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Comparative Analysis of Mesophilic and Thermophilic PETases Reveals Key Structural and Functional Features for Enhanced Plastic Degradation

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Abstract
The global issue of polyethylene terephthalate (PET) plastic pollution necessitates the development of sustainable degradation strategies. Enzymatic biodegradation using PET hydrolases, also known as PETases, presents a promising solution. While mesophilic PETases, such as the enzyme from *Ideonella sakaiensis* (*Is*PETase), have demonstrated effectiveness, their industrial application is often limited by low thermal stability. In contrast, thermophilic PETases, which function at elevated temperatures, offer the potential for enhanced catalytic efficiency. This study performs a comparative bioinformatics analysis to elucidate the key structural and functional features that differentiate thermophilic PETases from their mesophilic counterparts. Using amino acid sequence analysis, structural comparisons, and molecular docking simulations, we characterized *Is*PETase and two thermophilic PETases from *Thermobifida fusca* and *Thermobifida cellulosilytica*. Our findings reveal that thermophilic PETases possess a higher proportion of charged and aliphatic residues, which are crucial for maintaining structural stability at high temperatures. Structural superimposition confirmed a conserved catalytic core, but with notable differences in surface residue distribution, particularly an enrichment of aromatic and aliphatic residues within the binding pocket. Molecular docking further demonstrated that the thermophilic enzymes exhibit stronger binding affinities to the PET monomer, bis(2-hydroxyethyl) terephthalate (BHET), and a greater number of interacting residues. These results suggest that thermophilic PETases are structurally and functionally adapted for enhanced substrate binding and thermostability. This comparative analysis provides foundational insights for future enzyme engineering efforts aimed at developing more efficient and robust biocatalysts for large-scale PET biorecycling.
Keywords: hydrolases; PET; thermophilic PETase; mesophilic PETase; bioinformatics tools

Introduction

Polyethylene terephthalate (PET) is a widely used plastic polymer in various applications, including beverage bottles, food packaging, and textiles, due to its exceptional durability and versatility. However, the widespread use and improper disposal of PET have led to a global environmental crisis of plastic pollution (Cao et al., 2022). Traditional waste management methods, such as incineration and landfilling, are often unsustainable. Incineration releases toxic pollutants and greenhouse gases, while landfilling contributes to groundwater contamination and disrupts local ecosystems (Khairul Anuar et al., 2022; Koshti et al., 2018). These methods underscore the pressing need for more effective and environmentally friendly solutions for managing plastic waste.

In response to this challenge, enzymatic degradation has emerged as a promising and sustainable approach. This process utilizes microbial enzymes, known as PET hydrolases or PETases, to catalyze the breakdown of PET into its constituent monomers, such as bis(2-hydroxyethyl) terephthalate (BHET), mono-(2-hydroxyethyl) terephthalate (MHET), terephthalic acid (TPA), and ethylene glycol (EG) (Gao

et al., 2021; Kaushal et al., 2021). The discovery of a PETase enzyme from the mesophilic bacterium *Ideonella sakaiensis* 201-F6 marked a significant breakthrough, demonstrating the feasibility of using enzymes for the degradation of PET (Yoshida et al., 2016).

While mesophilic PETases show great potential, their application in large-scale industrial biorecycling processes is often limited by their low thermal stability. Bioreactors for PET degradation typically operate at temperatures above the polymer's glass transition temperature (around 65°C) to increase polymer chain flexibility and enzyme accessibility (Hong et al., 2023; Kawai, 2021). This requirement has shifted research focus toward thermophilic PETases, which are naturally adapted to function at elevated temperatures and may offer superior hydrolytic efficiency and thermal stability (Wei et al., 2022). Despite this, a comprehensive understanding of the specific sequence and structural features that differentiate thermophilic and mesophilic PETases and contribute to their distinct properties remains a knowledge gap (Kawai, 2021; Erickson et al., 2022).

This study aims to bridge this gap by leveraging bioinformatics tools to perform a detailed comparative analysis of a mesophilic PETase from *I. sakaiensis* and two thermophilic PETases from *Thermobifida fusca* and *Thermobifida cellulosilytica*. Using techniques such as amino acid sequence analysis, three-dimensional structural comparisons, and molecular docking simulations, we sought to characterize and compare their catalytic architectures and substrate-binding interactions. Our findings offer valuable insights into the functional and structural adaptations of thermophilic PETases, which can inform future enzyme engineering efforts aimed at developing highly efficient biocatalysts for sustainable plastic waste management.

Materials and Methods

This study employed a bioinformatics-driven approach to characterize and compare the PETase enzymes from a mesophilic bacterium, *Ideonella sakaiensis*, and two thermophilic bacteria, *Thermobifida fusca* and *Thermobifida cellulosilytica*. The workflow included sequence retrieval, physicochemical analysis, structural comparison, and molecular docking simulations.

Homologous Sequence Selection

The amino acid sequence of the mesophilic *I. sakaiensis* PETase (*Is*PETase) was retrieved from the UniProt Knowledgebase (UniProtKB) under the accession number A0A0K8P6T7. This sequence was then used as a query in a BLASTP search against the non-redundant protein database to identify homologous sequences. Thermophilic PETase candidates were selected from the BLASTP results based on high sequence similarity, low E-values, and annotations confirming their origin from thermophilic bacterial species, specifically *T. fusca* and *T. cellulosilytica*.

Amino Acid Sequence Analysis

The selected protein sequences were subjected to multiple sequence alignment (MSA) using tools available on the UniProt platform. The alignment was performed to identify conserved amino acid residues, particularly those within the active and binding sites. Furthermore, the physicochemical properties of the sequences were analyzed using the ProtParam tool on the ExPASy server (Gasteiger et al., 2005). This analysis calculated parameters such as amino acid composition (flexible, charged, aromatic, and aliphatic residues), which were used to infer structural adaptations to different temperature conditions.

Structural Analysis

The three-dimensional (3D) crystal structures of the selected PETases were retrieved from the Protein Data Bank (PDB). The PDB IDs used were 6EQD for *Is*PETase, 4CG1 for *T. fusca* PETase, and 5LUI for *T. cellulosilytica* PETase. These structures were chosen for their high resolution (below 2.0 Å), which ensured reliability for structural comparison. Pairwise structural alignments and superimposition were

performed using the tools available on the RCSB PDB platform to compare the overall protein folds, active site architectures, and the positions of catalytic triad residues.

Structure and Ligand Preparation

To prepare for molecular docking, the chemical structure of the PET monomer bis(2-hydroxyethyl) terephthalate (BHET) was downloaded from the PubChem database (CID 13739) in Structure Data File (SDF) format. The ligand file was then converted to Protein Data Bank (PDB) format using the Open Babel software to ensure compatibility with the docking platform. The retrieved protein structures were used directly in their PDB format for the simulations.

Molecular Docking Analysis

Molecular docking simulations were conducted using the online server CB-Dock2 (Liu et al., 2022). This server automatically predicts potential binding cavities within the protein and performs docking using AutoDock-based algorithms. The prepared protein and ligand files were submitted to CB-Dock2 for each PETase. The outputs, including the best binding affinity scores (reported in kcal/mol), 2D and 3D interaction diagrams, and lists of interacting residues, were then analyzed to compare the binding potential and key protein-ligand interactions across the mesophilic and thermophilic enzymes.

Results and Discussions

Comparison of Amino Acid and Structural Features

The comparative analysis of the mesophilic *I. sakaiensis* PETase (IsPETase) and the thermophilic PETases from *T. fusca* and *T. cellulosilytica* revealed both conserved features critical for function and distinct characteristics related to thermostability. Homologous sequences were selected from the UniProtKB database based on high alignment scores and low E-values (Figure 1). Multiple sequence alignment confirmed that the catalytic triad residues (Ser, Asp, and His) are highly conserved across all three enzymes, a finding consistent with the established mechanism of PET hydrolysis (Han et al., 2017; Joo et al., 2018) (Figures 2 and 3).

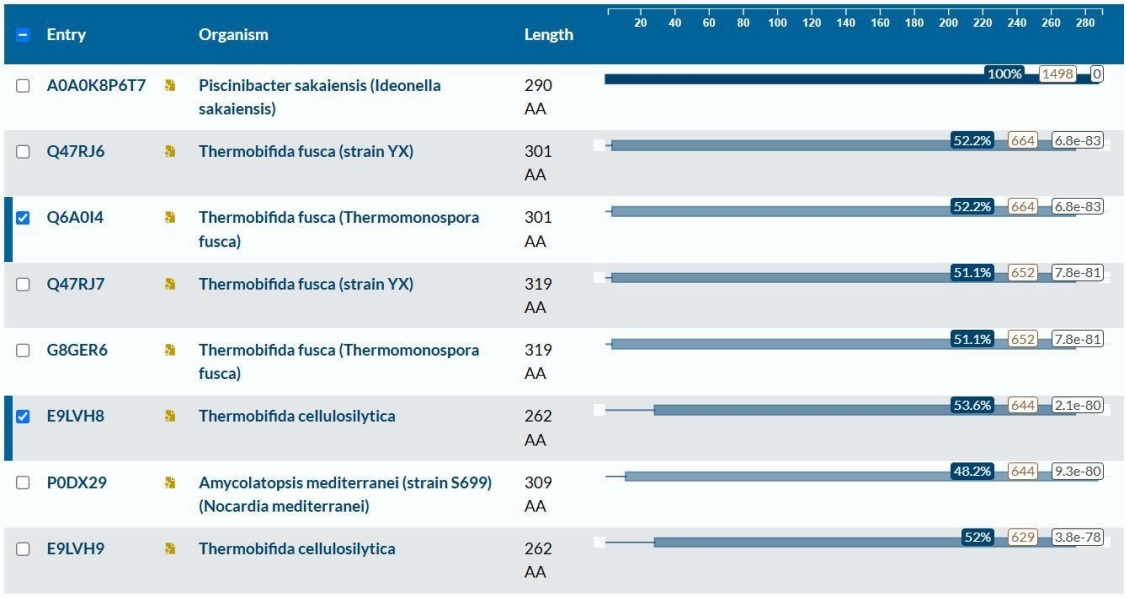


Figure 1 BLASTp results for the homologous PETase sequence from UniProt

Figure 2 Multiple sequence alignment of *Is*PETase, *T. fusca*, and *T.cellulosilytica* showing their active sites (yellow boxes) and binding sites (green boxes).

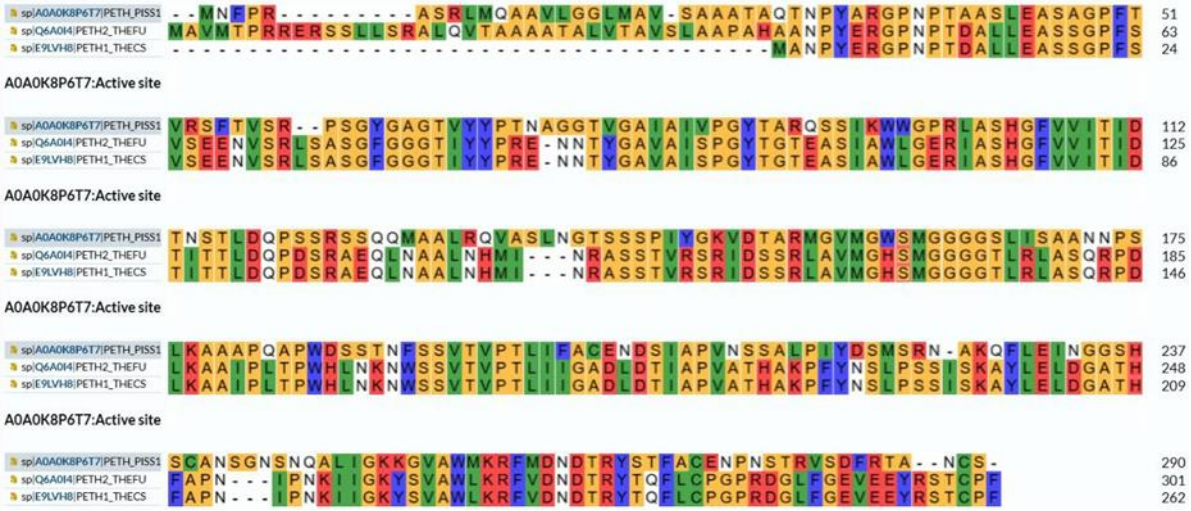


Figure 3 Multiple sequence alignment of *Is*PETase, *T. fusca*, and *T.cellulosilytica* showing the properties of their amino acid residues: charged (red), aromatic (blue), flexible (yellow), and aliphatic (green).

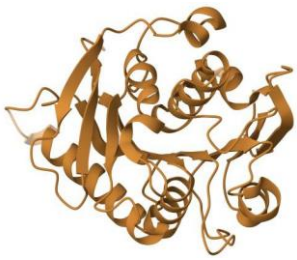
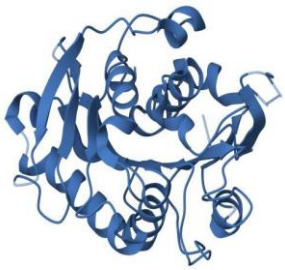
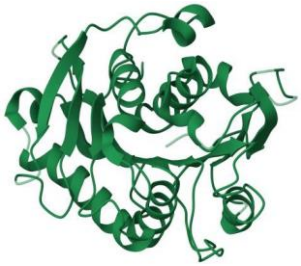
Analysis of the amino acid composition showed notable differences (Table 1). Thermophilic PETases from *T. fusca* and *T. cellulosilytica* contained a higher proportion of charged residues (Asp, Glu, Lys, Arg) and aliphatic residues compared to *Is*PETase. This enrichment of charged residues is a well-documented adaptation in thermophilic proteins, promoting the formation of salt bridges that enhance structural rigidity and stability at high temperatures (Tournier et al., 2020; Son et al., 2020). Similarly, the increased aliphatic content likely reinforces the hydrophobic core, further contributing to thermal stability by reducing protein flexibility (Zhao et al., 2022).

Table 1 : Amino acid composition of *I.sakaiensis*, *T. fusca*, and *T.cellulosilytica* PETases

Bacteria	**Mesophilic**	**Thermophilic**	
Organism	*I. sakaiensis*	*T. fusca*	*T. cellulosilytica*
Sequence Length	290	301	262
Amino Acid Composition	**Number and Percentage of Residues in Sequence (%)**		
Flexible Residues	143 (43.3%)	134 (44.6%)	112 (42.7%)
Positively-Charged Residues	24 (9.3%)	33 (10.9%)	28 (10.7%)
Negatively-Charged Residues	13 (4.5%)	25 (8.3%)	24 (9.2%)
Aromatic Residues	23 (7.9%)	23 (7.6%)	14 (8.7%)
Aliphatic Residues	57 (19.6%)	67 (22.2%)	57 (21.8%)

Structural comparisons, based on high-resolution crystal structures retrieved from the PDB (PDB IDs: 6EQD for *Is*PETase, 4CG1 for *T. fusca*, and 5LUI for *T. cellulosilytica* as detailed in Table 2), confirmed a high degree of similarity in the overall three-dimensional fold (Figure 4).

Table 2 : PETase Structure information retrieved from RCSB PDB for comparative analysis

Source Organism	**3D Structure**	**PDB ID**	**Resolution**
*Is*PETase		6EQD	1.70 Å
T. fusca		4CG1	1.40 Å
T. cellulosilytica		5LUI	1.50 Å

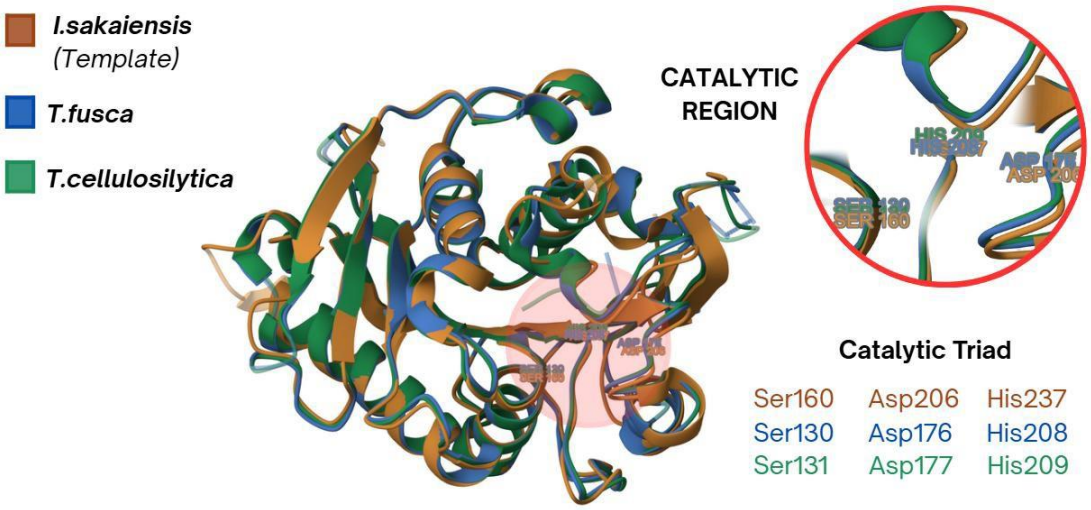


Figure 4 Superimposition of *I .sakaiensis, T.fusca,* and *T.cellulosilytica* PETases

Superimposition analysis resulted in low RMSD values and a high TM-score (Table 3), indicating that the core α/β-hydrolase fold is structurally conserved. The catalytic triad residues were precisely aligned, underscoring the evolutionary pressure to maintain the geometric arrangement required for enzymatic activity. Despite this, subtle modifications were observed in surface loops and residue distribution. Notably, surface analysis revealed that the thermophilic PETases exhibited a more pronounced clustering of aromatic and aliphatic residues, particularly within the binding pocket region (Table 4), which is essential for stabilizing interactions with the PET substrate (Austin et al., 2018; Wang et al., 2024).

Table 3 : Structural comparison between *Is*PETase and T. *fusca* and *T. cellulosilytica* PETases

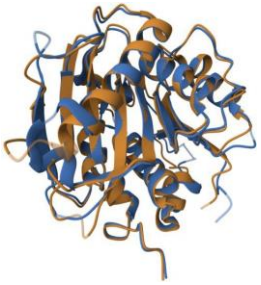
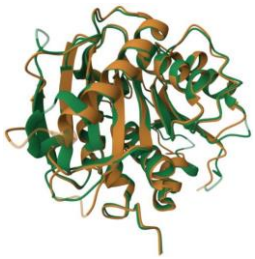
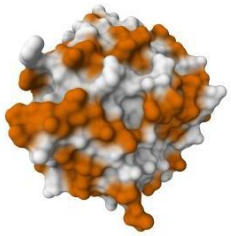
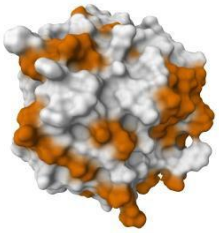
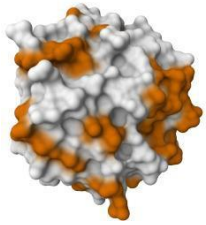
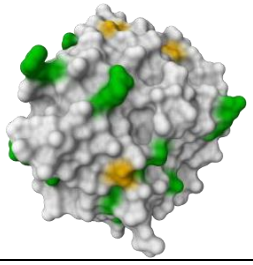
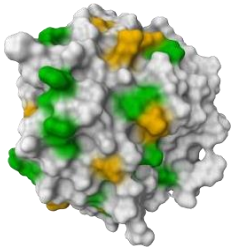
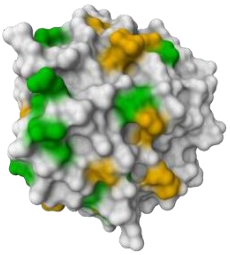
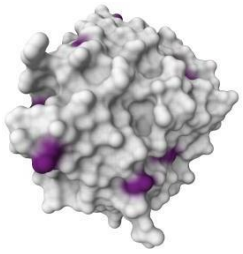
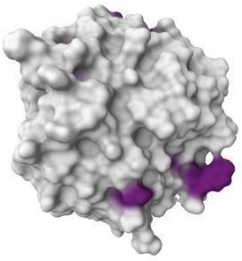
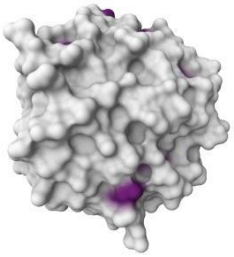
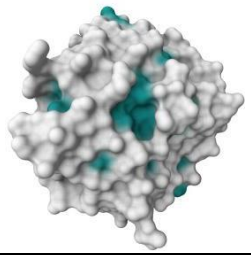
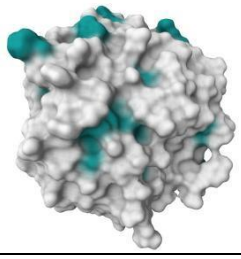
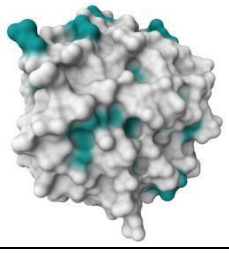
Comparison	Comparison	RMSD (Å)	TM-score	Catalytic Triad Conserved ?
	*Is*PETase vs. *T. fusca* PETase	1.37	0.94	Yes
	*Is*PETase vs. *T. cellulosilytica* PETase	1.44		

Table 4: Distribution of residue properties on surface 3D structures of *I. sakaiensis, T. fusca,* and *T. cellulosilytica* PETase

Enzyme	**PETase**		
Source Organism	***I.sakaiensis***	***T.fusca***	***T.cellulosilytica***
Flexible Residues			
Positively-charged (Green) and Negatively-charged (Yellow) Residues			
Aromatic Residues			
Aliphatic Residues			

Molecular Docking Analysis and Substrate Interactions

Molecular docking simulations using CB-Dock2 provided insights into the binding affinity and specific residue-level interactions between the enzymes and the PET monomer bis(2-hydroxyethyl) terephthalate (BHET). The results showed that the thermophilic PETases exhibited a stronger binding affinity to the substrate compared to the mesophilic *Is*PETase. Specifically, *T. fusca* PETase had the strongest binding affinity score (-5.7 kcal/mol), followed by *T. cellulosilytica* (-5.3 kcal/mol), with *Is*PETase showing the lowest score (-5.0 kcal/mol). These results suggest that thermophilic variants are better suited for forming stable enzyme-substrate complexes, a crucial factor for efficient catalysis under elevated temperatures (Liu et al., 2022). Further analysis of the interacting residues within the binding pockets revealed a greater number of contact points in the thermophilic enzymes (Table 5).

Table 5: Classification of interacting amino acid residues in the protein–ligand interaction of *Is*PETase, *T. fusca* PETase and *T. cellulosilytica* PETase based on residue type

Enzyme	**PETase**		
Bacteria	**Mesophilic**	**Thermophilic**	
Organism Source	*I. sakaiensis*	*T. fusca*	*T. cellulosilytica*
Total Contacted Residues	14	21	19
No. Of Flexible Residues Contacted (Ala, Gly, Pro, Ser, Thr)	5 (Ser207,Pro210, Gly234,Ser213, Ser282)	9 (Ala17, Ser19, Gly20, Ser23, Ala65, Ser66, Ala68, Ser76, Pro214)	9 (Pro145, Ala149, Thr166, Pro168, Ser196, Ser198, Thr259, Pro261, Ala263)
No. Of Charged Residues Contacted (Asp, Glu, His, Lys, Arg)	5 (Glu204,Asp206, Glu231, Asp283, Arg285)	5 (Glu16, Arg18, Glu72, Arg73, Lys216)	4 (Asp146,Lys148, Arg229, Glu265)
No. Of Aromatic Residues Contacted (Phe, Trp, Tyr)	1 (Phe229)	3 (Phe22, Tyr43, Trp69)	2 (Phe230, Phe262)
No. Of Aliphatic Residues Contacted (Cys, Ile, Leu, Met, Val)	3 (Cys203, Val211, Leu216)	4 (Val24, Ile67, Leu70, Ile213)	5 (Leu147,Val167, Ile197, Val231, Leu264)

The binding poses for *Is*PETase, *T. fusca* PETase, and *T. cellulosilytica* PETase are visualized in Figure 5. The binding pocket of the thermophilic PETases showed an increased frequency of hydrophobic residues, including aliphatic (e.g., Leu, Ile, Val) and aromatic (e.g., Phe, Trp, Tyr) amino acids. These residues are vital for forming π−π stacking interactions with the aromatic rings of the PET substrate, thus improving binding specificity and stability (Austin et al., 2018; Zhang et al., 2024). In contrast, *Is*PETase relied on a more balanced mix of charged and hydrophobic residues. The enrichment of hydrophobic residues and a greater number of flexible residues (e.g., Ser, Ala, Pro) in the thermophilic enzymes' binding pockets suggest a structural adaptation that enables a balance between rigidity for thermal stability and local flexibility for optimal substrate accommodation (Roth et al., 2024).

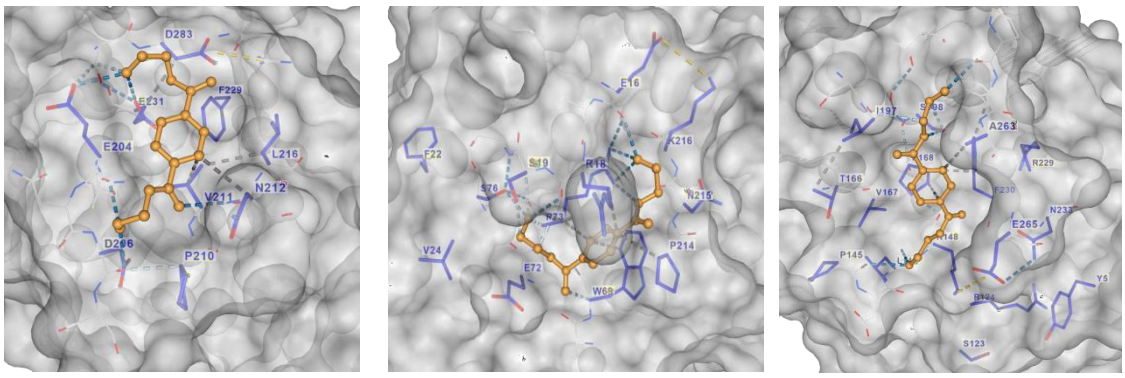


Figure 5 Visualization of the PET substrate (orange) bound within the active site of *Is*PETase (left), *T. fusca* PETase (middle), and *T. cellulosilytica* PETase (right). Key interacting residues are highlighted in blue, and the protein is shown in a grey surface view. The distinct binding poses and interacting residues reflect the varying substrate-binding characteristics of each enzyme.

Limitations

A primary limitation of this study is its reliance on computational and bioinformatics approaches. While these methods are powerful for screening and generating hypotheses, they cannot provide the definitive proof required for validation. The potential of these enzymes for plastic degradation must be confirmed through experimental biochemical validation, such as *in vitro* PET hydrolysis assays and site-directed mutagenesis. Due to the limited size of current databases containing plastics-degrading enzymes, the scope of this study was restricted, and the available annotations may not be comprehensive. Therefore, the findings presented here should be considered a foundation for further research, not a final conclusion on enzyme activity.

Conclusion

Based on the amino acid sequence analysis, thermophilic PETases were found to have a higher proportion of hydrophobic and aromatic residues within the predicted binding regions compared to the mesophilic *Is*PETase. These residues are known to enhance substrate binding and stability under high-temperature conditions. Structural analysis further supported these findings, revealing conserved catalytic triads across all PETases but with additional stabilizing features in thermophilic variants, such as increased aliphatic side chains and more compact folding. Molecular docking using CB-Dock2 demonstrated that thermophilic PETases showed stronger binding affinities toward the PET substrate than the mesophilic enzyme. Interacting residues in thermophilic enzymes also exhibited a greater number of hydrophobic and flexible amino acids, indicating enhanced substrate accommodation and potential thermostability. Collectively, these findings highlight the structural and functional features that differentiate thermophilic PETases. This supports their promising application in industrial-scale PET biodegradation and provides foundational insights for future efforts in enzyme engineering.

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