Supporting Information

Multiple Substrate Binding Mode-Guided Engineering of a Thermophilic PET Hydrolase

Lara Pfaff1, Jian Gao2,3, Zhishuai Li2,4, Anna Jäckering5,6, Gert Weber7, Jan Mican8, Yinping Chen9, Weiliang Dong9, Xu Han2,3, Christian G. Feiler7, Yu-Fei Ao1,10, Christoffel P.S. Badenhorst1, David Bednar8,11, Gottfried J. Palm12, Michael Lammers12, Jiri Damborsky8,11, Birgit Strodel5,6, Weidong Liu2,3,4*, Uwe T. Bornscheuer1*, Ren Wei1*

1Department of Biotechnology & Enzyme Catalysis, Institute of Biochemistry, University of Greifswald, Felix-Hausdorff-Str. 4, 17487 Greifswald, Germany

2Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, 32 West 7th Avenue, Tianjin Airport Economic Area, Tianjin 300308, China

3National Technology Innovation Center of Synthetic Biology, 32 West 7th Avenue, Tianjin Airport Economic Area, Tianjin 300308, China

4University of Chinese Academy of Sciences, 19A Yuquan Road, Beijing, 100049 China
5Institute of Biological Information Processing: Structural Biochemistry (IBI-7), Forschungszentrum Jülich, Wilhelm-Johnen-Straße, 52428 Jülich, Germany

6Institute of Theoretical and Computational Chemistry, Heinrich Heine University, Düsseldorf, Universitätsstr. 1, 40225 Düsseldorf, Germany

7Helmholtz-Zentrum Berlin für Materialien und Energie, Hahn-Meitner-Platz 1, 14109 Berlin, Germany

8Loschmidt Laboratories, Department of Experimental Biology and RECETOX, Faculty of Science, Masaryk University, Kamenice 5/C13, 625 00 Brno, Czech Republic

9State Key Laboratory of Materials-Oriented Chemical Engineering, College of Biotechnology and Pharmaceutical Engineering, Nanjing Tech University, Nanjing, China

10CAS Key Laboratory of Molecular Recognition and Function, Institute of Chemistry, Chinese Academy of Sciences, Zhongguancun North First Street 2, 100190 Beijing, China

11International Clinical Research Center, St. Anne's University Hospital, Pekarska 53, 656 91 Brno, Czech Republic

12Department Synthetic and Structural Biochemistry, Institute of Biochemistry, University of Greifswald, Felix-Hausdorff-Str. 4, 17487 Greifswald, Germany

*Email: liu_wd@tib.cas.cn; uwe.bornscheuer@uni-greifswald.de; ren.wei@uni-greifswald.de
**Materials and Methods**

**Materials**

Chemicals and consumables were purchased from Merck KGaA (Darmstadt, Germany), Sigma-Aldrich (Steinheim, Germany), Carl Roth (Karlsruhe, Germany), TCI (Tokyo, Japan), Thermo Fisher Scientific (Waltham, MA, USA) and New England Biolabs GmbH (Frankfurt am Main, Germany). Oligonucleotides (Table S8) were ordered from Thermo Fisher Scientific (Waltham, MA, USA) and Eurofins (Ebersberg, Germany). Amorphous PET film (250 µm thickness, product number ES301445) was purchased from Goodfellow GmbH (Bad Nauheim, Germany).

**Protein production for crystallization, structure determination, and refinement**

The genes encoding PES-H1 and PES-H2 were synthesized by GENE ray Biotech Co. (Shanghai, China) and cloned into the pET-32a vector between the EcoRI and NotI restriction sites. Detailed nucleotide sequences encoding the enzymes are given after the Material and Methods. The pET-32a-pes-h1 and pET-32a-pes-h2 plasmids were transformed into *Escherichia coli* BL21trxB (DE3). Cells from single colonies were used to inoculate 5 mL lysogeny broth (LB) medium and grown at 37 °C to an optical density at 600 nm (OD₆₀₀) of approximately 0.8 before induction using 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) at 16 °C for 18 h. Cells were harvested by centrifugation at 5,000 g for 15 min and then resuspended in lysis buffer consisting of 25 mM Tris-HCl (pH 7.5), 150 mM sodium chloride (NaCl), and 20 mM imidazole, followed by disruption using a French Press (800-1200 bar, 2-5 cycles). Cell debris were removed by centrifugation at 17,000 g for 1 h at 4 °C. The supernatant was then applied to a 5 mL Ni-NTA column on an FPLC system (GE Healthcare, Chicago, USA) for protein purification. Target proteins were eluted at 100 mM imidazole when a 20-250 mM imidazole
gradient was applied. Each protein was dialyzed against 25 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, and subjected to tobacco etch virus (TEV) protease digestion overnight to remove the hexahistidine (His\textsubscript{6})-tag. The solutions were then passed through an Ni-NTA column again. The untagged target protein was eluted with 25 mM Tris-HCl (pH 7.5) containing 150 mM NaCl. The purity of each protein (>95%) was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The purified proteins were concentrated to \(\sim 20\) mg mL\(^{-1}\) for screening crystallization conditions.

**Crystallization, data collection, structure determination, and refinement**

All crystallization experiments were conducted at 25 \(^\circ\)C using the sitting-drop vapor-diffusion method. In general, 1 \(\mu\)L of a PES-H1 or PES-H2 containing solution (15 mg mL\(^{-1}\) in 25 mM Tris-HCl (pH 7.5) containing 150 mM NaCl) were mixed with 1 \(\mu\)L of reservoir solution in 48-well Cryschem plates and equilibrated against 100 \(\mu\)L of the reservoir solution. The two optimized crystallization conditions for PES-H1 were: 17\% w/v polyethylene glycol (PEG) 6000, 0.1 M citric acid (cit) (pH 5.0), 30\% 2-methylpentane-2,4-diol (MPD) and 8\% Tacsimate (pH 4.0), 0.1 M citric acid (pH 5.0), 20\% PEG3350.

The two optimized crystallization conditions for PES-H2 were: 32\% PEG 2000 MME, 0.1 M KBr and 24\% PEG4000, 0.1 M citric acid (pH 5.6), and 9\% isopropanol. Within one week, the crystals reached sizes suitable for X-ray diffraction. The crystals in complex with 4-(2-hydroxyethylcarbamoyl) benzoic acid (MHETA) or bis(2-hydroxyethyl) terephthalate (BHET) were obtained by soaking them with their respective mother liquor containing substrate powder for 72 h. All X-ray diffraction data sets were collected at the Beamlines BL10U2(BL17U1)/BL17B/BL18U1/BL19U1 of the National Facility for Protein Science in
Shanghai (NFPS) at the Shanghai Synchrotron Radiation Facility (SSRF), and Beamline 14.1 at Berlin using the third-generation synchrotron radiation source (BESSY). The crystals were mounted in a CryoLoop and soaked with cryoprotectant solution prior to data collection at 100 K. The diffraction images were processed by using HKL2000.1 All crystal structures were solved by the molecular replacement (MR) method with the Phaser program2 from the Phenix3 suite using the structure of the IsPETase from *Ideonella sakaiensis* 201-F6 (PDB code: 5XG0) as the search model.4 Further refinement was carried out using the programs phenix.refine5 and Coot.6 Prior to the structural refinements, 5% randomly selected reflections were set aside for calculating $R_{\text{free}}$ as a monitor. Data collection and refinement statistics are summarized in Tables S1 and S2. All figures were prepared using PyMOL (http://pymol.sourceforge.net).

**Molecular dynamics simulations**

To model and analyze the PET-PES-H1 interactions, we used a PET trimer (3PET) to mimic the polymeric substrate of PES-H1. For correctly placing 3PET into the binding cleft, PyMOL8 was used to superimpose the central 3PET unit with the position of 1-2-(hydroxyethyl) 4-methyl terephthalate (HEMT) in complex with IsPETase (PDB code 5XH3).4 Generalized AMBER force field (GAFF) parameters9 were derived for 3PET by performing quantum mechanics calculations at the HF6-31G* level using Gaussian 0910. The partial charges were determined by restrained electrostatic potential (RESP) calculations11,12 using the antechamber tool of the Antechamber package9,13,14 available in AmberTools 2115 and redistributed to yield a net charge of zero for each 3PET unit using the Antechamber tool Prepgen (see GAFF parameters). All molecular dynamics (MD) simulations were carried out using GROMACS 2020.416 using the AMBER14SB17 protein force field with Parmbsc1 parameters18 and the newly derived PET parameters to determine the
potential energy and forces. After determining the pKa of titratable residues using Propka3 3.4.0, hydrogens were added to or removed from the PES-H1 crystal structure to mimic a protonation state at pH 7. The PES-H1-3PET complex was centered in a periodic dodecahedron box with at least 1 nm distance to the box edges, solvated with water described by the TIP3P water model\textsuperscript{19} and neutralized by adding Na\textsuperscript{+} ions, resulting in a system containing 32,990 atoms in total. Energy minimization of the system was conducted using the steepest descent algorithm\textsuperscript{20} prior to a two-step equilibration, first in the NVT ensemble for 0.2 ns, followed by a 1 ns equilibration simulation in the NpT ensemble to set the temperature to 303 K (30 °C, Nosé-Hoover thermostat)\textsuperscript{21,22} and the pressure at 1.0 bar (Parrinello-Rahman barostat).\textsuperscript{23} The final 100 ns production run was performed in triplicates using different randomly generated initial velocities. The electrostatic interactions were calculated using the particle-mesh Ewald method\textsuperscript{24,25} and a cutoff of 8 Å was applied to both the electrostatic and Lennard-Jones (LJ) interactions. The bonds were constrained using the LINCS algorithm\textsuperscript{26}, which allows for a time step of 2 fs. Coordinates were saved every 20 ps.

The three trajectories were concatenated and the 3PET conformations were clustered using the algorithm described by Daura et al.\textsuperscript{27} together with a cutoff of 2 Å applied to the root-mean-square deviation (RMSD) to assign the cluster membership. This resulted in the identification of four particularly stable binding poses corresponding to the four most populated clusters (Figure S3C). All MD snapshots belonging to each of the four clusters were retrieved and submitted to further analysis per binding pose. The protein residues putatively interacting with 3PET were identified using the Visual Molecular Dynamics (VMD)\textsuperscript{28} software and the strength of these residue-3PET interactions was energetically quantified.
**Enzyme production and purification for the enzymatic hydrolysis of PET substrates**

The genes encoding PES-H1 and the reference enzyme LCC ICCG were codon-optimized for expression in *E. coli*, synthesized and cloned into the pET-28a(+) vector and the pET-26b(+) vector by BioCat GmbH (Heidelberg, Germany) between the NcoI (for PES-H1) or NdeI (for LCC ICCG) and XhoI restriction sites. The plasmids (pET-28a(+)-*pes-h1* and variants) were used to transform competent *E. coli* SHuffle® T7 Express or *E. coli* BL21 (DE3). For the expression in *E. coli* SHuffle® T7 Express, the cells were grown in LB medium supplemented with kanamycin (50 µg mL⁻¹) at 30 °C to OD₆₀₀ of ~1 and then induced using 1 mM IPTG at 16 °C for 12 h. LCC ICCG and PES-H1 variants were expressed in autoinduction medium (ZYM-5052) supplemented with kanamycin (50 µg mL⁻¹) at 21 °C for 23 h and 20 h, respectively, as described previously.²⁹ Cells were harvested by centrifugation at 10,000 g for 30 min, resuspended in lysis buffer (50 mM sodium phosphate (pH 8.0), 300 mM NaCl) and lysed by ultrasonication (2×3 min, 50% power, 50% amplitude). Cell debris were removed by centrifugation at 10,000 g for 30 min and the enzymes were purified using cobalt-ion affinity chromatography (ROTI®Garose-His/Co Beads, Carl Roth, Karlsruhe, Germany). Unbound proteins were removed using lysis buffer (pH 8.0) supplemented with 20 mM or 50 mM imidazole. PES-H1 and LCC ICCG were eluted with elution buffer (50 mM sodium phosphate (pH 8.0), 300 mM NaCl, and 100 mM or 250 mM imidazole, respectively). The target proteins were desalted with 50 mM sodium phosphate buffer (pH 8.0) and concentrated using Vivaspin™ ultrafiltration devices (10 kDa MWCO, Cytiva, Freiburg, Germany). Enzyme purities were verified by SDS-PAGE analysis.
Site-directed mutagenesis

Enzyme variants were generated using the Q5® Site-Directed Mutagenesis Kit (New England Biolabs GmbH, Frankfurt am Main, Germany). The sequences of the mutagenesis oligonucleotides are listed in Table S8. Mutagenesis was verified by Sanger sequencing (Eurofins Genomics Germany GmbH, Ebersberg, Germany).

Melting temperature measurements

The $T_m$ values of the PES-H1 variants were determined by nano differential scanning fluorimetry (NanoDSF) using the Prometheus NT.48 (NanoTemper Technologies, Munich, Germany) and differential scanning calorimetry (DSC; TA Instruments, Newcastle, USA). The measurements were conducted using protein concentrations of 0.5 mg mL$^{-1}$ or 0.3 mg mL$^{-1}$ to determine temperature profiles from 20 °C to 95 °C and 20 °C to 100°C at 1 °C per minute, respectively. The nanoDSF instrument has a fixed excitation wavelength of 285 nm and records emitted light at 330 nm and 350 nm.

Enzymatic hydrolysis of PET film and PET powder

The degradation of amorphous Gf-PET PET film (7% crystallinity) was performed using an enzyme concentration of 0.5 mg$_{enzyme}$ g$_{PET}^{-1}$, 1 mg$_{enzyme}$ g$_{PET}^{-1}$, or 2 mg$_{enzyme}$ g$_{PET}^{-1}$ in 1.5 mL potassium phosphate buffer (1 M, pH 8.0) by shaking at 1,000 rpm and 72 °C on a ThermoMixer C (Eppendorf, Hamburg, Germany) for 12 h or 24 h. The reaction conditions were adapted from a published patent application. The hydrolysis reactions were performed in triplicate. Gf-PET films were cut into small pieces of ~2 cm × 1 cm and washed with 0.1% SDS solution, ultrapure H$_2$O and absolute ethanol before drying at room temperature for 24 h. Weight loss as a result of
enzymatic degradation was determined gravimetrically. The degradation of PET powder (13%, 26%, or 33% crystallinity) was conducted in the same way with an enzyme concentration of \(1 \text{ mg}_{\text{enzyme}} \text{ g}_{\text{PET}}^{-1}\) for 24 h. The degradation products were analyzed by reversed-phase high-performance liquid chromatography (HPLC) on a VWR Hitachi LaChrom Elite system (VWR International, Radnor, USA) equipped with a Kinetex\textsuperscript{®} column (5 \(\mu\)M EVO C18 100 Å, 150x4.6 mm; Phenomenex\textsuperscript{®}, Aschaffenburg, Germany).\textsuperscript{31} Samples (10 \(\mu\)l) were injected and separated at 30 °C with a gradient of acetonitrile in water containing 0.1% (v/v) formic acid. Acetonitrile was increased from 5% to 44% over 12 min and then to 70% over 3 min, after which the ratio remained constant for a further 3 min, using a flow rate of 0.8 mL min\(^{-1}\). Terephthalate (TPA), mono-(2-hydroxyethyl) terephthalate (MHET), and BHET were detected by UV absorbance at 240 nm and quantified based on a calibration curve obtained with commercially available TPA in the concentration range from 0 to 1 mM.

**Kinetic analysis**

For the kinetic analysis of PET nanoparticle (PET-NP) degradation by PES-H1 and its variants, PET-NPs were produced as previously described.\textsuperscript{32-34} For determining the kinetic parameters, a turbidimetric assay was applied as described before.\textsuperscript{35} A mixture of 3 mL ROTIPHORESE\textsuperscript{®} gel 40, 3 mL potassium phosphate buffer (1 M, pH 8.0), 120 \(\mu\)L tetramethylethylenediamine and PET-NP suspension (1.7 mg mL\(^{-1}\)) was prepared and filled up to a final volume of 15 mL with ultrapure H\(_2\)O. Ammonium persulfate (2 \(\mu\)L of a 40% (w/v) solution) and the enzyme solution were added to a total volume of 50 \(\mu\)L and mixed with 150 \(\mu\)L of the substrate- and acrylamide-containing mixture. The turbidimetric assay monitoring the enzymatic hydrolysis of PET-NP was performed at 600 nm in a microtiter plate at 70 °C for 40 min with 0.5 mg mL\(^{-1}\) PET-NP and varying enzyme
concentrations in the range of 0-2 µM. In addition, the degradation of PET-NP was also carried out with a constant enzyme concentration of 0.7 µM and substrate concentrations ranging from 0 to 0.5 mg mL⁻¹. All determinations were performed in triplicate.

A kinetic model (equation 1) based on previous studies,⁶⁻⁸ was used to analyze the heterogeneous kinetics of the PET-NP degradation, where $v_0$ is the initial reaction rate; $k_h$, the hydrolysis rate constant; $[S_0]$, the initial substrate concentration; $[E_0]$, enzyme concentration, and $K_A$, the adsorption equilibrium constant.

$$v_0 = k_h [S_0] \frac{K_A[E_0]}{1 + K_A[E_0]}$$  

(1)

**PET powder production and analysis**

*Pretreatment of waste PET bottles*

The bottle wall part of collected post-consumer PET plastic bottles was cut into small flakes of about 5 cm × 5 cm for more even melting and crushing processes. PET flakes were washed with ultrapure water and then dried in the drying oven. Before the melting experiment, the dried PET flakes were placed in an aluminum tray and sealed with tin foil to prevent oxidation. Then, the temperature was set to 280 °C in the high-temperature reactor for 40 min. This temperature exceeds the $T_m$ of PET (250-260 °C) and can thus result in homogeneous melts of PET. Low-crystallinity PET (lcPET) can be obtained by rapidly cooling the molten PET with ice-cold water within 10 s (quenching step). The melt quenched PET was crushed into powders using a portable herb grinder (Model 800Y, Yongkang Boou Hardware Products Co., Ltd., Yongkang, China) for 5 min or a planetary ball mill (Model PM2L, Shanghai Droide Instruments Ltd., Shanghai, China) for 30 min. The herb grinder uses a rotating blade to mechanically grind the melt-quenched PET. The temperature of the portable herb grinder could not be controlled and increased above room
temperature during grinding. In the ball milling process, the melt-quenched PET is mixed with steel milling balls, followed by continuous rotation at a speed of 400 rpm to grind the plastics through the collision of the beads. The temperature of the ball mill was constantly maintained at room temperature. Finally, PET powders with different particle sizes were collected and sieved with different mesh sizes. Similarly, Gf-PET films were crushed using the herb grinder and sieved in the same manner to produce lcPET powders for enzymatic degradation.

**Characterization of PET samples**

The crystallinity of PET was measured by differential scanning calorimetry (DSC) on an TAQ2000 instrument (TA instruments, Newcastle, USA). Thermograms were monitored from the first heating process from 50 to 300 °C at a rate of 10 °C min⁻¹ under a nitrogen atmosphere. Under these conditions, the crystallinity of virgin PET (waste PET bottles and commercial PET films), melt-quenched PET, and PET powders obtained by different pulverization methods were determined as described in a previous publication.³⁹

Additionally, gel permeation chromatography (GPC) measurements were performed using an Agilent PL-GPC50 (Agilent, California, USA) device to detect the molecular weight distributions of various PET samples dissolved in hexafluoro-2-propanol.

The crystallinity, the number average molecular weight (Mn), and the weight average molecular weight (Mw) of the PET samples are summarized in Table S6.
Using Ellman’s reagent to determine the presence of a disulfide bond

To quantify the number of free cysteine residues, Ellman’s reagent (5,5’-dithiobis-(2-nitrobenzoic acid, DNTB) was used. 25 µL of 1 mg mL⁻¹ R204C/S250C were incubated with 255 µL of freshly prepared Ellman’s reaction solution (2 mg mL⁻¹ DTNB in 0.1 M sodium phosphate buffer, pH 8.0) and 250 µL 0.1 M sodium phosphate buffer (pH 8.0) containing 0.1 M EDTA and incubated for 15 min. The absorption of TBN₂⁻ was measured at 412 nm. To calculate the concentration of free cysteine residues, a calibration curve prepared with cysteine solutions at concentrations from 0 to 1.5 mM was used.
Nucleotide sequences

PES-H1

Codon-optimized nucleotide sequence of PES-H1 cloned between the NcoI and XhoI restriction sites of the pET-28b(+) bacterial expression plasmid and between the EcoRI and NotI restriction sites of the pET-32a bacterial expression plasmid.

ATGGCTAAACCCTATGAAACGCGGCCCGGATCCGACCAGAAAGCAGCATTGAAGCGGT
GCGCGGCCCCTTTTGCGTGCGCAGACCACCCTCAGCCCGTCTGCAAGGCGGATGGCT
TTGGCGGCGGCCACCATTATTATCCGACCCGATACCCAGCCAGGACACCTTTTGCGCGG
TGGCGATCAGCCCGGCTTTTACCACCCTCGTCAAGAAAGCATTTGCCTGGCTGGGCCC
CGTATTGCAAGCCAGGCTTTTGTGATTACCATTGATACCCATACCCTTACGTCGTGAT
CAGCCCGGACAGCCCGCTCGTCAGTTGCAGCCGCGCGCTGGGATCATCTGCGTACCAA
CAGCGTGGTGTAACCTGATCAGCCGATACCCACCTGGAGCAGCGTGCGAGCCGAC
GCTGGTGGTTGCCGCGCTGCTGGATACCCGAAACACCTGGAGCAGCGTGCGAGCCGAC
GCTGGTGGTTGCCGCGCTGCTGGATACCCGAAACACCTGGAGCAGCGTGCGAGCCGAC
GCTGGTGGTTGCCGCGCTGCTGGATACCCGAAACACCTGGAGCAGCGTGCGAGCCGAC
PES-H2

Codon-optimized nucleotide sequence of PES-H2 cloned between the EcoRI and NotI restriction sites of the pET-32a bacterial expression plasmid.

ATGGAAAAACCCGTATGAACGTGGCCCCGGATCCGACCAGCAAGCAGCATTTGAAGCGAGTT
TCGCGGCCCCTTGGCAGTGGCCCCAGACCACCCTGAGTCGCTTACAGGCAGATGGTTTT
TGTTGGCCGCACTATTTATATCCAGGACCCGAGGAAGATATTGCGCTGGTTAGGTAGGTCGCCGT
GGCCATTCCCACGGGCTTTTACCCGAGGCCCCAGGAAGATATTGCGCTGGTTAGGTAGGTCGCCGT
TATTGCCTCTCAGGGCTTTGTTGTGATTACCATTTGATACCTACATTACCCGTACCTAGATCAG
CCGATTACGCCTCTGTCGTCAGTTACAGGCAGCAGCGAATCTGATCTGCTGACCGAGCCTTCTTCTCT
GTTGTTCTCTAATCGTATTGATCCGAATTGCAACGGCATGGCATTTATGAGGTCATAGCATGGGT
GGCGGCAGCGCCCTTAAAGTGCAAGCCGCTAAAATATGGGAAGTACGCTTGCAACCAGGCACCTAGTT
GTGGGCACGCGCATACCCGGTAAAAATTTGGAAGTACGCTTGCAACCAGGCACCTAGTT
AATTCACTGCGACGGATTTAGATAAAGCCTATATGAAACTGCGTGATGATGCTACACAT
TTTGTGCTAATACCAGCGATACCCAGACCACCCGCCAATATAGTATTGGCTGGCTGAAA
CGCTTTTGTGATAATGATCCTCGCATAGAAGTTTCTGTTGCTTGCGCCCGCGTGAGATT
TTGCAATTTAGCGAATATCGTGCAACCTGTCGGTTTAA
Table S1. Data collection and refinement statistics of PES-H1-apo, PES-H1-cit, PES-H2-apo and PES-H2-PEG crystals.

|                      | PES-H1-apo | PES-H1-cit | PES-H2-apo | PES-H2-PEG |
|----------------------|------------|------------|------------|------------|
| PDB code             | 7CUV       | 7E30       | 7W69       | 7E31       |
| Data collection      |            |            |            |            |
| space group          | P2₁        | P2₁2₁2₁    | C2         | P2₁2₁2₁    |
| -cell                |            |            |            |            |
| a [Å]                | 52.01      | 55.97      | 105.52     | 55.34      |
| b [Å]                | 69.54      | 97.08      | 55.34      | 94.86      |
| c [Å]                | 74.85      | 99.11      | 51.15      | 105.5      |
| α /β /γ (°)          | 90/90/90   | 90/90/90   | 90/3.36/90 | 90/90/90   |
| resolution [Å]       | 25.0-1.45  | 25.0-1.56  | 22.9-1.56  | 24.3-1.38  |
|                      | (1.50-1.45)| (1.62-1.56)| (1.62-1.56)| (1.43-1.38)|
| unique reflections   | 89271 (8550)| 77551 (7586)| 41976 (4156)| 114425 (11157)|
| redundancy           | 6.8 (3.8)  | 5.0 (3.2)  | 9.4 (6.4)  | 7.5 (4.3)  |
| completeness [%]     | 96.9 (93.5)| 99.9 (99.6)| 99.86 (99.43)| 99.78 (98.39)|
| average I/σ(I)       | 15.1 (2.5) | 18.5 (3.02)| 22.4 (2.80)| 49.3 (2.64)|
| Rmeans               | 0.18 (0.53)| 0.084 (0.48)| 0.12 (0.65)| 0.056 (0.52)|
| CC 1/2               | 0.994 (0.872)| 0.993 (0.838)| 0.993 (0.853)| 0.992 (0.829)|
| Refinement           |            |            |            |            |
| no. of reflections   | 89157 (8383)| 77449 (7581)| 41964 (4156)| 114421 (11157)|
|                      | 4457(419)| 3866 (373)| 2101 (210)| 5721 (557)|
| Rwork[a] (95 % of data) | 0.163 (0.214)| 0.152 (0.203)| 0.1838 (0.2487)| 0.1672 (0.2244)|
| Rfree[a] (5% of data)         | 0.180 (0.246) | 0.172 (0.225) | 0.1928 (0.2475) | 0.1772 (0.2426) |
|-------------------------------|---------------|---------------|-----------------|-----------------|
| RMSD bonds [Å]                | 0.009         | 0.013         | 0.013           | 0.008           |
| RMSD angles [°]               | 1.534         | 1.649         | 1.64            | 1.41            |
| dihedral angles               |               |               |                 |                 |
| most favored [%]              | 98.2          | 97.8          | 97.66           | 98.24           |
| allowed [%]                   | 1.8           | 2.2           | 2.34            | 1.76            |
| disallowed [%]                | 0             | 0             | 0               | 0               |

Average B-factor/ Number of non-hydrogen atoms

|              | Protein   | 16.25/3912 | 18.29/3924 | 20.37/3936 | 14.61/1968 |
|--------------|-----------|------------|------------|------------|------------|
| Ion/ligands  | 32.35/47  | 43.23/20   | 29.84/10   |            |            |
| solvent      | 31.10/653 | 32.03/555  | 33.86/773  | 29.75/313  |            |

Values in parentheses are for the highest resolution shell.
**Table S2.** Data collection and refinement statistics of PES-H1-MHETA, PES-H1-MHETA2, PES-H2-MHETA3 and PES-H2-BHET crystals.

|                           | PES-H1-MHETA1 | PES-H1-MHETA2 | PES-H1-MHETA3 | PES-H2-BHET |
|---------------------------|---------------|---------------|---------------|-------------|
| **PDB code**              | 7W6C          | 7W6O          | 7W6Q          | 7W66        |
| **Data collection**       |               |               |               |             |
| **space group**           | P2₁           | P2₁           | P2₁           | P2₁,2,2₁    |
| **cell**                  |               |               |               |             |
| a [Å]                     | 52.51         | 52.67         | 52.71         | 105.84      |
| b [Å]                     | 56.01         | 56.19         | 56.22         | 55.62       |
| c [Å]                     | 102.31        | 100.03        | 108.62        | 95.88       |
| α /β /γ (°)               | 90/94.41/90   | 90/93.91/90   | 90/93.78/90   | 90/90/90    |
| **resolution [Å]**        | 29.5-2.3      | 25.8-2.2      | 27.1-2.2      | 49.2-1.96   |
| (2.382-2.3)               | (2.279-2.2)   | (2.279-2.2)   | (2.03-1.96)   |             |
| **unique reflections**    | 26497 (2642)  | 29919 (2947)  | 30069 (2951)  | 39103 (4057)|
| **redundancy**            | 5.6 (5.4)     | 6.4 (6.7)     | 6.3 (6.3)     | 6.2 (5.2)   |
| **completeness [%]**      | 99.45 (98.80) | 99.84 (100.00)| 99.83 (99.97) | 94.36 (99.78)|
| **average I/σ(I)**        | 9.2 (6.0)     | 22.2 (18.6)   | 22.7 (17.6)   | 13.0 (2.5)  |
| **Rmeans**                | 0.19 (0.35)   | 0.09 (0.12)   | 0.083 (0.105) | 0.109 (0.75)|
| **CC 1/2**                | 0.979 (0.911) | 0.995 (0.985) | 0.996 (0.987) | 0.998 (0.797)|
| **Refinement**            |               |               |               |             |
| **no. of reflections**    | 26482 (2642)  | 29906 (2947)  | 30051 (2952)  | 39072 (4056)|
|                           | 1352 (122)    | 1479 (144)    | 1517 (147)    | 1954 (202)  |
| **Rwork[a] (95 % of data)** | 0.2487 (0.2789) | 0.2224 (0.2575) | 0.2250 (0.2567) | 0.1976 (0.2879)|
|                          | Rfree[a] (5 % of data) | 0.2791 (0.2733) | 0.2657 (0.3247) | 0.2550 (0.2916) | 0.2281 (0.3240) |
|--------------------------|------------------------|------------------|------------------|------------------|------------------|
| RMSD bonds [Å]           | 0.013                  | 0.006            | 0.006            | 0.005            |
| RMSD. angles [°]         | 1.82                   | 0.90             | 0.96             | 0.81             |
| dihedral angles          |                        |                  |                  |                  |
| most favored [%]         | 94.71                  | 95.49            | 94.51            | 97.65            |
| allowed [%]              | 5.29                   | 4.51             | 5.49             | 2.35             |
| disallowed [%]           | 0.00                   | 0.00             | 0.00             | 0.00             |

Average B-factor/ Number of non-hydrogen atoms

|                | Protein                 | 18.35/3912       | 15.09/3912       | 16.21/3912       | 24.89/3916       |
|----------------|-------------------------|------------------|------------------|------------------|------------------|
| Ion/ligands    | 38.57/30                | 30.84/15         | 31.07/15         | 36.50/18         |
| solvent        | 17.78/169               | 21.14/347        | 22.78/382        | 32.81/363        |

Values in parentheses are for the highest resolution shell.
Table S3. Backbone RMSD values calculated based on the crystal structures of selected homologous PET hydrolases to that of PES-H1 (PDB code: 7CUV). *Bur*PL: *Is*PETase-like enzyme from *Burkholderiales* bacterium RIFCSPLOWO2_02_FULL_57_36. *Rg*PETase: PETase from *Rhizobacter gummiphilus*.

| Enzyme                        | PDB ID | Main chain RMSD (Å) |
|-------------------------------|--------|---------------------|
| PES-H1                        | 7CUV   | 0.000               |
| PES-H1-cit                    | 7E30   | 0.259               |
| PES-H1-MHETA1                 | 7W6C   | 0.358               |
| PES-H1-MHETA2                 | 7W6O   | 0.209               |
| PES-H1-MHETA3                 | 7W6Q   | 0.232               |
| PES-H2                        | 7W69   | 0.207               |
| PES-H2-PEG                    | 7E31   | 0.208               |
| PES-H2-BHET                   | 7W66   | 0.258               |
| lipase                        | 1JFR   | 0.388               |
| Est119                        | 3VIS   | 0.541               |
| TfCut2                        | 4CG1   | 0.457               |
| LC-cutinase                   | 6THT   | 0.644               |
| Cut190<sup>522→P</sup>       | 4WFI   | 0.471               |
| *Thermobifida fusca* cutinase | 5ZOA   | 0.459               |
| *Is*PETase                    | 5XG0   | 0.512               |
| *Bur*PL                       | 7CWQ   | 0.578               |
| LCC cutinase from Biortus     | 7DS7   | 0.655               |
| PE-H                          | 6SBN   | 0.732               |
| PET2 F105R/E110K              | 7EC8   | 0.766               |
| *Rg*PETase                    | 7DZT   | 0.605               |
| PHL-7                         | 7NEI   | 0.285               |
**Table S4.** Characteristics of the four binding positions observed by clustering. In total, 252 clusters were found. The distance between the catalytic S130 and the carbonyl carbon of the central PET unit was averaged over all frames belonging to the respective cluster.

|                | Total / 1-4 | Cluster 1 | Cluster 2 | Cluster 3 | Cluster 4 |
|----------------|-------------|-----------|-----------|-----------|-----------|
| frames         | 15003 / 8765| 4253      | 2592      | 1136      | 784       |
| Fraction 1st   | 100% / 58.40%| 28.35%    | 17.28%    | 7.57%     | 5.23%     |
| Fraction 2nd   | 5001 / 3746 | 2074      | 101       | 1133      | 438       |
| Fraction 3rd   | 5001 / 2836 | 2041      | 790       | 3         | 2         |
| Distance [Å]   | Ser130-O - PET-C | 3.27 ± 0.30 | 4.12 ± 0.31 | 4.48 ± 0.34 | 3.58 ± 0.26 |
**Table S5.** Melting temperatures of PES-H1 and variants in potassium phosphate buffer. Values which were not determined are abbreviated with "n.d."

| Buffer                  | Tₘ [°C]     |
|-------------------------|-------------|
|                         | PES-H1 | L92F/Q94Y | R204C/S250C |
| 0.05 M potassium phosphate | 77.1   | 78.2     | 76.8        |
| 0.1 M potassium phosphate   | n.d.   | n.d.     | 79.6        |
| 0.5 M potassium phosphate   | n.d.   | n.d.     | 88.2        |
| 1 M potassium phosphate    | 84.9   | 86.7     | 91.3        |
Table S6. Crystallinity and molecular mass of PET materials before and after pretreatments determined by DSC and GPC, respectively. Values which were not determined are abbreviated with "n.d." $M_w$: weight average molecular mass, $M_n$: number average molecular mass.

| Sample name                                           | Crystallinity (%) by DSC | Molecular mass by GPC |
|-------------------------------------------------------|--------------------------|-----------------------|
| Goodfellow PET film untreated                          | 7%                       | $M_w$:39872           |
|                                                       |                          | $M_n$:19652           |
| PET nanoparticles prepared with Gf PET film           | n.d.                     | $M_w$:8864            |
|                                                       |                          | $M_n$:5770            |
| Crushed PET film powder (particle size: 177 µM mesh)  | 13%                      | $M_w$:26197           |
|                                                       |                          | $M_n$:20874           |
| Post-consumer PET bottle (untreated)                  | 27%                      | $M_w$:32304           |
|                                                       |                          | $M_n$:14885           |
| PET bottle after melt-quenching                       | 19%                      | $M_w$:30214           |
|                                                       |                          | $M_n$:14542           |
| crushed PET bottle powder (particle size: 177 µM mesh)| 33%                      | $M_w$:26667           |
|                                                       |                          | $M_n$:13290           |
| ball-milled PET bottle powder (particle size: 177 µM mesh) | 26%                       | $M_w$:22266           |
|                                                       |                          | $M_n$:16224           |
Table S7. Kinetic parameters for PET-NP hydrolysis by PES-H1, its variants, and LCC ICCG. $K_A$= adsorption equilibrium constant. $k_h$= hydrolysis rate constant, $k_h \times K_A$= pseudo-catalytic efficiency.

|                  | $K_A$ [µM$^{-1}$] | $k_h$ [mL mg$^{-1}$ min$^{-1}$] | $k_h \times K_A$ [mL µM$^{-1}$ mg$^{-1}$ min$^{-1}$]* |
|------------------|-------------------|---------------------------------|-----------------------------------------------------|
| PES-H1 wild-type | 4.710 ± 0.087     | 0.054 ± 0.001                   | 0.254                                               |
| L92F/Q94Y        | 8.259 ± 0.028     | 0.065 ± 0.002                   | 0.537                                               |
| R204C/S250C      | 4.063 ± 0.191     | 0.064 ± 0.001                   | 0.260                                               |
| LCC ICCG         | 5.953 ± 0.032     | 0.048 ± 0.001                   | 0.286                                               |

*We can clearly show a 2.1-fold higher “pseudo-catalytic efficiency” value (mathematically equivalent to the catalytic efficiency described by $k_{cat}/K_M$ based on the conventional Michaelis-Menten kinetics) with L92F/Q94Y compared to the wild-type enzyme. In addition, the ranking of the enzymes in terms of this value is remarkably consistent with that determined in the hydrolysis of amorphous PET materials as shown in Figures 4A and 4B. However, this pseudo-catalytic efficiency parameter based on the Langmuir-isotherm-based model is not yet properly defined and fully understood, which does not allow us to give a clear statement in the main text.
Table S8. Oligonucleotide primers used for the generation of PES-H1 variants.

| Variant        | Sequence (5’ → 3’) |
|----------------|--------------------|
| L92F/Q94Y-fw   | TTATCCGGACAGCCGCGGTCTG |
| L92F/Q94Y-rv   | TCAAAACGGGTGATGTTATCAATGGTAAATCACCAC |
| S204C-fw       | TATGGAACCTGTCGCTGCCAGCC |
| S204C-rv       | TAGGCTTTATTCCAGATCGCTCGG |
| S250C-fw       | TTTTGCCATCTGCAGATATCGCAGGCACCTGC |
| S250C-rv       | TCATCCGGCGCGGGGCCAC |
Figure S1. Structure of PES-H1. The structure of PES-H1 is represented as a cartoon. The catalytic triad (red dashed circle, top left) and disulfide bridge (red labels) are shown as sticks. The α-helices, β-sheets and loops are shown in light blue, purple, and cyan, respectively.

Figure S2. RMSD of the PES-H1 calculated against its crystal structure during the 3 × 100 ns MD simulations. The protein backbone atoms were used for this calculation. The three simulations were concatenated for this analysis and the start of the second and third simulation is indicated by vertical dotted lines. The RMSD values below 1 Å confirm the stability of the protein in the simulations.
Figure S3. RMSD of 3PET from its starting structure and the minimum distance between the catalytic S130 and the second carbonyl carbon of the 2nd repeating unit during the 3 × 100 ns MD simulations. (A) The RMSD values were determined for the whole 3PET substrate (black: all) as well as each 3PET unit (blue: 1st, red: 2nd and green: 3rd unit), respectively, after alignment of the protein structures. This provides a measure for the change in binding position of 3PET. (B) The evolution of the minimum distance between the γ-O atom of the catalytic S130 and the closest carbonyl carbon atom of the central repeating unit in 3PET was calculated. This analysis revealed that 3PET adopted a hydrolysis-competent orientation. Three simulations were concatenated for this analysis and the start of the second and third simulation is indicated by vertical dotted lines. (C) 3PET oligomer structure with the 1st, 2nd, and 3rd PET repeating units labeled from left to right and divided by dotted lines.
Figure S4. Binding positions of the 3PET clusters. Binding positions of all four clusters are given from a side view (A) and top view (B). The 3PET ligands are shown in green (cluster 1), orange (cluster 2), blue (cluster 3) and red (cluster 4). As an orientation marker, W155 is additionally shown as white sticks. The binding mode of cluster 1 (C) is colored from light green (1st unit) to dark green (3rd unit) and the most prominent interacting residues (sidechain/ backbone) are shown as orange sticks. (D) Superposition of the PES-H1 (light pink) and LCC ICCG with bound MHET (light orange, PDB code: 7VVE) structures. The 3PET ligand in the binding pose of cluster 1 is shown in light green, while MHET is colored in deep teal. For all images, the catalytic triad residues (S130, D176, and H208) and the oxyanion hole (F62 and M131) of PES-H1 are shown in light magenta. Nitrogen atoms are colored blue and oxygen atoms are colored red. The images were generated using PyMOL.
**Figure S5.** PET film degradation catalyzed by PES-H1 at 70 °C and 72 °C. Error bars indicate the standard deviations calculated from three replicates.

**Figure S6.** Phosphate dependence of PES-H1. PES-H1 was used to catalyze hydrolysis of Gf-PET film at 72 °C for 24 h in potassium phosphate buffer (pH 8.0). Phosphate concentrations ranged from 0.05 M to 1 M. Error bars indicate the standard deviations calculated from three replicates.
Figure S7. Cysteine calibration curve to determine the amount of free sulphydryl-groups and confirm the disulfide bond formation. The concentration of free cysteines in the R204C/S250C variant of PES-H1 (0.02 ±0 µM; red dot) was calculated based on the dotted calibration line obtained with linearly fitted absorbance data according to the experimentally determined absorbance values at 412 nm. No absorbance was measured for the R204C/S250C variant, demonstrating the formation of a new disulfide bond.
Figure S8. Electrostatic surfaces of PES-H1 (A) and LCC ICCG (B). Red areas indicate negatively charged residues whereas blue areas represent positively charged residues (-50 kT/e to +50 kT/e). The images were generated using PyMOL.
Figure S9. Hydrolysis of PET-NP by (A) PES-H1, (B) the L92F/Q94Y variant, (C) the R204C/S250C variant, and (D) LCC ICCG. The initial PET hydrolysis rates were determined by a turbidimetric assay by measuring apparent absorbance of light at 600 nm in a microtiter plate reader. Reactions in 1 M potassium phosphate buffer (pH 8.0) contained 0.5 mg mL⁻¹ PET-NP and 0-2 µM enzyme and were incubated at 70 °C. Data points represent the averages and error bars the standard deviations calculated from three replicates. The fitted data are shown as dotted curves.
Figure S10. Hydrolysis of PET-NP by (A) PES-H1, (B) the L92F/Q94Y variant, (C) the R204C/S250C variant, and (D) LCC ICCG. The initial PET hydrolysis rates were determined by a turbidimetric assay by measuring apparent absorbance of light at 600 nm in a microtiter plate reader. Reactions in 1 M potassium phosphate buffer (pH 8.0) contained 0.7 µM enzyme and 0-0.5 mg mL⁻¹ PET-NP and were incubated at 70 °C. Data points represent the averages and error bars the standard deviations calculated from three replicates. The fitted data are shown as dotted lines.
REFERENCES

(1) Otwinowski, Z.; Minor, W. [20] Processing of X-Ray Diffraction Data Collected in Oscillation Mode. In Methods in Enzymology; Elsevier, 1997; Vol. 276, pp 307–326.

(2) McCoy, A. J.; Grosse-Kunstleve, R. W.; Adams, P. D.; Winn, M. D.; Storoni, L. C.; Read, R. J. Phaser Crystallographic Software. J. Appl. Crystallogr. 2007, 40 (4), 658–674.

(3) Liebschner, D.; Afonine, P. V.; Baker, M. L.; Bunkóczi, G.; Chen, V. B.; Croll, T. I.; Hintze, B.; Hung, L.-W.; Jain, S.; McCoy, A. J.; Moriarty, N. W.; Oeffner, R. D.; Poon, B. K.; Prisant, M. G.; Read, R. J.; Richardson, J. S.; Richardson, D. C.; Sammito, M. D.; Sobolev, O. V.; Stockwell, D. H.; Terwilliger, T. C.; Urzhumtsev, A. G.; Videau, L. L.; Williams, C. J.; Adams, P. D. Macromolecular Structure Determination Using X-Rays, Neutrons and Electrons: Recent Developments in Phenix. Acta Crystallogr. D. Struct. Biol. 2019, 75 (10), 861–877.

(4) Han, X.; Liu, W.; Huang, J.-W.; Ma, J.; Zheng, Y.; Ko, T.-P.; Xu, L.; Cheng, Y.-S.; Chen, C.-C.; Guo, R.-T. Structural Insight into Catalytic Mechanism of PET Hydrolase. Nat. Commun. 2017, 8 (1), 2106.

(5) Afonine, P. V.; Grosse-Kunstleve, R. W.; Echols, N.; Headd, J. J.; Moriarty, N. W.; Mustyakimov, M.; Terwilliger, T. C.; Urzhumtsev, A.; Zwart, P. H.; Adams, P. D. Towards Automated Crystallographic Structure Refinement with Phenix.Refine. Acta Crystallogr. D. Biol. Crystallogr. 2012, 68 (4), 352–367.

(6) Emsley, P.; Cowtan, K. Coot: Model-Building Tools for Molecular Graphics. Acta Crystallogr. D. Biol. Crystallogr. 2004, 60 (12), 2126–2132.

(7) Brünger, A. T.; Adams, P. D.; Clore, G. M.; DeLano, W. L.; Gros, P.; Grosse-Kunstleve, R. W.; Jiang, J. S.; Kuszewski, J.; Nilges, M.; Pannu, N. S.; Read, R. J.; Rice, L. M.; Simonson,
T.; Warren, G. L. Crystallography & NMR System: A New Software Suite for Macromolecular Structure Determination. *Acta Crystallogr. D. Biol. Crystallogr.* **1998**, *54* (5), 905–921.

(8) *The PyMOL Molecular Graphics System, Version 1.8, 2015. Schrödinger, LLC.*

(9) Wang, J.; Wolf, R. M.; Caldwell, J. W.; Kollman, P. A.; Case, D. A. Development and Testing of a General Amber Force Field. *J. Comput. Chem.* **2004**, *25* (9), 1157–1174.

(10) M.J. Frisch, G.W. Trucks, H.B. Schlegel, G.E. Scuseria, M.A. Robb, J.R. Cheeseman, G. Scalmani, V. Barone, B. Mennucci, G.A. Petersson, H. Nakatsuji, M. Caricato, X. Li, H.P. Hratchian, A.F. Izmaylov, J. Bloino, G. Zheng, J.L. Sonnenberg, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, T. Vreven, J.A. Montgomery, J.E. Peralta, F. Ogliaro, M. Bearpark, J.J. Heyd, E. Brothers, K.N. Kudin, V.N. Staroverov, R. Kobayashi, J. Normand, K. Raghavachari, A. Rendell, J.C. Burant, S.S. Iyengar, J. Tomasi, M. Cossi, N. Rega, J.M. Millam, M. Klene, J.E. Knox, J.B. Cross, V. Bakken, C. Adamo, J. Jaramillo, R. Gomperts, R.E. Stratmann, O. Yazyev, A.J. Austin, R. Cammi, C. Pomelli, J.W. Ochterski, R.L. Martin, K. Morokuma, V.G. Zakrzewski, G.A. Voth, P. Salvador, J.J. Dannenberg, S. Dapprich, A.D. Daniels, Ö. Farkas, J.B. Foresman, J.V. Ortiz, J. Cioslowski, and D.J. Fox. Gaussian 09. Revision E.01, Gaussian Inc., Wallingford CT. **2009**.

(11) Bayly, C. I.; Cieplak, P.; Cornell, W.; Kollman, P. A. A Well-Behaved Electrostatic Potential Based Method Using Charge Restraints for Deriving Atomic Charges: The RESP Model. *J. Phys. Chem.* **1993**, *97* (40), 10269–10280.
(12) Cornell, W. D.; Cieplak, P.; Bayly, C. I.; Kollman, P. A. Application of RESP Charges to Calculate Conformational Energies, Hydrogen Bond Energies, and Free Energies of Solvation. *J. Am. Chem. Soc.* **1993**, *115* (21), 9620–9631.

(13) Wang, J.; Wang, W.; Kollman, P. A.; Case, D. A. Antechamber, An Accessory Software Package For Molecular Mechanical Calculations. *J. Am. Chem. Soc.* **2001**, 222.

(14) Wang, J.; Wang, W.; Kollman, P. A.; Case, D. A. Automatic Atom Type and Bond Type Perception in Molecular Mechanical Calculations. *J. Mol. Graph. Model.* **2006**, *25* (2), 247–260.

(15) K. Belfon, I.Y. Ben-Shalom, S.R. Brozell, D.S. Cerutti, T.E. Cheatham III, G.A. Cisneros, V.W.D. Cruzeiro, T.A. Darden, R.E. Duke, G. Giambasu, M.K. Gilson, H. Gohlke, A.W. Goetz, R. Harris, S. Izadi, S.A. Izmailov ,C. Jin K. Kasavajhala, M.C. Kaymak ,E. King, A. Kovalenko, T. Kurtzman, T.S. Lee, S. LeGrand, P. Li, C. Lin, J. Liu, T. Luchko, R. Luo, M. Machado, V. Man, M. Manathunga, K.M. Merz ,Y. Miao, O. Mikhailovskii, G. Monard, H. Nguyen, K.A. O’Hearn, A. Onufriev, F. Pan, S. Pantano, R. Qi A. Rahnamoun, D.R. Roe, A. Roitberg, C. Sagui, S. Schott- Verdugo, J. Shen ,C.L. Simmerling, N.R. Skrynnikov, J. Smith, J. Swails, R.C. Walker, J. Wang, H. Wei, R.M. Wolf, X. Wu, Y. Xue, D.M. York, S. Zhao, P.A. Kollman, D.A. Case, H.M. Aktulga. *Amber 2021*; University of California, San Francisco, 2021.

(16) Abraham, M. J.; Murtola, T.; Schulz, R.; Páll, S.; Smith, J. C.; Hess, B.; Lindahl, E. GROMACS: High Performance Molecular Simulations through Multi-Level Parallelism from Laptops to Supercomputers. *SoftwareX* **2015**, *1–2*, 19–25.
(17) Maier, J. A.; Martinez, C.; Kasavajhala, K.; Wickstrom, L.; Hauser, K. E.; Simmerling, C. Ff14SB: Improving the Accuracy of Protein Side Chain and Backbone Parameters from Ff99SB. *J. Chem. Theory Comput.* **2015**, *11*(8), 3696–3713.

(18) Ivani, I.; Dans, P. D.; Noy, A.; Pérez, A.; Faustino, I.; Hospital, A.; Walther, J.; Andrio, P.; Goñi, R.; Balaceanu, A.; Portella, G.; Battistini, F.; Gelpí, J. L.; González, C.; Vendruscolo, M.; Laughton, C. A.; Harris, S. A.; Case, D. A.; Orozco, M. Parmbscl: A Refined Force Field for DNA Simulations. *Nat. Methods* **2016**, *13*(1), 55–58.

(19) Jorgensen, W. L.; Chandrasekhar, J.; Madura, J. D.; Impey, R. W.; Klein, M. L. Comparison of Simple Potential Functions for Simulating Liquid Water. *J. Chem. Phys* **1983**, *79*(2), 926–935.

(20) Cauchy, M. A. Méthode Générale Pour La Résolution Des Systemes d’équations Simultanées. *CR Hebd. Acad. Sci.* **1847**, No. 25, 536–538.

(21) Nosé, S. A Molecular Dynamics Method for Simulations in the Canonical Ensemble. *Mol. Phys.* **1984**, *52*(2), 255–268.

(22) Hoover, W. G. Canonical Dynamics: Equilibrium Phase-Space Distributions. *Phys. Rev. A* **1985**, *31*(3), 1695–1697.

(23) Parrinello, M.; Rahman, A. Polymorphic Transitions in Single Crystals: A New Molecular Dynamics Method. *J. Appl. Phys.* **1981**, *52*(12), 7182–7190.

(24) Darden, T.; York, D.; Pedersen, L. Particle Mesh Ewald: An $N \cdot \log(N)$ Method for Ewald Sums in Large Systems. *J. Chem. Phys.* **1993**, *98*(12), 10089–10092.

(25) Essmann, U.; Perera, L.; Berkowitz, M. L.; Darden, T.; Lee, H.; Pedersen, L. G. A Smooth Particle Mesh Ewald Method. *J. Chem. Phys.* **1995**, *103*(19), 8577–8593.
(26) Hess, B.; Bekker, H.; Berendsen, H. J. C.; Fraaije, J. G. E. M. LINCS: A Linear Constraint Solver for Molecular Simulations. *J. Comput. Chem.* **1997**, *18* (12), 1463–1472.

(27) Daura, X.; Gademann, K.; Jaun, B.; Seebach, D.; van Gunsteren, W. F.; Mark, A. E. Peptide Folding: When Simulation Meets Experiment. *Angew. Chem. Int. Ed.* **1999**, *38* (1–2), 236–240.

(28) Humphrey, W.; Dalke, A.; Schulten, K. VMD: Visual Molecular Dynamics. *J. Mol. Graph.* **1996**, *14* (1), 33–38.

(29) Studier, F. W. Protein Production by Auto-Induction in High-Density Shaking Cultures. *Protein Expression Purif.* **2005**, *41* (1), 207–234.

(30) Zimmermann, W.; Wei, R.; Hille, P.; Oeser, T.; Schmidt, J. New Polypeptides Having a Polyester Degrading Activity and Uses Thereof. EP3517608A1, July 31, 2019.

(31) Palm, G. J.; Reisky, L.; Böttcher, D.; Müller, H.; Michels, E. A. P.; Walczak, M. C.; Berndt, L.; Weiss, M. S.; Bornscheuer, U. T.; Weber, G. Structure of the Plastic-Degrading *Ideonella sakaiensis* MHETase Bound to a Substrate. *Nat. Commun.* **2019**, *10* (1), 1717.

(32) Brott, S.; Pfaff, L.; Schuricht, J.; Schwarz, J.; Böttcher, D.; Badenhorst, C. P. S.; Wei, R.; Bornscheuer, U. T. Engineering and Evaluation of Thermostable *IspETase* Variants for PET Degradation. *Eng. Life Sci.* **2021**, *22* (3-4), 192-203

(33) Pfaff, L.; Breite, D.; Badenhorst, C. P. S.; Bornscheuer, U. T.; Wei, R. Fluorimetric High-Throughput Screening Method for Polyester Hydrolase Activity Using Polyethylene Terephthalate Nanoparticles. In *Methods in Enzymology*; Elsevier, 2021; Vol. 648, pp 253–270.

(34) Vogel, K.; Wei, R.; Pfaff, L.; Breite, D.; Al-Fathi, H.; Ortmann, C.; Estrela-Lopis, I.; Venus, T.; Schulze, A.; Harms, H.; Bornscheuer, U. T.; Maskow, T. Enzymatic Degradation of
Polyethylene Terephthalate Nanoplastics Analyzed in Real Time by Isothermal Titration Calorimetry. *Sci. Total Environ.* 2021, 773, 145111.

(35) Belisário-Ferrari, M. R.; Wei, R.; Schneider, T.; Honak, A.; Zimmermann, W. Fast Turbidimetric Assay for Analyzing the Enzymatic Hydrolysis of Polyethylene Terephthalate Model Substrates. *Biotechnol. J.* 2019, 14 (4), 1800272.

(36) Ronkvist, Å. M.; Xie, W.; Lu, W.; Gross, R. A. Cutinase-Catalyzed Hydrolysis of Poly(Ethylene Terephthalate). *Macromolecules* 2009, 42 (14), 5128–5138.

(37) Scandola, M.; Focarete, M. L.; Frisoni, G. Simple Kinetic Model for the Heterogeneous Enzymatic Hydrolysis of Natural Poly(3-Hydroxybutyrate). *Macromolecules* 1998, 31 (12), 3846–3851.

(38) Wei, R.; Oeser, T.; Barth, M.; Weigl, N.; Lübs, A.; Schulz-Siegmund, M.; Hacker, M. C.; Zimmermann, W. Turbidimetric Analysis of the Enzymatic Hydrolysis of Polyethylene Terephthalate Nanoparticles. *J. Mol. Catal., B-Enzym* 2014, 103, 72–78.

(39) Wei, R.; Breite, D.; Song, C.; Gräsing, D.; Ploss, T.; Hille, P.; Schwerdtfeger, R.; Matysik, J.; Schulze, A.; Zimmermann, W. Biocatalytic Degradation Efficiency of Postconsumer Polyethylene Terephthalate Packaging Determined by Their Polymer Microstructures. *Adv. Sci.* 2019, 6 (14), 1900491.