/10.1021/acscatal.0c05126>
- da Costa AM, de Oliveira Lopes VR, Vidal L, et al (2020) Poly(ethylene terephthalate) (PET) depolymerization by *Yarrowia lipolytica*: Investigations on cell growth, enzyme production and monomers consumption. *Process Biochemistry* 95:81–90. <https://doi.org/10.1016/j.procbio.2020.04.001>
- Dai L, Zhou N, Lv Y, et al (2022) Pyrolysis technology for plastic waste recycling: A state-of-the-art review. *Prog Energy Combust Sci* 93:101021. <https://doi.org/10.1016/J.PECS.2022.101021>
- Dantzig AH, Zuckerman SH, Andonov-Roland MM (1986) Isolation of a *Fusarium solani* Mutant Reduced in Cutinase Activity and Virulence
- Dherbécourt J, Falentin H, Canaan S, Thierry A (2008) A genomic search approach to identify esterases in *Propionibacterium freudenreichii* involved in the formation of flavour in Emmental cheese. *Microb Cell Fact* 7:. <https://doi.org/10.1186/1475-2859-7-16>
- Dickman MB, Patil SS (1986) Cutinase deficient mutants of *Colletotrichum gloeosporioides* are nonpathogenic to papaya fruit. *Physiol Mol Plant Pathol* 28:235–242. [https://doi.org/10.1016/S0048-4059\(86\)80067-4](https://doi.org/10.1016/S0048-4059(86)80067-4)
- Dimarogona M, Nikolaivits E, Kanelli M, et al (2015) Structural and functional studies of a *Fusarium oxysporum* cutinase with polyethylene terephthalate modification potential. *Biochim Biophys Acta Gen Subj* 1850:2308–2317. <https://doi.org/10.1016/j.bbagen.2015.08.009>
- Donald JE, Kulp DW, DeGrado WF (2011) Salt bridges: Geometrically specific, designable interactions. *Proteins: Structure, Function, and Bioinformatics* 79:898–915. <https://doi.org/10.1002/PROT.22927>
- Dong Q, Yuan S, Wu L, et al (2020) Structure-guided engineering of a *Thermobifida fusca* cutinase for enhanced hydrolysis on natural polyester substrate. *Bioresour Bioprocess* 7:. <https://doi.org/10.1186/s40643-020-00324-8>
- Erickson E, Gado JE, Avilán L, et al (2022) Sourcing thermotolerant poly(ethylene terephthalate) hydrolase scaffolds from natural diversity. *Nature Communications* 2022 13:1 13:1–15. <https://doi.org/10.1038/s41467-022-35237-x>
- Falkenstein P, Gräsing D, Bielytskyi P, et al (2020) UV Pretreatment Impairs the Enzymatic Depolymerization of Polyethylene Terephthalate. *Front Microbiol* 11:. <https://doi.org/10.3389/fmicb.2020.00689>
- Fecker T, Galaz-Davison P, Engelberger F, et al (2018) Active Site Flexibility as a Hallmark for Efficient PET Depolymerization by *I. sakaiensis* PETase. *Biophys J* 114:1302–1312. <https://doi.org/10.1016/j.bpj.2018.02.005>
- Frank R, Krinke D, Sonnendecker C, et al (2022) Real-Time Noninvasive Analysis of Biocatalytic PET Depolymerization. *ACS Catal* 12:25–35. <https://doi.org/10.1021/acscatal.1c03963>
- Gamerith C, Gajda M, Ortner A, et al (2017) Enzymatic hydrolysis of poly(ethyleneterephthalate) used for and analysed by pore modification of track-etched membranes. *N Biotechnol* 39:42–50. <https://doi.org/10.1016/j.nbt.2017.06.007>
- Geyer R, Jambeck JR, Law KL (2017) Production, use, and fate of all plastics ever made

- Ghodke VM, Puneekar NS (2022) Environmental role of aromatic carboxylesterases. *Environ Microbiol* 24:2657–2668
- Giangeri G, Morlino MS, De Bernardini N, et al (2022) Preliminary investigation of microorganisms potentially involved in microplastics depolymerization using an integrated metagenomic and biochemical approach. <https://doi.org/10.1016/j.scitotenv.2022.157017>
- Giltner CL, Van Schaik EJ, Audette GF, et al (2006) The *Pseudomonas aeruginosa* type IV pilin receptor binding domain functions as an adhesin for both biotic and abiotic surfaces. *Mol Microbiol* 59:1083–1096. <https://doi.org/10.1111/j.1365-2958.2005.05002.x>
- Haiko J, Westerlund-Wikström B (2013) The role of the bacterial flagellum in adhesion and virulence. *Biology (Basel)* 2:1242–1267
- Han M (2019) Depolymerization of PET Bottle via Methanolysis and Hydrolysis. In: *Recycling of Polyethylene Terephthalate Bottles*. Elsevier, pp 85–108
- Han X, Liu W, Huang JW, et al (2017) Structural insight into catalytic mechanism of PET hydrolase. *Nat Commun* 8:. <https://doi.org/10.1038/s41467-017-02255-z>
- He G, Huo G, Liu L, et al (2009) Enhanced cutinase production of *Thermobifida fusca* by a two-stage batch and fed-batch cultivation strategy. *Biotechnology and Bioengineering* 14:46–51. <https://doi.org/10.1007/S12257-008-0091-7/METRICS>
- Hegedűs T, Geisler M, Lukács G, Farkas B (2021) AlphaFold2 transmembrane protein structure prediction shines. *bioRxiv* 2021.08.21.457196. <https://doi.org/10.1101/2021.08.21.457196>
- Hurley CR, Leggett GJ (2009) Quantitative investigation of the photodepolymerization of polyethylene terephthalate film by friction force microscopy, contact-angle goniometry, and X-ray photoelectron spectroscopy. *ACS Appl Mater Interfaces* 1:1688–1697. <https://doi.org/10.1021/am900250q>
- Inderthal H, Tai SL, Harrison STL (2021) Non-Hydrolyzable Plastics – An Interdisciplinary Look at Plastic Bio-Oxidation. *Trends Biotechnol* 39:12–23
- Jabloune R, Khalil M, ben Moussa IE, et al (2020) Enzymatic Depolymerization of p-Nitrophenyl Esters, Polyethylene Terephthalate, Cutin, and Suberin by Sub1, a Suberinase Encoded by the Plant Pathogen *Streptomyces scabies*. *Microbes Environ* 35:. <https://doi.org/10.1264/jsme2.ME19086>
- Jarrett P, Benedict C v., Bell JP, et al (1984) Mechanism of the Biological depolymerization of Polycaprolactone. In: *Polymers as Biomaterials*. Springer US, Boston, MA, pp 181–192
- Joo S, Cho JJ, Seo H, et al (2018) Structural insight into molecular mechanism of poly(ethylene terephthalate) depolymerization. *Nat Commun* 9:. <https://doi.org/10.1038/s41467-018-02881-1>
- Kardas I, Lipp-Symonowicz B, Sztajnowski S (2011) The influence of enzymatic treatment on the surface modification of PET fibers. *J Appl Polym Sci* 119:3117–3126. <https://doi.org/10.1002/app.31724>
- Kato S, Sakai S, Hirai M, et al (2018) Long-term cultivation and metagenomics reveal ecophysiology of previously uncultivated thermophiles involved in biogeochemical nitrogen cycle. *Microbes Environ* 33:107–110. <https://doi.org/10.1264/jsme2.ME17165>
- Kawabata T, Oda M, Kawai F (2017) Mutational analysis of cutinase-like enzyme, Cut190, based on the 3D docking structure with model compounds of polyethylene terephthalate. *J Biosci Bioeng* 124:28–35. <https://doi.org/10.1016/j.jbiosc.2017.02.007>
- Kawai F, Oda M, Tamashiro T, et al (2014) A novel Ca²⁺-activated, thermostabilized polyesterase capable of hydrolyzing polyethylene terephthalate from *Saccharomonospora viridis* AHK190. *Appl Microbiol Biotechnol* 98:10053–10064. <https://doi.org/10.1007/s00253-014-5860-y>
- Kelly JJ, London MG, McCormick AR, et al (2021) Wastewater treatment alters microbial colonization of microplastics. *PLoS One* 16:. <https://doi.org/10.1371/journal.pone.0244443>
- Kenny ST, Runic JN, Kaminsky W, et al (2008) Up-cycling of PET (Polyethylene Terephthalate) to the biodegradable plastic PHA (Polyhydroxyalkanoate). *Environ Sci Technol* 42:7696–7701. <https://doi.org/10.1021/es801010e>
- Kim DE, Chivian D, Baker D (2004) Protein structure prediction and analysis using the Robetta server. *Nucleic Acids Res* 32:W526. <https://doi.org/10.1093/NAR/GKH468>
- Kleeberg I, Welzel K, VandenHeuvel J, et al (2005) Characterization of a new extracellular hydrolase from *Thermobifida fusca* degrading aliphatic-aromatic copolyesters. *Biomacromolecules* 6:262–270. <https://doi.org/10.1021/bm049582t>
- Köller W, Yao C, Trial F, Parker DM (2011) Role of cutinase in the invasion of plants. <https://doi.org/10.1139/b95-366> 73:1109–1118. <https://doi.org/10.1139/B95-366>
- Koshti R, Mehta L, Samarth N (2018) Biological Recycling of Polyethylene Terephthalate: A Mini-Review. *J Polym Environ* 26:3520–3529
- Kumar S, Bansal M (1998) Geometrical and sequence characteristics of α -helices in globular proteins. *Biophys J* 75:1935–1944. [https://doi.org/10.1016/S0006-3495\(98\)77634-9](https://doi.org/10.1016/S0006-3495(98)77634-9)

- Liu K, Xu Z, Zhao Z, et al (2023) A Dual Fluorescence Assay Enables High-Throughput Screening for Poly(ethylene terephthalate) Hydrolases. *ChemSusChem* 16:. <https://doi.org/10.1002/cssc.202202019>
- Liu Y, Yang X, Gan J, et al (2022) CB-Dock2: improved protein–ligand blind docking by integrating cavity detection, docking and homologous template fitting. *Nucleic Acids Res* 50:W159–W164. <https://doi.org/10.1093/NAR/GKAC394>
- Lopes DB, Fraga LP, Fleuri LF, Macedo GA (2011) Lipase and esterase-to what extent can this classification be applied accurately? *Lipases e esterases: como definir e classificar?* *Food Science and Technology* 31:603–611
- Lykidis A, Mavromatis K, Ivanova N, et al (2007) Genome sequence and analysis of the soil cellulolytic actinomycete *Thermobifida fusca* YX. *J Bacteriol* 189:2477–2486. <https://doi.org/10.1128/JB.01899-06/ASSET/3C6DE7B4-6AAB-49A7-84FF-5D5B95EC41EF/ASSETS/GRAPHIC/ZJB0060766130004.JPEG>
- Ma M, Wang L, Zhu H (2012) Enzymatic depolymerization of polyester-nanoparticles by lipases and adsorption of lipases on the polyester-nanoparticles. In: *Advanced Materials Research*. pp 2302–2307
- Maiti IB, Kolattukudy PE (1979) Prevention of Fungal Infection of Plants by Specific Inhibition of Cutinase. *Science* (1979) 205:507–508
- Malafatti-Picca L, Bucioli EC, de Barros Chaves MR, et al (2023) Fungal Screening for Potential PET Depolymerization. *Polymers (Basel)* 15:. <https://doi.org/10.3390/polym15061581>
- Martino P Di, Cafferini N, Joly B, Darfeuille-Michaud A (2003) *Klebsiella pneumoniae* type 3 pili facilitate adherence and biofilm formation on abiotic surfaces
- Maurya A, Bhattacharya A, Khare SK (2020) Enzymatic Remediation of Polyethylene Terephthalate (PET)–Based Polymers for Effective Management of Plastic Wastes: An Overview. *Front Bioeng Biotechnol* 8
- McMahon W, Birdsall HA, Johnson GR, Camilli CT (1959) PHYSICAL PROPERTIES EVALUATION OF COMPOUNDS AND MATERIALS Depolymerization Studies of Polyethylene Terephthalate. *UTC*
- Mecozzi M, Nisini L (2019) The differentiation of biodegradable and non-biodegradable polyethylene terephthalate (PET) samples by FTIR spectroscopy: A potential support for the structural differentiation of PET in environmental analysis. *Infrared Phys Technol* 101:119–126. <https://doi.org/10.1016/J.INFRARED.2019.06.008>
- Mehta A, Bodh U, Gupta R (2017) Fungal lipases: a review
- Miri S, Saini R, Davoodi SM, et al (2022) Biological depolymerization of microplastics: Better late than never. *Chemosphere* 286
- Molitor R, Bollinger A, Kubicki S, et al (2020) Agar plate-based screening methods for the identification of polyester hydrolysis by *Pseudomonas* species. *Microb Biotechnol* 13:274–284. <https://doi.org/10.1111/1751-7915.13418>
- Mueller RJ (2006) Biological depolymerization of synthetic polyesters-Enzymes as potential catalysts for polyester recycling. *Process Biochemistry* 41:2124–2128. <https://doi.org/10.1016/j.procbio.2006.05.018>
- Müller R-J, Kleeberg I, Deckwer W-D (2001) Biological depolymerization of polyesters containing aromatic constituents
- Müller RJ, Schrader H, Profe J, et al (2005) Enzymatic depolymerization of poly(ethylene terephthalate): Rapid hydrolyse using a hydrolase from *T. fusca*. *Macromol Rapid Commun* 26:1400–1405. <https://doi.org/10.1002/marc.200500410>
- Muringayil Joseph T, Azat S, Ahmadi Z, et al (2024) Polyethylene terephthalate (PET) recycling: A review. *Case Studies in Chemical and Environmental Engineering* 9:100673. <https://doi.org/10.1016/J.CSCEE.2024.100673>
- Nardini M, Dijkstra B (1999) α/β Hydrolase fold enzymes Nardini and Dijkstra 733. *Curr Opin Struct Biol* 9:732–737
- Nawrath C (2002) The Biopolymers Cutin and Suberin. *The Arabidopsis Book / American Society of Plant Biologists* 1:e0021. <https://doi.org/10.1199/TAB.0021>
- Nikolaivits E, Kanelli M, Dimarogona M, Topakas E (2018) A middle-aged enzyme still in its prime: Recent advances in the field of cutinases. *Catalysts* 8
- Nimchua T, Eveleigh DE, Sangwatanaroj U, Punnapayak H (2008) Screening of tropical fungi producing polyethylene terephthalate- hydrolyzing enzyme for fabric modification. *J Ind Microbiol Biotechnol* 35:843–850. <https://doi.org/10.1007/s10295-008-0356-3>
- Nimchua T, Punnapayak H, Zimmermann W (2007) Comparison of the hydrolysis of polyethylene terephthalate fibers by a hydrolase from *Fusarium oxysporum* LCH I and *Fusarium solani* f. sp. pisi. *Biotechnol J* 2:361–364. <https://doi.org/10.1002/biot.200600095>
- Nishida H, Tokiwa Y (1993) Distribution of Poly(p-hydroxybutyrate) and Poly(ϵ -caprolactone) Aerobic Degrading Microorganisms in Different Environments
- Numoto N, Kamiya N, Bekker GJ, et al (2018) Structural Dynamics of the PET-Degrading Cutinase-like Enzyme from *Saccharomonospora viridis* AHK190 in Substrate-Bound States Elucidates the Ca²⁺-Driven Catalytic Cycle. *Biochemistry* 57:5289–5300. <https://doi.org/10.1021/acs.biochem.8b00624>

- Olam M, Karaca H (2022) Characterization of products obtained of waste polyethylene terephthalate by pyrolysis. *Environ Prog Sustain Energy* 41:e13835. <https://doi.org/10.1002/EP.13835>
- O'Neill A, Cavaco-Paulo A (2004) Monitoring biotransformations in polyesters. *Biocatal Biotransformation* 22:353–356. <https://doi.org/10.1080/10242420400025760>
- Osman SF, Irwin P, Fett WF, et al (1999) Preparation, isolation, and characterization of cutin monomers and oligomers from tomato peels. *J Agric Food Chem* 47:799–802. <https://doi.org/10.1021/JF980693R/ASSET/IMAGES/LARGE/JF980693RF00003.JPEG>
- Palm GJ, Reisky L, Böttcher D, et al (2019) Structure of the plastic-degrading *Ideonella sakaiensis* MHETase bound to a substrate. *Nat Commun* 10:. <https://doi.org/10.1038/s41467-019-09326-3>
- Peña-García C, Martínez-Martínez M, Reyes-Duarte D, Ferrer M (2016) High Throughput Screening of Esterases, Lipases and Phospholipases in Mutant and Metagenomic Libraries: A Review. *Comb Chem High Throughput Screen* 19:605–615. <https://doi.org/10.2174/1386207319666151>
- Pereira P, Savage PE, Pester CW (2023) Neutral Hydrolysis of Post-Consumer Polyethylene Terephthalate Waste in Different Phases. *ACS Sustain Chem Eng* 11:7203–7209. https://doi.org/10.1021/ACSSUSCHEMENG.3C00946/ASSET/IMAGES/LARGE/SC3C00946_0006.JPEG
- Perz V, Baumschlager A, Bleymaier K, et al (2016) Hydrolysis of synthetic polyesters by *Clostridium botulinum* esterases; Hydrolysis of synthetic polyesters by *Clostridium botulinum* esterases. *Biotechnol Bioeng* 113:1024–1034. <https://doi.org/10.1002/bit.25874/abstract>
- Pfaff L, Breite D, Badenhorst CPS, et al (2021) Fluorimetric high-throughput screening method for polyester hydrolase activity using polyethylene terephthalate nanoparticles. In: *Methods in Enzymology*. Academic Press Inc., pp 253–270
- Ping LF, Chen XY, Yuan XL, et al (2017) Application and comparison in biosynthesis and biological depolymerization by *Fusarium solani* and *Aspergillus fumigatus* cutinases. *Int J Biol Macromol* 104:1238–1245. <https://doi.org/10.1016/j.ijbiomac.2017.06.118>
- Pinto A v., Ferreira P, Neves RPP, et al (2021) Reaction Mechanism of MHETase, a PET Degrading Enzyme. *ACS Catal* 11:10416–10428. <https://doi.org/10.1021/acscatal.1c02444>
- Pirillo V, Orlando M, Battaglia C, et al (2023) Efficient polyethylene terephthalate depolymerization at moderate temperature: a protein engineering study of LC-cutinase highlights the key role of residue 243. *FEBS J*. <https://doi.org/10.1111/FEBS.16736>
- PlasticsEurope (PEMRG) (2022) *Plastics - The Facts 2022*
- Podila GK, Dickman MB, Kolattukudy PE (1988) Transcriptional Activation of a Cutinase Gene in Isolated Fungal Nuclei by Plant Cutin Monomers. *Science* (1979) 242:922–925. <https://doi.org/10.1126/SCIENCE.242.4880.922>
- Purohit J, Chattopadhyay A, Teli B (2020) Metagenomic Exploration of Plastic Degrading Microbes for Biotechnological Application. *Curr Genomics* 21:253–270. <https://doi.org/10.2174/1389202921999200525155711>
- Puspitasari N, Tsai SL, Lee CK (2021) Fungal Hydrophobin RoIA Enhanced PETase Hydrolysis of Polyethylene Terephthalate. *Appl Biochem Biotechnol* 193:1284–1295. <https://doi.org/10.1007/s12010-020-03358-y>
- Pütz A (2006) Isolierung, Identifizierung und biochemische Charakterisierung Dialkylphthalat spaltender Esterasen
- Qi X, Yan W, Cao Z, et al (2022) Current advances in the biological depolymerization and bioconversion of polyethylene terephthalate. *Microorganisms* 10:. <https://doi.org/10.3390/microorganisms10010039>
- Qiu L, Yin X, Liu T, et al (2020) Biological depolymerization of bis(2-hydroxyethyl) terephthalate by a newly isolated *Enterobacter* sp. HY1 and characterization of its esterase properties. *J Basic Microbiol* 60:699–711. <https://doi.org/10.1002/jobm.202000053>
- Rajandas H, Parimannan S, Sathasivam K, et al (2012) A novel FTIR-ATR spectroscopy based technique for the estimation of low-density polyethylene biological depolymerization. *Polym Test* 31:1094–1099. <https://doi.org/10.1016/j.polymertesting.2012.07.015>
- Ribitsch D, Acero EH, Greimel K, et al (2012) A new esterase from *Thermobifida halotolerans* hydrolyses polyethylene terephthalate (PET) and polylactic acid (PLA). *Polymers (Basel)* 4:617–629. <https://doi.org/10.3390/polym4010617>
- Ronkvist ÅM, Xie W, Lu W, Gross RA (2009) Cutinase-Catalyzed hydrolysis of poly(ethylene terephthalate). *Macromolecules* 42:5128–5138. <https://doi.org/10.1021/ma9005318>
- Rosenberg M, Bayer EA, Delarea J, Rosenberg E (1982) Role of Thin Fimbriae in Adherence and Growth of *Acinetobacter calcoaceticus* RAG-1 on Hexadecane
- Roth C, Wei R, Oeser T, et al (2014) Structural and functional studies on a thermostable polyethylene terephthalate degrading hydrolase from *Thermobifida fusca*. *Appl Microbiol Biotechnol* 98:7815–7823. <https://doi.org/10.1007/s00253-014-5672-0>

- Sales JCS, de Castro AM, Ribeiro BD, Maria MA (2020) Supplementation of watermelon peels as an enhancer of lipase and esterase production by *Yarrowia lipolytica* in solid-state fermentation and their potential use as biocatalysts in poly(ethylene terephthalate) (PET) depolymerization reactions. *Biocatal Biotransformation* 38:457–468. <https://doi.org/10.1080/10242422.2020.1782387>
- Sharon M, Sharon C (2012) Studies on Biological depolymerization of Polyethylene terephthalate: A synthetic polymer. *J Microbiol Biotechnol Res* 2:248–257
- Shukla SR, Harad AM (2006) Aminolysis of polyethylene terephthalate waste. *Polym Degrad Stab* 91:1850–1854. <https://doi.org/10.1016/J.POLYMDEGRADSTAB.2005.11.005>
- Silva C, Da S, Silva N, et al (2011) Engineered *Thermobifida fusca* cutinase with increased activity on polyester substrates. *Biotechnol J* 6:1230–1239. <https://doi.org/10.1002/biot.201000391>
- Singh AK, Bedi R, Kaith BS (2021) Composite materials based on recycled polyethylene terephthalate and their properties – A comprehensive review. *Compos B Eng* 219:108928. <https://doi.org/10.1016/J.COMPOSITESB.2021.108928>
- Singh B, Sharma N (2008) Mechanistic implications of plastic depolymerization. *Polym Degrad Stab* 93:561–584
- Son HF, Cho IJ, Joo S, et al (2019) Rational Protein Engineering of Thermo-Stable PETase from *Ideonella sakaiensis* for Highly Efficient PET Depolymerization. <https://doi.org/10.1021/acscatal.9b00568>
- Sonnendecker C, Oeser J, Richter PK, et al (2022) Low Carbon Footprint Recycling of Post-Consumer PET Plastic with a Metagenomic Polyester Hydrolase. *ChemSusChem* 15:. <https://doi.org/10.1002/CSSC.202101062>
- Stanica-Ezeanu D, Matei D (2021) Natural depolymerization of waste poly(ethylene terephthalate) by neutral hydrolysis in marine water. *Scientific Reports* 2021 11:1 11:1–7. <https://doi.org/10.1038/s41598-021-83659-2>
- Stutzenberger F, Kahler G (1986) Cellulase biosynthesis during depolymerization of cellulose derivatives by *Thermomonospora curvata*. *Journal of Applied Bacteriology* 61:225–233
- Sulaiman S, Yamato S, Kanaya E, et al (2012) Isolation of a novel cutinase homolog with polyethylene terephthalate-degrading activity from leaf-branch compost by using a metagenomic approach. *Appl Environ Microbiol* 78:1556–1562. <https://doi.org/10.1128/AEM.06725-11>
- Syed-León R, Sandoval-Barrantes M, Trimiño-Vásquez H, et al (2020) Revisiting the fundamentals of p-nitrophenol analysis for its application in the quantification of lipases activity. A graphical update. *Uniciencia* 34:31–42. <https://doi.org/10.15359/RU.34-2.2>
- Thomsen TB, Almdal K, Meyer AS (2023) Significance of poly(ethylene terephthalate) (PET) substrate crystallinity on enzymatic depolymerization. *N Biotechnol* 78:162–172. <https://doi.org/10.1016/J.NBT.2023.11.001>
- Torena P, Alvarez-Cuenca M, Reza M (2021) Biological depolymerization of polyethylene terephthalate microplastics by bacterial communities from activated sludge. *Can J Chem Eng* 99:S69–S82. <https://doi.org/10.1002/CJCE.24015>
- Tournier V, Topham CM, Gilles A, et al (2020) An engineered PET depolymerase to break down and recycle plastic bottles. *Nature* 580:216–219. <https://doi.org/10.1038/s41586-020-2149-4>
- Walker TR (2021) (Micro)plastics and the UN Sustainable Development Goals. Elsevier B.V.
- Wang N, Guan F, Lv X, et al (2020) Enhancing secretion of polyethylene terephthalate hydrolase PETase in *Bacillus subtilis* WB600 mediated by the SPamy signal peptide. *Lett Appl Microbiol* 71:235–241. <https://doi.org/10.1111/lam.13312>
- Wang X, Lu D, Jönsson LJ, Hong F (2008) Preparation of a PET-hydrolyzing lipase from *Aspergillus oryzae* by the addition of bis(2-hydroxyethyl) terephthalate to the culture medium and enzymatic modification of PET fabrics. *Eng Life Sci* 8:268–276. <https://doi.org/10.1002/elsc.200700058>
- Webb HK, Arnott J, Crawford RJ, Ivanova EP (2012) Plastic Depolymerization and Its Environmental Implications with Special Reference to Poly(ethylene terephthalate). *Polymers* 2013, Vol 5, Pages 1-18 5:1–18. <https://doi.org/10.3390/POLYM5010001>
- Wei R, Oeser T, Barth M, et al (2014) Turbidimetric analysis of the enzymatic hydrolysis of polyethylene terephthalate nanoparticles. *J Mol Catal B Enzym* 103:72–78. <https://doi.org/10.1016/j.molcatb.2013.08.010>
- Wei R, Oeser T, Billig S, Zimmermann W (2012) A high-throughput assay for enzymatic polyester hydrolysis activity by fluorimetric detection. *Biotechnol J* 7:1517–1521. <https://doi.org/10.1002/biot.201200119>
- Woloshuk CP, Kolattukudy PE (1986) Mechanism by which contact with plant cuticle triggers cutinase gene expression in the spores of *Fusarium solani* f. sp. pisi. *Proceedings of the National Academy of Sciences* 83:1704–1708. <https://doi.org/10.1073/PNAS.83.6.1704>
- Wright RJ, Bosch R, Langille MGI, et al (2021) A multi-OMIC characterisation of biological depolymerization and microbial community succession within the PET plastisphere. *Microbiome* 2021 9:1 9:1–22. <https://doi.org/10.1186/S40168-021-01054-5>

- Xi X, Ni K, Hao H, et al (2021) Secretory expression in *Bacillus subtilis* and biochemical characterization of a highly thermostable polyethylene terephthalate hydrolase from bacterium HR29. *Enzyme Microb Technol* 143:. <https://doi.org/10.1016/j.enzmictec.2020.109715>
- Xin J, Zhang Q, Huang J, et al (2021) Progress in the catalytic glycolysis of polyethylene terephthalate. *J Environ Manage* 296:113267. <https://doi.org/10.1016/J.JENVMAN.2021.113267>
- Yoshida S, Hiraga K, Takehana T, et al (2016) A bacterium that degrades and assimilates poly(ethylene terephthalate). *Science* (1979) 351:1196–1199
- Zhang Y, Chen S, Xu M, et al (2010) Characterization of *thermobifida fusca* cutinase-carbohydrate-binding module fusion proteins and their potential application in bioscouring. *Appl Environ Microbiol* 76:6870–6876. <https://doi.org/10.1128/AEM.00896-10>
- Zhang Y, Wang L, Chen J, Wu J (2013) Enhanced activity toward PET by site-directed mutagenesis of *Thermobifida fusca* cutinase-CBM fusion protein. *Carbohydr Polym* 97:124–129. <https://doi.org/10.1016/j.carbpol.2013.04.042>
- Zhong-Johnson EZL, Voigt CA, Sinskey AJ (2021) An absorbance method for analysis of enzymatic depolymerization kinetics of poly(ethylene terephthalate) films. *Sci Rep* 11:. <https://doi.org/10.1038/s41598-020-79031-5>

Chapter 3

Objective 1

Pretreatment of polyethylene terephthalate (PET) using physicochemical methods and their effects on biological depolymerization

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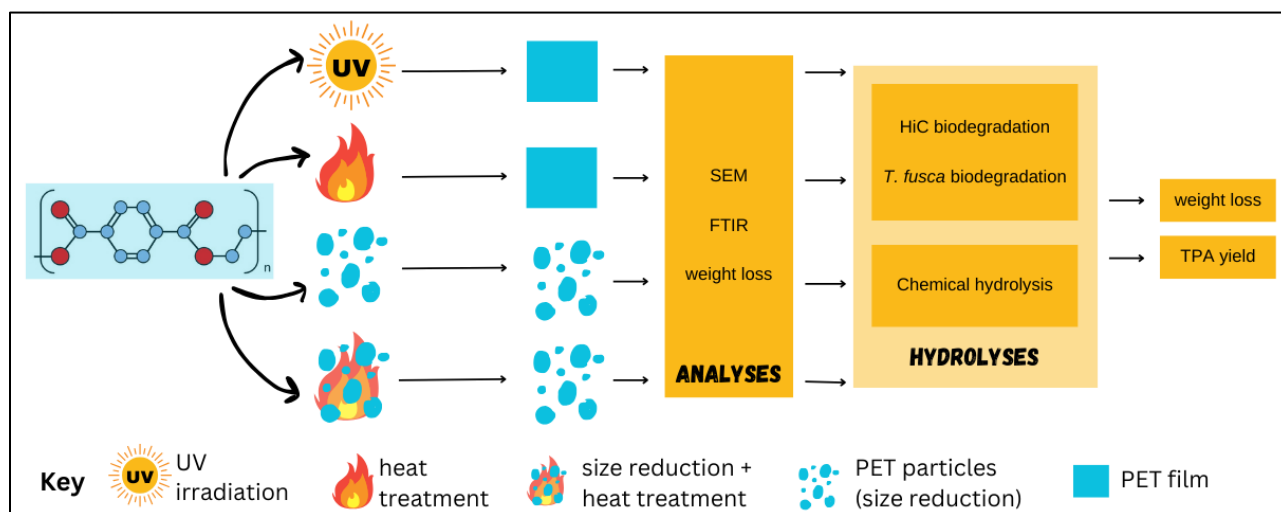
Abstract

Biological depolymerization has gained importance as a potential solution to alleviate pollution. This study involved the physicochemical transformations of polyethylene terephthalate (PET) by pretreatment to enhance biological depolymerization efficiency. PET films were subjected to pretreatments, including UV irradiation, thermal oxidation, size reduction, and thermal oxidation of size-reduced particles. These pretreated samples were then biodegraded using either an immobilized cutinase or whole-cell broths of *Thermobifida fusca* YX. The physicochemical effects of these treatments were evaluated through techniques such as attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy, scanning electron microscopy (SEM), and weight-loss analysis. The findings revealed that UV irradiation caused repetitive cycles of photo-oxidation over three hours, which impaired biological depolymerization due to increased crystallinity. Conversely, thermal oxidation improved biological depolymerization up to an optimal temperature of 80 °C. Higher temperatures were favorable for whole-cell biological depolymerization, while slightly lower temperatures (70–80 °C) were optimal for enzyme-mediated processes. A similar trend was observed for thermally oxidized size-reduced particles, with the smallest particle size exhibiting the highest biological depolymerization rates, $21.25 \pm 0.24\%$ with the immobilized enzyme and $16.61 \pm 0.63\%$ with whole cells. The study indicated that all pretreatments primarily targeted the ester linkage, specifically the C=O and C-H bonds. Additionally, the effects of pretreatments were tested by chemical hydrolysis under optimized conditions developed in our lab. Due to its inherently caustic nature, chemical hydrolysis did not require any pretreatment. These findings shed light on the interplay of physical and chemical factors influencing biological depolymerization, offering valuable insights into importance of pretreatments for biological hydrolysis of such polymers.

Keywords

PET pretreatments, UV irradiation, thermal oxidation, size reduction, PET biological depolymerization

Graphical abstract



3.1 Introduction

Since its invention in 1941, the production and consumption of polyethylene terephthalate (PET) based products has skyrocketed. The desirable properties of PET include its inertness, transparent nature, flexibility, and the availability of cheap monomers, albeit from fossil resources. Hence, it has been used for the mass-production of a list of products including water bottles, containers, textile fibres and packaging material (Nisticò, 2020). However, these very attributes have made PET inert to breakdown and ubiquitous in terrestrial and marine systems. Its accumulation has resulted in substantial environmental issues (Amanna et al., 2023; Webb et al., 2012). In 2021, 390.7 million metric tons of plastics were produced globally, of which 6.2% was PET (PlasticsEurope (PEMREG), 2021). In 2022, Canada recycled an average of 110,359 tonnes of the 243,743 tonnes of rigid PET waste, meaning that only 45% of the rigid PET was recycled (Canada Plastics Pact, 2024). Each year, more than 300 million metric tons of plastic waste are globally produced (Gourmelon, 2015). As a result of these challenges, many countries are looking for ways to solve the serious plastic pollution issue by developing methods to reuse the plastics, thereby coming closer to a circular economy (Amanna et al., 2021; PlasticsEurope (PEMREG), 2021). Many countries are increasingly using their unrecycled plastic waste for energy recovery (Walker, 2021). Unfortunately, these processes generate numerous toxic and hazardous compounds (Koshti et al., 2018; Paul et al., 2020; Revel et al., 2018; Shen et al., 2019; Wu et al., 2017). Managing over nine billion metric tons of plastics (Ian Tiseo, 2023) currently on our planet into a sustainable circular economy is a challenge that needs to be addressed urgently.

A circular economy requires the reuse of presently discarded plastics to produce new plastic items. The currently used recycling technology involves melting PET products after use, followed by its use for other lower value products. This is because melted PET flakes lead to plastics of varied functionality like reduced mechanical and optical properties and allows migration of

nonintentional added substances into food and pharmaceutical products, thus limiting the application of PET in this way (Brouwer et al., 2020; Thoden van Velzen et al., 2020; Welle, 2011, 2013). An efficient circular economic method would break down the waste PET into its monomeric constituents, terephthalic acid (TPA) and ethylene glycol (EG) which can be re-polymerized to get back products of required characteristics. This would eliminate the issue of migration of chemicals into PET, generating high quality recycled PET from waste PET.

Chemical hydrolysis is one such method that can depolymerise PET to TPA and EG. A recent study developed and optimized a chemical hydrolysis process that was able to hydrolyze used single-use PET bottles (Moges, 2022). This process was able to recover nearly 84% of TPA from amorphous PET films as well as a single-use PET bottle following ozonation pretreatment. Even though chemical hydrolysis working under caustic conditions may be able to recover higher yields of the monomers, processes which operate under near ambient conditions and require no toxic chemicals are preferred.

Biological depolymerization is a preferred route for its clean, near ambient conditions and green approach. In the last few decades, scientists have reported numerous enzymes and microorganisms with PET-hydrolyzing ability (Farzi et al., 2019; Q. S. Huang et al., 2022; Maheswaran et al., 2023; Qi et al., 2022; Torená et al., 2021b). Functional groups like C=O and -OH increase the wettability or the hydrophilicity of the polymer surface which in turn increases biological depolymerization (Benke et al., 2022; Donelli et al., 2010). Additionally, factors like adhesion, surface topology, molecular weight and additives used in the production of the polymer have a significant impact on biological depolymerization (Vague et al., 2019). Pretreatments help modify these intrinsic properties, making PET more accessible for biological depolymerization.

Pretreatments such as size reduction augment the overall surface area for reaction, while thermal and ultraviolet (UV) pretreatments induce polymer oxidation, subsequently leading to the generation of radicals (B. Singh & Sharma, 2008). Both, thermal and photo-oxidation, increase the incorporation of atmospheric oxygen into the polymer, decreasing the hydrophobicity (Hurley & Leggett, 2009; B. Singh & Sharma, 2008). The efficacy of UV pretreatment depends on its ability to penetrate through PET surface since the reaction is a surface phenomenon. Irradiation with UVA with natural weathering conditions resulted in chain scission of PET (Gok et al., 2015). Furthermore, Vague et al. (2019) reported change in functional groups after UVA irradiation with no further detail on its effects on biological depolymerization. Falkenstein et al., (Falkenstein et al., 2020) also reports an impairment in the biological depolymerization post UV irradiation. Different UV ranges have different energies and hence different penetrative and bond cleavage abilities. Similarly, temperature also affects biological depolymerization. Temperatures around the glass transition temperatures (amorphous: 67 °C and crystalline: 81 °C) allow movement of the polymer chains (Botelho et al., 2001; Panowicz et al., 2021). Most studies of exposing PET to heat follows thermal depolymerization and has not been used a method to modify the polymer's physical structure to augment biological depolymerization. Both the physical and chemical nature of PET affect biological depolymerization. Sepperumal et al. (2013) reported modification of the functional groups of powdered PET after biological depolymerization with *Penicillium* spps. Furthermore, Farzi et al. (2019) reported about 70% biological depolymerization of PET powder (size 212 µm) by *Streptomyces* spps after 18 days. Ma et al. (2012b), further reduced the size of PET to nanometers, and observed biological depolymerization up to 50%. Most studies of PET pretreatment and biological depolymerization focus on using one type of pretreatment with one type of biological depolymerization process. This makes the comparison of such processes

difficult. Therefore, a comprehensive study focussing on various pretreatments followed by enzymatic, whole cell and chemical hydrolyses was carried out to shed some light on the best combination required for PET biological depolymerization.

This study focusses on the effect of physicochemical pretreatments of PET for biological depolymerization using biological catalysts. Eighteen different pretreatments which include acid treatment, thermal oxidation, photooxidation, size reduction and a combination of thermal oxidation and size reduction were carried out. The effect of pretreatment on the physical and molecular structure of PET were analyzed and subsequently its impact on biological depolymerization were analyzed. PET samples were biodegraded using two types of bioagents, whole-cell broths of *Thermobifida fusca* YX and immobilized cutinase enzyme. The results of these experiments were compared to the chemical hydrolysis of PET.

3.2. Materials & Methods

3.2.1 Biological Agents and Chemicals

All experiments were carried out using amorphous PET films purchased from Goodfellow (Canada) (Product code: ES30-FM-000145). The actinomycete, *Thermobifida fusca* YX (ATCC BA-629), was purchased from Cedarlane (Canada). The immobilized enzyme Novozym® 51032 (1,3 Lipase from *Humicola insolens*) was purchased from Cedarlane (Canada) (Product code: IMML51-T1-350-2). The immobilized enzyme beads had the particle size ranging from 300 to 700 µm (immobilized by adsorption onto polymethacrylate divinylbenzene copolymer beads). All the other chemicals and solvents were purchased from Sigma-Aldrich (St. Louis, Missouri, USA) of reagent grade and used without modification unless specified otherwise.

3.2.2 Physicochemical pretreatments of PET

3.2.2.1 Ultraviolet (UV) pretreatment

The 1x1 inch amorphous PET film samples were exposed to two UV wavelengths, 254 nm (UVC) and 311 nm (UVB). The UVC pretreatment used was a modified protocol used by Hurley & Leggett (2009). The sample was kept 3 cm from the UVC light source inside a biosafety cabinet (maximal peak at 254 nm) for 2, 4, and 6 hours. The same protocol was used for comparison with the UVB light source using a Philips Narrowband purchased from Philips, Canada.

3.2.2.3 Size reduction

Amorphous PET was pulverized in a high-speed superfine pulveriser with 2 minutes intervals to reduce the effect of heat on the PET particles. The PET particles were separated with mesh sizes 1mm, 500 μ m, and 250 μ m. For subsequent experiments, 185 mg of the separated particles were used for each condition evaluated.

3.2.2.4 Thermal pretreatment of films and size-reduced PET

Amorphous PET film was cut into 1x1 inch samples and the size-reduced PET samples (1mm, 500 μ m, and 250 μ m) were incubated at 70 °C, 80 °C, and 90 °C in a hot air oven, respectively, for two weeks.

3.2.2.5 Acid pretreatment

A modified protocol adapted from Rajandas et al. (2012) was used for the acid pretreatment of PET films. The amorphous PET film was cut into 1x1 inch samples and submerged in 10 mL of 65% v/v nitric acid in 25 mL glass bottles with lids for two weeks. After every pretreatment, the PET films were washed with 0.1% SDS and subsequently washed three times with distilled water.

3.2.3 Analysis methods

3.2.3.1 Fourier Transform Infrared Spectrometry (FTIR)

The test film samples were washed thoroughly with deionized water. Spectra were collected in the 4000 to 600 cm^{-1} range at 2 cm^{-1} spectral resolution using a single-beam Bruker Tensor 37 FTIR which had the Bruker OPUS 5.5 software available at the Lakehead University Instrumentation Laboratory (LUIL), Lakehead University, Thunder Bay, Canada.

3.2.3.2 Differential Scanning Calorimetry

The untreated and pretreated PET film samples were cut into 2 x 2 mm squares. Differential scanning calorimetry was carried out in the DSC Q2000 TA Instruments (New Castle, USA) the temperature range was 20 to 270 $^{\circ}\text{C}$ and heating rate of 10 $^{\circ}\text{C}$ per min. The obtained values were viewed and analysed using TA Universal Analysis.

The degree of crystallinity (X_c) was calculated according to equation (1):

$$X_c = \frac{\Delta H_m - \Delta H_{CC}}{\Delta H_m^0} \times 100 \dots \text{Equation (1)}$$

Where, ΔH_{cc} is the cold crystallization enthalpy of the sample. ΔH_m is the heat of melting of the sample, while ΔH_m^0 is the heat of melting of a pure crystalline sample which is 140 J.g^{-1} according to (Mehta et al., 1978).

3.2.3.3 Scanning electron microscopy

The untreated and pretreated PET film samples were washed thrice with deionized water and air-dried before imaging. These samples were imaged using the SEM/EDS: Hitachi Su-70 Schottky Field Emission scanning electron microscope operated at an electron beam intensity of 5 kV at LUIL, Lakehead University, Thunder Bay, Canada. Before viewing under the microscope, the samples were carbon coated using the BOC Edwards Auto 306 to increase the electron density.

3.2.3.4 Gravimetric weight loss

The pretreated samples were weighed before and after the pretreatments using the Sartorius Quintix weighing balance. The samples were also weighed before and after biological hydrolysis and chemical hydrolysis.

3.2.4 Biological hydrolysis

Two types of biological hydrolyses, enzyme mediated and whole cell-based methods, were employed to determine the efficacy of pretreatment. After the biological hydrolysis, the PET films and particles were separated by vacuum filtration. The PET films and particles were then washed with deionized water three times and were dried at 50 °C in a hot air oven for 6 hours then weighed. Biological hydrolysis was done in triplicates and subsequently subjected to a t-test to determine significance.

3.2.4.1 Enzyme-mediated hydrolysis

The reaction system consisted of 700 mg immobilized Novozym® enzyme (with an activity of 10,000 $\mu\text{mol.g}^{-1}.\text{min}^{-1}$) and the pretreated sample in 10 mL of 200 mM Tris HCl buffer at pH 8.0. This system was incubated at 60 ± 2 °C at 150 rpm for three days. The enzyme was separated by vacuum filtration, and the PET samples were washed with deionized water thrice before drying in the hot air oven at 50 °C. These samples were then weighed to determine weight loss.

3.2.4.2 Whole-cell mediated hydrolysis

The actinomycete *T. fusca* YX was grown in 25 mL of Hagerdahl's for 5 days at 150 rpm at 50 °C. The enzyme activity of the extracellular broth was determined to be 28.36 $\mu\text{mol.ml}^{-1}.\text{min}^{-1}$. The pretreated sample was incubated with 100 μL of *T. fusca* YX inoculum in Hagerdahl's medium for two weeks.

3.2.5 Chemical hydrolysis by non-aqueous alkali

Chemical hydrolysis of PET was adapted from Bhogle & Pandit (2018). It was carried out by incubating amorphous PET samples (1x1 inch) in 20 mL of 8.5% w/w NaOH in methanol in a 125 mL Erlenmeyer flask in a CPX series ultrasonic bath (40 Hz, CAT: 15-337-419), Fisher Scientific (New Hampshire, USA) set at 60 °C for 2.9 hours. The reaction was stopped by the addition of an equal volume of chilled deionized water. The unhydrolyzed PET was separated using vacuum filtration, and then washed thrice with deionized water, followed by drying at 80 °C for 45 minutes to remove any residual methanol. After this, the weight of the unhydrolyzed PET was determined using the Sartorius Quintix weighing balance. The TPA in the filtrate was precipitated at pH 2.5 using undiluted sulphuric acid. The insoluble white precipitate was washed thrice with deionized water to remove soluble salts. The TPA salt was then dried in the hot air oven at 80 °C for 45 minutes. The pH of the filtrate was increased to 7 to analyze the non-precipitated oligomers by HPLC (See Section 3.2.4.4). The TPA yield was calculated using the TPA recovered after precipitation and the theoretical expected TPA. The theoretical calculations for expected TPA and EG were based on the equations from Bhogle & Pandit (2018). The total monomer yield was calculated using the formula in equation 2.

$$TPA\ yield = \frac{Quantified\ TPA}{Theoretical\ TPA} \times 100 \quad \text{---- Equation (2)}$$

Where,

Quantified TPA is the number of moles of TPA quantified after hydrolysis.

Theoretical TPA is the number of moles of TPA expected after complete hydrolysis.

3.3. Results & Discussion

3.3.1 Photo-oxidative depolymerization using ultraviolet radiation

Most previous studies report weathering of PET by exposure to most of the UV range (400 nm to 250 nm) (A. L. Day & Wiles, 1972; M. Day & Wiles, 1972; Falkenstein et al., 2020). In this study, to determine the effect of wavelength and exposure time of UV radiation, PET films were exposed to two specific wavelengths with different energies, 254 nm, and 311 nm. They were irradiated for 3, 4, and 6 hours at each wavelength. The surface structure after irradiation remained unchanged when imaged. However, with ATR-FTIR analysis, changes in the intensity of functional groups were observed. Untreated PET samples showed characteristic absorption peaks which include 2864 cm^{-1} and 2945 cm^{-1} for stretching of the asymmetrical/symmetrical C-H bond in a methylene group, 1714 cm^{-1} for C=O stretching in an α/β -unsaturated ester in a benzene ring, 1408 cm^{-1} for stretching of C=C in the benzene ring, 1257 cm^{-1} and 1242 cm^{-1} for stretching of C-O in a carboxylic acid, 1095 cm^{-1} for stretching of C-H bond in the benzene ring, 870 cm^{-1} for bending of C=C in cyclic or conjugated alkene and 725 cm^{-1} for C-H bending in a monosubstituted benzene ring (Alzuhairi & Al-Ghaban, 2016; Y. Huang et al., 2021; Zhu & Kelley, 2005). Furthermore, the degree of crystallinity of the untreated PET films, as calculated using Equation 1, was found to be 6.69%.

For PET samples irradiated with UVB or UVC, a general reduction in peak intensities was observed. UVC irradiation for 3 hours caused a decrease in peak intensities, followed by an increase at 4 hours, and then a return to the 3-hour levels after 6 hours. Specifically, the asymmetrical/symmetrical C-H bond in a methylene group at 2945 cm^{-1} and the C=O stretching in an α/β -unsaturated ester at 1714 cm^{-1} showed reductions, indicating bond cleavage near the aromatic moiety (see Fig. 3.1) (Smith, 2022). After 4 hours, the peak intensities increased, suggesting chain scission and exposure of more functional groups. By 6 hours, the intensities

decreased again, resembling the 3-hour spectrum (see Fig. 3.2). Photolysis initiated by UV disrupted C-O and C-H bonds at 2945 cm^{-1} and unsaturated sites at 1714 cm^{-1} (B. Singh & Sharma, 2008), are evident after 3 hours of UVC exposure. Furthermore, differential scanning calorimetry (DSC) analyses of the films subjected to UVB and UVC irradiation revealed a progressive increase in the degree of crystallinity over time. Specifically, the degree of crystallinity for the UVB irradiated samples was determined to be 7.87%, 8.44%, and 10.37% after 3, 4, and 6 hours of exposure, respectively (see Supplementary fig. 1). In comparison, UVC irradiated samples exhibited degrees of crystallinity of 8.83%, 9.07%, and 11.43% following 3, 4, and 6 hours of irradiation, respectively (see Supplementary fig. 2).

Propagation reactions are auto-oxidation cycles which can be observed by the increase after 4 hours of irradiation followed by a drop in the peak intensity after 6 hours of irradiation. Photolysis occurs through Norrish I and II reactions following the β -scission route. Norrish reactions in polyesters like PET commence by the formation of an excited terephthalic acid radical (DeCosta et al., 1999; Fechine et al., 2004; Zhu & Kelley, 2005). Due to the domination of type I reaction, carbonyl bonds are cleaved. These scissions occur in the amorphous domain creating two free ends that can restructure to increase the crystallinity of the polymer. The results obtained in this study with UV photolysis pretreatment are similar to that described previously by Singh & Sharma (B. Singh & Sharma, 2008). Irradiation with shorter wavelengths like 254 nm are known to reduce the molecular weight of PET, while 310 nm has a much-reduced extent of molecular weight reduction. Reduced molecular weight polymers can restructure with ease compared to their larger counterparts (M. Day & Wiles, 1972).

Following UV irradiation, the efficacy of biological depolymerization was assessed. There was an increase in *T. fusca*-mediated biological depolymerization with UVB-irradiated PET films. PET

films irradiated with UVB for 6 hours demonstrated nearly a 4 % weight loss compared to the untreated PET sample at 1 % (p-value: 0.0017). In comparison, enzyme-mediated biological depolymerization showed a 15% decrease compared to its untreated PET counterpart after 3 hours of UVB irradiation. With UVC irradiation, there was a decrease in the biological depolymerization performance with both enzyme-mediated, HiC, and whole-cell *T. fusca* biological depolymerization (see Fig. 3.3). Falkenstein et al. found that weathering of PET for 14 days with focus on UV exposure resulted in an increase in the abundance of C-O bonds due to intra-chain scissions (Falkenstein et al., 2020). However, this was accompanied by a decrease in the biological depolymerization efficiency. This was attributed to the increase in the crystallinity of the PET film after irradiation. In this study, a narrower range of wavelengths with peak intensities of 311 nm (UVB), and 254 nm (UVC) with shorter time exposures resulted in no new peaks, but a variation in the abundance of C=O and C-H which corroborates the findings of Falkenstein et al. Suggesting that the effects of UV exposure on PET films are demonstrated in 4 hours. This indicates that the 4-hour mark of UVC exposure is the critical time point beyond which impairment of biological depolymerization occurs. Hence, UVC exposure of more than 4 hours would not be a recommended pretreatment method.

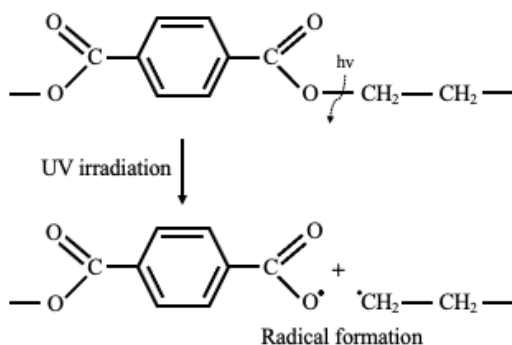


Figure 3.1 Schematic of the postulated photodegradative process as a result of UV irradiation

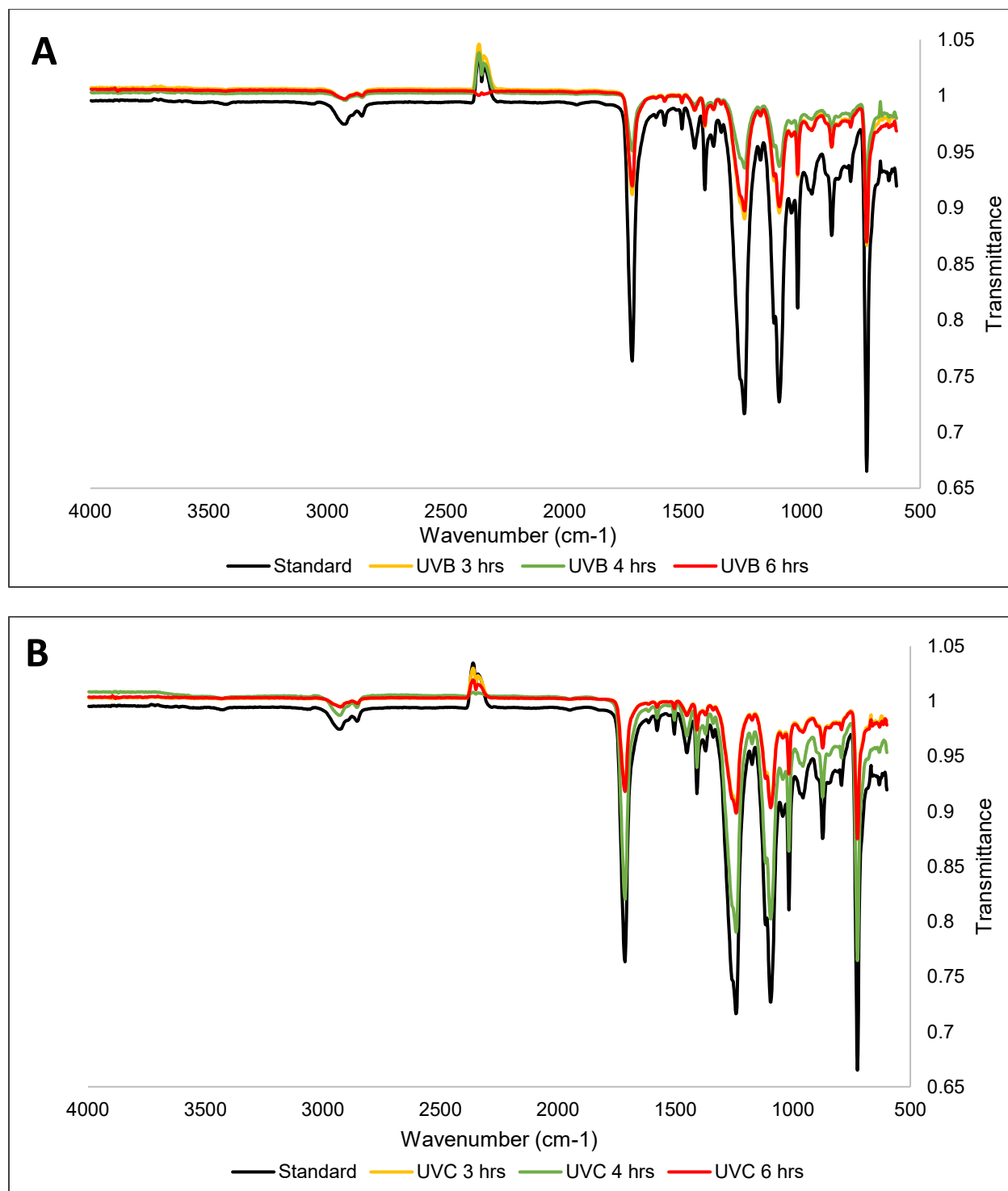


Figure 3.2 ATR-FTIR spectra of amorphous PET films exposed to (A) UVB and (B) UVC for 3, 4, and 6 hours versus untreated PET

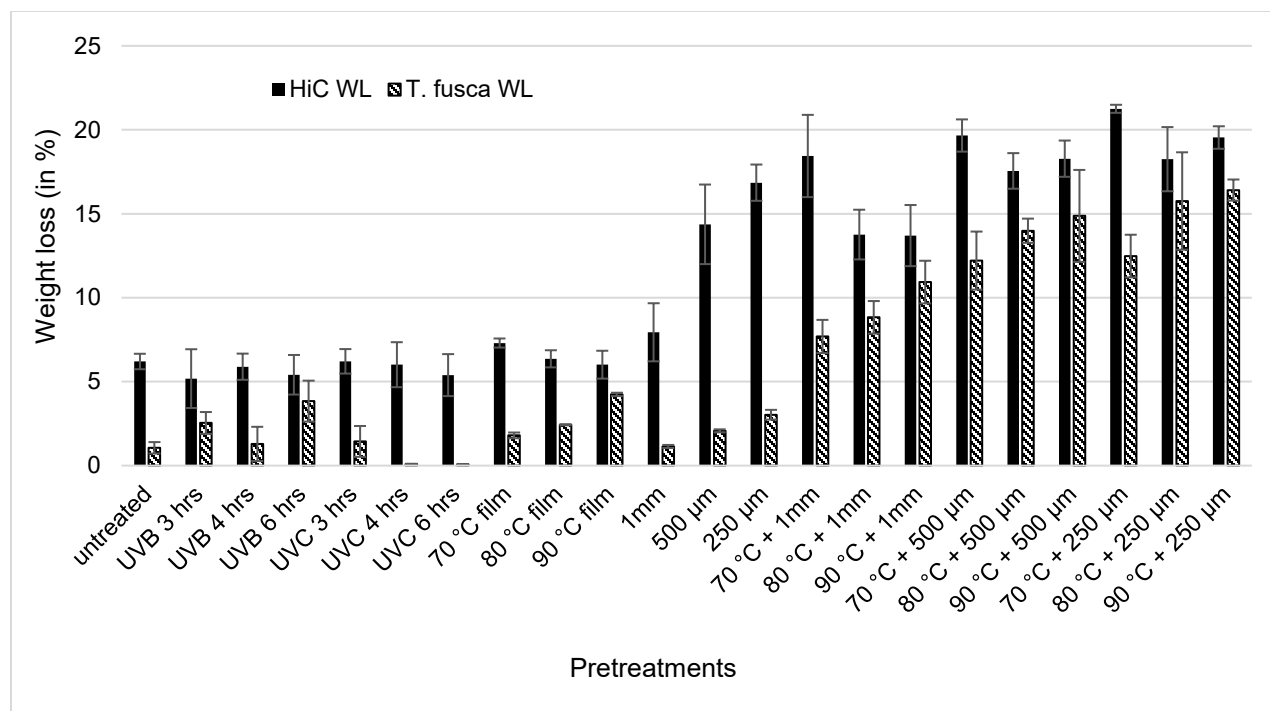


Figure 3.3 Weight loss of PET based on enzymatic biological depolymerization by immobilized HiC and whole-cell biological depolymerization by *Thermobifida fusca* YX following pretreatment by UVB and UVC irradiation, thermal oxidation, size reduction, and thermal oxidation of size reduced particles

3.3.2 Thermal oxidation

Thermal oxidation was assessed by exposing the PET film to dry heat at three temperatures, 70 °C, 80 °C, and 90 °C for two weeks. With each increase in temperature, there was a corresponding increase in the haziness of the films. Haze can be caused due to surface roughness, internal optical irregularities, level of crystallization, and porosity (Horváth et al., 2014; Molnár et al., 2020). At higher temperatures, the polymer chains can shift freely enough to be able to form crystals. DSC analysis of the treated films revealed a temperature-dependent increase in crystallinity. This was substantiated by the observed degrees of crystallinity at temperatures of 70 °C, 80 °C, and 90 °C,

which were measured to be 7.23%, 7.35%, and 7.51%, respectively (see Supplementary fig. 3). The effects of thermal treatment on the surface of the films were imaged using a scanning electron microscope. Multiple physical deformities like bulbous protrusions and cracks of varying widths were present on the film's surface. These deformities increased in frequency and intensity with increase in temperature (See Supplementary fig. 4).

There were no shifts in the hybridization state or electron distribution of the molecular groups within the fingerprint region when observed by ATR-FTIR. However, there was a variation in the abundance of the functional groups as indicated by a change in the peak intensity (Norman Jones et al., 1952). There was a slight increase in the stretching in the asymmetrical C-H bond at 2945 cm^{-1} when the PET films were exposed to $70\text{ }^{\circ}\text{C}$ and $80\text{ }^{\circ}\text{C}$ for two weeks. Meanwhile, there was a decrease when exposed to $90\text{ }^{\circ}\text{C}$ for two weeks (see Fig. 3.4). We observed a shift in the paradigm when exposed to $90\text{ }^{\circ}\text{C}$. This suggests a reduction in the C-H bonds upon an increase in temperature from 80 to $90\text{ }^{\circ}\text{C}$, which corresponds to the reduction in the bond between the asymmetrical C-atoms on either side of the aromatic ring with their corresponding H-atoms. This can be further confirmed by the reduction in the peak intensity at 1714.64 cm^{-1} which corresponds to the stretching of the bond between the C=O of an α/β -unsaturated ester in a benzene ring. This strongly indicates the cleavage of the bond or bonds between the asymmetrical carbons and the respective substitutions. Correspondingly, at peak 1714.64 cm^{-1} we observed a progressive increase in the stretching of C=O up to $80\text{ }^{\circ}\text{C}$ but observed a decrease when the temperature is increased to $90\text{ }^{\circ}\text{C}$. Thermal oxidation, similar to photo-oxidation follow similar initiation and propagation reactions (B. Singh & Sharma, 2008). Additionally, thermal oxidation of PET and its structural homolog, ethylene dibenzoate resulted in a radical formation across C-O in the ester bond (Botelho

et al., 2001). These results suggest that the initiation of thermal oxidation can occur with temperatures close to the glass transition temperature, that is as low as 70 °C.

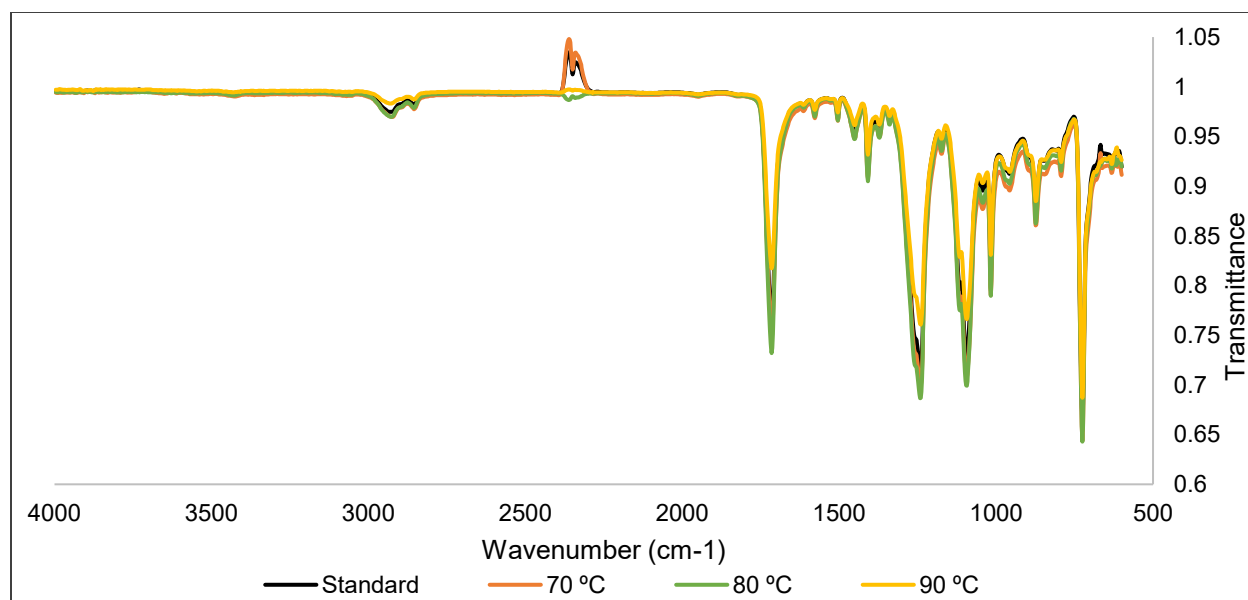


Figure 3.4 ATR-FTIR spectra of amorphous PET film exposed to exposed to 70 °C, 80 °C, and 90 °C for two weeks versus untreated PET

The effect of 90 °C was observed when the thermally treated samples were subjected to biological depolymerization. With both, the immobilized HiC and the whole-cell *T. fusca* there was a decrease in the weight loss compared to the untreated PET film (see Table 1). The increase in temperature above the glass transition temperature permits the movement of polymer chains which allow the formation of more crystalline regions. Biological depolymerization of thermally treated PET films with immobilized HiC and whole-cell *T. fusca* yielded contrasting results. PET films exposed to 70 °C resulted in $7.3 \pm 0.27\%$ weight loss with the immobilized enzyme HiC, which is minimal increase compared to the untreated PET film. Further increase in the pretreatment temperature (90 °C) impaired the biological depolymerization resulting in a decrease in weight loss with immobilized HiC. In contrast, there was a proportional increase in weight loss with an

increase in temperature when biodegraded with whole-cell *T. fusca*. Like cellulases, *T. fusca* YX could harbor multiple active PET-hydrolyzing enzymes as well as modifying enzymes that resulted in higher weight loss. This could contribute towards the increase in biological depolymerization of PET which has been thermally oxidized at 90 °C. Although the biological depolymerization of *T. fusca* YX increases with temperature, the energy consumption associated with a two-week thermal treatment is disproportionate to the observed biological depolymerization. While the method shows potential, its viability requires further evaluation through a comprehensive life cycle analysis.

3.3.3 Size-reduction

In order to determine the effect of particle size, PET samples were pulverized to 1 mm, 500 μm , and 250 μm . When imaged, the structure of the cross-sectional surface showed rough edges and several cracks resulting from pulverizing. In some cases, bulbous protrusions could be observed (see Supplementary fig. 2). Similar to the effect of thermal oxidation, there were no changes in the particles' hybridization states or functional groups, but there were variations in the abundance of the functional groups. A slight shift in peaks is observed upon size reduction compared to the standard PET film. Particle size 1 mm shows a peak shift to 2949 cm^{-1} , while 500 μm and 250 μm show a shift to 2927 cm^{-1} (see Supplementary fig. 6). These shifts still fall in the methyl C-H asymmetrical stretch. Furthermore, with progressive increase in size, there is corresponding increase in the peak intensities. This confirms the increase in the exposure of functional groups due to the increase in surface area.

Previous reports demonstrate an inverse correlation between particle size and biological depolymerization, i.e., a decrease in particle size has increased biological depolymerization (Farzi et al., 2019). Both enzyme-mediated and whole-cell-mediated biological depolymerization showed

a similar trend of increase in overall weight loss with a decrease in particle size. Weight loss was more substantial with HiC-mediated biological depolymerization compared to *T. fusca*-mediated biological depolymerization. The highest weight loss of 16.85 ± 1.08 % (absolute weight loss of 33.26 ± 1.96 mg) was observed with the smallest particle size, 250 μm with enzymatic biological depolymerization with immobilized HiC. This is 2.7 times more than the weight loss observed with untreated PET films, $6.2 \pm 0.46\%$ (absolute weight loss of 13 ± 0.5 mg) after three days of enzymatic hydrolysis. Furthermore, there was three times increase in biological depolymerization ($3.03 \pm 0.5\%$ weight loss) with whole cell mediated biological depolymerization with 250 μm particles than with untreated PET films. The reduction in particle size increases the surface area available per unit mass available for reaction increasing reaction rates. However, the energy input for the size reduction and the ability to bring about size reduction at higher volumes needs to be studied as at large scale this cost can be prohibitive.

3.3.4 Combination of thermal oxidation with size-reduction

As observed, reduction in particle size and thermally oxidized PET increases the overall biological depolymerization. To assess the combined effect, sized-reduced PET particles sizes, 1 mm, 500 μm , and 250 μm were thermally treated at three temperatures 70 $^{\circ}\text{C}$, 80 $^{\circ}\text{C}$, and 90 $^{\circ}\text{C}$ for two weeks. When imaged, all samples were observed to have an increased number of fine cracks throughout the surface. In addition, several broken-like structures were found toward the particles' sides. With an increase in temperature, various undulating protrusions were observed on the particle's surface compared to the untreated film (see Supplementary figs. 7-9).

After the assessment of changes in the physical structure, the change in the molecular bonds and functional groups were assessed. The variation in functional groups resulting from the combination of thermal oxidation and size reduction pretreatments were assessed using ATR-FTIR. The

combination of size reduction and temperature resulted in the variation of peak intensities following the thermally affected peaks, 2945 cm^{-1} and 1714 cm^{-1} . The peak at 2945 cm^{-1} corresponds to the stretching of the asymmetrical methyl C-H bond on either side of the aromatic ring and peak 1714 cm^{-1} corresponds to the stretching of the bond between the C=O of an α/β -unsaturated ester in a benzene ring respectively (see Supplementary fig. 10). The combination of size reduction with exposure to $70\text{ }^{\circ}\text{C}$ have increased overall peak intensities when compared to their corresponding controls of unheated size-reduced particles. An increase in the functional groups has been previously reported to be conducive to biological hydrolysis (Rajandas et al., 2012). This was confirmed in our study, where the combination of $250\text{ }\mu\text{m}$ size particles treated at $70\text{ }^{\circ}\text{C}$ for two weeks resulted in the highest weight loss of $21.25 \pm 0.24\%$. When exposed to $80\text{ }^{\circ}\text{C}$ for two weeks, interestingly, there was both an increase and decrease in peak intensities at different wavelengths. With decrease in size up to $500\text{ }\mu\text{m}$, there was a decrease in peak intensities. With further decrease in size to $250\text{ }\mu\text{m}$, there was an increase in peak intensities. This suggests an increase in the stretching of the C-H bond corresponding to the increase in abundance of the methylene group. The increase in the abundance of functional groups in the sample with particle size of $250\text{ }\mu\text{m}$ was due to the increased surface area. These results suggest the increase in bond cleavage with incremental decrease in size resulting in an increase in abundance of functional groups C=O and C-H. When combined with even increased temperature, $90\text{ }^{\circ}\text{C}$ there was a further reduction in the peak intensity. Following the earlier mentioned peaks, 2945 cm^{-1} and 1714 cm^{-1} , similar bond cleavage can be observed. The reduction in the asymmetrical C-bond on either side of the aromatic ring is indicative of its cleavage and the reduction in the stretching of the bond between the C=O of an α/β -unsaturated ester in a benzene ring which is indicative of the cleavage of the bond. There seemed to be a greater effect of bond cleavage with $250\text{ }\mu\text{m}$ exposed to $90\text{ }^{\circ}\text{C}$.

As mentioned earlier, size reduction and thermal treatment confer unique properties to the previously smooth polymer. Size reduction increases the overall surface area, roughness and exposure of functional groups due to the pulverizing process. Depending on the temperature there can either be an increase (at 70 °C and 80 °C) or decrease (at 90 °C) in the abundance of functional groups. The combination of these treatments resulted in both physical and chemical changes in PET, which augmented biological depolymerization. The combination of different sizes with different temperatures did not result in a synergistic effect for enzyme-mediated hydrolysis. However, there was an increase in weight loss compared to their non-temperature-treated counterparts. The highest weight loss with whole cell mediated hydrolysis was $16.41 \pm 0.63\%$ with 250 μm particles treated at 90 °C. As mentioned earlier, *T. fusca* YX could have a battery of PET-hydrolyzing enzymes (PHEs) as well as modifying enzymes. Meanwhile, the highest weight loss with immobilized enzyme was $21.25 \mp 0.24\%$ with 250 μm particles treated at 70 °C. Our results suggest that immobilized HiC as a better option for biological depolymerization of size-reduced PET particles. However, there was a synergistic effect of the combination of size reduction and temperature with whole cell mediated biological depolymerization with uninduced *T. fusca* YX. Induction of cutinases from *T. fusca* YX could potentially increase the overall biological depolymerization of PET particles.

The reduction of PET particle size presents inherent challenges, including the risk of microplastic and nanoplastic release into the environment (Amanna et al., 2023) and the substantial energy required for the size reduction process. Additionally, thermal treatment for two weeks would further increase energy consumption. However, the significant weight loss observed through both whole-cell and immobilized enzymatic biological depolymerization highlights the potential of the biological method. Optimizing the process to minimize energy consumption, followed by a

comprehensive life cycle analysis, could provide valuable insights into the feasibility and sustainability of this approach.

3.3.5 Acid pretreatment

PET films were treated with 65% v/v nitric acid to assess the effect of strong acid on the polyester. After 2 weeks, the films had turned completely white and were extremely brittle to handle. When viewed under the electron microscope, the surface of the films was completely deteriorated. There were craters and tiny holes throughout the surface of the film (See Supplementary fig. 11). Despite the visual indication of deterioration of the film, interestingly, the weight of the film increased by $5.73 \pm 0.71\%$ after the acid treatment. The assessment of functional groups by ATR-FTIR determined a change in the functional groups present, there was an additional broad peak ranging from 3206 cm^{-1} to 3583 cm^{-1} which suggests the presence of an -OH bond. Meanwhile, the other peaks remained unchanged (See Supplementary fig. 12). The drastic change in the physical structure and the formation of a new functional group did not augment biological depolymerization. In fact, there was no change in weight after enzymatic hydrolysis with HiC, indicating that no biological depolymerization occurred. With whole-cell hydrolysis, there was $5.68 \pm 0.38\%$ weight loss. This suggests that different enzymes were expressed by *T. fusca* YX that had a different cleavage mechanism than HiC. The actinomycete has been previously described to have a battery of enzymes for cellulolytic activity (Lykidis et al., 2007) and is currently being explored for other functions, like esterase activity. Additionally, the variation in the physical states of HiC (immobilized) and the whole cells (free suspension) could have affected the biological depolymerization.

3.3.6 Chemical hydrolysis by non-aqueous alkali

Chemical hydrolysis involving non-aqueous alkalis have been gaining interest due to their ability to hydrolyze in-use plastic that would otherwise end up in the landfill. Recent studies report the use of standard PET films as well as waste PET able to be hydrolyzed with non-aqueous alkali. One such study reports the chemical breakdown of amorphous untreated PET films results in over 80% TPA recovery (Moges, 2022). As observed earlier in this study, pretreatments cause chemical and, in some cases, physical changes. To determine the effect of pretreatment on TPA yield, each sample was exposed to individual and combined pretreatments prior to being chemically hydrolyzed using 8.5% NaOH in methanol in combination with ultrasonic waves for 2.9 hours. Under these conditions all PET samples had a 100% weight loss. Untreated PET sample resulted in as high as $90.85 \pm 0.84\%$ TPA recovered (using equation 2). As demonstrated by a previous study, HPLC analysis of the supernatant after the precipitation of TPA revealed trace amounts of TPA, MHET, BHET, and other oligomers (Moges, 2022). There was no significant difference between the TPA yields between the untreated film and pretreated samples (p-value: 0.96) (see Fig. 3.5). Due to the caustic nature of the treatment, no pretreatment would be required.

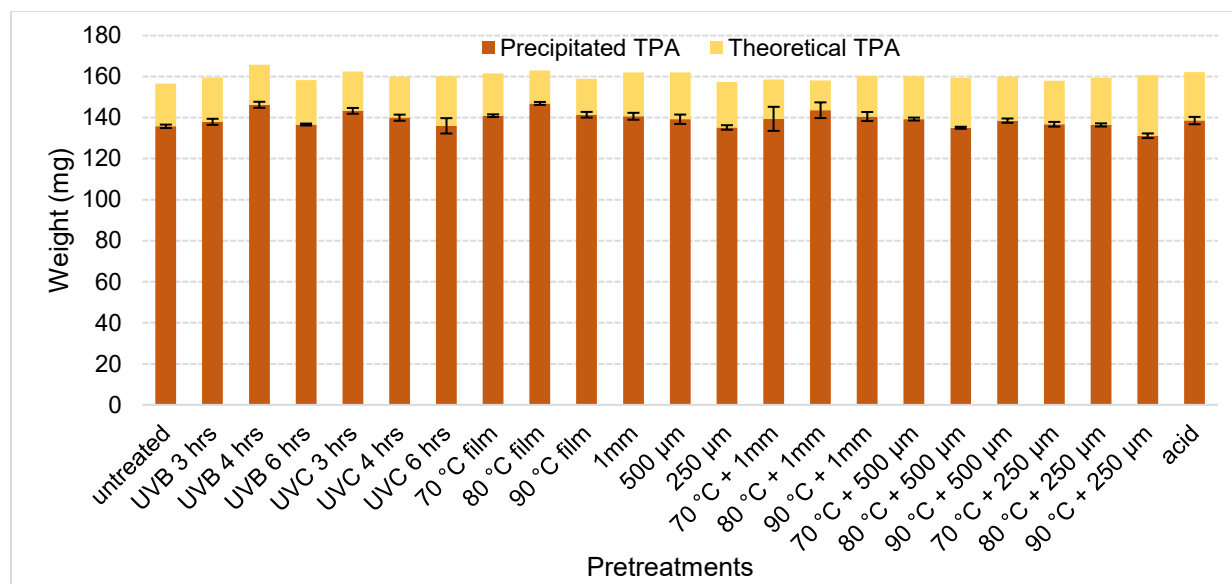


Figure 3.5 Precipitated terephthalic acid (TPA) after chemical hydrolysis compared to theoretical TPA yield

3.4 Conclusion

This study aimed to identify the physicochemical changes in amorphous PET films by pretreatment and their ability to augment biological depolymerization. Pretreatments like thermal oxidation with dry heat and photooxidation by UV irradiation, targeted C=O and C-H bonds. Despite targeting the same bonds, UV irradiation impaired biological depolymerization while thermal oxidation increased biological depolymerization. Interestingly, *T. fusca*-mediated biological depolymerization was better than a singular enzyme, HiC, when PET was exposed to higher temperatures like 90 °C. This is because of the free nature of the former as compared to hindrance to enzyme complex formation in the case of the enzyme anchored on a carrier in the case of the latter immobilized enzyme sample. While acid pretreatment demonstrated a change in the hybridization state of the functional groups, i.e., an additional alcohol (-OH) peak was observed. Despite the caustic nature of acid pretreatment, no large increase in biological depolymerization was observed compared to the other pretreatments. Size reduction produced the highest biological

depolymerization and can be attributed to the increased surface area of PET. The combination with the highest weight loss, 21.25 ± 0.24 % was with the smallest particle size, 250 μm at 70 °C with the immobilized HiC enzyme. However, this process is not only energy intensive but could also lead to the generation of several microplastics that could potentially enter the environment. These results suggest that whole-cell biological depolymerization is not just dependent on the shift in the hybridization peaks but also its abundance. Biological depolymerization is thus affected by the size of the particle, exposure to shorter wavelengths like UV, thermal treatments closer to glass transition temperatures and roughness of surface. Commercial PET films with increased crystallinity (20–30%) may exhibit behavior distinct from or analogous to the effects observed in the pretreatments discussed in this study, albeit at a different magnitude. This elevated crystallinity could potentially lead to a reduction in the biological depolymerization efficiency typically observed with amorphous PET, as the denser crystalline structure may further limit enzymatic or microbial access to the polymer chains. Biological depolymerization requires modification of PET via physicochemical pretreatments while chemical hydrolysis does not require any pretreatment. The caustic nature of the chemical process overcomes the need for any modification. Further life cycle assessments of the described physicochemical and biological depolymerization methods need to be studied to identify the most optimal route for reduction in pollution under milder conditions like biological reactions that can lead to a circular economy.

References

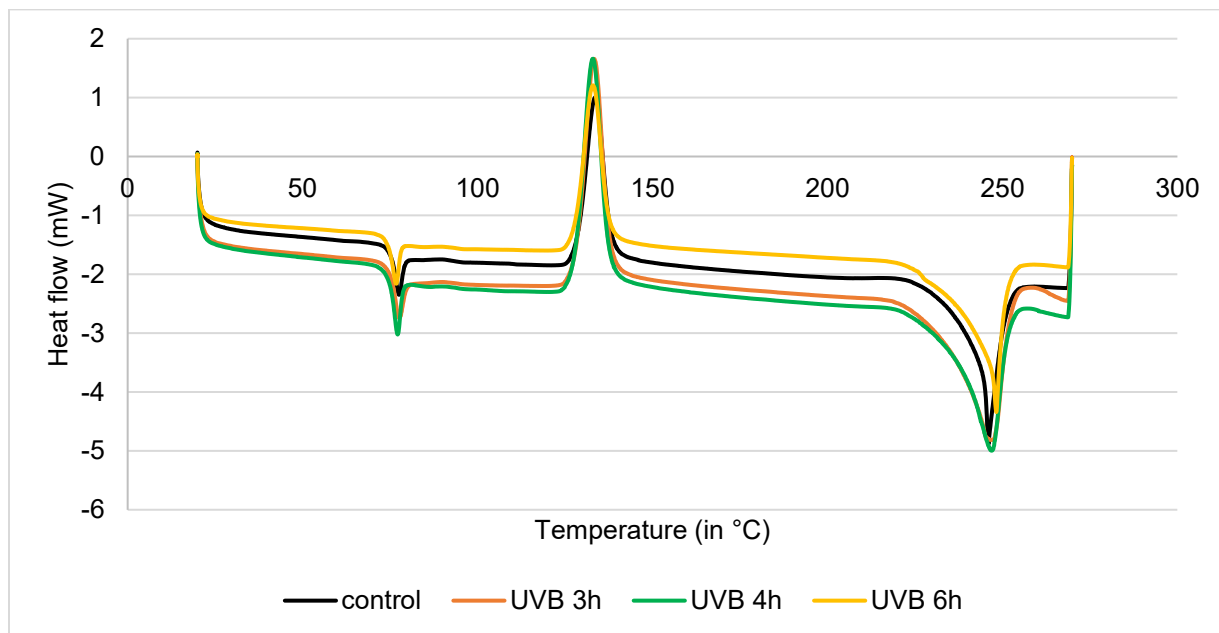
- Alzuhairi, M., & Al-Ghaban, A. (2016). *Chemical Recycling of Polyethylene Terephthalate (PET) as Additive for Asphalt*. <https://www.researchgate.net/publication/309420408>
- Amanna, R., Mahal, Z., Vieira, E. C. S., Samavi, M., & Rakshit, S. (2021). Plastics: Toward a Circular Bioeconomy. In *Biomass, Biofuels, Biochemicals* (pp. 781–811). Elsevier.
- Amanna, R., Samavi, M., & Rakshit, S. K. (2023). Biological depolymerization of microplastics and nanoplastics in water and wastewater. *Current Developments in Biotechnology and Bioengineering*, 293–314. <https://doi.org/10.1016/B978-0-323-99908-3.00002-6>

- Aslani, H., Pashmtab, P., Shaghghi, A., Mohammadpoorasl, A., Taghipour, H., & Zarei, M. (2021). Tendencies towards bottled drinking water consumption: Challenges ahead of polyethylene terephthalate (PET) waste management. *Health Promotion Perspectives*, 11(1), 60–68. <https://doi.org/10.34172/hpp.2021.09>
- Bhogle, C. S., & Pandit, A. B. (2018). Ultrasound-Assisted Alkaline Hydrolysis of Waste Poly(Ethylene Terephthalate) in Aqueous and Non-aqueous Media at Low Temperature. *Indian Chemical Engineer*, 60(2), 122–140. <https://doi.org/10.1080/00194506.2017.1310634>
- Blais, P., Day, M., & Wiles, D. R. (1973). Photochemical Depolymerization of Poly (ethylene Terephthalate). IV. Surface Changes. In *JOURNAL OF APPLIED POLYMER SCIENCE* (Vol. 17).
- Botelho, G., Queiro'squeiro's, A., Liberal, S., & Gijsman, P. (2001). Studies on thermal and thermo-oxidative depolymerization of poly(ethylene terephthalate) and poly(butylene terephthalate). *Polymer Depolymerization & Stability*, 74(1), 39–48. www.elsevier.com/locate/polydegstab
- Brouwer, M. T., Alvarado Chacon, F., & Thoden van Velzen, E. U. (2020). Effect of recycled content and rPET quality on the properties of PET bottles, part III: Modelling of repetitive recycling. *Packaging Technology and Science*, 33(9), 373–383. <https://doi.org/10.1002/PTS.2489>
- Canada Plastics Pact. (2024). *Canada-wide Plastic Packaging Flows: A Progress Report*. www.plasticspact.ca
- Day, 31, & Wiles, D. M. (1972). Photochemical Depolymerization of Poly(ethylene Terephthalate). 111. Determination of Decomposition Products and Reaction Mechanism. In *IED POJYMER SCIENCE* (Vol. 16).
- Day, A. L., & Wiles, D. 34. (1972). Photochemical Depolymerization of Poly(ethylene Terephthalate). I. Irradiation Experiments with the Xenon and Carbon Arc. In *JOURNAL OF APPLIED POLYMEH SCIENCE* (Vol. 16).
- Day, M., & Wiles, D. M. (1972). Photochemical Depolymerization of Poly (ethylene Terephthalate). 11. Effect of Wavelength and Environment on the Decomposition Process. In *JOURNAL OF APPLIED I'OLYMEK SCIENCE* (Vol. 16).
- Essaddam, H. (2017). *Polyethylene terephthalate depolymerization* (Patent PCT/US2016/041392).
- Falkenstein, P., Gräsing, D., Bielytskyi, P., Zimmermann, W., Matysik, J., Wei, R., & Song, C. (2020). UV Pretreatment Impairs the Enzymatic Depolymerization of Polyethylene Terephthalate. *Frontiers in Microbiology*, 11. <https://doi.org/10.3389/fmicb.2020.00689>
- Farzi, A., Dehnad, A., & Fotouhi, A. F. (2019). Biological depolymerization of polyethylene terephthalate waste using *Streptomyces* species and kinetic modeling of the process. *Biocatalysis and Agricultural Biotechnology*, 17, 25–31. <https://doi.org/10.1016/j.bcab.2018.11.002>
- Gourmelon, G. (2015). *Global Plastic Production Rises, Recycling Lags*.
- Horváth, Z., Menyhárd, A., Doshev, P., Gahleitner, M., Vörös, G., Varga, J., & Pukánszky, B. (2014). Effect of the molecular structure of the polymer and nucleation on the optical properties of polypropylene homo- and copolymers. *ACS Applied Materials and Interfaces*, 6(10), 7456–7463. https://doi.org/10.1021/AM5008535/ASSET/IMAGES/LARGE/AM-2014-008535_0013.JPEG
- Huang, Q. S., Yan, Z. F., Chen, X. Q., Du, Y. Y., Li, J., Liu, Z. Z., Xia, W., Chen, S., & Wu, J. (2022). Accelerated biological depolymerization of polyethylene terephthalate by *Thermobifida fusca* cutinase mediated by *Stenotrophomonas pavanii*. *Science of The Total Environment*, 808, 152107. <https://doi.org/10.1016/J.SCITOTENV.2021.152107>
- Huang, Y., Shang, C., & Li, L. (2021). Novel N-doped graphene enhanced ultrafiltration nano-porous polyvinylidene fluoride membrane with high permeability and stability for water treatment. *Separation and Purification Technology*, 267. <https://doi.org/10.1016/j.seppur.2021.118622>
- Hurley, C. R., & Leggett, G. J. (2009). Quantitative investigation of the photodepolymerization of polyethylene terephthalate film by friction force microscopy, contact-angle goniometry, and X-ray photoelectron spectroscopy. *ACS Applied Materials and Interfaces*, 1(8), 1688–1697. <https://doi.org/10.1021/am900250q>
- Ian Tiseo. (2023, February 8). *Plastic waste worldwide - statistics & facts*. <https://www.statista.com/topics/5401/global-plastic-waste/#topicOverview>
- Koshti, R., Mehta, L., & Samarth, N. (2018). Biological Recycling of Polyethylene Terephthalate: A Mini-Review. In *Journal of Polymers and the Environment* (Vol. 26, Issue 8, pp. 3520–3529). Springer New York LLC. <https://doi.org/10.1007/s10924-018-1214-7>
- Lykidis, A., Mavromatis, K., Ivanova, N., Anderson, I., Land, M., DiBartolo, G., Martinez, M., Lapidus, A., Lucas, S., Copeland, A., Richardson, P., Wilson, D. B., & Kyripides, N. (2007). Genome sequence and analysis of the soil cellulolytic actinomycete *Thermobifida fusca* YX. *Journal of Bacteriology*, 189(6), 2477–2486. <https://doi.org/10.1128/JB.01899-06/ASSET/3C6DE7B4-6AAB-49A7-84FF-5D5B95EC41EF/ASSETS/GRAPHIC/ZJB0060766130004.JPEG>

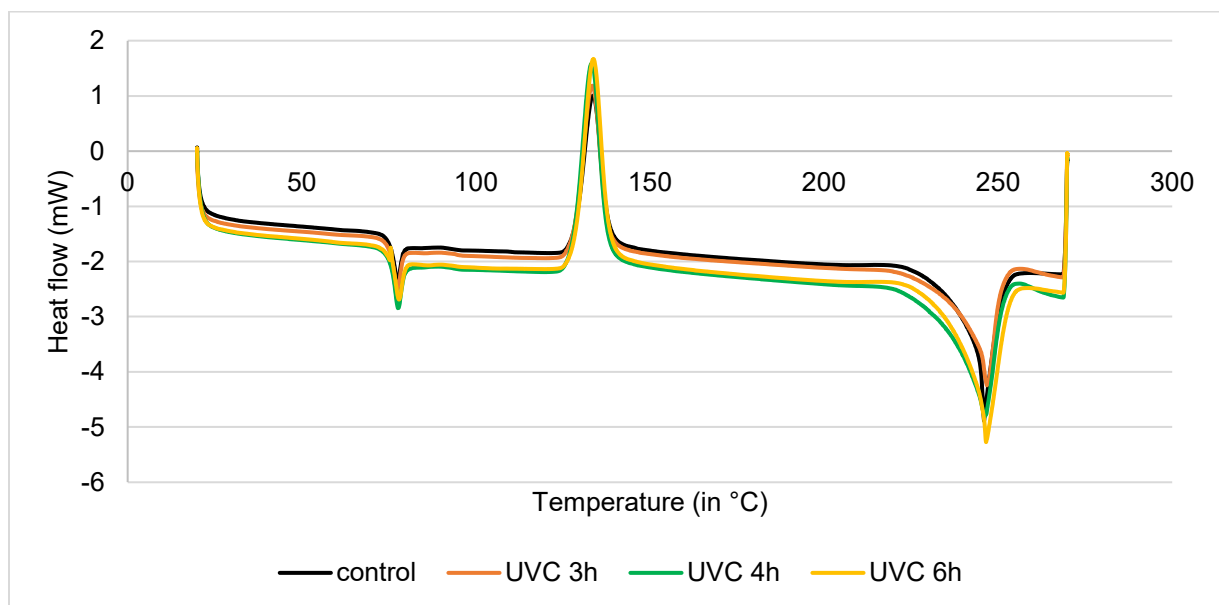
- Maheswaran, B., Al-Ansari, M., Al-Humaid, L., Sebastin Raj, J., Kim, W., Karmegam, N., & Mohamed Rafi, K. (2023). In vivo depolymerization of polyethylene terephthalate using microbial isolates from plastic polluted environment. *Chemosphere*, 310, 136757. <https://doi.org/10.1016/J.CHEMOSPHERE.2022.136757>
- Moges, B. (2022). *Chemical De-polymerization for reuse of Polyethylene Terephthalate (PET) towards a circular economy*.
- Molnár, J., Seps, Ö., Erdei, G., Lenk, S., Ujhelyi, F., & Menyhár, A. (2020). Modeling of light scattering and haze in semicrystalline polymers. *Journal of Polymer Science*, 58(13), 1787–1795. <https://doi.org/10.1002/POL.20200027>
- Norman Jones, R., Ramsay, D. A., Keir, D. S., & Dobriner, K. (1952). The Intensities of Carbonyl Bands in the Infrared Spectra of Steroids. *Journal of the American Chemical Society*, 74(1), 80–88. <https://doi.org/10.1021/ja01121a019>
- Panowicz, R., Konarzewski, M., Durejko, T., Szala, M., Łazińska, M., Czerwińska, M., & Prasula, P. (2021). Properties of Polyethylene Terephthalate (PET) after Thermo-Oxidative Aging. *Materials* 2021, Vol. 14, Page 3833, 14(14), 3833. <https://doi.org/10.3390/MA14143833>
- Paul, M. B., Stock, V., Cara-Carmona, J., Lisicki, E., Shopova, S., Fessard, V., Braeuning, A., Sieg, H., & Böhmert, L. (2020). Micro- And nanoplastics-current state of knowledge with the focus on oral uptake and toxicity. In *Nanoscale Advances* (Vol. 2, Issue 10, pp. 4350–4367). Royal Society of Chemistry. <https://doi.org/10.1039/d0na00539h>
- PlasticsEurope (PEMARG). (2021, June 1). *Annual production of plastics worldwide from 1950 to 2020 (in million metric tons)*. In Statista. <https://www-statista-com.ezproxy.lakeheadu.ca/statistics/282732/global-production-of-plastics-since-1950/>
- Qi, X., Yan, W., Cao, Z., Ding, M., & Yuan, Y. (2022). Current advances in the biological depolymerization and bioconversion of polyethylene terephthalate. *Microorganisms*, 10(1). <https://doi.org/10.3390/microorganisms10010039>
- Rajandas, H., Parimannan, S., Sathasivam, K., Ravichandran, M., & Su Yin, L. (2012). A novel FTIR-ATR spectroscopy based technique for the estimation of low-density polyethylene biological depolymerization. *Polymer Testing*, 31(8), 1094–1099. <https://doi.org/10.1016/j.polymertesting.2012.07.015>
- Revel, M., Châtel, A., & Mouneyrac, C. (2018). Micro(nano)plastics: A threat to human health? In *Current Opinion in Environmental Science and Health* (Vol. 1, pp. 17–23). Elsevier B.V. <https://doi.org/10.1016/j.coesh.2017.10.003>
- Shen, M., Zhang, Y., Zhu, Y., Song, B., Zeng, G., Hu, D., Wen, X., & Ren, X. (2019). Recent advances in toxicological research of nanoplastics in the environment: A review. In *Environmental Pollution* (Vol. 252, pp. 511–521). Elsevier Ltd. <https://doi.org/10.1016/j.envpol.2019.05.102>
- Singh, B., & Sharma, N. (2008). Mechanistic implications of plastic depolymerization. In *Polymer Depolymerization and Stability* (Vol. 93, Issue 3, pp. 561–584). <https://doi.org/10.1016/j.polymdegradstab.2007.11.008>
- Smith, B. C. (2022). Infrared Spectroscopy of Polymers, VIII: Polyesters and the Rule of Three. *Spectroscopy (Santa Monica)*, 37(10), 25–28. <https://doi.org/10.56530/SPECTROSCOPY.TA9383E3>
- Taghavi, N., Udugama, I. A., Zhuang, W. Q., & Baroutian, S. (2021). Challenges in biological depolymerization of non-degradable thermoplastic waste: From environmental impact to operational readiness. *Biotechnology Advances*, 49, 107731. <https://doi.org/10.1016/J.BIOTECHADV.2021.107731>
- Thoden van Velzen, E. U., Brouwer, M. T., Stärker, C., & Welle, F. (2020). Effect of recycled content and rPET quality on the properties of PET bottles, part II: Migration. *Packaging Technology and Science*, 33(9), 359–371. <https://doi.org/10.1002/PTS.2528>
- Torena, P., Alvarez-Cuenca, M., & Reza, M. (2021). Biological depolymerization of polyethylene terephthalate microplastics by bacterial communities from activated sludge. *The Canadian Journal of Chemical Engineering*, 99(S1), S69–S82. <https://doi.org/10.1002/CJCE.24015>
- Vague, M., Chan, G., Roberts, C., Swartz, N., & Mellies, J. (2019). Pseudomonas isolates degrade and form biofilms on polyethylene terephthalate (PET) plastic. *BioRxiv*, 647321. <https://doi.org/10.1101/647321>
- Walker, T. R. (2021). (Micro)plastics and the UN Sustainable Development Goals. In *Current Opinion in Green and Sustainable Chemistry* (Vol. 30). Elsevier B.V. <https://doi.org/10.1016/j.cogsc.2021.100497>
- Welle, F. (2011). Twenty years of PET bottle to bottle recycling - An overview. *Resources, Conservation and Recycling*, 55(11), 865–875. <https://doi.org/10.1016/J.RESCONREC.2011.04.009>
- Welle, F. (2013). A new method for the prediction of diffusion coefficients in poly(ethylene terephthalate). *Journal of Applied Polymer Science*, 129(4), 1845–1851. <https://doi.org/10.1002/APP.38885>
- Wu, W. M., Yang, J., & Criddle, C. S. (2017). Microplastics pollution and reduction strategies. *Frontiers of Environmental Science and Engineering*, 11(1). <https://doi.org/10.1007/s11783-017-0897-7>

Zhu, Z., & Kelley, M. J. (2005). *IR spectroscopic investigation of the effect of deep UV irradiation on PET films*.
<https://doi.org/10.1016/j.polymer.2005.05.135>

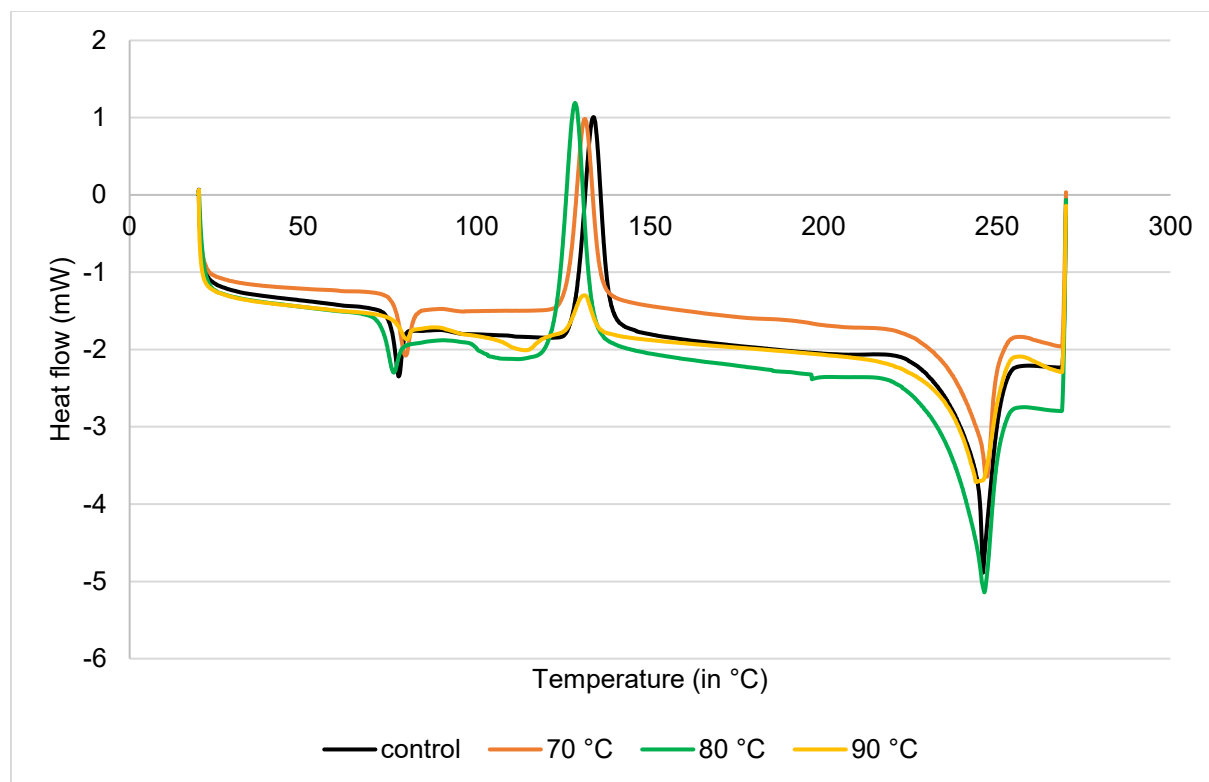
Appendix A



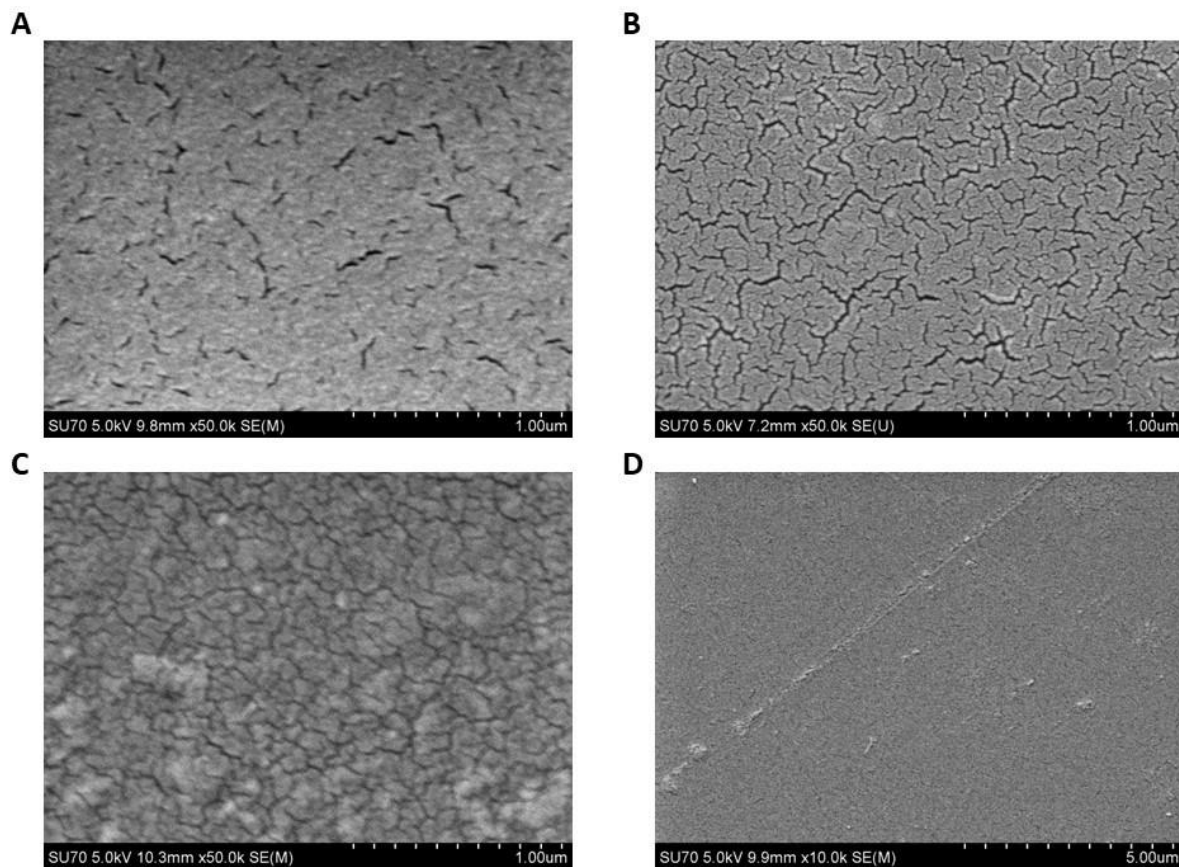
Supplementary Figure 3.1 Differential scanning calorimetric analysis of untreated PET (black) and UVB irradiated PET (3h (orange), 4h (green), 6h (blue))



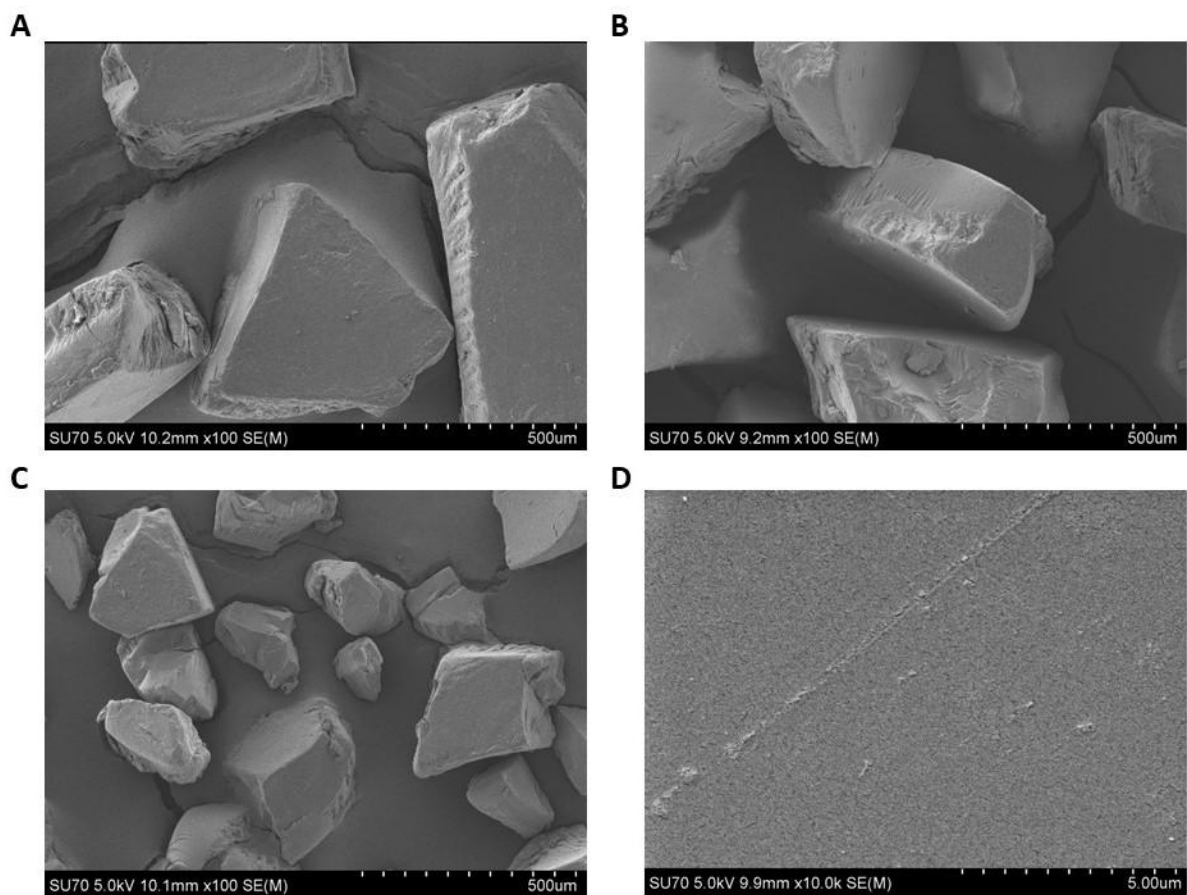
Supplementary Figure 3.2 Differential scanning calorimetric analysis of untreated PET (black) and UVC irradiated PET (3h (orange), 4h (green), 6h (blue))



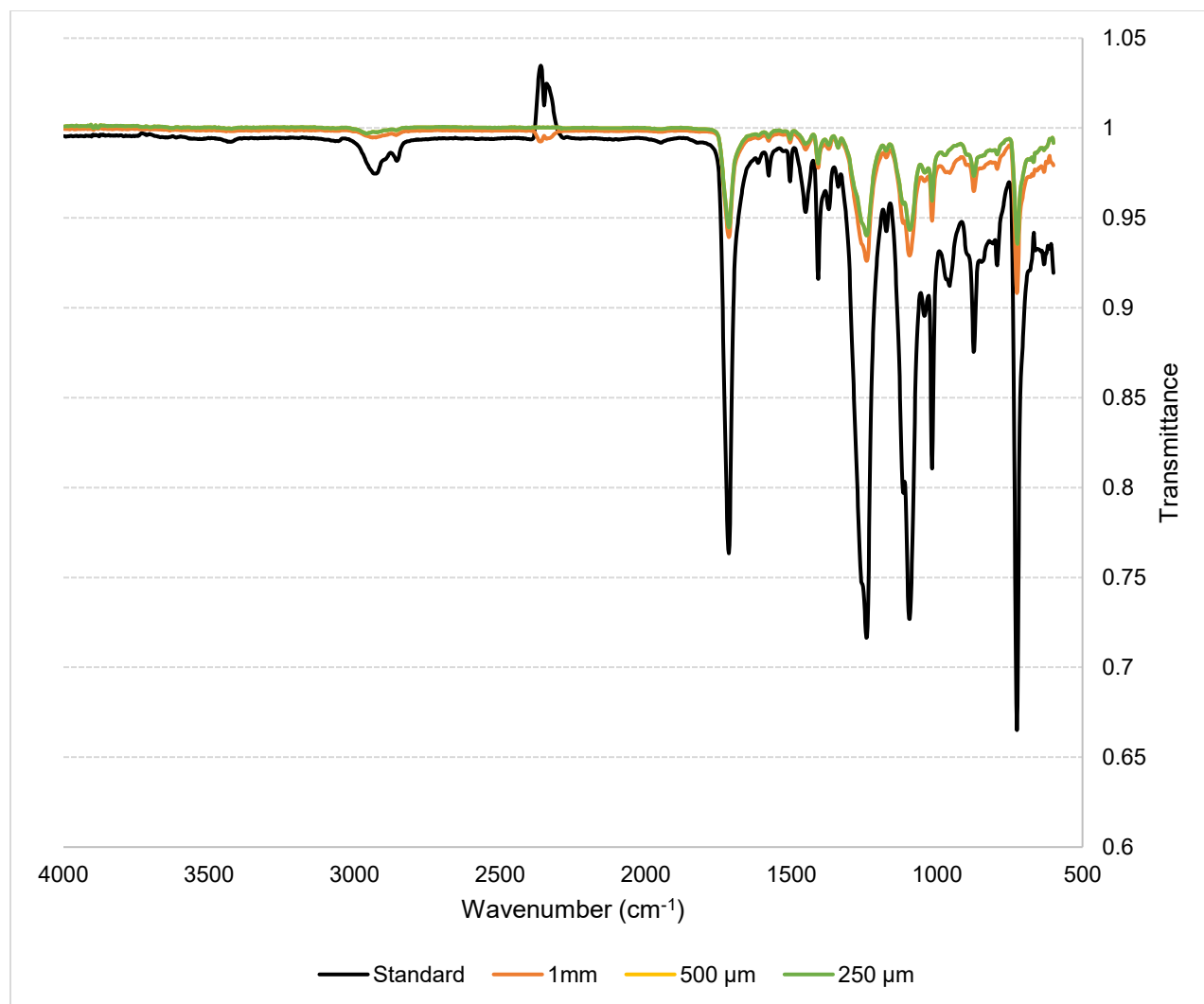
Supplementary Figure 3.3 Differential scanning calorimetric analysis of untreated PET (black) and thermally treated PET (70 °C (orange), 80 °C (green), and 90 °C (blue))



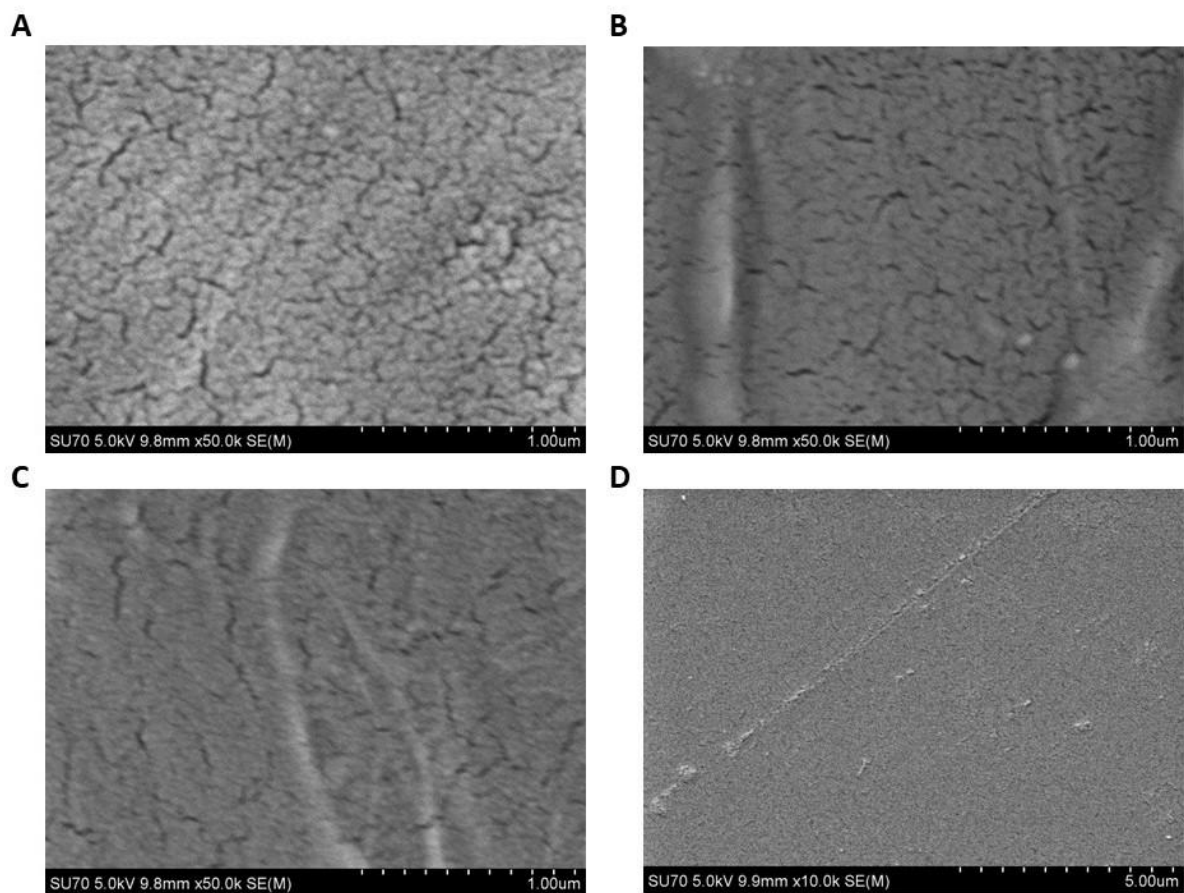
Supplementary Figure 3.4 Scanning electron images of amorphous PET films exposed to (A) 70 °C, (B) 80 °C, and (C) 90 °C for two weeks. Image of untreated PET film (D).



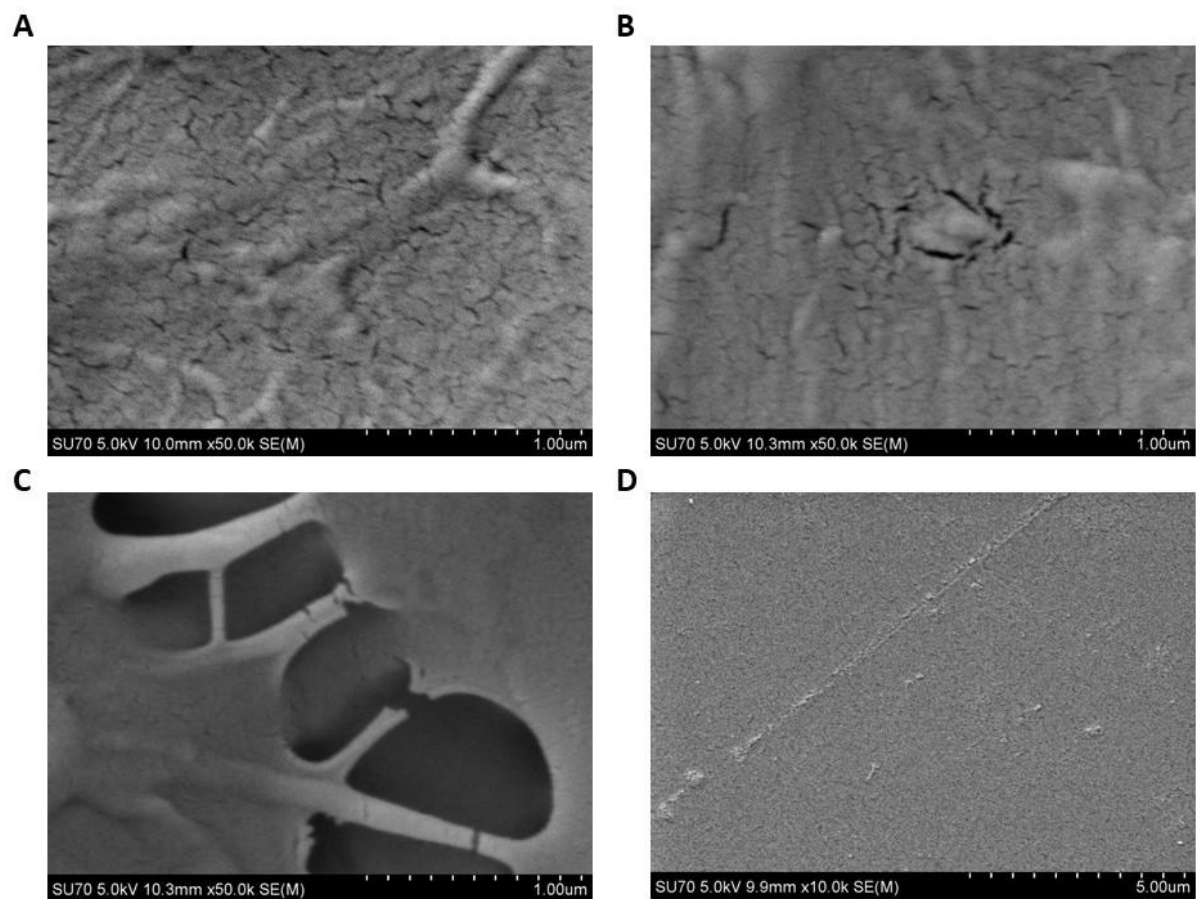
Supplementary Figure 3.5 Scanning electron images of PET particles at sizes (A) 1 mm, (B) 500 μm , and (C) 250 μm . Image of untreated PET film (D).



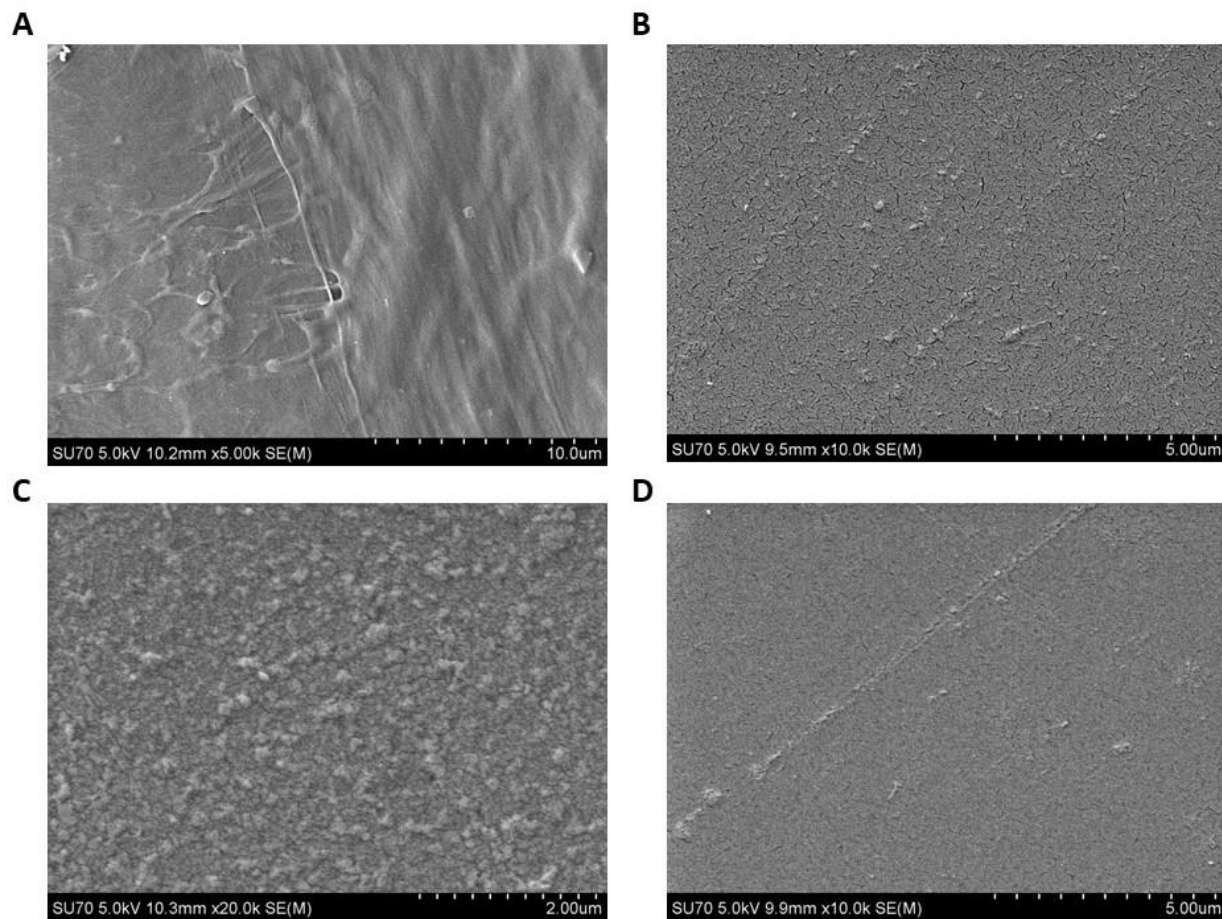
Supplementary Figure 3.6 FTIR spectra of PET particles at sizes 1 mm (orange), 500 μm (yellow), and 250 μm (green) versus untreated PET spectra (black).



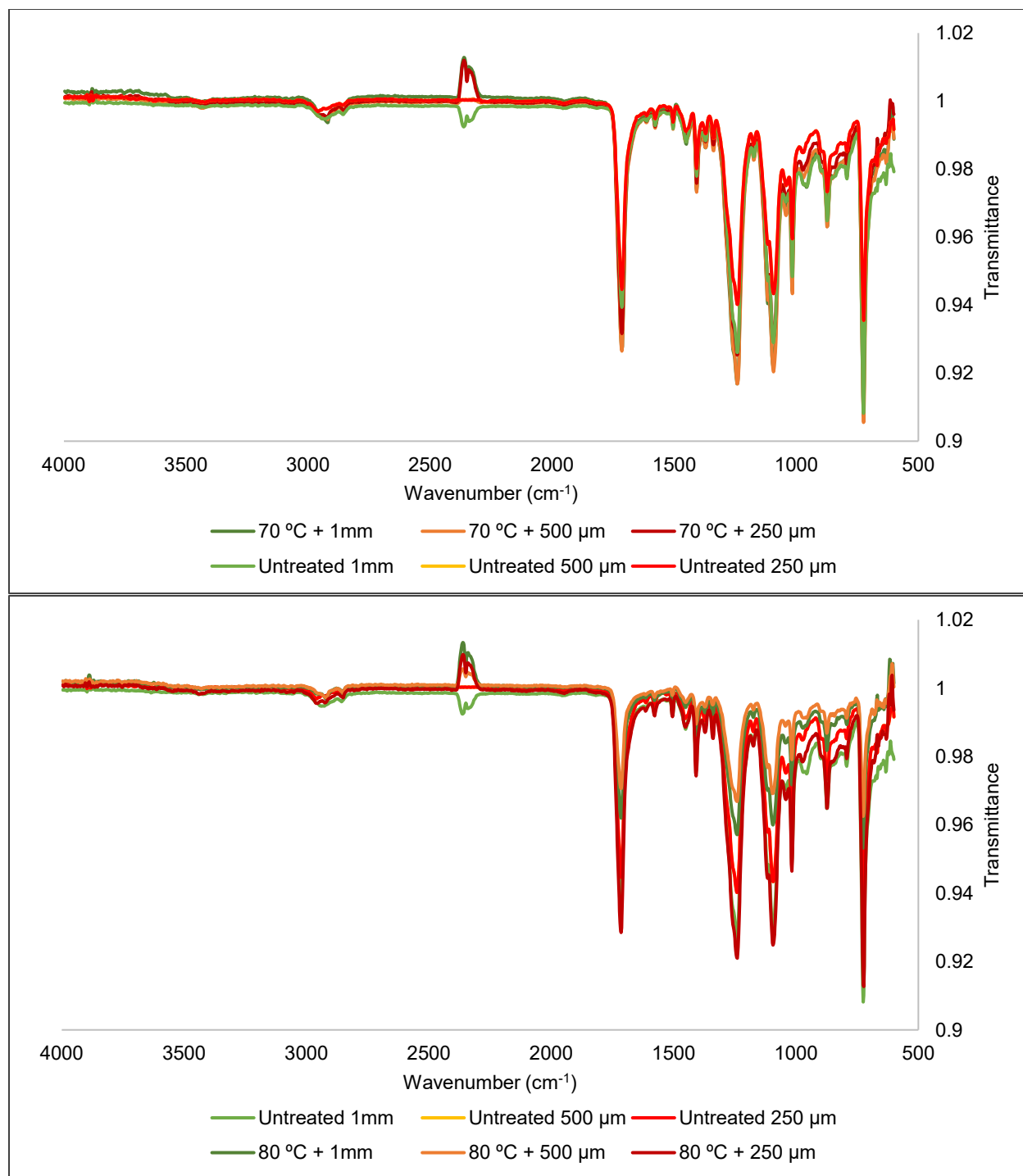
Supplementary Figure 3.7 Scanning electron images of PET particles at sizes (A) 1 mm, (B) 500 μm, and (C) 250 μm pretreated at 70 °C for two weeks. Image of untreated PET film (D).

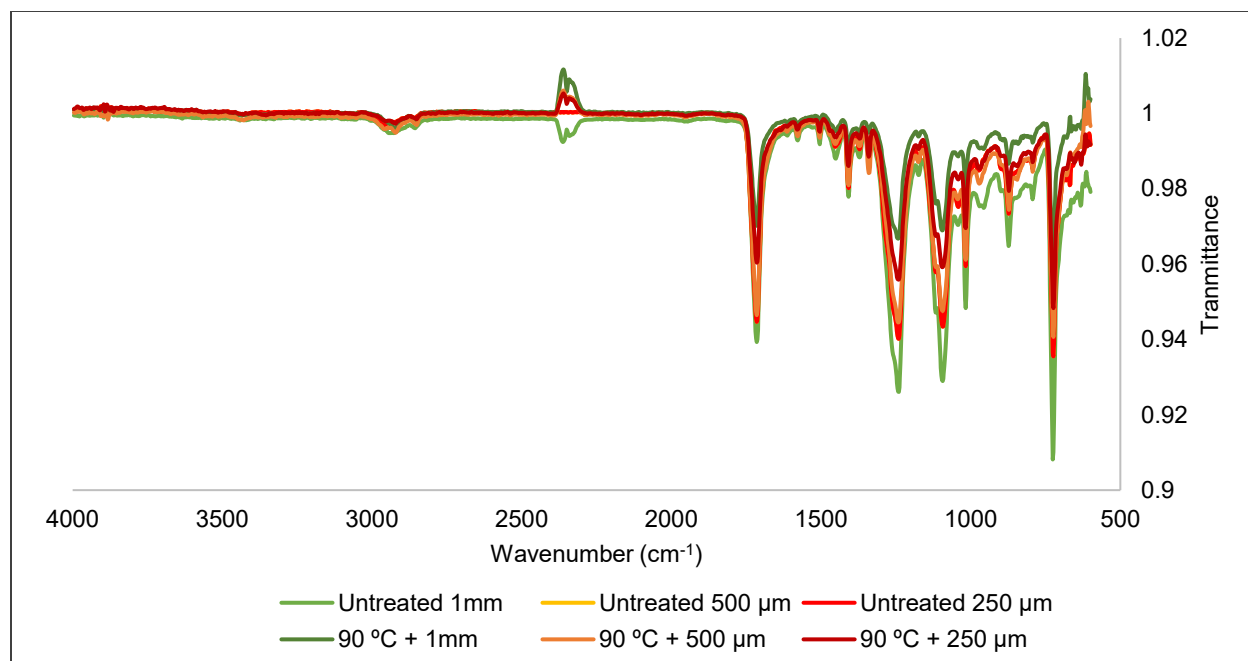


Supplementary Figure 3.8 Scanning electron images of PET particles at sizes (A) 1 mm, (B) 500 μm, and (C) 250 μm pretreated at 80 °C for two weeks. Image of untreated PET film (D).

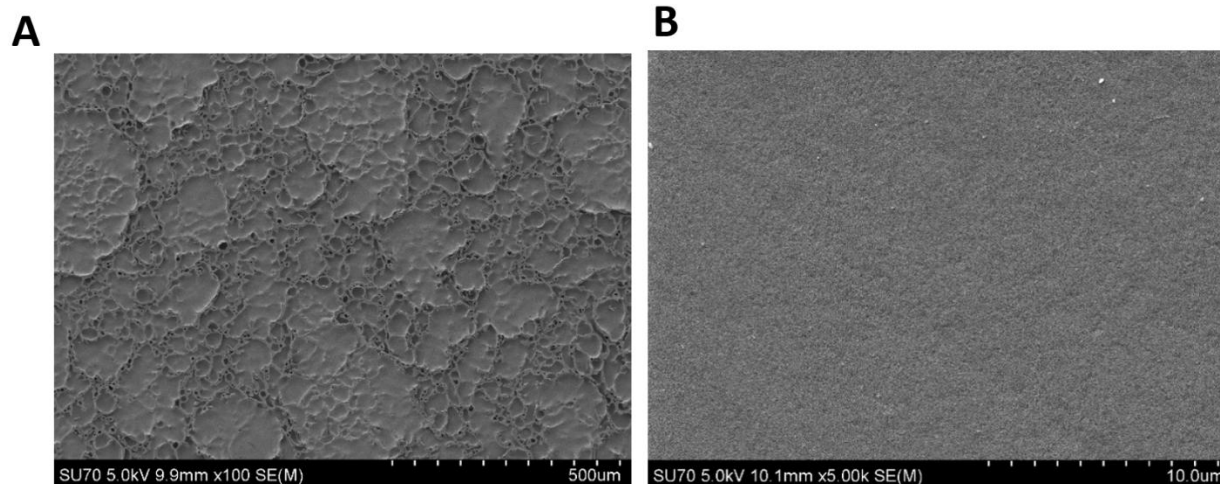


Supplementary Figure 3.9 Scanning electron images of PET particles at sizes (A) 1 mm, (B) 500 μm, and (C) 250 μm pretreated at 90 °C for two weeks. Image of untreated PET film (D).

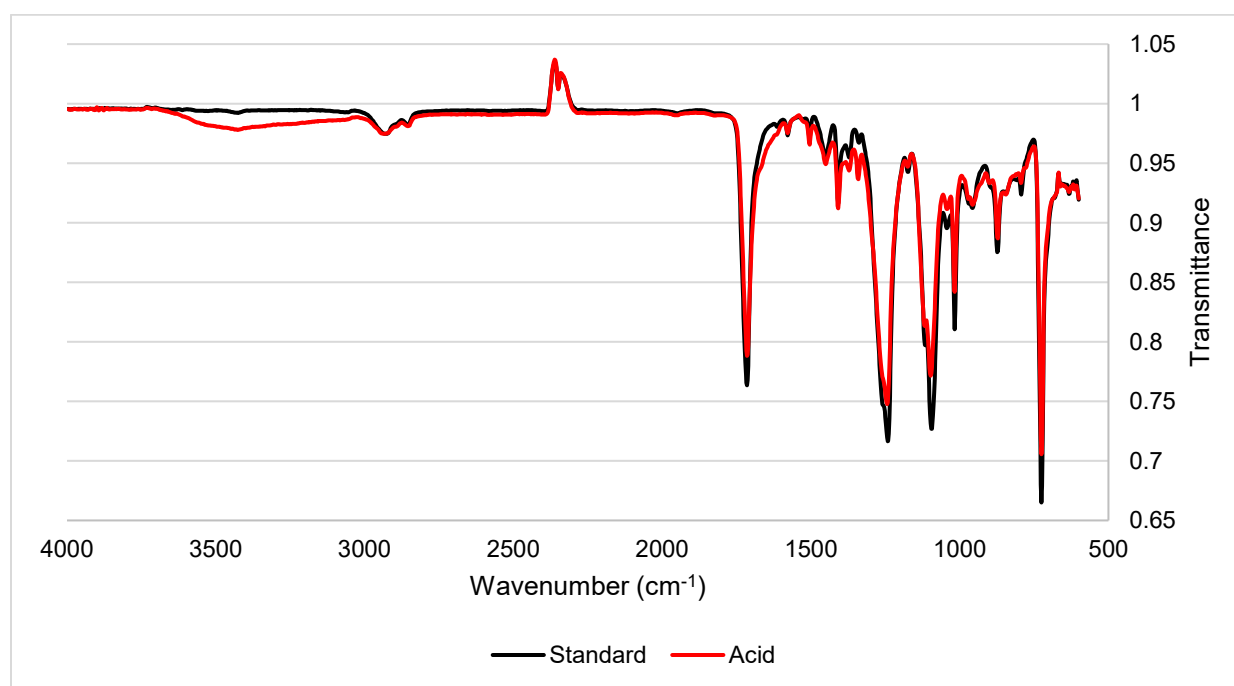




Supplementary Figure 3.10 ATR-FTIR spectra of size reduced particles thermally treated at (A) 70 °C, (B) 80 °C, and (C) 90 °C for two weeks. Untreated PET particles are depicted as follows: 1 mm (green), 500 μm (yellow), and 250 μm (red). **(A)** FTIR spectra of PET particles at sizes 1 mm (dark green), 500 μm (orange), and 250 μm (dark red) exposed to 70 °C for two weeks versus their corresponding untreated samples. **(B)** FTIR spectra of PET particles at sizes 1 mm (dark green), 500 μm (orange), and 250 μm (dark red) exposed to 80 °C for two weeks versus their corresponding untreated samples. **(C)** FTIR spectra of PET particles at sizes 1 mm (dark green), 500 μm (orange), and 250 μm (dark red) exposed to 90 °C for two weeks versus their corresponding untreated samples



Supplementary Figure 3.11 Scanning electron images of PET films treated with (A) acid and (B) image of untreated PET film



Supplementary Figure 3.12 FTIR spectra of acid treated PET film (red) versus untreated PET spectra (black)

Chapter 4

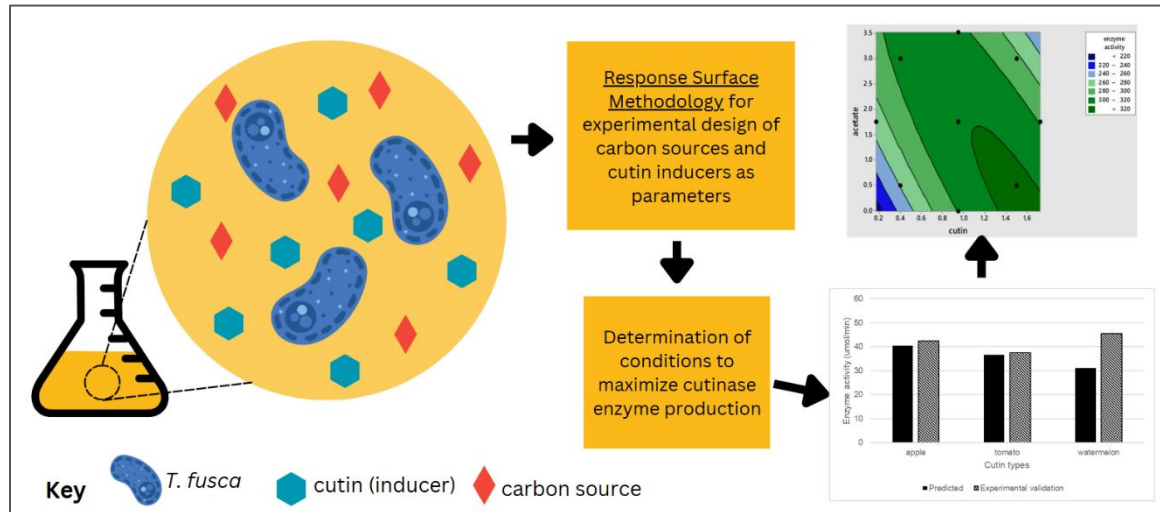
Objective 2

**Response surface methodology optimization for maximum cutinase production from
Thermobifida fusca YX by modification of carbon and cutin sources**

Abstract

Cutinase, an enzyme with significant industrial potential for applications in bioremediation related to the textile, and polymer industries and the biological depolymerization of polyethylene terephthalate (PET) plastics. Optimization of its production conditions need to be carried out to meet commercial demands. Several studies focus on genetically engineering the enzyme. However, its subsequent translation to larger scale applications is limited due to the complications generated as a result of genetic engineering. This study focuses on maximizing cutinase production using the actinomycete *Thermobifida fusca* YX through the application of Response Surface Methodology (RSM). RSM is a statistical technique that helps in the optimization of process parameters using experimental design to determine the relationships between multiple variables and their combined effects. A Central Composite Design (CCD) was utilized to systematically vary the levels of these critical factors and obtain a predictive model based on experimental results. The model's adequacy and predictive capability were assessed using Analysis of Variance (ANOVA) and regression analysis. Optimization results indicated a 42% increase in cutinase yield when grown under the predicted optimal conditions of 0.98 g/L sodium butyrate, 0.98 g/L sodium lactate and 1.6 g/L of Roma tomato cutin, highlighting the efficiency of RSM in enhancing cutinase production. The cutinase obtained as a result was used for the biological depolymerization of PET. This study demonstrates the use of RSM for determining the global maximum for enzyme production taking into account the critical parameters which effect enzyme production using rigorous optimization methods.

Graphical abstract



Keywords:

Cutinase, Response Surface Methodology, Optimization of critical enzyme production parameters, *Thermobifida fusca*.

4.1 Introduction

Of the multitude of global challenges our society currently faces, plastic accumulation has attracted considerable attention to invoke a change in our current environmental management system. Single-use plastics like polyethylene terephthalate (PET or PETE) are one of the largest contributors of disposed plastics in our environment (Geyer et al., 2017). The scientific community has made significant effort for the biological depolymerization of PET using PET-hydrolyzing enzymes (PHEs) (Z. Chen et al., 2020; Pirillo et al., 2023; Puspitasari et al., 2021; Sagong et al., 2020; Sulaiman et al., 2012; Tournier et al., 2020). Cutinase is one such enzyme that has attracted attention for its PET biological depolymerization abilities.

Cutinase enzyme production by microbes can be induced by cutin, a naturally occurring aliphatic polyester that consists of long chain fatty acid (Fett et al., 1999). Several plant pathogens and opportunistic pathogens secrete cutinase to cleave the hydrophobic cutin. Cutin is the protective layer over most leaves and fruits and usually consists of different monomeric units depending on its origin (Fich et al., 2016b; Nawrath, 2002). Studies have reported variations in monomeric constituents even between species/cultivars of certain fruits like apples. Cutin from apples and tomatoes have been shown to increase cutinase production in *Thermobifida fusca* spp (Fett et al., 1999). Cutins from apples and tomatoes have high concentrations of the monomer 10,16-dihydroxydecanoic acid (Arrieta-Baez et al., 2020; Osman et al., 1999). This monomer has been speculated to be an important inducer for cutinase production. Additionally, watermelon cutin has been reported to increase cutinase production in *Streptomyces* spp. (Chaudhari & Singhal, 2015; Sales et al., 2020). In addition to inducers, carbon sources used in microbial media also play a role in cutinase production. Glucose has been known to have catabolite repression and sometimes is not good for enzyme production (Brückner & Titgemeyer, 2002). Other carbon sources have been

shown to increase enzyme production under fermentation conditions. Sodium acetate, and a combination of butyrate and lactate have been previously shown to increase the production of cutinase in a batch fermenter (He et al., 2009). However, there are very few studies that focus on how carbon sources affect enzyme production (Dalmau et al., 2000; Kakde & Chavan, 2011; Kanwal & Reddy, 2011). A combination of carbon sources and inducers which can lead to manifold increase in enzyme production.

Genetic engineering has emerged as one of the most extensively researched strategies for enhancing PET biological depolymerization, as demonstrated by numerous studies (Cui et al., 2021; Puspitasari et al., 2021; Silva et al., 2011; Son et al., 2019; Tournier et al., 2020; Wei et al., 2016). However, despite its promise, this approach comes with significant challenges that cannot be overlooked (Gero et al., 1997). Central to genetic engineering is the use of marker genes found in plasmids, which are transmissible elements whose mobility allows them to transfer across various bacterial species and sometimes, even genera. These marker genes, which may include metabolic breakdown indicators, antibiotic resistance genes, or chromophore producers, raise serious concerns regarding environmental safety. The potential for these plasmids to inadvertently spread traits among different organisms poses a risk that could have far-reaching ecological implications. Moreover, the process of developing such genetic constructs is complex, demands substantial financial resources, time and skilled labour (Berry, 2007; Gero et al., 1997). Additional complications like predictability of genetic stability in different environmental conditions, complex interactions with the existing microorganisms in the environment, the risk of disrupting the existing microbial balance in the environment, challenges controlling the population growth of the genetically modified bacterium, complicate the path to effective and reliable solutions (Berry, 2007; Gero et al., 1997). Given these challenges, it is crucial to explore alternative strategies that

might yield safer and more sustainable outcomes. One promising avenue is the optimization of the bacterium's environment to enhance enzyme production naturally. This approach not only circumvents the risks associated with genetic manipulation but also embraces a more holistic method of fostering biological depolymerization processes. By focusing on environmental modifications, we can potentially maximize efficiency while minimizing ecological impact, paving the way for a more sustainable solution in PET biological depolymerization.

Most studies for optimization of fermentation control variables follow a “One Variable At a Time (OVAT)” approach, which does not consider interacting variables. Response Surface Methodology (RSM) allows the study of relationships between several interacting response variables using methods like central composite design (CCD), Box Behnken design or Pareto chart. One of the first studies for cutinase production from *Fusarium oxysporum* optimized all the media components required for maximal production of cutinase using RSM (Alves Macedo & Fontes Pio, 2005). Another group isolated *Aspergillus* sp. RL2Ct which demonstrated high esterolytic activity and optimized the cutin concentration, temperature, and calcium chloride concentration in the medium using CCD (Kumari et al., 2016). In a more recent study, cutinase production from *Fusarium verticillioides* was optimized for its application as a potential insecticide degrader using RSM (Parmar & Rahi, 2023).

This study aims to focus on the optimization of the environment (bacterial growth medium) of *Thermobifida fusca* YX for the maximal production of cutinase. We explore combinations of the carbon sources sodium acetate, sodium butyrate and sodium lactate and cutin (inducer) obtained from Royal gala apples, Roma tomatoes and watermelons. The interacting variables of cutin sources and carbon sources were optimized using Response Surface Methodology with a Central

Composite Design to find the optimal concentration for maximal cutinase production. The predicted values obtained by optimization for the maximum production of cutinase were then experimentally validated. Cutinase was produced at larger volumes so that partial purification of the extracellular enzyme could be carried out. The partially purified enzyme was tested for PET biological depolymerization.

4.2 Materials and Methods

4.2.1 Chemical, microorganism and cultivation

The actinomycete, *Thermobifida fusca* strain YX (ATCC BA-629), was purchased from Cedarlane (Canada). All the other chemicals and solvents were purchased from Fisher Scientific (New Hampshire, USA) at reagent grade and used without further modification unless specified otherwise. The strain was cultivated in 50 mL of tryptone yeast extract (TYE) broth in an unbaffled 100 mL Erlenmeyer flask for 3 days at 50 °C at 150 rpm in a rotary shaker. Amorphous PET film used for the hydrolysis studies were purchased from Goodfellow (Canada) (Product no.: ES301445).

4.2.2 Carbon source experiments

Studies for determination of media conditions to maximize production of cutinase began with selecting the carbon sources. The carbon sources selected, based on literature review, were sodium acetate, sodium butyrate, sodium lactate, and glucose. First, the range of each of the carbon sources was determined. For this, *T. fusca* YX was grown in Tryptone Yeast Extract (TYE) medium with one of the chosen carbon sources. Additionally, combinations of the carbon sources were assessed for additive or synergistic effect on cutinase production. The concentration was up to 10 g/L of each carbon source with 0.5 g/L increments. Glucose was used as a negative control due to

catabolite repression and its ability to reduce inducible enzyme production (Brückner & Titgemeyer, 2002). After the determination of the upper limit for the carbon sources, *T. fusca* YX was grown in the presence of 0.4% w/v cutin from Royal Gala apple peels (see Section 2.3). The supernatant was assessed for cutinase activity (see Section 2.5).

4.2.3 Cutin extraction

The protocol for cutin extraction was adapted from Fett et al. (1999). Royal gala apple peels, tomato peels and watermelon peels were placed in the -20 °C freezer for 4 hours. An oxalate buffer was prepared by mixing 16 g/L ammonium oxalate and 4 g/L oxalic acid. The peels were then added to the oxalate buffer solution (pH 3.5) and incubated at 80 °C for 2 hours. After 2 hours, the cutin containing films were peeled off the rinds and rinsed with deionized water twice before being dried in the hot air oven at 50 °C overnight. The dried films were powdered using a mortar and pestle for further experiments.

4.2.4 Experimental design using Response Surface Methodology (RSM) with Central Composite Design (CCD)

In order to improve the effectiveness of cutinase production, the environmental conditions of *Thermobifida fusca* were optimized for maximum cutinase expression and secretion. The experimental design was carried out in central composite design technique under Response Surface Methodology in Minitab v.17. This software was used for experimental design, statistical analysis, quadratic model buildings, and generation of graph plots. The two main affecting factors for cutinase expression are carbon and inducer sources and concentrations. As previously mentioned, the carbon sources selected after preliminary experiments were sodium acetate, and a combination of sodium butyrate and sodium lactate. The range for these were 0.5 – 3.5 g/L. Cutin is a polymer, whose constituent monomers vary slightly depending on its origin. Cutin from Roma tomatoes,

Royal Gala apples and watermelons were chosen as inducers. From the experiments described in Section 2.2, the range for inducers was 0-3.5 g/L based on Fett et al. (1999). The range of the independent variables and their coded and actual levels are listed in Table 4.1. The end points of the ranges are coded as α and $-\alpha$, while 1 and -1 are the midpoint values between the endpoint and zero. Six combinations were used in this study: Combination 1, 2 and included sodium acetate as carbon source and Roma tomato, Royal Gala apple and watermelon cutin respectively, Combination 4, 5 and 6 included sodium butyrate and sodium lactate combination source and Roma tomato cutin, Royal Gala apple cutin and watermelon cutin respectively. The data was analyzed using a one-way analysis of variance (ANOVA) using Minitab v.17 software.

Table 4.1. The experimental range and levels of independent variables for maximum cutinase production. (Combinations 1,2 and 3 used sodium acetate as the carbon source with cutin from Roma tomato, Royal Gala apple and watermelon respectively. Combinations 4,5 and 6 had sodium butyrate and sodium lactate as carbon sources with the cutin from Roma tomato, Royal Gala apple and watermelon respectively)

Combinations	Variables	Coded factor levels (in g/L)				
		$-\alpha$	-1	0	1	α
1, 2, & 3	Na-acetate	0*	0.5	1.75	3	3.518
	Cutin	0.172	0.4	0.95	1.5	1.728
4, 5, & 6	Na-butyrate	0.98	0.5	2.25	3	3.51
	Na-lactate	0.98	0.5	2.25	3	3.51
	Cutin	0.025	0.4	0.95	1.5	1.87

*At this point, the statistical model gave a value of -0.018 g/L of Na-acetate, hence, the experiment was carried out without adding the carbon source.

4.2.5 Analysis of esterase activity by para-nitrophenyl butyric acid (pNPB) hydrolysis assay

Cutinase (esterase enzyme) activity was quantified using pNPB (10 μ L of 10 mM) as the substrate in 1 mL of 200 mM Tris-Cl (pH 8.0). The reaction was started by the addition of 100 μ L of extracellular enzyme supernatant obtained after fermentation and incubated at 60 °C for 8 minutes. The generation of para-nitrophenol was quantified at 405 nm using the xMark™ Microplate Absorbance Spectrophotometer, Bio-Rad Laboratories Inc. Protein concentration in the supernatant was determined by measuring absorbance at 280 nm (Stoschek, 1990) using a calculated molar coefficient of 16,180 M⁻¹.cm⁻¹.

4.2.6 Partial purification and concentration of cutinase

The production of cutinase was scaled up 10 times to a level of 250 mL with TYE along with carbon and cutin concentrations at level determined by optimization. The actinomycete, *T. fusca* YX was grown for 5 days at 50 °C at 150 rpm. The secreted cutinase was precipitated using ammonium sulphate (60% saturation). The precipitated protein was separated by centrifugation and resuspended in 50 mL of 200 mM Tris-Cl buffer pH 8.0. The partially purified enzyme was tested for PET biological depolymerization, which is described in Section 4.2.7.

4.2.7 PET biological depolymerization

The reaction system consisted of amorphous PET films (1x1 inch) and 100 μ L of crude enzyme extract that were incubated with 10 mL of 200 mM Tris HCl buffer at pH 8. This system was incubated at 60 $\pm$ 2 °C at 150 rpm. After 3 days, the PET samples were washed with deionized water thrice before drying in the hot air oven at 40 °C. These samples were then weighed to determine weight loss.

4.3 Results and Discussion

4.3.1 Effect of carbon source on cutinase production

Cutinase production is affected by the kinds and amounts of carbon and nitrogen sources (Du et al., 2007) as well as cutin sources and concentration (Fett et al., 1999). To ascertain the optimal concentration range of carbon source for cutinase production, *Thermobifida fusca* YX was cultivated using either a single carbon source and a mixture of carbon sources, viz., glucose, sodium acetate, sodium butyrate and sodium lactate. Bacteria grown in the presence of organic acids exhibit a change in their metabolic behavior to accommodate the uptake and utilization of a non-sugar carbon source (Dalmau et al., 2000; Deng, 2011). Additionally, organic acids like acetic acid, lactic acid and butyric acid are end products of fermentation pathways that can inhibit bacterial growth at higher concentrations (Nakai & Siebert, 2003; Pinhal et al., 2019). However, when used as a primary carbon source at optimal concentrations, it can increase enzyme production.

The actinomycete did not grow in in any medium with concentrations above 5 g/L for both a single carbon source and two carbon sources. However, *T. fusca* YX did grow in the medium without any carbon source indicating that the secondary nutrient sources in the medium, tryptone and yeast extract, supported the growth of the actinomycete. Additionally, the combination of sodium butyrate with sodium lactate or sodium acetate inhibited the growth of *T. fusca* YX (p-value: 0.0070). Furthermore, to determine the optimal combinations of carbon sources that yield elevated levels of cutinase, *T. fusca* YX was cultivated using maximum tolerable concentrations of various carbon sources in conjunction with Royal Gala apple cutin. Cutinase production increased up to 15-fold for all carbon source combinations tested in the presence of cutin. Carbon sources had an influence on enzyme production as well. Two combinations of carbon sources with cutin showed

an increase compared to the control (TYE + cutin), viz., acetate, and the combination of butyrate and lactate. The combination of butyrate, lactate and 0.4% w/v cutin derived from Royal Gala apple peels resulted in 37.56 $\mu\text{mol}/\text{min}$ enzyme activity while acetate and with the same level and concentration of cutin resulted in 35.85 $\mu\text{mol}/\text{min}$ enzyme activity (p-value: 4.407E-15) (see Fig. 4.1). The range selected for the optimization experiments were 0.5-3g/L for each carbon source, thus allowing a total of 6 g/L for two carbon source combinations.

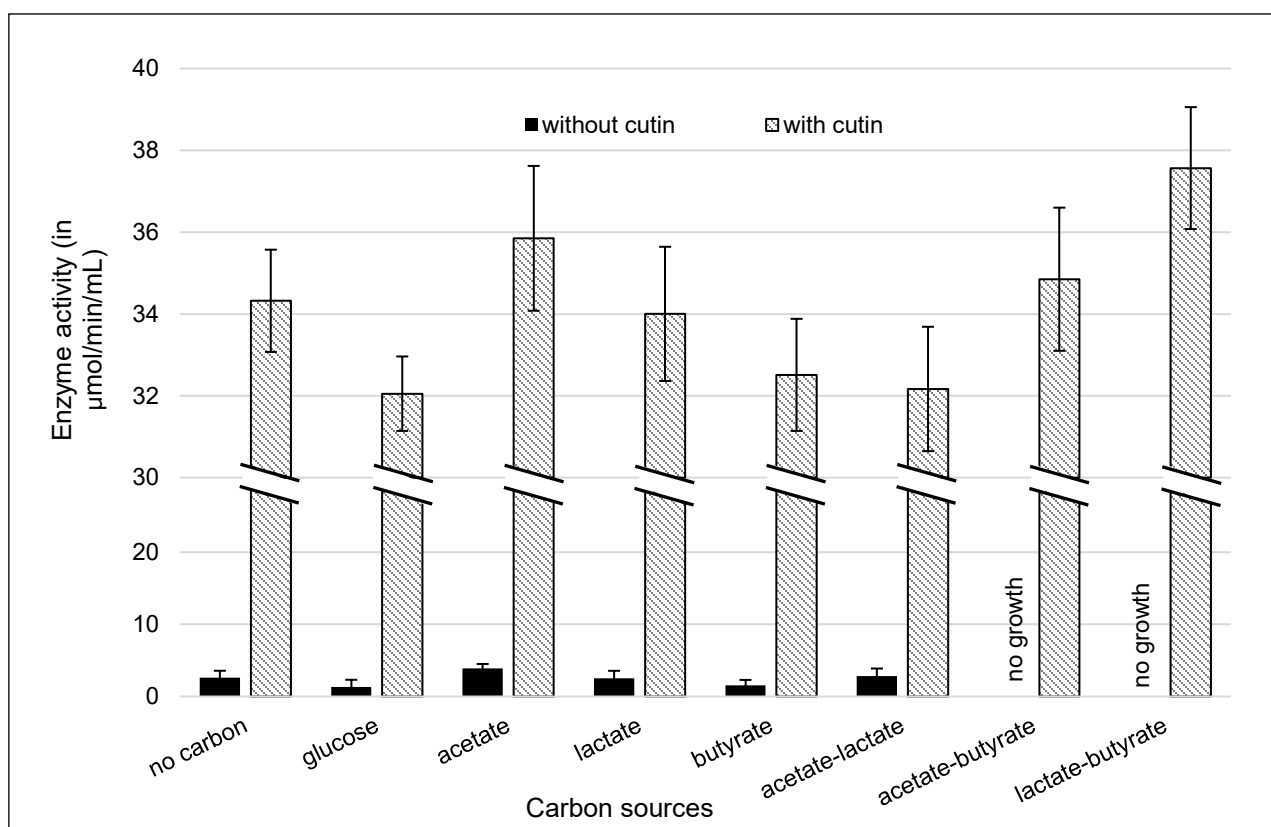


Figure 4.1. Comparison of cutinase expression when grown in the presence of Royal Gala apple cutin and different carbon sources, glucose (5g/L), acetate (5g/L), lactate (5g/L), butyrate (5g/L), acetate-lactate (2.5g/L + 2.5g/L), butyrate-acetate (2.5g/L + 2.5g/L), and butyrate-lactate (2.5g/L + 2.5g/L).

After the preliminary experiments, the carbon sources acetate, and the combination of butyrate and lactate were further optimized for maximum production of cutinase using RSM (see next section). Interestingly, *T. fusca* YX grown with butyrate and a second carbon source resulted in no growth and hence no enzyme production. However, *T. fusca* YX grew with the two carbon sources in the presence of cutin. This suggests that cutin negates the inhibitory effect of the combination of butyrate and lactate. In fact, the *T. fusca* YX grown with butyrate, lactate in the presence of cutin, gave the highest enzyme activity among the combinations studied. In a similar study, *T. fusca* WSH03-11, was grown on various ratios of acetate, lactate and butyrate along with 1.5 g/L apple cutin. Highest cutinase activity was reported with a 4:1:3 ratio of butyrate, acetate, and lactate, indicating that higher quantities of butyrate and lactate are preferred for cutinase production in the presence of apple cutin. Subsequently, *T. fusca* WSH03-11 was cultivated using a two-stage fermentation strategy, batch cultivation using carbon sources acetate and lactate, followed by a fed-batch cultivation with a 12 mL/h feed rate of butyrate which resulted in a 70% increase in cutinase activity (He et al., 2009).

Despite grown in the presence of cutin, there was low levels of enzyme production when *T. fusca* YX was grown on glucose. Glucose being a readily consumable sugar causes catabolite repression. Catabolite repression occurs via the PEP uptake system that inhibits several other metabolic pathways by inhibiting their uptake from the extracellular environment (Brückner & Titgemeyer, 2002; Doelle et al., 1982). Additionally, since all the metabolic pathways of *T. fusca* YX have not yet been elucidated, it can be speculated that the reduction in enzyme activity observed with sodium lactate and sodium butyrate could be due to catabolite repression.

4.3.2 Optimization of carbon and cutin sources using Response Surface Methodology

A central composite design was utilized to identify the combination with the maximum enzyme production by analyzing two factors: the concentration of carbon source and inducer. Cutin from various sources have varying monomeric constituents that could contribute towards induction of enzyme production. Hence, enzyme production was optimized by selecting two carbon sources, Na-acetate, and a combination of Na-butyrate and Na-lactate, with the addition of cutin from three sources, Roma tomatoes, Royal Gala apples and watermelon peels. The optimizations using these six combinations were assessed using the Statgraphics Centurion XV software. Using RSM and the limits listed in Table 1, experimental design generated a series of combinations to be conducted experimentally (Tables 4.2 & 4.3). A series of thirteen experiments were performed for the two variable combinations, acetate and cutin, and twenty experiments for the three variable combinations, butyrate, lactate and cutin. After determining the enzyme activity for each combination, an approximate regression model of the enzyme activity based on the experimental results was evaluated and expressed as quadratic equations (see Table 4.4). The graphical representation of the response surface is displayed as the three-dimensional contour plot. The generated model was analyzed using ANOVA (see Supplementary Tables 1-6 in Appendix B). The second-order response surface plot for optimizing three variables based on the suggested statistically optimized model is displayed in Figs. 4.2 and 4.3. Each three-dimensional response plot represents the maximum points within the area of experimentation. Additionally, the optimal values for each of the variables are described in Table 4.5. The optimal values for each variable predicted for every combination were then validated.

The graphs presented in Figure 2 demonstrate that, across all cutin sources, enzyme activity tends to be higher at lower concentrations of Na-acetate. These findings align with the research by He et al. (2009), who cultivated *T. fusca* YX using varying concentrations of Na-acetate while

maintaining a constant concentration of 4 g/L Na-butyrate. Their results indicated that both cell growth and enzymatic activity diminished as the acetate concentration increased. In a similar vein, Peng et al., (2024) observed that the growth of a genetically modified *Fusarium verticillioides*, along with its ability to transcribe cutinase, was significantly impacted when Na-acetate was utilized as a carbon source.

When examining the mixture of Na-butyrate and Na-lactate as carbon sources, only tomato cutin exhibited behavior similar to that observed with Na-acetate as the sole carbon source, showing increased production at lower carbon source concentrations. Conversely, enzyme activity was enhanced with apple and watermelon cutin as inducers. Overall, the enzyme activity with combinations 4, 5, and 6 (Na-butyrate and Na-lactate) exceeded that observed with combinations 1, 2, and 3 (Na-acetate), as previously mentioned. Graphical representations of the response surface are displayed as the three-dimensional contour plot in Fig. 4.2. The second-order response surface plot for optimizing three variables based on the suggested statistically optimized model is displayed. Each three-dimensional response plot represents the maximum points within the area of experimentation. Additionally, the optimal values for each of the variables are displayed in Fig. 4.3 and Table 4.5. The optimal points for each variable predicted for each combination were then experimentally validated.

Table 4.2. Experimentally determined enzyme activity using the experimental design provided by RSM for Combinations 1, 2, and 3. (Combination 1,2 and 3 used sodium acetate as the carbon source with cutin from Roma tomato, Royal Gala apple and watermelon respectively)

Run	Na-acetate (g/L)	Cutin (g/L)	Enzyme activity with tomato cutin ($\mu\text{mol/min/mL}$)	Enzyme activity with apple cutin ($\mu\text{mol/min/mL}$)	Enzyme activity with watermelon cutin ($\mu\text{mol/min/mL}$)
1	1.75	0.95	39.42	41.45	36.98
2	1.75	0.95	33.46	46.5	30.74
3	0.5	1.5	33.14	41.79	29.14
4	3.0	1.5	32.83	34.83	30.17
5	0	0.95	33.87	35.94	30.56
6	1.75	0.95	30.53	38.46	27.35
7	1.75	0.95	32.05	35.78	27.87
8	1.75	0.95	34.77	36.68	30.88
9	1.75	1.72	33.13	36.86	31.03
10	1.75	0.17	32.07	33.76	28.39
11	3.51	0.95	34.34	39.32	30.44
12	3.0	0.4	35.82	37.04	27.91
13	0.5	0.4	37.75	33.71	37.51

Table 4.3. Experimentally determined enzyme activity using the experimental design provided by RSM for Combinations 4, 5, and 6. (Combination 4,5 and 6 had sodium butyrate and sodium lactate as carbon sources with the cutin from Roma tomato, Royal Gala apple and watermelon respectively)

Run	Na- butyrate (g/L)	Na- lactate (g/L)	Cutin (g/L)	Enzyme activity with tomato cutin ($\mu\text{mol/min/mL}$)	Enzyme activity with apple cutin ($\mu\text{mol/min/mL}$)	Enzyme activity with watermelon cutin ($\mu\text{mol/min/mL}$)
1	2.25	2.25	0.95	38.28	38.11	37.6
2	1.5	1.5	0.4	27.23	29.85	33.48
3	2.25	2.25	0.95	30.43	33.76	27.18
4	2.25	2.25	1.87	37.19	34.58	33.76
5	2.25	0.98	0.95	35.57	33.76	33.24
6	1.5	3.0	0.4	32.49	30.22	32.09
7	2.25	2.25	0.95	34.65	32.86	31.87
8	0.98	2.25	0.95	33.57	33.83	30.51
9	2.25	2.25	0.95	32.28	33.15	35.66
10	2.25	2.25	0.95	31.52	32.81	34.27
11	1.5	3.0	1.5	33.71	31.59	32.35
12	2.25	2.25	0.95	33.73	31.82	32.37
13	3.0	1.5	1.5	28.41	32.01	34.79
14	3.0	1.5	0.4	28.57	25.31	30.06
15	2.25	3.51	0.95	28.9	30.01	32.74
16	2.25	2.25	0.025	11.42	13.35	1.57
17	3.0	3.0	1.5	36.28	33.17	40.52
18	1.5	1.5	1.5	36.72	25.93	29.04
19	3.0	3.0	0.4	32.39	24.51	29.94
20	3.51	2.25	0.95	29.14	36.33	39.04

Table 4.4. Optimized quadratic equations for enzyme activity of the predicted regression models (Combination 1,2 and 3 used sodium acetate as the carbon source with cutin from Roma tomato, Royal Gala apple and watermelon respectively. Combination 4,5 and 6 had sodium butyrate and sodium lactate as carbon sources with the cutin from Roma tomato, Royal Gala apple and watermelon respectively)

Combination	Equation for enzyme activity in terms of the carbon and cutin sources
1 ^a	$36.4369 - 1.67096*\text{acetate} - 0.281024*\text{cutin} + 0.271188*\text{acetate}^2 + 0.589091*\text{acetate}*\text{cutin} - 1.12445*\text{cutin}^2$
2 ^b	$22.841 + 5.86114*\text{acetate} + 22.2887*\text{cutin} - 0.625144*\text{acetate}^2 - 3.74182*\text{acetate}*\text{cutin} - 7.05792*\text{cutin}^2$
3 ^c	$38.7673 - 4.89521*\text{acetate} - 5.68758*\text{cutin} + 0.0980244*\text{acetate}^2 + 3.86545*\text{acetate}*\text{cutin} - 0.851088*\text{cutin}^2$
4 ^d	$24.0343 - 2.80935*\text{butyrate} - 4.66044*\text{lactate} + 31.8229*\text{cutin} - 0.237328*\text{butyrate}^2 + 2.09778*\text{butyrate}*\text{lactate} - 2.11515*\text{butyrate}*\text{cutin} + 0.315798*\text{lactate}^2 - 1.27879*\text{lactate}*\text{cutin} - 8.68112*\text{cutin}^2$
5 ^e	$20.6082 - 4.18578*\text{butyrate} + 7.77033*\text{lactate} + 12.399*\text{cutin} + 0.449443*\text{butyrate}^2 - 1.26*\text{butyrate}*\text{lactate} + 5.42727*\text{butyrate}*\text{cutin} - 1.55874*\text{lactate}^2 + 2.19697*\text{lactate}*\text{cutin} - 12.1551*\text{cutin}^2$
6 ^f	$50.5048 - 17.5632*\text{butyrate} - 11.5036*\text{lactate} + 16.5276*\text{cutin} + 2.73851*\text{butyrate}^2 + 0.82*\text{butyrate}*\text{lactate} + 5.90606*\text{butyrate}*\text{cutin} + 1.61656*\text{lactate}^2 + 3.19697*\text{lactate}*\text{cutin} - 14.9054*\text{cutin}^2$

Footnotes:

^a Combination 1: Roma tomato cutin and sodium acetate

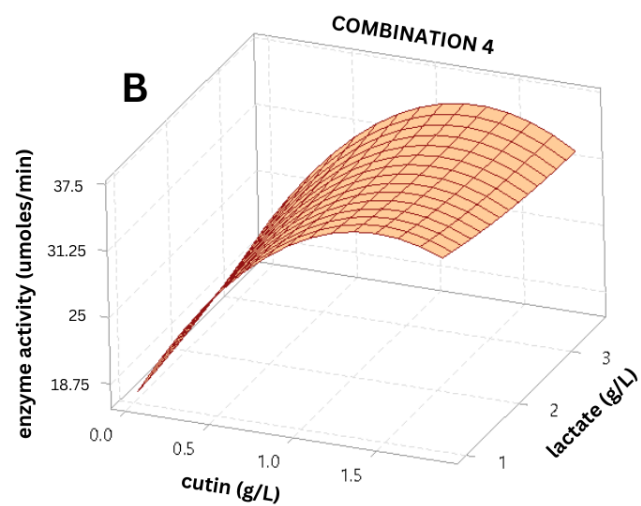
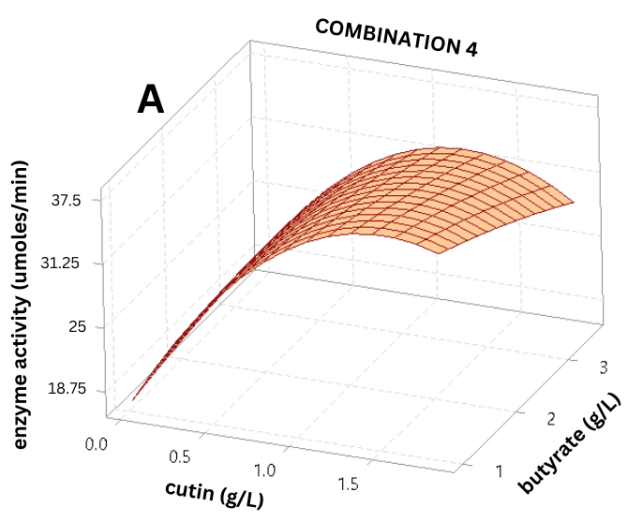
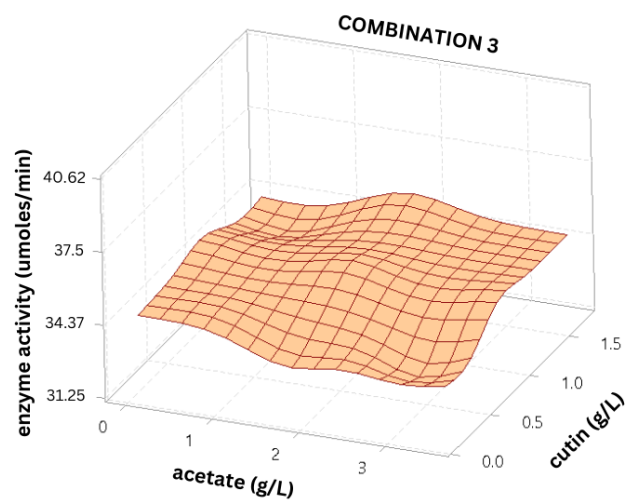
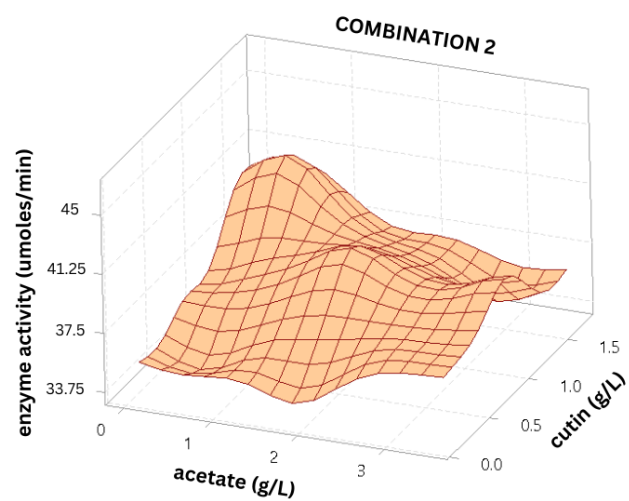
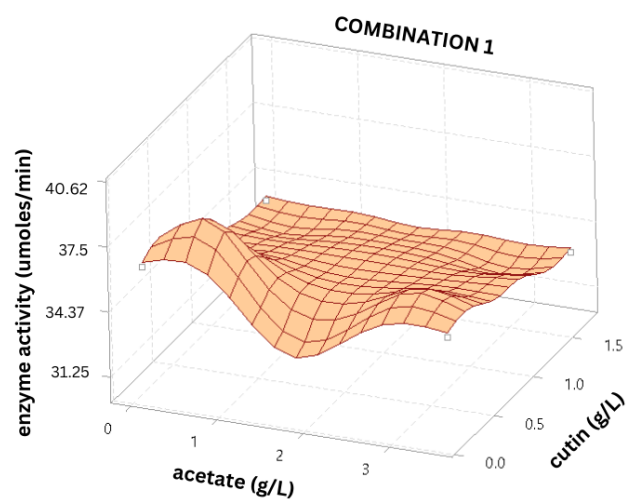
^b Combination 2: Royal Gala apple cutin and sodium acetate

^c Combination 3: Watermelon cutin and sodium acetate

^e Combination 4: Roma tomato cutin, sodium butyrate and sodium lactate

^f Combination 5: Royal Gala apple cutin, sodium butyrate and sodium lactate

^g Combination 6: Watermelon cutin, sodium butyrate and sodium lactate



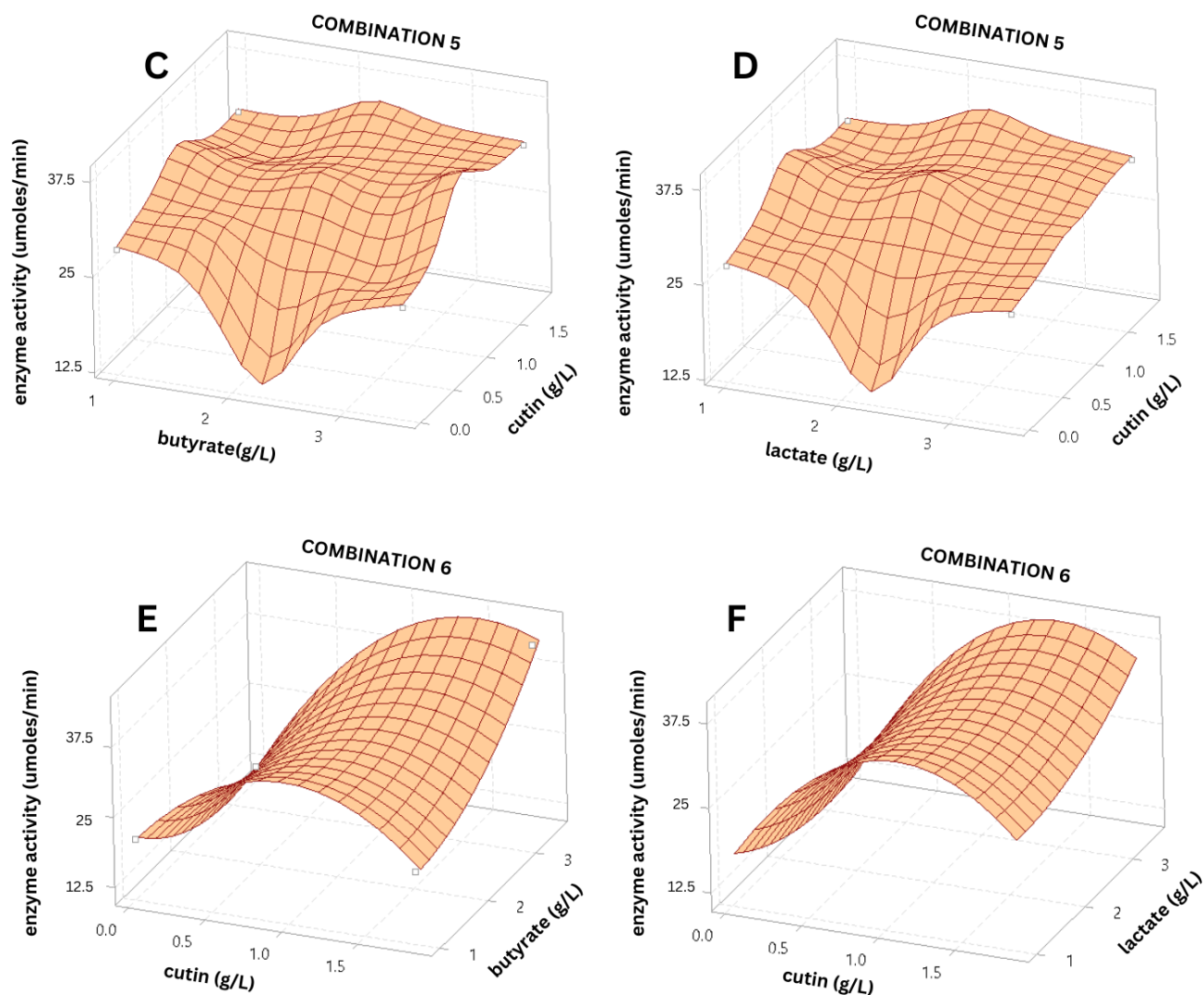
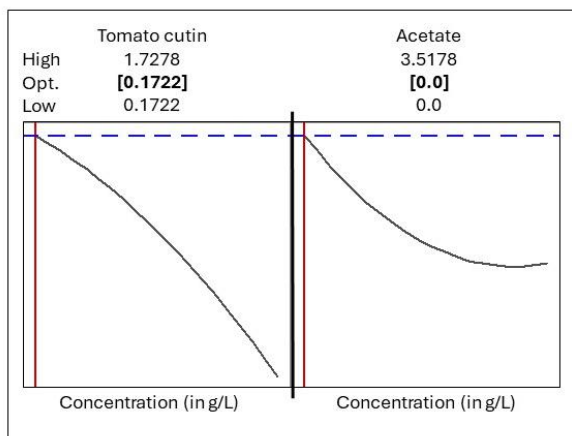
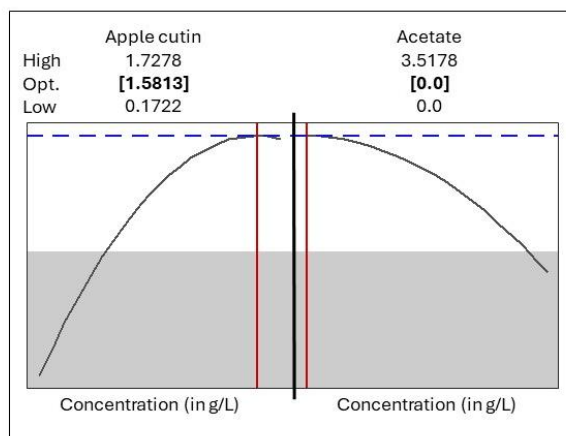


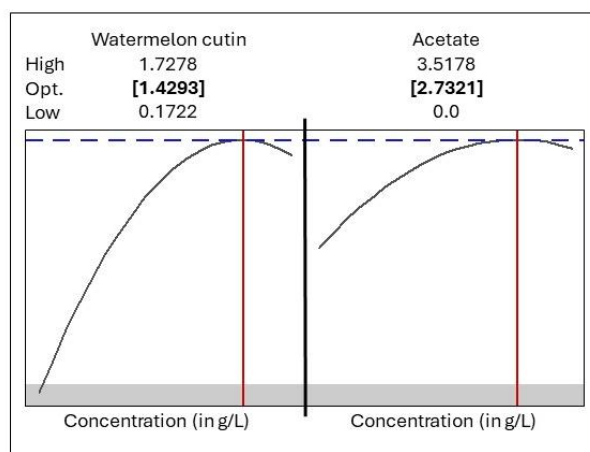
Figure 4.2. Graphical representation of the response surface plots for each combination. The surface responses for both lactate and butyrate with Roma tomato cutin (A & B), Royal Gala apple cutin (C & D), and watermelon cutin (E & F). Combination 1,2 and 3 used Sodium acetate as the carbon source with cutin from Roma tomato, Royal Gala apple and watermelon respectively. Combination 4,5 and 6 had Na-butyrate and Na-lactate as carbon sources with the cutin from Roma tomato, Royal Gala apple and watermelon respectively



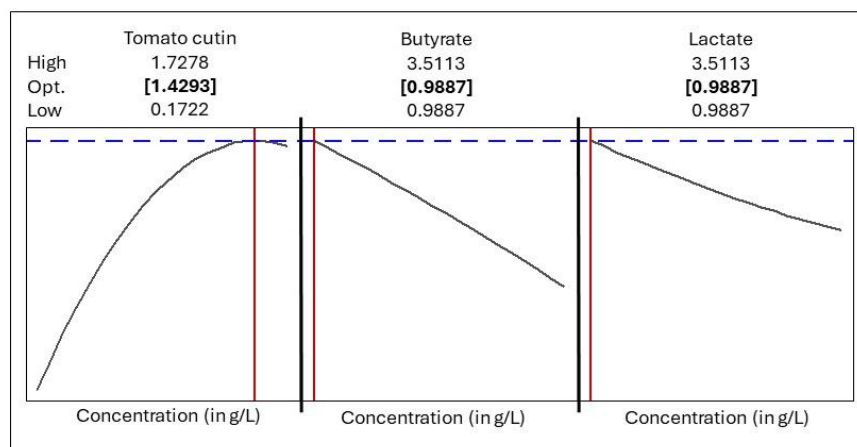
Combination 1: Acetate & Roma tomato cutin
Maximum enzyme activity: 36.67 $\mu\text{moles/min}$



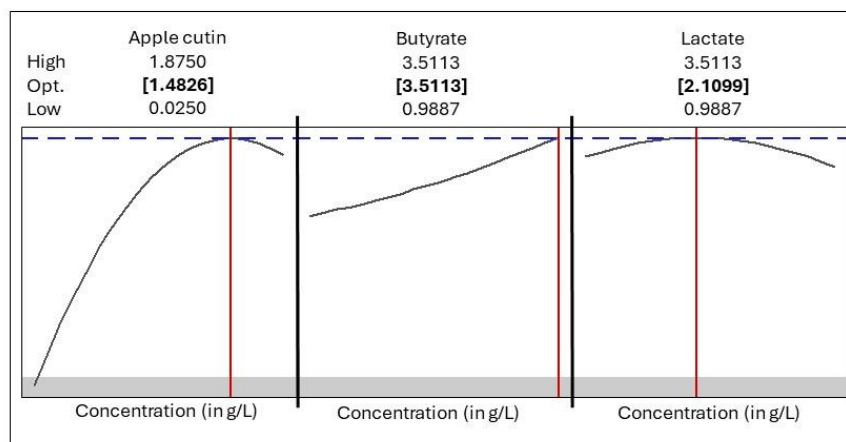
Combination 2: Acetate & Royal Gala apple cutin
Maximum enzyme activity: 40.45 $\mu\text{moles/min}$



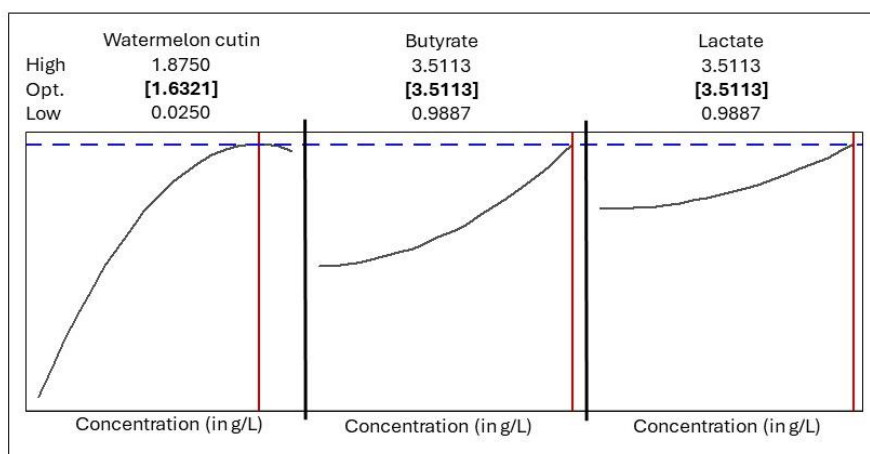
Combination 3: Acetate & watermelon cutin
Maximum enzyme activity: 30.98 $\mu\text{moles/min}$



Combination 4: Butyrate, lactate & Roma tomato cutin
Maximum enzyme activity: 42.11 $\mu\text{moles/min}$



Combination 5: Butyrate, lactate & Royal Gala apple cutin
Maximum enzyme activity: 38.37 μ moles/min



Combination 6: Butyrate, lactate & watermelon cutin
Maximum enzyme activity: 51.70 μ moles/min

Figure 4.3 Optimization plots of variables for the predicted maximum cutinase production for each combination. Combination 1,2 and 3 used Sodium acetate as the carbon source with cutin from Roma tomato, Royal Gala apple and watermelon respectively. Combination 4,5 and 6 had Na-butyrate and Na-lactate as carbon sources with the cutin from Roma tomato, Royal Gala apple and watermelon respectively

Table 4.5. The predicted optimized factors for maximal enzyme activity using *Thermobifida fusca* YX. (Combination 1,2 and 3 used Sodium acetate as the carbon source with cutin from Roma tomato, Royal Gala apple and watermelon respectively. Combination 4,5 and 6 had Na-butyrate and Na-lactate as carbon sources with the cutin from Roma tomato, Royal Gala apple and watermelon respectively)

Combination	Predicted optimized factors
1	0 g/L sodium acetate 0.17 g/L of Roma tomato cutin
2	0 g/L sodium acetate 1.58 g/L of Royal Gala apple cutin
3	2.73 g/L sodium acetate 1.42 g/L watermelon cutin
4	0.98 g/L sodium butyrate 0.98 g/L sodium lactate 1.6 g/L Roma tomato cutin
5	3.51 g/L sodium butyrate 2.11 g/L sodium lactate 1.48 g/L Royal Gala apple cutin
6	3.51 g/L sodium butyrate 3.51 g/L sodium lactate 1.63 g/L watermelon cutin

4.3.3 Validation experiments and adequacy of the regression models

The second order polynomial regression equation models and the optimized values obtained were then experimentally validated. In order to confirm the validity of the statistical experimental strategies and to gain a better understanding of factors influencing enzyme activity, six confirmation runs were performed, one for each combination. In most of the combinations, the experimental enzyme activity exceeded the predicted enzyme activity (see Fig. 4.4). The optimized

combination 4 produced the highest enzyme activity of 48.74 $\mu\text{mol}/\text{min}$ at 50 °C (see Fig. 4.4). There was some increase in the enzyme activity in cutin combinations when acetate was used as a carbon source. Despite Combination 2 not providing the highest enzyme activity, the fact that it used less carbon and only a moderate level of cutin could suggest that use of this combination would prove to be beneficial from an economic standpoint for applicative purposes. However, because the optimized Combination 4 produced the highest enzyme activity, it was used for further experiments. The optimization of two factors, viz., carbon source and inducer resulted in a maximum of 42% increase in enzyme activity. Similar results are reported in literature using other organisms. Optimization of ten physicochemical factors using RSM led to a 2.5-fold increase in the cutinase activity of *Aspergillus* sp. RL2Ct (Kumari et al., 2016). The optimization of four factors of the bacterial growth medium of *Pseudomonas cepacia* NRRL B 2320 led to a two-fold increase in the cutinase activity (Dutta et al., 2013). Our results using *Thermobifida fusca*, the most researched organism for cutinase enzyme genetic engineering and enzyme production, could be value inputs for further study and increase in production of this important enzyme.

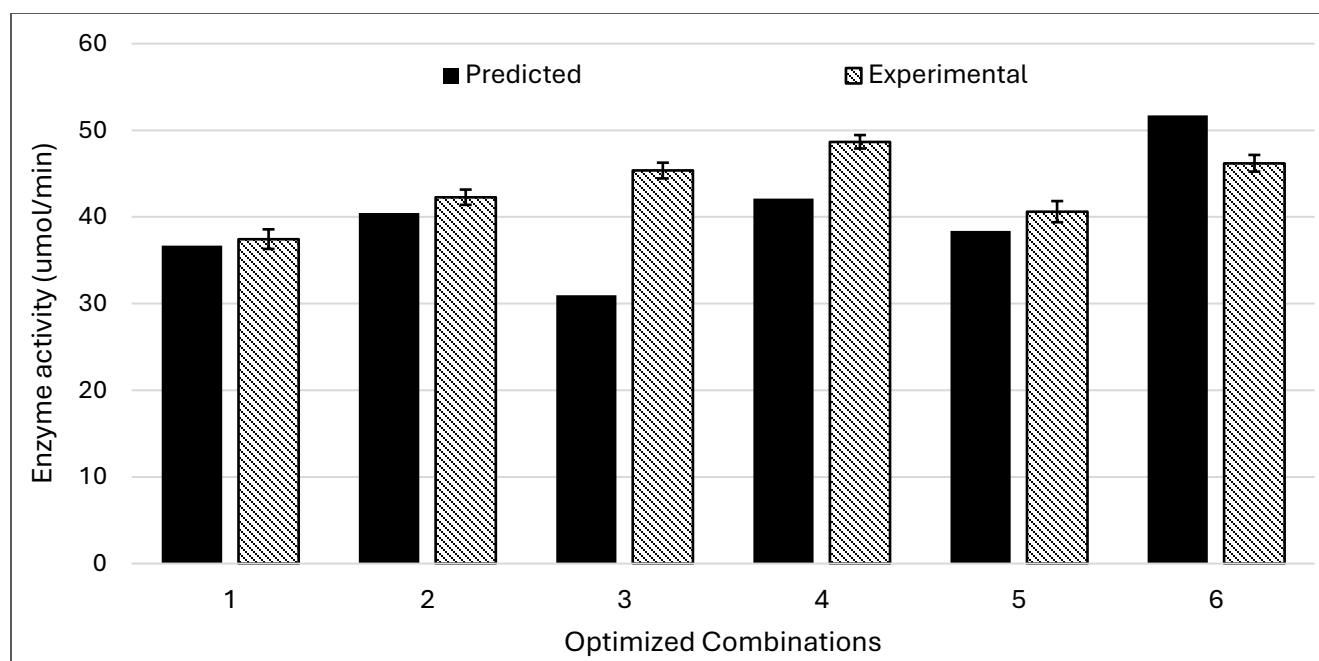


Figure 4.4 The enzyme activity of the predicted optimized values obtained after RSM optimization (in solid) versus the experimental value of the predicted optimized combinations (in striped). (Combination 1,2 and 3 used Sodium acetate as the carbon source with cutin from Roma tomato, Royal Gala apple and watermelon respectively. Combination 4,5 and 6 had Na-butyrate and Na-lactate as carbon sources with the cutin from Roma tomato, Royal Gala apple and watermelon respectively)

For Combinations 1, 2, and 3, sodium acetate was not an influencing factor for cutinase production. The secondary carbon sources, tryptone and yeast extract, in the growth medium proved to be sufficient for cutinase production. Additionally, lower concentrations of the inducer, cutin, was sufficient for maximum production of cutinase in Combinations 1 and 3. These combinations could be a more economically feasible way to mass produce cutinase with minimum carbon source and inducer. For Combination 6, the predicted optimum carbon sources are the boundary value, 3.51 g/L. Compared to the other combinations, Combination 6 would require a higher quantity of

resources. The optimized Combination 4 produced the highest enzyme activity of 48.74 $\mu\text{mol/min}$ (see Fig. 4.4). There was some increase in the enzyme activity in cutin combinations when acetate was used as a carbon source. Overall, the optimization of two factors, viz., carbon source and inducer, followed by partial purification resulted in a maximum of 42% increase in enzyme activity as mentioned earlier. In comparison, Pio & Macedo, (2007) reported a 22.68 $\mu\text{mol/min/mL}$ cutinase production by *Fusarium oxysporum* after 48 hours by optimizing the inducer, flaxseed oil, carbon source and nitrogen source. The optimization of the bacterial growth medium of *Pseudomonas cepacia* NRRL B 2320 led to a 2-fold increase in cutinase activity (Dutta et al., 2013). Optimization of the cutinase production from *Thermobifida fusca* has been studied using *Escherichia coli* BL21 as an expression chassis after genetic engineering with the genes encoding for cutinase, *tfu_0883* (Hegde & Veeranki, 2013; Su et al., 2015). This is the first study that reports optimization of cutinase expression from *T. fusca* YX without any genetic engineering.

As seen in Figure 4.4, cutin from varying sources affects the cutinase production in *T. fusca* YX. As mentioned earlier, cutin from different sources can have different monomers or different quantities of similar monomers. Tomato cutin has been previously shown to significantly increase cutinase production due to its monomeric constituents. The most commonly occurring monomers for tomato cutin are 10,16-dihydroxyhexadecanoic acid and 16-hydroxyhexadecanoic acid (Osman et al., 1999). Similarly, apple cutin is rich in 10,16-dihydroxyhexadecanoic acid, as well as 9,10,18-trihydroxyoctadecanoic acid (Arrieta-Baez et al., 2020). Watermelon cutin has been shown to induce cutinase production in *Streptomyces spp*s and *Yarrowia lipolytica* (Chaudhari & Singhal, 2015; Sales et al., 2020). The effect of specific major components of cutins from different sources can be studied further. This can also be done with other organisms capable of producing cutinase enzymes and lead to further increase in enzyme production.

4.3.5 PET biological depolymerization

The crude enzyme extract obtained after partial purification with ammonium sulphate was tested for its esterolytic activity on a real application, the biological depolymerization of PET. The partially purified enzyme degraded $26.01 \pm 0.71\%$ (weight loss quantity: 48.1 ± 0.88 mg) of the untreated PET film (original weight: ~ 185 mg)(see Fig. 4.5). An overall 4.26-fold increase in the weight loss of PET films due to biological depolymerization by the crude enzyme obtained under unoptimized conditions. Meanwhile, Sales et al. (2021) reported only 11.5 ± 0.7 $\mu\text{mol/L}$ of TPA, along with MHET and BHET by depolymerization of PET (original weight used: 10 mg) after 3 days of incubation. In another study, cutinase production from a yeast, *Fusarium falciforme* was optimized using CCD to increase the hydrophilicity of PET fabric (Sooksai et al., 2019). Most studies report on the optimization of growth media and environmental conditions for expression bacterial chassis like, *E. coli* BL21 DE3. Here, we demonstrate that large quantities of PET degrading enzyme can be produced by the non-engineered bacteria.

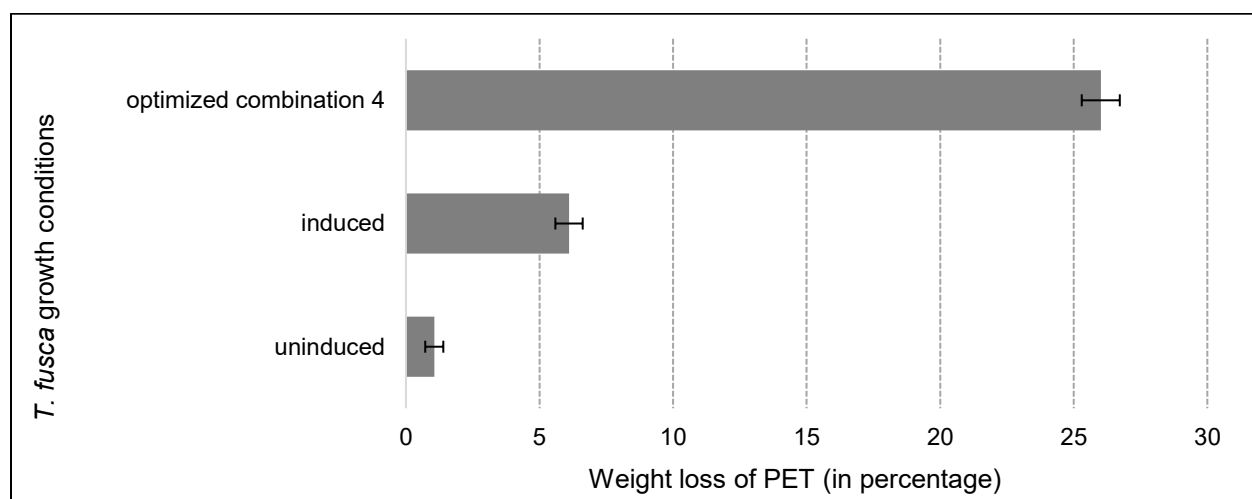


Figure 4.5. Comparison of the weight loss of PET when biodegraded with broth from uninduced *Thermobifida fusca* YX, *T. fusca* YX induced with Royal Gala apple cutin (0.4% w/v) and *T.*

fusca YX induced with the optimized Combination 4 (0.98 g/L sodium butyrate, 0.98 g/L sodium lactate, and 1.6 g/L Roma tomato cutin).

4.4 Conclusion

In conclusion, the study successfully demonstrated that Response Surface Methodology (RSM) is an effective and efficient approach for optimizing the production of cutinase from *Thermobifida fusca* YX. This is the first study on the optimization of two key factors, carbon source and inducer, for the maximum production of cutinase from *Thermobifida fusca* YX. Through the systematic evaluation and adjustment of carbon sources and cutin sources we achieved a significant increase in esterolytic activity. The parameters affecting esterase production were cutins from all three sources, Royal Gala apple, Roma tomato and watermelon, and the combination of sodium butyrate and sodium lactate. This optimization process provides a valuable framework for scaling up cutinase production in industrial applications, where maximizing yield is crucial for cost-effectiveness and process efficiency. Additionally, this study circumvents the need for genetic engineering by demonstrating the increase in PET biological depolymerization using RSM. Furthermore, an increase in the production of enzymes without the use of genetic engineering provides us insight into the effect of the environment with regard to gene expression. Gene expression is not a one-to-one relation but is a congruence of multiple affecting factors like the environment, inducer, and gene copies. Future research can build on these findings by exploring other factors affecting enzyme production like metal ions and alternative substrates to further enhance cutinase production and its applicability in various biotechnological fields.

References

- Arrieta-Baez, D., Flores, M. de J. P., Méndez-Méndez, J. V., León, H. F. M., & Gómez-Patiño, M. B. (2020). Structural Studies of the Cutin from Two Apple Varieties: Golden Delicious and Red Delicious (*Malus domestica*). *Molecules* 2020, Vol. 25, Page 5955, 25(24), 5955. <https://doi.org/10.3390/MOLECULES25245955>
- Brückner, R., & Titgemeyer, F. (2002). Carbon catabolite repression in bacteria: choice of the carbon source and autoregulatory limitation of sugar utilization. *FEMS Microbiology Letters*, 209(2), 141–148. <https://doi.org/10.1111/J.1574-6968.2002.TB11123.X>
- Chaudhari, S. A., & Singhal, R. S. (2015). Cutin from watermelon peels: A novel inducer for cutinase production and its physicochemical characterization. *International Journal of Biological Macromolecules*, 79, 398–404. <https://doi.org/10.1016/j.ijbiomac.2015.05.006>
- Chen, Z., Wang, Y., Cheng, Y., Wang, X., Tong, S., Yang, H., & Wang, Z. (2020). Efficient biological depolymerization of highly crystallized polyethylene terephthalate through cell surface display of bacterial PETase. *Science of the Total Environment*, 709. <https://doi.org/10.1016/j.scitotenv.2019.136138>
- Dalmau, E., Montesinos, J. L., Lotti, M., & Casas, C. (2000). Effect of different carbon sources on lipase production by *Candida rugosa*. *Enzyme and Microbial Technology*, 26(9–10), 657–663. [https://doi.org/10.1016/S0141-0229\(00\)00156-3](https://doi.org/10.1016/S0141-0229(00)00156-3)
- Deng, Y. (2011). *Characterization and genetic analysis of the cellulolytic microorganism Thermobifida fusca*. <https://www.proquest.com/openview/159859c25c3646a38f46c43177db80bc/1?pq-origsite=gscholar&cbl=18750>
- Doelle, H. W., Ewings, K. N., & Hollywood, N. W. (1982). *Regulation of glucose metabolism in bacterial systems*. 1–35. https://doi.org/10.1007/3540116982_1
- Du, G. C., Zhang, S. L., Hua, Z. Z., Zhu, Y., & Chen, J. (2007). Enhanced cutinase production with *Thermobifida fusca* by two-stage pH control strategy. *Biotechnology Journal*, 2(3), 365–369. <https://doi.org/10.1002/BIOT.200600122>
- Fett, W. F., Wijey, C., Moreau, R. A., & Osman, S. F. (1999). Production of cutinase by *Thermomonospora fusca* ATCC 27730. In *Journal of Applied Microbiology* (Vol. 86).
- Fich, E. A., Segerson, N. A., & Rose, J. K. C. (2016). The Plant Polyester Cutin: Biosynthesis, Structure, and Biological Roles. *Annual Review of Plant Biology*, 67, 207–233. <https://doi.org/10.1146/ANNUREV-ARPLANT-043015-111929>
- Gero, J. S., Kazakov, V. A., & Schnier, T. (1997). Genetic Engineering and Design Problems. *Evolutionary Algorithms in Engineering Applications*, 47–68. https://doi.org/10.1007/978-3-662-03423-1_3
- He, G., Huo, G., Liu, L., Zhu, Y., Du, G., & Chen, J. (2009). Enhanced cutinase production of *Thermobifida fusca* by a two-stage batch and fed-batch cultivation strategy. *Biotechnology and Bioprocess Engineering*, 14(1), 46–51. <https://doi.org/10.1007/S12257-008-0091-7/METRICS>
- Hegde, K., & Veeranki, V. D. (2013). Production optimization and characterization of recombinant cutinases from *thermobifida fusca* sp. NRRL B-8184. *Applied Biochemistry and Biotechnology*, 170(3), 654–675. <https://doi.org/10.1007/s12010-013-0219-x>
- Kakde, R. B., & Chavan, A. M. (2011). *Effect of Carbon, Nitrogen, Sulphur, Phosphorus, Antibiotic and Vitamin Sources on Hydrolytic Enzyme Production by Storage Fungi* (Vol. 3, Issue 5). www.recent-science.com
- Kanwal, H. K., & Reddy, M. S. (2011). Effect of carbon, nitrogen sources and inducers on ligninolytic enzyme production by *Morchella crassipes*. *World Journal of Microbiology and Biotechnology*, 27(3), 687–691. <https://doi.org/10.1007/S11274-010-0507-3/TABLES/2>
- Kumari, V., Kumar, V., Chauhan, R., Asif, • Mohammad, Tek, •, & Bhalla, C. (2016). Optimization of medium parameters by response surface methodology (RSM) for enhanced production of cutinase from *Aspergillus* sp. RL2Ct. 3 *Biotech*, 6(149). <https://doi.org/10.1007/s13205-016-0460-4>
- MacLeod, M., Arp, H. P. H., Tekman, M. B., & Jahnke, A. (2021). The global threat from plastic pollution. *Science*, 373(6550), 61–65. <https://doi.org/10.1126/SCIENCE.ABG5433>
- Nakai, S. A., & Siebert, K. J. (2003). Validation of bacterial growth inhibition models based on molecular properties of organic acids. *International Journal of Food Microbiology*, 86(3), 249–255. [https://doi.org/10.1016/S0168-1605\(02\)00551-2](https://doi.org/10.1016/S0168-1605(02)00551-2)
- Nawrath, C. (2002). The Biopolymers Cutin and Suberin. *The Arabidopsis Book / American Society of Plant Biologists*, 1, e0021. <https://doi.org/10.1199/TAB.0021>
- Osman, S. F., Irwin, P., Fett, W. F., O'Connor, J. V., & Parris, N. (1999). Preparation, isolation, and characterization of cutin monomers and oligomers from tomato peels. *Journal of Agricultural and Food Chemistry*, 47(2), 799–802. <https://doi.org/10.1021/JF980693R/ASSET/IMAGES/LARGE/JF980693RF00003.JPEG>

- Parmar, A. S., & Rahi, D. K. (2023). Cutinase production from *Fusarium verticillioides* using response surface methodology and its application as potential insecticide degrader. *Environmental Science and Pollution Research*, 30(36), 86484–86497. <https://doi.org/10.1007/S11356-023-28635-1/FIGURES/7>
- Peng, M., Wang, J., Lu, X., Wang, M., Wen, G., Wu, C., Lu, G., Wang, Z., Shim, W. B., & Yu, W. (2024). Functional analysis of cutinase transcription factors in *Fusarium verticillioides*. *Phytopathology Research*, 6(1), 1–18. <https://doi.org/10.1186/S42483-024-00267-4/FIGURES/7>
- Pinhal, S., Ropers, D., Geiselmann, J., De Jong, H., & Metcalf, W. W. (2019). Acetate Metabolism and the Inhibition of Bacterial Growth by Acetate. In *jb.asm.org 1 Journal of Bacteriology* (Vol. 201). <https://journals.asm.org/journal/jb>
- Pio, T. F., & Macedo, G. A. (2007). Optimizing the production of cutinase by *Fusarium oxysporum* using response surface methodology. *Enzyme and Microbial Technology*, 41(5), 613–619. <https://doi.org/10.1016/J.ENZMICTEC.2007.05.008>
- Pinillo, V., Orlando, M., Battaglia, C., Pollegioni, L., & Molla, G. (2023). Efficient polyethylene terephthalate depolymerization at moderate temperature: a protein engineering study of LC-cutinase highlights the key role of residue 243. *The FEBS Journal*. <https://doi.org/10.1111/FEBS.16736>
- Puspitasari, N., Tsai, S. L., & Lee, C. K. (2021). Fungal Hydrophobin RoIA Enhanced PETase Hydrolysis of Polyethylene Terephthalate. *Applied Biochemistry and Biotechnology*, 193(5), 1284–1295. <https://doi.org/10.1007/s12010-020-03358-y>
- Sagong, H. Y., Seo, H., Kim, T., Son, H. F., Joo, S., Lee, S. H., Kim, S., Woo, J. S., Hwang, S. Y., & Kim, K. J. (2020). Decomposition of the PET Film by MHETase Using Exo-PETase Function. *ACS Catalysis*, 10(8), 4805–4812. <https://doi.org/10.1021/acscatal.9b05604>
- Sales, J. C. S., de Castro, A. M., Ribeiro, B. D., & Coelho, M. A. Z. (2021). Improved production of biocatalysts by *Yarrowia lipolytica* using natural sources of the biopolyesters cutin and suberin, and their application in hydrolysis of poly (ethylene terephthalate) (PET). *Bioprocess and Biosystems Engineering*, 44(11), 2277–2287. <https://doi.org/10.1007/S00449-021-02603-W/TABLES/3>
- Sales, J. C. S., de Castro, A. M., Ribeiro, B. D., & Maria, M. A. (2020). Supplementation of watermelon peels as an enhancer of lipase and esterase production by *Yarrowia lipolytica* in solid-state fermentation and their potential use as biocatalysts in poly(ethylene terephthalate) (PET) depolymerization reactions. *Biocatalysis and Biotransformation*, 38(6), 457–468. <https://doi.org/10.1080/10242422.2020.1782387>
- Sooksai, T., Bankeeree, W., Sangwatanaroj, U., Lotrakul, P., Punnapayak, H., & Prasongsuk, S. (2019). Production of cutinase from *Fusarium falciforme* and its application for hydrophilicity improvement of polyethylene terephthalate fabric. *3 Biotech*, 9(11), 1–11. <https://doi.org/10.1007/S13205-019-1931-1/TABLES/4>
- Su, L., Hong, R., & Wu, J. (2015). Enhanced extracellular expression of gene-optimized *Thermobifida fusca* cutinase in *Escherichia coli* by optimization of induction strategy. *Process Biochemistry*, 50(7), 1039–1046. <https://doi.org/10.1016/J.PROCBIO.2015.03.023>
- Sulaiman, S., Yamato, S., Kanaya, E., Kim, J. J., Koga, Y., Takano, K., & Kanaya, S. (2012). Isolation of a novel cutinase homolog with polyethylene terephthalate-degrading activity from leaf-branch compost by using a metagenomic approach. *Applied and Environmental Microbiology*, 78(5), 1556–1562. <https://doi.org/10.1128/AEM.06725-11>
- Tournier, V., Topham, C. M., Gilles, A., David, B., Folgoas, C., Moya-Leclair, E., Kamionka, E., Desrousseaux, M. L., Texier, H., Gavalda, S., Cot, M., Guémard, E., Dalibey, M., Nomme, J., Cioci, G., Barbe, S., Chateau, M., André, I., Duquesne, S., & Marty, A. (2020). An engineered PET depolymerase to break down and recycle plastic bottles. *Nature*, 580(7802), 216–219. <https://doi.org/10.1038/s41586-020-2149-4>

Appendix B

Supplementary Table 1. Analysis of Variance of the regression model of combination 1 (sodium acetate and tomato cutin)

Source	DF	Adj SS	Adj MS	F-value	P-value
Model	5	547.84	109.57	0.2	0.951
Linear	2	410.8	205.4	0.38	0.698
cutin	1	391.18	391.18	0.72	0.424
acetate	1	19.62	19.62	0.04	0.855
Square	2	95.25	47.62	0.09	0.917
cutin*cutin	1	21.74	21.74	0.04	0.847
acetate*acetate	1	62.29	62.29	0.11	0.745
2-Way Interaction	1	41.8	41.8	0.08	0.789
cutin*acetate	1	41.8	41.8	0.08	0.789
Error	7	3795.64	542.23		
Lack-of-Fit	3	848.06	282.69	0.38	0.771
Pure Error	4	2947.58	736.9		
Total	12	4343.49			

Supplementary Table 2. Analysis of Variance of the regression model of combination 2 (sodium acetate and apple cutin)

Source	DF	Adj SS	Adj MS	F-value	P-value
Model	5	4782.6	956.52	1.23	0.386
Linear	2	854.4	427.2	0.55	0.600
cutin	1	843.1	843.1	1.09	0.332
acetate	1	11.3	11.3	0.01	0.907
Square	2	2233.4	1116.71	1.44	0.300
cutin*cutin	1	2017.6	2017.6	2.60	0.151
acetate*acetate	1	412.8	412.8	0.53	0.490
2-Way Interaction	1	1695.8	1695.8	2.18	0.183
cutin*acetate	1	1695.8	1695.8	2.18	0.183

Error	7	5432.9	776.14		
Lack-of-Fit	3	608.7	202.90	0.17	0.913
Pure Error	4	4824.2	1206.06		
Total	12	10215.5			

Supplementary Table 3. Analysis of Variance of the regression model of combination 3 (sodium acetate and watermelon cutin)

Source	DF	Adj SS	Adj MS	F-value	P-value
Model	5	638.37		0.23	0.938
Linear	2	225.15		0.20	0.821
cutin	1	213.77	213.77	0.39	0.554
acetate	1	11.38	11.38	0.02	0.890
Square	2	261.06		0.24	0.796
cutin*cutin	1	235.15	235.15	0.42	0.536
acetate*acetate	1	49.66	49.66	0.09	0.773
2-Way Interaction	1	152.15	152.15	0.27	0.617
cutin*acetate	1	152.15	152.15	0.27	0.617
Error	7	3883.71	554.82		
Lack-of-Fit	3	132.88	44.29	0.05	0.985
Pure Error	4	3750.83	937.71		
Total	12	4522.08			

Supplementary Table 4. Analysis of Variance of the regression model of combination 4 (sodium butyrate, sodium lactate and tomato cutin)

Source	DF	Adj SS	Adj MS	F-value	P-value
Model	9	24153.3	2683.7	1.7	0.210
Linear	3	16345.8	5448.6	3.45	0.059
cutin	1	15639.3	15639.3	9.92	0.01
butyrate	1	669.3	669.3	0.42	0.529
lactate	1	37.2	37.2	0.02	0.881
Square	3	6564.4	2188.1	1.39	0.303
cutin*cutin	1	6372.4	6372.4	4.04	0.072

butyrate*butyrate	1	16.7	16.7	0.01	0.920
lactate*lactate	1	31.5	31.5	0.02	0.891
2-Way Interaction	3	1243.2	414.4	0.26	0.851
cutin*butyrate	1	388.4	388.4	0.25	0.630
cutin*lactate	1	142.6	142.6	0.09	0.770
butyrate*lactate	1	712.2	712.2	0.45	0.517
Error	10	15771.3	1577.1		
Lack-of-Fit	5	13272.4	2654.5	5.31	0.045
Pure Error	5	2498.9	499.8		
Total	19	39924.6			

Supplementary Table 5. Analysis of Variance of the regression model of combination 5 (sodium butyrate, sodium lactate and apple cutin)

Source	DF	Adj SS	Adj MS	F-value	P-value
Model	9	27410.5	3045.6	4.09	0.019
Linear	3	11050.3	3683.4	4.94	0.023
cutin	1	11033.9	11033.9	14.80	0.003
butyrate	1	12.0	12.0	0.02	0.902
lactate	1	4.4	4.4	0.01	0.940
Square	3	13109.6	4369.9	5.86	0.014
cutin*cutin	1	12342.3	12342.3	16.56	0.002
butyrate*butyrate	1	68.8	68.8	0.09	0.767
lactate*lactate	1	832.6	832.6	1.12	0.315
2-Way Interaction	3	3250.7	1083.6	1.45	0.285
cutin*butyrate	1	2572.2	2572.2	3.45	0.093
cutin*lactate	1	421.8	421.8	0.57	0.469
butyrate*lactate	1	256.6	256.6	0.34	0.570
Error	10	7455.1	745.5		
Lack-of-Fit	5	5870.6	1174.1	3.71	0.088
Pure Error	5	1584.4	316.9		
Total	19	34865.6			

Supplementary Table 6. Analysis of Variance of the regression model of combination 6 (sodium butyrate, sodium lactate and watermelon cutin)

Source	DF	Adj SS	Adj MS	F-value	P-value
Model	9	50342.1	5593.6	2.39	0.096
Linear	3	22545.6	7515.2	3.21	0.07
cutin	1	19913.1	19913.1	8.50	0.015
butyrate	1	2427.2	2427.2	1.04	0.33
lactate	1	205.2	205.2	0.09	0.773
Square	3	23740.2	7913.4	3.38	0.062
cutin*cutin	1	18760.7	18760.7	8.01	0.018
butyrate*butyrate	1	2187.7	2187.7	0.93	0.357
lactate*lactate	1	759.5	759.5	0.32	0.582
2-Way Interaction	3	4056.4	1352.1	0.58	0.643
cutin*butyrate	1	3063.1	3063.1	1.31	0.279
cutin*lactate	1	880.3	880.3	0.38	0.554
butyrate*lactate	1	113.0	113.0	0.05	0.831
Error	10	23420.6	2342.1		
Lack-of-Fit	5	19244.1	3848.8	4.61	0.060
Pure Error	5	4176.5	835.3		
Total	19	73762.7			

Chapter 5
Research Objective 3

Genomic study of *Thermobifida fusca* YX for novel PET-hydrolyzing enzymes and their subsequent molecular docking with polyethylene terephthalate and cutin ligands

Abstract

Polyethylene terephthalic acid (PET or PETE) plastic has been a major pollutant of the environment for several decades. This has resulted in the substantial increase in the number of studies focusing on enzymatic hydrolysis of PET. The biological depolymerization of PET has been of considerable interest because of its non-toxic nature and because it can be carried at nearly ambient conditions. Considerable scientific research has been focused on genetically manipulating organisms for enhanced production of a few enzymes. This in turn has limited our focus on exploring the potential battery of enzymes already present in bacteria that are primarily hydrolytic in nature. The actinomycete *Thermobifida fusca* YX is one such bacteria, with the singular enzyme (protein encoded by Tfu_0883) being the focus of recent research. The present study addresses this gap by studying other potential PET-hydrolyzing enzymes (PHEs) in *T. fusca* YX based on structural and functional homology. The study discovered six potential PHEs, of which three were extracellular, two were transmembrane proteins and one was intracellular. Using a series of software and web servers, the primary, secondary, and tertiary structures of the potential PHEs were analyzed for their stability, both structurally and thermally. Molecular docking studies of the enzymes revealed exergonic binding energies with PET ligands and their variants. Additionally, we studied the *in silico* binding of constructed cutin dimers with enzymes, making it the first study understanding the intricate binding interactions between cutin ligands and potential PHEs.

Keywords: molecular docking, PET-hydrolyzing enzymes, cutin, PET, ligand, *Thermobifida fusca* YX

5.1. Introduction

Recently, plastic consumption has skyrocketed causing a corresponding increase in the waste generated, this particularly true for single-use plastics (Heidbreder et al., 2019; Jia et al., 2019; Walker & Xanthos, 2018; Xanthos & Walker, 2017). Polyethylene terephthalic acid (PET) is one of the main polymers for single-use products that need to be eliminated over time (Law & Narayan, 2021). Along with constant improvement in recycling technology, there has been considerable effort in developing enzymes that efficiently biodegrade PET plastics (Tournier et al., 2020; Yoshida et al., 2016). These PET-hydrolyzing enzymes (PHEs) have been the focus of considerable interest due to the inertness of PET, and its implications as an environmental hazard. Research groups around the world have focused on studying and modifying a few PHEs that have demonstrated potential (Brott et al., 2022; Fecker et al., 2018; Son et al., 2019; Sulaiman et al., 2012; Tournier et al., 2020). There is considerable scope for looking beyond these few enzymes to other enzymes with the same functionality but higher efficiency. In addition, the methods used to isolate the enzymes further limit our understanding of all the possible hydrolyzing and modifying enzymes present within microorganisms. For example, few enzymes from microorganisms like *Thermobifida fusca*, *Ideonella sakaiensis*, and *Humicola insolens* have been genetically studied in detail (Carniel et al., 2017; Yoshida et al., 2016; Zimmermann & Billig, 2011). Despite having their entire genomes sequenced, the scientific community has not looked beyond the already studied PET-hydrolyzing enzymes. In order to harness potential enzymes that better fit our needs we need to explore the battery of potential PHEs within each of those genomes. Our current understanding of PHEs involves the classes cutinases, lipases, esterases, a few hydrolases, and PETases (Amanna & Rakshit, 2023). Cutinases have been a prime interest as the formation of the enzyme-substrate complex with PET and cutin involve the ester bond that joins the monomers in PET. In the recent past, there has been an increase in the discovery of PHEs that

belong to these classes (Ho et al., 2023; X. Huang et al., 2018; Puspitasari et al., 2021; Son et al., 2019; N. Wang et al., 2020). Despite these discoveries, PET biological depolymerization research has narrowed its mission by focusing on the development of a single, efficient enzyme to hydrolyze PET by mutating cutinases from *Ideonella sakaiensis* and Leaf-Compost Cutinase (Tournier et al., 2020; Yoshida et al., 2016). Although the development of such a desirable enzyme is valuable, its environmental application would need to be thoroughly assessed due to the potential risk of safety to both humans and the environment by such genetically modified enzymes (Wesseler et al., 2023). This would further impede the progress towards elimination of plastic in the environment.

The actinomycete found in compost soil, *Thermobifida fusca*, is known for its battery of hydrolyzing enzymes and has been extensively studied for its cellulases (Gomez Del Pulgar & Saadeddin, 2014; Moreira et al., 1981; Stutzenberger & Kahler, 1986; Wilson, 2004). Considering the degradative nature of the actinomycete, *Thermobifida fusca* YX could potentially contain a battery of cutinases as well within its genome. Our attempts to fill this gap using *in silico* protein structure determination and other bioinformatic tools to study the genome of *T. fusca* YX for enzymes with PET-hydrolyzing capabilities. Additionally, these enzymes were analyzed for their binding interactions with PET based on its molecular structure and conformation.

The availability of the genome sequence of *Thermobifida fusca* YX opens an avenue for studying all the potential PHEs and PET-modifying enzymes present in the actinomycete. Such studies have been carried out for cellulases (Lykidis et al., 2007). With this background, we are convinced that *T. fusca* YX could have a battery of enzymes with potential to hydrolyze or modify PET. Hence, the actinomycete's whole genome (GenBank ID: CP000088.1) was assessed for potential PHEs. Proteins structurally similar to cutinase (gene: Tfu_0883; experimentally validated 3D structure PDB IS: 5ZOA (Q. L. Dong et al., 2018)) belonging to classes like aminopeptidases, lipases,

esterases, and hydrolases were selected for the study. In order to broaden the scope of data mining, two approaches were adopted, a combination of BLASTs, and the SUPFAM web server. The resultant enzymes were subjected to a series of analyses to predict their primary, secondary, and tertiary structures. Finally, the predicted structures of the potential PHEs were docked with PET to understand the molecular interaction with the predicted catalytic triad in the active site.

5.2. Materials and Methods

5.2.1 Identification of potential PHEs

The first approach for mining sequences was to identify similar sequences by using tblastn (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). This software compares a query protein sequence against translated nucleotide, thereby finding similar proteins to the query protein sequence (Madden et al., 1996). The amino acid sequence and the experimentally determined 3D structure of the protein expressed by the gene, Tfu_0883, was retrieved from RCSB PDB, in both FASTA and PDB formats. The RCSB PDB contains experimentally validated three-dimensional structures of proteins. The protein structure of 5ZOA does not contain hexahistidine epitope purification tag nor the signal peptide, meanwhile, protein structure of 4CG1, contains both the signal peptide as well as the histidine tag. Proteins 5ZOA and 4CG1 were used as queries to mine for other potential PHEs within the *Thermobifida fusca* YX genome (NCBI Accession ID: CP000088.1) (Lykidis et al., 2007), using tblastn. The filters and masking parameters and general parameters were set to default (Scoring Parameters were as follows, Gap Costs: Existence: 15 Extension: 2, and Word Size: 5), while the scoring parameter matrix was set to BLOSUM45 to broaden the range of alignment. The second method used a structural approach using the protein encoded by the Tfu_0883 gene to shortlist protein families from the α/β -hydrolase superfamily for potential PHEs.

SUPFAM is a superfamily database of structural and functional proteins. Using the SUPFAM web server (<https://supfam.org>), structurally significant domains compared to the protein encoded by Tfu_0883 were shortlisted. The proteins belonging to families with previously known PET-hydrolyzing activity were shortlisted. The cellular location of the proteins was predicted using SignalP 6.0 (Teufel et al., 2022).

5.2.2 Primary structure collection

The primary sequence of the shortlisted PHEs were retrieved from the sequenced genome of *Thermobifida fusca* YX (NCBI Accession No. CP000088.1), NCBI. The retrieved sequences were input into web servers ProtParam (Gasteiger et al., 2005) and Sequence Manipulation Suite (Stothard, 2000) for analysis. Both ProtParam and Sequence Manipulator Suite compute the physical and chemical parameters of a protein. Using the protein sequence of the shortlisted proteins, these tools were used to predict the theoretical molecular weight, pI, instability index, aliphatic index, and grand average hydropathy (GRAVY) that are crucial attributes to understanding the nature of the proteins. Additionally, the protein sequences of the shortlisted proteins were aligned using ClustalW (<https://www.ebi.ac.uk/jdispatcher/msa/clustalo>) (Madeira et al., 2024) with the default settings (transition matrix used was Gonnet, gap opening penalty used was 6 bits, gap extension used was 1 bit, and the HHalign algorithm). The HHalign used two Hidden Markov Models (HMMs) to find similarities between protein sequences or structures.

5.2.3 Secondary structure analysis

Secondary structures consist of α -helices, β -sheets, β -turns and random coils. These were predicted using SOPMA (Geourjon & Deléage, 1995) web server using the FASTA files containing the

amino acid sequences of the proteins of interest. Its subsequent visualization was studied using the Heatmapper (<http://heatmapper.ca/>) web server.

5.2.4 Tertiary structure prediction

Prediction of the tertiary structures was done using the Robetta web server (Kim et al., 2004) (<https://robetta.bakerlab.org/>), which uses the improved deep learning based RoseTTAFold Modeling Method algorithm. This software predicts tertiary structure based on principles that govern protein folding energetics, statistical tendencies of conformational features that native structures acquire, and structural homology (Rohl et al., 2004). The predicted models were structurally validated by assessing their QMEAN using QMEANDisCo (Studer et al., 2020) and using Saves v6.0 web server (<https://saves.mbi.ucla.edu/>). The Saves v6.0 web server consists of multiple programs like ERRAT (Colovos & Yeates, 1993), 3D-1D (Verify 3D) score (Bowie et al., 1991) and Ramachandran plots (using PROCHECK (Laskowski et al., 1993)) that theoretically validate a predicted tertiary structure of a protein. These were further compared with the number of folds and sheets predicted by the secondary structure predicting software. In addition, salt bridges and their combinations were determined in the potential PHEs using *Evaluating the Salt Bridges* (ESBRI) web server (Costantini et al., 2008).

5.2.5 Molecular docking analysis

5.2.5.1 Ligand Preparation

The substrate ligands sourced from PubChem (<https://pubchem.ncbi.nlm.nih.gov/>) in .sdf format, includes PET (CID PubChem 42608431), BHET (CID PubChem 13739), MHET (CID PubChem 22062452), 10,16-dihydroxyhexadecenoic acid (CID PubChem 441449) and 9,10-epoxy-12-

octadecenoic acid (CID PubChem 5283018). The remaining ligands were constructed using Avogadro v1.1.1 39 software equipped with a force field (Hanwell et al., 2012). An MMFF94 force field designed by Merck with the steepest descent was applied to minimize the total energy of the system (Flores-Castañón et al., 2022).

5.2.5.2 Molecular docking

The binding sites of the proteins were predicted by CB-Dock 2 (Cavity-detection guided Blind Docking) server (Y. Liu et al., 2022). Using a novel curvature-based cavity detection, this server predicts the potential cavities of the protein and calculates its center and sizes. Additionally, CB-Dock2 provides an extensive list of all the interacting amino acids in the detected cavity. The software works along with AutoDock Vina which has been optimized to achieve a success rate of over 70% in the models. The protein structure files were submitted in .pdb format and the ligands in .sdf format. The program predicts five cavity-ligand interactions based on Vina Score, cavity volume, center, and docking size. Each interacting cavity was checked for the presence of the nucleophilic serine that is part of the catalytic triad for all PHEs. The resulting docking interactions between ligands and proteins were visualized with the Licorice and Surface options, respectively. Due to the limitations of CB-Dock2, the large cutin molecules were docked using USCF Chimera software with the AutoDock Vina function (Pettersen et al., 2004). The grid box was centered at x: -4.26, y: 25.106 and z: 15.893 with sizes of 109.067, 102.151 and 129.587 Å, respectively. The non-covalent docking using the AutoDock Vina function was performed, and ten output poses were generated with their calculated binding free energy. The docking model with the lowest binding energy was selected. The selected binding model was evaluated for the proper distances of the oxyanion hole.

5.3. Results and Discussion

5.3.1 Identification of potential PHEs

The whole genome of *T. fusca* YX has been partially annotated and has been predicted to encode 3117 proteins (Lykidis et al., 2007). Only 68% (2181 genes) have been assigned a function, 26% (830 genes) display sequence similarity with no known function, and the remaining 3.3% (106 genes) seem to be unique to *T. fusca* YX. The protein sequence encoded by the gene Tfu_0883 from UniProtKB in FASTA format was used as a query sequence for all database searches unless mentioned otherwise. The UniProtKB code of the primary structure of the protein encoded by Tfu_0883 (without signal peptide) was determined to be Q47RJ6. Using the 90% identity function on UniProtKB, similar proteins were identified and shortlisted based on their origin, *Thermobifida* spp. Protein sequences Q6A0I4 (E-value: 1.6E-121), G8GER6 (E-value: 5.9E-114), Q47RJ7 (E-value: 5.9E-114), E9LVH8 (E-value: 1.6E-106), E9LVH9 (E-value: 4.2E-100), A0A9P2TBN9 (E-value: 1.0E-107) and E5BBQ2 (E-value: 3.8E-103) were thus shortlisted. Each of the mined UniProtKB amino acid sequences were aligned with the whole genome of *T. fusca* YX (CP000088.1) using the tblastn function. To broaden the scope of alignment, BLOSUM45 was used to find similar regions/domains within proteins with an e-value set at 0.001. The UniProtKB enzymes aligned with three gene sequences from the whole genome, Tfu_0883 (with and without signal peptide), Tfu_0882 (with and without signal peptide) and Tfu_0267 (partially aligned with an identity of 38/135) (see Table 1). Tfu_0883 and Tfu_0882 have been annotated as triacylglycerol lipases which have been previously demonstrated to have cutinase activity (Lykidis et al., 2007). Hence, to broaden the scope of mining proteins with esterase activity, the protein sequence encoded by Tfu_0267 (annotated as serine aminopeptidase – hypothetical protein) in FASTA format was used as a query in tblastn against the whole genome of *T. fusca* YX.

Table 5.1. Pairwise alignment using tblastn of the proteins mined from UniProtKB against the whole genome of *Thermobifida fusca* YX (CP000088.1)

UniProtKB ID	Gene matches in <i>T. fusca</i> YX	E-value	Identities	Percent identity	Percent positive	Gaps
Q6A0I4	Tfu_0883 (without signal peptide)	0	301/301	100%	100%	0
	Tfu_0882 (without signal peptide)	3E-169	245/263	93%	95%	0
	Tfu_0267	2E-04	38/135	28%	43%	3
G8GER6	Tfu_0883 (with signal peptide)	0	319/319	100%	100%	0
	Tfu_0882 (with signal peptide)	4E-171	245/263	93%	95%	0
	Tfu_0267	2E-04	38/135	28%	43%	3
Q47RJ7	Tfu_0883 (with signal peptide)	0	319/319	100%	100%	0
	Tfu_0882 (with signal peptide)	4E-171	245/263	93%	95%	0
	Tfu_0267	2E-04	38/135	28%	43%	3
E9LVH8	Tfu_0883 (without signal peptide)	0	301/301	100%	100%	0
	Tfu_0882 (without signal peptide)	3E-169	245/263	93%	95%	0
	Tfu_0267	2E-04	38/135	28%	43%	3
E9LVH9	Tfu_0883 (without signal peptide)	0	301/301	100%	100%	0
	Tfu_0882 (without signal peptide)	3E-169	245/263	93%	95%	0
	Tfu_0267	2E-04	38/135	28%	43%	3
A0A9P2TBN9	Tfu_0883 (without signal peptide)	0	301/301	100%	100%	0
	Tfu_0882 (without signal peptide)	3E-169	245/263	93%	95%	0
	Tfu_0267	2E-04	38/135	28%	43%	3
E5BBQ2	Tfu_0883 (with signal peptide)	0	319/319	100%	100%	0
	Tfu_0882 (with signal peptide)	4E-171	245/263	93%	95%	0
	Tfu_0267	2E-04	38/135	28%	43%	3

Then et al. (2015) reported the presence of five enzymes in *T. fusca* KW3 with cutinase activity, viz., Tfh (NCBI ID: AAZ54921), BTA2 (NCBI ID: CAH17554), Tfu_0882 (NCBI ID: AAZ54920.1), Tf_Cut1 (NCBI ID: CBY05529) and Tf_Cut2 (BTA-1) (NCBI ID: CBY05530). The protein sequences in FASTA format were aligned against *T. fusca* YX genome using tblastn. Pairwise alignment resulted in two matching genes in *T. fusca* YX, Tfu_0883 (with or without signal peptide) and Tfu_0882 (with or without signal peptide) (see Table 5.2).

Table 5.2. Pairwise alignment using tblastn of the cutinases in *T. fusca* KW3 against the whole genome of *T. fusca* YX (CP000088.1).

<i>T. fusca</i> KW3 cutinase	Gene matches in <i>T. fusca</i> YX	E-value	Identities	Percent identity	Percent positive	Gaps
Tfh	Tfu_0883 (without signal peptide)	0	319/319	100%	100%	0
	Tfu_0882 (without signal peptide)	3E-169	245/263	93%		0
	Tfu_0267 (partial)	2E-04	38/135	28%	43%	5
BTA2	Tfu_0882 (with partial signal peptide)	0	260/262	99%	100%	0
	Tfu_0883 (with partial signal peptide)	6E-174	242/262	92%	95%	0
	Tfu_0267 (partial)	1E-06	36/131	27%	44%	7
Tfu_0882	Tfu_0882 (with signal peptide)	0	319/319	100%	100%	0
	Tfu_0883 (with signal peptide)	2E-175	285/309	92%	95%	0
	Tfu_0267 (partial)	6E-07	36/131	27%	44%	7
Tf_Cut1	Tfu_0882 (with signal peptide)	0	308/319	97%	98%	0
	Tfu_0883 (with signal peptide)	6E-176	292/309	94%	97%	0
	Tfu_0267 (partial)	1E-04	40/145	28%	42%	7
Tf_Cut2 (BTA-1)	Tfu_0883 (without signal peptide)	5E-180	259/261	99%	99%	0

Tfu_0882 (without signal peptide)	3E-168	243/261	93%	96%	0
Tfu_0267 (partial)	1E-04	38/135	28%	43%	5

In addition to the previous method, a structural search was adopted to widen the breadth to incorporate every active candidate. Using the SuperFamily 2.0 web server, the protein sequence of Tfu_0883 was used as a query. SuperFamily is a database of structural and functional annotations of which the annotation is based on a hidden Markov model. A total of 40 structurally and functionally significant domains were present in 39 proteins in *T. fusca* YX (see Supplementary Figure 5.1), of which 12 additional proteins were shortlisted based on the catalytic function, cellular location and product of the gene. These included enzymes belonging to the families carboxylesterase (Tfu_0155, Tfu_1487), lipase (Tfu_0882, Tfu_0883, Tfu_0267, Tfu_0899), carbon-carbon bond hydrolase (Tfu_2815, Tfu_0513, Tfu_1333), epoxide hydrolase (Tfu_0132, Tfu_0162, Tfu_1239) and PHB depolymerase (Tfu_0082). The cellular location of enzymes was predicted based on the presence of a signal peptide or a transmembrane domain which was predicted using the SignalP 6.0 web server which predicts the presence of five types of signal peptides (Teufel et al., 2022). The enzymes mentioned were further shortlisted based on their cellular location and their predicted activity (see Supplementary Table 1). Of all the mined proteins, three were predicted to be extracellular, viz., Tfu_1487, Tfu_0882 and Tfu_0883, while Tfu_0746 and Tfu_0082 were predicted to be transmembrane proteins. Based on the catalytic function and homology to Tfu_0883, enzymes Tfu_0267, Tfu_0882, and Tfu_0746 could have PET hydrolyzing activity. Hence, the shortlisted enzymes for all further analysis were Tfu_0082, Tfu_0267, Tfu_0746, Tfu_0882, Tfu_0883 and Tfu_1487.

5.3.2 Validation of the methodology

Before proceeding with the analysis of the shortlisted potential PHEs, the methodology had to be optimized and validated. The only cutinase of interest for structural studies in *T. fusca* YX so far has been Tfu_0883 which has been annotated as a triacylglycerol hydrolase (GenBank accession no. AAZ54921.1). To optimize and validate the methodology flow, this enzyme was used as a standard. The enzyme structure used for the study was the Tfu_0883 protein without the signal peptide, expressed in *E. coli* BL21, and determined using X-ray crystallography at a resolution of 1.54 Å (PDB ID: 5ZOA)(Q. L. Dong et al., 2018). This structure was then docked with three-dimensional standard ligand structures PET (CID PubChem 42608431), BHET (CID PubChem 13739) and MHET (CID PubChem 22062452) using the CB-Dock2 web server (Y. Liu et al., 2022) (see Fig 5.1). The catalytic site was studied by assessing the interacting amino acids and the AutoVina score obtained with the AutoDocking mode of the web server. This was compared with the experimentally validated catalytic triad and active site determined in the PET hydrolase from *Ideonella sakaiensis* (IsPETase) by X-ray crystallography (Joo et al., 2018). In IsPETase, the distance between the -OH group of Ser160 and the -NH group of His237 is 3.01 Å, and between the -NH group of His237 and the -OH group of Asp206 the distance is 3.21 Å (RCSB PDB IS: 5XJH) (Joo et al., 2018). These values were used to assess the docking of PET with 5ZOA to understand the spatial arrangement of the catalytic triad and active site.

5.3.3 Primary structure determination and their physicochemical properties

Owing to its molecular size, the location of the shortlisted enzymes is important in understanding the potential interaction with PET. Being polymeric and branched in nature, large molecules of PET are unable to traverse across the cellular membrane of most microorganisms. Using SignalP v6.0 web server (Teufel et al., 2022), Tfu_0267 was predicted to not have a signal peptide, potentially being cytosolic in nature. Tfu_0082 and Tfu_0746 were predicted to have transmembrane domains, and Tfu_0882, Tfu_0883 and Tfu_1487 were predicted to be extracellular enzymes after analyzing their signal peptides. The primary structures of the potential PHEs were analyzed for their polypeptide length, molecular weight, theoretical pI, extinction coefficient, instability index, GRAVY (*grand average* of hydropathy) and aliphatic index using the ExPASy ProtParam web server (see Table 3)(Gasteiger et al., 2005). Both transmembrane proteins have been predicted to be the largest enzymes, with a predicted molecular weight of 53.57 kDa for Tfu_0746 followed by 43.93 kDa for Tfu_0082. The aliphatic index is an indicator of the relative volume of aliphatic amino acids alanine, valine, leucine, and isoleucine in a protein. It is also indicative of thermal stability. Proteins with an aliphatic index ≥ 60 have been reported to be thermally stable up to 60 °C (Ikai, 1980). Each of the enzymes has an aliphatic index ranging from 79-89. The GRAVY value of each of the enzymes predicted them to be hydrophilic in nature. Instability index measures the stability of the protein in a test tube, which has been statistically determined to have a value ≤ 40 . Generally, unstable enzymes have a half-life of up to 5 hours (Guruprasad et al., 1990). The instability index of all the enzymes with their respective signal peptides were below 40 indicating their stability. All the proteins were predicted to have stable half-lives. Enzymes Tfu_0082, Tfu_0882, Tfu_0883 and Tfu_1487 were predicted to have two extinction coefficients under the presumption of cysteines and cystines in the polypeptides. Cystines serve an important function in proteins by allowing them to retain their three-dimensional

structure by disulphide linkage (Wiedemann et al., 2020). Furthermore, all the enzymes showed the presence of the GX SXG motif (see Fig. 5.2 and Supplementary Table 5.2). This structural motif is a conserved motif which is representative of triacylglycerol lipases that are characteristic of hydrolytic activity due to the presence of the nucleophilic serine within the motif (Akoh et al., 2004; Rajakumari & Daum, 2010). The nucleophilic serine is an amino acid within the catalytic triad that actively participates in cleaving the ester bond between cutin monomers, and the ester bond between terephthalic acid and ethylene glycol.

Table 5.3. Potential PHEs mined from *Thermobifida fusca* YX (GenBank Accession ID: CP000088.1) that are part of the alpha/beta hydrolase superfamily.

Gene	Tfu_0082	Tfu_0267	Tfu_0746	Tfu_0882	Tfu_0883	Tfu_1487
Annotation	Esterase, PHB depolymerase	Conserved hypothetical protein	Conserved hypothetical protein	Triacylglycerol lipase/cutinase	Triacylglycerol lipase/cutinase	Esterase/lipase
Length	414 aa	318 aa	504 aa	260 aa	260 aa	305 aa
Mol. Wt.	43.93 kDa	35.11 kDa	53.37 kDa	28.33 kDa	32.21 kDa	32.42 kDa
pI	5.59	9.29	5.68	7.14	7.82	5.47
Protein ID	AAZ54120.1	AAZ54305.1	AAZ54784.1	AAZ54920.1	AAZ54921.1	AAZ55523.1
Catalytic Triad	Ser119-Asp197-His256	Ser25-Asp27-His3	Ser113-Asp219-His247	Ser130-Asp176-His208	Ser130-Asp176-His208	Ser157-Asp216-His211
GXSXG motif	GLSAG	GSSLG	GISYG	GHSMG	GHSMG	GDSAG
GRAVY	-0.180	-0.312	-0.041	-0.247	-0.120	-0.018
Extinction coefficient (M ⁻¹ cm ⁻¹)	97790 (cystines) or 97290 (cysteines)	61,420	104,850	38515 (cystines) or 38390 (cysteines)	37025 (cystines) or 36900 (cysteines)	37025 (cystines) or 36900 (cysteines)
Instability index	37.52	28.45	36.04	36.12	38.53	38.28
Aliphatic index	71.47	89	79.06	84.39	84.39	90.39
Location	transmembrane	intracellular	transmembrane	extracellular	extracellular	extracellular

AAZ55523.1	PEQARARVAAGNSSCANGPDLRSVRDIRLPVAD--GQIGARVYTPHA-APRATFVYLHGG	83
AAZ54305.1	-----V-----TGQTRREQVSFD\$AGVACAGILYHPAEPAPGRQPCVMAH	55
AAZ54784.1	-----V-----GSTTQKVRIPVDD-GTELSALVITP-TGTEGPHLLVMPS	67
AAZ54120.1	LTQV-----SAFGSNPGNLMYVYRPAT-LPDNAPLVLLH	75
AAZ54920.1	---P-----YERGNPTDALLE-----ARSGPFSVS--	83
AAZ54921.1	---P-----YERGNPTDALLE-----ASSGPFSVS--	65
:		
AAZ55523.1	GWVTGDLDYSD-ELCRFLADRLAWTVVSDYRL-----APEHP-	120
AAZ54305.1	GFTGTMDR-LS-HYAQRFA-DAGLTVLTFDYRN-----FGASG-	90
AAZ54784.1	AWSTNKLLYVG-AAWKLAR-ESGYQVISYTSRG-----FHESG-	103
AAZ54120.1	GCSQDAATYHAHSGWAKYADSLGFALVYAEQKS-----ANNSSSCFNWFQKSDT	124
AAZ54920.1	---EERASRFG-----ADGFGGGTIYYPRENNTYGAVAI\$PGYTGTQASVAWLGERIA	133
AAZ54921.1	---EENVSRLS-----ASGFGGGTIYYPRENNTYGAVAI\$PGYTGT\$EASIAWLGERIA	115
. : .		
AAZ55523.1	-----F-PTPVNDAYAAVQHV-----A-ATIAANGPLGVG\$GDSAG\$GNL	156
AAZ54305.1	--G-----QPRQLVDI-AGQQADWHAAIRFA-----RSRDDIDPDRIALW\$SSLG\$GGH	135
AAZ54784.1	--G-----RIEV--AG-PRDVADARAII\$DWA-----LANTDADPDRI\$GAGI\$YGAGI	146
AAZ54120.1	ARG-----SGEAQSIRSMVDYAVRTY-----SLDEERVYIS\$GL\$SAG\$GAM	163
AAZ54920.1	SHGFVVITIDTNTTLDQPDSRARQLNAALDYMINDASSAVRSRIDSSRLAVM\$GHS\$M\$GGG	193
AAZ54921.1	SHGFVVITIDTITTL\$DQPDSRAEQLNAALNHMINRASSTVRSRIDSSRLAVM\$GHS\$M\$GGG	175
. : : : : *		
AAZ55523.1	AAACTLRA--RDEHGPR-----LDFQV	176
AAZ54305.1	VLTVAADDQRIAAAVAQ-----IPWLG	157
AAZ54784.1	SLLTAAEDARIKAVGAM-----SGWAD	168
AAZ54120.1	ASEMLAAYPDVFAGGSIVAGIPTGCASSLLDATT\$CMFSGRNLTPKQW\$GDLVRAKNPGWQ\$G	223
AAZ54920.1	TLRLASQRPDLKA-----AIPLT	211
AAZ54921.1	TLRLASQRPDLKA-----AIPLT	193
:		
AAZ55523.1	LLYPVTDHDFTRASYQTHATAFPIGVAEMRWFDHYLPDPERRDLPLASPLRAELAGLPP	236
AAZ54305.1	DWRTTRDKLRSLADPSTRKL----AVAAVRDAWRAWR-----GRAPLLVKV\$GQPG	204
AAZ54784.1	L----TASIYPNETFSTQAV---ELL\$S-----SAHLT\$GRPG	198
AAZ54120.1	PWPRVAIWHGT-----ADAVTPKNAQSSRDQW\$TNVWG-----IGQTP	261
AAZ54920.1	PWHLNKNWSSV-----RVPTLI-----IGADL	233
AAZ54921.1	PWHLNKNWSSV-----TVPTLI-----IGADL	215
*		

Figure 5.2. Multiple protein sequence alignment of the alpha/beta hydrolases using the software ClustalW (Madeira et al., 2024). The red box indicates the presence of the GX\$XG motif, the catalytic serines are highlighted in red while the identical amino acids are highlighted in yellow.

5.3.4 Secondary structure analysis

Secondary structures consist of α -helices, β -turns, extended strands and random coils which were analyzed using the SOPMA web server (Geourjon & Deléage, 1995). Alpha-helices contribute towards the thermostability of the enzyme based on intrinsic stability (Kumar & Bansal, 1998; Petukhov et al., 1998). Each protein contains more α -helices (over 25%) compared to β -turns ($5 \pm 1.5\%$). Tfu_0267 has the highest percentage of α -helices part of its structure (35.53%) compared to its counterparts (see Fig. 5.3). Roughly half of their secondary structures are made of random coils, with the lowest being 38.67% random coils in Tfu_0267. The higher number of random coils in enzymes contributes to the flexibility of conformational changes. The percentage of the secondary structures of all the enzymes in ascending order were beta turns, extended strands, alpha helices, and the highest, random coils.

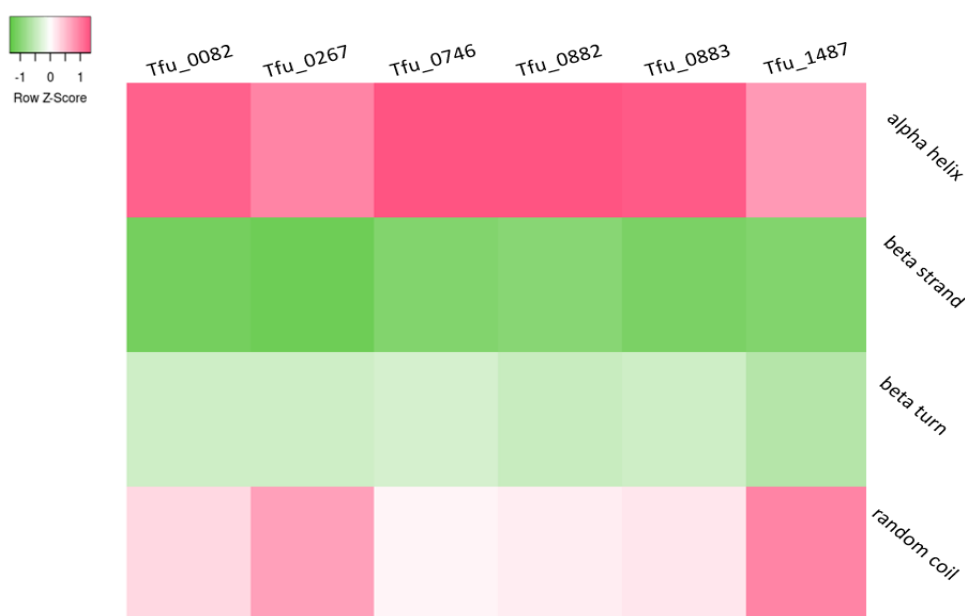


Figure 5.3. Heatmap comparing the secondary structures like alpha helices, beta-turns, beta strands and random coils of the protein sequences derived from the genes Tfu_0082, Tfu_0267, Tfu_0746, Tfu_0882, Tfu_0883 and Tfu_1487 from *T. fusca* YX

5.3.5 Functional and structural motifs

Functional analysis begins with the classification of the enzyme into its homologous superfamily based on their shared similarity in tertiary structures. This was done using InterPro web server (<https://www.ebi.ac.uk/interpro/>) (Paysan-Lafosse et al., 2023). InterPro classifies proteins into families and predicts domains and important sites that provide functional analysis based on the amino acid sequence. Enzymes Tfu_0082, Tfu_0883, Tfu_0882, Tfu_0746 and Tfu_1487 were classified as belonging to the α/β -hydrolase superfamily (InterPro ID: IPR029058). Three of the five, viz. Tfu_0746, Tfu_0882 and Tfu_0883 belong to the chlorophyllase/cutinase family (InterPro ID: IPR017395). This family consists of chlorophyllase and cutinase which share the PET hydrolase fold, which is an important determinant of PET-hydrolyzing activity (Ribitsch et al., 2017).

Protein domains of the potential PHEs were assessed using the Conserved Domain Database (CDD) web server (J. Wang et al., 2023). Tfu_0082 was classified into the α/β hydrolase fold superfamily (cl21494). Tfu_0267 were classified into the hydrolase 4 superfamily (cl38349). The protein Tfu_0746 was classified into the α/β hydrolase superfamily and the x-prolyl-dipeptidyl aminopeptidase (cl35312). Tfu_0883 was classified into the diene lactone hydrolase superfamily (cl43135) which is similar to the α/β hydrolase fold superfamily (cl21494). Tfu_1487 was classified as an acetyl esterase lipase (cl27028). Each of these families is related to or structurally similar to the α/β hydrolase superfamily. In addition, each of these families has a nucleophilic serine that actively participates and is part of the catalytic triad.

The domains were also assessed with GenomeNet web server (<https://www.genome.jp/>) (Tokimatsu et al., 2011), to confirm the results obtained by CDD. Tfu_0082 was classified into the esterase_PHB, AB Hydrolase 1, Hydrolase 4 families. Tfu_0267 was classified into Hydrolase 4, AB hydrolase and diene lactone hydrolase superfamilies. Hydrolase 4 is a serine aminopeptidase

that contains a nucleophilic serine along with histidine and aspartic acid. AB hydrolase (or α/β hydrolase) superfamily (InterPro ID: IPR029058) suggests the structural motif that sheds light on the arrangement of the alpha helices and beta strands. Tfu_0746 was classified into the x-prolyl-dipeptidyl aminopeptidase and alpha/beta hydrolase superfamilies. In addition to the mentioned superfamilies, both Tfu_0882 and Tfu_0883 were classified into the chlorophyllase/cutinase family (InterPro ID: IPR017395). The chlorophyllase/cutinase family domain contains the PET hydrolase fold along with other determinants like α/β -folds and the Ser-His-Asp catalytic triad, which share the PET hydrolase fold, which is an important determinant. Lastly, Tfu_1487 was classified into the AB hydrolase 3 and Hydrolase 4 families.

5.3.6 Determination of tertiary structure and validation

An important contributor towards structural and thermal stability in tertiary structures of proteins are salt bridges. Salt bridges are noncovalent interactions that link a positively charged amino acid with a negatively charged amino acid within 4 Å (400 pm) and are crucial for structural and thermal stability. The number of salt bridges present within the enzymes and their distances were predicted using the ESBRI web server. The most dominant salt bridge in all the enzymes was Asp-Arg with an average distance of 3.29 Å in Tfu_0082, 3.19 Å in Tfu_0267, 3.21 Å in Tfu_0746, 3.36 Å in Tfu_0882, 3.32 Å in Tfu_0883 and 3.14 Å in Tfu_1487. Asp/Arg is a stabilizing salt bridge which means the interacting residues are spaced and ordered such that they favor helix formation by up to 50% and simultaneously slow down the unfolding by 50% (Meuzelaar et al., 2016). Simulation studies indicate that salt bridges create kinetic barriers for unfolding the protein, thereby “locking” the folded structure of the native protein. Arg-Asp and Arg-Glu play a more critical role in the correct folding of the native structure than its stabilization (Meuzelaar et al., 2016). The salt bridge

between arginine and aspartic acid is known to display well-defined geometric preferences that contribute towards the protein's overall stability (Donald et al., 2011). It must be considered that there can be salt bridges that are between 4 – 7 Å, however, those have not been considered due to the limitation of the web server's algorithm. Due to varying polypeptide length, the ratio of salt bridges to the length of the polypeptide was determined to be 1 salt bridge per 15 amino acids for Tfu_0267, Tfu_0882 and Tfu_0883, while Tfu_0746 was assessed to have 1 salt bridge per 20 amino acids and Tfu_1487 was assessed to have 1 salt bridge per 12 amino acids (see Fig. 5.4).

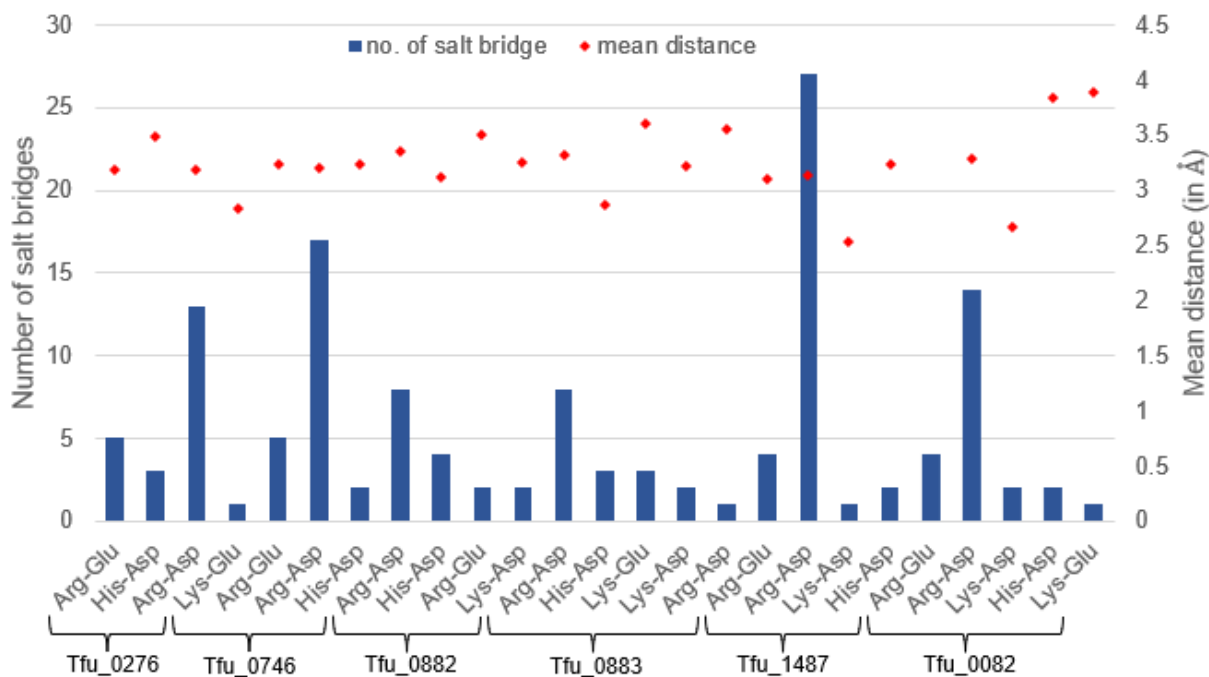


Figure 5.4. Number of salt bridges present in the PHEs and their mean distance in angstrom.

The tertiary structure was determined using the Robetta web server developed by Baker Lab uses the de novo RosETTAFold prediction algorithm which is based on fragment insertion (Kim et al., 2004). Five model structures were predicted for each of the submitted enzyme amino acid

sequences with or without the signal peptide to predict the structure of the PHEs in their predicted cellular location. Each of these predicted structures were assessed for their QMEANDiSco, a composite scoring system for both global and local residues (Studer et al., 2020). The score indicated stability of the structure (see Table 5.4).

Additional analyses like ERRAT, Verify 3D and PROCHECK (Ramachandran plots) (Laskowski et al., 1993) were performed using the SAVES v6.0 web server (<https://saves.mbi.ucla.edu/>). ERRAT analyses the statistics of non-bonded interactions between different atom types and plots the value of the error function versus position of a 9-residue (Colovos & Yeates, 1993). Verify3D determines the compatibility of an atomic model (3D) with its own amino acid sequence (1D) by assigning a structural class based on its location and environment (alpha, beta, loop, polar, nonpolar etc.)(Bowie et al., 1991). A value above 80% is indicative of a stable structure and hence, “passes” the test. PROCHECK checks the stereochemical quality of a protein structure by analyzing residue by residue geometry and overall structure geometry (Laskowski et al., 1993). Every predicted model displayed a disallowed serine in the Ramachandran plots (see Supplementary Figures 5.2-5.7). In some cases, amino acids deviate from their ideal conformation for the optimization of the tertiary structure of the protein like hydrogen bonding keeping hydrophobic residues buried, interactions with the substrate or ligand at the active site (Pal & Chakrabarti, 2002). Using the catalytic triad predicted that falls within the measured distance for optimal catalytic activity (described further in Section 5.3.8.1), the below mentioned models were used for the docking studies. Models 2 and 4 for protein Tfu_0082, model 2 for protein Tfu_0276, model 4 for protein Tfu_0746, model 1 for protein Tfu_0882, model 1 for protein Tfu_0883, and model 3 for protein Tfu_1487 were used for docking studies (see Fig. 5.5).

Table 5.4. Structural or mathematical validation of the predicted tertiary structures of potential PHEs using QMEANDisCo (Studer et al., 2020), Ramachandran plots (Laskowski et al., 1993), ERRAT Score (Colovos & Yeates, 1993), and Verify3D (Bowie et al., 1991).

Gene	Model	ERRAT	Verify3D	PROCHECK (Ramachandran plot)	QMEANDisCo
Tfu_0082	1	88.36	88.36% (Pass)	Core region: 87% Additional allow region: 12% Generous allow region: 0.3% Disallow region: 0.6%	0.67 ± 0.05
	2	Error	90.57% (Pass)	Core region: 87.3% Additional allow region: 10.7% Generous allow region: 1.3% Disallow region: 0.6%	0.68 ± 0.05
	3	86.42	89.33% (Pass)	Core region: 86.4% Additional allow region: 11.4% Generous allow region: 1% Disallow region: 1.3%	0.69 ± 0.05
	4	89.61	88.27% (Pass)	Core region: 86.7% Additional allow region: 11.4% Generous allow region: 1% Disallow region: 1%	0.67 ± 0.05
	5	88.36	89.07% (Pass)	Core region: 84.4% Additional allow region: 13.3% Generous allow region: 2.3% Disallow region: 0%	0.69 ± 0.05
Tfu_0267	1	96.10	78.3% (Fail)	Core region: 90.7% Additional allow region: 8.2% Generous allow region: 0.4% Disallow region: 0.7%	0.62 ± 0.05
	2	98.38	83.33% (Pass)	Core region: 90.3% Additional allow region: 6.7% Generous allow region: 1.5% Disallow region: 1.5%	0.62 ± 0.05
	3	97.08	79.87% (Fail)	Core region: 90.3% Additional allow region: 8.9% Generous allow region: 0.4% Disallow region: 0.4%	0.62 ± 0.05
	4	97.39	76.73% (Fail)	Core region: 90% Additional allow region: 8.6% Generous allow region: 1.1% Disallow region: 0.4%	0.62 ± 0.05
	5	95.11	83.65% (Pass)	Core region: 88.1% Additional allow region: 11.2%	0.62 ± 0.05

				Generous allow region: 0.0% Disallow region: 0.7%	
Tfu_0746	1	94.54	88.1% (Pass)	Core region: 87.2% Additional allow region: 11.6% Generous allow region: 0.0% Disallow region: 1.2%	0.72 ± 0.05
	2	91.51	97.87% (Pass)	Core region: 86% Additional allow region: 10.4% Generous allow region: 1.2% Disallow region: 2.4%	0.72 ± 0.05
	3	94.35	88.89% (Pass)	Core region: 84.7% Additional allow region: 12.1% Generous allow region: 1.7% Disallow region: 1.5%	0.71 ± 0.05
	4	92.71	89.09% (Pass)	Core region: 87.2% Additional allow region: 9.7% Generous allow region: 1.2% Disallow region: 1.9%	0.71 ± 0.05
	5	89.89	90.48% (Pass)	Core region: 88.4% Additional allow region: 8.7% Generous allow region: 1.5% Disallow region: 1.5%	0.72 ± 0.05
Tfu_0882	1	92.82	92.31% (Pass)	Core region: 89.4% Additional allow region: 9.3% Generous allow region: 1.4% Disallow region: 0.0%	0.83 ± 0.05
	2	95.63	92.69% (Pass)	Core region: 89.8% Additional allow region: 7.9% Generous allow region: 2.3% Disallow region: 0.0%	0.83 ± 0.05
	3	93.97	92.69% (Pass)	Core region: 84.7% Additional allow region: 12.5% Generous allow region: 1.9% Disallow region: 0.9%	0.84 ± 0.05
	4	94.44	86.15% (Pass)	Core region: 87% Additional allow region: 9.7% Generous allow region: 2.8% Disallow region: 0.5%	0.83 ± 0.05
	5	94.69	92.31% (Pass)	Core region: 90.3% Additional allow region: 6.9% Generous allow region: 1.9% Disallow region: 0.9%	0.84 ± 0.05
Tfu_0883 (validation)	1	94.35	88.85% (Pass)	Core region: 88.9% Additional allow region: 9.2% Generous allow region: 1.4% Disallow region: 0.5%	0.84 ± 0.05

	2	94.4	88.08% (Pass)	Core region: 87.1% Additional allow region: 11.1% Generous allow region: 1.4% Disallow region: 0.5%	0.84 ± 0.05
	3	96.42	88.46% (Pass)	Core region: 88% Additional allow region: 9.2% Generous allow region: 1.4% Disallow region: 1.4%	0.83 ± 0.05
	4	93.22	87.69% (Pass)	Core region: 88% Additional allow region: 10.1% Generous allow region: 1.4% Disallow region: 0.5%	0.84 ± 0.05
	5	96.42	81.92% (Pass)	Core region: 88.5% Additional allow region: 9.2% Generous allow region: 1.4% Disallow region: 0.9%	0.84 ± 0.05
Tfu_1487	1	93.48	90.07% (Pass)	Core region: 88.5% Additional allow region: 10.6% Generous allow region: 0.4% Disallow region: 0.4%	0.79 ± 0.05
	2	95.05	89.71% (Pass)	Core region: 89.4% Additional allow region: 9.3% Generous allow region: 0.9% Disallow region: 0.4%	$0.79. \pm 0.05$
	3	96.59	92.65% (Pass)	Core region: 87.2% Additional allow region: 11.5% Generous allow region: 0% Disallow region: 1.3%	0.78 ± 0.05
	4	98.09	91.91% (Pass)	Core region: 90.3% Additional allow region: 6.6% Generous allow region: 1.3% Disallow region: 1.8%	0.78 ± 0.05
	5	94.25	91.18% (Pass)	Core region: 88.5% Additional allow region: 8.4% Generous allow region: 1.3% Disallow region: 1.8%	0.78 ± 0.05

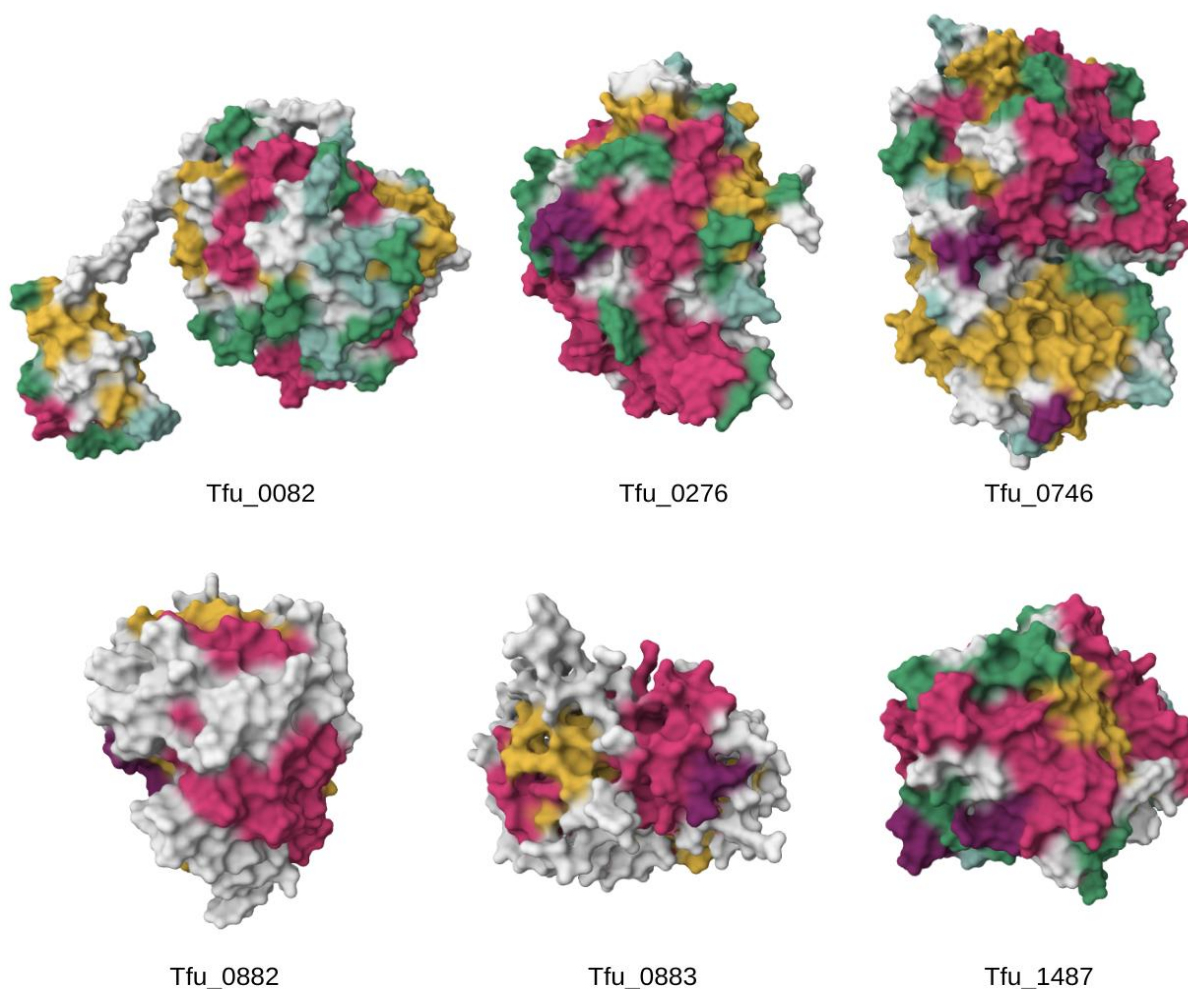


Figure 5.5. Predicted tertiary structure models of Tfu_0082, Tfu_0267, Tfu_0746, Tfu_0882, Tfu_0883 and Tfu_1487.

5.3.7 Cutin monomers

In addition to the standard ligands used in the previous molecular docking, the enzyme's primary substrate is the cutin which is a naturally occurring polymer that protects the plant surface. Cutin from different sources have varying monomers. Cutin from apples primarily consists of 10,16-dihydroxyhexadecanoic acid, 10,20-dihydroxyeicosanoic acid, 9,10,18-trihydroxyoctadecanoic acid (also known as phloionolic acid), 9,10-epoxy-12-octadecenoic acid and 9,10-epoxy-18-

hydroxy-12-octadecenoic acid (Arrieta-Baez et al., 2020). Cutin from tomatoes primarily consists of 10,16-dihydroxyhexadecanoic acid, 16-hydroxyhexadecanoic acid (also known as juniperic acid), 9,10,16-trihydroxyhexadecanoic acid and 9,10,18-trihydroxyoctadecanoic acid. The monomers whose three-dimensional structure was not available were constructed using Avogadro v.1.1.1 software (see Fig. 5.6). In addition, the dimers of the commonly occurring monomers were constructed for docking with the three-dimensional constructed proteins. The heavily branched cutin polymers for both tomato and apple cutins were constructed by joining the two monomers at the carbonyl oxygen of one monomer with the oxygen of the hydroxyl group of the second monomer (Fich et al., 2016b) (see Fig. 5.7). The polymers branch outward, i.e., away from the xylogalacturonan, which is how it is naturally present in plant cuticles. No cross-linking has been shown in this depiction of cutin polymer. Due to the limitation of the docking softwares, the heavily branched polymers could not be used for docking. Instead, dimers of the most commonly occurring monomers were constructed (in Avogadro v.1.1.1) and docked with the potential PHEs. The most commonly occurring monomers for tomato cutin are 10,16-dihydroxyhexadecanoic acid and 16-hydroxyhexadecanoic acid. The most commonly occurring monomers for apple cutin are 10,16-dihydroxyhexadecanoic acid and 9,10,18-trihydroxyoctadecanoic acid (Arrieta-Baez et al., 2020).

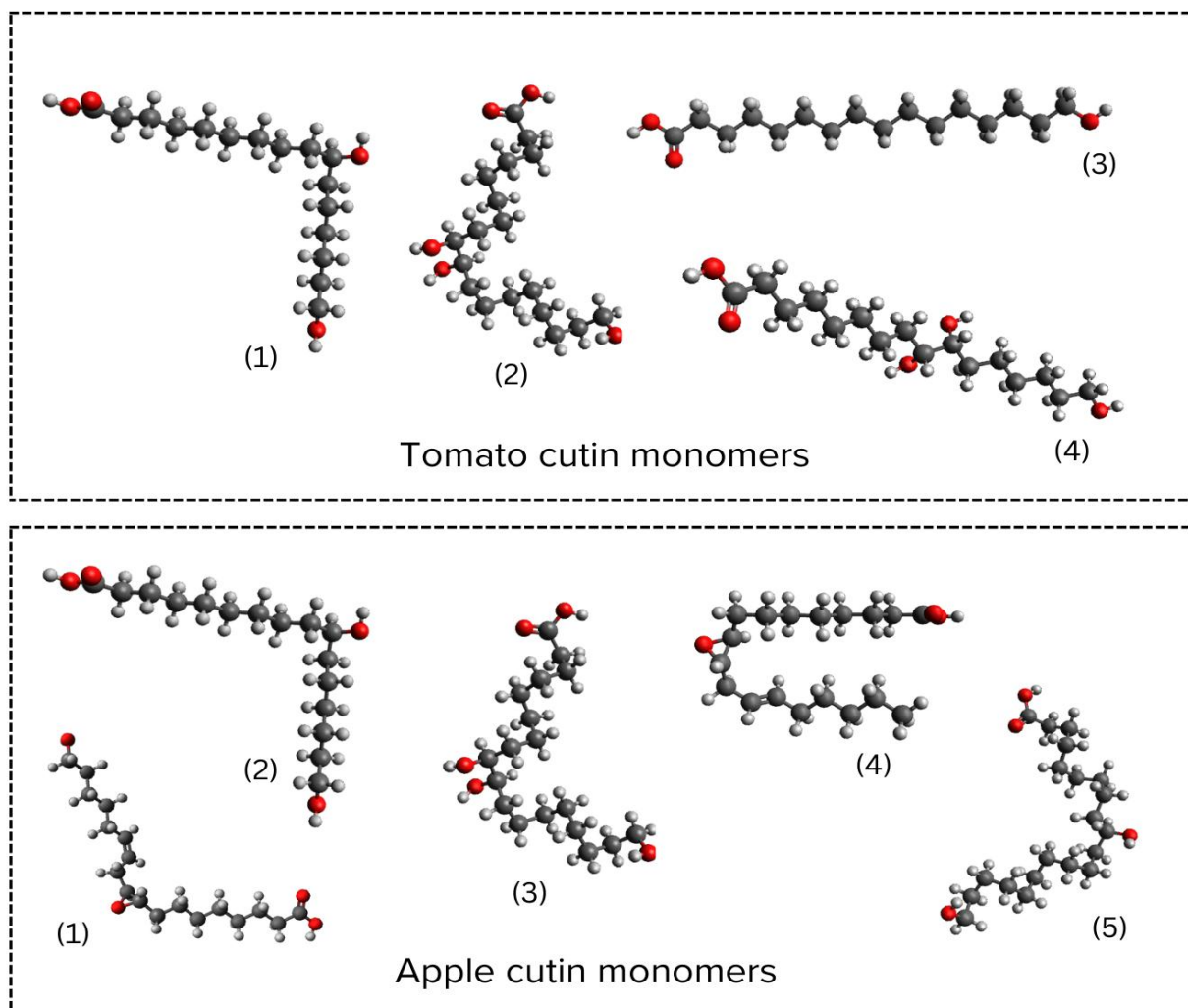


Figure 5.6. The constructed three-dimensional structures of cutin monomers from tomato and apple. Cutin monomers from tomato include (1) 10,16-dihydroxyhexadecanoic acid, (2) 9,10,18-trihydroxyoctadecanoic acid, (3) 16-hydroxyhexadecanoic acid, and (4) 9,10,16-trihydroxyhexadecanoic acid. Cutin monomers from apple include (1) 9,10-epoxy-18-hydroxy-12-octadecenoic acid, (2) 10,16-dihydroxyhexadecanoic acid, (3) 9,10,18-trihydroxyoctadecanoic acid, (4) 9,10-epoxy-12-octadecenoic acid, and (5) 10,20-dihydroxyeicosanoic acid.

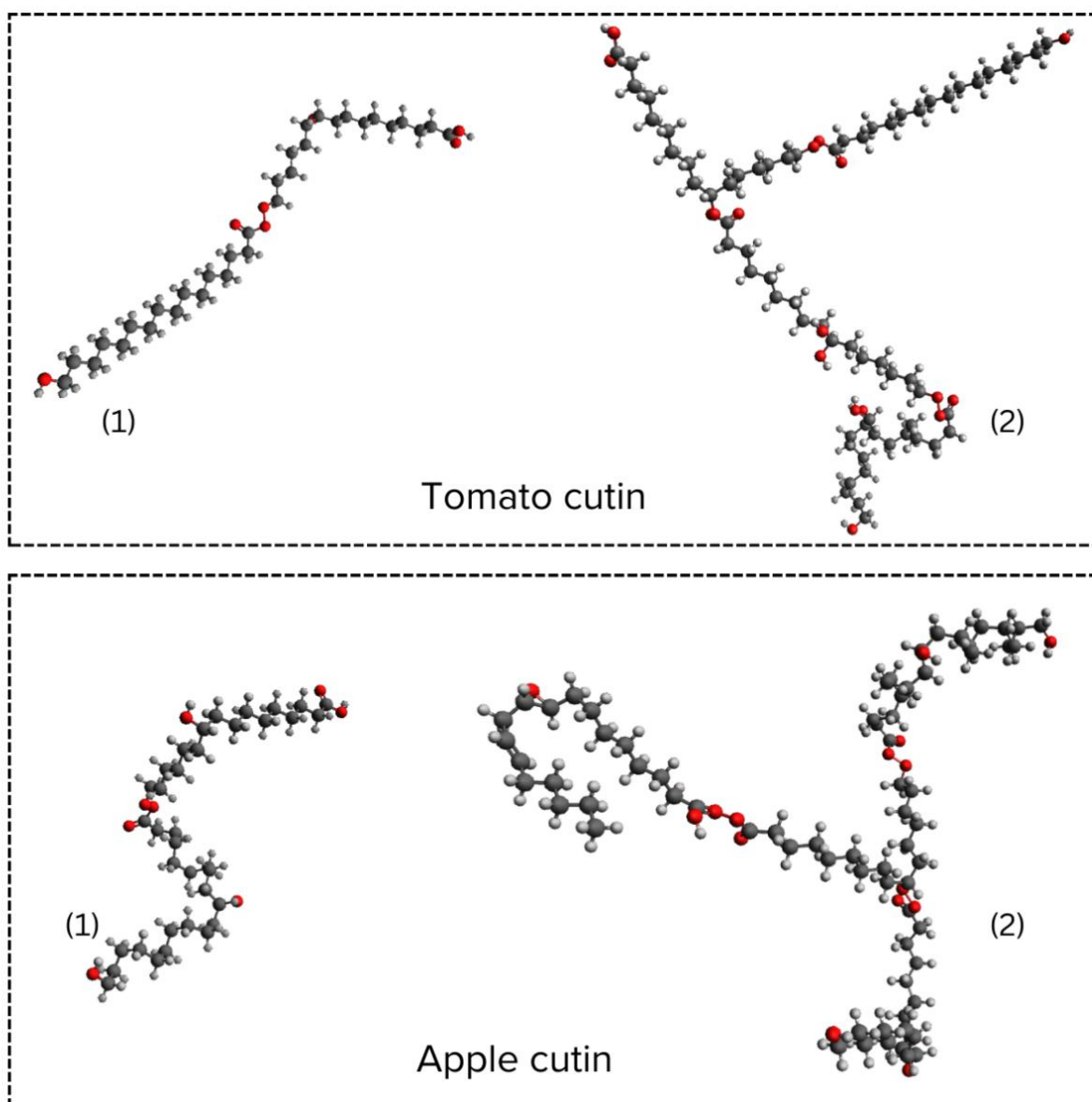


Figure 5.7. Three-dimensional constructed cutin dimers and polymer from tomato and apple. The dimers used for docking studies are depicted in structures (1) while the heavily branched polymer is depicted in structures (2).

5.3.8 Molecular docking of Tf_PHEs

The determined structure 4CG1 (thermostable polyethylene terephthalate degrading hydrolase from *Thermobifida fusca* YX) was docked with PET (CID PubChem 42608431), BHET (CID

PubChem 13739) and MHET (CID PubChem 22062452) ligands using CB-Dock2 (created by Cao Lab, Sichuan University). Five potential interaction frameworks derived from a curvature-dependent surface area model for the prediction of protein-ligand binding affinity pockets were suggested (Y. Liu et al., 2022). A cavity of interest was selected based on fulfilment of three criteria. Firstly, the obtained binding energy values (Vina Score) for each pocket were assessed for negative values suggesting an exergonic reaction. Secondly, the cavity volume was taken into consideration to accommodate polymers PET and cutin. Lastly, the expected interacting catalytic triad, viz., histidine, aspartic acid and the nucleophilic serine were present in the cavity of interest. Every model predicted by Robetta web server for each was docked with PET. Using the previously mentioned criteria, one model for each potential PHE was selected. Among the potential predicted PET hydrolases, Tfu_0082 had the largest cavity size while Tfu_1487 had the smallest cavity size (see Table 5.5). The previously studied enzyme, Tfu_0883 had a cavity size of 219 with -5.8 kJ/mol binding energy. All the cavities of interest demonstrated negative binding energies, which is ideal for substrate-ligand interactions. The predicted enzyme structures were coloured according to their secondary structures, where, alpha helices were magenta, and beta sheets were yellow, meanwhile, the ligands were coloured according to their elements, where, nitrogen was blue, oxygen was red, carbon was gray, and hydrogen was white.

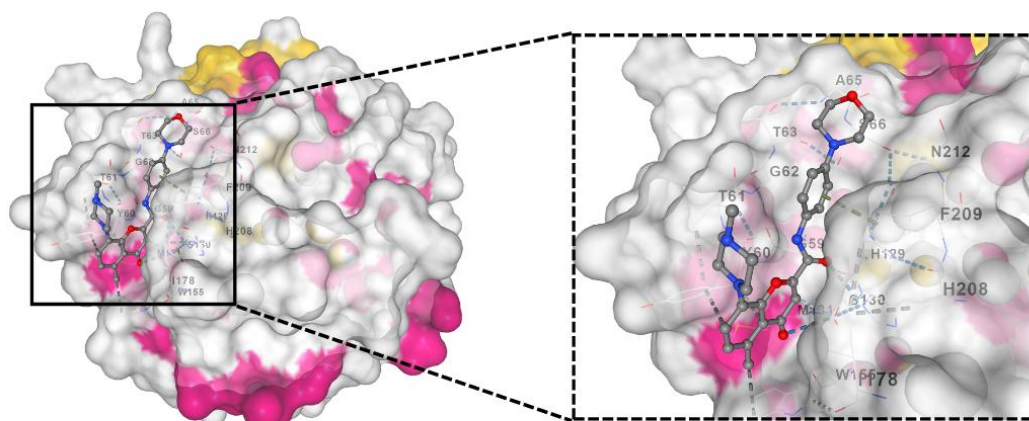
Additionally, the experimentally determined structures of 5ZOA (three-dimensional structure of the protein encoded by the gene Tfu_0883 without the signal peptide) and 7QJS (three-dimensional structure of the protein encoded by the gene Tfu_0882 with the signal peptide) were docked with PET, BHET and MHET ligands (see Figures 5.1 & 5.8). Structures 4CG1 and 5ZOA have a 100% identity in their polypeptide sequence, the structure of 4CG1 contains the signal peptide as well as a His-tag that was used for its purification. Meanwhile, 5ZOA neither has a signal peptide nor a

His-tag, therefore being the closest to an accurate representation of the extracellular PHE. The sequence of 7QJS was 100% identical to Tfu_0882. The potential PHEs were docked with the ligands to assess the potential binding interaction between the predicted catalytic triad and the ligands (see Figures 5.9-5.18). Ligands, PET, BHET and MHET were docked with the potential PHEs using CB-Dock2 (Y. Liu et al., 2022). The cutin dimers of both apple and tomato origin were docked using the AutoDock Vina function in the UCSF Chimera due to their large size (Pettersen et al., 2004). Despite its accommodation on USCF Chimera, only 32 torsions of the dimer could be docked. The PET, BHET and MHET ligands interact with the catalytic triad analogous to the already studied Tfu_0883. The cutin ligands interact similarly.

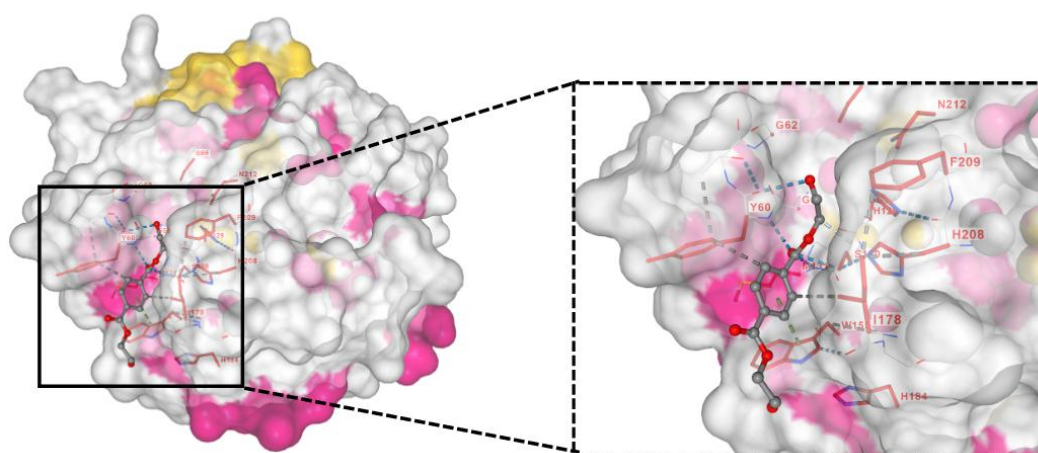
Table 5.5. Amino acid residues of the predicted active site cavity of the shortlisted models of the potential PHEs from *Thermobifida fusca* YX using CB-Dock2

Enzyme & Model	Cavity volume	Vina Score (kJ/mol)	Amino acid residues in the active site
Tfu_0082 (model 2)	654	-7.5	GLY37 CYS38 SER39 GLN40 TYR45 ASN72 SER73 SER74 CYS76 PHE77 TRP79 PHE80 LEU118 SER119 ALA120 ILE145 LEU153 LEU154 ALA156 THR157 MET160 PHE161 ALA196 ASP197 ALA198 VAL199 VAL200 MET254 GLY255 HIS256 TYR275
Tfu_0267 (model 2)	269	-8.0	MET1 ASN2 HIS3 GLY4 THR5 ARG6 GLY7 PRO8 PRO9 GLN23 VAL24 SER25 ASP 27 ALA32 ALA34 GLY87 ALA88 SER89 GLY90 ARG94 THR226 LEU227
Tfu_0746 (model 4)	467	-8.3	ALA40 TRP41 SER42 THR43 LEU46 LEU47 TYR48 ILE112 SER113 TYR114 LEU158 SER162 ASP 219 HIS247 ALA248 GLU251 MET358 GLN365

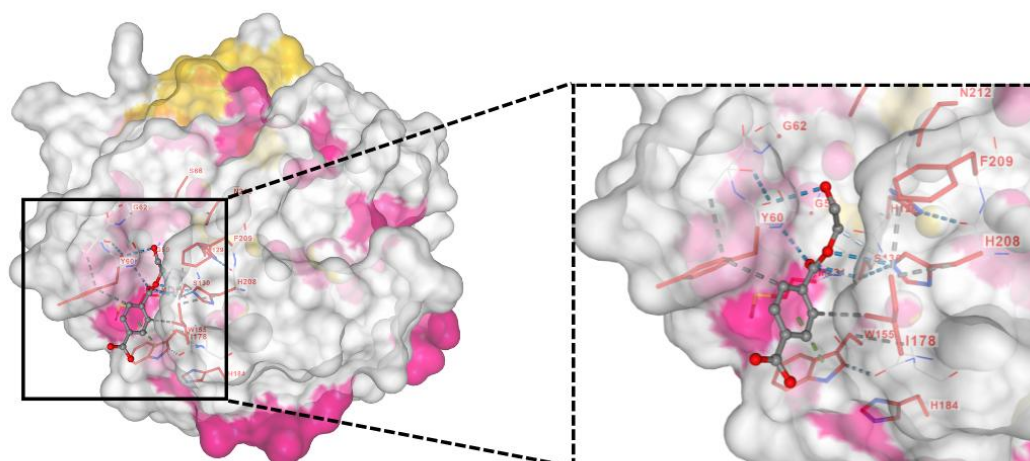
Tfu_0882 (model 1)	113	-8.7	PRO58 GLY59 TYR60 THR61 GLY62 THR63 ALA65 SER66 HIS129 SER130 MET131 TRP155 ASP176 ILE178 HIS208 PHE209 ASN212 ILE213
Tfu_0883 (model 1)	219	-5.8	GLY59 TYR60 THR61 GLY62 ALA65 SER66 HIS129 SER130 MET131 TRP155 ASP 176 ILE178 HIS208 PHE209 ASN212
Tfu_1487 (model 3)	100	-5.8	THR154 ARG155 ALA156 SER157 GLN159 THR160 HIS161 THR163 ALA164 PHE165 GLY210 HIS211 ARG215 ASP216 VAL219 ALA220 GLU236 PRO238



7QJS docked with PET



7QJS docked with BHET



7QJS docked with MHET

Figure 5.8. 7QJS docked with PET, BHET and MHET using CB-Dock2 web server

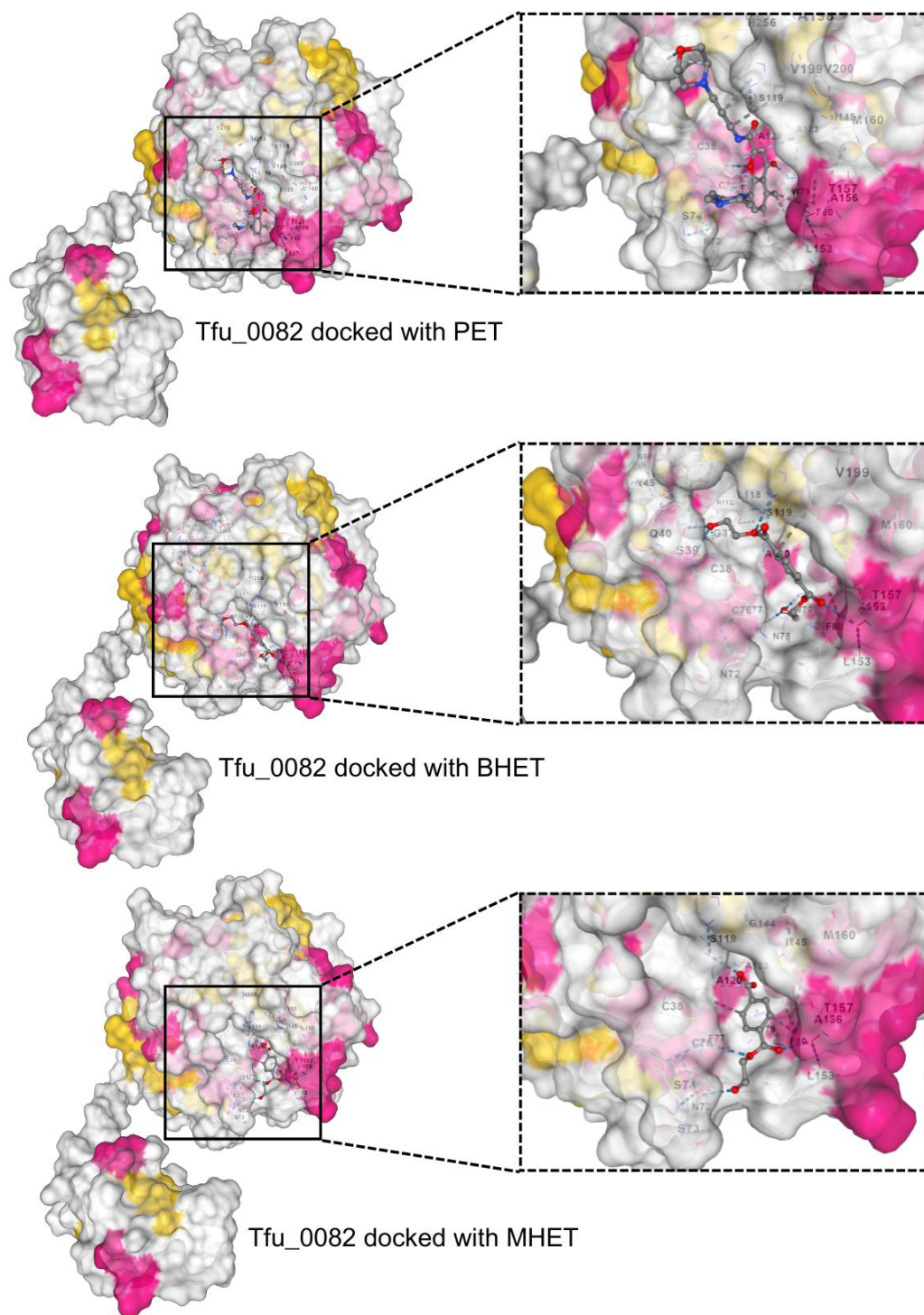


Figure 5.9. Potential PHE Tfu_0082 docked with PET, BHET and MHET using CB-Dock2 web server

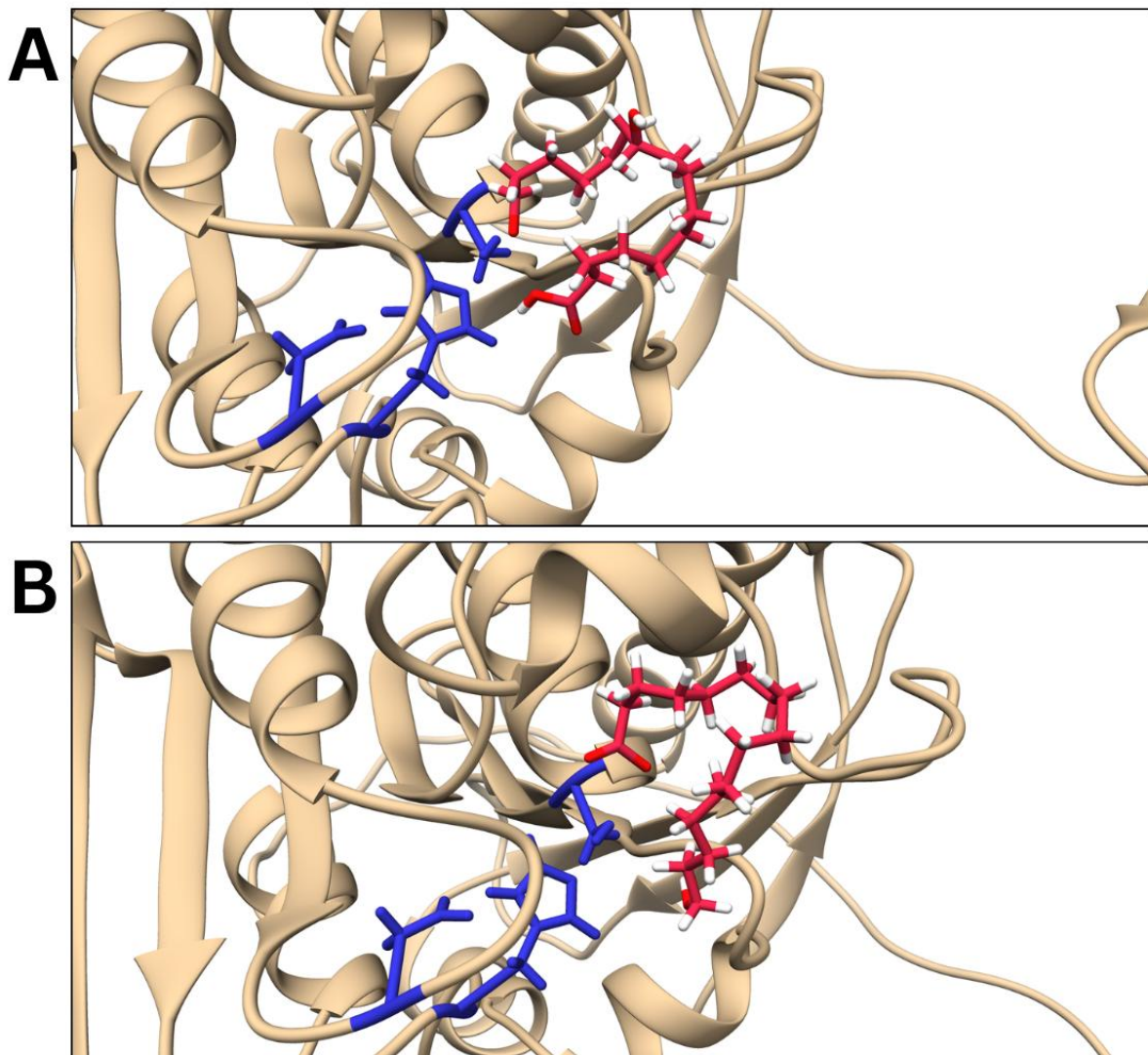
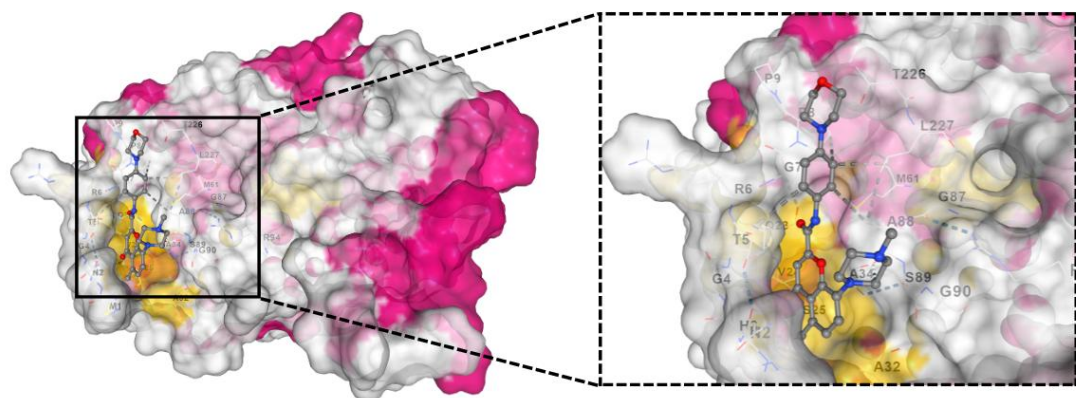
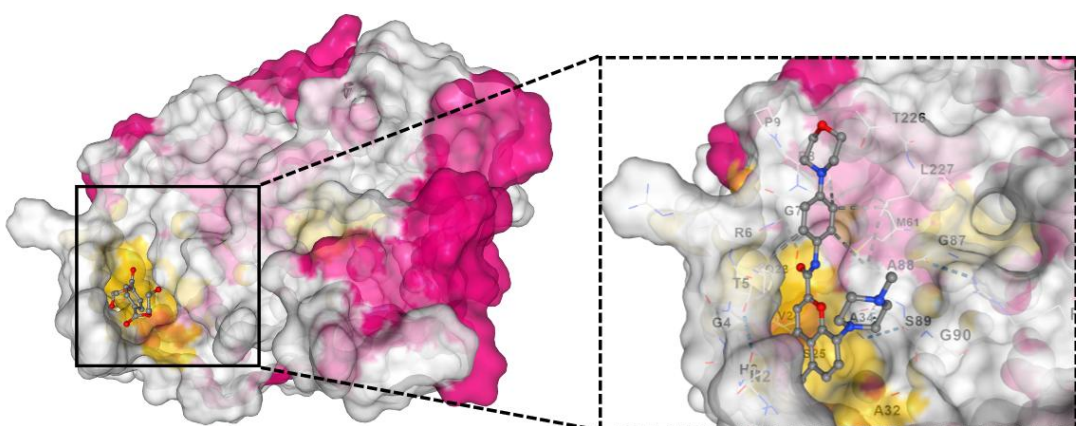


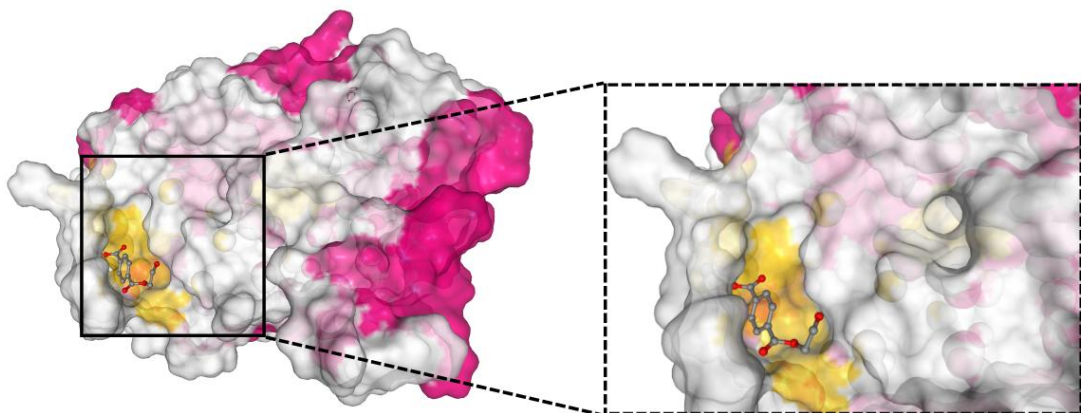
Figure 5.10. Potential PHE Tfu_0082 docked with apple (A) and tomato (B) cutin dimers using the AutoDock Vina function on UCSF Chimera software. The catalytic triad is denoted as blue and the cutin ligand is denoted as red.



Tfu_0276 docked with PET



Tfu_0276 docked with BHET



Tfu_0276 docked with MHET

Figure 5.11. Potential PHE Tfu_0276 docked with PET, BHET and MHET using CB-Dock2

web server

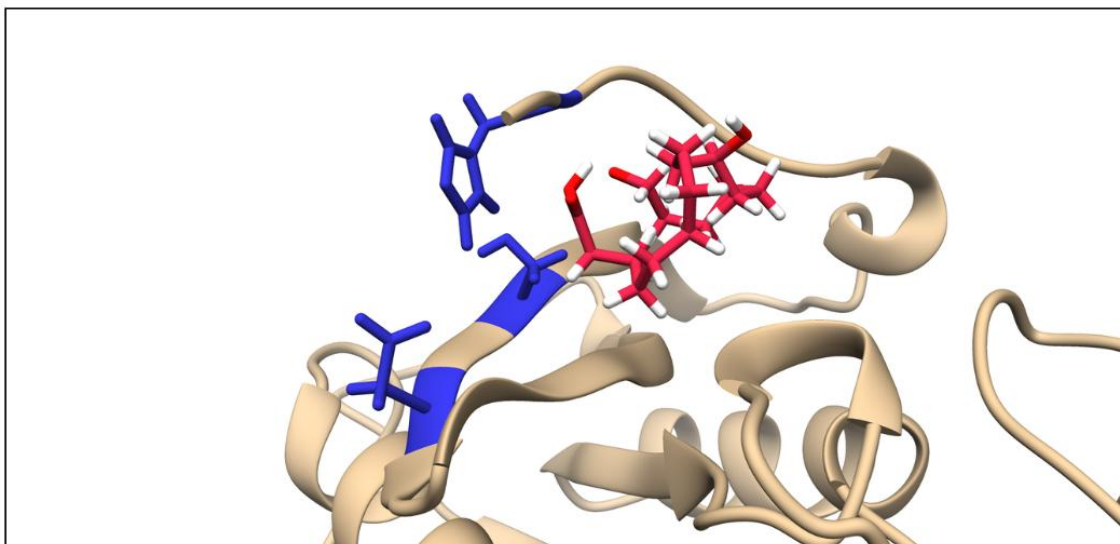
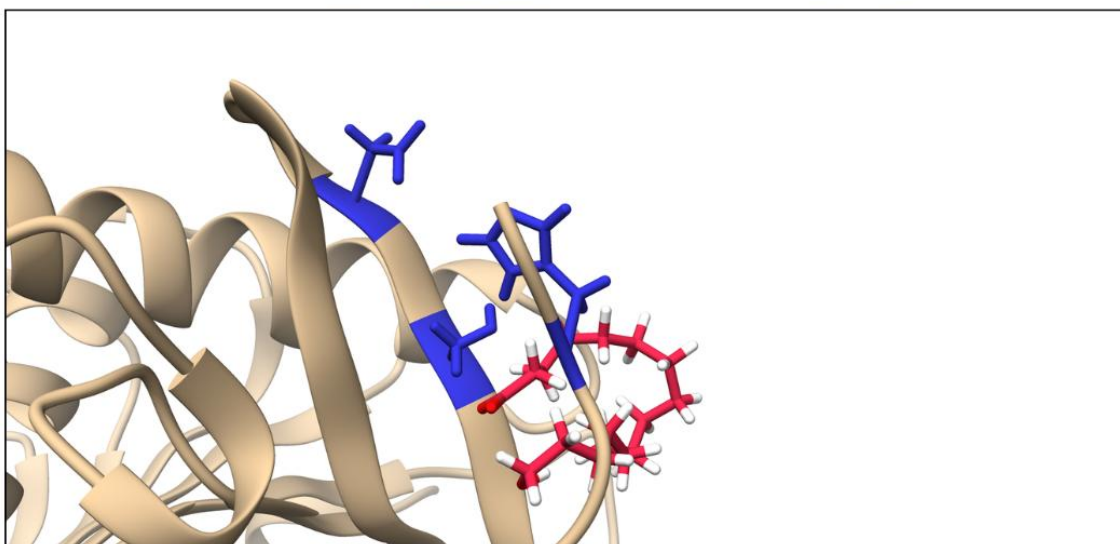
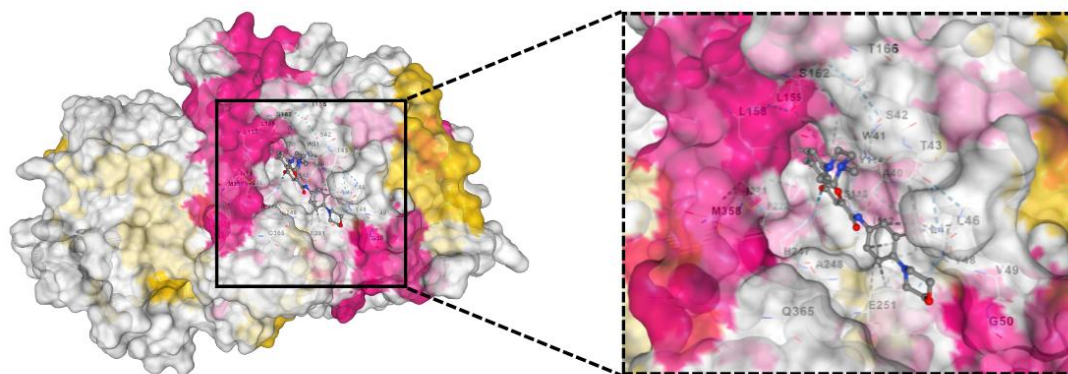
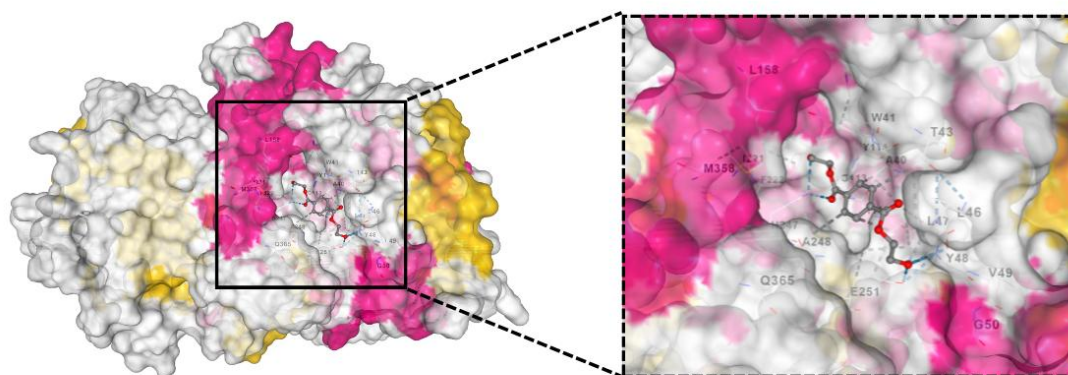
A**B**

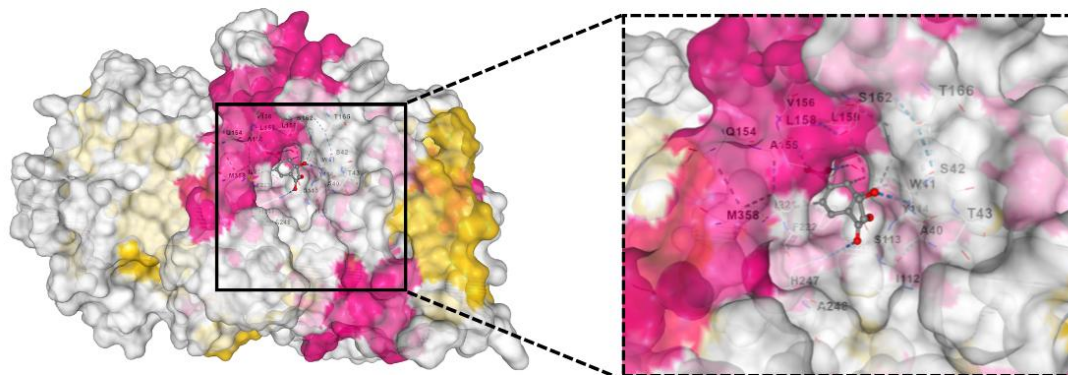
Figure 5.12. Potential PHE Tfu_0276 docked with tomato and apple cutin dimers using the AutoDock Vina function on UCSF Chimera software. The catalytic triad is denoted as blue and the cutin ligand is denoted as red.



Tfu_0746 docked with PET



Tfu_0746 docked with BHET



Tfu_0746 docked with MHET

Figure 5.13. Potential PHE Tfu_0746 docked with PET, BHET and MHET using CB-Dock2 web server

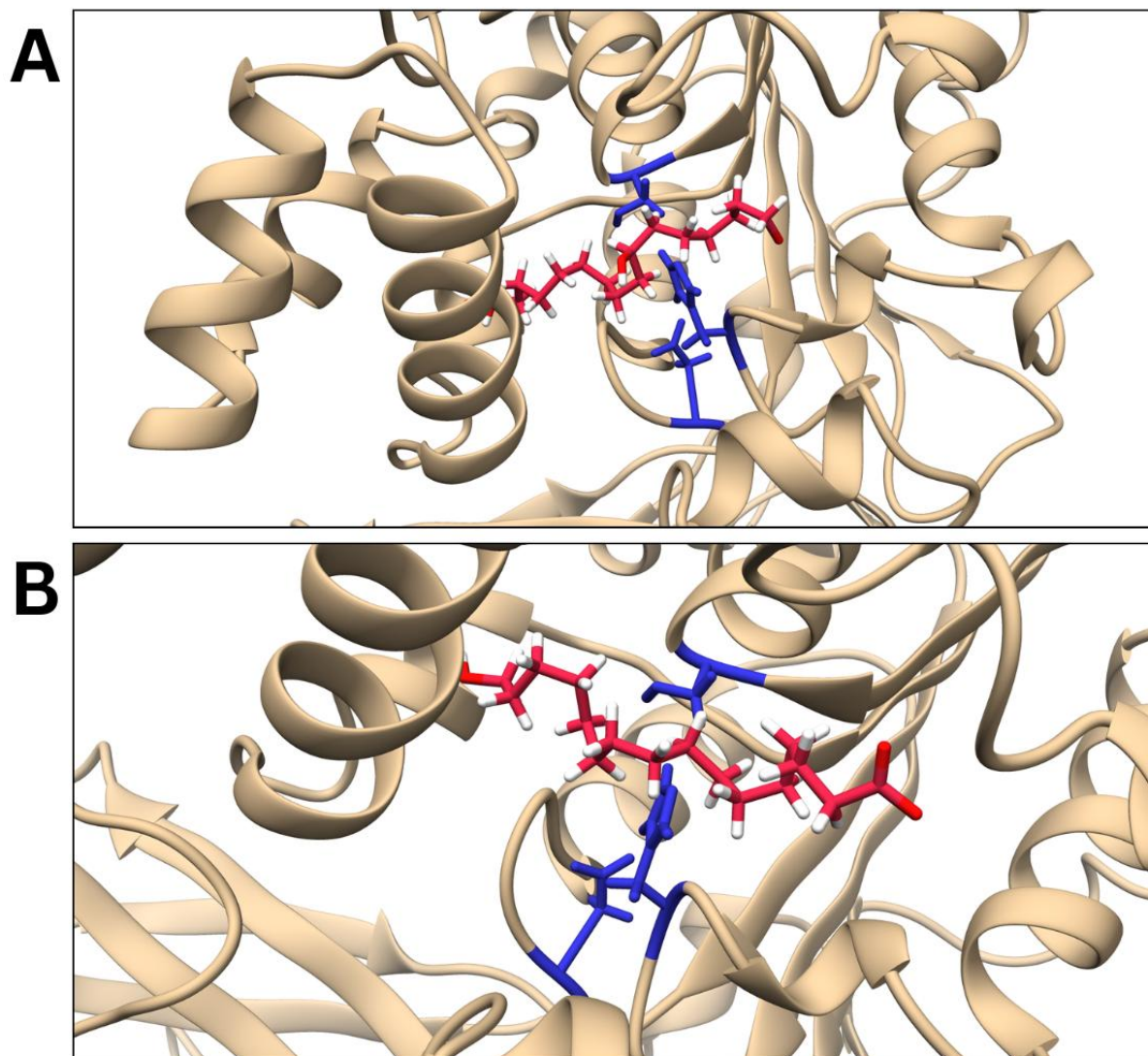


Figure 5.14. Potential PHE Tfu_0746 docked with tomato and apple cutin dimers using the AutoDock Vina function on UCSF Chimera software. The catalytic triad is denoted as blue and the cutin ligand is denoted as red.

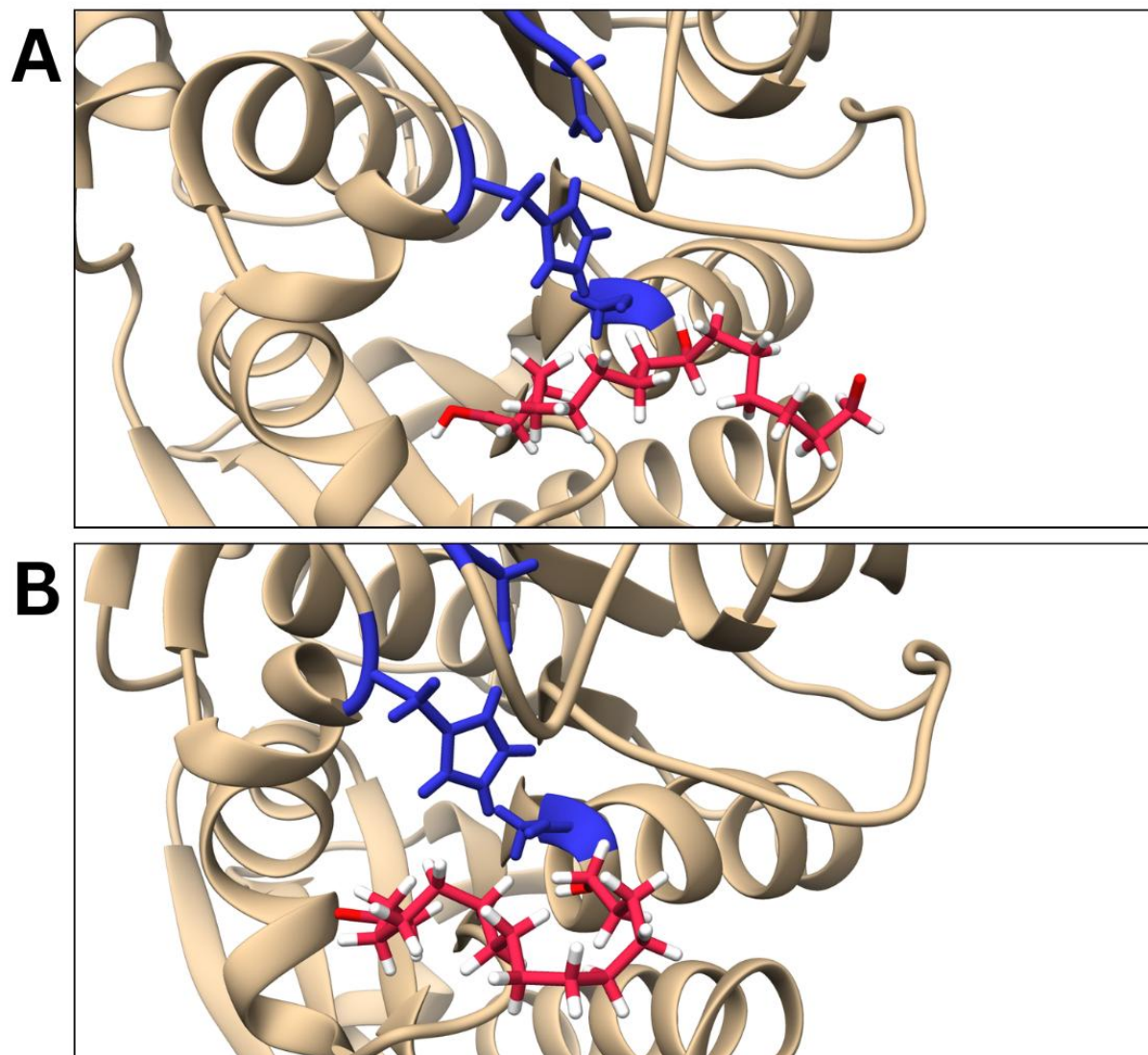


Figure 5.15. PHE Tfu_0882 docked with tomato and apple cutin dimers using the AutoDock Vina function on UCSF Chimera software. The catalytic triad is denoted as blue and the cutin ligand is denoted as red.

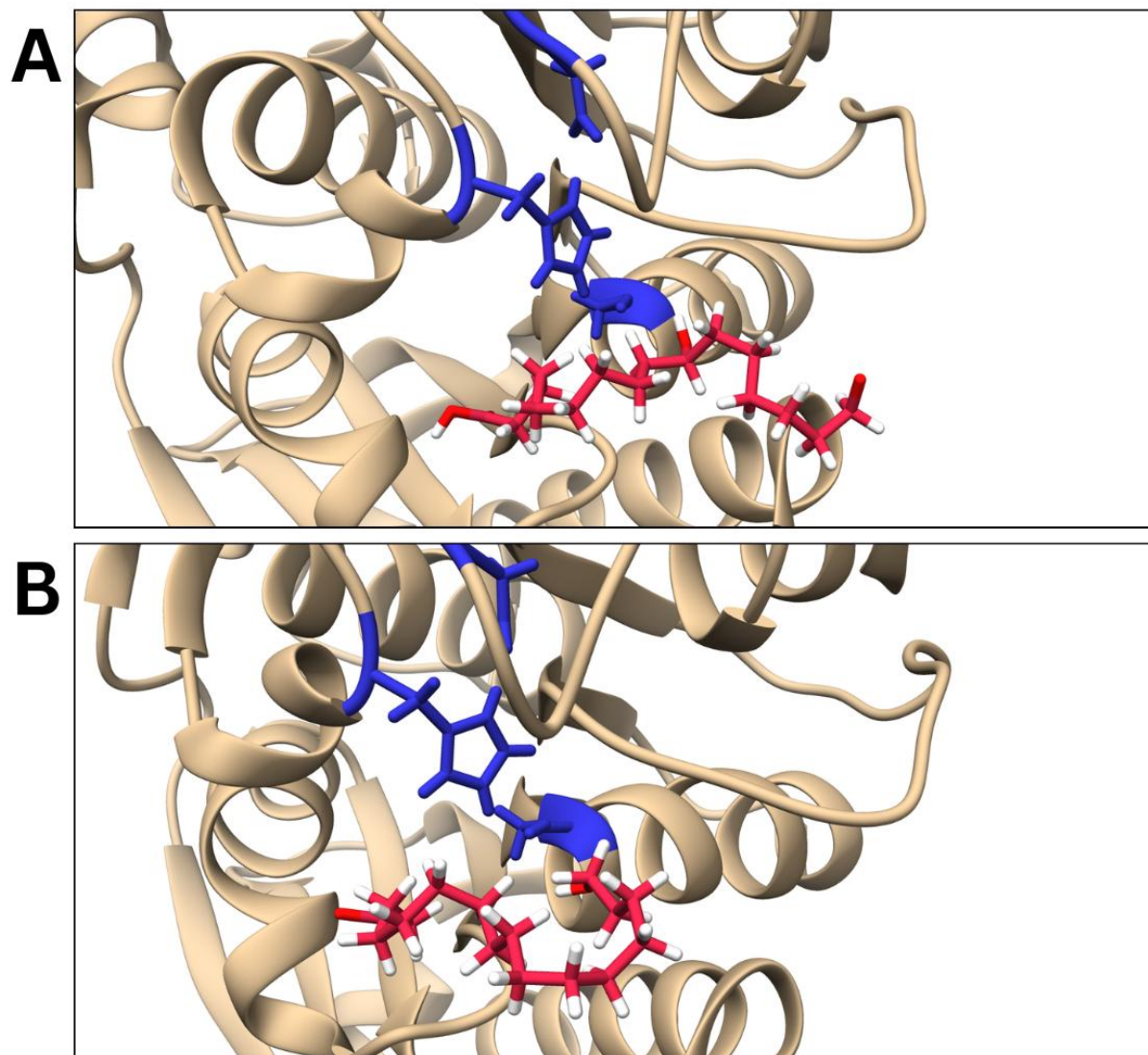
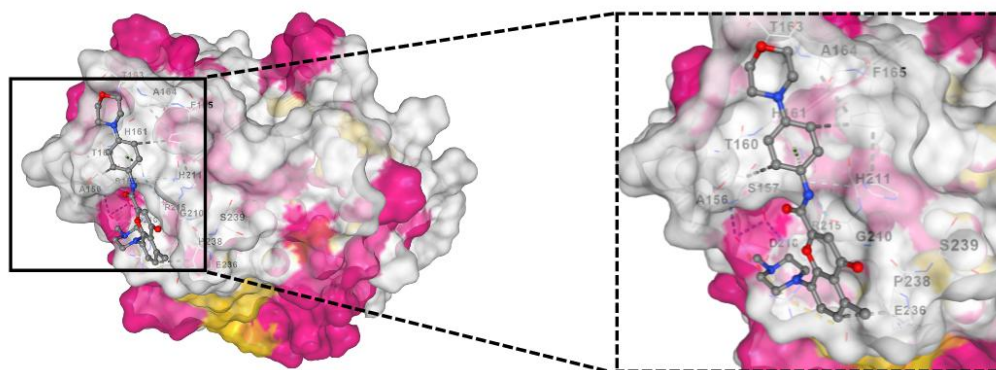
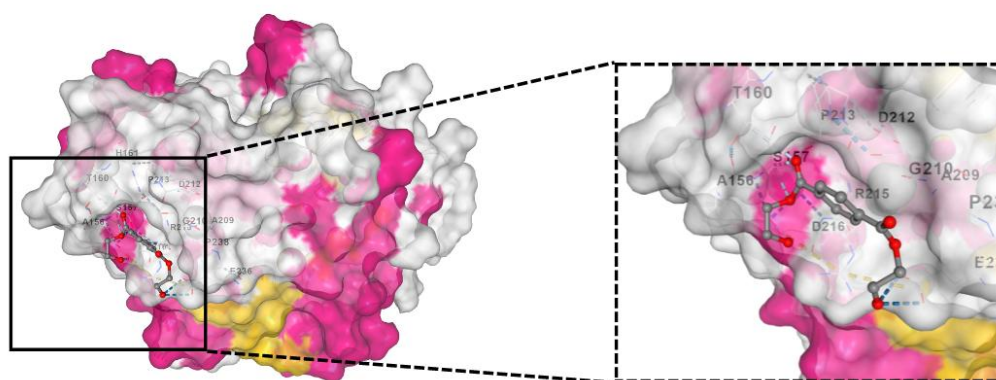


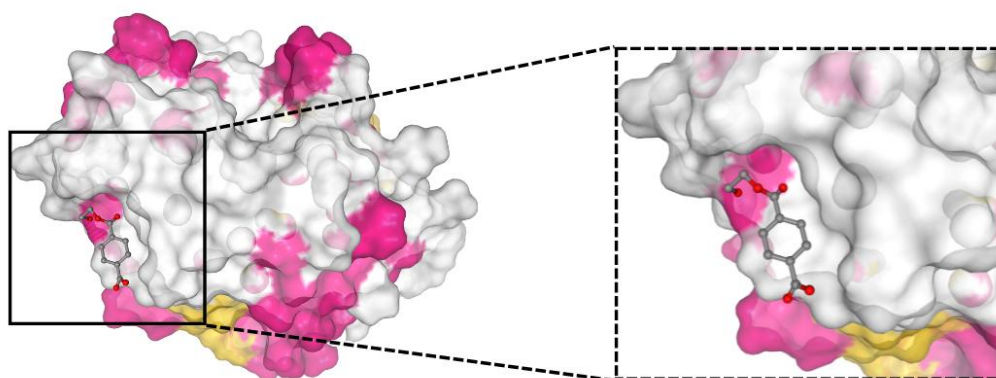
Figure 5.16. PHE Tfu_0883 docked with tomato and apple cutin dimers using the AutoDock Vina function on UCSF Chimera software. The catalytic triad is denoted as blue and the cutin ligand is denoted as red.



Tfu_1487 docked with PET



Tfu_1487 docked with BHET



Tfu_1487 docked with MHET

Figure 5.17. Potential PHE Tfu_1487 docked with PET, BHET and MHET using CB-Dock2 web server.

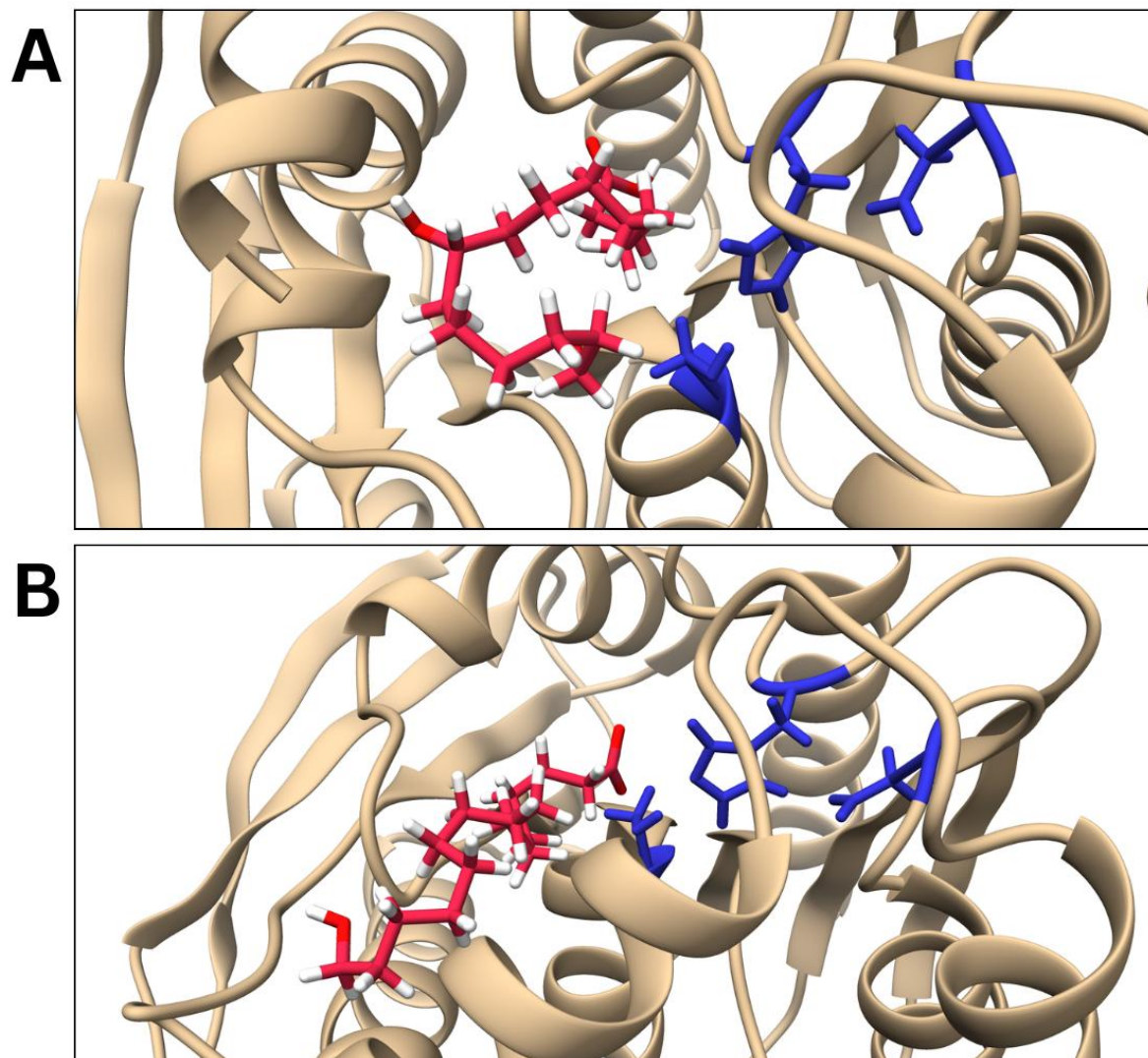


Figure 5.18. PHE Tfu_1487 docked with tomato and apple cutin dimers using the AutoDock Vina function on UCSF Chimera software. The catalytic triad is denoted as blue and the cutin ligand is denoted as red.

5.3.8.1 Determination of catalytic triad

Determination of the catalytic triad was done with the help of docking a standard ligand, PET, with the models and assessing the interacting serines in the five predicted cavities (using CB-Dock2) of the potential PHEs. The interacting amino acids in each of the predicted active site

cavities were assessed for the presence of the disallowed serine, aspartic acid and histidine (see Table 5.5) (Nardini & Dijkstra, 1999). To further confirm the predicted amino acids in the catalytic triad, two independent web servers were used to predict the catalytic triad, PrankWeb webserver and COACH webserver. P2Rank (<https://prankweb.cz/>) is a machine learning method that does not rely on templates, designed to predict ligandability by analyzing the local chemical environment around points located on the solvent-accessible surface of proteins (Jendele et al., 2019). While COACH is a meta-server, template-based approach to binding prediction between protein and ligand (Yang et al., 2013). The same binding cavity was determined for each protein using both the template-dependent and the template-independent, i.e., the lowest binding energy in every prediction consisted of the conserved amino acids: serine, histidine and aspartic acid. After this partial confirmation, the spatial geometry of the catalytic triad was assessed using UCSF Chimera software (Pettersen et al., 2004). The distance between the side chains of each amino acid (-OH for serine and aspartic acid, and both delta and epsilon -NH for histidine) were within the 3 Å (Nardini & Dijkstra, 1999), which is necessary for the oxyanion hole formation. Thus, indicating that the predicted active site pockets and their corresponding catalytic triads could be the true active site and catalytic triad (see Fig. 5.19).

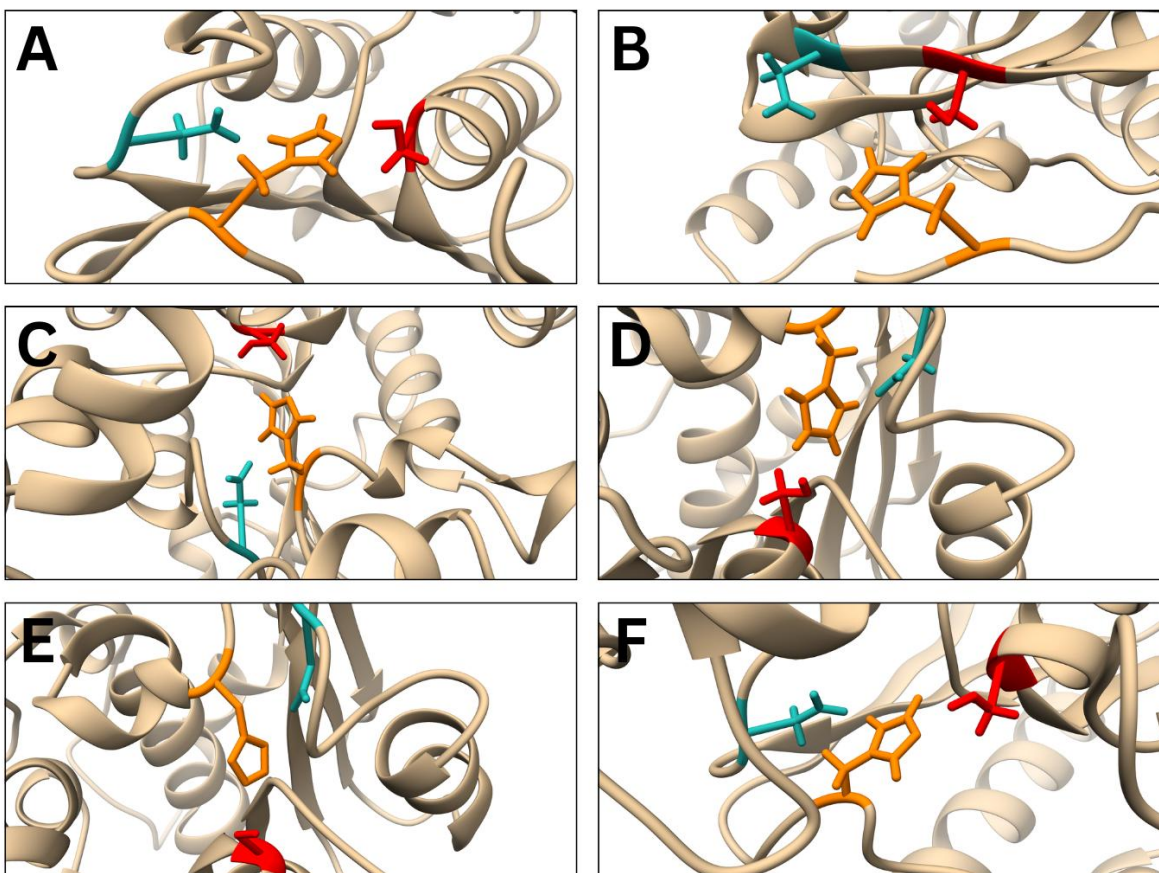


Figure 5.19. The catalytic triad of the speculated active site of the potential PHEs. Serine (shown in red), aspartic acid (shown in teal), and histidine (shown in orange) are shown in proteins Tfu_0082 (A), Tfu_0267 (B), Tfu_0746 (C), Tfu_882 (D), Tfu_0883 (E), and Tfu_1487 (F).

5.4 Conclusion

One intracellular enzyme, two transmembrane enzymes and three extracellular enzymes from *Thermobifida fusca* YX were identified in this *in silico* study, based on an α/β superfamily structural and functional analysis. The potential PHEs from *Thermobifida fusca* YX were predicted to be structurally and thermally stable. Each enzyme displayed the characteristic α/β -superfamily barrel assembly of their α -helices and β -sheets as well as the characteristic GX SXG motif. Three-

dimensional structure prediction revealed the catalytic triad (Ser-His-Asp) within the desired distances. Ramachandran plot analysis of the predicted three-dimensional structures displayed another key feature of PHEs, which is the presence of a serine in a disallowed region of the plot. The molecular docking interactions with PET and cutin ligands were found to have stable exergonic binding energies. This suggests that the shortlisted enzymes could potentially hydrolyze or modify PET, which can be further confirmed with experimental validation. This could steer PET-hydrolysis research in a direction that does not involve extensive genetic manipulation to obtain enzymes suitable for the biological depolymerization of PET. For example, with the right combination of augmentation, these bacteria could be potentially used for composting PET plastics. Additionally, PET-degrading enzymes have been gaining attraction for their use in the textile and detergent industries due to their thermal stability. Furthermore, this study displays the repetitive nature of data on databases.

References

- Akoh, C. C., Lee, G. C., Liaw, Y. C., Huang, T. H., & Shaw, J. F. (2004). GDSL family of serine esterases/lipases. *Progress in Lipid Research*, 43(6), 534–552. <https://doi.org/10.1016/J.PLIPRES.2004.09.002>
- Amanna, R., & Rakshit, S. K. (2023). Review of nomenclature and methods of analysis of polyethylene terephthalic acid hydrolyzing enzymes activity. In *Biological depolymerization*. Springer Science and Business Media B.V. <https://doi.org/10.1007/s10532-023-10048-z>
- Arrieta-Baez, D., Flores, M. de J. P., Méndez-Méndez, J. V., León, H. F. M., & Gómez-Patiño, M. B. (2020). Structural Studies of the Cutin from Two Apple Varieties: Golden Delicious and Red Delicious (*Malus domestica*). *Molecules* 2020, Vol. 25, Page 5955, 25(24), 5955. <https://doi.org/10.3390/MOLECULES25245955>
- Bowie, J. U., Lüthy, R., & Eisenberg, D. (1991). A method to identify protein sequences that fold into a known three-dimensional structure. *Science (New York, N.Y.)*, 253(5016), 164–170. <https://doi.org/10.1126/SCIENCE.1853201>
- Brott, S., Pfaff, L., Schuricht, J., Schwarz, J. N., Böttcher, D., Badenhorst, C. P. S., Wei, R., & Bornscheuer, U. T. (2022). Engineering and evaluation of thermostable IsPETase variants for PET depolymerization. *Engineering in Life Sciences*, 22(3–4), 192–203. <https://doi.org/10.1002/ELSC.202100105>
- Carniel, A., Valoni, É., Nicomedes, J., Gomes, A. da C., & Castro, A. M. de. (2017). Lipase from *Candida antarctica* (CALB) and cutinase from *Humicola insolens* act synergistically for PET hydrolysis to terephthalic acid. *Process Biochemistry*, 59, 84–90. <https://doi.org/10.1016/j.procbio.2016.07.023>
- Colovos, C., & Yeates, T. O. (1993). Verification of protein structures: patterns of nonbonded atomic interactions. *Protein Science : A Publication of the Protein Society*, 2(9), 1511. <https://doi.org/10.1002/PRO.5560020916>
- Costantini, S., Colonna, G., & Facchiano, A. M. (2008). ESBRI: a web server for evaluating salt bridges in proteins. *Bioinformation*, 3(3), 137–138. <https://doi.org/10.6026/97320630003137>

- Donald, J. E., Kulp, D. W., & DeGrado, W. F. (2011). Salt bridges: Geometrically specific, designable interactions. *Proteins: Structure, Function, and Bioinformatics*, 79(3), 898–915. <https://doi.org/10.1002/PROT.22927>
- Dong, Q. L., Wu, L., Wu, J., & Zhou, J. H. (2018, December 4). RCSB PDB - 5ZOA: The crystal structure of a *Thermobifida fusca* cutinase. <https://www.rcsb.org/structure/5ZOA>
- Fecker, T., Galaz-Davison, P., Engelberger, F., Narui, Y., Sotomayor, M., Parra, L. P., & Ramírez-Sarmiento, C. A. (2018). Active Site Flexibility as a Hallmark for Efficient PET Depolymerization by *I. sakaiensis* PETase. *Biophysical Journal*, 114(6), 1302–1312. <https://doi.org/10.1016/j.bpj.2018.02.005>
- Fich, E. A., Segerson, N. A., & Rose, J. K. C. (2016). The Plant Polyester Cutin: Biosynthesis, Structure, and Biological Roles. *Annual Review of Plant Biology*, 67, 207–233. <https://doi.org/10.1146/ANNUREV-ARPLANT-043015-111929>
- Flores-Castañón, N., Sarkar, S., & Banerjee, A. (2022). Structural, functional, and molecular docking analyses of microbial cutinase enzymes against polyurethane monomers. *Journal of Hazardous Materials Letters*, 3, 100063. <https://doi.org/10.1016/J.HAZL.2022.100063>
- Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M. R., Appel, R. D., & Bairoch, A. (2005). Protein Identification and Analysis Tools on the ExPASy Server. *The Proteomics Protocols Handbook*, 571–607. <https://doi.org/10.1385/1-59259-890-0:571>
- Geourjon, C., & Deléage, G. (1995). SOPMA: significant improvements in protein secondary structure prediction by consensus prediction from multiple alignments. *Bioinformatics*, 11(6), 681–684. <https://doi.org/10.1093/BIOINFORMATICS/11.6.681>
- Gomez Del Pulgar, E. M., & Saadeddin, A. (2014). The cellulolytic system of *Thermobifida fusca*. *Critical Reviews in Microbiology*, 40(3), 236–247. <https://doi.org/10.3109/1040841X.2013.776512>
- Guruprasad, K., Reddy, B. V. B., & Pandit, M. W. (1990). Correlation between stability of a protein and its dipeptide composition: a novel approach for predicting in vivo stability of a protein from its primary sequence. *Protein Engineering, Design and Selection*, 4(2), 155–161. <https://doi.org/10.1093/PROTEIN/4.2.155>
- Hanwell, M. D., Curtis, D. E., Lonie, D. C., Vandermeersch, T., Zurek, E., & Hutchison, G. R. (2012). Avogadro: An advanced semantic chemical editor, visualization, and analysis platform. *Journal of Cheminformatics*, 4(8), 1–17. <https://doi.org/10.1186/1758-2946-4-17/FIGURES/14>
- Heidbreder, L. M., Bablok, I., Drews, S., & Menzel, C. (2019). Tackling the plastic problem: A review on perceptions, behaviors, and interventions. *Science of The Total Environment*, 668, 1077–1093. <https://doi.org/10.1016/J.SCITOTENV.2019.02.437>
- Ho, N. H. E., Effendi, S. S. W., Ting, W. W., Yi, Y. C., Yu, J. Y., Chang, J. S., & Ng, I. S. (2023). Heterologous expression and characterization of *Aquabacterium parvum* lipase, a close relative of *Ideonella sakaiensis* PETase in *Escherichia coli*. *Biochemical Engineering Journal*, 197, 108985. <https://doi.org/10.1016/J.BEJ.2023.108985>
- Huang, X., Cao, L., Qin, Z., Li, S., Kong, W., & Liu, Y. (2018). Tat-Independent Secretion of Polyethylene Terephthalate Hydrolase PETase in *Bacillus subtilis* 168 Mediated by Its Native Signal Peptide. *Journal of Agricultural and Food Chemistry*, 66(50), 13217–13227. <https://doi.org/10.1021/acs.jafc.8b05038>
- Ikai, A. (1980). Thermostability and Aliphatic Index of Globular Proteins. *The Journal of Biochemistry*, 88(6), 1895–1898. <https://doi.org/10.1093/OXFORDJOURNALS.JBCHEM.A133168>
- Jendele, L., Krivak, R., Skoda, P., Novotny, M., & Hoksza, D. (2019). PrankWeb: a web server for ligand binding site prediction and visualization. *Nucleic Acids Research*, 47(W1), W345–W349. <https://doi.org/10.1093/NAR/GKZ424>
- Jia, L., Evans, S., & Linden, S. van der. (2019). Motivating actions to mitigate plastic pollution. *Nature Communications* 2019 10:1, 10(1), 1–3. <https://doi.org/10.1038/s41467-019-12666-9>
- Joo, S., Cho, I. J., Seo, H., Son, H. F., Sagong, H. Y., Shin, T. J., Choi, S. Y., Lee, S. Y., & Kim, K. J. (2018). Structural insight into molecular mechanism of poly(ethylene terephthalate) depolymerization. *Nature Communications*, 9(1). <https://doi.org/10.1038/s41467-018-02881-1>
- Kim, D. E., Chivian, D., & Baker, D. (2004). Protein structure prediction and analysis using the Robetta server. *Nucleic Acids Research*, 32(Web Server issue), W526. <https://doi.org/10.1093/NAR/GKH468>
- Kumar, S., & Bansal, M. (1998). Geometrical and sequence characteristics of α -helices in globular proteins. *Biophysical Journal*, 75(4), 1935–1944. [https://doi.org/10.1016/S0006-3495\(98\)77634-9](https://doi.org/10.1016/S0006-3495(98)77634-9)
- Laskowski, R. A., MacArthur, M. W., Moss, D. S., & Thornton, J. M. (1993). PROCHECK: a program to check the stereochemical quality of protein structures. *Urn:Issn:0021-8898*, 26(2), 283–291. <https://doi.org/10.1107/S0021889892009944>
- Law, K. L., & Narayan, R. (2021). Reducing environmental plastic pollution by designing polymer materials for managed end-of-life. *Nature Reviews Materials* 2021 7:2, 7(2), 104–116. <https://doi.org/10.1038/s41578-021-00382-0>

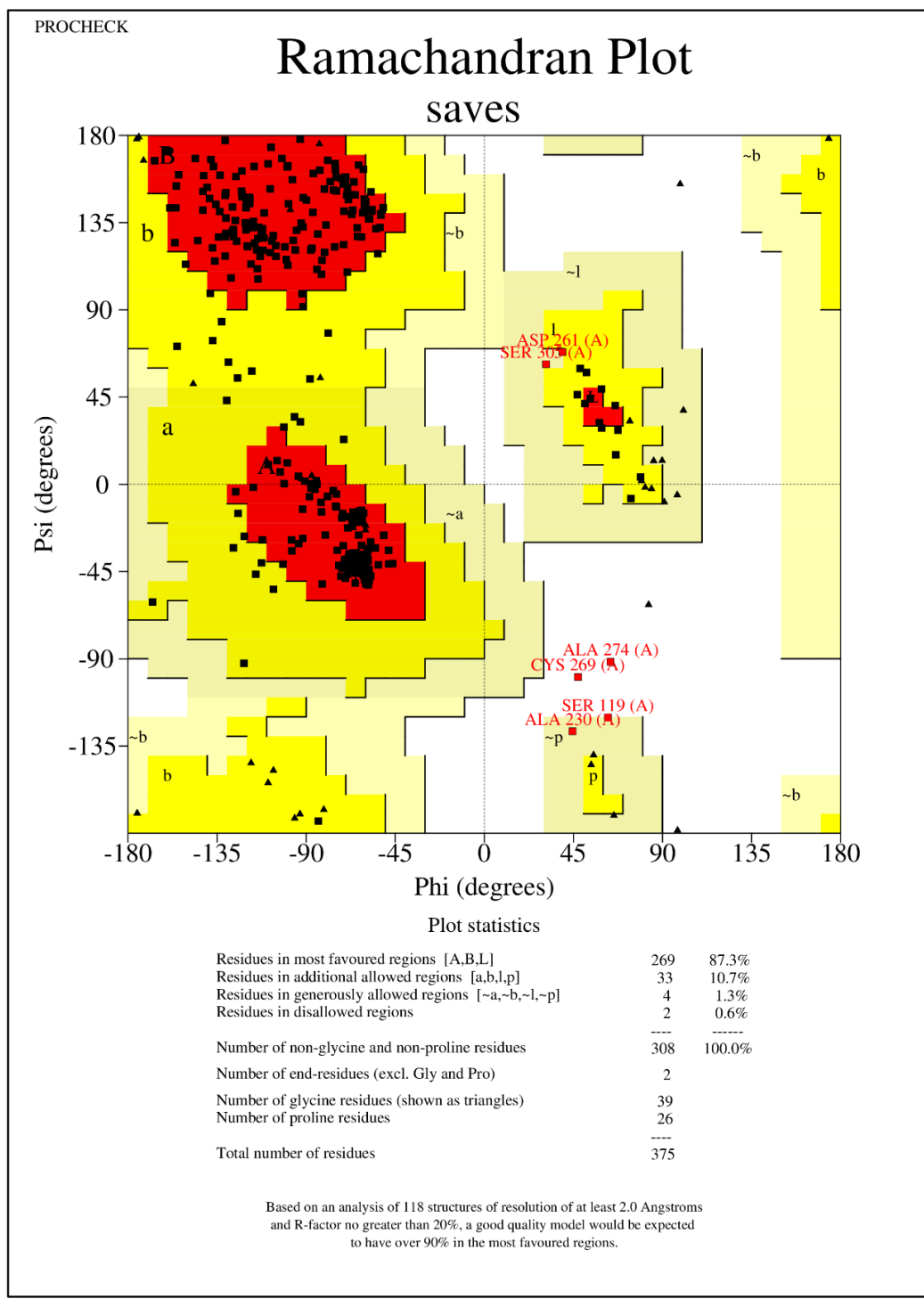
- Liu, Y., Yang, X., Gan, J., Chen, S., Xiao, Z. X., & Cao, Y. (2022). CB-Dock2: improved protein–ligand blind docking by integrating cavity detection, docking and homologous template fitting. *Nucleic Acids Research*, 50(W1), W159–W164. <https://doi.org/10.1093/NAR/GKAC394>
- Lykidis, A., Mavromatis, K., Ivanova, N., Anderson, I., Land, M., DiBartolo, G., Martinez, M., Lapidus, A., Lucas, S., Copeland, A., Richardson, P., Wilson, D. B., & Kyrpides, N. (2007). Genome sequence and analysis of the soil cellulolytic actinomycete *Thermobifida fusca* YX. *Journal of Bacteriology*, 189(6), 2477–2486. <https://doi.org/10.1128/JB.01899-06/ASSET/3C6DE7B4-6AAB-49A7-84FF-5D5B95EC41EF/ASSETS/GRAPHIC/ZJB0060766130004.JPEG>
- Madden, T. L., Tatusov, R. L., & Zhang, J. (1996). [9] Applications of network BLAST server. *Methods in Enzymology*, 266, 131–141. [https://doi.org/10.1016/S0076-6879\(96\)66011-X](https://doi.org/10.1016/S0076-6879(96)66011-X)
- Madeira, F., Madhusoodanan, N., Lee, J., Eusebi, A., Niewielska, A., Tivey, A. R. N., Lopez, R., & Butcher, S. (2024). The EMBL-EBI Job Dispatcher sequence analysis tools framework in 2024. *Nucleic Acids Research*, 52(W1), W521–W525. <https://doi.org/10.1093/NAR/GKAE241>
- Meuzelaar, H., Vreede, J., & Woutersen, S. (2016). Influence of Glu/Arg, Asp/Arg, and Glu/Lys Salt Bridges on α -Helical Stability and Folding Kinetics. *Biophysical Journal*, 110(11), 2328–2341. <https://doi.org/10.1016/J.BPJ.2016.04.015>
- Moreira, A. R., Phillips, J. A., & Humphrey, A. E. (1981). Production of Cellulases by *Thermomonospora* sp. *Biotechnology and Bioengineering*, 23(6), 1339–1347.
- Nardini, M., & Dijkstra, B. (1999). $\alpha\beta$ Hydrolase fold enzymes Nardini and Dijkstra 733. *Current Opinion in Structural Biology*, 9(6), 732–737.
- Pal, D., & Chakrabarti, P. (2002). On residues in the disallowed region of the Ramachandran map. *Biopolymers*, 63(3), 195–206. <https://doi.org/10.1002/bip.10051>
- Paysan-Lafosse, T., Blum, M., Chuguransky, S., Grego, T., Pinto, B. L., Salazar, G. A., Bileschi, M. L., Bork, P., Bridge, A., Colwell, L., Gough, J., Haft, D. H., Letunić, I., Marchler-Bauer, A., Mi, H., Natale, D. A., Orengo, C. A., Pandurangan, A. P., Rivoire, C., ... Bateman, A. (2023). InterPro in 2022. *Nucleic Acids Research*, 51(D1), D418–D427. <https://doi.org/10.1093/NAR/GKAC993>
- Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., & Ferrin, T. E. (2004). UCSF Chimera—a visualization system for exploratory research and analysis. *Journal of Computational Chemistry*, 25(13), 1605–1612. <https://doi.org/10.1002/JCC.20084>
- Petukhov, M., Muñoz, V., Yumoto, N., Yoshikawa, S., & Serrano, L. (1998). Position dependence of non-polar amino acid intrinsic helical propensities. *Journal of Molecular Biology*, 278(1), 279–289. <https://doi.org/10.1006/JMBI.1998.1682>
- Puspitasari, N., Tsai, S. L., & Lee, C. K. (2021). Fungal Hydrophobin RolA Enhanced PETase Hydrolysis of Polyethylene Terephthalate. *Applied Biochemistry and Biotechnology*, 193(5), 1284–1295. <https://doi.org/10.1007/s12010-020-03358-y>
- Rajakumari, S., & Daum, G. (2010). Multiple Functions as Lipase, Steryl Ester Hydrolase, Phospholipase, and Acyltransferase of Tgl4p from the Yeast *Saccharomyces cerevisiae*. *Journal of Biological Chemistry*, 285(21), 15769–15776. <https://doi.org/10.1074/JBC.M109.076331>
- Ribitsch, D., Hromic, A., Zitzenbacher, S., Zartl, B., Gamerith, C., Pellis, A., Jungbauer, A., Łyskowski, A., Steinkellner, G., Gruber, K., Tscheliessnig, R., Herrero Acero, E., & Guebitz, G. M. (2017). Small cause, large effect: Structural characterization of cutinases from *Thermobifida cellulolytica*. *Biotechnology and Bioengineering*, 114(11), 2481–2488. <https://doi.org/10.1002/BIT.26372>
- Rohl, C. A., Strauss, C. E. M., Misura, K. M. S., & Baker, D. (2004). Protein Structure Prediction Using Rosetta. *Methods in Enzymology*, 383, 66–93. [https://doi.org/10.1016/S0076-6879\(04\)83004-0](https://doi.org/10.1016/S0076-6879(04)83004-0)
- Son, H. F., Cho, I. J., Joo, S., Seo, H., Sagong, H.-Y., Choi, S. Y., Lee, S. Y., & Kim, K.-J. (2019). Rational Protein Engineering of Thermo-Stable PETase from *Ideonella sakaiensis* for Highly Efficient PET Depolymerization. <https://doi.org/10.1021/acscatal.9b00568>
- Stothard, P. (2000). The Sequence Manipulation Suite: JavaScript Programs for Analyzing and Formatting Protein and DNA Sequences. *BioTechniques*, 28(6), 1102–1104. <https://doi.org/10.2144/00286IR01>
- Studer, G., Rempfer, C., Waterhouse, A. M., Gummienny, R., Haas, J., & Schwede, T. (2020). QMEANDisCo—distance constraints applied on model quality estimation. *Bioinformatics*, 36(6), 1765–1771. <https://doi.org/10.1093/BIOINFORMATICS/BTZ828>
- Stutzenberger, F., & Kahler, G. (1986). Cellulase biosynthesis during depolymerization of cellulose derivatives by *Thermomonospora curvata*. *Journal of Applied Bacteriology*, 61(3), 225–233.
- Sulaiman, S., Yamato, S., Kanaya, E., Kim, J. J., Koga, Y., Takano, K., & Kanaya, S. (2012). Isolation of a novel cutinase homolog with polyethylene terephthalate-degrading activity from leaf-branch compost by using a

- metagenomic approach. *Applied and Environmental Microbiology*, 78(5), 1556–1562. <https://doi.org/10.1128/AEM.06725-11>
- Teufel, F., Almagro Armenteros, J. J., Johansen, A. R., Gíslason, M. H., Pihl, S. I., Tsirigos, K. D., Winther, O., Brunak, S., von Heijne, G., & Nielsen, H. (2022). SignalP 6.0 predicts all five types of signal peptides using protein language models. *Nature Biotechnology* 2022 40:7, 40(7), 1023–1025. <https://doi.org/10.1038/s41587-021-01156-3>
- Then, J., Wei, R., Oeser, T., Barth, M., Belisário-Ferrari, M. R., Schmidt, J., & Zimmermann, W. (2015). Ca²⁺ and Mg²⁺ binding site engineering increases the depolymerization of polyethylene terephthalate films by polyester hydrolases from *Thermobifida fusca*. *Biotechnology Journal*, 10(4), 592–598. <https://doi.org/10.1002/biot.201400620>
- Tokimatsu, T., Kotera, M., Goto, S., & Kanehisa, M. (2011). KEGG and GenomeNet Resources for Predicting Protein Function from Omics Data Including KEGG PLANT Resource. *Protein Function Prediction for Omics Era*, 271–288. https://doi.org/10.1007/978-94-007-0881-5_14
- Tournier, V., Topham, C. M., Gilles, A., David, B., Folgoas, C., Moya-Leclair, E., Kamionka, E., Desrousseaux, M. L., Texier, H., Gavalda, S., Cot, M., Guémard, E., Dalibey, M., Nomme, J., Cioci, G., Barbe, S., Chateau, M., André, I., Duquesne, S., & Marty, A. (2020). An engineered PET depolymerase to break down and recycle plastic bottles. *Nature*, 580(7802), 216–219. <https://doi.org/10.1038/s41586-020-2149-4>
- Walker, T. R., & Xanthos, D. (2018). A call for Canada to move toward zero plastic waste by reducing and recycling single-use plastics. In *Resources, Conservation and Recycling* (Vol. 133, pp. 99–100). Elsevier B.V. <https://doi.org/10.1016/j.resconrec.2018.02.014>
- Wang, J., Chitsaz, F., Derbyshire, M. K., Gonzales, N. R., Gwadz, M., Lu, S., Marchler, G. H., Song, J. S., Thanki, N., Yamashita, R. A., Yang, M., Zhang, D., Zheng, C., Lanczycki, C. J., & Marchler-Bauer, A. (2023). The conserved domain database in 2023. *Nucleic Acids Research*, 51(D1), D384–D388. <https://doi.org/10.1093/NAR/GKAC1096>
- Wang, N., Guan, F., Lv, X., Han, D., Zhang, Y., Wu, N., Xia, X., & Tian, J. (2020). Enhancing secretion of polyethylene terephthalate hydrolase PETase in *Bacillus subtilis* WB600 mediated by the SPamy signal peptide. *Letters in Applied Microbiology*, 71(3), 235–241. <https://doi.org/10.1111/lam.13312>
- Wesseler, J., Kleter, G., Meulenbroek, M., & Purnhagen, K. P. (2023). EU regulation of genetically modified microorganisms in light of new policy developments: Possible implications for EU bioeconomy investments. *Applied Economic Perspectives and Policy*, 45(2), 839–859. <https://doi.org/10.1002/AEPP.13259>
- Wiedemann, C., Kumar, A., Lang, A., & Ohlenschläger, O. (2020). Cysteines and Disulfide Bonds as Structure-Forming Units: Insights From Different Domains of Life and the Potential for Characterization by NMR. *Frontiers in Chemistry*, 8, 509891. <https://doi.org/10.3389/FCHEM.2020.00280/BIBTEX>
- Wilson, D. B. (2004). Studies of *Thermobifida fusca* plant cell wall degrading enzymes. *The Chemical Record*, 4(2), 72–82. <https://doi.org/10.1002/TCR.20002>
- Xanthos, D., & Walker, T. R. (2017). International policies to reduce plastic marine pollution from single-use plastics (plastic bags and microbeads): A review. *Marine Pollution Bulletin*, 118(1–2), 17–26. <https://doi.org/10.1016/J.MARPOLBUL.2017.02.048>
- Yang, J., Roy, A., & Zhang, Y. (2013). Protein-ligand binding site recognition using complementary binding-specific substructure comparison and sequence profile alignment. *Bioinformatics*, 29(20), 2588–2595. <https://doi.org/10.1093/bioinformatics/btt447>
- Yoshida, S., Hiraga, K., Takehana, T., Taniguchi, I., Yamaji, H., Maeda, Y., Toyohara, K., Miyamoto, K., Kimura, Y., & Oda, K. (2016). A bacterium that degrades and assimilates poly(ethylene terephthalate). *Science*, 351(6278), 1196–1199. <http://science.sciencemag.org/>
- Zimmermann, W., & Billig, S. (2011). Enzymes for the Biofunctionalization of Poly(Ethylene Terephthalate). *Advances in Biochemical Engineering/Biotechnology*, 125, 97–120. https://doi.org/10.1007/10_2010_87

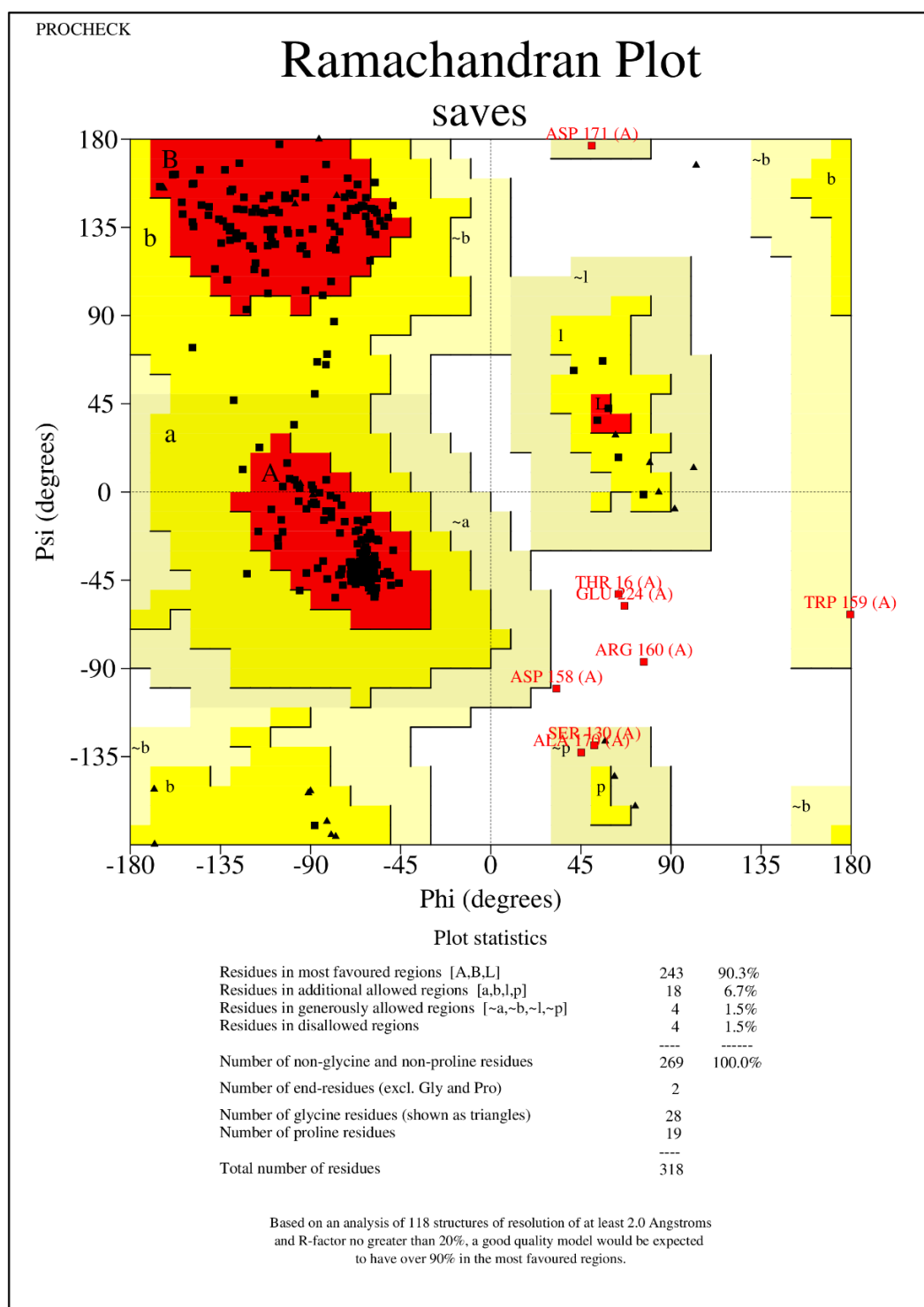
Appendix C

Sequence ID	Evalue	Region	Family
gi 72160483 ref YP_288140.1 	8.31e-41	5-267	Aclacinomycin methylesterase RdmC
gi 72160486 ref YP_288143.1 	7.48e-45	140-375	PHB depolymerase-like
	1.6e-19	25-139	PHB depolymerase-like
gi 72160536 ref YP_288193.1 	1.02e-65	19-305	Epoxide hydrolase
gi 72160546 ref YP_288203.1 	1.97e-17	24-166,195-286	Aclacinomycin methylesterase RdmC
gi 72160559 ref YP_288216.1 	2.75e-67	43-313	Carboxylesterase
gi 72160566 ref YP_288223.1 	4.4e-57	8-254	Epoxide hydrolase
gi 72160671 ref YP_288328.1 	1.11e-52	14-163,214-317	Lipase
gi 72160889 ref YP_288546.1 	4.69e-62	462-708	DPP6 catalytic domain-like
gi 72160917 ref YP_288574.1 	3.41e-42	25-286	Carbon-carbon bond hydrolase
gi 72161150 ref YP_288807.1 	4.16e-52	30-332	PepX catalytic domain-like
gi 72161154 ref YP_288811.1 	4.77e-26	71-130,169-233,316-460	Haloalkane dehalogenase
gi 72161210 ref YP_288867.1 	4.17e-63	388-640	Acylamino-acid-releasing enzyme, C-terminal donain
gi 72161278 ref YP_288935.1 	8.18e-71	3-253	Epoxide hydrolase
gi 72161286 ref YP_288943.1 	8.31e-44	62-300	Lipase
gi 72161287 ref YP_288944.1 	2.2e-44	44-282	Lipase
gi 72161303 ref YP_288960.1 	0.00000378	531-658	Fungal lipases
gi 72161643 ref YP_289300.1 	1.33e-86	4-386	Epoxide hydrolase
gi 72161737 ref YP_289394.1 	3.11e-41	20-283	Carbon-carbon bond hydrolase
gi 72161751 ref YP_289408.1 	1.32e-45	18-140,198-269	Haloperoxidase
gi 72161866 ref YP_289523.1 	7.55e-37	79-207,234-397	Acetyl xylan esterase-like
gi 72161889 ref YP_289546.1 	3.27e-71	20-298	Carboxylesterase
gi 72161942 ref YP_289599.1 	4.05e-38	20-244	Biotin biosynthesis protein BioH
gi 72161960 ref YP_289617.1 	4.05e-22	94-375	Thioesterase domain of polypeptide, polyketide and fatty acid synthases
gi 72162054 ref YP_289711.1 	3.74e-61	12-276	Haloperoxidase
gi 72162100 ref YP_289757.1 	4.63e-52	18-292	Haloperoxidase
gi 72162147 ref YP_289804.1 	2.21e-30	225-432	Dienelactone hydrolase
gi 72162255 ref YP_289912.1 	2.17e-39	409-661	Prolyl oligopeptidase, C-terminal domain
gi 72162264 ref YP_289921.1 	2.22e-55	1055-1321	Thioesterase domain of polypeptide, polyketide and fatty acid synthases
gi 72162283 ref YP_289940.1 	1.53e-18	232-429	Thioesterases
gi 72162611 ref YP_290268.1 	2.41e-47	433-690	Prolyl oligopeptidase, C-terminal domain
gi 72162715 ref YP_290372.1 	4.93e-35	14-240	Thioesterase domain of polypeptide, polyketide and fatty acid synthases
gi 72162748 ref YP_290405.1 	2.32e-69	2-310	Proline iminopeptidase-like
gi 72162796 ref YP_290453.1 	3.4e-31	9-219	Atu1826-like
gi 72162826 ref YP_290483.1 	3.74e-115	3-495	Acetylcholinesterase-like
gi 72162834 ref YP_290491.1 	6.24e-38	15-147,191-258	Atu1826-like
gi 72163179 ref YP_290836.1 	1.9e-25	60-125,175-229,335-465	Proline iminopeptidase-like
gi 72163214 ref YP_290871.1 	8.11e-56	7-287	Carbon-carbon bond hydrolase
gi 72163221 ref YP_290878.1 	2.73e-99	30-378	O-acetyltransferase
gi 72163383 ref YP_291040.1 	2.43e-18	53-260	Haloperoxidase

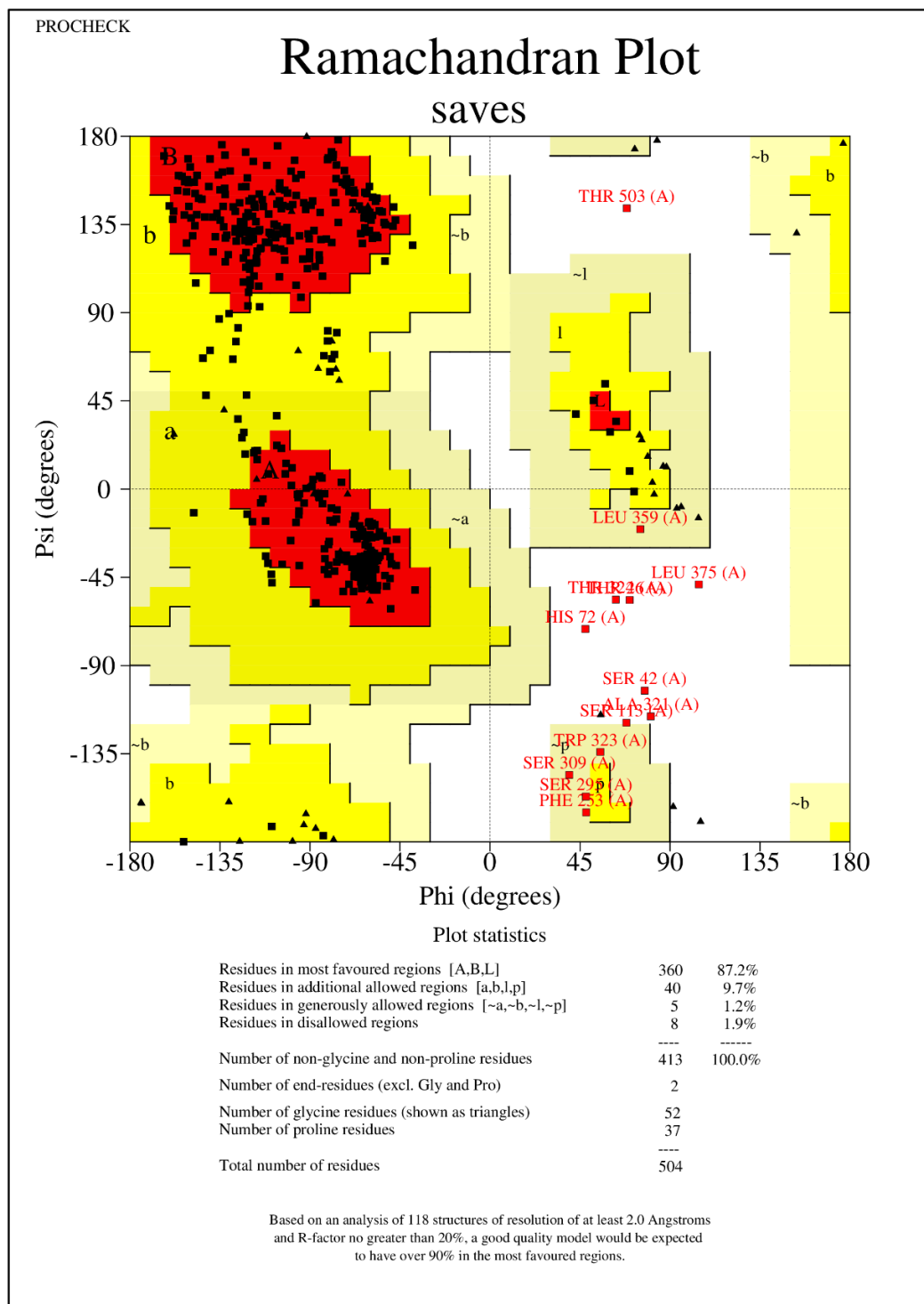
Supplementary Figure 5.1 The result of SuperFamily 2.0 using the protein sequence of Tfu_0883 (give NCBI ID) as the query sequence where 39 proteins belonging to the α/β -hydrolase superfamily in *Thermobifida fusca* YX.



Supplementary Figure 5.2. Ramachandran plot of model 2 of the predicted structure of Tfu_0082

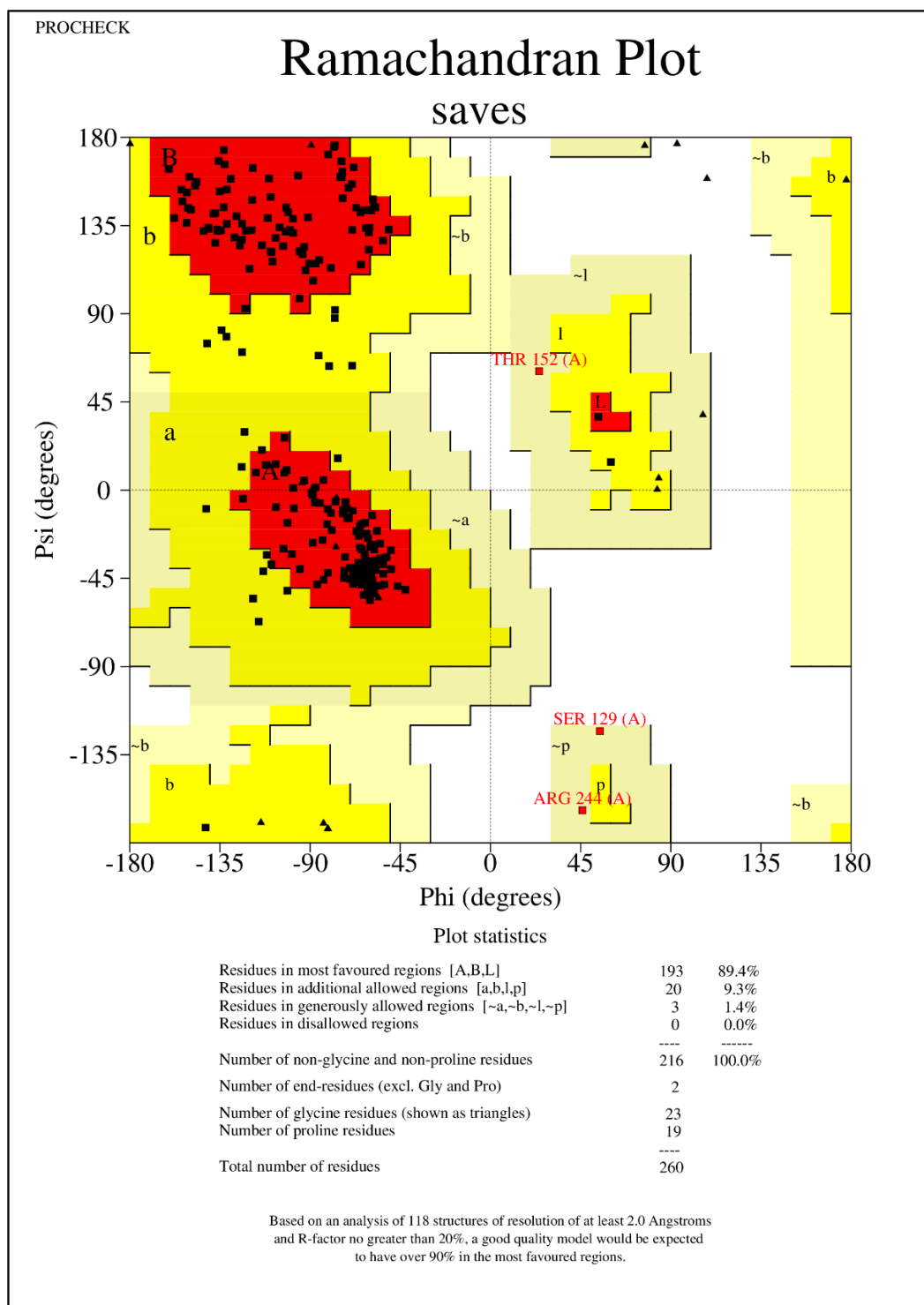


Supplementary Figure 5.3. Ramachandran plot of model 2 of the predicted structure of Tfu_0276

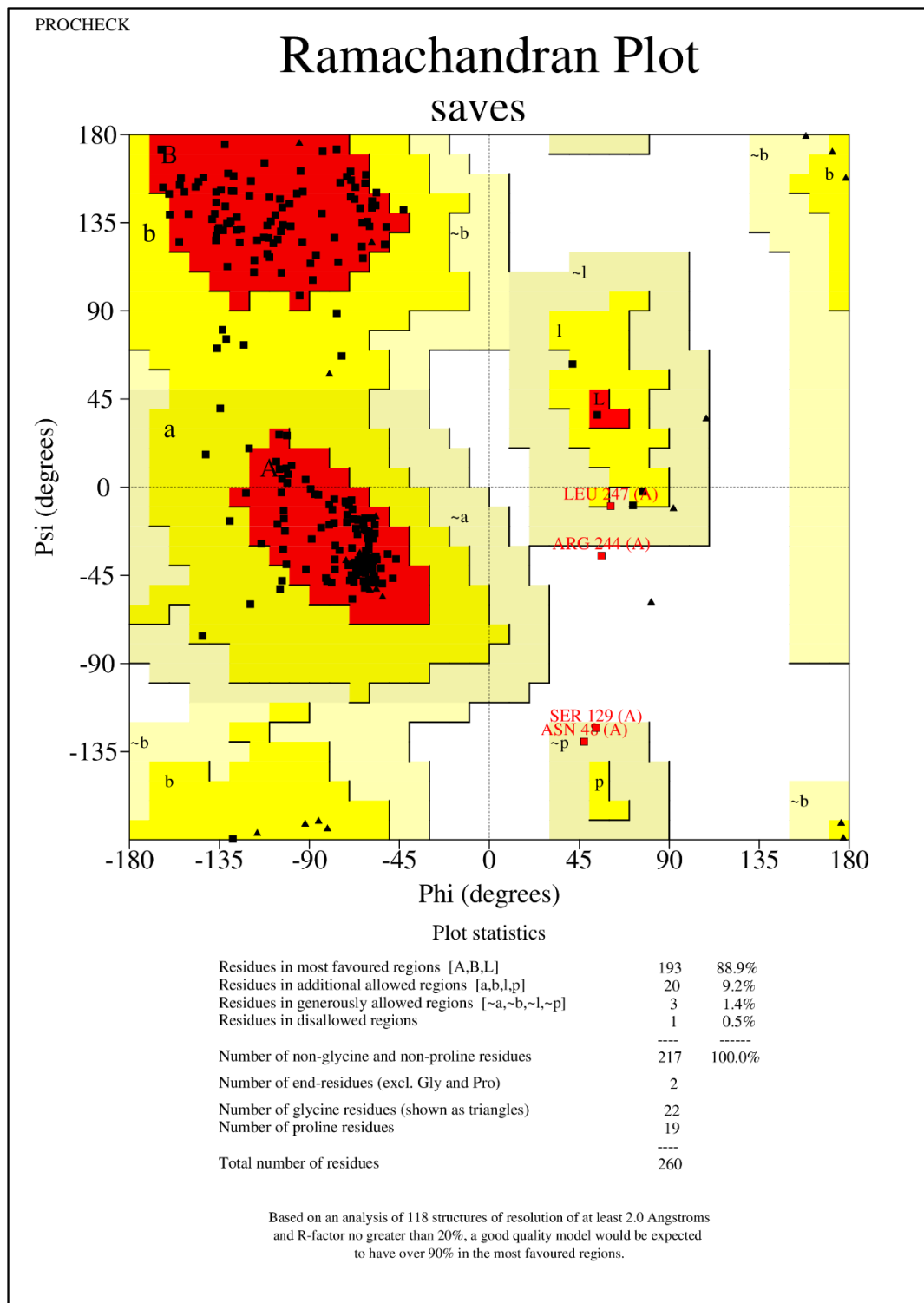


Supplementary Figure 5.4. Ramachandran plot of model 4 of the predicted structure of

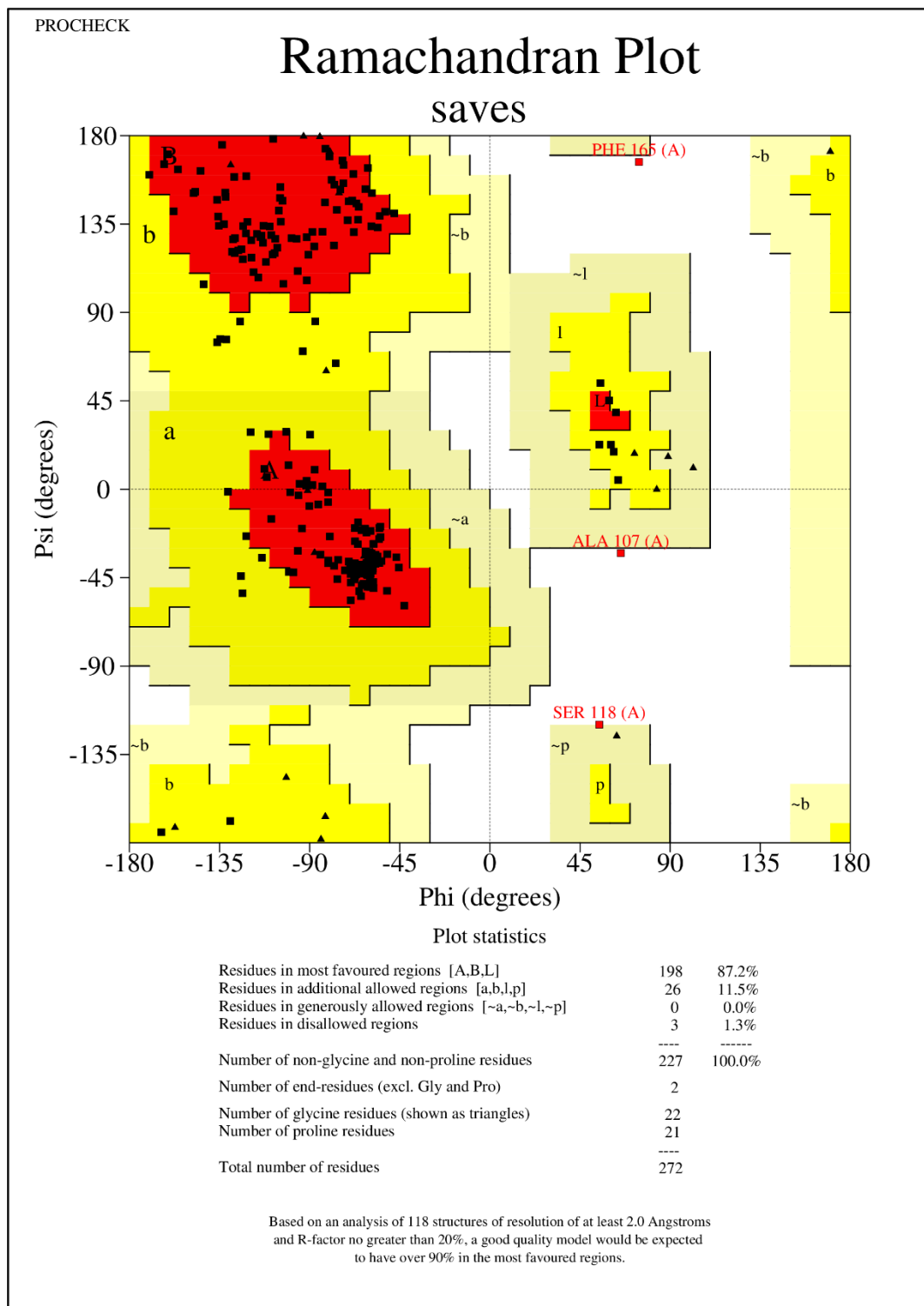
Tfu_0746



Supplementary Figure 5.5. Ramachandran plot of model 1 of the predicted structure of Tfu_0882



Supplementary Figure 5.6. Ramachandran plot of Tfu_0883 (PDB ID: 5ZOA – experimentally determined three-dimensional structure by Dong et al., 2018)



Supplementary Figure 5.7. Ramachandran plot of model 3 of the predicted structure of

Tfu_1487

Supplementary Table 5.1. Characteristics of the thirteen shortlisted proteins based on the superfamily analysis by SUPFAM. The product of the gene and catalytic function was obtained from the annotated whole genome of *T. fusca* YX (Lykidis et al., 2007)

Family	Gene	Product	Catalytic function	Location
Carboxylesterase	Tfu_0155	Esterase/lipase	undefined	Intracellular
	Tfu_1487	Esterase/lipase	Hydrolase	Extracellular
Lipase	Tfu_0267	Hypothetical protein	$(ET^1)_n + H_2O = (ET)_{n-1} + 4-[(2\text{-hydroxyethoxy})$ carbonyl] benzoate	Intracellular
	Tfu_0882	Triacylglycerol lipase	$(ET)_n + H_2O = (ET)_{n-1} + 4-[(2\text{-hydroxyethoxy})$ carbonyl] benzoate	Extracellular
	Tfu_0883	Triacylglycerol lipase	$(ET)_n + H_2O = (ET)_{n-1} + 4-[(2\text{-hydroxyethoxy})$ carbonyl] benzoate	Extracellular
	Tfu_0899		Endopeptidase	Intracellular
	Tfu_0746	Hypothetical protein	$(ET)_n + H_2O = (ET)_{n-1} + 4-[(2\text{-hydroxyethoxy})$ carbonyl] benzoate	Transmembrane
Carbon-carbon bond hydrolase	Tfu_0513	Hydrolase	Hydrolase	Intracellular
	Tfu_1333	Hypothetical protein	Undefined	Intracellular
	Tfu_2815	Hydrolase	Acyltransferase, C-C bond hydrolase	Intracellular
Epoxide hydrolase	Tfu_0132	Hydrolase	Hydrolase	Intracellular
	Tfu_0162		Hydrolase	Intracellular
	Tfu_1239	Hydrolase	Hydrolase	Intracellular
PHB depolymerase	Tfu_0082	Esterase, PHB depolymerase	PHB depolymerase	Transmembrane

Footnotes: ¹ET Ethylene terephthalate

Supplementary Table 5.2. Percent Identity Matrix of the multiple protein sequence alignment using ClustalW (Madeira et al., 2024) of the shortlisted potential PHEs from *Thermobifida fusca* YX.

	AAZ55523.1 (Tfu_1487)	AAZ54305.1 (Tfu_0267)	AAZ54784.1 (Tfu_0746)	AAZ54120.1 (Tfu_0082)	AAZ54920.1 (Tfu_0882)	AAZ54921.1 (Tfu_0883)
AAZ55523.1	100.00	17.70	23.01	18.80	13.33	13.33
AAZ54305.1	17.70	100.00	25.82	19.67	18.00	19.50
AAZ54784.1	23.01	25.82	100.00	22.82	19.25	18.31
AAZ54120.1	18.80	19.67	22.82	100.00	21.74	20.55
AAZ54920.1	13.33	18.00	19.25	21.74	100.00	93.02
AAZ54921.1	13.33	19.50	18.31	20.55	93.02	100.00

Chapter 6

Summary & Future Recommendations

Summary of Work Done

While most PET biological depolymerization research has focused on engineering polyethylene terephthalate-hydrolyzing enzymes (PHEs), this study explores alternative factors influencing PET depolymerization using the actinomycete *Thermobifida fusca* YX. Three primary objectives were addressed: (1) enhancing PET biodegradability through physicochemical pretreatment, (2) optimizing culture conditions for improved production of the PET-degrading enzyme cutinase, and (3) identifying and characterizing potential PHEs through a genome-based in silico approach.

In the first phase, PET films were modified by various physicochemical methods, including UV-induced photooxidation (UVB and UVC), thermal oxidation at 70 °C–90 °C, and size reduction. Among these, particle size reduction had the most pronounced effect, with 250 µm particles showing the highest weight loss during enzymatic hydrolysis (21.25%) and whole-cell biological depolymerization (16.61%). Thermal treatment at 70–80 °C improved biological depolymerization; however, 90 °C impaired enzyme activity. UVB exposure enhanced whole-cell depolymerization, while UVC was detrimental. Chemical hydrolysis showed high recovery of terephthalic acid (TPA ~85%), but pretreatments had limited additional impact, highlighting the limitations of caustic depolymerization methods. Overall, pretreatments such as size reduction and thermal oxidation can improve PET biodegradability, although their effectiveness is constrained under certain conditions. The study advocates for an integrated pretreatment strategy to enhance PET depolymerization efficiency.

In the second phase, the production of cutinase by *T. fusca* YX was optimized by varying two key media components: carbon sources (sodium acetate, sodium butyrate, and sodium lactate) and natural cutin inducers (derived from Royal Gala apples, Roma tomatoes, and watermelon peels). Using Central Composite Design and Response Surface Methodology (RSM), six combinations were tested. The optimal formulation—0.98 g/L sodium butyrate, 0.98 g/L

sodium lactate, and 1.6 g/L Roma tomato cutin—resulted in a 42% increase in cutinase yield. This enzyme facilitated a 26.01% weight loss of untreated PET films, confirming its enhanced biological depolymerization potential.

In the final phase, bioinformatic analysis of the *T. fusca* YX genome was conducted to explore its enzymatic repertoire for PET depolymerization. Six candidate PHEs were identified, including three extracellular, two transmembrane, and one intracellular enzyme. All contained the conserved catalytic triad (Ser-His-Asp) characteristic of hydrolases. Protein structure prediction and molecular docking (using SOPMA, Robetta, CB-Dock2, and AutoDock Vina) revealed favorable exergonic binding interactions with PET ligands, indicating promising PET-degrading capabilities. These findings support the use of *T. fusca* YX as a natural, non-genetically modified platform for PET biological depolymerization.

Future Recommendations

Building on these findings, future research should prioritize the development of scalable and efficient enzymatic and microbial systems for PET biological depolymerization under ambient conditions. Further investigation into the environmental safety, ecological impact, and economic feasibility of deploying *T. fusca* YX in large-scale or in situ applications (e.g., landfills or waste treatment facilities) is recommended. If proven safe and effective, this monoculture could accelerate plastic breakdown in real-world settings.

Moreover, advancements in integrated chemical-biological recycling methods could enhance the circularity of PET waste management. Combining optimized pretreatment protocols, enzyme engineering, and fermentation strategies with emerging recycling technologies may reduce reliance on virgin plastic production and mitigate environmental pollution.

Finally, the six putative PHEs identified from the *T. fusca* YX genome warrant further experimental validation. Their esterase and PET-hydrolyzing activities should be

biochemically characterized to assess their suitability for industrial applications. Such studies could unlock novel biocatalysts for sustainable plastic depolymerization and contribute meaningfully to global efforts in managing plastic waste.