Supporting Information

Rational Protein Engineering of Thermo-Stable PETase from *Ideonella sakaiensis* for Highly Efficient PET Degradation

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1. Supporting Tables

Table S1. Primers used for cloning and site-directed mutagenesis

Name	Primer
*Is*PETase_F	5'-GCGCGCATATGCGCGGCCCGAACCCGACAG -3'
*Is*PETase_R	5'- GCGCGCTCGAGGCTGCAATTCGCTGTACGAAAATC -3'
*Is*PETase^{S121D}_F	5'-ACGTTAGACCAGCCAGATAGTCGGAGTTCGCAA -3'
*Is*PETase^{S121D}_R	5'-TTGCGAACTCCGACTATCTGGCTGGTCTAACGT -3'
*Is*PETase^{S121E}_F	5'-ACGTTAGACCAGCCAGAAAGTCGGAGTTCGCAA -3'
*Is*PETase^{S121E}_R	5'-TTGCGAACTCCGACTTTCTGGCTGGTCTAACGT -3'
*Is*PETase^{D186H}_F	5'-CCTCAAGCACCATGGCATTCTTCGACAAATTTT -3'
*Is*PETase^{D186H}_R	5'-AAAATTTGTCGAAGAATGCCATGGTGCTTGAGG -3'
*Is*PETase^{P181A}_F	5'-CTGAAAGCAGCGGCGGCTCAAGCACCATGGGAT -3'
*Is*PETase^{P181A}_R	5'-ATCCCATGGTGCTTGAGCCGCCGCTGCTTTCAG -3'
*Is*PETase$^{P181A/D186H}$_F	5'-AGCGGCGGCTCAAGCACCATGGCATTCTTCGAC-3'
*Is*PETase$^{P181A/D186H}$_R	5'-GTCGAAGAATGCCATGGTGCTTGAGCCGCCGCT-3'
*Is*PETase^{P181G}_F	5'-CTGAAAGCAGCGGCGGGTCAAGCACCATGGGAT-3'
*Is*PETase^{P181G}_R	5'-ATCCCATGGTGCTTGACCCGCCGCTGCTTTCAG-3'
*Is*PETase^{P181S}_F	5'-CTGAAAGCAGCGGCGTCGCAAGCACCATGGGAT-3'
*Is*PETase^{P181S}_R	5'-ATCCCATGGTGCTTGCGACGCCGCTGCTTTCAG-3'
*Is*PETase^{D186H}_F	5'-CCTCAAGCACCATGGCATTCTTCGACAAATTTT -3'
*Is*PETase^{D186H}_R	5'-AAAATTTGTCGAAGAATGCCATGGTGCTTGAGG -3'
*Is*PETase^{D186F}_F	5'-CCTCAAGCACCATGGTTCTCTTCGACAAATTTT-3'
*Is*PETase^{D186F}_R	5'-AAAATTTGTCGAAGAGAACCATGGTGCTTGAGG-3'
*Is*PETase^{D186L}_F	5'-CCTCAAGCACCATGGCTATCTTCGACAAATTTT-3'
*Is*PETase^{D186L}_R	5'-AAAATTTGTCGAAGATAGCCATGGTGCTTGAGG-3'
*Is*PETase^{D186I}_R	5'-CCTCAAGCACCATGGATCTCTTCGACAAATTTT-3'
*Is*PETase^{D186I}_F	5'-AAAATTTGTCGAAGAGATCCATGGTGCTTGAGG-3'
*Is*PETase^{D186V}_R	5'-CCTCAAGCACCATGGGTATCTTCGACAAATTTT-3'
*Is*PETase^{D186V}_F	5'-AAAATTTGTCGAAGATACCCATGGTGCTTGAGG-3'
*Is*PETase^{R280A}_F	5'-AACCCGAATAGCACCAGAGTGTCTGATTTTCGT -3'
*Is*PETase^{R280A}_R	5'-ACGAAAATCAGACACTCTGGTGCTATTCGGGTT -3'

Table S2. Data collection and refinement statistics of *Is*PETase variants

	*Is*PETase$^{S121D/D186H}$	*Is*PETase$^{S121E/D186H}$	*Is*PETase^{P181A}	*Is*PETase$^{S121E/D186H/R280A}$
PDB code	6IJ3	6IJ4	6IJ5	6IJ6
Data collection				
Wavelength (Å)	0.97934	0.97934	0.97934	0.97934
Unit cell (*a*, *b*, *c;* γ) (Å; °)	115.43, 50.63, 41.49; 90.0, 93.1, 90.0	116.20, 50.86, 41.49; 90.0, 92.7, 90.0	115.48, 50.94, 41.26; 90.0, 92.6, 90.0	114.91, 51.14, 51.16; 90.0, 109.7, 90.0
Space group	*C*121	*C*121	C121	C121
Solvent content (%)	47.20	47.80	47.27	54.84
Protein chains in AU	1	1	1	1
Resolution range (Å)	50.00-1.40	50.00-1.85	50.00-1.72	50.00-1.95
Highest resolution shell (Å)	1.42-1.40	1.88-1.85	1.75-1.72	1.98-1.95
Unique reflections	46596	19977	25150	19893
Redundancy	3.2(2.9)	3.3(2.9)	3.2(2.7)	3.5(3.1)
Completeness (%)	99.0(98.5)	98.3(96.2)	98.8(96.9)	97.4(94.2)
R_{merge} (%)	12.2(38.1)	10.8(34.9)	7.1(36.1)	9.7(19.5)
Average I/σ(I)	25.99(3.64)	25.56(3.53)	27.31(3.92)	33.04(9.90)
Refinement				
R (%)	19.4	20.7	20.9	15.1
R_{free} (%)	23.3	24.7	26.2	19.3
Mean B value (Å^2)*	16.4	28.6	25.0	19.6
B from Wilson plot (Å^2)	12.3	24.1	20.2	19.2
RMS deviation bond lengths (Å)	0.013	0.013	0.013	0.013
RMS deviation bond angles (°)	1.889	1.592	1.638	1.638
Number of amino acid residues	261	262	261	261
Number of water molecules	225	102	100	188

*Mean B value is for both protein atoms and the solvent molecules.

2. Supporting Figures

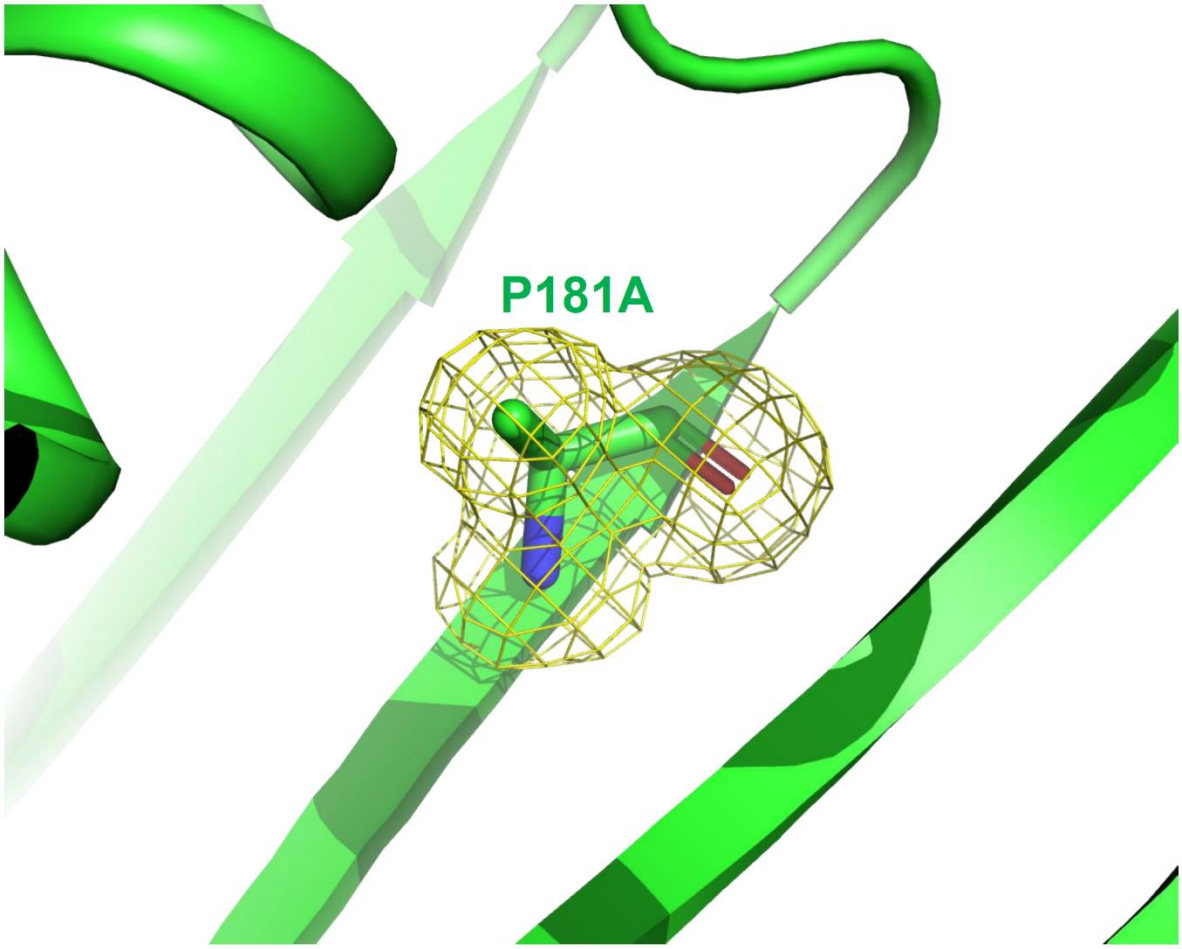

Figure S1. The electron density map of the *Is*PETase^{P181A} variant. The Fo-Fc map of the mutated residue in the *Is*PETase^{P181A} variant is shown as a yellow-colored mesh and is contoured at 2.0 σ. The structure of *Is*PETase^{P181A} is presented with a green colored cartoon, and the mutated P181A residue is shown as a stick model.

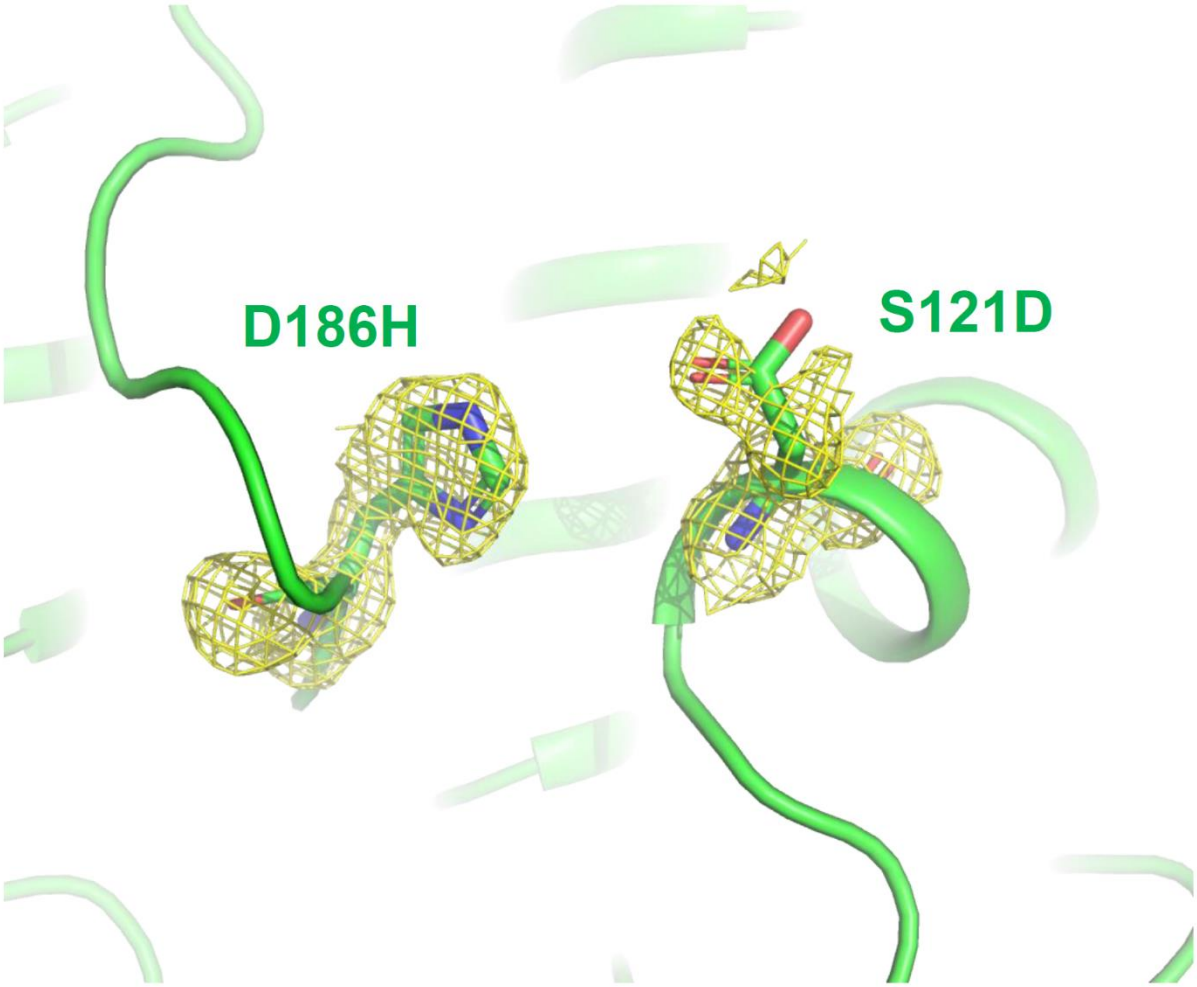

Figure S2. The electron density map of the *Is*PETase$^{S121D/D186H}$ variant. The Fo-Fc map of the mutated residues in the *Is*PETase$^{S121D/D186H}$ variant is shown as a yellow-colored mesh and is contoured at 2.0 σ. The structure of *Is*PETase$^{S121D/D186H}$ is presented with a green colored cartoon, and the mutated S121D and D186H residues are shown as stick models.

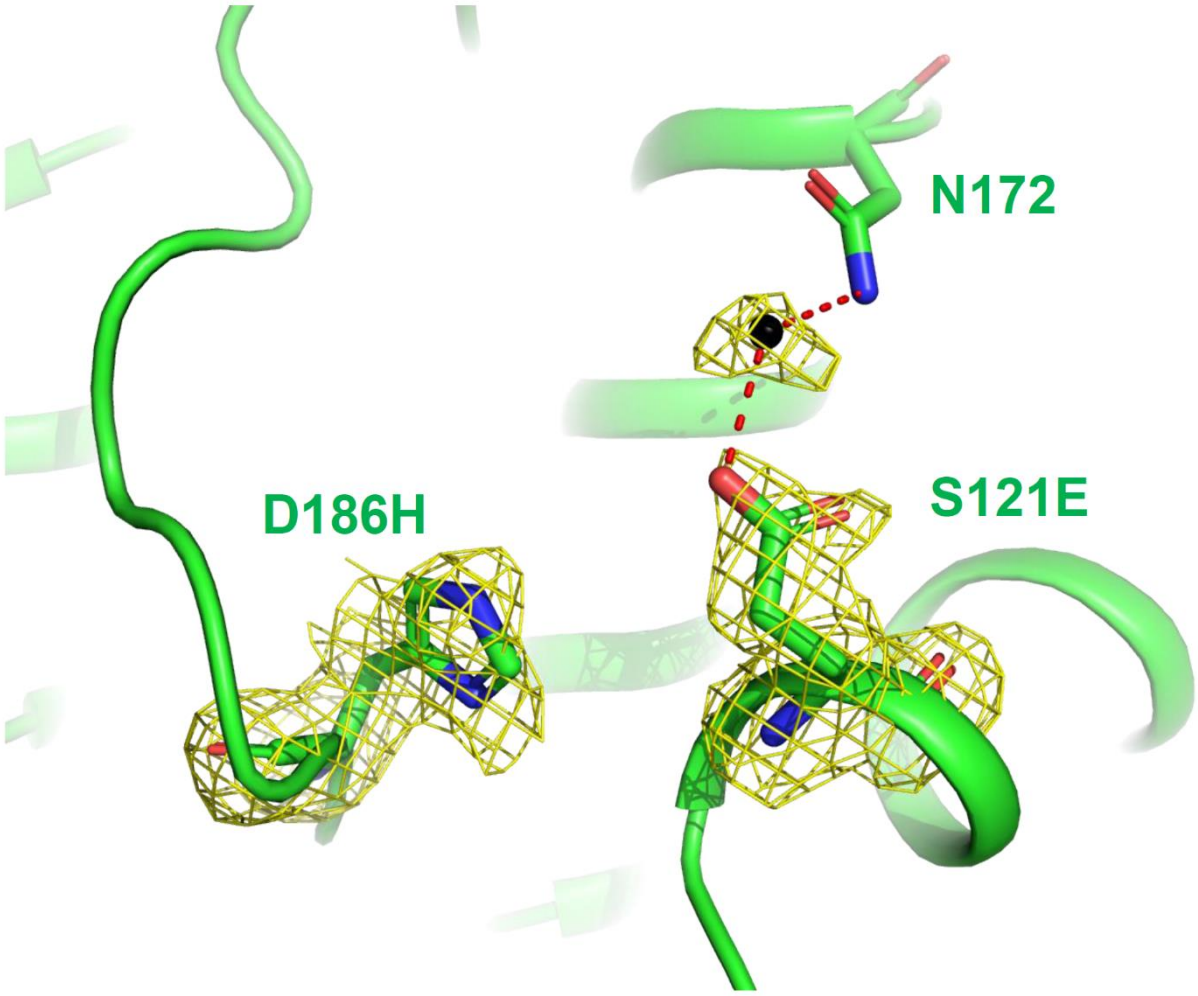

Figure S3. The electron density map of the *Is*PETase$^{S121E/D186H}$ variant. The Fo-Fc map of the mutated residues in the *Is*PETase$^{S121E/D186H}$ variant is shown as a yellow-colored mesh and is contoured at 2.0 σ. The structure of *Is*PETase$^{S121E/D186H}$ is presented with a green colored cartoon, and the mutated S121E and D186H residues are shown as stick models. A water molecule is presented as a black-colored sphere and hydrogen bonds are as red-colored dotted-lines.

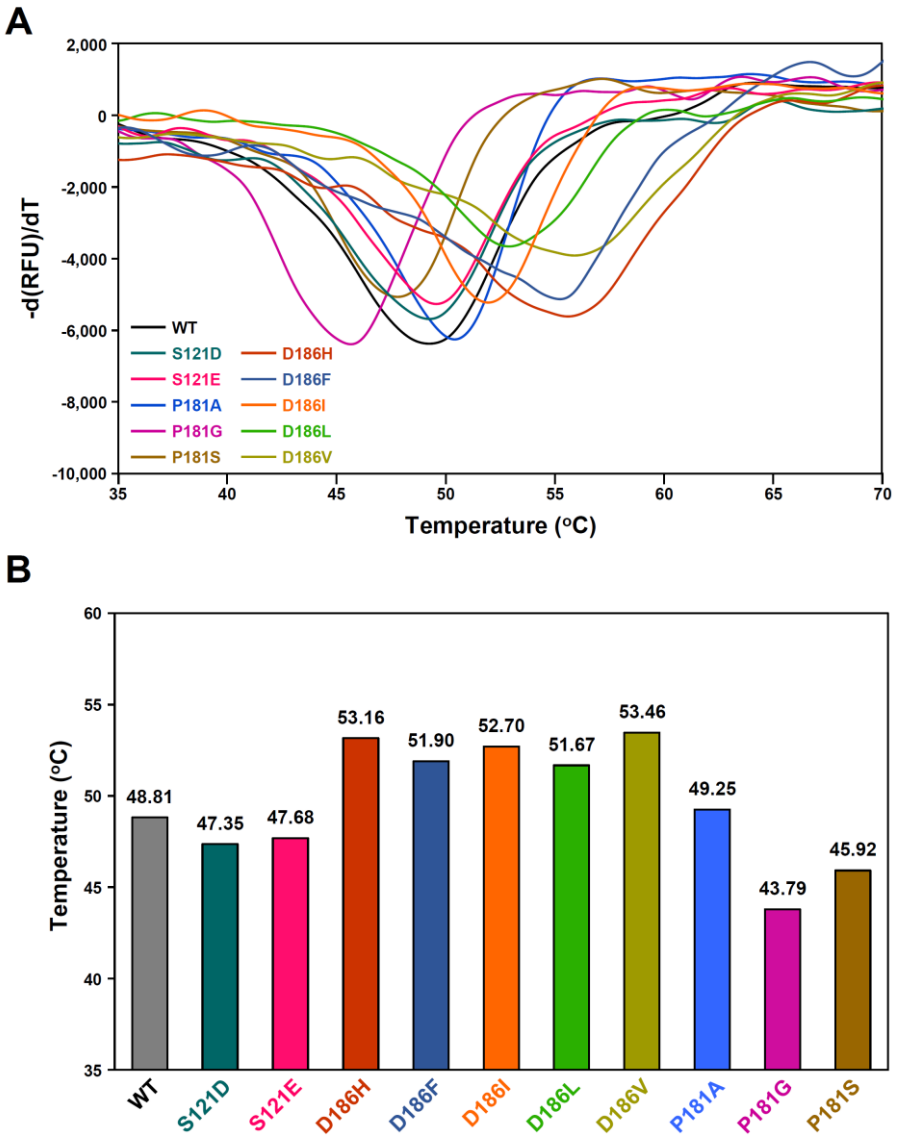

Figure S4. Thermal stability measurement of *Is*PETase^{WT} and variants. (A) Thermal stability measurement of *Is*PETase^{WT} and variants. **(B)** Comparison of *Tm* values of *Is*PETase^{WT} and variants.

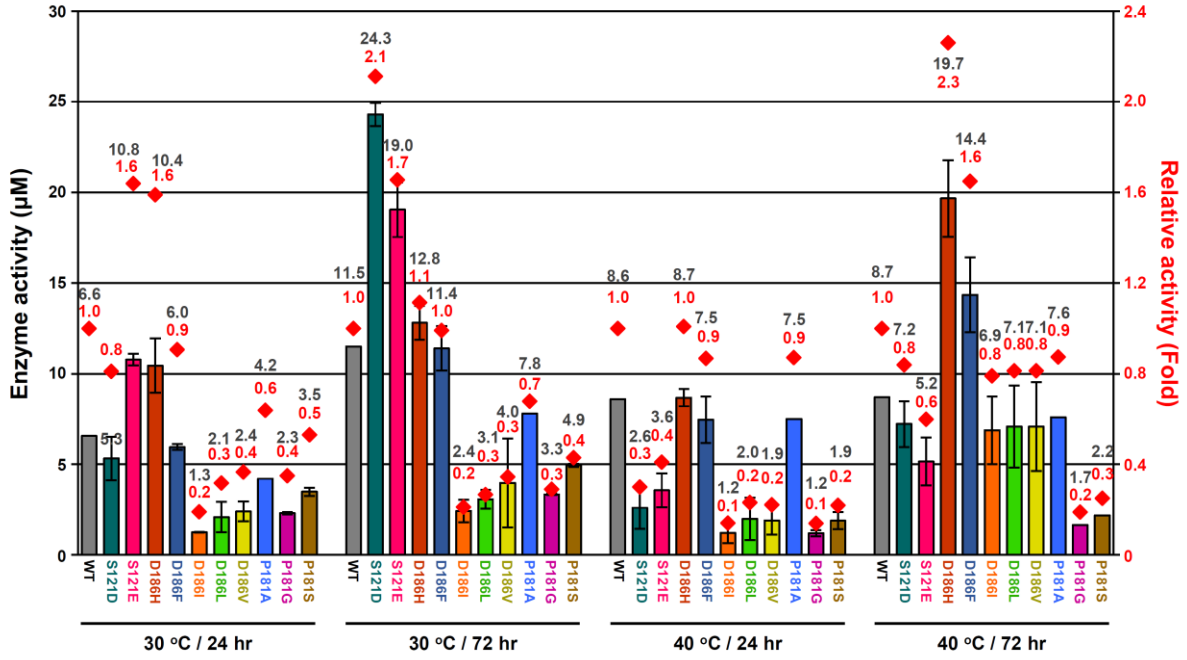

Figure S5. PETase activity of the variants. PET degradation activity of *Is*PETase^{WT} and variants. The enzyme activity is the sum of MHET and TPA.

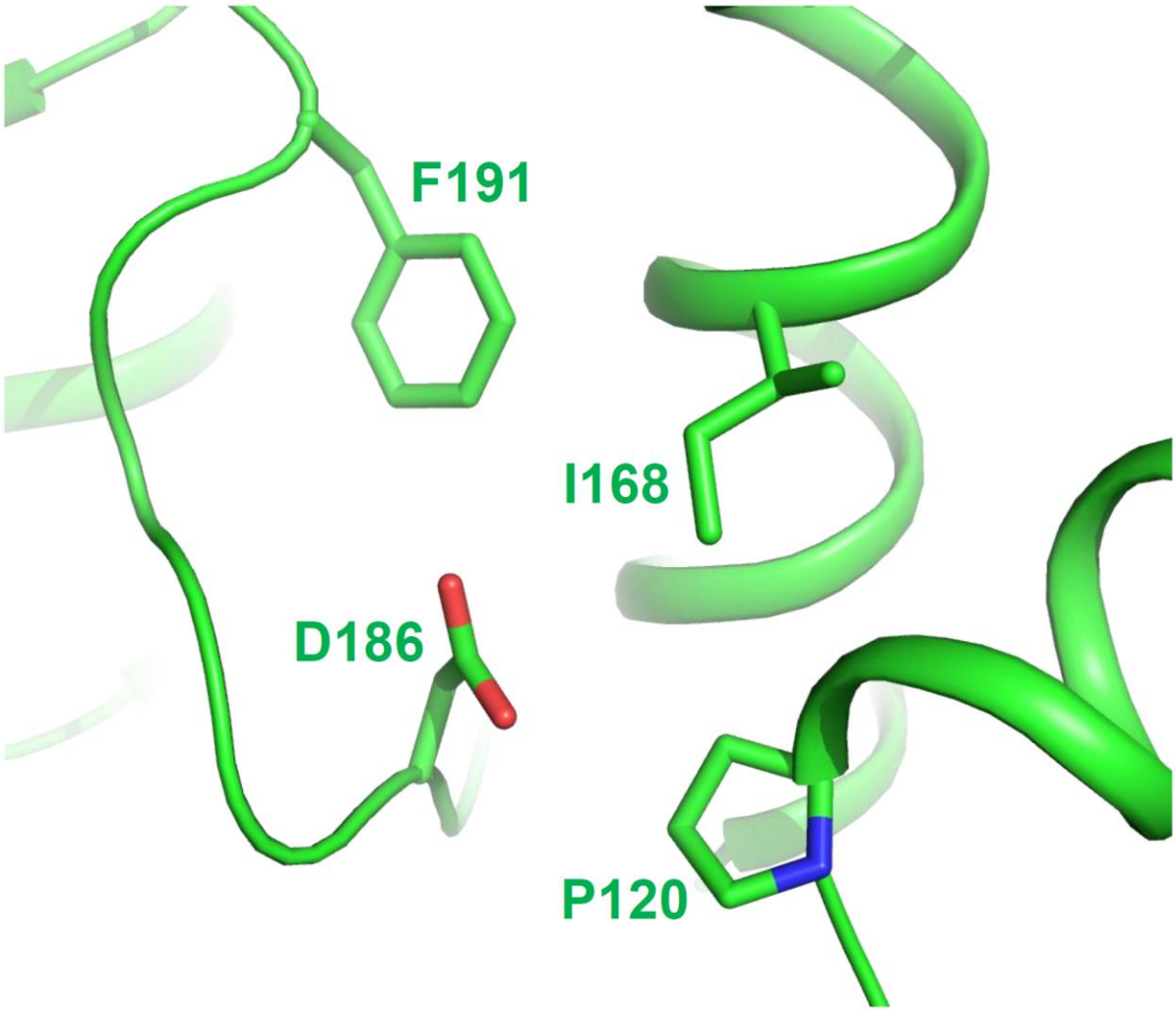

Figure S6. Collision of polarity between Asp186 and the surrounding hydrophobic residues. The *Is*PETase structure is shown as a cartoon diagram with a green color. Asp186 and the surrounding hydrophobic residues are presented with stick models, and labeled appropriately.

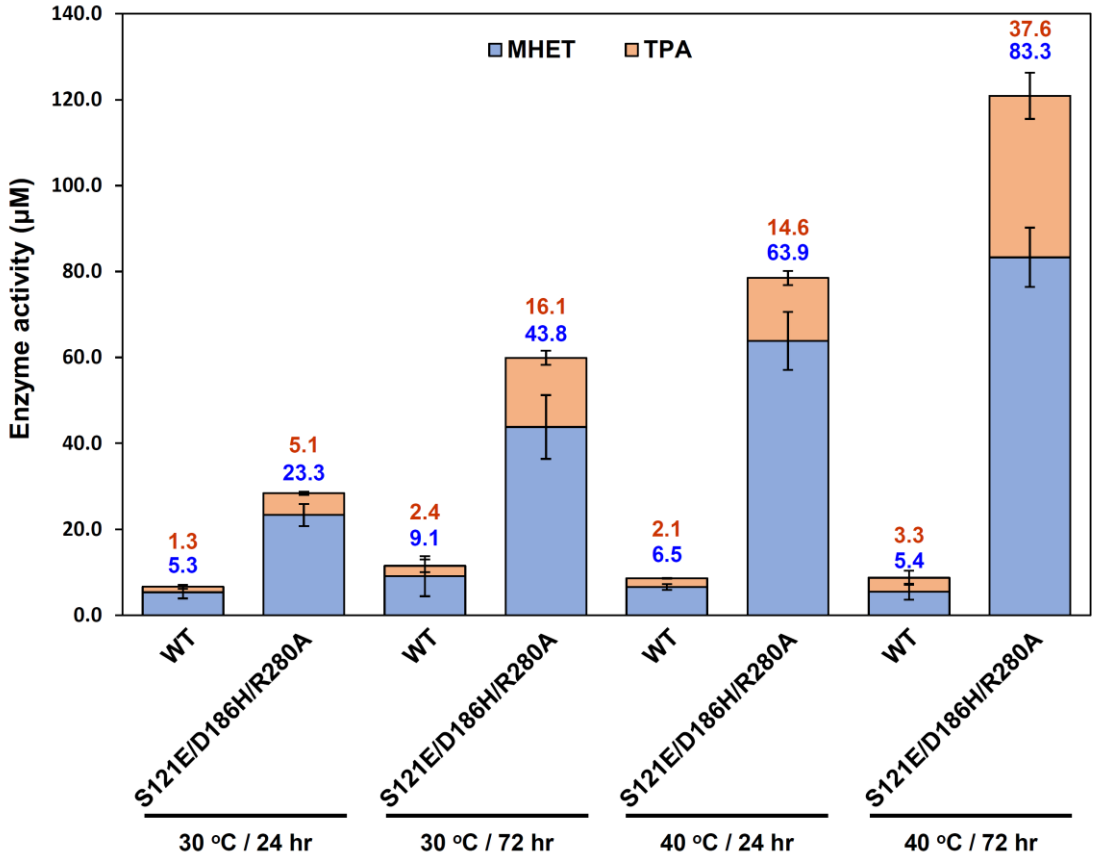

Figure S7. PETase activity of *Is*PETase^{WT} and *Is*PETase$^{S121E/D186H/R280A}$. The PET film degradation ability of *Is*PETase^{WT} and *Is*PETase$^{S121E/D186H/R280A}$. The quantity of MHET and TPA are labelled with blue and orange colors, respectively.

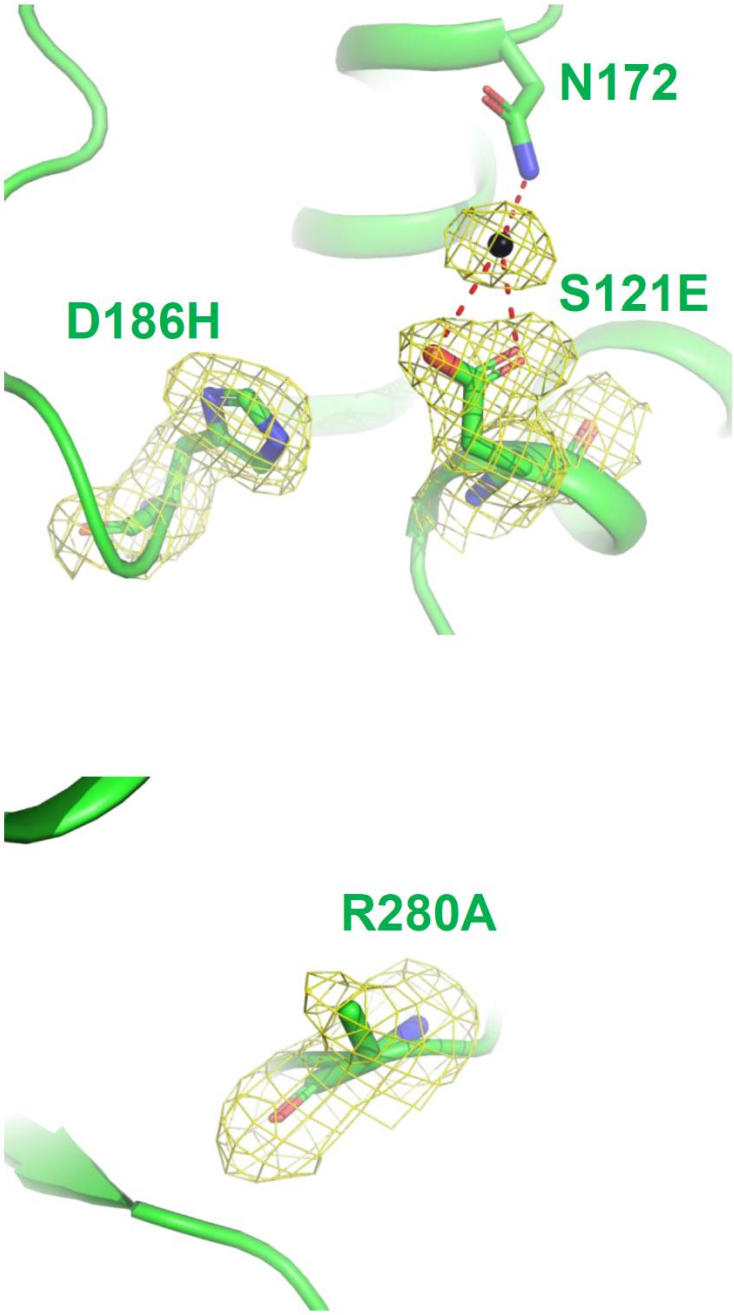

Figure S8. The electron density map of the *Is*PETase$^{S121E/D186H/R280A}$ variant. The Fo-Fc map of the mutated residue in the *Is*PETase$^{S121E/D186H/R280A}$ variant is shown as a yellow-colored mesh and is contoured at 2.0 σ. The structure of *Is*PETase$^{S121E/D186H/R280A}$ is presented with a green colored cartoon, and the mutated S121E, D182H, and R280A residues are shown as stick models. A water molecule is presented as a black-colored sphere and hydrogen bonds are as red-colored dotted-lines.

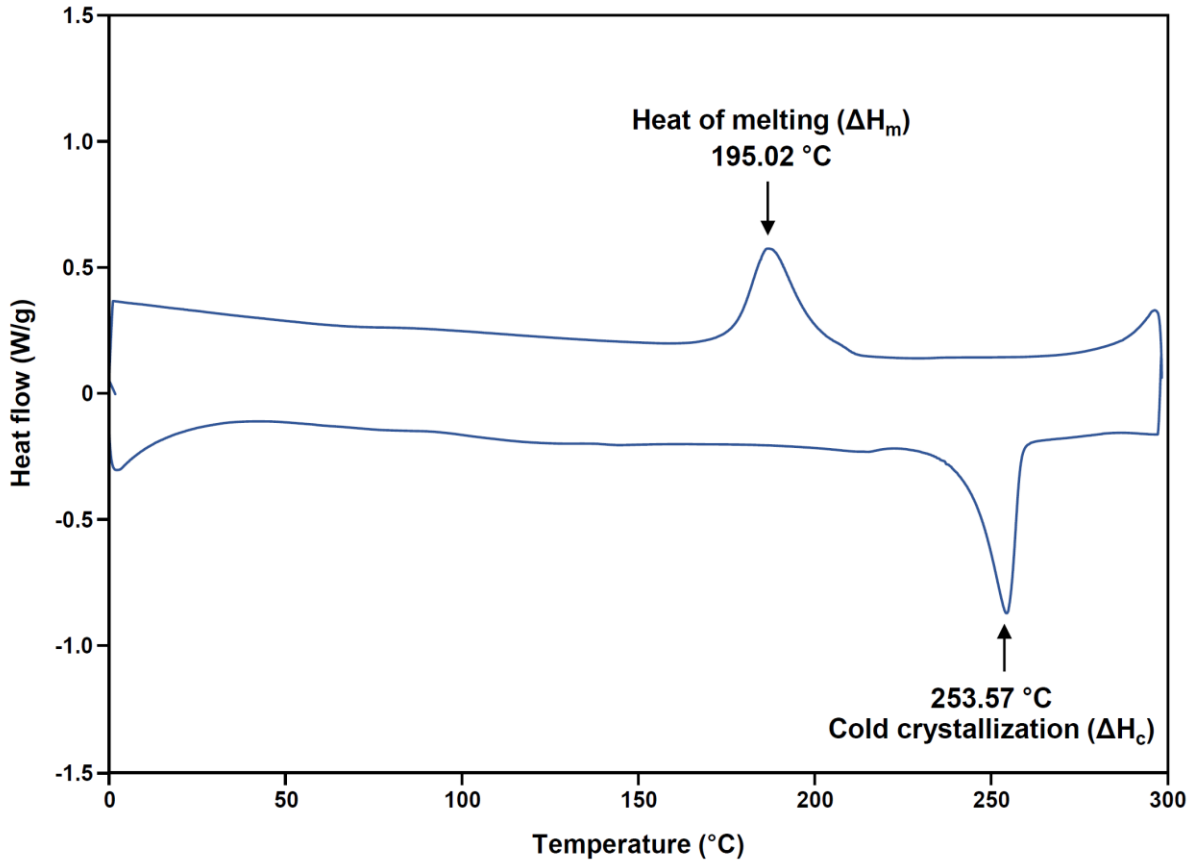

Figure S9. Differential scanning calorimetry (DSC) for crystallinity of PET film. The crystallinity of PET film was calculated by DSC experiments. The heat of melting (ΔH_m) and cold crystallization (ΔH_c) values were determined as 195.02 and 253.57 °C, respectively. Finally calculated crystallinity of PET film was 41.79%.

3. Experimental procedures

3.1 Protein preparation

The *Is*PETase gene was amplified by polymerase chain reaction (PCR) using synthesized gene with codon optimization for expression *Escherichia coli* cells as a template. The nucleotide sequence corresponding to the signal peptide was removed from the synthetic DNA. The PCR product was then subcloned into pET15b expression vector, and the pET15b:*Is*PETase plasmid was transformed into the *E. coli* strain Rosetta gami-B, which was grown in fresh LB medium containing 100 μg mL^{-1} ampicillin at 37 °C. After induction by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), the culture was further incubated for 16 h at 18 °C. The cells were then harvested by centrifugation at 4,000 x g for 10 min at 20 °C. The cell pellet was resuspended in buffer A (50 mM Na_2HPO_4-HCl, pH 7.0) and then disrupted by ultrasonication. The cell debris was removed by centrifugation at 13,500 g for 30 min, and the supernatant was applied to a Ni-NTA agarose column (Qiagen). After washing with buffer A containing 30 mM imidazole, the bound proteins were eluted with 300 mM imidazole in buffer A. Finally, trace amounts of contaminants were removed by size-exclusion chromatography using a HiPrep 26/60 Sephacryl S-300 HR column (GE Healthcare Life Sciences) equilibrated with buffer A. All purification steps were performed at 4 °C. The degree of protein purity was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The purified protein was concentrated to 28 mg mL^{-1} in 50 mM Na_2HPO_4-HCl, pH 7.0. Site-directed mutagenesis experiments were performed using the Quick Change kit (Agilent). Mutant proteins were purified in the same manner as wild type *Is*PETase. Primers used for cloning and site-directed mutagenesis are listed in Table S1.

3.2 Melting temperature (*Tm*) measurement

Thermal stability of *Is*PETase was determined by measuring melting curves with the protein thermal shift dye (Applied Biosystems) in StepOnePlus Real-Time PCR (Thermo Fisher Scientific) according to manufacturer's instructions. Briefly, 5 μg protein was mixed with 1 x protein thermal shift dye (Applied Biosystems) in 20 μl and signal changes reflecting protein denaturation were monitored by increasing temperature from 25 to 99 °C. Melting temperatures were determined from the first derivative curve.

3.3 Degradation of PET film

To analyze the degradation of PET by *Is*PETase^{WT} and variants, commercial PET film (UBIGEO, Republic of Korea), which has 41.79% crystallinity (Figure. S9), was used as the substrate for enzyme assay. The PET film was prepared in a circular form with 6 mm diameter. The PET film was soaked in 300 μl of glycine/NaOH, pH 9.0 with 500 nM of *Is*PETase enzyme. The reaction mixture was incubated at 30 °C and 40 °C for 24 and 72 hours, and long-time enzyme activities for *Is*PETase^{WT} and *Is*PETase$^{S121E/D186H/R280A}$ were measured during 1 to 10 days at 30 °C and 40 °C. After PET film was removed from reaction mixture, the enzyme reaction was terminated by heating at 85 °C for 15 min. Then, the samples were centrifuged at 13,500 x g for 10 min, and the supernatant was analyzed by LC.

3.4 Protein heat-inactivation

Protein heat-inactivation experiments were performed using *Is*PETase^{WT} and *Is*PETase$^{S121E/D186H/R280A}$ mutant. *Is*PETase reaction mixtures (500 nM *Is*PETase in 300 ul glycine/NaOH, pH 9.0) were inactivated at 30 ^{o}C (3 hour to 10 days), 40 ^{o}C (30 min to 5 days), and 50 ^{o}C (1 to 60 min), and then cooled on ice for 10 minutes. The 6 mm PET film was soaked into inactivated reaction mixtures and incubated at 30 ^{o}C for 24 hours. After PET film was

removed from reaction mixture, the enzyme reaction was terminated by heating at 85 °C for 15 min. Then, the samples were centrifuged at 13,500 x g for 10 min, and the supernatant was analyzed by LC.

3.5 HPLC analysis

The in vitro assay samples were analyzed by CMB-20A, (Shimadzu) connected to a UV/Vis detector (SPD-20A) and C18 column (SunFire^{TM} C18, 5 μm, 4.6 x 250 mm) was used. Mobile phase A was water containing 0.1% formic acid, and mobile phase B was acetonitrile, and the flow rate was fixed at 0.8 ml min^{-1}. The mobile phase was changed gradually from 1% to 5% buffer B for 5 min and then 100% buffer B was flowed for 10 min. The detection wavelengths was 260 nm. All experiments were performed triplicate.

3.6 Crystallization, data collection and structure determination

Crystallizations of the purified *Is*PETase$^{S121D/D186H}$, *Is*PETase$^{S121E/D186H}$, *Is*PETase^{P181A}, and *Is*PETase$^{S121E/D186H/R280A}$ mutant proteins were initially performed with commercially available spares-matrix screens from Rigaku and Molecular Dimensions by using the hanging-drop vapor-diffusion method at 20 °C. Each experiment consisted of mixing 1.0 μL of protein solution (28 mg mL^{-1} in 50 mM Na_2HPO_4, pH 7.0) with 1.0 μL of reservoir solution and equilibrating the drop against 0.5 mL of reservoir solution. *Is*PETase$^{S121D/D186H}$, *Is*PETase$^{S121E/D186H}$, and *Is*PETase^{P181A} mutants were crystallized in the condition of 28% PEG 3K, 0.1 M Tris, pH 6.5 and 0.2 M sodium chloride. *Is*PETase$^{S121E/D186H/R280A}$ mutant were crystallized in the condition of 17% PEG 10K, 0.1 M Bis-tris, pH 5.0 and 0.15 M Ammonium acetate. The crystals were transferred to a cryo-protectant solution composed of the corresponding conditions described above with 30% (v/v) glycerol, fished out with a loop

larger than the crystals, and flash-frozen directly in a nitrogen stream. All data were collected at the 7A beamline of the Pohang Accelerator Laboratory (PAL, Republic of Korea), using a Quantum 270 CCD detector (ADSC, USA)[1]. *Is*PETase$^{S121D/D186H}$, *Is*PETase$^{S121E/D186H}$, *Is*PETase^{P181A}, and *Is*PETase$^{S121E/D186H/R280A}$ crystals diffracted to 1.40 Å, 1.85 Å, 1.72 Å, and 1.95 Å resolutions, respectively. All data were indexed, integrated, and scaled using the HKL-2000 software package[2]. The structures of *Is*PETase$^{S121D/D186H}$, *Is*PETase$^{S121E/D186H}$, *Is*PETase^{P181A}, and *Is*PETase$^{S121E/D186H/R280A}$ mutants were determined by molecular replacement with CCP4 version of MOLREP[3], using the structure of wild type *Is*PETase as a search model. Model building was performed manually using the program WinCoot[4] and structure refinement was performed with CCP4 refmac5[5]. Data collection and refinement statistics are summarized in Table S2. The structures of *Is*PETase$^{S121D/D186H}$, *Is*PETase$^{S121E/D186H}$, *Is*PETase^{P181A}, and *Is*PETase$^{S121E/D186H/R280A}$ mutants were deposited in the Protein Data Bank with PDB codes of 6IJ3, 6IJ4, 6IJ5, and 6IJ6, respectively.

3.7 PET film crystallinity

The thermal crystallinity of PET film was measured using differential scanning calorimetry instrument (DSC Q2000). For the DSC thermographs, 6 mg of PET film sample was equilibrated at 0 °C, heated to 300 °C at 10 °C min^{-1}, held at 300 °C for 1 min, and then cooled to 0 °C at 10 °C min^{-1}. The heat of melting (ΔH_m) and cold crystallization (ΔH_c) values were determined as 195.02 and 253.57 °C, respectively (Figure. S9). The heat of melting if the PET has 100% crystallinity ($\Delta H_m°$) is 140.1 J g^{-1}, and the percent crystallinity can be calculated using the following equation.

$$\%\ crystallinity = \left[\frac{\Delta H_m - \Delta H_c}{\Delta H_m{}^\circ}\right] \times 100$$

References

1. Park, S.-Y.; Ha, S.-C.; Kim, Y.-G., The protein crystallography beamlines at the pohang light source II. *Biodesign* **2017,** *5*, 30-34.

2. Otwinowski, Z.; Minor, W., Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol.* **1997**, 276, 307-326.

3. Vagin, A.; Teplyakov, A., Molecular replacement with MOLREP. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2010,** *66*, 22-25.

4. Emsley, P.; Cowtan, K., Coot: model-building tools for molecular graphics. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2004,** *60*, 2126-2132.

5. Murshudov, G. N.; Vagin, A. A.; Dodson, E. J., Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **1997,** *53*, 240-255.