Directed evolution of an efficient and thermostable PET depolymerase

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The recent discovery of IsPETase, a hydrolytic enzyme that can deconstruct poly(ethylene terephthalate) (PET), has sparked great interest in biocatalytic approaches to recycle plastics. Realization of commercial use will require the development of robust engineered enzymes that meet the demands of industrial processes. Although rationally engineered PETases have been described, enzymes that have been experimentally optimized via directed evolution have not previously been reported. Here, we describe an automated, high-throughput directed evolution platform for engineering polymer degrading enzymes. Applying catalytic activity at elevated temperatures as a primary selection pressure, a thermostable IsPETase variant (HotPETase, \( T_m = 82.5^\circ C \)) was engineered that can operate at the glass transition temperature of PET. HotPETase can depolymerize semicrystalline PET more rapidly than previously reported PETases and can selectively deconstruct the PET component of a laminated multiform. Structural analysis of HotPETase reveals interesting features that have emerged to improve thermostability and catalytic performance. Our study establishes laboratory evolution as a platform for engineering useful plastic degrading enzymes.

Poly(ethylene terephthalate) (PET) is among the most abundantly produced synthetic polymers. Demand for this material has grown substantially due to a heightened global appetite for convenient single-use containers1,2, with an estimated 1 million PET bottles being produced every minute3. Although mechanical recycling methods are available for PET, recycling rates remain low due to difficulties in collecting and sorting mixed postconsumer waste streams4, and declining polymer properties after repeated processing cycles5. In light of these challenges, depolymerization of PET into its component monomers has attracted substantial interest as a means of circularizing the PET life cycle6,7. This can be achieved using chemical recycling techniques, including solvolysis methods such as hydrolysis and glycolysis8,9. More recently, enzymatic depolymerizations have emerged as a potentially attractive alternative10,11. Techno-economic analysis and life-cycle assessments predict that biocatalysis can offer a cost-effective and energy efficient approach to PET recycling. Furthermore, enzymatic recycling could also facilitate selective depolymerizations of complex mixed feedstock waste streams that are particularly challenging to recycle effectively.

For enzymatic PET recycling to be feasible, suitable biocatalysts must first be discovered and then engineered to tailor their properties for target applications. Unfortunately, while microorganisms are extremely well-equipped to deconstruct biological polymers such as proteins, DNA and carbohydrates, they are generally not well-adapted to achieve efficient depolymerization of synthetic polymers12. Nevertheless, some cutinases have been shown to have promiscuous PET degradation abilities13-15. These enzymes typically display poor activity towards PET materials with high crystallinities, akin to those commonly found in postconsumer waste. To function effectively, even engineered cutinases require the extensive preprocessing of PET substrates to amorphize the material16, a process that compromises the economic and environmental sustainability of biocatalytic plastic recycling approaches17.

The recent discovery of an organism, Ideonella sakaiensis, the ability to use PET as a carbon source18, revealed a naturally evolved, PET-hydrolysing enzyme (IsPETase) that has an enhanced ability to depolymerize more crystalline forms of PET19. There are interesting structural differences between IsPETase and homologous cutinases, which are thought to be linked to this improved activity19, including a conformationally flexible Trp185 that has been proposed to aid polymer binding20,21. The unique catalytic properties of IsPETase make it an attractive candidate as a biocatalyst for PET recycling. Unfortunately, the wild-type enzyme suffers from low thermostability18, meaning that biotransformations must be run at ambient temperatures far below the glass transition temperature (\( T_g \)) of PET (\( T_g \) of approximately 60–70°C), which compromises polymer deconstruction rates22,23.

In an effort to address these limitations, improvements in PETase stability have been achieved using a variety of rational engineering approaches24-27. In contrast, experimental optimization of IsPETase using directed evolution, which typically offers a more comprehensive approach to enzyme engineering28,29, remains under-explored, probably due to the lack of suitable protocols for monitoring the deconstruction of insoluble plastics with sufficient throughput30. Here we establish an automated, high-throughput directed evolution platform for engineering plastic deconstructing enzymes and showcase its use by engineering a thermostable variant of IsPETase that can operate at the glass transition temperature of PET. This engineered biocatalyst can efficiently depolymerize semicrystalline PET and can selectively deconstruct real-world laminated packaging materials.

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Directed evolution of a thermostable PETase. We selected a rationally designed, thermostabilized variant of IsPETase containing three mutations, S121E, D186H, R280A (IsPETaseTS, melting temperature ($T_m$) of 56.8 °C), as a starting point for engineering. Consistent with previous reports, IsPETaseTS displays superior PET degradation activity compared to the wild-type enzyme at 40 °C; however, this activity decreases dramatically at more elevated temperatures (Supplementary Fig. 1). Our first objective was to develop a high-throughput screening workflow to underpin the directed evolution study. To this end, individual enzyme variants were evaluated as crude cell lysates arrayed in 96-deep-well plates, using amorphous PET film (amoPET, 6.7% crystallinity, sourced from Goodfellow as a preform sheet) as the reaction substrate. The amoPET substrate allowed us to prepare uniform 6 mm diameter discs that could be easily placed into individual wells to allow fair comparison of variant activity (Fig. 1). We found that the addition of our standard chemical cell lysis reagents, lysozyme and polymyxin B, resulted in the production of a lysate with substantially reduced activity (Supplementary Fig. 2), hence, we developed a method for 96-well plate cell lysis using the commercial reagent, BugBuster. The accumulation of mono(2-hydroxyethyl) terephthalate (MHET) and terephthalic acid (TPA) in the reaction supernatant was monitored by ultra performance liquid chromatography (UPLC); these two products result from the partial and complete hydrolysis of the PET backbone, respectively, and are the known major products of IsPETase-mediated depolymerizations. Using the UPLC method developed here, the MHET and TPA produced by a single degradation reaction can be analysed in under 2 minutes (Supplementary Fig. 2). Using our integrated, automated system, over 2,000 enzyme variants can be assessed for plastic deconstruction activity in around 2 days.

The evolutionary strategy comprised sequential rounds of saturation mutagenesis, using degenerate NNK codons to individually randomize between 24–30 residue positions per cycle. In total, 106 of the 264 residues present in IsPETase were targeted for mutation throughout evolution. Residues were selected for randomization on the basis of a number of considerations, including their identification by online protein stability-enhancing tools, visual inspection of the protein crystal structure or previous reports of their involvement in substrate binding or thermostability (Supplementary Table 1). In each round of evolution, around the top 3% of hits were assessed as purified enzymes. Beneficial mutations found were then combined by DNA shuffling, and the resulting variants assayed as purified proteins to identify the most active sequence, which was then used as a template for the next round of evolution. Between
rounds 2 and 3, an additional disulfide bridge (N233C, S282C) wasrationally generated in the protein, following reports that the inclusion of this structural feature increased protein stability in homologous, promiscuous PET-degrading cutinases, leading to a 5.5°C increase in Tm (Extended Data Fig. 1 and Supplementary Fig. 3).

To simultaneously improve both thermostability and activity, the evolutionary pressures applied were gradually changed across rounds by raising both the reaction temperature and extending the reaction time. For rounds 1–4, the primary focus was on improved catalysis at elevated temperatures. Cell lysates were pre-incubated at sequentially higher temperatures (from 55–75°C) for 1 h, before conducting PET depolymerization reactions for 3 h. The reaction temperature also increased from 55–70°C during these rounds (Supplementary Table 1). Once a satisfactory level of thermostability was achieved, additional selection pressures of catalyst longevity and activity on more crystalline material were added. To this end, reactions in rounds 5 and 6 were conducted at 70°C, with reaction times of 5 and 7 h, respectively, initially using amoPET as the substrate. The top 3% of clones identified during rounds 5 and 6 were then screened as purified enzymes against a commercially available semicrystalline PET powder (cryPET, 29.8% crystallinity, sourced from Goodfellow) that has a crystallinity level more reminiscent of material dominant in postconsumer waste streams.

The most thermostable and active variant to emerge following six rounds of evolution, HotPETase, contains 21 mutations compared to lPETase WT, three from the starting protein template lPETase TS, two from the rational insertion of an additional disulfide bridge and a further 16 found through directed evolution (Fig. 1 and Extended Data Fig. 1). HotPETase has a melting temperature of 82.5°C, the highest Tm recorded so far of an active lPETase derivative. This elevated thermostability means that the enzyme can be incubated before reaction at 75°C for 90 min with only a 6% loss of activity over 24 h (Supplementary Fig. 4). Enzyme pre-incubation at 80°C for 90 min resulted in a more substantial 35% reduction in activity. Assessment of variants along the evolutionary trajectory demonstrated that evolution led to progressive improvements in both thermostability and activity in cryPET deconstruction assays performed at 60°C (Fig. 2a and Supplementary Fig. 3). While lPETase WT and lPETase TS have minimal activity at 60°C, HotPETase operated well under these conditions.

Biochemical characterization. We next determined the activity of HotPETase across a range of temperatures by monitoring the release of MHET and TPA over time (Fig. 2b). For comparison, analogous experiments were performed using the starting template, lPETase TS, and the engineered thermostable cutinase LCCICCG (ref. ). Comparisons between LCCICCG and lPETase TS variants were carried out under the optimal buffer conditions for each individual protein, using cryPET powder as the substrate (Supplementary Fig. 5). At 40°C, slightly improved initial reaction rates were achieved by HotPETase versus lPETase TS (Fig. 2b), demonstrating that the evolution of thermostability has not compromised activity at ambient temperatures. While the activity of lPETase TS was severely compromised at higher temperatures, the rate of PET hydrolysis by HotPETase is substantially improved by operating at temperatures approaching the reported Tm of PET in aqueous solutions (around 60–65°C). At 65°C, each mole of HotPETase releases 2.7 x 10^4 M of monomers in 1 hour, a time-course over which reaction progression is linear. At the same temperature LCCICCG produced 5.7 x 10^3 moles of monomer product in the same time frame, highlighting the superior catalytic activity of this engineered lPETase. For both HotPETase and LCCICCG, the reaction rates were slightly reduced at 70 versus 65°C.

Comparison of reactions with HotPETase and lPETase TS at 40°C show that evolution has afforded a more robust catalyst with increased longevity (Fig. 2c and Extended Data Fig. 2). For lPETase TS, soluble product formation essentially ceases after 8 h. In contrast, for reactions with HotPETase, monomeric products continue to accumulate for more than 48 h. Consistent with previous studies, the reaction profile is non-linear with faster initial phase for roughly 8 h, followed by a slower phase from 8–48 h. Similar, but more pronounced, non-linear reaction profiles are observed at elevated temperatures (from 60–70°C, Extended Data Fig. 2). The time-course of reactions with HotPETase at 65°C demonstrate that product accumulation rises rapidly for the first 3 h of reaction (1.51 mM of MHET + TPA), but then slows substantially after this time, producing 1.61 mM of soluble monomers over 48 h (Fig. 2d).

As a result, while PET depolymerization with HotPETase is substantially faster at 65°C, the extent of depolymerization at longer time frames is greater at 40°C (Fig. 2c,d).

Product accumulation over time is also non-linear for LCCICCG in reactions at 65°C, with 0.68 and 1.78 mM of monomers produced over 5 and 48 h, respectively (Extended Data Fig. 3). It is interesting to note that HotPETase operating at 40°C deconstructs cryPET more efficiently than LCCICCG at 65°C, both with respect to initial rate and extent of depolymerization over 48 h. HotPETase also depolymerizes amoPET discs (used for library screening) more effectively than LCCICCG across a range of temperatures from 40 to 65°C (Supplementary Fig. 6). At 70°C, although HotPETase produces more soluble monomers than LCCICCG over 3 h, at this temperature over 24 h, LCCICCG is a more effective depolymerase of amoPET due to its enhanced longevity.

To understand the origins of the non-linear reaction profiles of HotPETase, particularly at elevated temperatures, we conducted experiments to supply additional enzyme or substrate once reaction progression had ceased. Addition of fresh HotPETase, following cryPET depolymerization for 24 h at 60°C, leads to similar product accumulation versus time trends as observed at the outset of the reaction (Extended Data Fig. 4). In contrast, addition of fresh PET substrate does not give rise to any additional soluble products. These observations suggest that reactions stall due to catalyst deactivation, not as a result of inhibition by soluble released products or exhaustion of available plastic substrate. It is interesting to note that during evolution, lPETase libraries were analysed over time frames ranging from 3 to 7 h, meaning that limited selection pressure was applied to catalyst longevity at elevated temperatures. We anticipate that adapting selection pressures during future rounds of evolution will lead to improved variants capable of operating efficiently at elevated temperatures for more extended periods.

To further explore the use of HotPETase, we next attempted to deconstruct commercial-grade PET materials. HotPETase can depolymerize milled bottle-grade PET (bPET, 41.9% crystallinity, full material characterization can be found in Extended Data Table 1 and Supplementary Figs. 7 and 8), albeit with a reduced conversion compared to that observed with cryPET powder (Fig. 3) (9.7 and 2.8% with cryPET and bPET, respectively). To showcase the selectivity achievable with biocatalytic depolymerizations, HotPETase was used to deconstruct a common laminated packaging tray lid composed of PET and polyethylene (PE) (1.6% crystallinity, thickness of 325 μm PET and 40 μm PE, Extended Data Table 1). This PET/PE laminate is challenging to recycle mechanically, and indeed is considered a pollutant in commercial recycling streams. The HotPETase enzyme is adept at selectively deconstructing the PET portion of this material. In this instance, the extent of depolymerization after 24 h is substantially improved at 60 versus 40°C (9.2 versus 2.9 mM of soluble monomer products released, corresponding to a degree of depolymerization of 48.1 and 15.3%, respectively, Fig. 3a and Extended Data Fig. 5). Scanning electron microscopy (SEM) reveals significant pitting of the PET surface, whereas the PE surface appears unchanged, compared to control reactions run in the absence of enzyme (Fig. 3b and Supplementary Fig. 9). The patterns of PET surface erosion differ in samples depolymerized at 40 versus...
Fig. 2 | Directed evolution of \(\text{IsPETase}^{15}\) afforded a more thermostable and active catalyst. 

a, Bar chart demonstrating a snapshot of protein variants along the evolutionary trajectory from \(\text{IsPETase}^{WT}\) to HotPETase. Protein variants are on the x axis; the mutations added to each variant in the bar chart compared to the previous variant are represented as follows: 1 is S121E, D186H, R280A; 2 is P181V, S207R, S214Y; 3 is Q119K, S213E, N233C, S282C; 4 is R90T, Q182M, N212K, R224L and 5 is S58A, S61V, K95N, M154G, N241C, K252M, T270Q. Solid coloured bars represent the mean \(T_m\) of each variant (left y axis). Hashed bars represent the mean concentration of MHET and TPA produced by each enzyme variant over the course of 48 h at 60 °C, using 0.4% cryPET by mass (4 g l\(^{-1}\)) and 0.29 mg enzyme per g PET (corresponding to 0.04 \(\mu\)M enzyme).

b, Bar chart showing the mean enzyme turnovers per hour after 1 h of reaction, with cryPET substrate at different temperatures with \(\text{IsPETase}^{WT}\) (yellow), HotPETase (pink) or \(\text{LCCICCG}\) (green) as the biocatalyst, using 0.4% cryPET substrate loading (4 g l\(^{-1}\)) and 0.29 mg g\(^{-1}\) enzyme loading (0.04 \(\mu\)M).

c, 48 h time-course reactions, with either HotPETase (pink) or \(\text{IsPETase}^{WT}\) (yellow), showing the mean total concentration of released MHET and TPA (left y axis) and mean percentage substrate depolymerized (right y axis) in reactions at 40 °C, using 0.4% cryPET substrate loading (4 g l\(^{-1}\)) and 0.29 mg g\(^{-1}\) enzyme loading (0.04 \(\mu\)M).

d, 48 h time-course reactions with HotPETase (pink) or \(\text{LCCICCG}\) (green), showing the mean total concentration of released MHET and TPA (left y axis) and the mean percentage substrate depolymerized (right y axis) in reactions at 65 °C, using 0.4% cryPET substrate loading (4 g l\(^{-1}\)) and 0.29 mg g\(^{-1}\) enzyme loading (0.04 \(\mu\)M). For all reactions presented in this figure, \(\text{IsPETase}\) and its derivatives were assayed in the library screening buffer: pH 9.2, 50 mM Gly-OH with 4% BugBuster; \(\text{LCCICCG}\) was assayed in its reported optimal operating buffer: pH 8, 100 mM K-Pi. Error bars represent the s.d. of triplicate measurements, each replicate measurement is represented with a black circle.
6.07 mM of soluble monomer products were formed (MHET:TPA ratio of 1:0.29) within 5 h at 60 °C, corresponding to a degree of depolymerization of 36.4%. Assays were conducted under the library screening buffer conditions: pH 9.2, 50 mM Gly-OH buffer with 4% BugBuster. Reactions were carried out in triplicate; error bars represent the s.d. of the total MHET and TPA produced in the replicates; each replicate measurement is represented as a black circle. The y axis is on a logarithmic scale. Physical characterization of the substrates without treatment, incubated with buffer-only and postenzymatic deconstruction, is detailed in Extended Data Table 1.

To understand how HotPETase interacts with PET oligomers, we performed in silico docking using distance restraints to the Ser160 catalytic nucleophile and the backbone amides of the oxyanion hole (Ser160 catalytic nucleophile and the backbone amides of the oxyanion hole). The best docking pose is shown in Fig. 4a, with the PET oligomer (2-hydroxyethyl- (monohydroxyet hyl terephthalate), 4PET) occupying a shallow, extended binding cleft. The ‘wobbling’ tryptophan, Trp185, a feature that is thought to aid substrate binding and catalysis in the wild-type enzyme20,35, is present as a single conformer in apo-HotPETase and is suitably positioned to accommodate the docked 4PET in a productive pose for catalysis (Fig. 4b). Extensive remodelling of the loop region connecting β7-α5, including introduction of a bulky Tyr214, leads to a new β-stacking interaction with Trp185 that restricts its conformational freedom (Fig. 4b and Extended Data Fig. 7d). A hydrogen bonding network involving Trp185 and Tyr214 and the terminal hydroxyl group of 4PET also contributes to the stabilization of the docked oligomer within the binding cleft.

**Structural analysis.** To gain insights into the origins of HotPETase thermostability and its improved activity, the crystal structure of the enzyme was solved and refined to a resolution of 2.2 Å for comparison to the starting variant IsPETase15. The structures of HotPETase (Protein Data Bank (PDB) 7QV1) and IsPETase15 (PDB 6J6) superimpose well, with a root-mean-square-deviation of 1.18 Å (Extended Data Fig. 7a). In HotPETase, the disulfide bridge between the Cys233 and Cys282 pair is formed as intended, with an S-S interatomic distance of 2.03 Å (Extended Data Fig. 7b). The P181V mutation results in an additional hydrogen bond between Val118 and Leu199 leading to better packing of the central β-sheet region compared to IsPETase (Extended Data Fig. 7c and Supplementary Fig. 14). Analysis of the surface charge distributions of HotPETase and IsPETase15 reveals substantial changes, including in the putative polymer binding cleft (Supplementary Fig. 15). Ensemble refinements of IsPETase15 and HotPETase demonstrates that regions Ala183 to Asn190 and Cys203 to Leu216 have substantially decreased flexibility in the evolved enzyme (Supplementary Fig. 16).

60 °C, with defined pits observed at 40 °C compared with a more rugged surface at the higher temperature (Supplementary Fig. 9). These differences could plausibly arise due to different rates and extents of polymer deconstruction at the two temperatures, or due to increased chain mobility at 60 versus 40 °C.

Biocatalytic deconstruction of a range of PET-based materials by HotPETase. a Bar chart showing the mean concentration of released PET degradation products, MHET (blue) and TPA (yellow), produced following reaction of HotPETase at 60 °C over the course of 24 h, using 0.4% total substrate loading (4 g l⁻¹) and 0.29 mg g⁻¹ enzyme loading (0.04 μM). HotPETase has activity on crystalline PET powder (cryPET), milled bottle-grade PET (bgPET) and a PET/PE composite packaging tray lid (thickness of 325 μm PET and 40 μm PE, estimated 3.6 g l⁻¹ PET substrate loading). Assays were performed in silico docking using distance restraints to the Ser160 catalytic nucleophile and the backbone amides of the oxyanion hole (Ser160 catalytic nucleophile and the backbone amides of the oxyanion hole). The best docking pose is shown in Fig. 4a, with the PET oligomer (2-hydroxyethyl- (monohydroxyet hyl terephthalate), 4PET) occupying a shallow, extended binding cleft. The ‘wobbling’ tryptophan, Trp185, a feature that is thought to aid substrate binding and catalysis in the wild-type enzyme20,35, is present as a single conformer in apo-HotPETase and is suitably positioned to accommodate the docked 4PET in a productive pose for catalysis (Fig. 4b). Extensive remodelling of the loop region connecting β7-α5, including introduction of a bulky Tyr214, leads to a new β-stacking interaction with Trp185 that restricts its conformational freedom (Fig. 4b and Extended Data Fig. 7d). A hydrogen bonding network involving Trp185, Tyr214 and the terminal hydroxyl group of 4PET also contributes to the stabilization of the docked oligomer within the binding cleft.

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To understand how HotPETase interacts with PET oligomers, we performed in silico docking using distance restraints to the Ser160 catalytic nucleophile and the backbone amides of the oxyanion hole (Tyr87 and Met161). The lowest energy docking pose is shown in Fig. 4a, with the PET oligomer (2-hydroxyethyl- (monohydroxyet hyl terephthalate), 4PET) occupying a shallow, extended binding cleft. The ‘wobbling’ tryptophan, Trp185, a feature that is thought to aid substrate binding and catalysis in the wild-type enzyme20,35, is present as a single conformer in apo-HotPETase and is suitably positioned to accommodate the docked 4PET in a productive pose for catalysis (Fig. 4b). Extensive remodelling of the loop region connecting β7-α5, including introduction of a bulky Tyr214, leads to a new β-stacking interaction with Trp185 that restricts its conformational freedom (Fig. 4b and Extended Data Fig. 7d). A hydrogen bonding network involving Trp185, Tyr214 and the terminal hydroxyl group of 4PET also contributes to the stabilization of the docked oligomer within the binding cleft.
To explore the functional significance of the altered environment around Trp185 in HotPETase, residues installed in the β7–α5 connecting loop during evolution were reverted back to the amino acids present in the wild-type enzyme (HotPETase K212N, E213S, Y214S (HotPETaseLR)). These modifications led to a substantial 7.5 °C reduction in $T_m$ and compromised catalytic performance at elevated temperatures (Extended Data Fig. 8). Catalytic activity at low temperatures is minimally affected, suggesting that in the heavily engineered HotPETase, the fixed conformation of Trp185 is not detrimental to catalysis. Combined, these results indicate that a flexible Trp185 is not a prerequisite for efficient PET deconstruction.

Conclusions

The catalytic performances of PETases have previously been improved through rational engineering using computational methods, providing an important basis towards the development of commercially viable PET depolymerases. However, the engineering of industrial biocatalysts is most commonly achieved through directed evolution. The notable lack of PETases engineered using laboratory evolution probably reflects the challenges of developing suitable high-throughput, quantitative methods for analysing the catalytic deconstruction of insoluble polymers. Here, we have developed an automated directed evolution platform for engineering plastic deconstructing enzymes and showcase its use through the development of an evolved thermostable PETase (HotPETase, $T_m = 82.5 \, ^\circ \text{C}$), that can operate at the glass transition temperature of PET and depolymerizes semicrystalline PET more rapidly than previously reported PETases. HotPETase is able to deconstruct commercial bottle-grade PET and can selectively deconstruct PET in a PET/PE laminated packaging material, highlighting the potential benefits of enzymatic depolymerizations for real-world samples with minimal pretreatment or processing. Structural characterization of HotPETase highlights formation of the intended Cys233-Cys282 disulfide bridge and improved packing of the central β-sheet region, which probably aids thermostability, alongside the presence of a single well-defined conformer of Trp185, indicating that flexibility of this tryptophan is not a prerequisite for effective catalysis. To maximize the use of our platform moving forward, it will be important to interface our evolution methods with alternative strategies for augmenting biocatalyst function, including computationally guided engineering, introduction of polymer binding domains and the development of multi-enzyme complexes. Likewise, combining and optimizing biocatalytic deconstructions with enzymatic monomer upcycling methods will be an important avenue for exploration.

In all cases, detailed techno-economic and life-cycle analysis will play a crucial role in assessing commercial viability, as well as defining target parameters for future biocatalyst engineering.

In the future, we anticipate that by adapting the selection pressures of our directed evolution workflows, we will be able to engineer a suite of useful biocatalysts with complementary functions and improved activities under process-relevant conditions. For example, we can extend catalyst stability and lifetime by increasing reaction times and temperatures, optimize biocatalysts to act on alternative plastic substrates or enhance enzyme specificities in order that they operate on single polymer components from mixed plastic waste streams. In doing so, our laboratory evolution platform will contribute to a biocatalytic recycling strategy to recover value from plastic waste.

Methods

Gene construction. The genes encoding IsPETase26 (IsPETase S121E, D186H, R280A, signal sequence removed as by Son et al.26) and LCCK233 (LCC F243I, D238C, S283C, Y127G, signal sequence removed as by Tournier et al.26) were commercially synthesized by Integrated DNA Technologies as gBlock fragments with codon optimization for expression in Escherichia coli cells. The IsPETase23 gene was cloned into the Ndel (5’ end) and Xhol (3’ end) sites of a pBBBEBK vector modified to contain a C-terminal hexa-histidine tag coding sequence following the Xhol restriction site26, to form pBBBEBK_IsPETase23. The gene encoding LCCK233 was cloned into the Ndel (5’ end) and Xhol (3’ end) sites of pET-22b vector (Novagen) leading to fusion to a C-terminal hexa-histidine tag coding sequence, to form pET-22b_LCCK233. Nucleotide sequences and expressed amino acid sequences of the genes used and plasmid maps of the vector constructs are provided in Supplementary Figs. 17–19.

Library construction. Rounds 1–6: iterative saturation mutagenesis. In each round, 24–30 residues were selected and individually randomized using cassette mutagenesis. Positions were chosen for mutation on the basis of a range of factors, detailed in Supplementary Table 1. For residue identification via the Protein One Stop Repair Shop webserver27, IsPETase26 was used as the input protein (PDB 5X1H), with all constraints fixed to the default settings; positions identified more than twice by the software were selected for mutation. For residue identification via the R-fitter software28, IsPETase26 was again used as the input protein (PDB 5X1H): the 15 top positions ranked by highest B-factor were selected for mutation. DNA libraries at chosen residue positions were constructed via standard overlap-extension PCR, using degenerate primer pairs (containing an NNK codon at the position to be mutated) and pBBBEBK_IsPETase23 as the template for round 1, with the most active clone discovered at the end of each directed evolution cycle serving as the template for subsequent rounds. Primer sequences are provided in Supplementary Table 2.

Shuffling by overlap-extension PCR. After each round of evolution, beneficial diversity was combined by a process of DNA shuffling. Fragments were generated by overlap-extension PCR using designed primers that encoded for either an identified beneficial mutation or the parental amino acid. Using these primers,
up to six short fragments were created, DpnI digested, PCR-purified and mixed in appropriate combinations in overlap-extension PCRs. The resulting genes contained all possible combinations of mutations (from two to five mutations per gene) and were subsequently cloned into the pBBE8K vector as described previously.

**Variant gene construction.** HotPETase K212N, E213S, Y214S (HotPETaseLR), was created via overlap-extension PCR with HotPETase as the template protein and primers designed to encode the wild-type residues at positions 212–214. Primer sequences are provided in Supplementary Table 3. The resulting gene was cloned into the pBBE8K vector as described previously.

**Protein production for library screening.** For all protein expression and screening of libraries, transfer and aliquoting steps were performed using a Hamilton liquid-handling robot. UPLC libraries were expressed in chemically competent Origami 2 E. coli cells. Single colonies from a fresh transformation were used to inoculate 180 µl of Luria–Bertani (LB) media supplemented with 25 µg ml⁻¹ kanamycin (to maintain the pBBE8K_PETase plasmid) and 2.5 µg ml⁻¹ tetracycline (to maintain the glutathione reductase [gor] gene-containing plasmid present in Origami cells), in a 96-deep-well plate. Each plate contained six positive controls consisting of clones of the parent template, and two negative controls consisting of clones containing pBBE8K_RFP (red fluorescent protein). Plates were incubated overnight at 30 °C, 80% humidity in a shaking incubator (950 r.p.m.). The soluble fraction was subjected to affinity chromatography via HotPETase K212N, E213S, Y214S (HotPETaseLR), was previously.

**Variation in the expression and screening of Origami 2 cells, in 96-deep-well plates. Each plate contained six positive controls consisting of clones of the parent template, and two negative controls consisting of clones containing pBBE8K_RFP (red fluorescent protein). Plates were incubated overnight at 30 °C, 80% humidity in a shaking incubator (950 r.p.m.).** The soluble fraction was subjected to affinity chromatography via HotPETase K212N, E213S, Y214S (HotPETaseLR), was previously.

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μ added to form a uniform, colourless solution that was filtered through a 0.24 μm polytetrafluoroethylene filter. SEC analysis was conducted on a system composed of an Agilent 1260 Infinity II LC system equipped with an Agilent guard column (PLGel 5 μm, 50 × 7.5 mm) and two Agilent Mixed-C columns (PLGel 5 μm, 300 × 7.5 mm). The mobile phase used was HPLC-grade CHCl3 at 35 °C at flow rate of 1.0 ml min⁻¹. SEC samples were calibrated against linear polystyrene standards (162–2.4 × 10⁶ g mol⁻¹).

SEM analysis of enzymatic depolymerizations of PET/PE composite packaging. A section of a PET/PE packaging lid (710 mg) was fully submerged in reaction buffer (pH 9.2, 50 mM Gly-OH, 4% BugBuster, 50 ml total) in a glass bottle, and HotPETase (0.04 μM final concentration) added to initiate the reaction. Reactions were incubated at either 40 or 60 °C with agitation at 1200 r.p.m. The PET/PE packaging lid portion was washed and a fresh buffer and enzyme solution added each day over the course of 6 days. The control reactions were run in an identical manner, but with no enzyme added. The percentage depolymerization of the PET packaging of each of the replicate was measured by the release of MTPA monomers, determined by UPLC analysis of the reaction supernatant taken each day, assuming an estimated 12.6 g l⁻¹ PET substrate loading. The extent of depolymerization was further confirmed by weight loss analysis of samples before and after biotransformations. Samples were analysed by SEM as follows: polymer samples were sputter coated with Au/Pd (thickness 5 nm) to prevent charging during SEM imaging and were observed using secondary electron imaging in a Tescan SC Mira, FEG-SEM with an accelerating voltage of 5 kV and probe current of approximately 2 nA.

Tescan SC Mira, FEG-SEM with an accelerating voltage of 5 kV and probe current of approximately 2 nA.

Protein melting temperature (Tm) analysis. The melting temperatures (Tm) of HotPETase and its variants were determined using differential scanning fluorimetry. For each protein, a 50 μl sample of 5 mM protein was prepared in buffer (pH 9.2, 50 mM Gly-OH) with a final concentration of 10X SYPRO Orange dye stock solution (Sigma-Aldrich) in an optically clear, lidded PCR tube (Bio-rad). Differential scanning fluorimetry melt-curve experiments were conducted using a Bio-rad CFX Connect 96 Real-Time PCR system set on 95 °C with an increment of 0.3 °C s⁻¹. Each protein’s Tm was determined from a mean value for the peak of the first derivative of the melt curve from three replicate measurements.

Structure determination of HotPETase. Protein crystallization of HotPETase was achieved by sitting drop vapour diffusion of 20 nl of 6 mg ml⁻¹ protein mixed with an equal volume of reservoir solution and incubated at 20 °C. Crystals were observed after 72 h incubation with a reservoir solution comprising 0.85 M sodium citrate tribasic dehydrate, 0.1 M Tris, pH 8.0 and 0.1 M sodium chloride (LMB screen IT96117 Molecular Dimensions). Before data collection, crystals were cryogently protected with the addition of 20% PEG 200 to the mother liquor and plunged cooled in liquid nitrogen. All data were collected at Diamond Light Source. Data reduction was performed with Dials and the structure solved by molecular replacement using a search model derived from HotPETasewt structure PDB 5XHL. Iterative rounds of model building and refinement were performed in COOT and Phenix using phenix.refine and phenix.ensemble_refinement. Validation with MOLPROBITY and PDBREDO were incorporated into the iterative rebuild and refinement process. Data collection and refinement statistics are shown in Supplementary Table 4. The HotPETase coordinates and structure factors have been deposited in the PDB under accession number 7QVH. The 4PET docking simulations were performed in ICM-Pro and resulted into the iterative rebuild and refinement process. Data collection and refinement were conducted using a DSC 2500 TA instrument. Samples were run in triplicate, in series over a ∼50 to 300 °C temperature range under a nitrogen atmosphere, at a heating rate of ±10 °C min⁻¹ in a 40 μl aluminium crucible. The number and weight average molecular weights (Mn and Mw) of polymer chains were determined by SEC. Samples (4 mg) were dissolved in hexafluoro-2-propanol (120 μl) at room temperature. Once dissolved, HPLC-grade chloroform (1,880 T of approximately 2 nA.

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Author contributions

A.P.G and E.L.B designed and directed the research. E.L.B carried out molecular biology, enzyme characterization, assay development, directed evolution experiments and interpreted and presented the data. E.L.B., R.S and J.F carried out protein production and purification and performed biochemical assays. R.S carried out protein crystallization. P.J.R.D. discussed interpretations of biochemical assays. S.K. and M.P.S. carried out polymer characterization measurements and analysed and presented the associated data. S.R and S.J.H. performed microscopy and interpreted the data. A.A.T. and A.A.G carried out substrate milling. F.J.H and C.L. interpreted, analysed and presented structural data, and carried out molecular docking studies. A.P.G., E.L.B., M.P.S. and S.K. wrote the manuscript. All authors discussed the results and reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Supplementary information

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Extended Data Fig. 1 | Overview of the IsPETase directed evolution progression. The crystal structures represent an overview of the evolution, with the IsPETase<sup>Ts</sup> protein represented as a turquoise ribbon. The catalytic triad and W185 are shown in ball and stick representation coloured by all atoms, with blue and grey carbon atoms, respectively. The three mutations in IsPETase<sup>Ts</sup> compared to IsPETase<sup>Wt</sup> are highlighted as yellow spheres, mutations installed in directed evolution rounds 1-2 as light blue spheres, the rationally inserted additional disulphide bridge (DSB) as black spheres and mutations installed in rounds 3–6 as pink spheres. More details on the evolution outcomes can be found in Supplementary Table 1.
Extended Data Fig. 2 | Comparison of reactions with IsPETase$^{Ts}$ and HotPETase over a range of temperatures. 48 h time-courses of cryPET reactions, showing the mean total concentration of released MHET and TPA, with HotPETase (yellow-pink) and IsPETase$^{Ts}$ (blues) over time, using 0.4% cryPET substrate loading (4 g L$^{-1}$) and 0.29 mg g$^{-1}$ enzyme loading (0.04 μM). Reactions were performed in pH 9.2, 50 mM Gly-OH buffer, 4% BugBuster, in triplicate, with error bars representing the s.d. of the replicate measurements.
Extended Data Fig. 3 | Comparison of reactions with HotPETase and LCC44G over a range of temperatures. 48 h time-courses of cryPET reactions, showing the mean total concentration of released MHET and TPA, with HotPETase (yellows-pinks) and LCC44G (greens) over time, at a range of temperatures, using 0.4% cryPET substrate loading (4 g L⁻¹) and 0.29 mg g⁻¹ enzyme loading (0.04 μM). LCC44G was assayed in its reported optimal operating buffer: pH 8, 100 mM K-Pi; isPETase and its derivatives were assayed under the library screening buffer conditions: pH 9.2, 50 mM Gly-OH buffer, 4% BugBuster. Reactions were carried out in triplicate, with error bars representing the s.d. of the replicate measurements.
Extended Data Fig. 4 | Comparison of reactions with additional enzyme or substrate added. 48 h time-courses showing the mean total concentration of released MHEt and tPA, where following reaction with HotPETase at 60 °C for 24 h (pink) (using 0.4% cryPET substrate loading (4 g L⁻¹) and 0.29 mg g⁻¹ enzyme loading (0.04 μM)), either 0.29 mg g⁻¹ fresh enzyme (0.04 μM) (blue) or 4 g L⁻¹ fresh cryPET substrate (yellow) was added. Reactions were performed in pH 9.2, 50 mM Gly-OH buffer, 4% BugBuster, in triplicate, with error bars representing the s.d. of the replicate measurements.
Extended Data Fig. 5 | Comparison of HotPETase activity with different substrates at 40 °C and 60 °C under library screening conditions and optimised reaction conditions. Bar chart showing the mean total concentration of released MHET and TPA accumulated over 3 h (light pink) and 24 h (dark pink) at either 40 °C or 60 °C in reactions using HotPETase with different PET substrates (crystalline PET powder (cryPET), milled bottle-grade PET (bgPET), PET/PE composite film lid (PET/PE), 0.4% total substrate loading (4 g L⁻¹)). Reactions were performed using either library hit screening conditions (A): 0.29 mg g⁻¹ enzyme loading (0.04 μM), pH 9.2, 50 mM Gly-OH, 4% BugBuster or optimised conditions (B): 3.62 mg g⁻¹ enzyme loading (0.5 μM), pH 9.7, 50 mM Gly-OH buffer, 4% BugBuster. Reactions were carried out in triplicate; error bars represent the s.d. of the replicate measurements; each replicate measurement is represented as a black circle.
Extended Data Fig. 6 | Comparison HotPETase activity under standard and optimised reaction conditions. 24 h time-courses of reactions conducted at 60 °C with HotPETase, showing the mean percentage of cryPET depolymerized (0.4% cryPET substrate loading (4 g L⁻¹)), calculated using the concentration of MHEt and tPA produced. Standard conditions were: 0.29 mg g⁻¹ enzyme loading (0.04 μM), library screening buffer: pH 9.2, 50 mM Gly-OH, 4% BugBuster (dashed line). Optimised reaction conditions were: 3.62 mg g⁻¹ enzyme loading (0.5 μM), pH 9.7, 50 mM Gly-OH, 4% BugBuster (solid line). Reactions were carried out in triplicate; error bars represent the s.d. of the replicate measurements.
Extended Data Fig. 7 | Comparison of crystal structures and features of HotPETase and IsPETase. (a) A global superposition of IsPETase (yellow) and HotPETase (light blue). Mutations in IsPETase compared to IsPETase are highlighted with yellow spheres. Mutations installed during directed evolution are highlighted with pink spheres. The rationally inserted disulphide bridge is highlighted with black spheres. The catalytic triad and W185 are in ball and stick representation coloured by all atoms, with blue and grey carbon atoms, respectively. (b) The disulphide bridge is correctly formed between C233 and C282 in HotPETase. Electron density is 2Fo-Fc contoured at 1 sigma (blue) and 2 sigma (yellow). (c) The conversion of P181 in IsPETase to V181 in HotPETase (highlighted pink) results in extension of β-sheet 6, and the formation of an additional hydrogen bond (dashed lines) to L199. (d) In HotPETase, the wobbling tryptophan (W185, grey sticks), forms a π-stacking interaction (dashed line) with the installed Y214 (pink sticks).
Extended Data Fig. 8 | Comparison of HotPETase and HotPETase<sup>LR</sup>. (a) Protein melt curves for HotPETase and HotPETase<sup>K212N, E213S, Y214S</sup> (HotPETase<sup>LR</sup>). Melt curve readings were carried out in triplicate. (b) 24 h time-courses, showing the mean total concentration of released MHET and tPA, in reactions at either 40 °C or 65 °C with either HotPETase or HotPETase<sup>LR</sup>, using 0.4% cryPET substrate loading (4 g L<sup>−1</sup>) and 0.29 mg g<sup>−1</sup> enzyme loading (0.04 μM). Reactions were performed in pH 9.2, 50 mM Gly-OH buffer, 4% BugBuster, in triplicate; error bars represent the s.d. of the replicate measurements.
## Extended Data Table 1 | PET substrate characterization before and after enzyme reactions

| Substrate                  | Treatment          | % Crystallinity | $M_w$ (kDa) | $M_n$ (kDa) | Dispersity |
|----------------------------|--------------------|-----------------|-------------|-------------|------------|
| Amorphous PET film (amoPET) | No Treatment       | 6.7             | 58.0        | 22.4        | 2.51       |
|                            | Buffer-Only Control | 6.6             | 57.1        | 21.1        | 2.71       |
|                            | Enzyme Reaction    | 8.4             | 57.3        | 20.5        | 2.79       |
| Crystalline PET powder (cryPET) | No Treatment       | 29.8            | 83.3        | 34.6        | 2.40       |
|                            | Buffer-Only Control | 32.7            | 77.9        | 29.7        | 2.62       |
|                            | Enzyme Reaction    | 38.2            | 80.5        | 30.2        | 2.78       |
| Milled bottle-grade PET (bgPET) | No Treatment       | 39.9            | 72.2        | 23.9        | 2.77       |
|                            | Buffer-Only Control | 40.4            | 74.1        | 27.8        | 2.65       |
|                            | Enzyme Reaction    | 41.3            | 76.1        | 30.2        | 2.51       |
| PET/PE composite film      | No Treatment       | 1.5             | 56.3        | 21.0        | 2.73       |
|                            | Buffer-Only Control | 6.1             | 52.9        | 19.4        | 2.73       |
|                            | Enzyme Reaction    | 13.6            | 52.1        | 19.7        | 2.65       |

PET substrates were characterised pre-reaction (no treatment), following incubation in reaction buffer (buffer-only control) or following enzymatic depolymerization (enzyme reaction). Enzymatic reactions were conducted for 48 h with HotPETase at 60 °C, using 0.4% cryPET substrate loading (4 g L⁻¹) and 0.29 mg g⁻¹ enzyme loading (0.04 μM). Reactions were performed in pH 9.2, 50 mM Gly-OH buffer, 4% BugBuster. The negative, buffer-only control was prepared in a similar fashion without the addition of HotPETase. Crystallinity was assessed by DSC and molecular weights by SEC.
Extended Data Table 2 | Characterisation of cryPET substrate depolymerization under optimised conditions

| Substrate             | Treatment          | % Crystallinity | $M_w$ (kDa) | $M_n$ (kDa) | Dispersity |
|-----------------------|--------------------|-----------------|-------------|-------------|------------|
| Crystalline PET powder (cryPET) | No Treatment       | 29.8            | 83.3        | 34.6        | 2.40       |
|                       | Buffer-Only Control| 32.7            | 77.9        | 29.7        | 2.62       |
|                       | Enzyme Reaction    | 41.7            | 78.2        | 28.1        | 2.78       |

CryPET substrate was characterised pre-reaction (no treatment), following incubation in reaction buffer (buffer-only control) or following enzymatic depolymerization (enzyme reaction). Enzymatic reactions were conducted for 48 h with HotPETase at 60°C, using 0.4% cryPET substrate loading (4 g L$^{-1}$), 3.62 mg g$^{-1}$ enzyme loading (0.5 μM), pH 9.7, 50 mM Gly-OH, 4% BugBuster. The negative, buffer-only control was prepared in a similar fashion without the addition of HotPETase. Crystallinity was assessed by DSC and molecular weights by SEC.
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Policy information about availability of computer code

Data collection

Residues of interest were identified using PROSS; the Protein Repair One-Stop Shop, a publically available web server tool. Goldenzeig, A. et al. Automated Structure- and Sequence-Based Design of Proteins for High Bacterial Expression and Stability. Mol. Cell 2016, 63 (2), 397–346, https://pross.weizmann.ac.il/step/pross-terms/, and B-Fitter a publically available protein analysis software: Reetz, M. T. & Carballera, J. D. Iterative saturation mutagenesis (ISM) for rapid directed evolution of functional enzymes. Nature Protocols 2, 891–903 (2007), https://www.kofo.mpq.de/en/research/biocatalysis. These are cited within the text.

X-ray crystallography data were collected at Diamond Light Source (Harwell, UK).

NMR data was collected with a 400 MHz Bruker spectrometer.

SEM imaging was carried out using a Tescan SC Mira, FEG-SEM with an accelerating voltage of 5 kV and probe current of ~2 nA.

DSF melt-curve experiments were conducted using Biorad CFX Connect 96 Real-Time PCR system with the manufacturer’s software.

DSC data was obtained from using a DSC 2500 TA instrument.

SEC was performed on a system composed of an Agilent 1260 Infinity II LC system.

UPLC analysis was performed on a 1290 infinity II Agilent LC system.

Data analysis

Data reduction was performed with Dials and the structure solved by molecular replacement using a search model derived from IsPETaseWT structure SXIH. Iterative rounds of model building and refinement were performed in COOT and Phenix using phenix.refine and phenix.ensemble.refinement. Validation with MOLPROBITY and PDBREDO were incorporated into the iterative rebuild and refinement process.

HPLC peak integrations were carried out using Agilent OpenLab software, HPLC peak area data were analyzed and visualized in Microsoft Excel.

NMR Data were analyzed using Mestrenova software.

DSF melt-curves were analyzed using Biorad CFX Manager software.

DSC data were analyzed using TRIOS software (TA instruments).

Crystallography visualisation was carried out in Chimera and ICM Browser (Molsoft LLC).

The graphical abstract, Figure 1 and Supplementary Figure 9 were created using Biorender.com.
Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

Coordinates and structure factors for HotPETase have been deposited in the Protein Data Bank under accession number PDB: 7QVH. PDB 6UJ6 and PDB 5XH were also used in the study for comparisons.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences
- Behavioural & social sciences
- Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

**Sample size**
For enzymatic assays and Tm measurements the standard sample size was n=3, to allow the calculation of a mean and standard deviation. For some experiments in the Supplementary information n=2 was used to allow the calculation of an average. Results were reproducible, standard deviations were small and the replication of experiments was successful.

**Data exclusions**
None excluded

**Replication**
All results in triplicate or duplicate and stated, from which a mean and standard deviation were calculated. Standard deviations were small and replication of experiments was successful.

**Randomization**
Data was not subjected to randomization as it was not applicable to the experiments carried out.

**Blinding**
For substrate characterization, SEM imaging and HPLC analysis, the researchers were blinded as to which samples were being analyzed.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

**Materials & experimental systems**

| n/a | Involved in the study |
|-----|-----------------------|
| X   | Antibodies            |
| X   | Eukaryotic cell lines |
| X   | Palaeontology and archaeology |
| X   | Animals and other organisms |
| X   | Human research participants |
| X   | Clinical data         |
| X   | Dual use research of concern |

**Methods**

| n/a | Involved in the study |
|-----|-----------------------|
| X   | ChIP-seq              |
| X   | Flow cytometry        |
| X   | MRI-based neuroimaging |