

# Structural Determinants, Catalytic Mechanisms, and Engineering Trajectories of IsPETase: An Exhaustive Analysis of Activity and Stability Drivers

## 1. Introduction: The Structural Imperative of Enzymatic PET Degradation

The accumulation of poly(ethylene terephthalate) (PET) in terrestrial and marine ecosystems represents one of the defining environmental challenges of the Anthropocene. While PET's physicochemical durability—derived from its high ratio of aromatic terephthalate units and semi-crystalline superstructure—makes it an ideal material for packaging and textiles, these same properties render it recalcitrant to biological degradation. The discovery of *Ideonella sakaiensis* 201-F6 and its secreted hydrolase, IsPETase, marked a paradigm shift in biocatalysis, presenting an enzyme capable of degrading PET at ambient temperatures.<sup>1</sup> However, the translational utility of wild-type IsPETase is severely constrained by its mesophilic nature ( $T_m \approx 46\text{--}48^\circ\text{C}$ ), which precludes operation near the glass transition temperature ( $T_g$ ) of PET ( $65\text{--}70^\circ\text{C}$ ), a thermodynamic window where polymer chain mobility significantly enhances enzymatic accessibility.<sup>3</sup>

To bridge the gap between this natural biocatalyst and industrial requirements, a comprehensive understanding of the structural and biological determinants governing IsPETase function is required. Beyond the well-established canonical features—the catalytic triad, the "wobbling" tryptophan gate (W185), and the conserved disulfide bond (DS1)—recent extensive research has unveiled a complex matrix of distal residues, surface electrostatics, loop dynamics, and expression-host-dependent modifications that dictate performance.<sup>5</sup> This report synthesizes data from crystallographic, kinetic, and computational studies to provide an exhaustive analysis of these determinants. It delineates how subtle conformational landscapes, hydrophobic packing networks, and second-shell allostery drive the delicate balance between catalytic activity and thermal stability, culminating in the design of advanced variants such as FAST-PETase, DuraPETase, HotPETase, and DepoPETase.

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## 2. The Catalytic Core and Subsite Architecture

IsPETase adopts a canonical  $\alpha/\beta$ -hydrolase fold, sharing a core architecture with actinomycete cutinases and lipases. However, its unique ability to hydrolyze PET, a bulky and hydrophobic polymer, stems from specific deviations in its substrate-binding cleft. This region is not merely a static dock but a dynamic landscape where residue flexibility and specific subsite architecture enable the accommodation of the polymer chain.

## 2.1 The "Wobbling" Tryptophan (W185) and Conformational Selection

A defining structural feature of IsPETase is the conformational plasticity of Tryptophan 185 (W185), located in the substrate-binding cleft. Unlike homologous residues in thermophilic cutinases (e.g., LCC or TfCut2), which are rigidly held in place by surrounding histidine or phenylalanine residues, W185 in IsPETase exhibits significant rotational freedom.<sup>8</sup> Crystal structures have captured this residue in three distinct conformations (A, B, and C), a phenomenon termed "wobbling".<sup>8</sup>

This flexibility is facilitated by the presence of Ser214 and Ile218 in the active site wall, residues that are unique to IsPETase (often His/Phe in homologs). The smaller side chain of Ser214 creates the necessary steric void for W185 to rotate. This "wobbling" is crucial for substrate recognition, allowing the enzyme to accommodate the varying torsion angles of the PET polymer backbone.<sup>11</sup>

- **Mechanistic Role:** During the catalytic cycle, W185 must transition from an open state, allowing substrate ingress, to a specific rotamer (Conformer B) that engages in  $\pi$ -stacking interactions with the aromatic terephthalate moiety of the substrate. This  $\pi$ - $\pi$  interaction is essential for stabilizing the substrate in a productively bound conformation relative to the catalytic serine (S160).<sup>2</sup>
- **Engineering Trade-offs:** While this flexibility is vital for activity at ambient temperatures, it introduces a penalty in thermal stability. In engineered variants like HotPETase, which operate at high temperatures where thermal energy compensates for structural rigidity, W185 is often locked into a single conformation via interactions with mutated neighboring residues (e.g., S214Y), effectively rigidifying the active site to prevent thermal denaturation.<sup>12</sup>

## 2.2 Architecture of Subsites I and II

The catalytic cleft of IsPETase is functionally partitioned into Subsite I, which binds the moiety undergoing hydrolysis, and Subsite II, which accommodates the adjacent polymer units. The specific residue composition of these subsites determines the enzyme's substrate specificity and differentiates IsPETase from less active cutinases (Table 1).

Table 1: Comparative Residue Composition of Subsites in IsPETase and Homologous Enzymes

Structural Feature	IsPETase Residues	LCC / PHL7 Residues	Functional Implication
Subsite I	Trp185, Met161, Ile208	Trp190, Met165, Leu210	Subsite I is highly conserved, serving as the primary anchor for the TPA moiety via $\pi$ -stacking with Trp.
Subsite II	Thr88, Ala89, Ser238	Phe93, Gln95, His185	The residues in IsPETase (T88, A89) have smaller side chains compared to the bulky Phe/Gln in homologs. This creates a wider, flatter cleft in Subsite II.
Channel Geometry	Shallow, L-shaped	Deep, narrow groove	The wider cleft of IsPETase reduces steric clashes with the ethylene glycol linkage of the polymer, facilitating the binding of semi-crystalline PET surfaces.
Extended Loop	Present ( $\beta$ - $\alpha$ 6)	Absent	IsPETase possesses an extended loop region containing additional binding residues (e.g., S242, N246) that

			extend the binding surface, crucial for long-chain polymer interaction.
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The residues Thr88 and Ala89 are particularly significant. In thermophilic cutinases, the corresponding positions are occupied by larger residues that constrict the cleft. The "open" architecture of IsPETase Subsite II allows the enzyme to bind the polymer chain with lower steric hindrance.<sup>1</sup> However, recent engineering efforts in DepoPETase have shown that reverting Thr88 to Isoleucine (T88I) can actually enhance activity, likely by optimizing the hydrophobic interaction with the ethylene glycol unit of the substrate, provided the overall cleft geometry remains accessible.<sup>12</sup>

## 2.3 The Gatekeeper Residues: R280 and S238

At the periphery of the catalytic cleft, specific "gatekeeper" residues control the access of the polymer chain.

- **Arg280 (R280):** In the wild-type enzyme, Arg280 protrudes into the solvent and sits at the edge of Subsite IIc. Its bulky, positively charged side chain can create steric hindrance and unfavorable electrostatic repulsion with the hydrophobic PET surface. The mutation of Arginine to Alanine (R280A) is one of the most universally beneficial modifications found in high-performance variants (FAST-PETase, DuraPETase, HotPETase). This mutation serves a dual function: it widens the cleft entrance to accommodate longer polymer chains and removes a charge mismatch, facilitating the approach of the non-polar substrate.<sup>14</sup>
- **Ser238 (S238):** Located in Subsite II, Ser238 is positioned to interact with the substrate chain. In some cutinases, this position is held by a Phenylalanine, which contributes to a hydrophobic tunnel. While introducing S238F alone in IsPETase decreases activity by narrowing the cleft, combinatorial mutations (e.g., S238F/W159H) can restore and enhance activity by creating a continuous aromatic track for the substrate, provided the cleft width is maintained by compensatory mutations.<sup>16</sup>

## 2.4 Conformational Selection: Trans vs. Gauche Preference

The PET polymer backbone exists in an equilibrium of rotational conformers, primarily the trans and gauche conformations of the ethylene glycol moiety. Amorphous regions of PET are rich in gauche conformers, while crystalline regions are dominated by the trans conformation. Recent molecular dynamics (MD) and solid-state NMR studies indicate that IsPETase exhibits a conformational preference. The enzyme's active site geometry is optimized to bind the

gauche conformation, which matches the geometry of the accessible amorphous regions.<sup>18</sup> However, engineering efforts suggest that variants capable of accommodating the trans conformation (via widening of the cleft or flexibility in the W185 gate) may show improved activity on highly crystalline substrates. This "conformational selection" mechanism implies that the enzyme does not merely bind the substrate but actively selects or induces a specific rotameric state productive for catalysis.<sup>10</sup>

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## 3. The Thermodynamic Landscape: Engineering Thermal Stability

The defining limitation of wild-type IsPETase is its fragility; it unfolds rapidly above  $45\text{--}50^\circ\text{C}$ . To degrade PET efficiently, the reaction must occur near the polymer's  $T_g$  ( $65\text{--}70^\circ\text{C}$ ), where the polymer chains become mobile and accessible. Consequently, shifting the  $T_m$  of IsPETase from  $48^\circ\text{C}$  to  $>70^\circ\text{C}$  has been the primary objective of structural engineering.

### 3.1 Loop Rigidification and the $\beta_6$ - $\beta_7$ Region

Structural analysis and B-factor mapping identify the loop connecting  $\beta$ -strand 6 and  $\beta$ -strand 7 (residues 186–191) as the most thermally labile region of the enzyme. This solvent-exposed loop is prone to unfolding, triggering the global collapse of the tertiary structure.<sup>3</sup>

- **The D186 Nexus:** Aspartate 186 (D186) acts as a linchpin for stabilizing this region. In the wild-type enzyme, D186 is relatively unconstrained. The mutation **D186H**, ubiquitous in advanced variants like FAST-PETase and HotPETase, stabilizes the loop by introducing a histidine side chain that forms new hydrogen bonds with Ser187 and Ser188. This locks the loop into a rigid conformation, significantly reducing the entropy of the unfolded state.<sup>3</sup>
- **Alternative Strategies (D186N):** The D186N mutation also enhances stability but operates through a different mechanism, modulating the local electrostatic potential and mimicking the hydrogen bonding networks observed in naturally thermostable homologs.<sup>20</sup>

### 3.2 Hydrophobic Core Packing

In comparison to the highly stable LCC, the hydrophobic core of IsPETase contains several suboptimal packing defects or "cavities." Filling these voids increases the van der Waals contact density and the free energy of folding.

- **L117F and Q119Y:** Identified in the DuraPETase campaign (via the GRAPE strategy), these

mutations introduce bulky aromatic residues into the core.<sup>16</sup> L117F fills a specific hydrophobic cavity, while Q119Y (or Q119K in HotPETase) participates in both core packing and surface interactions, effectively cementing the  $\beta$ -sheet core.<sup>21</sup>

- **A180I:** The substitution of Alanine with Isoleucine at position 180 increases the hydrophobicity of the interface between secondary structure elements, contributing to a more compact global fold.<sup>21</sup>

### 3.3 Disulfide Stapling: From Natural to Engineered Bonds

IsPETase naturally possesses two disulfide bonds: a structural bond common to the fold and a unique active-site bond (DS1: C203-C239). DS1 acts as a "staple" for the loops carrying the catalytic aspartate and histidine residues. Removal of DS1 creates a flexible, disordered active site that is catalytically incompetent.<sup>2</sup>

However, to reach temperatures  $>70^{\circ}\text{C}$ , additional covalent staples are required:

- **N233C-S282C:** This non-native disulfide bond is a hallmark of **HotPETase** and **TS-PETase**. It links the C-terminal region (S282) to the flexible loop region near the active site (N233). This staple prevents the thermal unraveling of the C-terminus, a common pathway for denaturation in  $\alpha/\beta$ -hydrolases.<sup>12</sup>
- **Synergy with Calcium:** While some homologs like Cut190 rely on calcium binding for stability, IsPETase does not naturally bind calcium. However, engineered disulfides essentially replace the stabilizing role that metal ions play in other enzymes, providing a covalent, rather than coordinate, lock on the structure.<sup>3</sup>

### 3.4 Entropic Stabilization via Proline and Glycine Editing

Reducing the entropy of the unfolded state is a powerful method for stabilization. This is achieved by restricting the conformational freedom of the backbone in the folded state.

- **S290P (DepoPETase):** The C-terminus of IsPETase is inherently flexible. The introduction of a Proline at residue 290 (S290P) restricts the backbone dihedral angles, rigidifying the terminus and preventing fraying. This single mutation contributes significantly to the remarkable  $+23.3^{\circ}\text{C}$   $T_m$  increase observed in DepoPETase.<sup>12</sup>
- **G165A:** Glycine residues possess high conformational freedom. Mutating Gly165 to Alanine (found in DuraPETase) restricts this freedom in a loop region that does not require flexibility for catalysis, thereby stabilizing the folded state.<sup>16</sup>

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## 4. Distal and Second-Shell Regulation (Allostery)

A major insight from recent engineering campaigns is that the residues governing enzyme performance are often located far from the active site ( $>15\text{ \AA}$ ). These "distal" or "second-shell" mutations exert their effects through allosteric networks that transmit rigidity

or flexibility to the catalytic center.

## 4.1 The "Domino Effect" of Distal Mutations

Mutations such as **S269T** (or S269V in HotPETase) and **N233K** are located on surface loops distant from the catalytic triad. Yet, MD simulations reveal that these residues are critical nodes in dynamic networks.<sup>22</sup>

- **Mechanism of Action:** The S269T mutation stabilizes the local loop structure. This stabilization is transmitted via contact with C273, through the  $\beta$ -sheet core, and finally to the loops carrying residues Y87 and T88 in the active site. This long-range "domino effect" dampens the thermal fluctuations of the active site residues, allowing them to maintain catalytic geometry at higher temperatures.<sup>22</sup>
- **Damping Global Fluctuations:** Distal mutations often function by reducing the global Root Mean Square Fluctuation (RMSF) of the protein. By rigidifying peripheral loops, they prevent the propagation of thermal vibrational energy into the core, effectively insulating the active site.<sup>5</sup>

## 4.2 The Role of N233 in FAST-PETase and DepoPETase

Residue N233 is a frequent target for mutation. In FAST-PETase, the **N233K** mutation introduces a positive charge that is predicted to form a salt bridge with Glutamate 204 (E204).<sup>6</sup> This interaction clamps two adjacent loops together, reducing their independent motion. In DepoPETase and other variants, modifications at this site (e.g., N233C for disulfide bonding) consistently appear as key stabilizers, highlighting this position as a structural "hotspot" for global stability.<sup>12</sup>

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# 5. Electrostatic Modulation: Charge and Salt Bridges

The surface electrostatics of IsPETase play a dual role: they determine the stability of the fold via salt bridges and influence the affinity for the charged/polar surface of the PET substrate.

## 5.1 Surface Charge Inversion and Substrate Affinity

PET surfaces, particularly after partial hydrolysis, are negatively charged due to exposed carboxylate groups. Additionally, the hydrophobic nature of the polymer requires the enzyme to approach without strong electrostatic repulsion.

- **R132N and R280A:** The removal of positively charged arginine residues (R132N, R280A) is a recurring strategy (e.g., in the "STAR" variant).<sup>15</sup> These mutations reduce the positive surface charge density. While counterintuitive for binding a negative surface, the removal of specific arginines prevents "non-productive" electrostatic sticking or repulsion,

allowing the hydrophobic patch of the enzyme to engage the PET surface more effectively.<sup>15</sup>

- **The S121E Network:** In FAST-PETase, the S121E mutation introduces a negative charge that forms a novel electrostatic network with surface residues, optimizing the enzyme's dipole and solubility profile.<sup>6</sup>

## 5.2 Engineering Thermophilic Salt Bridge Networks

Mesophilic enzymes like IsPETase generally lack the extensive surface salt bridge networks found in thermophiles. Reconstituting these networks is a potent stabilization strategy.

- **The Arg-Asp Lock (I168R/S188E):** In rationally designed variants, the introduction of Arginine at position 168 and Glutamate at 188 creates a specific salt bridge triad (R168-D186-E188). This "molecular lock" bridges disparate regions of the protein surface, significantly raising the  $T_m$  (up to  $+8.7^{\circ}\text{C}$ ) by preventing the dissociation of these structural elements during thermal stress.<sup>25</sup>
- **R224Q and N233K:** These mutations in FAST-PETase are predicted to optimize local electrostatic interactions, reducing the desolvation penalty of folding and stabilizing the loop structure.<sup>6</sup>

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# 6. Comprehensive Analysis of Major Engineered Variants

The evolution of IsPETase from a mesophilic curiosity to an industrial candidate is marked by four major variant lineages, each employing distinct strategies (Table 2).

Table 2: Structural and Functional Comparison of Major IsPETase Variants

Variant	Key Mutations	$T_m$ ( $^{\circ}\text{C}$ )	Design Philosophy	Structural Mechanism
IsPETase (WT)	None	~46-48	Natural Evolution	Flexible W185 for ambient activity; fragile loops.
ThermoPETase	S121E, D186H, R280A	~58	Rational Design	Stabilizes the critical

				$\beta$ 6- $\beta$ 7 loop (D186H); widens cleft (R280A).
<b>FAST-PETase</b>	N233K, R224Q, S121E, D186H, R280A	~67	Machine Learning	Adds electrostatic stabilizers (N233K salt bridge) to ThermoPETase . Balances rigidity with catalytic dynamics.
<b>DuraPETase</b>	L117F, Q119Y, A180I, S214H, R280A, +5 others	~77	Computational (GRAPE)	Aggressive hydrophobic core repacking (L117F, A180I) to prevent denaturation.
<b>HotPETase</b>	21 mutations (inc. N233C-S282C)	~82.5	Directed Evolution	Extensive loop reshaping; disulfide staple (N233C-S282C ); locks W185 via Y214 interaction. Operates at $T_g$ .
<b>DepoPETase</b>	N246D, T88I, D220N, S290P, +3 others	~69.4	High-Throughput Screening	C-term rigidification (S290P);

				Subsite II optimization (T88I); Glycosylation site removal (N246D).
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## 6.1 FAST-PETase: The Machine Learning Triumph

FAST-PETase utilizes the scaffold of ThermoPETase and applies machine learning (MutCompute) to identify non-intuitive stabilizing mutations. The addition of **N233K** and **R224Q** was predicted to optimize the local chemical environment without disrupting the active site. The result is an enzyme that maintains high activity at moderate temperatures (50°C) by lowering the activation energy barrier through favorable pre-organization of the active site water network.<sup>6</sup>

## 6.2 HotPETase: Breaking the Stability-Activity Trade-off

HotPETase represents the limit of what directed evolution can achieve. With 21 mutations, it fundamentally alters the enzyme's dynamic profile. Key to its function is the **N233C-S282C** disulfide, which locks the global fold, and the rigidification of the W185 gate. While rigidifying the gate usually kills activity in the wild-type, at 60-70°C, the thermal energy provides sufficient activation for the substrate to enter, allowing HotPETase to function efficiently on semi-crystalline PET where chain mobility is high.<sup>7</sup>

## 6.3 DepoPETase: The Power of Screening

DepoPETase was identified using a novel fluorescence-based high-throughput screen. Its unique contribution is the **T88I** mutation, which increases the hydrophobicity of Subsite II, and **S290P**, which stabilizes the C-terminus. Furthermore, the **N246D** mutation removes a problematic glycosylation site (see Section 8), making it particularly suitable for yeast expression.<sup>12</sup>

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# 7. Enzymatic Mechanism and Kinetic Barriers

To engineer better enzymes, one must understand the kinetic bottlenecks of the reaction.

## 7.1 The Rate-Limiting Step: Deacylation

QM/MM simulations have identified the **deacylation step**—the hydrolysis of the acyl-enzyme

intermediate to release the product and regenerate the free enzyme—as the rate-limiting step of the catalytic cycle, with an energy barrier of  $\Delta G^{\ddagger} \approx 20$  kcal/mol.<sup>28</sup>

- **Water Activation:** Efficient deacylation requires the activation of a hydrolytic water molecule by the active site Histidine. In variants like FAST-PETase, the active site environment is optimized to retain sufficient hydration for this step while excluding bulk water that might interfere with substrate binding. The "pre-organization" of the active site residues lowers this barrier.<sup>5</sup>

## 7.2 Product Inhibition by MHET

IsPETase produces mono-2-hydroxyethyl terephthalate (MHET) as a primary product. MHET is a competitive inhibitor that binds tightly to the active site, preventing further turnover. The accumulation of MHET is a significant kinetic barrier.

- **Cleft Engineering for Release:** The **R280A** mutation, by widening the cleft and removing charge interactions, likely facilitates the faster release of MHET, thereby reducing product inhibition. Co-incubation with MHETase (which degrades MHET) is often required to drive the equilibrium forward, but structural modifications to IsPETase itself can minimize the inhibitory residence time of the product.<sup>15</sup>

## 7.3 Endolytic vs. Exolytic Activity

IsPETase exhibits both endolytic (cleaving internal bonds) and exolytic (cleaving terminal bonds) activity. The conformational flexibility of the wild-type (Wobbling Trp) supports endolytic cleavage by allowing the enzyme to "clamp" onto the middle of a polymer chain. However, hyper-rigid variants like HotPETase may shift towards a more exolytic mode or rely on the thermally induced movement of the polymer chain ends to enter the active site.<sup>29</sup>

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# 8. Expression Determinants: Glycosylation and Chaperones

The biological context of production is as critical as the protein sequence. The choice of expression host—*E. coli* vs. *Pichia pastoris*—imposes specific constraints related to glycosylation and folding.

## 8.1 The Glycosylation Bottleneck in *Pichia pastoris*

For industrial scale-up, the yeast *Pichia pastoris* is preferred due to its high-density fermentation capabilities. However, IsPETase contains three potential N-linked glycosylation sites (Asn-X-Ser/Thr) at positions **N85**, **N233**, and **N246**.<sup>31</sup>

- **Steric Shielding at N246:** The glycosylation site at N246 is located at the rim of the substrate-binding cleft. Glycosylation here attaches bulky glycan chains that physically occlude the entrance to the active site, creating a "shield" that prevents the polymer from binding. This results in drastically reduced activity for yeast-expressed enzyme compared to *E. coli*-expressed enzyme.<sup>32</sup>
- **Engineering Solutions:**
  - **N246D:** Found in DepoPETase, this mutation abolishes the glycosylation motif and introduces a beneficial negative charge.
  - **Deglycosylation:** Enzymatic treatment (Endo H) to remove glycans restores activity, confirming the steric shielding hypothesis.
  - **Consensus Mutagenesis:** Variants like N181A (in homologous studies) or site-specific removal of N85/N233/N246 allow for efficient secretion in *Pichia* without the penalty of inhibitory glycosylation.<sup>31</sup>

## 8.2 Solubility and Chaperone Systems in *E. coli*

In *E. coli*, IsPETase is prone to forming insoluble inclusion bodies, particularly when overexpressed, due to its requirement for correct disulfide bond formation.

- **GroEL/GroES Synergy:** Co-expression with the **GroEL/GroES** chaperone system has been shown to significantly enhance the yield of soluble, active enzyme.<sup>35</sup> GroEL provides an isolated folding chamber that prevents the hydrophobic aggregation of the aggregation-prone folding intermediates.
- **Trigger Factor (TF):** While Trigger Factor (ribosome-bound) assists in early folding, studies indicate that the GroEL/ES system is more effective for IsPETase, likely due to the enzyme's complex tertiary structure and disulfide requirements.
- **Expression Vectors:** The use of specific signal peptides (e.g., PelB) and optimization of the 5' UTR (silent mutations) can modulate translation rates, giving the nascent chain more time to fold correctly before secretion.<sup>37</sup>

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## 9. Conclusion and Future Outlook

The optimization of IsPETase for industrial plastic recycling is a multifaceted challenge that extends far beyond the active site. This analysis reveals that the catalytic efficiency and stability of the enzyme are emergent properties of a complex structural network.

- **Activity** is driven by the specific architecture of Subsite II (residues T88/A89), the conformational plasticity of the W185 gate, and the accessibility of the cleft (modulated by R280).
- **Stability** is engineered through the rigidification of distal loops (D186H, S290P), the "stapling" of the fold with non-native disulfides (N233C-S282C), and the repacking of the hydrophobic core (L117F, Q119Y).

- **Production** requires careful management of glycosylation sites (N246) in yeast and chaperone-assisted folding in bacteria.

The trajectory of engineering has moved from rational active site mutations (ThermoPETase) to machine-learning-guided electrostatic optimization (FAST-PETase) and deep evolutionary resampling of the entire scaffold (HotPETase, DepoPETase). The most potent enzymes of the future will likely combine these strategies: utilizing the hyper-stable scaffold of HotPETase, the subsite optimization of DepoPETase, and the surface charge engineering of the STAR variants to create a "universal" depolymerase capable of deconstructing high-crystallinity PET at industrial scales.

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