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Perspective

Mechanism-Based Design of Efficient PET Hydrolases

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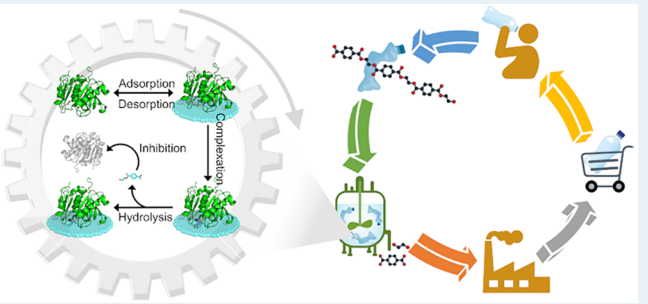
ABSTRACT: Polyethylene terephthalate (PET) is the most widespread synthetic polyester, having been utilized in textile fibers and packaging materials for beverages and food, contributing considerably to the global solid waste stream and environmental plastic pollution. While enzymatic PET recycling and upcycling have recently emerged as viable disposal methods for a circular plastic economy, only a handful of benchmark enzymes have been thoroughly described and subjected to protein engineering for improved properties over the last 16 years. By analyzing the specific material properties of PET and the reaction mechanisms in the context of interfacial biocatalysis, this Perspective identifies several limitations in current enzymatic PET degradation approaches. Unbalanced enzyme−substrate interactions, limited thermostability, and low catalytic efficiency at elevated reaction temperatures, and inhibition caused by oligomeric degradation intermediates still hamper industrial applications that require high catalytic efficiency. To overcome these limitations, successful protein engineering studies using innovative experimental and computational approaches have been published extensively in recent years in this thriving research field and are summarized and discussed in detail here. The acquired knowledge and experience will be applied in the near future to address plastic waste contributed by other mass-produced polymer types (e.g., polyamides and polyurethanes) that should also be properly disposed by biotechnological approaches.



KEYWORDS: *Hydrolase, enzymatic degradation, interfacial biocatalysis, plastic recycling, protein engineering, polyethylene terephthalate (PET), product inhibition, thermostability*

1. INTRODUCTION

Scientists began to study biological degradation of plastic waste in the early 1970s.[1] Around the same time, the distribution of plastic waste in the Pacific Ocean was first quantified.[2] After nearly 50 years, climate change and environmental pollution caused by mass production, mass consumption, and improper end-of-life management of petroleum-based plastic products have become unprecedented challenges for human society.[3−5] Consciousness of these issues spreading among governments and policymakers, industries producing or relying on plastics, and end-users of plastic products, has recently boosted research and development of novel plastic replacement materials and waste plastic valorization strategies to enable a transition from a linear to a circular plastic economy.[6−9]

To this end, biotechnological plastic recycling has become a thriving research area in recent years.[6,9,10] The biodegradation of hydrophobic vinyl polymers, such as polyolefins and polystyrene, which represent over 80% of all conventional plastics produced,[11] is still an intensively debated topic.[9,12,13] In contrast, the biocatalytic degradation of polyester-type plastics, such as polyethylene terephthalate (PET), has evolved in the last two decades from verifying trace amounts of released monomers after weeks of incubation to highly efficient depolymerization within several hours.[14−17] PET is a heteroatomic polymer consisting of terephthalic acid (TPA) and ethylene glycol (EG) connected by ester bonds. It is a semicrystalline thermoplastic composed of crystalline regions with uniformly packed molecules and amorphous regions with randomly arranged microstructures.[18] With a first patent filed for its synthesis in 1941 and commercialization starting in the early 1950s,[19] particularly as synthetic polyester fabric for the textile industry and as packaging materials for food and beverages,[20,21] PET has become one of the most important mass-produced petrochemical plastics.[22] The majority of PET products have a high crystalline fraction (usually 30−40% crystallinity)[23,24] benefiting their durability against mechanical and chemical stress.[25] Post-consumer PET accounts for a considerable fraction of the global solid waste stream, and its

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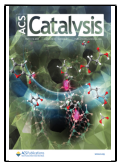

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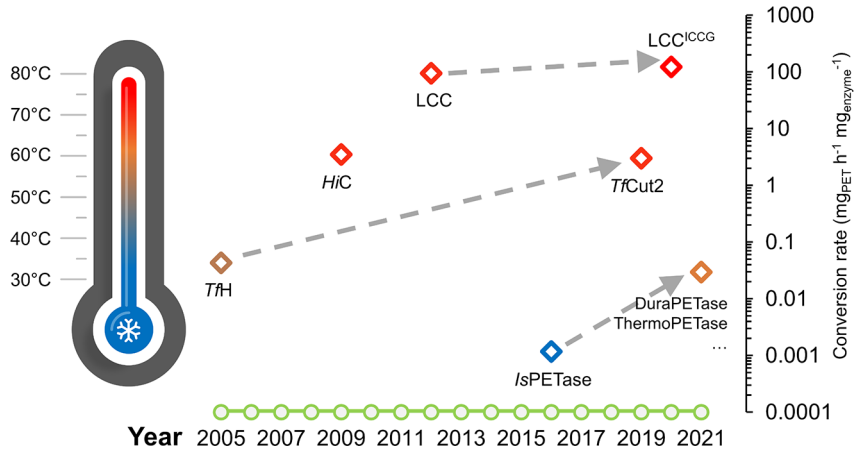

Figure 1. Selected milestones of a 16-year-long history of identifying and engineering PET hydrolases. Both optimal reaction temperatures (in varying colors) for PET degradation and normalized maximal conversion rates (diamonds corresponding to logarithmic values on the vertical axis) calculated based on various publications are denoted, regardless of the material properties of applied PET substrates. The successes in raising the degradation performance using certain benchmark enzymes by both protein and process engineering are indicated by arrows with broken lines. *Tf*H: hydrolase from *T. fusca* DSM43793;[31] *Hi*C: *Humicola insolens* cutinase;[42] LCC: leaf-branch compost cutinase;[33,52] *Is*PETase: *I. sakaiensis* PET hydrolase;[16,34] *Tf*Cut2: *T. fusca* KW3 cutinase;[53] LCC^{ICCG}: LCC variant with indicated quadruple substitutions;[16] DuraPETase, ThermoPETase, etc.: various thermostabilized *Is*PETase variants.[54−56]

breakdown is a source of microplastics and microfibers that further pollute the environment.[11,26−28]

Tokiwa et al. suggested in the 1970s that aromatic polymers are resistant to enzymatic and microbial attack.[29] Later research found, however, that crude enzyme preparations from specific fungal species can induce surface modifications on high-crystallinity PET materials.[30] The first breakthrough was achieved with the cutinase *Tf*H (from the filamentous actinomycete *Thermobifida fusca*) which caused >50% weight loss of melt-quenched post-consumer PET bottles (10% crystallinity) following 3 weeks of incubation.[31] During 16 years of extensive research, the initial low PET degradation activity of *T. fusca* PET hydrolase has been considerably enhanced by more than an order of magnitude (Figure 1) as a result of both protein and process engineering.[32] The development of metagenomic approaches has allowed for the identification of a more active leaf-branch-compost cutinase LCC,[33] which has emerged as the most promising benchmark thermophilic PET hydrolase. In 2020, *Nature* published a breakthrough article on engineered LCC variants which can rapidly depolymerize amorphized (i.e., by lowering the crystallinity through a thermomechanical process to allow for enzymatic degradation) PET waste at an industrially relevant scale within 10 h, and the recovered monomers were readily used to synthesize virgin polymers, thereby closing the recycling loop.[16] *Is*PETase and the related monoester-hydrolyzing *Is*MHETase, both derived from the bacterium *Ideonella sakaiensis*, which was isolated from plastic-contaminated sediment samples, have also been intensively studied.[34,35] The uniqueness of this bacterium in degrading and metabolizing highly amorphous PET led not only to the discovery of a tandem polymer degradation pattern by two distinct enzyme classes,[36] but also to its utility in the efficient bio-upcycling of PET waste to other useful biopolymers when it was applied as a whole cell-based catalyst.[37]

Based on >65 crystal structures so far elucidated,[38] these bacterial PET hydrolases exhibit conserved structural properties and can be classified into a single subclass of the α/β-hydrolase fold enzyme superfamily as represented by the first solved *Streptomyces exfoliates* lipase structure (PDB ID: 1JFR).[39,40] This subclass is distinct from the polyester-hydrolyzing fungal cutinases (EC 3.1.1.74) with shorter polypeptides and more compact structures.[41] *Thermomyces insolens* (formerly named *Humicola insolens*) cutinase *Hi*C is a prominent member of the fungal PET hydrolase family. It has been successfully commercialized and also received broad attention in recent years by researchers working on PET waste degradation. However, here the emphasis was more on process engineering and improvement rather than protein engineering.[42−45]

This Perspective intends to provide a deeper understanding of important obstacles in the engineering of effective industrially applicable PET hydrolases, as well as to provide guidance for further efforts in this intriguing research field. Previous research has indicated that enzymatic polyester hydrolysis occurs preferentially in its amorphous parts rather than the well-ordered crystalline regions with poor chain mobility.[46] Understanding the accessibility of amorphous polymer chains around the polymer glass transition temperature, in conjunction with tailoring the thermostability of biocatalysts, has thus become a focus of contemporary research.[38,47] As a typical surface erosion process, it is not yet fully understood how the adsorption and desorption of biocatalysts onto the polymer surface influence the overall degradation kinetics and performance. This problem is reminiscent of the decades spent investigating the relationship between the binding of cellulases onto crystalline cellulose and catalytic activity.[48] Altering their affinity to polymer substrates by protein engineering, on the other hand, has proven to be a viable approach to maximize the catalytic capacity of both cellulose and PET depolymerases. Similar to the aforementioned natural polymer-degrading enzymes such as cellulases, mass transfer of certain degradation intermediates that can function as inhibitors of the biocatalysts, has been identified as the third main bottleneck that must be addressed with a low-cost solution for adequate reaction efficiency.[49−51] This Perspective summarizes and discusses special efforts devoted to the engineering of thermostability (Section 2), to study the de/adsorption properties, and to address the product inhibition (Sections 3 and 4).

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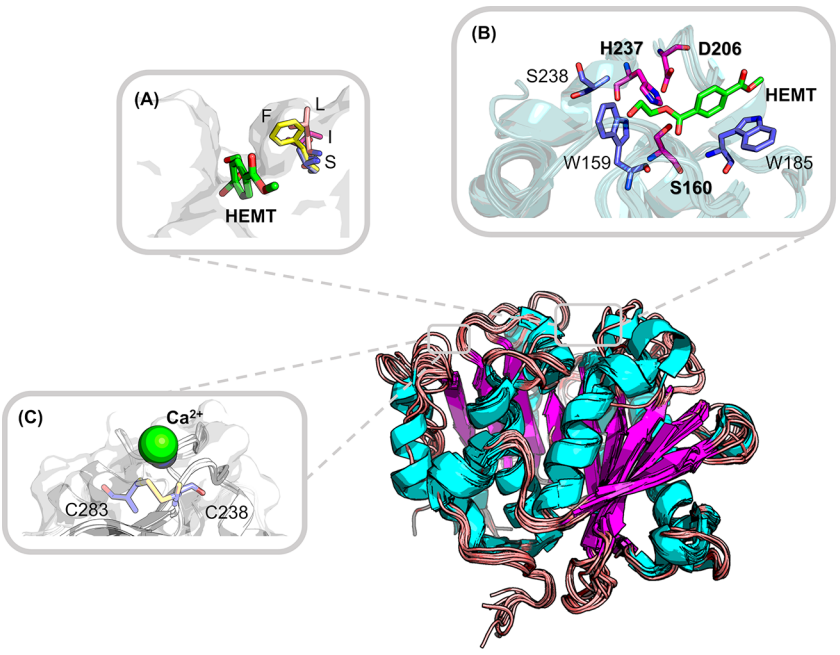

Figure 2. Frequently reported mutation hot spots illustrated on the superposed crystal structures of known bacterial PET hydrolases. Backbones shown in the cartoon are derived from the structural superposition with selected homologous enzymes. The catalytic triad (S160, D206, and H237) as well as two aromatic residues (W159 and W185) are involved in the interaction with the monomer analogue 1-(2-hydroxyethyl)-4-methyl terephthalate (HEMT) (A, B) based on the *Is*PETase structure (PDB ID: 5XH3; the numbering of residues is modified consistently with other structures solved later for easy comprehension). (A) One frequently reported mutation hotspot equivalent to S209 (B) in *Is*PETase can adopt various residues which might influence the widths of the binding pocket:[65,90] F found in 4CG1, 4EB0, and 7OSB and also conserved in many other PET hydrolases; S found in the *Is*PETase structure 5XH3; I found in an LCC mutant 6THT; L found in another PET hydrolase 7CUV. (C) One of the putative Ca^{2+} binding sites revealed by cocrystallized structures such as 4WFJ, 5LUL, and 5ZNO can be replaced by a disulfide bridge (6THT, 7CTS, and 7CTR) to thermostabilize several PET hydrolases.

2. DESIGN OF THERMOSTABLE PET HYDROLASES BASED ON MATERIAL PROPERTIES

Earlier research on enzymatic degradation of aromatic and aliphatic copolyesters suggested that increasing the proportion of aromatic moieties raises the melting point, thus lowering the polymer chain mobility and biodegradability at the optimal reaction temperatures of the applied hydrolases.[57,58] For a long time, enzymatic depolymerization of PET with a melting point over 260 °C was thought to be unachievable according to this idea, until, in 2005, Müller et al. showed significant weight loss of amorphized PET waste using *Tf*H.[31] This discovery drew attention to the preferential (or perhaps exclusive) degradation of amorphous PET, as well as the importance of its glass transition temperature (T_g) in understanding enzymatic degradation.[42,46] T_g is the temperature range in which the glass transition process occurs, i.e., the amorphous polymer structure transitions from a "glassy" (hard and brittle) state into a sticky-liquid or rubbery one as a function of increasing temperature.[18,59] The T_g of bulk amorphous PET is in the range of 65−71 °C, estimated based on data collected with differential scanning calorimetry (DSC) at zero heating rate.[60−62] This has so far been accepted as a standard value by research communities working on enzymatic PET hydrolysis.[31,42,53,63−65] In addition to its role as both a solvent and a reactant in enzymatic PET degradation, water has a plasticization effect on the PET polymer, as reported since the early 1980s,[66,67] by lowering the T_g of bulk polymer by up to 16 °C.[60,68,69] This influence is more pronounced on the surface polymer layer of about 13 nm which has an intrinsically lower T_g (~48.1 °C) than that of the bulk polymer even when water is not present.[70] With both effects taken into account, the superficial PET layer exposed to enzymatic degradation in an aqueous milieu can indeed have a biocatalysis-relevant $T_g \lesssim 40$ °C, explaining the considerably fast degradation of amorphous PET by, for example, *I. sakaiensis* and its *Is*PETase at ambient temperature.[34] The enhanced segmental mobility of the surface polymer, on the other hand, may decrease to a level similar to that of the bulk polymer as a result of superficial crystallization.[71] For example, as a result of UV-treatment-induced crystallization, the overall degradation performance could be significantly impaired.[72]

Based on these PET material characteristics, the advantage of using thermophilic and thermostable enzymes in depolymerization, over their mesophilic counterparts, is obvious and evident from preliminary studies.[42,56,73,74] In this regard, searching for new thermophilic enzymes, such as those found in metagenomic libraries,[33,75] as well as known thermostabilizing ones, provides viable options for improving degradation performance.[74] In the presence of selected bivalent ions such as Ca^{2+} or Mg^{2+}, the overall thermostability of many bacterial PET hydrolases has been improved, as indicated by increased midpoints of thermal denaturation (T_m) by 10−16 °C and enhanced optimal temperatures (T_{opt}) for PET degradation by at least 10 °C.[52,76−78] The probable Ca^{2+} binding site was found to be close to the catalytic triad based on cocrystallized structures (Figure 2) and molecular dynamics simulations.[76−81] The interaction with Ca^{2+} controls the process of opening and closing the active site during substrate binding and unbinding by Cut190 from *Saccharomonospora viridis*.[78,80] Because Ca^{2+} will precipitate terephthalate to generate insoluble byproducts from the reaction super-

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Table 1. Selected *Is*PETase Engineering Studies Designed to Improve Thermal Stability

nomenclature and introduced mutations	improved stability[a]	design approach and interpretation	refs
S238F/W159H	T_m = 56.5 °C, ΔT_m = +9.7 °C	Structural and sequence comparison with homologous PET hydrolases	65,90
ThermoPETase: S121E/D186H/R280A	T_m = 57.6 °C, ΔT_m = +8.8 °C	Structure-based design; Water-mediated hydrogen bond between E121 and H186	54
DuraPETase: A214H/I168R/W159H/S188Q/R280A/A180I/G165A/Q119Y/L17F/T140D	T_m = 77.0 °C, ΔT_m = +31.0 °C	Structure-based design and machine learning approach; Multiple stabilizing interactions	55
W159H/F229Y	ΔT_m = +10.4 °C	Sequence comparison with other homologous PET hydrolases	91
DuraPETase+N233K	T_m = 83.5 °C, ΔT_m = +38.4 °C	Machine learning; Introduction of salt bridge between K233 and E204	88
FAST-PETase: ThermoPETase+R224Q/N233K	T_m= 67.4 °C, ΔT_m = +22.3 °C	Machine learning; Introduction of a hydrogen bond between Q224 and S192	88
TS-PETase: ThermoPETase+N233C/S282C	T_m = 69.4 °C, ΔT_m = +22.3 °C	Structural comparison with other homologous PET hydrolases	83
TM3: ThermoPETase+K95N/F201I/N233C/S282C	T_m = 70.8 °C, ΔT_m = +25.8 °C	Random mutagenesis based on error prone PCR and structural comparison with LCC^{ICCG} mutant	56
D1: DuraPETase+N233C/S282C	T_m = 81.1 °C, ΔT_m = +36.1 °C	Structural comparison with LCC^{ICCG} mutant	56
HotPETase: TS-PETase+P181V/S207R/S214Y/Q119K/S213E/R90T/Q182M/N212K/R224L/S58A/S61V/K95N/M154G/N241C/K252M/T270Q	T_m= 82.5 °C, ΔT_m = +37.5 °C	Directed evolution	89

[a] ΔT_m was estimated compared to T_m of the wild-type *Is*PETase either determined in the same publication or that of 45.1 °C determined by Brott et al.[56] T_m values were determined by circular dichroism, differential scanning calorimetry, or differential scanning fluorimetry.

natants,[82] relying on Ca^{2+} salts for high degradation efficiency is undesirable and can be minimized by tailoring the Ca^{2+}-binding sites of the biocatalysts. Earlier research was carried out by substituting one major Ca^{2+}-binding site with a salt bridge or a putative disulfide bond, resulting in an increased T_m of the *T. fusca* cutinase *Tf*Cut2 by up to 25 °C.[77,79] The latter strategy was then adopted in engineering other homologous PET hydrolases, resulting in increased T_m by up to 26 °C,[16,56,81,83] although the formation of this disulfide bond was indeed validated by protein crystallography for only two enzymes.[16,81] More recently, Nakamura et al. have also introduced cysteine pairs to form putative disulfide bonds at alternative positions in the homologous hydrolase PET2, but this led only to a marginal increase of T_m by less than 3.1 °C.[84]

Enhanced thermostability of PET hydrolases has also been reported when they were recombinantly expressed in alternative hosts other than *Escherichia coli*, for example, *Bacillus subtilis*[53,85] or *Pichia pastoris*.[86] Compared to the wild-type LCC obtained from *E. coli*, an increased T_m of >4 °C or >12 °C has been described when it was expressed in *B. subtilis* or *P. pastoris*, respectively.[85,86] The remarkable improvement with the latter host was attributed to glycosylation which mitigated the enzyme thermal aggregation at high reaction temperatures.[86]

Despite the fact that *Is*PETase is distinguished by its high PET hydrolyzing activity at ambient temperature, an effect which is presumably due to its more flexible and open substrate binding cleft,[87] efforts to thermostabilize this enzyme have been widely reported recently, with the goal of outperforming other cutinase-like PET hydrolases also at higher temperature ranges (Table 1). A ThermoPETase mutant with three residue substitutions, generated based on a structure-based engineering strategy, exhibited an increased T_m by 8.8 °C and up to 14-fold improved PET hydrolyzing activity compared to the wild-type *Is*PETase at 40 °C.[54] DuraPETase is an *Is*PETase variant with ten mutated residues, discovered by a novel GRAPE (*greedy accumulated strategy for protein engineering*) computational method.[55] Compared to wild-type *Is*PETase, DuraPETase showed a T_m increase of 31 °C and over 300-fold higher hydrolytic activity against high-crystallinity PET powder. By applying a convolutional neural network, MutCompute, trained for stability optimization, Lu et al. reported in a recent preprint the most thermostable *Is*PETase variant based on a single N233K substitution in addition to the DuraPETase, resulting in a T_m of 83.5 °C.[88] FAST-PETase, the most promising variant based on additional mutations of ThermoPETase, demonstrated significantly improved PET degradation activity against low-crystallinity PET waste at 50 °C compared to the wild-type and other known mutants of *Is*PETase. By screening a randomized *Is*PETase library based on error-prone PCR, at least one of their computationally targeted stability-related hot spots was also discovered.[56] Further substitutions related to thermostabilizing *Is*PETase found by other protein engineering strategies have been reported to be distributed across the entire sequence (Table 1). In another recent preprint, >13,000 *Is*PETase variants were evaluated by applying catalytic activity at elevated temperatures as a primary selection pressure. This directed evolution procedure afforded a HotPETase variant with 21 mutations compared to wild-type *Is*PETase and a T_m of 82.5 °C.[89] Therefore, the thermostability of *Is*PETase and other homologous enzymes depends on the interplay of many effects, necessitating further comprehensive research.

By raising the reaction temperature from 65 to 75 °C, the transition of the polymer microstructure from a less-ordered amorphous state to a nondegradable crystalline state can occur significantly earlier and faster.[16] This "physical aging" reaction competes with the enzymatic depolymerization and thus becomes the main determining factor of the total achievable degradation level.[53] As a result, using biocatalysts with only very high thermostability, such as the BhrPETase with a T_m of 101 °C,[85] is not always a good way to improve the degradation performance. As thermostabilizing an enzyme usually comes at the expense of lowering the flexibility of certain catalysis-relevant structural segments, a more balanced protein engineering strategy focusing on a thermo-active biocatalyst with T_{opt} at 75 °C and sufficient long-term robustness will hold promise for industrial applications. A recent techno-economic

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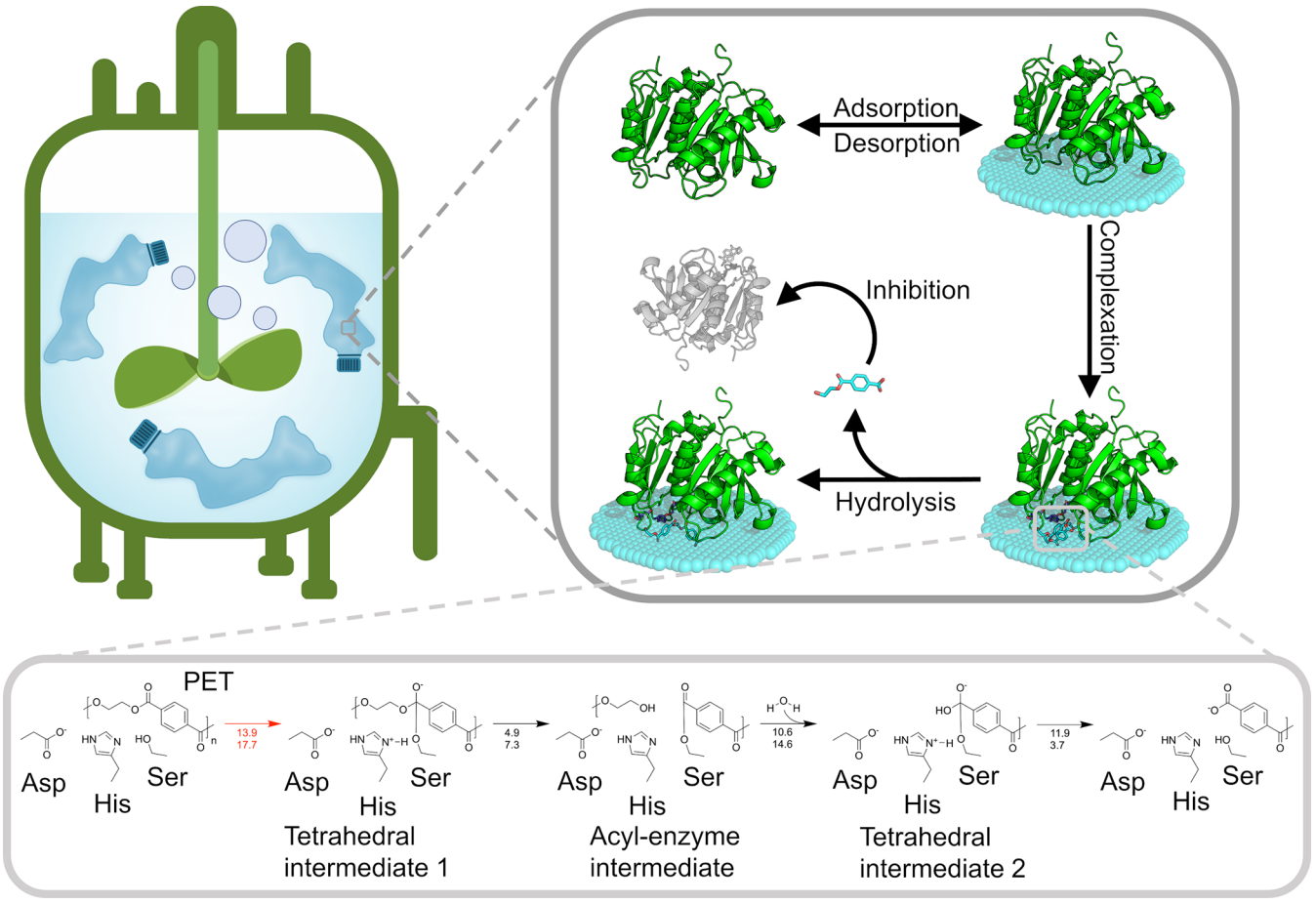

Figure 3. Interfacial biocatalytic hydrolysis of PET and its reaction mechanism. The states of a PET hydrolase are schematically illustrated in the upper right panel. In the lower panel, individual steps of the hydrolysis reaction are schematically shown in line with their activation free-energy barriers in kcal·mol^{-1} summarized based on different studies.[107,108] The reaction is initiated by a nucleophilic attack by a catalytic serine resulting in a tetrahedral intermediate stabilized by a catalytic histidine, an aspartic acid, and the oxyanion hole, followed by breakdown of the tetrahedral intermediate 1 into an acyl−enzyme intermediate and release of an alcohol. The aspartate−histidine pair activates the water for attack on the acyl−enzyme intermediate carbonyl, resulting in the formation of the second tetrahedral intermediate. The deacylation of this tetrahedral intermediate releases the carboxylic acid product. The rate-limiting step is regarded as the initial nucleophilic attack and highlighted in red with two free-energy activation barriers denoted. The top number is the Boltzmann-weighted average from 20 QM/MM MD simulations,[108] and the bottom number comes from adiabatic mapping studies.[107]

analysis of a simulated enzymatic PET recycling process revealed that the reaction duration and enzyme price have a greater influence on process costs than the energy cost to maintain an operating temperature of up to 80 °C.[92] Therefore, a commercially viable biocatalytic PET degradation process will rely on highly efficient enzymes rather than more heat-stable ones, which have, for example, T_{opt} in a higher temperature range but are less active at 75 °C.

3. UNDERSTANDING INTERFACIAL ENZYMATIC PET HYDROLYSIS FROM A STRUCTURE−FUNCTION PERSPECTIVE

Enzymatic PET hydrolysis is a surface erosion process characterized by the primary degradation of the exterior polymer to expose the inside of the material.[42,46,93] Because neither water (as both a solvent and a reactant) nor the biocatalysts can permeate the inner core of the polymer, only a limited number of superficial ester bonds can be accessed, implying that the reaction occurs primarily under conditions using an excess of enzyme.[94] As a consequence of this, research on PET hydrolysis kinetics has commonly employed an inverse Michaelis−Menten equation[90,95,96] or its mathematically equivalent expression based on the derivation of the Langmuir adsorption isotherm.[42,63,97−99] By simultaneous analysis using the conventional Michaelis−Menten equation, which is typically used to analyze bulk reactions on soluble substrates in great excess to enzymes, the accessible enzyme attack sites on the PET surface can be estimated using the ratio of parameters derived from these swapped kinetic models.[90,96]

The degradation-relevant binding of these PET hydrolases and variants was investigated by various biophysical approaches, such as quartz crystal microbalance,[100−102] chemiluminescence,[100] and fluorescence.[103] Binding isotherms have been directly estimated based on the concentrations of free enzymes determined in the supernatant after incubation with PET.[104−106] Selected PET hydrolases were shown to have a high affinity for the PET surface, as evidenced by the rapid formation of a monolayer, although this was thought to be mainly contributed by nonspecific adsorption.[106] A related kinetic study indicated that PET polymers were hydrolyzed at a remarkably lower rate than PET oligomers, regardless of their water solubility.[96] The conversion rates of these oligomers were found to be comparable to those of small-molecule *para-*

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Table 2. Selected PET Hydrolase Engineering Studies in the Presence of Binding Modules

binding module	enzyme	fold improvement	reaction condition	ref
PcAA14A	ThermoPETase	1.3	PET granules (3 g/L) incubated with 8.3 mg_{Enzyme}/g_{PET} in glycine-NaOH buffer (pH 9) for 5 days	137
RolA	*Is*PETase	1.2 (by weight loss) 1.5 (by HPLC)	Preincubation with RolA for 3 h, then degradation of PET fiber waste and high-crystallinity PET powder (15 g/L) at pH 8, 30 °C with 1.3 g_{Enzyme}/g_{PET} for 5 days	136
HGFI	*Is*PETase	1.3 (by weight loss) 1.6 (by HPLC)		
Zwitterionic polypeptide (EK)	*Is*PETase	11	Degradation of amorphous Goodfellow PET film and high-crystallinity (45.2%) beverage bottle with 200 nM enzyme in glycine-NaOH-buffer at pH 9 and 30−40 °C for up to 6 days	138
CBM	A cutinase from *T. fusca*	1.4−1.5 (only affinity)	Incubated with PET fiber and 50 μM enzyme in 50 mM Tris-buffer (pH 8) at 50 °C for 24 h	104
ChBD	LCC^{ICCG}	1.2	With 0.6 g/L amorphous Goodfellow PET film, post-consumer waste PET (16% crystallinity), or high crystallinity (40%) PET at 65 °C for 12 h	103
CBM	LCC^{ICCG}	1.3		
CBM	ThC_Cut1	1.4	With amorphous Goodfellow PET film and 25 mM enzyme in 100 mM potassium phosphate buffer (pH 7) at 50 °C	100
PBM	ThC_Cut1	3.8		

nitrophenyl esters. This suggests that the complexation or dissociation with the degradable polyester strands requires large activation barriers, determining the overall conversion rate rather than the chemical catalysis itself, which showed, in addition, a similar hydrolysis enthalpy to those reported in the literature for other readily accessible esters.[95] Recently, the hydrolysis reaction mechanism of several PET hydrolases against PET-related oligomers was investigated using QM/MM molecular dynamics (MD) simulations or adiabatic mapping to resemble polyester degradation.[107−110] Because there is only one cocrystallized *Is*PETase structure in a complex with a PET monomer analogue available so far, molecular docking of oligomeric aromatic esters has been widely used to study the substrate interaction in the binding groove as well as with the catalytic triad of various PET hydrolases.[16,65,87,111−113] The protein surface landscapes of bacterial PET hydrolases are comparable in close vicinity to the catalytic triad, requiring a high conformational selectivity of accommodated PET repeating units directly around the target ester bond at favored twisting angles.[112−114] In contrast, it is unclear and unlikely if distal PET repeating units are involved in a catalysis-related interaction with additional surface residues which requires a long PET strand to adopt a specific conformation.[113,115] Nonetheless, the defined polymer segment conformation directly next to the target ester bond will require a steric reconfiguration of the polymer chains with adequate mobility in order to form the productive tetrahedral intermediate via the initial nucleophilic attack by the catalytic serine.[115] This is evidenced by the highest activation free-energy barrier determined by QM/MM adiabatic mapping, suggesting the nucleophilic attack as a probable rate-limiting step (Figure 3).[107,108] Further reaction steps of the PET hydrolysis mechanism are canonically the same as for other conventional ester hydrolases, in agreement with the recent findings in kinetic studies.[96]

PET hydrolases are thought to possess dynamic catalytic sites with aromatic subsites which are locally stabilized upon substrate binding.[65,112,116] For example, the conserved W185 in *Is*PETase was also found in many other known PET hydrolases (Figure 2B).[116,117] A substitution of this residue usually resulted in a drastically reduced hydrolytic activity on PET.[112,118] Recently, Chen et al. identified that S214 and I218 are uniquely present in *Is*PETase to reduce the steric hindrance of W185, therefore enabling a higher flexibility of the indole side chain, allowing a higher polymer hydrolysis rate.[119] Introduction of the corresponding double-residue substitutions into homologous polyester hydrolases indeed boosted PET hydrolyzing activity, albeit at the expense of stability at temperatures >60 °C.

Large polymer segments may be unable to fit into a binding cleft that is too narrow. The overall catalytic properties of several PET hydrolases can be changed by widening or narrowing the binding clefts.[97,120] Interestingly, the free energy barrier and hydrolytic reaction thermodynamics do not differ significantly between the thermophilic $LCCI^{ICCG}$ mutant and the mesophilic *Is*PETase.[107] This suggests that the high activity of thermostable PET hydrolases is attributable mostly to the increased accessibility of PET polymer to nucleophilic attack at elevated temperatures, rather than to the fundamental interaction affinity. Austin et al. measured the width of the substrate binding cleft of *Is*PETase via the residue pair T88/S238.[65] By substituting S238 to F, which is highly conserved in homologous cutinase-like PET hydrolases, increased hydrolytic activity on PET was determined when accompanied by the mutation W159H, although the binding cleft width appeared to be narrowed. This phenomenon suggests that the polymer binding in the surface groove is more likely a dynamic rather than static process, as supported by an NMR analysis of PET chain mobility in the context of enzymatic hydrolysis.[115,121] The residue equivalent of the *Is*PETase S238 is F in *Tf*Cut2 and LCC.[52,111] By substitution to A, I, and W, significantly increased PET hydrolysis activity was reported.[16,122] These findings suggest that further systematic iterative engineering of all variable residues in the PET hydrolase binding pocket may be beneficial for gaining a thorough understanding of the rate-limiting interactions with aromatic polyesters and, as a result, improving the overall degradation performance.

Mono(2-hydroxyethyl) terephthalate (MHET) and bis(2-hydroxyethyl) terephthalate (BHET) are degradation intermediates well-known to inhibit the efficiency of depolymerization catalyzed by various PET hydrolases, except for *Hi*C and Cut190.[50,78,123,124] MHET has a stronger inhibitory effect and can hardly be hydrolyzed by the wild-type *Is*PETase.[96] Thus, *I. sakaiensis* produces a distinct MHETase to yield TPA and EG for its growth.[34] This serves as a template for developing a dual enzyme system for effective PET degradation containing an oligo-ester-specific helper enzyme.[123] Recently, a chimeric dual-enzyme system with MHETase linked to *Is*PETase resembling their natural microbial host was established, resulting in PET degradation efficiency improved by at least

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2.8-fold.[125] Additional MHETase-like enzymes from *Comamonas thiooxydans* and *Hydrogenophaga sp.* PML113[125] and an enzyme from a marine consortium[126] with similar structures but considerably lower substrate affinity for MHET than the *Is*MHETase[127] were also identified. This distinction in catalytic characteristics may provide a wide range of choices for combining with other depolymerizing enzymes, particularly for the use in whole cell-based multiple enzyme systems.[128,129] While *Is*PETase has been successfully stabilized, additionally thermostabilizing MHETase-like enzymes would provide a robust two-enzyme system with industrial promise. Other technical solutions have been proposed in this regard, such as employing enzyme mutants that are less susceptible to product inhibition[99] or a membrane reactor for continuous removal of the inhibitors.[130]

4. ENGINEERING ENZYMATIC DE/ADSORPTION ONTO PET SURFACES BY INCLUDING BINDING MODULES

To resemble other natural polymer degrading enzymes, specific polymer binding modules were either directly added in the reaction mixture or covalently fused to appropriate PET hydrolases. Among these, the cellulose-binding domains (CBM) CenA from *Cellulomonas fimi*[104] and that of cellobiohydrolase I from *Trichoderma reesei*,[100,131] the polyhydroxyalkanoate (PHA)-binding module of PHA depolymerase from *Alcaligenes faecalis*,[100] and the chitin-binding module from the chitinase CmChi1 from *Chitinolyticbacter meiyuanensis* SYBCH1[103] increased the degradation performance when they were fused to the C-termini of PET hydrolases (Table 2). Selected tryptophan residues in CBMs were identified as mutation hot spots for enhanced hydrogen bond formation with aromatic moieties at the PET surface.[104,132]

Hydrophobins are small amphiphilic proteins (about 20 kDa) produced by filamentous fungi with surfactant-like activity.[133] Although the highest boosting effect with hydrophobins was discovered when fused to a PET hydrolase, they can already improve the enzymatic binding affinity to the PET surface when introduced to the degradation mixtures as free molecules or applied to material as a pretreatment.[134−136]

In this regard, the lytic polysaccharide monooxygenase (LPMO) from the white-rot fungus *Pycnoporus coccineus* (PcAA14A)[137] and a zwitterionic polymer based on positively charged lysine and negatively charged glutamate[138] were both recently shown to improve PET degradation performance when added to *Is*PETase. LPMO and the so-called EKylation, on the other hand, appear to promote the enzymatic degradation via different mechanisms than hydrophobins or other binding modules.

While the necessity of binding modules for effective cellulase-catalyzed cellulose degradation has been questioned,[139] their presence was recently found to alter the binding and hydrolysis kinetics at various solid loading levels.[140] The CBM was shown to favor low solid loading level in an adsorption-controlled scenario where higher catalytic efficiency is associated with tighter substrate binding but disfavored at high substrate concentrations, as shown by a so-called "volcano plot" of specific reaction rates against the binding strengths.[140] Accordingly, optimal PET hydrolases are suspected to also follow this Sabatier principle and will function at an intermediate substrate binding strength. Future research should be conducted to confirm this hypothesis based on comprehensive binding and hydrolysis kinetic studies with various PET hydrolases, as well as those containing artificially introduced polymer binding modules, to clarify their roles for the interfacial biocatalysis, which is obviously missing so far. This is of great interest for potential industrial applications, where a very high solid loading level is generally preferred, in terms of an economical use of biocatalysts.

5. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

More than 16 years have elapsed since the first microbial PET hydrolase was reported. Many research efforts have been expended in the quest for new PET hydrolases as well as in the optimization of known enzymes. A large number of high-quality studies have been conducted using a few benchmark enzymes, such as cutinase(-like) enzymes from *Thermobifida* species,[141] the plant compost-derived LCC cutinase, and *Is*PETase. Different research groups employed varying experimental conditions, such as pH, buffer compositions (different salts) and concentrations (different ionic strengths), and agitation techniques and speeds, making a balanced comparison of the degradation data very challenging, even when the same benchmark enzyme was used. Selected experimental parameters can also have a significant impact on the degradation performance as well as the related data interpretation in terms of interfacial biocatalysis mechanisms. For example, buffer salts can influence the enzyme performance[142] and the solubility of degradation products or inhibitors,[143] and they can also fundamentally modify the water interaction with polymeric substrates.[144] When simultaneous mechanical treatment is occurring, the ideal water volume for enzymatic PET degradation can vary dramatically.[44] More crucially, studies employed PET materials from various suppliers, with varying crystallinity (even that of the widely used amorphous Goodfellow PET film may differ from batch to batch) and in various forms (powder, foil, sheet, etc.). As a result, standardization of PET hydrolysis tests and conditions using polymer samples with defined material sources and properties should be applied in future investigations to allow for a straightforward data comparison. Several recent studies used manually picked post-consumer low-crystallinity PET packaging in order to achieve striking enzymatic degradation performance at a small laboratory scale.[88,145] However, in a real-world scenario, post-consumer PET packaging with all crystallinity patterns ends up in mixed household plastic waste which is collected and then separated in an industrial plastic sorting system according to polymer type and color, but not crystallinity.[146,147] To remove the contaminants which may hamper the subsequent recycling process, a washing step (e.g., with hot soda water) is usually applied prior to thermomechanical reprocessing (e.g., shredding and drying).[148] These processes will age the sorted waste polymer considerably and consequently eliminate the low-crystallinity fraction of PET. Following additional amorphization and micronization steps, these waste plastic flakes can be readily used for biotechnological recycling.[16,92] Therefore, for lab-scale research in the field of PET hydrolases, using equally pretreated real-world PET waste (e.g., with the same amorphization and micronization equipment and protocols) instead of biased selection of a low-crystallinity fraction will deliver more reliable standardized data on the enzymatic degradation performance of industrial relevance.

As an alternative to the energy-consuming amorphization step,[92] the challenge in lowering the polymer crystallinity is

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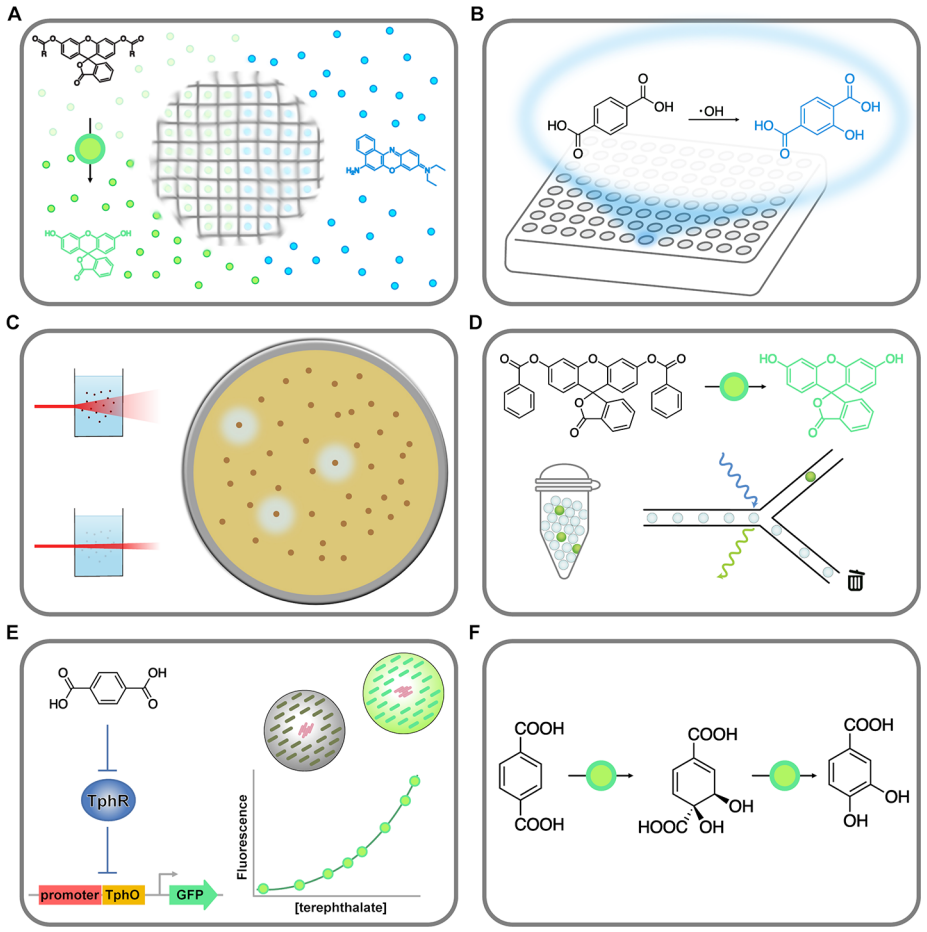

Figure 4. Potential PET hydrolase screening methods arranged in order of increasing potential throughput. (A) Fluorogenic substrates like fluorescein dilaurate can be trapped in polyester films or particles[175,176] and can be released and hydrolyzed upon polymer hydrolysis, generating a fluorescence signal. (B) Fluorimetric method based on the reaction of terephthalic acid with hydroxyl radicals to form the fluorophore 2-hydroxyterephthalic acid.[177−179] Tens of thousands of clones can be screened using microtiter plate-based assays (A, B). (C) Agar plate assay based on the hydrolysis of polyester (PET) nanoparticles.[75,180,181] Clear zones (halos) form around clones expressing active polyester-hydrolyzing enzymes, allowing simple visual identification. Millions of clones can easily be screened using this method. (D) Recently reported ultra-high-throughput droplet-based assay for PETase activity.[182] The use of the fluorogenic surrogate substrate fluorescein dibenzoate indicates a low selectivity, since many other esterases would also be identified using this assay. Tens of millions of clones could be analyzed using this method. Combinations of the turbidimetric assay (C) and droplet-based methods (D) seem promising. (E) Ultra-high-throughput assay based on a terephthalic acid biosensor.[183] Cells could be entrapped in hydrogel beads[184] containing reporter cells that express GFP in response to terephthalic acid formed by clones expressing active PET-hydrolyzing enzymes. Because fluorescence-activated cell sorting (FACS) can be used to sort the beads, the throughput of this method is potentially in the hundreds of millions. (F) Envisaged growth selection approach based on the conversion of terephthalic acid to protocatechuic acid, which could be catabolized by engineered strains of *E. coli* or other model organisms.[183,185] The throughput of this method would be limited only by library size and transformation efficiencies, making it one of the most attractive methods.

envisaged to be possibly solved with a specific crystalline PET depolymerase, like those found in natural cellulase systems to decrystallize cellulose.[149] To this end, continuously growing PET hydrolase sequence data from large-scale sequencing projects can be used for exploring this missing enzyme activity and other novel PET hydrolases in genomic and metagenomic databases. EnzymeMiner and other available *in silico* tools provide useful computational platforms for automated identification of promising enzyme candidates for experimental validation.[150] Pan-genome analysis focusing on microbes which produce known PET hydrolases, e.g., in the *Thermobifida* and *Ideonella* genera, could facilitate finding yet-unknown MHETase-like enzymes to address the product-inhibition problem. Nevertheless, as the substrates of identified PET hydrolases and carbon sources for the host strains are unlikely to be synthetic polyester in their natural habitats, the probability of success of such a research activity is hard to predict. The explored sequence space can also be used to predict enzymes with improved properties by machine learning and ancestral sequence reconstruction.[151−153] Robust proteins can be designed by sequence-based approaches and fully automated computational workflows, like FireProt^{ASR}, making complex protocols of ancestral inference accessible to nonexperts.[154] Recent developments of highly accurate protein structure prediction methods, like AlphaFold2 and RoseTTA-Fold, can assist a structure-based design of novel PET hydrolases identified by database searches.[155−157] While hydrolytic biocatalysis might fundamentally be unable to crack the well-ordered crystalline PET, oxidative enzymes, which may have a mechanistically comparable function to LPMOs known from lignin and cellulose degrading systems,[158] can offer further options for mining for polyester decrystallizing activities.

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High-quality structures incorporating PET relevant ligands will be required for further understanding of the structure−function relationship and related engineering of PET hydrolases. Application of novel cryoEM techniques to examine the binding of bigger polymer substrates may be required for a thorough understanding of the substrate−enzyme interaction at the solid−liquid interface. To date, these mechanistic insights have primarily been derived from docking and simulation experiments using flexible PET oligomers, which overlook the inherent mobility and steric constraints given by neighboring polymers under realistic reaction conditions. The utilization of particular force fields designed for interface reactions, as well as taking into account real-world material properties, will help *in silico* protein engineering even more.[159] The calculated energy barriers for PET hydrolysis are balanced for individual reaction stages (Figure 3), implying that more than one catalytic step would need to be enhanced at the same time. This is typically difficult to address rationally, providing room for directed evolution and machine learning. Hence, the future development of suitable screening assays applicable to large enzyme libraries is of particular relevance in this regard. Due to technical limitations of individual approaches, achieving ultra-high-throughput screening specifically for PET degradation activity still remains a challenge (Figure 4).

Furthermore, researchers may investigate the power and specific advantages of using biocatalysts over chemical catalysts, such as when combined with living cells as whole-cell catalysts, which can potentially allow for a one-pot process that simultaneously includes an enzyme cascade not only to degrade the plastics but also to convert the degradation intermediates into a variety of products with added value.[32] The utility of mesophilic enzymes can potentially be reemphasized using such a procedure, because numerous metabolic pathways converting monomers to other value-added compounds are currently only available/engineerable in mesophilic microbial frameworks. Aside from the well-engineered frameworks such as *Pseudomonas putida*, *E. coli*, and *P. pastoris*,[160−164] *I. sakaiensis* has recently emerged as a new promising strain for genomic engineering with considerably high conversion efficiency from PET substrates to PHA biopolymers.[37,129] On the other hand, thermophilic whole-cell biocatalysts such as *Clostridium thermocellum* can be easily employed for faster PET depolymerization, for example, targeting textile waste also containing cellulose, which may be degraded and valorized simultaneously.[21,165] For more details regarding the biotechnological potential of enzymatic textile recycling, readers are referred to a recent comprehensive review by Jönsson et al.[21] In this regard, designing the PET degrading multienzyme complex by combining the advantages of various enzyme classes to resemble an architecture similar to the natural cellulosome might be a viable option.[128]

A photosynthetic microalga, *Phaeodactylum tricornutum*, has been engineered to functionally express *Is*PETase and is able to degrade selected PET-related materials in a saltwater environment at 21−30 °C.[166] This has been suggested to be a potential bioremediation approach for seawater polluted by PET microplastics, although the conversion rate was very low and the application of genetically engineered microorganisms in an open environment is currently strictly forbidden worldwide.[9] Although a recent study has reported wild-type marine microbes with PET-metabolizing activity,[167] microplastics present in aquatic ecosystems can hardly support microbial growth and will therefore remain as persistent xenobiotics that cannot be remediated easily.[168]

Scientists have thus far succeeded in identifying and creating biocatalysts that appear to fit the need for PET waste depolymerization on an industrial and commercial scale. The fundamental understanding of interfacial biocatalysis on PET should be extended and transferred to address the challenges associated with the biotechnological degradation of other more abundant plastics such as polyolefins or more similar plastics such as polyamides (PA) and polyurethanes (PUR) with hydrolyzable backbones.[21,32,169] While the breakdown of carbon−carbon backbones in polyolefins can be energetically very challenging,[170,171] chemical and thermal pretreatments have been shown to enable subsequent biochemical transformation[172,173] and thus should be extensively studied in future research. On the other hand, the identification of putative PUR or PA hydrolases is envisioned as a result of collaborative large-scale research activities (e.g., the MIX-UP project[174] and the upPE-T project) on plastic recycling. A combination of these novel biocatalysts will pave a new path for the valorization of unsorted mixed plastic waste that cannot be efficiently recycled via other disposal approaches at an industrial scale.

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Notes

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