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Recent advances in enzyme engineering for improved deconstruction of poly(ethylene terephthalate) (PET) plastics



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In the last ~20 years, a multitude of natural enzymes have been discovered that can catalyze the breakdown of the common plastic poly(ethylene terephthalate) (PET). While enzymatic PET recycling is an attractive alternative end-of-life route for this waste plastic, the enzymes are not yet optimized for efficient and economical industrial use. Here, we discuss recent advances in engineering these PET-degrading enzymes, which include PET, bis(2-hydroxyethyl) terephthalate (BHET), and 2-hydroxyethyl terephthalic acid (MHET) hydrolases, toward industrially-relevant engineering goals. We place emphasis on trends from past efforts in rational and semi-rational design and emerging areas in directed evolution/high throughput screening and computational design for engineering these enzymes.

Many of the same properties that make plastics attractive in use—e.g., durability, non-reactivity, chemical, light, and thermal resistance—allow them to persist and accumulate in the environment¹. Coupled with low recycling rates² (i.e., high discard rates into landfills and the environment) and limited end-of-life pathways^{3–5}, plastic pollution has become a cause for global concern, with mounting recognition of its environmental and health effects^{6–8}.

Chemical recycling offers an alternative end-of-life pathway for plastics, in contrast to conventional, mechanical recycling^{9,10}. In mechanical recycling, old plastics can be reformed to new materials, but characteristically, with diminished properties, and with a limit on the number of times they can be mechanically recycled¹¹. Mechanical recycling typically involves the steps of: sorting, washing, shredding/grinding, and melting, followed by re-extrusion of new materials¹². In chemical recycling, plastics are broken down to constituent chemical species, either monomers or oligomers, which can then be used to create like-new plastics, or as feedstocks for plastics upcycling^{13,14}. Chemical recycling methods include glycolysis, methanolysis, pyrolysis, gasification, and hydrolysis^{12,13}.

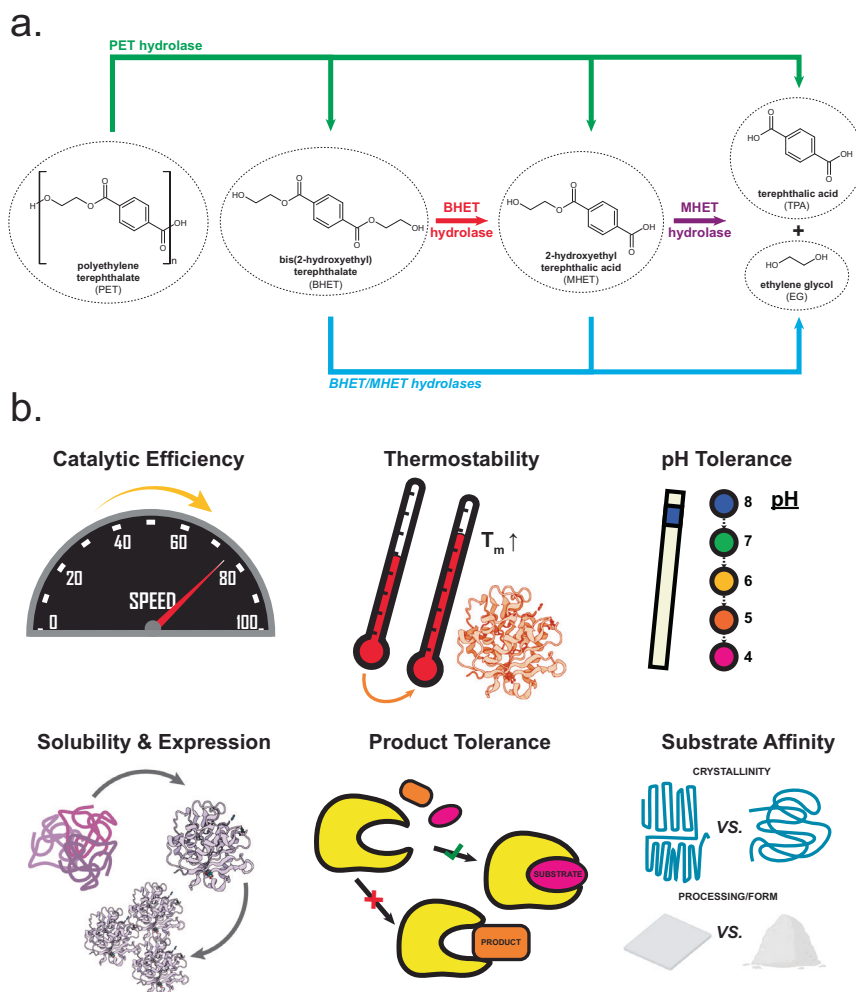
Enzymatic recycling (or biodegradation), via enzymatic hydrolysis, is a promising route for plastic recycling. Of particular promise is using enzymatic recycling for contaminated or colored plastic waste streams, as enzymatic recycling is more agnostic to feedstock quality vs. other methods, such as mechanical recycling^{15,16}. Thus, enzymatic recycling could complement other types of recycling by creating and expanding value in waste feedstocks. Recent years have witnessed the discovery of naturally-occurring enzymes capable of breaking down manmade plastics to their building block, chemical constituents^{17,18}. Hence, enzymatic recycling has gained increased attention, leading some to envision a biorefinery approach to

dealing with post-consumer plastic waste streams^{13,18}. Arguably the most successful and prolific efforts in enzymatic plastic recycling have been with poly(ethylene terephthalate) (PET)—common in single-use packaging and textiles, and one of the most-produced plastics—as the target^{17,19}. The first observations of enzymes (in this case, the thermophilic soil bacterium *Thermobifida fusca*²⁰, which can efficiently degrade plant cell walls²¹) degrading PET (Fig. 1a) were reported almost 20 years ago; since then, PET depolymerization with enzymes has increased orders of magnitude in efficiency and speed, from reactions taking several weeks to several hours¹⁹.

However, issues remain with enzymatic recycling. Natural enzymes are, generally, not yet optimized for industrial scale biodegradation^{17–19}. Several properties lacked by these enzymes that will be essential to their industrial adoption include: high catalytic activity, high substrate and product tolerances, high thermostability, high expression and solubility, and acidic pH tolerance (Fig. 1b).

Recent efforts have been made to relieve the shortcomings of these enzymes by protein engineering. In this Review, we first overview the state of industrial enzymatic PET recycling, to frame and motivate efforts in enzyme engineering. Next, we highlight the current state-of-the-art approaches used to engineer enzymes toward improved PET depolymerization efficiency, focusing on PET hydrolases (also called PETases), bis(2-hydroxyethyl) terephthalate (BHET) hydrolases (also called BHETases), and 2-hydroxyethyl terephthalic acid (MHET) hydrolases (also called MHETases) (Fig. 1a). Further, we discuss future opportunities for researchers, toward advancing protein engineering of these classes of enzymes past existing bottlenecks. We anticipate this summarization and guidance will facilitate continuing work in the field, toward improved, economically- and environmentally-competitive industrial enzymatic PET deconstruction and recycling.

Fig. 1 | How enzymes break down PET plastic and future goals for enzyme engineering. **a.** Reaction scheme for PET breakdown. Multiple classes of enzymes can participate in enzymatic breakdown of PET. PET hydrolases depolymerize PET to BHET, MHET, terephthalic acid (TPA), and ethylene glycol (EG), though may not effectively use intermediate products as substrates. BHET hydrolases, MHET hydrolases, and dual-function BHET/MHET hydrolases are active on intermediate substrates and may facilitate full conversion to TPA and EG products. **b.** Examples of protein engineering goals for enzymes involved in PET breakdown. Fulfillment of these engineering goals could improve the cost and environmental impact competitiveness of enzymatic PET degradation.



Prospects for industrial enzymatic PET recycling

Current approaches for chemical PET recycling

Viable chemical recycling approaches for PET include glycolysis, methanolysis, and enzymatic hydrolysis^{12,13}. As previous Reviews, such as the thorough analysis by McNeeley and Liu^{22,23} have thoroughly discussed current chemical recycling approaches, and are not the focus of this Review, we will provide only an abridged overview here. Briefly, glycolysis uses ethylene glycol (EG), in the presence of a catalyst, to break down PET to BHET, which can then be repolymerized to PET^{12,22}. Industrial glycolysis reactions are performed at 170–300 °C and 1–5 atm, with a typical solvent to PET ratio of 1:1 to 10:1 (w/w), for ~1–3 h, to recover ~60–90% BHET (of purities of 63–99%)^{12,22}. Methanolysis uses vaporized methanol, in the presence of a catalyst, to break down PET to dimethyl terephthalate (DMT) and EG, which can then be repolymerized to PET using a multi-step process¹². Industrial methanolysis reactions are performed at 240–300 °C and 10–200 atm, with a typical solvent to PET ratio of 2:1 to 8:1 (w/w), for ~1–10 h, to recover ~70–99% DMT^{12,22}. The Technology Readiness Levels (TRLs) of glycolytic and methanolytic PET depolymerization are 5 and 7, respectively¹². These are in comparison to mechanical recycling (typically used in municipal recycling programs) with a TRL of 9¹².

Enzymatic hydrolysis uses PET hydrolase enzymes (which have been discovered from a diversity of hosts and metagenomes^{24–28}) to hydrolyze the ester bonds in PET to yield MHET and EG (Fig. 1a). MHET can be further hydrolyzed by MHET hydrolases to yield the monomers, terephthalic acid (TPA), and EG (Fig. 1a). PET oligomers or small molecules, such as BHET, may accumulate as byproducts, requiring the use of other enzymes, such as BHET hydrolases, for complete hydrolysis (Fig. 1a). PET depolymerizations

with merely a PET hydrolase may directly yield TPA as a product and MHET may self-hydrolyze to TPA and EG. Enzymatic hydrolysis reactions are performed at 40–72 °C and 1 atm and a solvent (aqueous buffer) to PET ratio of 4:1 (w/w), for ~10–48 h, to recover 90% or more pure TPA^{12,22,29}. Enzymatic PET hydrolysis has a TRL of 4¹².

As stated, a thorough comparison of current chemical recycling approaches are not the focus of this Review. We refer readers to the work of McNeeley and Liu^{22,23}, who particularly highlight the specific unit operations and advantages and disadvantages of each process, and Uekert, Singh, and colleagues^{12,15,16}, who perform technoeconomic analyses (TEAs) and life cycle analyses (LCAs) on the processes and provide recommendations for improvement, particularly for enzymatic PET hydrolysis. However, briefly, enzymatic hydrolysis is attractive because it *i*) uses relatively mild process conditions, *ii*) the enzymes are selective for the PET ester, and *iii*) the product stream (predominantly TPA and some MHET) is of high purity and can be directly incorporated into polymerization processes. Further, this TPA is relatively easy to recover by crystallization²³. However, the enzymatic reactions require significantly longer residence times than other chemical recycling approaches, it is difficult to recover the EG product, and the process requires large amounts of water, base, and acid to ensure stability of the enzymes and precipitate products^{12,15,16,23}. These issues present motivation for improvements via protein and process engineering.

Process development for industrial enzymatic PET hydrolysis

In a proposed enzymatic PET recycling process by Singh and colleagues¹⁵, post-consumer PET (typically, as shredded flakes) is first pre-treated, where it is amorphized and its size is reduced, decreasing the polymer crystallinity

and increasing its surface area—together, increasing its ability to be broken down by the enzyme^{29–31}. The PET is then depolymerized in a stirred-tank, batch reactor, with the enzyme, in aqueous buffer¹⁵. Base addition (i.e., NaOH) is necessary to maintain the pH of the reaction, as generation of TPA causes acidification, while the optimal pH of many promising PET hydrolases is around 8 to 9^{24–26}. Following depolymerization, the hydrolysate is purified of remaining solids, enzymes, and other contaminants by passing through filters and columns. Acid (i.e., H₂SO₄) is then added to drop the pH to ~2.5 to precipitate the TPA, which can be passed through a continuous crystallizer, yielding high purity TPA. To recover the other reaction products, a base is added to neutralize the liquor, which then passes through a membrane unit. The retentate, which contains EG and the salt of the conjugate acid and base (i.e., Na₂SO₄) is then sent to a continuous crystallizer to recover the salt as a co-product for sale. The permeate is sent to wastewater. Finally, the EG is recovered by distillation of the EG/water liquor (with some distilled water sent to recycle in the depolymerization reactor)¹⁵.

The modeled process by Singh, et al., envisioned using a set of seven 950 m³ reactors at 60 °C and pH 8 with 5 mg enzyme per g of PET with 15% PET solids loading for 96 h to achieve a depolymerization of 90%¹⁵. Here, 1 kg of PET (consuming 0.17 kg H₂O) yields 0.78 kg TPA and 0.29 kg EG (with 0.1 kg leftover PET), with the conceptual plant able to process ~50 million kg of PET per year. This is the approximate capacity of the PET biorecycling plant currently under construction by Carbios³², with Carbios currently operating a demonstration plant with a 20 m³ reactor able to process 2000 kg (~100,000 bottles) per cycle³³. Process parameters may change from those modeled by Singh, et al., depending on optimal enzyme conditions and emerging recommendations to increase economics. Currently, small-scale (lab- and pilot-scale) bioreactors in literature are run at 65–72 °C and pH 7–8 with 0.5–3 (typically 1) mg enzyme per g of PET with recommended PET loadings of 16.5–20% (w/v), altogether ideally achieving ≥90% depolymerization in ≤12 h^{31,34}. These lab-scale bioreactors are typically 250–500 mL^{29,31,34,35}, although could be miniaturized for inexpensive validation testing in ~30 mL bioreactors³⁶. Small-scale laboratory tests, meanwhile, are typically performed on the 100–1000 µL scale^{34,35}.

Process considerations for enzymatic PET recycling

Current and proposed pilot- and industrial-scale enzymatic PET recycling processes include PET pre-treatment steps^{15,29–31,37,38}. Post-consumer PET should be pre-treated to achieve a high efficiency of depolymerization. Pre-treatment involves steps for amorphization, whereby the crystallinity of the polymer is reduced, followed by steps to increase the specific surface area (SSA) of the PET^{22,30,37,38}, typically by size reduction (e.g., pelletization^{29,30} and/or micronization by cryo-grinding/milling^{15,30}) or foaming^{22,29,37}. Typical particle sizes are 200–500 µm^{17,29,34,35}, while pellets are <1 mm diameter^{34,38}. Increasing the surface area of the polymer increases enzymatic reaction rates³⁹, while amorphization is critical to overcoming PET's recalcitrance to enzymatic hydrolysis^{29,40}. Post-consumer PET is characteristically semi-crystalline—containing regions of highly-ordered, tightly-packed polymer chains^{40–42}—which impedes entry of enzymes and the hydrolysis reactant, water. While crystallinity is important for some PET material properties (such as gas permeability), it severely inhibits enzyme accessibility^{40,41}. The crystallinity of PET packaging can be ~1–6%⁴¹, although regions of PET bottles can be upwards of 34%⁴¹, while PET fibers from textiles can be ~16–40%⁴³. PET hydrolases are significantly more efficient in depolymerizing amorphous PET, in which the polymer chains are disordered and randomly-oriented^{17,29,42}. Amorphization is performed by melting the PET (above 260 °C) in an extruder, allowing the polymer chains to randomly disorder, then rapidly cooling it (below the glass transition temperature, T_g, ~70 °C) to preserve the amorphous structure^{30,40}. The amorphized polymer can then be pelletized, ground, and/or milled. Patents assigned to Carbios describe a process whereby PET, melted in an extruder, is mixed with a foaming agent—a pressurized gas, volatile liquid, or gas-releasing chemical—then quickly quenched in water to create a PET foam with high surface area and low crystallinity³⁷. A recent report describes a scalable melt fiber spinning system that rapidly extrudes and spools PET

fibers with increased surface area and decreased crystallinity that does not require active cooling⁴⁴.

Industrially-relevant enzymatic PET depolymerization reactions are typically run at temperatures of 65–72 °C^{17,29,30,45,46}. At these elevated temperatures, using thermostable enzymes, these reactions have been most optimal^{17,27,45,46}. This is, in part, due to the reaction temperature approaching the T_g, the temperature at which the polymer transitions from a hard and brittle state to one that is rubbery and viscous⁴⁷, of PET: in the range of 65–81 °C, although water-soaking PET has been reported to decrease the surface (interfacial) T_g to as low as 40 °C⁴⁸. Near the T_g, the polymer chain mobility is increased for enzymatic accessibility. However, increasing the temperature over T_g will induce crystallization, as polymer chains are able to reorder, which will impede significant PET hydrolysis^{17,29,45,46,49}. Tournier, et al., reported that incubating post-consumer PET at 72 °C for ~15 h increased its crystallinity from ~15% to ~40%, while this happened in less than 6 h at 75 °C and over 24 h at 70 °C²⁹. Thus in PET enzymatic hydrolysis, there is a competition between depolymerization and recrystallization, the bounds of which depend on varied factors including: temperature, enzyme loading, enzyme stability/deactivation, PET particle size or film thickness, polymer molecular weight, amorphization/extrusion parameters, and the availability of water/buffer^{45,48,49}. All things considered, the need for polymer chain mobility, enzyme activation and adhesion, and propensity for recrystallization, must be balanced and the optimal temperature, T_{opt}, may vary based on the enzyme and process conditions. Notwithstanding, the T_{opt} for many recent studies reporting high-performance enzymes is 68–70 °C^{29,31,35,50–53}.

Process innovations for improved enzymatic PET recycling

Process innovations have the potential to increase the efficiency and cost-competitiveness of enzymatic PET hydrolysis. Kaabel, et al., for instance, developed a moist-solid reactor system, whereby highly-crystalline, non-pre-treated PET flakes were added at a high (40%) solids loading to a reactor with enzyme and minimal buffer, which were then mixed in a ball mill, then incubated for 7 days at 55 °C⁵⁴. They found high selectivity for TPA over MHET. In this process, TPA precipitates and no pH control is necessary. Overall, moist-solids reactions could offer a route to more environmentally benign enzymatic PET recycling¹⁶.

Enzyme immobilization has not seen widespread use with PET degradation, but could offer improved enzyme longevity by mitigating the effects of extreme temperature and pH and allowing enzymes to be reused^{16,55}. Major concerns are limited substrate accessibility and mass transport to/from an immobilized enzyme and detrimental effects to the enzyme caused by conjugation. López-Teijeiro, et al., recently demonstrated the immobilization of a PET hydrolase in protein nanospheres, which allowed activity at a broader range of temperature and pH vs. the non-immobilized enzyme and reusability for up to 10 cycles, with little loss to enzyme activity⁵⁶. Fritzsche, et al., attempted a suite of immobilization strategies—fixation to solid carriers, formation of cross-linked enzyme aggregates, and attachment to stimulus-responsive polymers—and found that linkage to a pH-responsive polymer resulted in recyclability over 5 cycles with meager decreases in activity⁵⁷. Schwaminger, et al., additionally reported that the attachment of a PET hydrolase to an iron oxide nanoparticle through a His-tag allowed for magnetic separation and reuse over 10 cycles with up to 50% decrease in activity⁵⁸.

Likewise, the use of continuous reactor schemes has not seen significant use in enzymatic PET recycling, although continuous reactors generally offer superior yields, process control, scalability, and throughput vs. batch reactors. For industrial enzymatic PET recycling, a continuous reaction scheme could remove reaction products that drop reaction pH and/or inhibit enzyme hydrolysis, which could improve efficiency of reactions and reduce base additions required for pH maintenance. Toward continuous PET depolymerization, Barth, et al., demonstrated that using ultrafiltration to remove inhibitory PET depolymerization reaction

intermediates improved the hydrolysis of PET by 70% over 24 h compared to a standard batch reactor⁵⁹. Recently, Ayafor, et al., employed a membrane reactor for in situ product removal and showed over 2-fold improvements in PET depolymerization and TPA yields. However, this depended upon using a high enough enzyme loading that would significantly drop reaction pH and, further, they demonstrated the need for optimization of how much the reaction is diluted⁶⁰. As it is posited that at least some PET hydrolases first break down PET to small oligomers, then break down these soluble oligomers to monomers^{61,62}, removing oligomers from the reaction before full conversion can decrease yields of TPA⁶⁰.

Life-cycle and technoeconomic analyses of enzymatic PET recycling processes

Performing TEA, LCA, and socioeconomic impact analyses on their modeled enzymatic PET recycling process, Singh, et al., reported a minimum selling price (MSP) of \$1.93/kg for recycled TPA (rTPA), compared to virgin TPA (vTPA), which fluctuated in 2010–2020 from ~\$1–1.5/kg¹⁵. The key cost drivers for the process were feedstock cost and electricity, which was dominated by the pre-treatment steps for extrusion and cryo-grinding. Other major cost drivers included the costs of base and acid for hydrolysis reactor pH maintenance and TPA crystallization, as well as water/steam usage for EG recovery via distillation. Sensitivity analyses indicated that variability of the MSP of rTPA was most affected by solids loading and extent of conversion. Enzyme loading, enzyme cost, and reaction residence time were less impactful. In the most demonstrative case, a process without the need for pre-treatment steps would reduce the MSP by 12%. With that said, Singh, et al., reported the rTPA process would have up to an 83% lower energy use and produce up to 43% less greenhouse gases (GHGs) and an economy-wide assessment showed the process would generate 45% more socioeconomic benefits and reduce environmental impacts by 95%, all compared to vTPA.

A follow-up in-depth LCA by Uekert, et al., using an altered methodology and more comprehensive system boundaries and parameters, however, found that the modeled rTPA process and rPET process (involving the closed-loop manufacturing of PET using rTPA) were generally worse than their virgin counterparts (excepting fossil fuel use and ecotoxicity), with up to 17-fold higher impacts to analyzed natural environment, natural resource, and human health metrics¹⁶. Similar to the study by Singh, et al., major impact drivers were base consumption (for pH maintenance) and electricity, water, and acid use^{15,16}, as well as collection and shredding of PET and discarding of waste (including unconverted PET)¹⁶. Sensitivity analyses indicated that variability of the impacts were most affected by solids loading, PET depolymerization extent, sorting yield, and TPA recovery, with enzyme loading, pH, residence time, and temperature having lesser effects¹⁵.

A recent study by Murphy, et al.⁶³, motivated by the shortcomings of the rPET process identified by Singh, et al.¹⁵, and Uekert, et al.¹⁶, showed that several process changes to the original process modeled by Singh, et al.¹⁵, could decrease the MSP of rPET to \$1.51, compared to a 5-year U.S. average of \$1.87 for vPET. An LCA of the altered process also showed reductions in GHG emissions, ecotoxicity, fossil resources use, and water use compared to the original rPET process and vPET⁶³. Namely, Murphy, et al., modified (and experimentally-validated) the process by: implementing pre-treatment to be extrusion followed by rapid quenching and palletization, rather than extrusion and cryo-milling; using a fed-batch reactor scheme, where enzyme and PET were periodically added to existing reactor liquor, to concentrate EG prior to distillation, enabling economical EG recovery; using mechanical vapor recompression in the distillation; and using NH₄OH, rather than NaOH, for base additions, forming (NH₄)₂TPA, which can be thermally cracked to NH₃ and TPA, eliminating the need for stoichiometric acid/base additions. The fed-batch scheme also caused the precipitation of TPA salts in the depolymerization reactor, allowing for easier separation⁶³.

Engineering PET hydrolases

Design goals for PET hydrolase engineering

Most of the work in protein engineering toward enzymatic PET depolymerization has focused on PET hydrolases (Fig. 1a). Informed by process

goals and challenges and lessons learned from TEA/LCA^{15,16} (*vide supra*), key engineering goals for PET hydrolases include: increased activity, thermostability, expression/solubility, and improved pH and substrate/product tolerance (Fig. 1b)^{17,19,31,35}. Meeting these goals will allow for efficient depolymerization in reactions with high PET loadings to high extents, while minimizing enzyme and waste costs, which have been identified as cost and impact drivers of modeled processes. An ideal PET hydrolase would exhibit high thermostability, allowing for longevity in reactions and potential reuse, and could hydrolyze varied substrate types and forms, including semi-crystalline PET and un-treated/minimally-treated post-consumer PET, e.g., as shredded flakes. Resistance to product inhibition would increase depolymerization efficiency while also facilitating new process innovations, such as fed-batch reactions, while stability and activity at a range of pH, down to acidic pHs, could dramatically improve process costs by reducing the needs for pH maintenance.

Rational design strategies

A selection of the rationally-designed PET hydrolases discussed in this Review are shown in Table 1, while a structural depiction of the rational design strategies that have been used to engineer PET hydrolases is shown in Fig. 2a. Additionally, an overview of key PET hydrolase functional domains and residues is shown in Fig. 3. Regions are classified according to Richter, et al.⁵⁰. Subsite I refers to residues that directly interact with the substrate and residues that comprise a second interaction sphere that influences the interacting residues. Subsite II is suggested to contribute to PET binding and guidance toward the active site⁵⁰.

Economically viable PET hydrolases must be thermostable at high temperatures for long reaction times (Fig. 1b), but not at the expense of enzyme activity. Efforts have been made to increase enzyme thermostability of varied natural PET hydrolase scaffolds, with the goal to confer wild-type PET hydrolases with increases in melting temperature (T_m)^{17,19,27}. A recommended guideline is the enzyme having a T_m that is at least 10 °C higher than the reaction temperature¹⁹. A rational design strategy has been used in several scaffolds successfully—based off early work in *Thermobifida fusca* (*T. fusca*) cutinase II (*TjCut2*), where Ca²⁺ binding sites were modified to install salt bridges⁶⁴ or disulfide bonds⁶⁵ (Fig. 2a)—to increase T_m by up to 25 °C. Analogous salt bridges and disulfide bonds have been created in homologous PET hydrolases based on existing metal binding sites, in *Ideonella sakaiensis* (*IsPETase*)^{41,52}, leaf-branch compost cutinase (LCC)^{29,41}, Polyester Hydrolase Leipzig #7 (PHL7)^{50,51}, and others^{66,67}. Son, et al., pursued structure-guided rational engineering to target a flexible, high B-factor region away from the active site, of *IsPETase* (Fig. 2a). The S121E/D186H mutations designed to stabilize the loops in the region were found to result in a T_m increase of over 7.2 °C and up to 6-fold higher activity than wild-type. The mutations R280A, S121E, D186H compose the mutant ThermoPETase⁶⁸.

Fusion of polymer binding domains to PET hydrolases have been used as a means to increase enzyme activity, by increasing the rates of enzyme adsorbing to the hydrophobic polymer. This has been extensively reviewed by Wei, et al.¹⁹, and Tournier, et al.¹⁷. Natural and engineered cellulose-binding domains have been fused to the C-termini of PET hydrolases (Fig. 2a) for increased degradation activities^{69–71}. However, at higher, more industrially-relevant PET solids loadings, in levels past the adsorption-limited regime, the beneficial effects of the cellulose-binding domain were observed to be diminished by Graham, et al.⁷². Thus, further exploration is necessary to determine if domain fusions are necessary to increase the activity of PET hydrolases in industrial settings. However, tethering of *IsPETase* to *Ideonella sakaiensis* MHETase (*IsMHETase*) to maintain the proximity of the enzyme active sites (Fig. 2a) showed significantly improved catalytic turnover vs. the simple addition of both enzymes separately to the same reaction⁷³. Fusions with hydrophobins^{74,75} and zwitterionic peptides⁷⁶ (Fig. 2a) are two additional methods shown to be successful in increasing substrate affinity.

Tuning enzyme surface properties has been proposed as an additional solution to enhancing PET hydrolysis, via improved association with the hydrophobic, noncharged polymer^{17,19}. Creation of hydrophobic patches

Table 1 | Exemplary engineered PET hydrolases discussed

Enzyme Name	Mutations	Performance	Reaction Conditions/Substrate	Engineering Method	Citation
ThermoPETase	/sPETase S121E/D186H/R280A	13.9-fold more monomers released vs. /sPETase	72 h rxn, 40 °C, 6 mm dia. crystalline PET, 500 nM enzyme, pH 9 (small scale)	Rational design	Son, et al. ⁶⁸
TS-PETase	ThermoPETase + N233C/S282C	Estimated 6.8-fold higher A_{560} vs. /sPETase	72 h rxn, 30 °C, 0.63 cm ² amorphous PET, 400 nM enzyme, pH 9 (small scale)	Rational design (disulfide bond)	Zhong-Johnson, et al. ¹¹⁹
HotPETase	ThermoPETase + N233C/S282C + 16 other mutations*	~4-fold more monomers released vs. ThermoPETase	48 h rxn, 40 °C, 0.4% crystalline PET, 0.29 mg enzyme/g PET, pH 9.2 (small scale)	Semi-rational design of mutational hotspots	Bell, et al. ⁸²
/sPETase-W159H/S238F	/sPETase W159H/S238F	~4% more monomers released vs. /sPETase, 4% higher crystallinity change	96 h rxn, 30 °C, 6 mm dia. crystalline PET, 50 nM enzyme, pH 7.2 (small scale)	Rational design	Austin, et al. ⁸²
DuraPETase	/sPETase + 10 mutations** (including W159H and R280A)	260-fold more monomers released vs. /sPETase	72 h rxn, 50 °C, 8 mm dia. crystalline PET, 0.01 mg/mL enzyme, pH 9 (small scale)	Computational GRAPE ³⁰ strategy	Cui, et al. ⁹⁰
/sPETase-W159H/F229Y	/sPETase W159H/F229Y	40-fold more monomers released vs. /sPETase	24 h rxn, 40 °C amorphous PET floccule, 1 mg/mL enzyme, pH 9.5 (small scale)	Computational Premuse ¹⁰⁷ tool	Meng, et al. ¹⁰⁷
FAST-PETase	ThermoPETase + R224Q/N233K	38-fold more monomers released vs. ThermoPETase	96 h rxn, 50 °C, 6 mm dia. amorphous PET, 200 nM enzyme, pH 8 (small scale)	MutCompute ¹¹ ML algorithm	Lu, et al. ⁴¹
DopoPETase	/sPETase + 7 mutations*** (including D186H and N233K)	1,400-fold more monomers released vs. /sPETase	48 h rxn, 50 °C, 6 mm dia. amorphous PET, 0.5 mg/mL enzyme, pH 9 (small scale)	Directed evolution (error-prone PCR)	Shi, et al. ¹²²
LCC-ICCG	LCC Y127G/D238C/F241I/S283C	1.6-fold higher depolymerization vs. LCC	24 h rxn, 72 °C, 20% pre-treated post-consumer PET, 1 mg enzyme/g PET, pH 8 (bioreactor)	Rational design (disulfide bond) + semi-rational design	Tournier, et al. ²⁸
LCCICOG _{16M}	LCC-ICCG + S32L/D187/S98R/T157P/E173Q/N213P	~2.4-fold more monomers released vs. LCC-ICCG	24 h rxn, 75 °C, post-consumer crystalline PET powder, 5 µM enzyme, pH 8 (small scale)	Optimal temperature ML algorithm ¹⁰⁸ + evolutionary analysis ¹⁰⁹	Ding, et al. ¹¹⁰
LCC-YGA	LCC S29W/L124G/H183Y	~1.15-fold more monomers released vs. LCC-ICCG	24 h rxn, 70 °C, 6 mm dia. amorphous PET, 500 nM enzyme, pH 8 (small scale)	Cross-correlation-based accumulated mutagenesis (CAM) ¹¹¹ method	Zheng, et al. ¹¹¹
LCC-RIP	LCC-ICCG + A59R/V63I/N248P	~2-fold more monomers released vs. LCC-ICCG	7 d rxn, 85 °C, pieces of reinforced PET film, 0.01 mg/mL enzyme, pH 8 (small scale)	Rational design	Zeng, et al. ⁹¹
LCC-A2	LCC-ICCG + H218Y/N248D	~1.1-fold higher depolymerization vs. LCC-ICCG	8 h rxn, 72 °C, 20% pre-treated post-consumer PET, 3 mg enzyme/g PET, pH 8.5 (bioreactor)	Affinity analysis based on dynamic docking (ADD) ¹⁰⁶ method	Zheng, et al. ¹⁰⁶
LCC-ICCG-H218Y	LCC-ICCG + H218Y	2.6-fold more monomers released vs. LCC-ICCG	6 h rxn, 70 °C, 8 mg amorphous PET disks, 500 nM enzyme, pH 8 (small scale)	Directed evolution (error-prone PCR)	Cribari, et al. ¹³³
LCC-LANL	LCC-ICCG + P38L/Y61C/M91I/L117P/A149V/H218Y/Q224H/S247L/T256I	1.11-fold higher depolymerization vs. LCC-ICCG	48 h rxn, 68 °C, 16.5% amorphous PET coupons, 1 mg enzyme/g PET, pH 8 (bioreactor)	Directed evolution (DNA shuffling)	Groseclose, et al. ³⁵
TurboPETase	BhrPETase + H218S/F222I/A109R/D238K/A251C/A281C/W104L/F243T	1.36-fold higher depolymerization vs. LCC-ICCG	8 h rxn, 65 °C, 20% pre-treated PET powder, 2 mg enzyme/g PET, pH 8 (bioreactor)	Protein language model + GRAPE ³⁰ strategy	Cui, et al. ¹⁰²
PES-H1-L92F/Q94Y	PHL7 L93F/Q95Y	2.9-fold more monomers released vs. PHL7; 2.2-fold more vs. LCC-ICCG	24 h rxn, 72 °C, amorphous PET powder, 1 mg enzyme/g PET, pH 8 (small scale)	Rational design	Pfaff, et al. ⁵¹
PHL7-Jemez	PHL7 A35V/Q95Y/T121/Q175E/H185N	3.7-fold higher depolymerization vs. PHL7	48 h rxn, 65 °C, 20% amorphous PET coupons, 1 mg enzyme/g PET, pH 8 (bioreactor)	Directed evolution (DNA shuffling)	Groseclose, et al. ³³
TfCut2 L32E/S113E/T237Q	TfCut2 L32E/S113E/T237Q	5.3-fold more monomers released vs. TfCut2	48 h rxn, 65 °C, 1% crystalline PET powder, 1 µmol/L enzyme, pH 8 (small scale)	MutCompute ¹¹ ML algorithm	Meng, et al. ¹⁰⁴
Kubu-P ^{M12}	Kubu-P with 12 mutations****	~2.6-fold higher depolymerization vs. LCC-ICCG	48 h rxn, 70 °C, 30% amorphized PET powder, 0.58 mg enzyme/g PET, pH 8 (bioreactor)	Landscape profiling ¹¹² + rational design	Seo, et al. ¹¹²

P181V/S207R/S214Y/Q119K/S213E/R80T/Q182M/N212K/R224L/S58A/S61V/K95N/M154G/N241C/K252M/T270Q.

**S214H/I186R/W159H/S188Q/R280A/A180I/G165A/A119Y/L117F/T140D.

***T88I/D186H/D220N/N233K/N246D/R260Y/S290P.

****T95R/T119N/E131Q/Q127S/A173C/V184I/K197C/D190H/D239S/A236C/S281C/A279S.

The name and corresponding mutations are given, along with the performance of that enzyme variant vs. relevant benchmarks and the conditions at which the performance was measured. The method of engineering for each variant is included. Fold change is calculated based on the performance of the variant divided by that of the benchmark enzyme. Monomers released indicates analysis by HPLC quantification, while depolymerization indicates that measurement was performed by calculating extent of reaction based on the stoichiometry of base titration required to maintain pH through the reaction in a bioreactor.

near the active site by, for example, truncating one of the termini (Fig. 2a), has been demonstrated successfully, even conferring observable activity when the wild-type enzyme showed none⁷⁷. Concerning surface electrostatics, a study of two highly related PET hydrolases, Thc_Cut1 and Thc_Cut2, revealed that mutating a residue away from the active site from a charged to a noncharged residue (Fig. 2a) resulted in increased hydrolysis⁷⁸. Considering that, during PET enzymatic hydrolysis, the polymer becomes locally negatively charged due to anionic carboxyl groups, the use of surfactants have been proposed as a way to mitigate charge repulsion between the polymer and enzyme⁷⁹. However, from a purely protein engineering perspective, introduction of positively charged residues (Fig. 2a) can increase the binding constant of the enzyme to PET, as demonstrated in PET2, a thermostable enzyme found in a metagenomic library⁶⁷.

Alteration of the binding cleft (Figs. 2a and 3) has been shown to affect the catalytic properties of PET hydrolases. In *Fusarium solani* cutinase (FcC), enlarging the active site (Fig. 2a) was shown to have 4- to 5-fold increases in activity on PET fibers compared to wild-type⁸⁰. In IsPETase, conversely, structure-guided mutagenesis of residues to narrow the opening of the substrate binding site were shown to decrease activity⁸¹. However, narrowing of the binding cleft (Fig. 2a) also in IsPETase by introducing a set of mutations, S238F/W159H to mimic *T. fusca* cutinase, was shown by Austin, et al., to increase both catalytic activity and reduction in crystallinity of the substrate⁸². The reduction in crystallinity is presumed to be due to surface erosion and pitting of the plastic by the enzyme, which disrupts ordered crystalline structures, leading to a relative increase of amorphous regions⁸². Notwithstanding, this finding by Austin, et al., is perhaps contrary to the thought that a larger binding cleft may allow funneling of larger polymer chains, but was explained through more favored aromatic interactions of the substrate with F238, whereby the polymer is better positioned and distanced from the catalytic triad (Fig. 3c)⁸². Hence, activity could decrease if substrate affinity decreases at the expense of larger binding cleft. In LCC and TjCut2, there were likewise increases in activity from mutation of the residue analogous to S238^{29,83}. Joo, et al. observed, in IsPETase, that polar R280, in the binding cleft, may inhibit large-chain substrate binding and saw that making the R280A mutation increased activity over 30% compared to the wild-type⁸⁴. Similarly, in IsPETase, W185 can wobble, adopting multiple conformations to facilitate ligand-induced opening and widening of the substrate-binding groove, permitted by the small side chain of S214^{82,84,85}. Mutation of this Ser to a His (i.e., S214H) dramatically decreased activity⁸⁵—corroborated by the observation that the equivalent His residue in homologs packs against the Trp residue, preventing its rotation⁸⁵. However, the equivalent residue to IsPETase S214, in PHL7, H185, when mutated to a serine (i.e., H185S) resulted in a dramatic decrease of protein stability and activity⁵⁰. Mutation to an asparagine, meanwhile (i.e., H185N) resulted in increased activity⁵³. Efforts to transplant the mechanism of the wobbling W185 in mesophilic IsPETase to thermophilic LCC and TjCut2 by mutation of the surrounding, smaller residues mimicking IsPETase S214/I218 (i.e., creating LCC-H218S/F222I) (Fig. 2a) showed that activity was increased at low temperatures, but not at higher temperatures⁸⁶. With that said, transferal of the unique IsPETase S214/I218 to several other PET hydrolases which natively have His/Phe at the analogous sites were reported to enhance activity for those that function at less than 60 °C⁸⁶. It is postulated that the unique Ser/Ile in heat-labile⁶⁸ IsPETase contributes to the flexibility of the substrate-binding pocket, allowing improved binding to PET, although this flexibility may be detrimental at higher temperatures⁸⁶. Taken together, this indicates that key residues, located proximal to the PET active site and suspected to play important roles in the mechanism of PET binding and catalysis, may not yet be optimized for PET degradation, and it may be difficult to map homologous residues across different scaffolds (notably, between mesophilic vs. thermophilic enzymes). Each enzyme scaffold may require its own specific tuning.

Additionally, there exist tradeoffs in altering the substrate binding cleft size and affinity. While a larger substrate binding cleft (Fig. 3c) may, for example, increase catalytic rates via improved PET substrate binding, it may also affect specificity, affinity, and/or stability. For example, the I224A

mutation in Cut190*, attempting to improve substrate binding by affecting hydrophobicity, was shown to increase activity (higher $V_{\max}$ and k_{cat}) but decrease affinity (higher K_M) toward the PET model substrate poly(butylene succinate-co-adipate) (PBSA)⁸⁷. The I224A/Q138A double mutant showed a further increase in K_M and a lower $V_{\max}$ than the I224A single mutant, indicating potentially excessive space near the active site, at the detriment of activity and affinity. Molecular modeling showed that the I224A and Q138A mutants indeed had lower binding energies and increased binding surface areas compared to wild-type Cut190*⁸⁷. Conversely, binding affinity that is too high can impede enzyme activity by the Sabatier principle: if the enzyme complexes too tightly to the PET interface and cannot dissociate, there is a cost to turnover⁸⁸. Arnling Bååth, et al., found that LCC and TfC may bind PET too tightly for maximum activity and turnover could be increased by adding small amounts of surfactant. Presumably, mutations that enhance binding affinity could decrease activity; an intermediate binding affinity could be optimal⁸⁸. On the other hand, enhanced activity and thermostability could be at the expense of one another. The Y87E mutation in IsPETase, which was made to a loop adjacent to the binding site, showed a 12 °C increase in T_m , but the mutant showed practically no enzyme activity, suggested to be due to unfavorable structural rigidity⁸⁹. As discussed, increasing the flexibility of the binding cleft may decrease stability at high temperatures⁸⁶. Increased affinity may more directly correlate with thermostability, as PET hydrolases are thought to be more stabilized upon substrate binding, as aromatic residues around the catalytic site associate with aromatic PET moieties^{84,85}. Substrate selectivity could also be affected through altering substrate binding cleft size and affinity (*vide infra*).

Examining the binding of a model substrate to LCC using molecular docking and enzyme contact-surface analysis, Tournier, et al. identified 15 residues in the first contact shell, near the active site (Fig. 3a, c). Upon mutating 11 of the residues using site-saturation mutagenesis, it was found that most mutations either decreased activity, or did not increase it more than 48%²⁹. However, several variants were discovered with increased PET depolymerization activity or increased T_m . Several of these mutations, T96M, Y127G, F243I, F243W (Fig. 3a), were combined with a rationally-designed disulfide bond, D238C/S283C (Fig. 3a), to create a handful of high performance PET hydrolases, including LCC-ICCG²⁹. Other PET hydrolase scaffolds have been subjected to mutations of their analogous residues, showing significant increases in catalytic activity. For example, the PHL7-L93F/Q95Y variant, which showed over a 2-fold increase on PET substrates over wild-type, was created by mutation at these hotspots⁵¹, as have other PHL7 variants⁵⁰, to resemble LCC-ICCG²⁹ or DuraPETase⁹⁰ (which is discussed below). Such mutagenesis is typically a good starting point for improving activity, especially considering that PET hydrolases generally share high structural and sequence homology (Fig. 3b) and minimal knowledge of enzyme structure is required. In a recent study, Zeng, et al., used the structure of LCC-ICCG co-crystallized with a PET analog to guide the engineering of hydrophilic interactions on the protein surface and internal hydrophobic interactions to yield LCC-RIP, increasing T_m to nearly 99 °C and activity by up to 100% vs. LCC-ICCG in reactions with reinforced PET over a week at 85 °C⁹¹. However, it is questionable whether reaction at such high temperatures is advantageous; highly-active enzymes would be required to outcompete presumably rapid re-crystallization at 85 °C.

Product (i.e., MHET, BHET, TPA, EG) accumulation has been shown to inhibit depolymerization in PET hydrolase scaffolds including IsPETase⁹², TjCut2⁹³, FcC⁹⁴, and PHL7⁵³ and will likely impede industrial use of these enzymes³¹ (Fig. 1b). Rational design of the substrate binding site to lower affinity of breakdown products has been proposed as one method to relieve inhibition. Wei, et al., saw up to a 2.7-fold increase in activity over wild-type by exchanging binding site residues in TjCut2 with those of LCC, whereby the G62A mutation was made. From molecular modeling, it was found that the G62A decreased the binding strength interaction (Fig. 2a) between the residue and the PET dimer by 5.5-fold⁹⁵. Creation of tethered chimeras of PET hydrolase with an enzyme able to efficiently break down intermediates has been shown to increase depolymerization greater than the two enzymes alone (Fig. 2a), as discussed^{73,96}, potentially, in part, due to

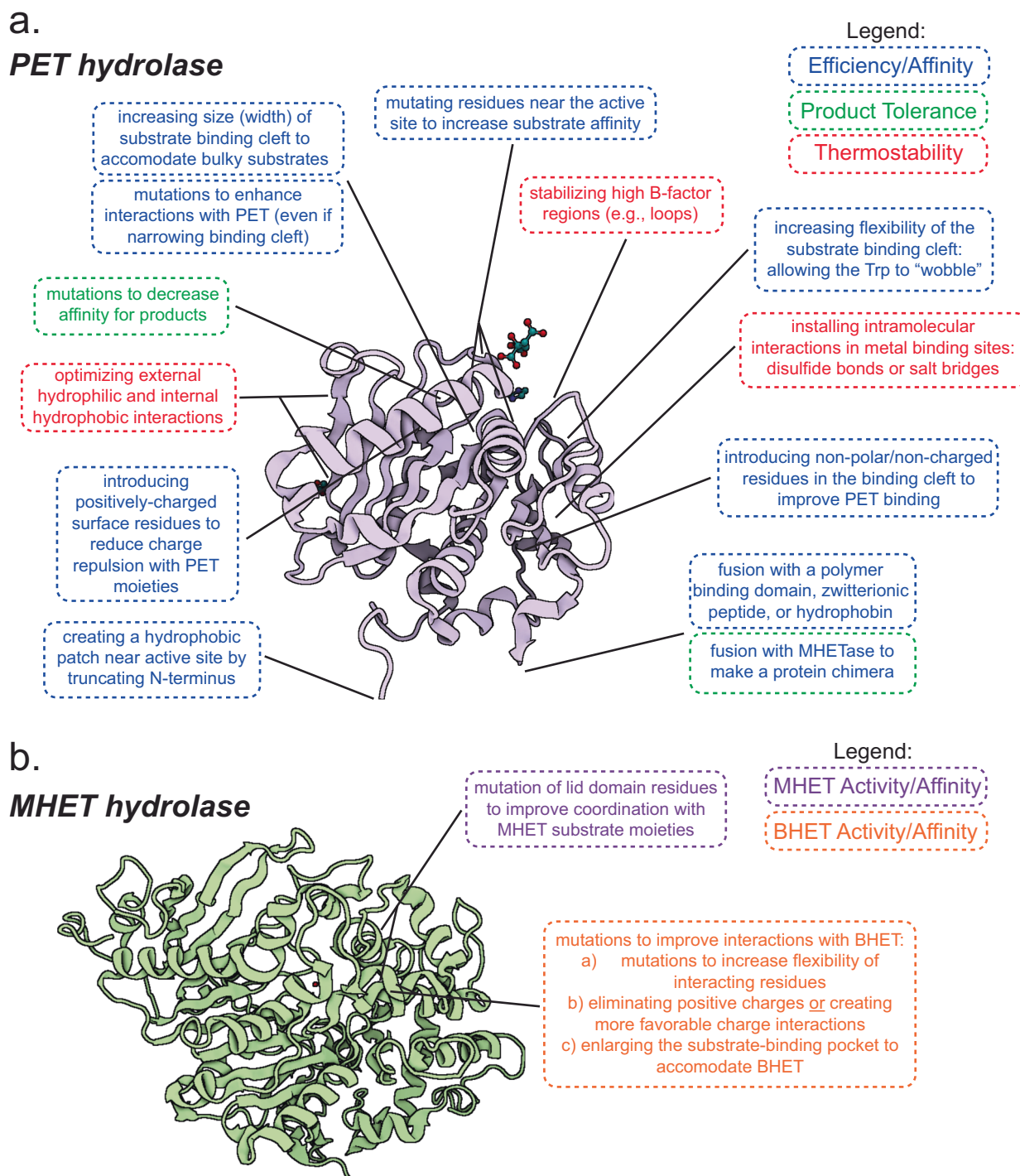


Fig. 2 | Strategies used in rational engineering of PET degrading enzymes. Strategies are selected from those discussed in this Review for engineering. **a** PET hydrolases and **b** MHET hydrolases. Modeled structures are shown only to be representative, with lines indicating approximate locations on the enzyme scaffold (or analogous location mapped from homologous scaffold) where mutation(s)

would be made. The modeled PET hydrolase is LCC-ICCG (PDB: 6THT³⁹) and the modeled MHET hydrolase is IsMHETase (PDB: 6QZ4⁷³). Color(s) of boxes and text indicate the design goal the mutation(s) aim to achieve; two colors indicate the mutation(s) may affect multiple properties.

relieved substrate inhibition via rapid elimination of inhibitory intermediates. In another study, Avilan, et al.⁹⁷, discovered that the IsPETase W159H/S238F variant created by Austin, et al.⁸², showed relieved product inhibition compared to wild-type, with the suggestion that these mutations may stabilize a key loop, and thereby reduce the flexibility, where the active site (catalytic) histidine is located (Fig. 3a). The IsPETase HotPETase variant⁵² shares this relieved product inhibition, potentially due to fewer cavities and decreased flexibility near the active site⁹⁷. Hence, Avilan, et al.,

argued that increasing enzyme thermostability may also help to relieve product inhibition⁹⁷.

Emerging computational design approaches

Computationally-aided design has recently made significant headways into improving PET hydrolases toward a variety of design targets. Here, we aim to highlight several recent methods that function by a range of diverse approaches. A selection of the computationally designed PET hydrolases

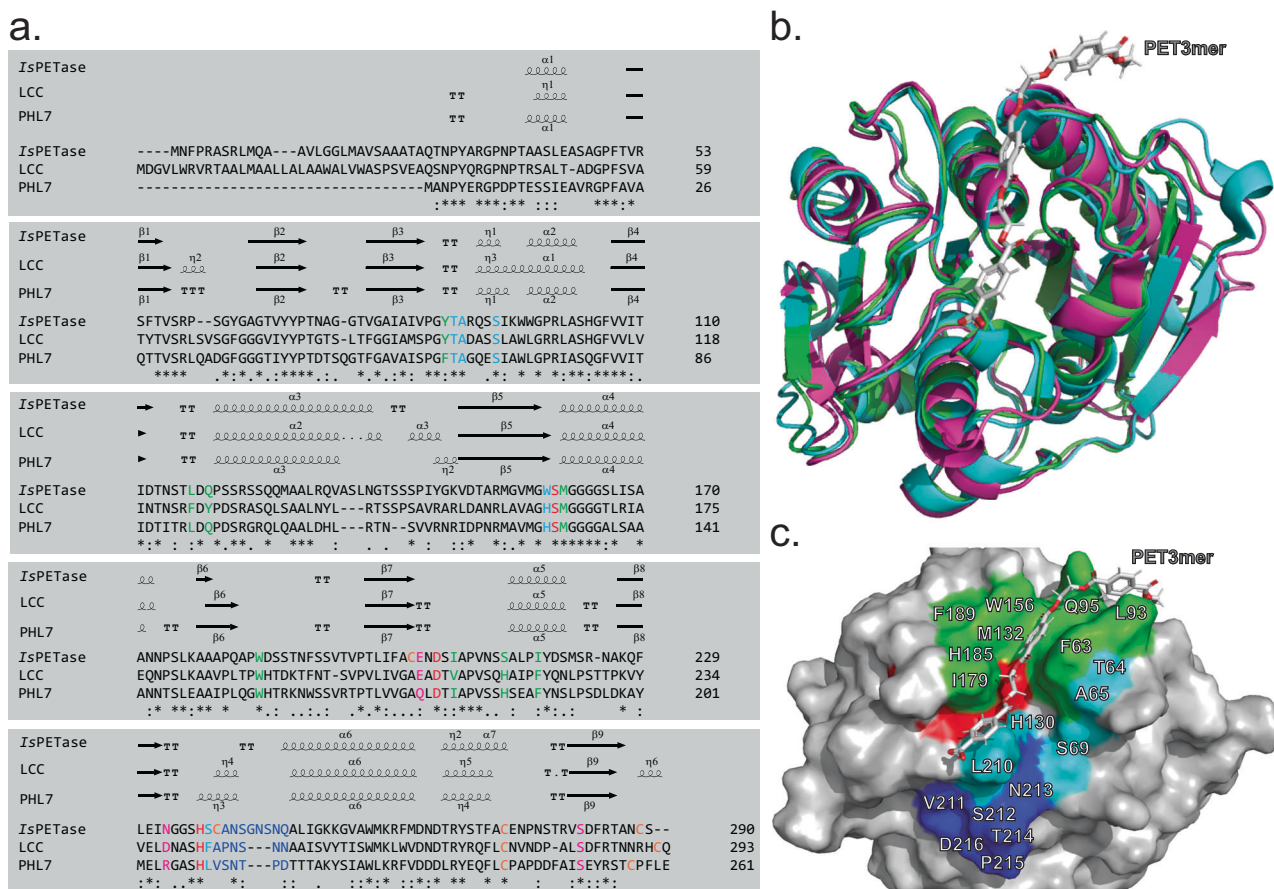


Fig. 3 | Sequence and structural analysis of PET hydrolases. **a** Annotated multiple sequence alignment (MSA) of IsPETase²⁴, LCC²⁵, and PHL7²⁶. In each panel, an MSA is shown for the 3 protein sequences performed by Clustal Omega¹⁵⁷ on the EMBL-EBI Webserver. The 5 panels span the entire protein sequences, with residue numbers at the right. Above the MSAs are images showing the secondary structure, performed using ESPrnt 3.0¹⁵⁸, from the crystal structures for IsPETase (PDB: 5XJH⁸⁴), LCC (PDB: 4EB0²⁵), and PHL7 (PDB: 7NE1²⁶). Nomenclature for the MSA is standard: * denotes identical residues, : denotes conserved residues, and . denotes semi-conserved residues. Images for secondary structure represent: α -helices as numbered α /squiggles, 3_{10} helices as numbered η /squiggles, β -strands as numbered β /arrows, and β -turns as TT. Residues are colored as follows: orange for native cysteine residues (which are part of native disulfide bonds), green for residues corresponding to subsite I (classified by Richter, et al.⁵⁰), cyan for residues corresponding to subsite II (classified by Richter, et al.⁵⁰), red for the catalytic triad, blue for the extended loop region, and magenta for residues that could form disulfide bonds or salt bridges with other magenta residues, as discussed in the text. **b** Modeled structures of IsPETase (magenta), LCC (cyan), and PHL7 (green) overlaid and docked to the PET3mer. **c** A surface representation of PHL7 docked to the PET3mer, focusing on the substrate binding groove, with protein regions highlighted according to the colors scheme in (a) and residues labeled.

discussed in this Review are shown in Table 1 and an overview of the strategies is shown in Fig. 4.

Cui, et al., developed a methodology termed GRAPE⁹⁰ (Greedy Accumulated strategy for Protein Engineering) toward the goal of discovering the most efficient paths of accumulated mutations—namely, that avoid uncooperative epistatic effects, while identifying coupled synergistic effects (Fig. 4a). First, mutations predicted to be stabilizing in IsPETase were generated in silico (from ABACUS⁹⁸, FoldX⁹⁹, Rosetta_ddg¹⁰⁰, and Skyline32¹⁰¹) which were then down-selected to avoid any observed biophysical pitfalls (e.g., mutations creating hydrophobic surface residues). Potential candidates were then experimentally examined and mutations of variants that expressed well were clustered according to their positions and presumed effects, reducing the total number of combinations. A workflow then tested and accumulated mutations in each cluster before adding the mutations in other clusters, systematically combining mutations until the final variant, DuraPETase was discovered⁹⁰. DuraPETase had a 31 °C higher T_m and over 300-fold higher activity compared to wild-type on high-crystallinity PET powder⁹⁰. In a second study, Cui, et al., discovered TurboPETase¹⁰² (based on the BhrPETase scaffold¹⁰³) by first employing an artificial intelligence (AI) based language model with a Transformer encoder trained on datasets of homologous PET hydrolase sequences to predict amino acid variation probability across the evolutionary landscape.

Positions that the wild-type residues fit less well than substitutions were identified, mutated, and experimentally tested. After finding that some accumulated mutations beneficial for activity had decreased T_m , they employed GRAPE. TurboPETase achieved nearly complete depolymerization of 20% (w/w) PET in 8 h¹⁰².

Lu, et al., developed a self-supervised, structure-based machine learning (ML) 3D convolutional neural network, MutCompute (Fig. 4b)⁴¹. MutCompute was trained on 19,000 structures from the Protein Data Bank (PDB) to identify stabilizing mutations in the IsPETase/ThermoPETase scaffold based on those wild-type residues not optimized for their micro-environments. Predictions were ranked by predicted probabilities and mutations were combined stepwise, with variants that showed higher activity and thermostability explored further. After identifying four mutations which showed the highest improvements (S121E, T140D, R224Q, and N233K), they experimentally tested the combinations to discover the best variant, FAST-PETase (S121E, R224Q, and N233K)⁴¹. FAST-PETase had up to 38-fold higher activity compared to ThermoPETase⁴¹. Meng, et al., later employed MutCompute to identify residues in TjCut2 to increase hydrolytic activity and stability¹⁰⁴. Stepwise recombination and verification gave new enzyme variants with 2.9-fold and 5.3-fold improvements in activity on amorphous PET film and crystalline PET powder, respectively, and a T_m increase of 5.7 °C compared to the wild-type¹⁰⁴.

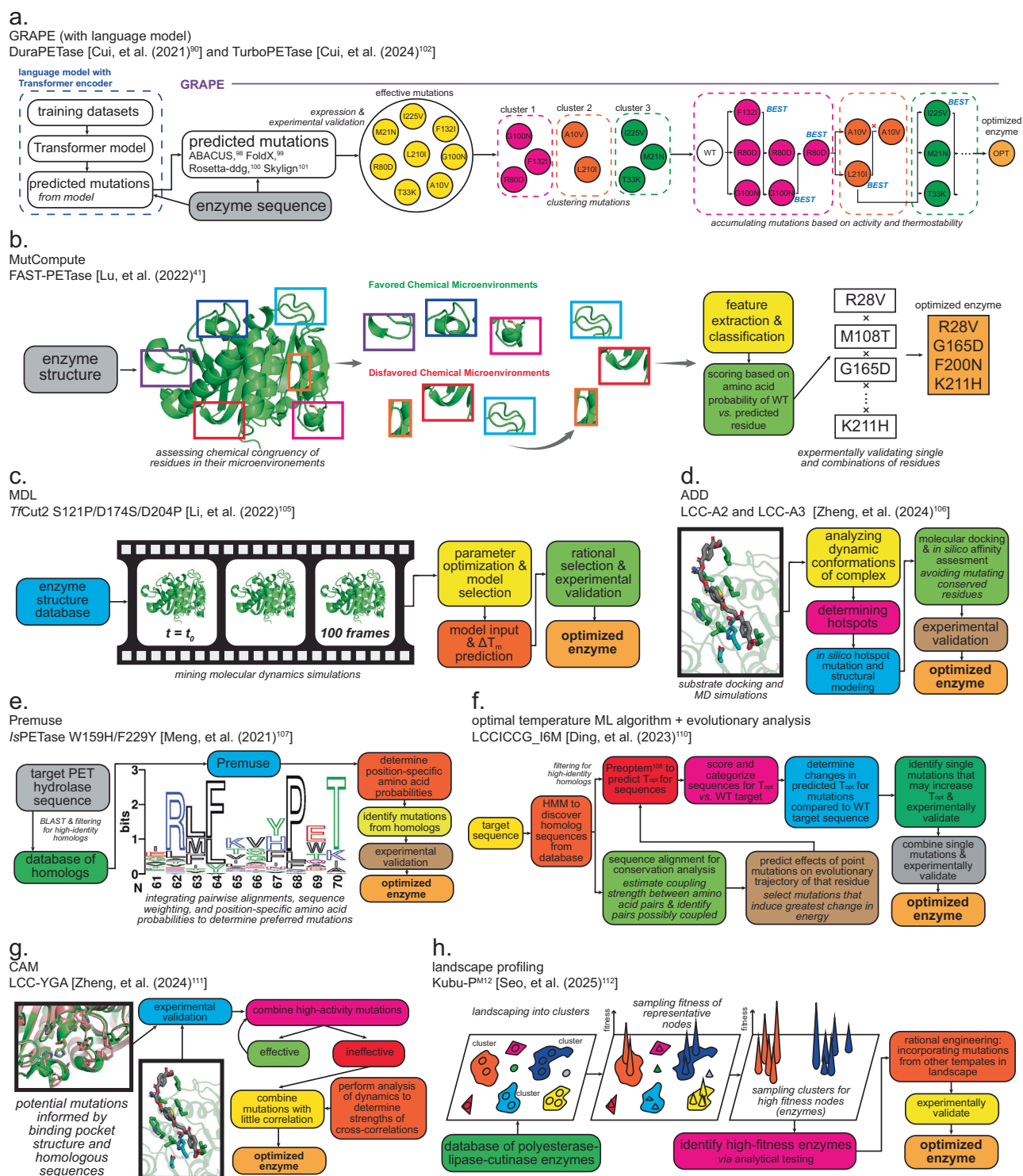


Fig. 4 | Overview of selected computational design methods. Any sequences, mutations, or structures are only for demonstration and are not specifically taken from the study in question. **a** GRAPE⁹⁰ method, which could feature a language model with a Transformer encoder prior to the GRAPE workflow¹⁰². **b** MutCompute⁴¹. The representative structure is LCC-ICCG (PDB: 6THT²⁹). **c** MDL¹⁰⁵. The representative structure

is LCC-ICCG (PDB: 6THT²⁹). **d** ADD¹⁰⁶. The representative structure is the PET 3mer docked to PHL7 (PDB: 7NEI²⁶). **e** Premuse¹⁰⁷. The WebLogo was based on ref. 159. **f** Optimal temperature ML and evolutionary analysis algorithms¹¹⁰. **g** CAM¹¹¹. The representative structures are from LCC-ICCG²⁹ and PHL7²⁶ (overlaid) and PHL7 docked to the PET 3mer. **h** Landscape profiling method¹¹².

Qi and colleagues used ML methods to mine molecular dynamics (MD) trajectories in a method they termed MDL (Fig. 4c) to engineer TfCut¹⁰⁵. They used MD to simulate the structural changes in the enzyme at reaction conditions, coupled with ML, to analyze relationships between the enzymes' characteristics and their thermostability. After verifying that the MD simulations provided sufficient structural features for ML algorithm

input—from negative and positive data—and the regression results performed well, they predicted variants with improved thermostability. Their TfCut2 variant had a T_m that was 9.3 °C higher and an activity that was increased by over 45-fold compared to wild-type¹⁰⁵. They then extended this idea to develop a PET ligand affinity analysis based on dynamic docking (termed ADD) workflow (Fig. 4d) to improve the activity of LCC-ICCG over 3

rounds of engineering. Their top variants were named LCC-A2 and LCC-A3¹⁰⁶. ADD involves analyzing the ligand affinity energy of enzymes associating with PET chains by molecular docking and examining dynamic protein conformations to determine hotspot residues for mutation. A hotspot residue would have longer duration of binding to the substrate under dynamic conformational sampling¹⁰⁶.

The Premuse tool was designed to integrate sequence alignments and quantitative selection of consensus-preferred mutations based on natural sequence evolution (Fig. 4e)¹⁰⁷. Using pairwise sequence alignments and calculated position-specific amino acid probabilities, the IsPETase W159H/F229Y variant was identified, which was conferred with over a 10 °C increase in T_m compared to the wild-type enzyme¹⁰⁷. In another attempt to engineer LCC-ICCG, Ding, et al., pursued two strategies using an ML optimal temperature prediction tool, Preoptem¹⁰⁸, combined with co-evolutionary sequence analysis, EVcouplings¹⁰⁹, (Fig. 4f) to create LCCICCG_16M, which showed 3.6-fold higher activity than LCC-ICCG on high-crystallinity, untreated PET¹¹⁰. Zheng, et al., improved the activity of LCC-ICCG by 2.1-fold to create LCC-YGA by using a cross-correlation-based accumulated mutagenesis (CAM) strategy (Fig. 4g)¹¹¹. With the goal to mitigate epistatic effects in combinatorial mutation sets, they devised a strategy to first explore single mutations via remodeling of the binding pocket and incorporating mutations from homologs, then exploring combinations of beneficial mutations, before finally accumulating additional mutations with little dynamic correlation. In this way, accumulated mutations can increase activity until nonadditive effects are seen; then, additional mutations with low cross-correlation are added. Mutations with low-cross correlation can mitigate epistatic effects, as they can rescue conflicting dynamics between protein residues¹¹¹.

In a recent study, Seo, et al., developed a landscape profiling method (Fig. 4h) to determine those enzymes with the highest fitness for PET degradation activity and thermostability¹¹². First, putative, natural sequences across the polyesterase-lipase-cutinase families were collected and clustered into related neighborhoods. Then, representative nodes (sequences) from clusters were sampled for fitness on the basis of activity (in terms of monomer release from bottle-derived PET powder at 30 °C), T_m , and production yield. This indicated which clusters had the highest fitness. Finally, high-fitness clusters were thoroughly sampled to identify high-fitness nodes¹¹². Analytical testing identified two high-fitness nodes (sequences/enzymes), Mipa-P and Kubu-P. Following discovery of these two enzymes, cross-template engineering was performed, whereby rationally-designed mutations were chosen for the two scaffolds by alignment to homologous high-fitness nodes. This yielded the final variants, Mipa-P^{M19} and Kubu-P^{M12}. Notably, Kubu-P^{M12} exhibited significantly enhanced PET degradation activity compared to LCC-ICCG at high PET loadings (20% and 30% (w/w)) and increased tolerance to reactions in ethylene glycol¹¹².

Collectively, computational approaches show promise in significantly enhancing PET hydrolase activity for industrial use. Recent methods have demonstrated successfully engineering PET hydrolases with improved thermostability and/or activity, as has been common with laboratory-based rational design, although some recent studies have begun considering additional goals, including protein production¹¹², as additional selection criteria. Other goals, such as function at a range of pH, may be amenable future targets for computational design. Gado, et al., recently reported EpHod, a tool to predict the optimal pH of an enzyme¹¹³. EpHod showcases language model embeddings and optimized hyperparameters for training, allowing it to learn complex structural and biophysical features—such as proximity of residues from the catalytic center and accessibility of solvent—from sequence data¹¹³. Tools such as EpHod may be impactful when used to complement other discussed methods—i.e., those targeting traditional activity and thermostability goals. Further, these tools span a range of principal approaches, including: systematic workflows to accumulate functional mutations^{90,102,111}; AI/ML methods to determine amino acid variation across evolutionary landscapes¹⁰², analyze MD simulations¹⁰⁵, design for optimal temperature¹¹⁰, and optimize residues' structural

microenvironments^{41,104}; analyze dynamic binding of ligand to enzyme via docking simulations¹⁰⁶; sequence-based consensus design and co-evolutionary analysis^{107,110}; and thorough sequence fitness landscape profiling for enzyme identification and engineering¹¹².

Screening methods for PET hydrolase engineering: from rational to semi-rational design

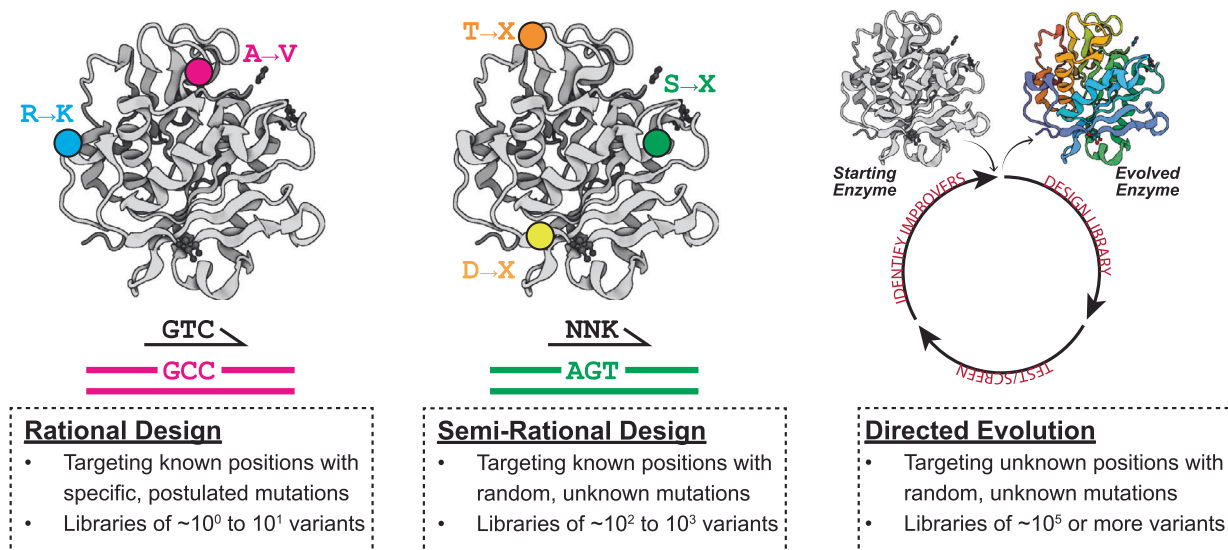
Past screening methods for PET hydrolase engineering have varied significantly in scale and scope—e.g., from assays meant to characterize single rational design mutants (Fig. 5a), to microtiter plate assays sufficient to screen semi-rational design site-saturation libraries (Fig. 5a) over several days. Weight loss of polymer before vs. after reaction (gravimetric analysis)^{26,51,64} and release of fluorogenic probes from polymer structures^{114,115} were some of the first screening assays developed—and have been employed to characterize ~10 enzyme variants at a time (Fig. 5b).

Microtiter plate-based screens (Figs. 5b and 6a) have been used to indirectly measure reaction products (or characteristic byproducts), enabling the efficient screening of up to 10³ enzyme variants at a time. Belisário-Ferrari, et al., developed a plate-based assay to measure turbidity changes from breakdown of the 2PET model substrate in suspensions¹¹⁶. Lusty Beech, et al., developed an assay to measure activity with colorimetric readout by detecting pH changes from the H⁺ released by PET hydrolysis¹¹⁷. Arnling Bååth, et al.¹¹⁸, and Zhong-Johnson, et al.¹¹⁹, developed plate-based screens to measure aggregate aromatic breakdown products from enzymatic PET hydrolysis via UV absorbance readout. Work by Wei and colleagues demonstrated use of reaction products with hydroxyl radicals to generate the fluorophore 2-hydroxyterephthalic acid to quantify TPA in microtiter plates^{120,121}. Similarly, Shi, et al., demonstrated using the hydrolysis of the model substrate bis (2-hydroxyethyl) 2-hydroxyterephthalate (BHET-OH), which PET hydrolases can convert to the fluorescent 2-hydroxyterephthalate (TPA-OH), to measure activity (Fig. 6a). They then screened error-prone PCR libraries of IsPETase over 3 rounds of directed evolution to yield DepoPETase, which had an over 1,400-fold increase in PET hydrolysis products compared to wild-type at 50 °C and a 23.3 °C higher T_m ¹²². Others have used direct product quantification using ultra high performance liquid chromatography (UHPLC) (Fig. 5b)^{27,52}. Using a high-throughput reaction setup and HPLC screening workflow in microtiter plates, Bell, et al., significantly improved the activity and thermostability of IsPETase through targeted, semi-rational directed evolution (Fig. 6b)⁵². Screening approximately 2,000 variants per round over 6 rounds of increasing selection pressures, mutational hotspots from literature were site-saturated, and mutations from the best variants from each round (based on improved monomer product release) were combined to finally discover HotPETase. HotPETase showed a T_m increase of 37.5 °C and approximately a 3.5-fold increase in activity over ThermoPETase^{52,68}.

High-throughput screens to enable directed evolution of PET hydrolases

Until recently, PET hydrolase engineering focused exclusively on rational and semi-rational design^{17,19}, (Fig. 5a) in which, single mutations (~10¹ variants) and small mutant libraries (<10³ variants) were constructed based upon structural inspection, homology, domain swapping, and modeling. While directed evolution is a powerful technique for engineering enzymes (and other proteins) with improved and/or new functions^{123,124}, it has lagged behind rational design for engineering PET hydrolases. The key limitation has been in the lack of high-throughput screening assays that can effectively evaluate the large enzyme libraries required for directed evolution by random mutagenesis (Fig. 5b)¹⁹. Indeed, directed evolution is able to better probe a protein's sequence space—in scale and scope—for rare, beneficial, and surprising mutations than more constrained libraries¹²³, (Fig. 5a) and thus future advances in PET hydrolase engineering may be enabled or accelerated via directed evolution. Rational design may be insufficient to, for example, enhance multiple catalytic steps simultaneously¹⁹, account for epistatic effects and incompatible mutations, and requires extensive

a.



b.

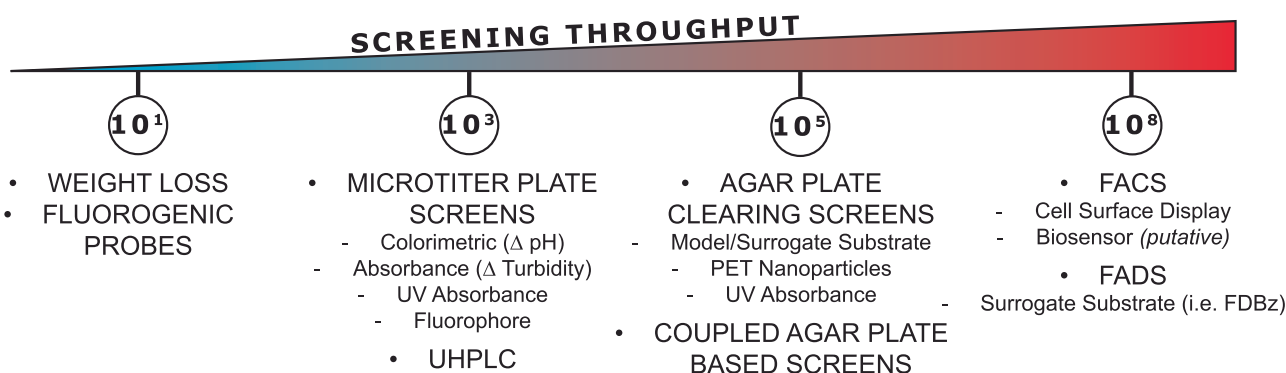


Fig. 5 | Enzyme library types and methods to screen them. **a** Rational design, semi-rational design, and directed evolution are the three key laboratory approaches to engineering PET hydrolases. However, a screening approach to evaluate the library

associated with the method is required. **b** Screening methods used to evaluate PET hydrolase libraries.

structural information and a rational basis for engineering. Now, screening assays have been developed that can evaluate 10^5 or more variants in several days (Fig. 5b). A selection of the PET hydrolases engineered using high-throughput screening, as well as the semi-rationally designed PET hydrolases, discussed in this Review are shown in Table 1 and an overview of screening assays used to evaluate libraries for semi-rational design and random mutagenesis are shown in Fig. 6.

Agar plate-based screening methods are one possibility to increasing the screening throughput to 10^5 variants at a time (Fig. 5b). Substrates can be embedded in agar plates, with visible signals indicating breakdown of the substrate. PET nanoparticle suspensions have been used in several studies, where halos, or clearing zones, indicate that the variant can hydrolyze the PET^{125–128}. However, the need for high hydrolytic activity can render identification of improved enzyme variants at the colony-level difficult on PET substrates.

Fluorescence-activated droplet sorting (FADS) approaches¹²⁹ offer one high-throughput alternative (Fig. 5b). Qiao, et al., developed an ultra-high-throughput method where bacteria expressing PET hydrolases were enclosed in droplets, then the droplets were injected with the model substrate fluorescein dibenzoate (FDBz) (Fig. 6c). Breakdown of FDBz by a PET

hydrolase creates fluorescent products, which allows the droplets to be sorted and bacteria to be isolated. Recent advances demonstrate that biosensors could provide for ultra-high-throughput screening approaches, as well^{130–132}. Pardo, et al., developed a TPA-responsive biosensor, which can sense and respond to levels of TPA to express green fluorescent protein (GFP). Fluorescent activated cell sorting (FACS) of libraries of cells encapsulated with extracellularly-expressed PET hydrolases that can then import the TPA (as demonstrated in the work) could rapidly screen 10^8 variants or greater at a time¹³².

A recent report by Cribari, et al., developed a novel, ultra-high-throughput screening platform for PET hydrolase engineering using yeast display with FACS (Fig. 5b)¹³³. On cell surfaces tagged with probes comprised of an aromatic ester and biotin, a PET hydrolase was expressed (Fig. 6d). In this screening assay, if a PET hydrolase was able to efficiently cleave the aromatic ester, then biotin, which is later detected with a fluorescent streptavidin conjugate, was present in lower amounts. Hence, cells expressing a high-activity PET hydrolase will exhibit low fluorescence, which can be isolated with FACS. This screening assay could process 10^7 variants or greater. From 3 rounds of sorting of error-prone PCR libraries, the screen discovered LCC variants with enriched mutations at H218, with

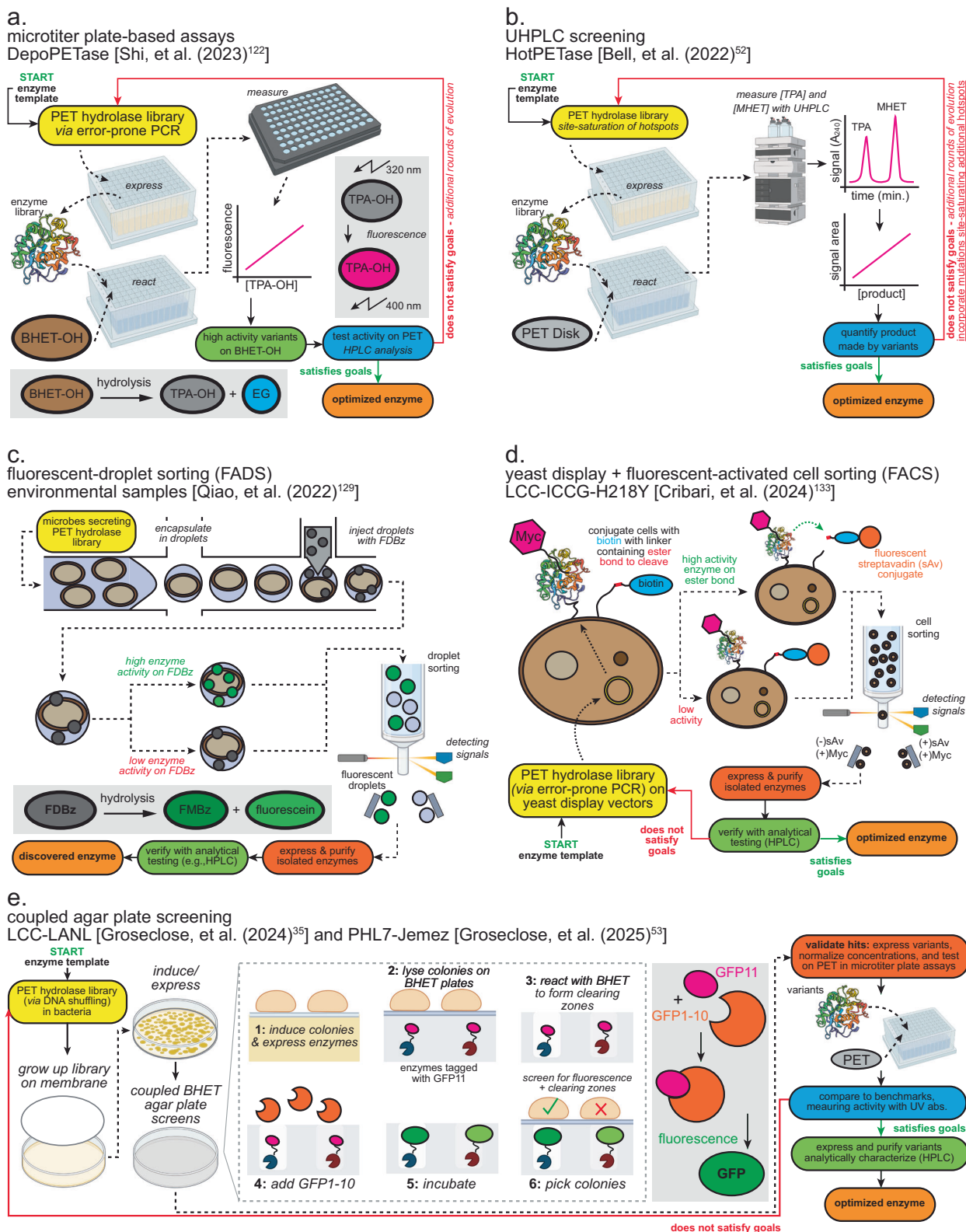


Fig. 6 | Overview of selected high-throughput screening methods. **a** Microtiter plate-based assays. The overview is based on the screen by Shi, et al., to engineer DepoPETase¹²², but similar workflows could be applied to using absorbance (change in turbidity)¹¹⁶, color (change in pH)¹¹⁷, or UV absorbance (aromatic product release)^{118,119} as readouts. **b** UHPLC screening⁵². **c** FADS¹²⁹. Note: the authors did not

use the screen to engineer enzymes, but the screen could be adapted to screen enzymes using directed evolution similarly to the other methods. A similar workflow could be used with a TPA biosensor¹³², where the cells would need to be encapsulated for the reaction and the TPA would need to be imported. **d** Yeast display coupled with FACS¹³³. **e** Coupled BHET and split-GFP screens on agar plates^{35,53,135}.

H218Y being the exemplary variant. LCC-ICCG-H218Y exhibited 2.6-fold higher activity compared to LCC-ICCG. The authors postulated that mutation at H218 could improve substrate binding, as H218 is adjacent to W190 (Fig. 3a), which helps form part of the substrate binding pocket. W190 and the W185 analog in IsPETase are, as mentioned, critical for activity^{82,84,85,133,134}. While the authors screened 27 million variants in their work, however, a downside is that the screening must be performed at ambient temperature, where yeast grows, so it is not yet optimized to screen for thermostable variants. The same issue would present with the FADS approach by Qiao, et al., using bacteria in microdroplets¹²⁹. An ideal screening assay for PET hydrolases should screen for improved variants with a selection pressure mandating activity near the T_{opt} of PET, about 70 °C.

In two other recent works, Groseclose and colleagues developed a high-throughput coupled plate-based screening assay (Fig. 5b) to identify improved PET hydrolases from directed evolution (Fig. 6e). The assay can evaluate variants simultaneously for activity, thermostability, and protein solubility/expression^{35,53,135}. In their screening, libraries of *Escherichia coli* (*E. coli*) containing variants of PET hydrolases (10^5 or greater at a time) with C-terminal GFP11 tags^{136–138} were grown on semi-permeable membranes. The libraries were then induced to express the enzyme by moving the membrane to a plate with isopropyl β -D-thiogalactopyranoside (IPTG). The membrane was then again moved to an agar plate containing the BHET model substrate, where the cells were partially lysed, and the membrane was removed. The BHET plate was then incubated at the reaction temperature (~70 °C). Appearances of halos, or clearing zones, indicated enzyme variants active on BHET. Following this, the GFP11 tag on the enzymes was complemented by incubation with GFP1-10 solution on the plates, showing green fluorescent signal based on the amount of enzymes from the lysed colony (indicating solubility/expression). Thermostability was reported to act as a selection pressure via heat treatment prior to reaction, or reaction at high temperature.

Following the co-screening assay, potential hits were validated by small-scale expression and reaction with PET substrates in solution, measuring aromatic products in throughput of $\sim 10^2$ with UV absorbance screens^{118,119}. After rounds of directed evolution, with libraries diversified from random mutation and shuffled from previous, validated hits, final enzymes were expressed, purified, and tested in vitro. An advantage of the assay is that it allows normalization of activity to solubility/expression through the high-throughput screening steps, without laborious, low-throughput expression, purification, and quantification. Further, it uses only widely-accessible reagents and equipment and is adaptable to a variety of selection pressures (e.g., pH, temperature, PET substrate type)¹³⁵. Groseclose and colleagues used the high-throughput screening platform to engineer LCC³⁵ and PHL7⁵³ using directed evolution, with evolved LCC-LANL exhibiting 11% higher conversion after 48 h in a bioreactor with 16.5% amorphous PET loading vs. LCC-ICCG³⁵ and evolved PHL7-Jemez exhibiting 270% higher conversion after 48 h in a bioreactor with 20% amorphous PET loading vs. PHL7-WT⁵³. Final enzyme variants had a diversity of mutations, at least 3 amino acid mutations each, after at least 3 rounds of directed evolution, with increasing stringency of thermostability and activity selection pressures^{35,53}.

Considerations for PET hydrolase screening

A key consideration in high-throughput screening is the evaluation metric. That is, the criteria for which an enzyme variant is chosen from the library to be characterized further or proceed to additional rounds of directed evolution. This is typically a lesser consideration with rational design, as the pools of variants are significantly smaller and most/all of the variants are pursued through thorough screening and characterization. The evaluation metric could also relate to how many rounds of directed evolution are undertaken: How improved must potential enzyme variants be before they are declared final? Commonly, performances of PET hydrolases engineered in the lab are reported in terms of fold change for activity (e.g., 3-fold higher products released by a certain time) and change in T_m temperature for

thermostability (e.g., +3 °C). Evaluation on PET is commonly performed in small-scale (<1500 μ L), either in test tubes or 96-well plates, using commercially-available (i.e., from Goodfellow) PET substrates, in sheet or powder forms, of different percent crystallinities (from ~9% sheets³⁵, product ES301445, to ~42% powders¹³⁹, product ES306031), at <10% solids loading³⁴—but typically lower, from 0.4%⁵² to 2.9%^{27,35,53}. Toward improved transparency and reproducibility in this rapidly-growing, global field of research, it is critical for researchers to report standardized details of their assessment methods, ultimately permitting fair comparison between enzymes and groups³⁴. This includes source, property, and treatment parameters of PET^{34,139}, as well as enzyme characteristics and reaction conditions: notably, enzyme and substrate loading³⁴. Mass and/or concentration of enzyme and substrate should be disclosed, not simply volume of enzyme or piece or size of substrate, as has been common in previous studies (Table 1).

Often the key evaluation metric for PET hydrolase screening is increased depolymerization activity—i.e., of a set PET substrate type and loading—under the relevant reaction conditions—i.e., at a set temperature and pH—at a certain endpoint—e.g., 48 h. However, it may be desired to also select enzyme variants that display some interesting, emergent property depending on engineering goals, not merely those variants that have higher fold changes in depolymerization activity (in terms of higher UV absorbance^{35,118,119} or monomer release via HPLC⁵²) vs. the starting point enzyme. For illustration, a researcher may choose to investigate a variant with a high initial rate (within 4 h reaction) that plateaus (after 12 h) or a variant that exhibits lower, but sustained activity (over 24 h and beyond). These variants could provide breakthrough insights into engineering faster or more temperature- and pH-stable enzymes. Even if these variants may not be optimized themselves, combining these interesting mutations with others (e.g., using DNA shuffling¹⁴⁰) can provide paths to enzymes that simultaneously achieve many identified engineering goals (Fig. 1b). This also motivates potential advantages of taking different measurements to allow for data-rich decision-making. Rather than merely measuring end-point activity at 48 h, reactions can be measured over time, or parallel reactions can be set up at high temperature or low pH to use more extreme conditions as selection pressures. Ultimately, evaluation metrics may vary somewhat depending on specific engineering goals and the number of enzyme variants a researcher or team can reasonably handle.

To provide a sense of throughput and evaluation metrics, we will focus on two recent screening methods developing high-performance PET hydrolases: Bell, et al., using high-throughput UHPLC screening⁵² (Fig. 6b) to develop HotPETase⁵² and Groseclose, et al., using a high-throughput plate-based screening assay^{35,135} (Fig. 6e) to develop PHL7-Jemez⁵³. Bell, et al. performed 6 rounds total of evolution. Each round, 1,930 to 2,000 variants were screened and 3 to 13 best variants (bearing single mutations) were selected. Within rounds, up to 3 identified mutations were shuffled together. Selected variants had 1.13- to 2.96-fold increases in activity compared to their parents at reaction conditions of increasing pressure (in terms of increasing heat treatment temperature, reaction temperature and time, and decreasing enzyme volume). In each round, ~3% of the top hits were assessed as purified proteins, and in Rounds 5 and 6, the top 3% of variants were selected to be tested on crystalline PET (only amorphous PET used in Rounds 1–4). As mentioned above, the final HotPETase variant showed an approximate 3.5-fold increase in activity over ThermoPETase⁵². Groseclose, et al. performed 4 rounds total of evolution. Each round, approximately 20,000 variants were screened on BHET agar plates, of which roughly 25 to 50 were selected for screening on PET. In Rounds 1–3, 8 to 14 variants were selected in reactions of increasing pressure (in terms of increasing reaction time and substrate loading, with increasing BHET concentration and higher/longer heat treatment temperatures used in the high-throughput screening stage), yielding variants with 1.03- to 12.74-fold increases in activity and up to 3.51-fold higher expression vs. PHL7-WT. The final four

enzyme variants from Round 4 had 2.0- to 3.2-fold higher activities and 1.7- to 3.1-fold higher expression levels compared to PHL7-WT. Groseclose, et al., stopped evolution after Round 4 as screened enzyme variants were achieving approximately 2-fold higher activities and expression levels under relevant reaction conditions compared to the starting parent. The exemplary variant, PHL7-Jemez, showed approximately a 4-fold higher product release compared to PHL7-WT in small-scale reactions. As a heuristic, Bell, et al., and Groseclose, et al., were selecting the top 0.05% to 0.5% of their variants in their successful directed evolution campaigns.

In comparison, the large-scale, computationally-guided rational design study by Cui, et al., began with 253 mutations predicted to be stabilizing, which were down-selected to 85 to avoid biophysical pitfalls⁹⁰. These 85 mutations were further filtered to 21 variants (mutations) that expressed and increased stability (≥ 1.5 °C increase in T_m). Then, in each of 10 rounds of shuffling, 4 to 12 new variants were made by creating combinations of single mutations with the best parent from the previous round. One best variant was selected per round (based off of degradation performance), but mutations that were not selected in a round were allowed to appear in later rounds. In each round, degradation performance was increased by up to 2-fold, with 65 total combination mutants tested. The final DuraPETase mutant, as mentioned, had a 31 °C higher T_m and over 300-fold higher activity compared to *IsPETase*⁹⁰.

Engineering BHET and MHET hydrolases

Design goals for MHET and BHET hydrolases

While PET hydrolases appear to exhibit promiscuous activity to hydrolyze the intermediate products BHET and MHET, MHET hydrolases and, recently, BHET hydrolases, have been shown to increase the efficiency of PET depolymerization reactions when coupled with PET hydrolases (Fig. 1a)^{141,142}.

An ideal process scheme would involve the simultaneous hydrolysis of all PET-derived substrates to TPA and EG in one pot. One advantage of MHET and BHET hydrolases is that they exhibit significantly higher turnover on their small-molecule, partially-soluble MHET and BHET substrates compared to PET hydrolases on PET. Additionally, due to their small-molecule substrates, MHET and BHET do not possess many of the properties that could inhibit PET hydrolase activity, such as crystallinity, limited interfacial substrate surface area, and a heterogeneous interface. However, to date, no MHET or BHET hydrolase has been discovered that can survive at the optimal temperatures of PET reactions, ~70 °C. Hence, a critical design goal for these enzymes is increasing their thermostability so they can be used in tandem reactions near the T_{opt} of PET hydrolases (Fig. 1b).

Moreover, MHET hydrolases express in significantly lower amounts than PET hydrolases—and its low expression may currently hinder industrial application¹⁴³. Although concentrations of MHET hydrolases in reactions could likely be permitted to be lower compared to PET hydrolases due to their increased turnover, the expression of these enzymes must be significantly increased to improve enzyme cost and process economics (Fig. 1b). This could be mitigated with improved enzyme activity, a perennial design goal for industrial enzymes.

Engineering MHET hydrolases

Palm, et al., were the first to determine the structure of *IsMHETase*, and established the foundation for MHET hydrolase (Fig. 1a) rational engineering. In general, they determined that *IsMHETase* and *IsPETase* are structurally conserved (Fig. 2), except for a large lid domain in *IsMHETase* that confers high activity on MHET^{73,144}. They determined that *IsMHETase* specificity is highly dictated by contact between the substrate and lid domain, including critical residue R411, which coordinates with the carboxylic groups, and, further, that substrate binding is through an induced-fit mechanism facilitated by F415¹⁴⁴. Palm, et al., then conducted mutagenesis of the active site residues, discovering abolishment of activity for several residues by mutation to alanine and a W397A variant that caused increased activity at high substrate concentrations, but lowered substrate affinity

(decreasing the K_M). Toward improved BHET hydrolysis by *IsMHETase*, Palm, et al., further discovered that the active site mutations S416A and S419G retained MHET activity while also allowing for BHET activity (Fig. 2b), possibly due to the increased flexibility of R411 to bind BHET. The F424Q and F424N mutations, which potentially confer more space within the active site and provide for hydrogen bonding partners, also increase BHET activity. Eliminating the positive charge of R411, either by mutating to alanine or glutamine, increased BHET activity, as well. As discussed with PET hydrolases, it should be acknowledged that altering the selectivity of an MHET hydrolase, for example by widening the active site binding cleft to better accommodate the BHET substrate, has potential tradeoffs: activity, affinity, and/or thermostability may be affected. A structural depiction of the rational design strategies that have been used to engineer MHET hydrolases is shown in Fig. 2b.

Knott, et al., further investigated active site and structural features of *IsMHETase* to confer high MHET activity. Motivated by sequence alignments with proteins across the tannase family, mutations were made to active site residues. While *IsPETase* has a serine at position 131, 91% of tannase family sequences have a glycine at the analogous position. However, they observed activity of the S131G mutant was decreased by 97% compared to wild-type⁷³. Similarly, the F495I mutation, to mimic that of other tannase family members, showed significantly decreased activity, as did E226T. Collectively, as witnessed by mutagenesis of PET hydrolases, mutation of residues around the active site selected to enhance activity may not be outright predictable based on computational design or homology mapping. Knott, et al., additionally confirmed the necessity of the lid domain for MHETase activity. Addition of the *IsMHETase* lid domain to *IsPETase* conferred a small level of activity on MHET and the deletion of the lid domain from *IsMHETase* resulted in 1000-fold less activity than wild-type⁷³.

Inspired by the work by Palm, et al., Sagong, et al., sought to increase the BHET hydrolysis activity of *IsMHETase*¹⁴⁵. To affect BHET binding, they created an R411K mutant, aiming to allow more beneficial hydrogen bonding vs. R411, when BHET is the substrate (Fig. 2b). They also created F424N/H/D/E/T/V/L/I (mutation to smaller residues) mutants to enlarge the substrate-binding pocket to better accommodate the BHET substrate (Fig. 2b), as suggested by Palm, et al. The R411K mutant showed 1.7-fold higher activity on BHET than wild-type and the F424 mutants showed up to 3.9-fold higher activity (for F424N), and, further, combined mutants R411K/F424N, R411K/F424V, and R411K/F424I showed up to 11.1-fold higher activity. The authors also determined that the mutants had decreased activity for MHET, while several, including R411K/F424I and R411K/F424V, had higher activity against the PET5 oligomer. A summary of the engineered MHET hydrolases (specifically, *IsMHETase*) discussed in this Review is shown in Table 2.

Discovery and engineering of BHET hydrolases

Li, et al., discovered two BHET hydrolases (Fig. 1a), BsEst and ChryBHE-Tase, via enzyme mining from environmental landfill samples and screening with stepwise straining with diethyl terephthalate (DET), PET powder, and MHET substrates¹⁴¹. Modeling of these two enzymes' structures with substrates showed solvation of the substrate binding cleft, but no presence of BHET. Using a mechanism-guided approach, potential barriers to BHET access were removed, truncating identified regions with flexible glycine linkers. Following in silico scoring, two top variants, Δ BsEst and Δ ChryBHE-Tase, were identified. Δ BsEst hydrolyzed 100% of BHET in 3 h, a 2-fold improvement over wild-type, and Δ ChryBHE-Tase hydrolyzed 100% in 3 h less than its wild-type. MD simulations verified that the increased flexibility of the removed barriers in the enzymes allowed improved BHET substrate access. The BHET hydrolases additionally were conferred with increased thermostability, presumably via creation of more favorable physiochemical properties including internal H-bonds and SASA. A summary of the engineered BHET hydrolases discussed is shown in Table 3.

Miao, et al., recently discovered an enzyme from the UniParc database they termed BMHETase for its ability to hydrolyze both MHET and BHET¹⁴². It was reported to have higher activity on BHET than both

Table 2 | Exemplary engineered MHET hydrolases discussed

IsPETase Position	Mutations	Performance	Reaction Conditions/Substrate	Engineering Method	Citation
W937	A	~2.2-fold higher MHET turnover vs. IsMHETase	30 min rxn, 30 °C, 6 nM enzyme, pH 7.5, 1 mM MHET	Rational design	Palm, et al. ¹⁴⁴
R411	A	~0.05-fold lower MHET turnover, ~5-fold higher BHET turnover, vs. IsMHETase	MHET: 30 min rxn, 30 °C, 6 nM enzyme, 1 mM MHET, pH 7.5; BHET: 19.25 h rxn, 30 °C, 100 nM enzyme, 1 mM BHET, pH 7.5	Rational design	Palm, et al. ¹⁴⁴
R411	Q	~0.1-fold lower MHET turnover, ~4.5-fold higher BHET turnover, vs. IsMHETase		Rational design	Palm, et al. ¹⁴⁴
R411	K	~0.42-fold lower MHET turnover, 1.7-fold higher BHET turnover, vs. IsMHETase	MHET: 30 °C, 0.1–2 nM enzyme, 100 µM MHET, pH 8; BHET: 30 °C, 0.1–2 nM enzyme, 100 µM BHET, pH 8	Rational design	Sagong, et al. ¹⁴⁵
F415	H	~1.6-fold higher MHET turnover vs. IsMHETase	30 min rxn, 30 °C, 6 nM enzyme, pH 7.5, 1 mM MHET	Rational design	Palm, et al. ¹⁴⁴
S416	A	~0.9-fold lower MHET turnover, ~8.5-fold higher BHET turnover, vs. IsMHETase	MHET: 30 min rxn, 30 °C, 6 nM enzyme, 1 mM MHET, pH 7.5; BHET: 19.25 h rxn, 30 °C, 100 nM enzyme, 1 mM BHET, pH 7.5	Rational design	Palm, et al. ¹⁴⁴
S419	G	~0.8-fold lower MHET turnover, ~7.5-fold higher BHET turnover, vs. IsMHETase		Rational design	Palm, et al. ¹⁴⁴
F424	Q	~0.3-fold lower MHET turnover, ~20-fold higher BHET turnover, vs. IsMHETase		Rational design	Palm, et al. ¹⁴⁴
F424	N	~0.5-fold lower MHET turnover, ~20.5-fold higher BHET turnover, vs. IsMHETase		Rational design	Palm, et al. ¹⁴⁴
F424	N/H/D/E/T/V/L/I	For F424N: ~0.2-fold lower MHET turnover, 3.9-fold higher BHET turnover, vs. IsMHETase	MHET: 30 °C, 0.1–2 nM enzyme, 100 µM MHET, pH 8; BHET: 30 °C, 0.1–2 nM enzyme, 100 µM BHET, pH 8	Rational design	Sagong, et al. ¹⁴⁵
S416A/F424N		~0.19-fold lower MHET turnover, 10.2-fold higher BHET turnover, vs. IsMHETase		Rational design	Sagong, et al. ¹⁴⁵
R411K/F424N		~0.2-fold lower MHET turnover, 8.7-fold higher BHET turnover, vs. IsMHETase		Rational design	Sagong, et al. ¹⁴⁵
F411K/F424V		~0.3-fold lower MHET turnover, 10.5-fold higher BHET turnover, vs. IsMHETase		Rational design	Sagong, et al. ¹⁴⁵
R411K/F424I		~0.32-fold lower MHET turnover, 11.1-fold higher BHET turnover, vs. IsMHETase		Rational design	Sagong, et al. ¹⁴⁵
R411K/S416A/F424I		~0.25-fold lower MHET turnover, 15.3-fold higher BHET turnover, vs. IsMHETase		Rational design	Sagong, et al. ¹⁴⁵

Positions and mutations to that position are listed for each enzyme variant. The performance of the enzyme variants vs. relevant benchmarks and the conditions at which the performance was measured is listed, as well as the method of engineering for each variant. Fold change is calculated based on the performance of the variant divided by that of the benchmark enzyme. Turnover is derived from the authors' calculation of the enzyme's kinetic rate. Many variants were tested to measure the activities with both MHET and BHET as substrates, which are listed separately.

Table 3 | Exemplary engineered BHET hydrolases and dual-function MHET/BHET hydrolases discussed

Enzyme Name	Mutations	Performance	Reaction Conditions/Substrate	Engineering Method	Citation
Δ BsEst	BsEst Δ 410–418	2-fold faster conversion of BHET, 2.6-fold higher turnover, vs. BsEst	Activity: 30 °C, 10% (v/v) purified enzyme, 5 mM BHET, pH 7.5; kinetics: 0.8 to 15 mM BHET	Rational design (eliminating active site barrier)	Li, et al. ¹⁴¹
Δ ChryBHETase	ChryBHETase Δ 66–76	100% conversion of BHET 3 h earlier, 1.7-fold higher turnover, vs. ChryBHETase	Activity: 30 °C, 10% (v/v) purified enzyme, 5 mM BHET, pH 7.5; kinetics: 0.8 to 15 mM BHET	Rational design (eliminating active site barrier)	Li, et al. ¹⁴¹
BMHETase ^{BM}	BMHETase G133S/R247G/R275D/A373Y/A400S/G406S	~7.5-fold higher MHET activity, ~2.25-fold higher BHET activity, vs. BMHETase	MHET: 1 h, 60 °C, 500 nM enzyme, 2.5 mM MHET, pH 8; BHET: 10 min, 60 °C, 2 mM enzyme, 2 mM BHET, pH 8	Rational design (homology mapping)	Miao, et al. ¹⁴²
TfCa I69W	TfCa I69W	~2.4-fold higher activity vs. TfCa (converting BHET to TPA)	45 °C, 2 mM BHET, 550 nM enzyme, pH 7.5	Semi-rational design of targeted sites	von Haugwitz, et al. ¹⁴⁸
TfCa V376A	TfCa V376A	~1.75-fold higher activity vs. TfCa (converting BHET to TPA)	45 °C, 2 mM BHET, 550 nM enzyme, pH 7.5	Semi-rational design of targeted sites	von Haugwitz, et al. ¹⁴⁸
TfCa R428A	TfCa R428A	~1.5-fold higher activity vs. TfCa (converting BHET to TPA)	45 °C, 2 mM BHET, 550 nM enzyme, pH 7.5	Semi-rational design of targeted sites	von Haugwitz, et al. ¹⁴⁸
TfCa I69W/V376A/R428A	TfCa I69W/V376A/R428A	~3.75-fold higher activity vs. TfCa (converting BHET to TPA)	45 °C, 2 mM BHET, 550 nM enzyme, pH 7.5	Semi-rational design of targeted sites	von Haugwitz, et al. ¹⁴⁸

If a unique name is given for an enzyme, the name is given alongside the mutation(s). Otherwise, the position is listed alongside the mutations to that position. The performance of the enzyme variants vs. relevant benchmarks and the conditions at which the performance was measured is listed, as well as the method of engineering for each variant. Fold change is calculated based on the performance of the variant divided by that of the benchmark enzyme. Turnover is derived from the authors' calculation of the enzyme's kinetic rate. Some variants were tested to measure the activities with both MHET and BHET as substrates, which are listed separately. BsEst and ChryBHETase are BHET hydrolases, while BMHETase and TfCa have dual-function MHET/BHET hydrolase activity.

LCC-ICCG and FAST-PETase at temperatures of 50 to 70 °C. Rational engineering to mimic the residues in IsMHETase, to increase MHET activity, resulted in a 6 point-mutation variant that had 8-fold higher activity on MHET, 2-fold higher activity on BHET, and a T_{opt} increased by 5 °C. It was posited that this was due to improved interactions with the MHET substrate and a larger substrate binding pocket. TfCa, a carboxylesterase from *T. fusca*, also has activity on both MHET and BHET, as well as a cyclic PET oligomer^{146,147}. After determination of TfCa's structure in complex with an MHET substrate analog, von Haugwitz, et al., conducted alanine scanning and site-saturation mutagenesis of identified residues to discover conferred improved activities on BHET by the R428A and V376A mutations, which may allow for more bulky substrate binding, and the I69W mutation, which may better interact with aromatic moieties on the substrate (Fig. 2a)¹⁴⁸. A summary of the engineered MHET/BHET hydrolases discussed is shown in Table 3.

Screening methods for MHET and BHET hydrolase engineering

Given the prospect of MHET and BHET hydrolases to enhance the efficiency of enzymatic PET recycling processes and the growth of discoveries of novel MHET and BHET hydrolase scaffolds—such as the MHET hydrolase Mle046¹⁴⁹, the BHET hydrolase ScLipA¹⁵⁰, and those enzymes discussed in the previous sections—engineering of MHET and BHET hydrolases could lead to future improvements in enzymatic PET recycling. Just as with PET hydrolases, we anticipate that coupling rational design, computational design, and directed evolution approaches will prove to be successful in developing new MHET and BHET hydrolases.

With that said, the development of screening assays enabling directed evolution of MHET and BHET hydrolases has been scarce. Currently, the use of HPLC (Fig. 5b) in screening MHET and BHET hydrolase activity widely prevails over other methods^{73,141,142,144}. However, many of the high-throughput screening assays developed for PET hydrolases could feasibly be co-opted for use with MHET and BHET hydrolases. Lusty Beech, et al., for example, designed their colorimetric microtiter plate assay to detect PET degradation through protons released by BHET and MHET hydrolysis (Fig. 5b)¹¹⁷.

One key consideration is that MHET hydrolases have high specificity toward MHET and little activity toward substrate analogs typically used for PET hydrolase engineering, such as p-nitrophenols¹⁴⁴. As such, screening assays for MHET hydrolases should use MHET as the substrate to be most efficient. Recently, Saunders, et al., reported a medium-throughput screening assay for MHET hydrolases, whereby the enzymes are first screened on the model substrate 1-naphthyl terephthalate (1NT). If the MHET hydrolase exhibits activity toward this substrate, 1-naphthol and TPA are produced. Addition of the Fast Blue B Salt, which reacts with the 1-naphthol, will produce a brown azo dye, which can be detected with absorbance at 610 nm¹⁴³. Saunders, et al., then used consensus design of IsMHETase, where the most common (consensus) amino acids were introduced at various positions of the protein, which has been shown to improve protein stability, folding, and expression^{143,151}. They saw an approximate order of magnitude increase in soluble expression, postulated to be due to improved protein folding. The variants did have reduced activity on MHET¹⁴³, likely in part due to screening on 1NT.

Altering the model substrates used in PET hydrolase screening assays to MHET or BHET can give a direct route to screen for MHET and BHET hydrolases, provided that the assays can specifically detect the desired product. For this reason, using UV absorbance based screening, in which there would be high background from the soluble substrate, would cause it to be difficult to detect product species. However, the use of the screening assay developed by Shi, et al.¹²², (Fig. 6a) to screen for hydrolysis of BHET-OH to TPA-OH, could likely be employed to discover high activity BHET hydrolases. The TPA biosensor developed by Pardo, et al.¹³², could putatively screen for BHET or MHET activity, if there is minimal promiscuity of the biosensor to detect larger PET subunits. Likewise, the yeast-display screening assay pioneered by Cribari, et al.¹³³, (Fig. 6d) could be used to detect BHET and MHET hydrolases in ultra-high throughput, as the model

Table 4 | Summary of PET recycling strategies discussed

PET Recycling Strategy			
	Mechanical	Chemical (Methanolysis/Glycolysis)	Enzymatic
Strengths	- In some cases (i.e., using high-quality, low-contamination PET), it has the lowest cost and environmental impacts - Infrastructure is already in place	- Can handle contamination and mixed waste streams - Creates new presursors for high-quality PET synthesis	- Enzymes are highly-selective, able to handle low-quality, highly-contaminated waste - Creates direct presursors for high-quality PET synthesis
Limitations	- Struggles with contaminants, mixed wastes, and some types of PET (e.g., thermoforms) - Downcycling puts a limit on the max number of recycling cycles per material	- Harsh process conditions are resource- and energy-intensive - Requires new infrastructure - Potential issues with product separation and purification	- Has mild reaction conditions, but is water- and energy-intensive, from base/acid additions, required PET pre-treatment, and EG distillation - Requires new infrastructure
Contexts	- Could be improved by advanced sorting technologies - Can be complemented with chemical recycling for hard-to-recycle materials	Could be improved through improved catalyst design, reagent recycle, and separations to improve yields and minimize energy and resource use	Could be improved through enzyme engineering and process innovations (as discussed throughout the text)

Strengths, limitations, and contexts are shown for mechanical, chemical (i.e., methanolytic and glycolytic), and enzymatic PET recycling processes. See ref. 12. and ref. 23. for additional information.

substrate to detect ester bond cleavage is universal across PET, BHET, and MHET. Additionally, agar plate based screening (Fig. 3b) could be adapted toward MHET and BHET hydrolase directed evolution, whereby the plates could be cast with MHET or BHET, rather than PET nanoparticles. By detecting halos on the plates, enzyme variants could be effectively identified. Adaptation of the platform by Groseclose, et al.³⁵, (Fig. 6e) either by using the BHET agar plates disclosed, or instead using MHET as the substrate, could then additionally couple protein expression/solubility and thermostability screening to activity screening.

The evaluation criteria for MHET and BHET hydrolases will be generally similar to that for PET hydrolases (see above). That is, for rational design and small semi-rational design campaigns (<100 variants total), all variants will be thoroughly tested and characterized, with the best variants chosen to be reported. Multiple mutations can be combined (maintaining <100 variants screened), with engineering continuing until all combinations have been attempted or no further improvements are observed⁹⁰. For larger libraries—large semi-rational design campaigns and random mutagenesis libraries for directed evolution (numbering >100 variants, and preferably 10³ and greater)—the top 0.05%⁵³ to 0.5%⁵² of variants should be chosen. However, this could depend on the ability to handle and characterize more (or less) variants and, as with PET hydrolases, we recommend selecting variants with any interesting properties for further study, even if they do not outright have higher activity under the measured conditions.

Concluding Remarks

This Review summarizes and discusses current state-of-the-art and emerging approaches for engineering enzymes involved in enzymatic PET degradation—namely, PET, MHET, and BHET hydrolases. The goal is to bridge *i*) lessons learned in the last ~10 years of rapidly-growing PET-degrading enzyme discovery and engineering—which has focused on PET hydrolases—with *ii*) next-generation directed evolution/high-throughput screening and computational design approaches, along with *iii*) the development of other classes of PET-degrading enzymes, MHET and BHET hydrolases. We motivate these engineering efforts with lessons learned from recent process modeling, technoeconomic, and life cycle analyses, to further bridge ongoing laboratory-based testing with considerations for scaleup and industrial deployment, as enzymatic PET recycling matures as a technology. Achieving these analysis-driven design goals will be critical to improving the competitiveness of enzymatic PET recycling vs. other PET recycling strategies.

In some cases, i.e., with clean, high-quality PET, mechanical recycling is the most energy-efficient, low-cost, and least environmentally-impacting recycling method (Table 4)¹². However, there is some PET waste that should not be recycled mechanically and is currently landfilled^{12,152,153}. These are materials such as thermoformed containers (for salads, berries, etc.), colored bottles, and textiles. Further, there is a limit on the number of times a material can be mechanically recycled¹¹. Hence, in a future PET economy,

there can be a niche for chemical recycling (Table 4). For example, enzymatic (hydrolytic) recycling is more agnostic to mixed and contaminated waste streams compared to mechanical recycling^{15,16}. With that said, the chemical recycling approach that should be pursued will depend on technical constraints (e.g., feedstock quality) and process priorities (e.g., cost, environmental impact, quality of resin)¹². Advanced chemical recycling technologies are active areas of scientific research and, as such, process innovations could significantly affect future decision-making¹³. A summary of the PET recycling strategies discussed in this Review are shown in Table 4.

Several challenges currently experienced by enzymatic PET recycling center around the PET pre-treatment process, base addition for pH maintenance in depolymerization reactions, and difficulty in EG/water distillation, leading to high costs (MSPs), energy and water use, emissions, and toxicity/waste (Table 4)^{15,16}. While Murphy, et al., recently proposed key process improvements to mitigate these challenges and make the cost and impacts of rPET better relative to vPET⁶³, opportunities remain in process and enzyme engineering to strengthen the industrial feasibility of enzymatic PET recycling on an industrial scale.

Toward industrially-relevant design goals for PET hydrolase enzyme engineering (Fig. 1b), researchers have traditionally relied upon rational design (Table 5) and screening of several variants at a time to confer improvements in properties such as: thermostability, via creation of disulfide bonds and salt bridges^{29,41,52,64–67}; activity, via enhanced substrate binding^{29,67,80,83,84}; and product inhibition, via tethering of a PET hydrolase and an MHET hydrolase^{73,96}. More recently, the development of higher throughput screens has facilitated the screening of larger, semi-rational design libraries (Table 5), allowing researchers to improve activity by targeting residues surrounding active sites²⁹ and mutational hotspots⁵² identified previously with a range of amino acid substitutions. Now, varied computational approaches (Table 5)—which may be guided by structure^{41,104}, sequence^{107,110,112}, or simulations^{105,106}—are emerging to rapidly design potential enzymes prior to any laboratory testing. Likewise, as high-throughput screening assays (Table 5) are at last developed for PET hydrolase engineering^{35,53,129,133}, directed evolution is now possible^{35,53,133}, enabling the screening of large enzyme libraries to discover rare, highly-optimized enzyme variants. A summary of the enzyme engineering strategies discussed in this Review is shown in Table 5. In parallel, primarily rational^{73,144,145} and semi-rational¹⁴⁸ design approaches have been used to increase improve the MHET and BHET hydrolysis activities of MHET and BHET hydrolases, although recent advances (i.e., in computational design and high-throughput screening) and lessons learned in PET hydrolase engineering could accelerate the engineering of these other classes of enzymes.

Outlook

Directed evolution efforts, either by large, semi-rational design libraries or random mutagenesis libraries, have lagged behind rational design efforts due to the lack of high-throughput screening methods that can operate near

Table 5 | Summary enzyme engineering strategies discussed

Enzyme Engineering Strategy				
	Rational Design	Computational Design	Semi-Rational Design	Random Mutagenesis
Strengths	• Precisely targets protein residues/regions that are predicted to be most impactful • Small libraries need only simple, low-effort screening methods	• Can rapidly screen mutants in silico prior to any laborious experimentation • Can potentially create de novo function with limited previous data	• Precisely targets protein residues/regions that are predicted to be most impactful • Tests varied chemistry at positions • Screening can be done without ultra-high-throughput methods	• Can probe entire sequence space, finding rare, surprising mutations • Requires limited structural and evolutionary information • Efficiently navigates good vs. bad mutation combinations (epistatic effects)
Limitations	• Requires knowledge of enzyme structure and/or homologous enzymes and mutations • Can only probe limited sequence space: no surprising mutations	• Requires experimental validation: predictions may be inaccurate • Requires reliable models • Screening large, combinatorial mutation spaces may be resource-intensive	• Requires knowledge of enzyme structure and/or homologous enzymes and mutations • Limited to mutation at the positions selected • Requires a high-throughput screen	• Requires a high-throughput screen to find improved variants • Mechanistic insight into mutations may not be readily apparent
Contexts	• Can complement other strategies, especially for first-generation engineering of newly discovered enzyme scaffolds • Many mutants are currently known that improve key functions (e.g., T_m)	• Can complement other strategies, e.g., using experimental datasets to train better models • Models and methods can be generalized to many different scaffolds	• Can complement other strategies, e.g., by targeting hotspots found through random mutagenesis • Screens exist for its successful execution	• Can complement other strategies, e.g., by gathering large-scale sequence-function data for AI/ML models • Screens exist for its successful execution

Strengths, limitations, and contexts are shown for enzyme engineering using rational design, computational design, semi-rational design, and engineering by random mutagenesis approaches.

the optimal temperature of most efficient PET depolymerization reactions, ~70 °C. However, recent advances in the development of high-throughput screening assays, such as microtiter plate screens^{52,122}, surface display technologies¹³³, and coupled plate-based assays^{35,53,135}, have successfully demonstrated the directed evolution on PET hydrolases (Fig. 5). Anticipated future developments, such as in ultra-high-throughput biosensor screening¹³², will accelerate PET hydrolase engineering further. Future advances in protein engineering of PET-degrading enzymes should also complement emerging process innovations. While the engineering goals discussed here (Fig. 1b) will be valuable for reducing costs of industrial enzymatic PET recycling processes, for instance, Murphy, et al., suggest that enzyme longevity, tolerance to EG, and ability to depolymerize alternative forms of pre-processed PET will be most impactful in their modeled process⁶³. We posit these forward-looking engineering goals can be approached using strategies adapted from those discussed here.

As new PET hydrolases are discovered, especially those with interesting native features, such as preference for high-crystallinity substrates²⁷—allowing them to serve as better footholds for engineering—we envision a workflow whereby rational design and semi-rational design would be the first focus. Here, site-saturation mutagenesis in proximity to the active site and mutation of other hotspot residues can be easily explored, via homology mapping of these highly-conserved enzymes based on literature, by structural inspection guided by previous successes, or computational approaches (Fig. 3). Directed evolution strategies can then be employed with these first-generation evolved PET hydrolases as templates, whereby larger sequence spaces can be explored to engineer the enzymes toward improved catalytic activity under specific selection pressures. Next-generation selection pressures should include screening for properties currently underexplored with PET hydrolases, but these could be aided by existing work with similar enzymes. Huang, et al., for example, engineered a lipase using error-prone PCR for high pH tolerance¹⁵⁴. Identified hotspots through directed evolution may then be used in future rounds of rational or semi-rational design, with mutations fed into ML models, that would further accelerate discovery of high-performance PET hydrolases.

Toward the application of AI and ML in engineering new PET-degrading enzymes (Fig. 4), large quantities of high-quality sequence/function relationship data will be extremely critical in training and building models^{155,156}. While both positive and negative enzyme performance data would be informative for training AI/ML models, current studies with PET-degrading enzymes focus primarily on mutations that improve the performance of the final enzyme variants. To increase the transparency of future studies and facilitate the success of forthcoming AI/ML assisted protein design, we encourage the community to openly present the results of their mutational studies including both positive and negative effects of single, double, triple, etc., mutations on the performance of enzyme variants. This type of data will become increasingly rich and valuable as future high-throughput screening and directed evolution studies generate large amounts of random and combinatorial mutation data for diverse enzyme scaffolds, although a wealth of data currently exists from dozens of studies on engineering these enzymes. A selection of mutational data is shown in Table 6, where several studies have identified single mutation and combinations of mutations that increase/decrease enzyme activity and/or thermostability. Other metrics, such as expression/solubility, may be beneficial to be considered as well. The high-throughput screening assay recently developed by Groseclose and colleagues is well-suited to acquire such multi-dimensional datasets, where one can examine multiple enzyme properties including hydrolytic activities, thermostability and expression/solubility levels without the need for enzyme purification^{35,53,135}.

>With that said, this data should be gathered and reported using standardized protocols. To ensure reproducibility across research groups and the fair comparison of evolved enzymes, the complete details of reaction conditions and parameters must be disclosed. Arnal, et al.³¹, and Wei, et al.³⁴ have recently proposed protocols and considerations to better standardize PET hydrolase testing. Proposals by Arnal, et al., suggest using a standardized substrate (cryo-milled Goodfellow

Table 6 | Selection of mutations across PET and MHET hydrolase scaffolds that resulted in an increase or decrease in enzyme activity and thermostability

Scaffold Name	Detrimental Mutations		Beneficial Mutations		Conditions / Substrate	Citation
	Activity	Thermostability	Activity	Thermostability		
IsPETase	R100E, T113P, R123V, P181A, E204K, G254V	A74T, A82V, Q91P, A183T, A215P, T286V	V134L, A152S, S166T, T198V	T77E, Q119Y, W159H, I168R, D186H, S188Q, S214H, P181A	200 µL PET nanoparticles, 10 µL of 0.1 mg/mL enzyme stock, 37 °C, 1 h, 300 µL rxn, pH 9	Cui, et al. ⁸⁰
	I168R/D186H/S214H		I168R/S214H, D186H/S214H, W159H/I168R/S188Q/S214H			
LCC	T96M, Y127G, D238C/S283C, D238C/F243W/ N246D/S283C	F243I	F243I/W, N246D	T96M, Y127G, N246D/M, D238C/S283C	100 mg preform PET, 1 mL of 0.69 µM enzyme stock, 70 °C, 100 mL rxn, pH 8	Tournier, et al. ²⁸
	D238C/F243I/N246D/S283C			D238C/F243I/S283C, Y127G/D238C/F243I/S283C, D238C/F243I/N246M/S283C		
PHL7	F63Y/A, M132W, L210F	L210V/I, L93A	L210T/V, L93A	F63Y/A, Q95G, M132W, L210F	~45 mg amorphous PET coupons, 0.55 mg enzyme/g PET, 70 °C, 1.8 mL rxn, 16 h, pH 8	Richter, et al. ⁵⁰
	H130W, H185S, F189I		L93F, Q95Y, L210T/A/S, D233K			
IsMHETase	S225A, R411A/Q, 416 A/G, F424Q/N, D492A, H528A, R411A/S416A, R411A/S416G, R411A/S419G, R411Q/S416A, R411Q/S416G	n.d.	W297A, F415A	n.d.	30 min, 30 °C, 6 nM enzyme, 1 mM MHET, pH 7.5	Palm, et al. ¹⁴⁴

The list is not exhaustive. One reference which disclosed thermostability and activity data for single and multiple mutation variants was chosen for each scaffold. Mutations (single or multiple) comprising a variant were classified whether or not they improved or decreased the activity upon comparison to the wild-type scaffold under the tested conditions. For the PET hydrolase scaffolds, IsPETase, LCC, and PHL7, activity refers to PET hydrolysis, while for IsMHETase, activity refers to MHET hydrolysis. Thermostability of variants was not measured by Palm, et al.¹⁴⁴, so the corresponding column is marked "n.d." for not determined.

amorphous powder), a fixed enzyme to substrate ratio, and fixed substrate loading for bioreactor experiments, with small-scale (laboratory) experiments measured by TPA formed after a certain time and activity reported as specific rates (i.e., $\text{g}_{\text{TPA,eq}} \text{h}^{-1} \text{mg}_{\text{enzyme}}^{-1}$)³¹. Wei, et al. suggest reporting a range of polymer and enzyme properties and reaction parameters³⁴.

Emerging methods in high-throughput screening and computational design may have the power to alleviate some bottlenecks in PET-degrading enzyme engineering. The additive (epistatic) effects of mutations in a protein can be difficult to discern and require trial and error, which may be intractable using low-throughput techniques for large mutational spaces. This is additionally convoluted as some mutations may decrease enzyme activity while increasing thermostability or vice versa (see Table 6). However, some combinations of mutations may be synergistic, while others are incompatible. Screening using high-throughput experimental and computational methods can enable the efficient testing of combinatorial mutation spaces and could help avoid rationally-designed dead-ends.

For instance, Cui, et al.⁹⁰, in their development of DuraPETase, extensively experimentally tested the thermostability and activity of 85 single mutants, which were then combinatorially combined in several rounds. While P181A was identified as a potential beneficial mutation, it was discovered that this variant had increased thermostability, but decreased activity, and this mutation was not propagated in later rounds (Table 6). Son, et al.⁶⁸ had previously rationally designed P181A to fix a disruption in a β -sheet and also found an increase in stability, but postulated that this came with a collapse of the active site. Interestingly, Bell, et al., discovered P181V in their final HotPETase variant⁵². Cui, et al., additionally discovered synergistic and incompatible mutations in their engineering⁹⁰. While S188Q behaved synergistically with the IsPETase W159H/I168R/S214H mutation combination, they saw that while I168R/S214H and D186H/S214H improved activity and thermostability compared to IsPETase wild-type, the addition of D186H to I168R/S214H resulted in a lower-performing variant. Similarly, Tournier, et al.²⁹, discovered that some single mutations in LCC, such as F243I and Y127G, displayed an improvement to either thermostability or activity (Table 6). However, upon combination of the mutations (along with a rationally-designed disulfide bond), synergistic effects gave highly improved enzymes such as D238C/F243I/N246M/S283C (LCC-ICCM) and Y127G/D238C/F243I/S283C (LCC-ICCG)²⁹. Other combinations of the mutations, though, such as D238C/F243I/N246D/S283C, showed incompatibilities, resulting in reduced activity and stability (Table 6). In the PHL7 scaffold, it is notable that Pfaff, et al., discovered the PES-H1-L92F/Q94Y⁵¹ mutant by combination of the mutations L93F and Q95Y also identified by Richter, et al.⁵⁰ as improving activity and thermostability (Table 6).

Although work on BHET and MHET hydrolases has been outnumbered by that for PET hydrolases, we anticipate that these enzymes will become increasingly important in industrial PET recycling. Fortunately, some of the structural and methodological insights gained from PET hydrolases seem to be able to be extended to BHET and MHET hydrolases (Fig. 2), including that many of the high-throughput screening assays for PET hydrolases can be adapted for these enzymes. This should enable the rapid engineering of BHET and MHET hydrolases toward industrial desired goals.

Similarly, adapting existing high-throughput screening assays for PET polymer and subunit hydrolysis to other plastic polymers targeted for enzymatic recycling (with hydrolyzable polymer backbones), such as poly(urethane) and nylon, will enable the rapid screening and engineering of enzymes degrading these polymers. Altering the model substrates in those high-throughput screening assays discussed here to include amide or urethane bonds will be the first step to development of new screening assays for these enzymes. Hence, as enzyme cocktails become developed that can break down mixed, post-consumer plastic waste streams in one pot reactions, a complete biorefinery may be developed where mixed plastic can be recycled, or valorized, enabling an alternative end-of-life strategy for plastics that may be more feasible than current, conventional approaches.

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Competing interests

The authors declare competing financial interests. A high-throughput screening platform, methods, and enzyme variants that are discussed are the subject of domestic and foreign patent applications by Los Alamos National Laboratories on behalf of the Department of Energy and Triad National Security, L.L.C.

Additional information

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