Supporting Information

Depolymerization of Post-Consumer PET Bottles with Engineered Cutinase 1 from *Thermobifida Cellulosilytica*

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1. Experiment

1.1 Cutinases preparation

The codon-optimized wild-type ThcCut1 and its variants (relevant nucleotide sequences are listed in Nucleotide Sequences) were subcloned between the NdeI and XhoI restriction enzyme sites of the pET-22b (+) bacterial expression plasmid and transformed into *E. coli* BL21 (DE3) competent cells (TransGen Biotech, China). The strains were cultured in Luria-Bertani (LB) medium that containing 0.1 mg/mL Ampicillin, and were grown in the shake flask at 37 °C until the absorbance (A600 nm) reached 0.8. Then, 0.2 mM of isopropyl β-D-thiogalactoside (IPTG) was added to the medium and the strains were induced at 16 °C for 21 h, followed by harvest of the cells by centrifugation (2,040 × *g*, 15 min, and 4 °C). The collected cells were resuspended in lysis buffer (100 mM K_2HPO_4-KH_2PO_4, pH 8.0, 300 mM NaCl) and broken up in ice-water by an ultrasonic cell crusher (SCIENTZ, JY92-IIN, China). Subsequently, cell fragments were removed by centrifugation (2,040 × *g*, 15 min, 4 °C). The supernatant was added into a His-accept nickel column and unbound proteins were washed out with the buffer (100 mM K_2HPO_4-KH_2PO_4, pH 8.0, 300 mM NaCl, 20 mM imidazole), while the bound proteins were eluted with the buffer (100 mM K_2HPO_4-KH_2PO_4, pH 8.0, 300 mM NaCl, 250 mM imidazole). The eluate was concentrated by ultrafiltration (Millipore, 10 kDa, USA) and the process was repeated until the imidazole was completely removed. The purity of the obtained cutinases was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the protein concentration was determined using NanoPhotometer-N50 (IMPLEN, Germany) at 280 nm.[1]

1.2 Preparation of PET substrates

The PET nanoparticles (PET-NPs) were prepared as follows:[2,3] 50 mg of amorphous PET film (AGfF-PET, Goodfellow, product number 252-144-75, UK) was dissolved in 5 mL of

hexafluoroisopropanol (HFIP). The polymer solution (10 mg/mL) was dispersed well in deionized water (200 mL) by ultrasound (180 W). HFIP and excess water were removed from the dispersion by vacuum rotary evaporation, followed by solid-liquid separation by filtration. The obtained PET-NPs was characterized by a dynamic light scattering size-analyzer (HORIBA, SZ-100, Japan) at 25 °C. The aforementioned protocol was repeated for triplicate, and the average particle size of PET-NPs was 100.2 ± 0.3 nm (Table S1). Subsequently, the concentration of PET-NPs was diluted to 0.25 mg/mL with deionized water for the enzyme activity of the cutinases.

As to produce PET particles (PcBP-PET) from post-consumer PET bottles (Body only, removed neck and base of the bottle), the initial pieces were to be cut (1 cm × 1 cm) from the commercial bottles (C'estbon, China). Then, the PET pieces were shredded into particles using a high-speed rotary mill equipped with a 1.0 mm trapezoidal hole sieve ring. PcBP-PET with ~1 mm diameter can be obtained after shredding.

The crystallinity and glass transition temperature of PET particles were measured by a differential scanning calorimeter (NETZSCH, DSC 200 F3, Germany). Generally, 7 mg of PET particle was equilibrated at 40 °C, heated to 300 °C at a rate of 10 °C/min, followed by holding at 300 °C for 5 min and cooling down to 40 °C at a rate of 10 °C/min. Finally, the surrounding environment was heated to 300 °C at a rate of 10 °C /min to obtain triple heat flow-temperature curves for heat up/down.

The crystallinity was calculated according to the following equation:

$$Crystallinity\ (\%) = \frac{\Delta H_m - \Delta H_{rc}}{\Delta H_m^*} \times 100\% \quad \text{(S1)}$$

where ΔH_m is the melting enthalpy (J/g), which can be determined by integrating the heat-absorbing melting peak over the first heat-up curve. ΔH_{rc} is the recrystallization enthalpy (J/g), which can be determined by integrating the exothermic recrystallization peak over the first heat-up curve. ΔH_m^* is the melting of 100% crystalline PET enthalpy, which is 140.1 J/g.[4-6]

The glass transition temperature was determined by the second heat-up curve and taken as the midpoint temperature of the glass transition process.[7]

1.3 Analysis of the mutant cutinases

The crystal structure of ThcCut1 (PDB ID: 5LUI) was used as a template of the mutant homology using the SWISS-MODEL online tool.[8,9] 2PET composing of 2 repeating units of PET was used for the model substrate, docked by using Autodock 4 and run for 250,000 energy evaluations. Molecular dynamics (MD) simulations were conducted using GROMACS 5.1.4 with charmm36-mar2019 force field parameters.[10] The enzyme-substrate complex was placed in a rectangular water cassette with a distance of 10 Å between the protein and the water cassette using TIP3P water molecules. Energy minimization was performed by the most rapid descent method before MD simulation. Two independent MD simulations were performed for each enzyme-substrate complex, starting with different initial atomic velocities. The NVT phase was performed using simulated annealing to warm the system, with the temperature gradually increasing from 5 K to 333 K within 300 ps, followed by stabilization at 333 K for 50 ps. 100 ps was equilibrated before the 10 ns product simulation. The time step is 2 fs, and the MD simulation is dynamically sampled every 1 ps. Throughout the MD simulations, periodic boundary conditions are used and the temperature is kept constant using a modified Berendsen thermostat.[11] Long-range interactions are handled using a particle mesh Ewald (PME) algorithm method, in which bonds involving hydrogen atoms were constrained by the LINCS algorithm and the long-range electrostatic interactions were calculated using the Ewald algorithm.[12,13] Snapshots of enzyme interactions with 2PET were analyzed and visualized with Pymol 2.3.3 software (https://pymol.org/).

The crystal structure of ThcCut1 (PDB: 5LUI) was used as a template for MD simulations using GROMACS 5.1.4 software with charmm36-mar2019 force field parameters. The

concentration of ligand $CaCl_2$ was 10 mM. The simulations were repeated 3 times independently at 300 K for 10 ns (2 fs time steps). The distance between the calcium ion and the hydroxyl oxygen atom in amino acid was counted (the binding distance was 1.95-2.35 Å). The number of frames with the distance between the calcium ion and the carboxyl oxygen atom in the amino acid in the binding distance range as a percentage of the total number of frames was counted separately as the binding probability. Amino acids with higher binding probabilities (the top 3) are shown in Fig. S7.

NanoDSF (NanoTemper, Prometheus NT.48, Germany) was adopted to determine the thermal stability of the cutinases. The device has an excitation wavelength of 280 nm and emission wavelengths of 330 nm and 350 nm. Wild-type ThcCut1 and its variants were diluted to 0.1 mg/mL using lysis buffer. Samples were heated from 25 °C to 100 °C at the rate of 1 °C/min, under excitation power of 100%. Melting temperature (T_m) was reported from Prometheus software (I_{330nm}/I_{350nm} ratio with maximum slope value corresponding to the temperature), and the measurement was repeated in triplicate.[14,15]

The enzyme activity of the cutinases towards PET particles can be determined as follow: the purified cutinases were diluted to 50 μg/mL in the reaction buffer (100 mM K_2HPO_4-KH_2PO_4, pH 8.0). Then, 300 μL of the solution was mixed thoroughly with 300 μL of PET nanoparticles (PET-NPs, 0.25 mg/mL) in a 2 mL centrifuge tube. The centrifuge tubes were shaken at 60 °C and 400 rpm for 2 h. The hydrolysis was terminated by adding 600 μL of termination buffer (160 mM H_3PO_4-KH_2PO_4 (pH 2.5) that contained 20% (v/v) of dimethyl sulfoxide (DMSO)).[16] The supernatant was collected by centrifugation (9,700 × *g*, 3 min) and the product was analyzed by high-performance liquid chromatography (HPLC). One unit of enzyme activity (U) was defined as the amount of enzyme required to generate 1 μmol terephthalic acid (TPA) per 1 h at 60 °C.

1.4 Effects of different additives on enzymatic hydrolysis of AGfF-PET

AGfF-PET (0.9 × 0.9 cm^2, ~25 mg) slice was suspended into 3 mL of the reaction buffer (blank control, additional 0.3% of Tween 80, or 0.3% of Triton X100, or 30 ppm dodecyltrimethylammonium bromide (DTAB),[17] or H_3PO_4 solution (100 mM), or KOH solution (186 mM)) was added into a 5 mL glass vial and pretreated at 60 °C for 2 h before the enzymatic hydrolysis carried out. After pretreatment, except for the acid or alkali pretreatment groups that need to be adjusted to the reaction pH value, other groups do not need to be adjusted. The reaction was initiated by adding ThcCut1-G63A (final concentration adjusted to 25 μg/mL). Finally, AGfF-PET was washed, dried, and weighed before and after the reaction to calculate the weight loss rate.

1.5 Enzymatic hydrolysis of the AGfF-PET and PcBP-PET

A slice of AGfF-PET (0.9 × 0.9 cm^2, ~25 mg) was placed in a 5 mL glass vial that containing 3 mL of reaction buffer (with or without 30 ppm DTAB) and 75 μg of the purified cutinase. Then, hydrolysis was performed by shaking the reaction flask at different temperatures on a dry thermostat (Ruicheng, DC10, China). After 96 h, the reaction was terminated by adding an equal volume of termination buffer. The supernatant was collected by centrifugation (9,700 × *g*, 3 min) and the product was analyzed by HPLC. The solid AGfF-PET residual remaining in the final reactants was washed, dried, and weighed to calculate the weight loss.

As for the evaluation of the availability of the mutant cutinases, PcBP-PET (~20 mg) was used following the similar protocol for AGfF-PET hydrolysis.

1.6 Analysis of the hydrolysis products of PET particles

The hydrolysis products of PET were analyzed using a high-performance liquid chromatography instrument (Thermo Fisher, UltiMate 3000, USA) equipped with an Acclaim

120 C18 column (Thermo Fisher, product number 059149, USA). The products mainly consisted of bis(2-hydroxyethyl) terephthalate (BHET), mono (hydroxyethyl) terephthalate (MHET), and terephthalic acid (TPA). Phosphate buffer (20 mM H_3PO_4-KH_2PO_4, pH 2.5) was applied as the A-phase and methanol as the B-phase of HPLC. The flow rate was 1 mL/min, and the elution was carried out in gradients of different concentrations: 25 % methanol for 15 min, methanol in a gradient from 25 % to 100 % for 10 min, 100 % methanol for 1 min, and finally methanol content in a gradient from 100 % to 25 % for 4 min. The effluent was monitored at 240 nm.[16] The morphology of the residual solid fraction of PET after enzymatic hydrolysis can be analyzed by scanning electron microscopy (HITACHI, E-1010, Japan) equipped with an electron beam intensity of 10 kV.

2. Supporting Tables

Table S1 Particle sizes of PET-NPs.

Sample	Z-average Size (nm)	Polydispersity Index
1	99.9	0.280
2	100.3	0.304
3	100.4	0.295

Table S2 Crystallinity and glass transition temperature of PET specimens.

Specimens	Melting enthalpy (J/g)	Recrystallization enthalpy (J/g)	Glass transition temperature (°C)	Crystallinity (%)
AGfF-PET	30.8	20.9	81.0	7.1
PcBF-PET	37.1	0	81.7	26.5
PcBP-PET	29.0	16.3	81.7	9.1

3. Supporting Figures

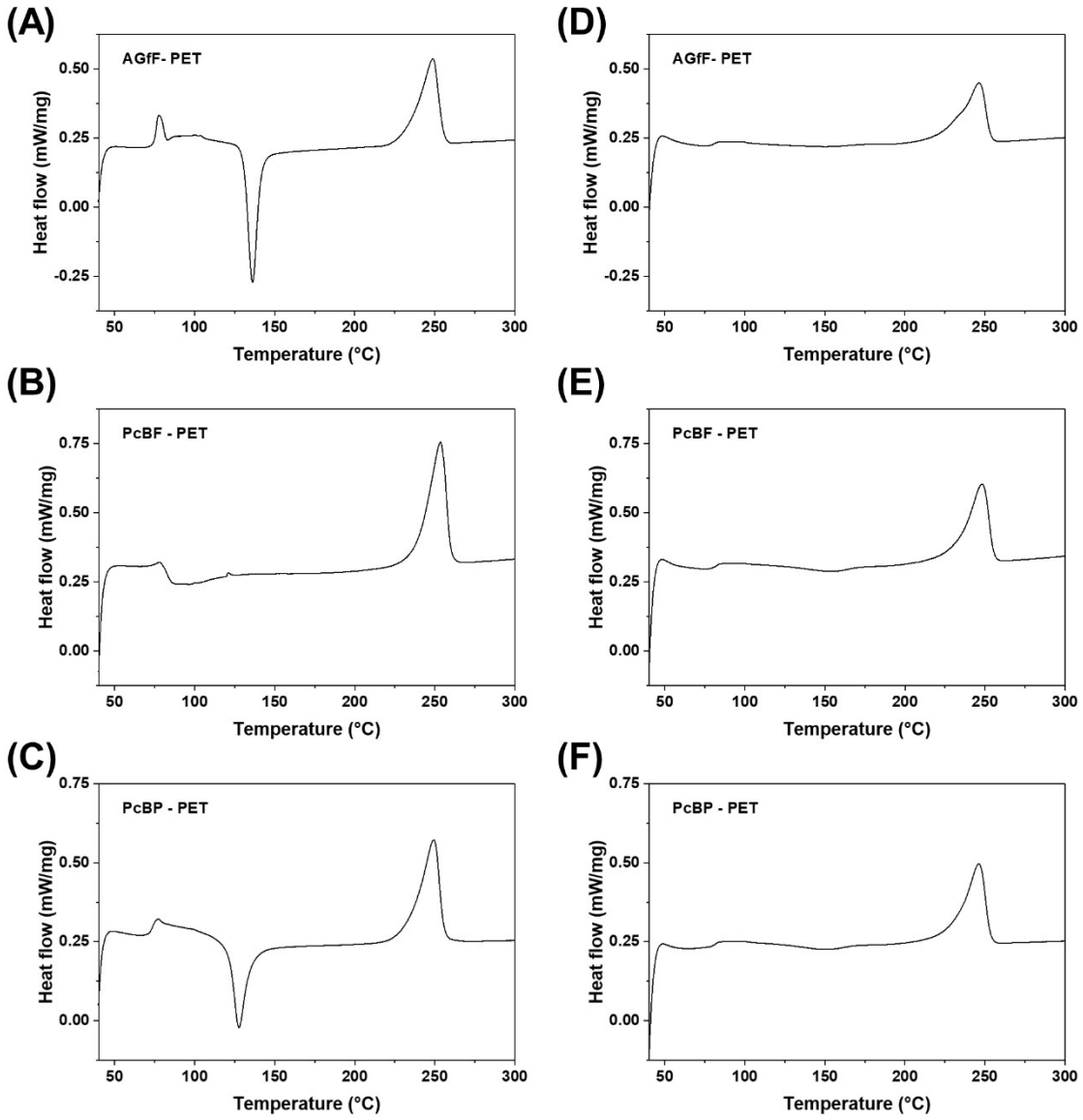

Fig. S1 Heat flow-temperature curve for the first heat-up of (A) AGfF-PET, (B) PcBF-PET, and (C) PcBP-PET, and the second heat-up of (D) AGfF-PET, (E) PcBF-PET, and (F) PcBP-PET by DSC.

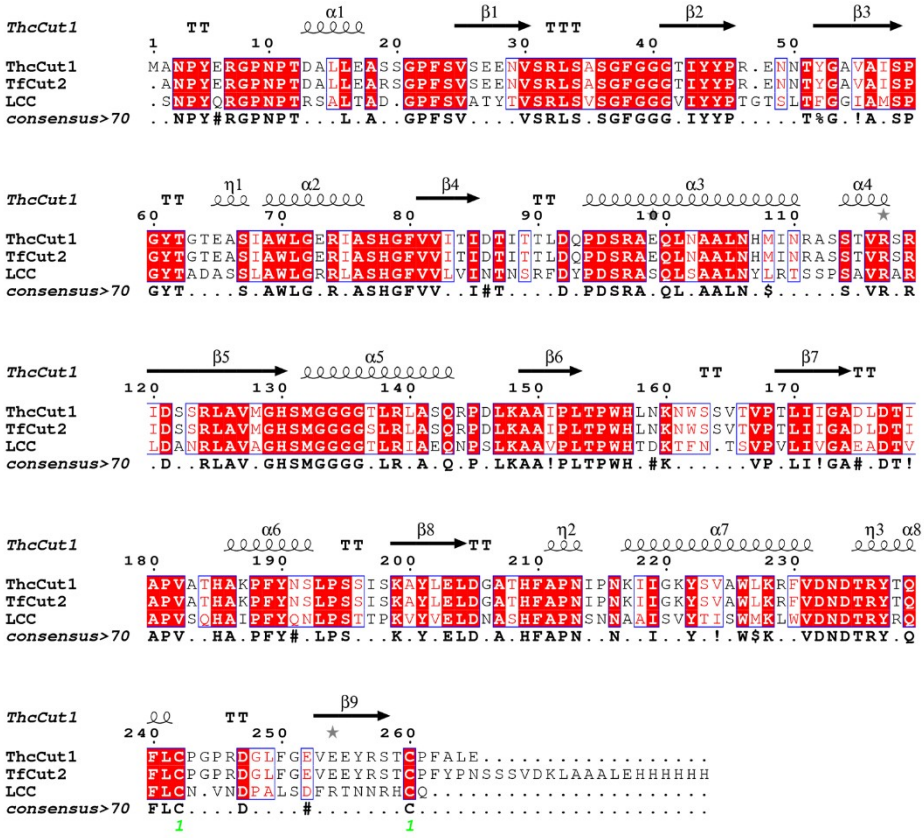

Fig. S2 Sequence alignment of cutinases for PET hydrolysis. The amino acid sequences of ThcCut1 (PDB: 5LUI), TfCut2 (PDB: 4CG1), and LCC (PDB: 4EB0) were aligned. Sequence analysis is carried out based on ClustalX and aligned with ESPript.[181,19] Squares shaded in red refer to the same region, and residues in red text are moderately conserved. The cartoon above the alignment refers to the secondary structure, while the helix refers to the α-helix. The arrow refers to the β-strand, "T" refers to the turn, and the green number below the sequence is the position of the residue forming the disulfide bond.

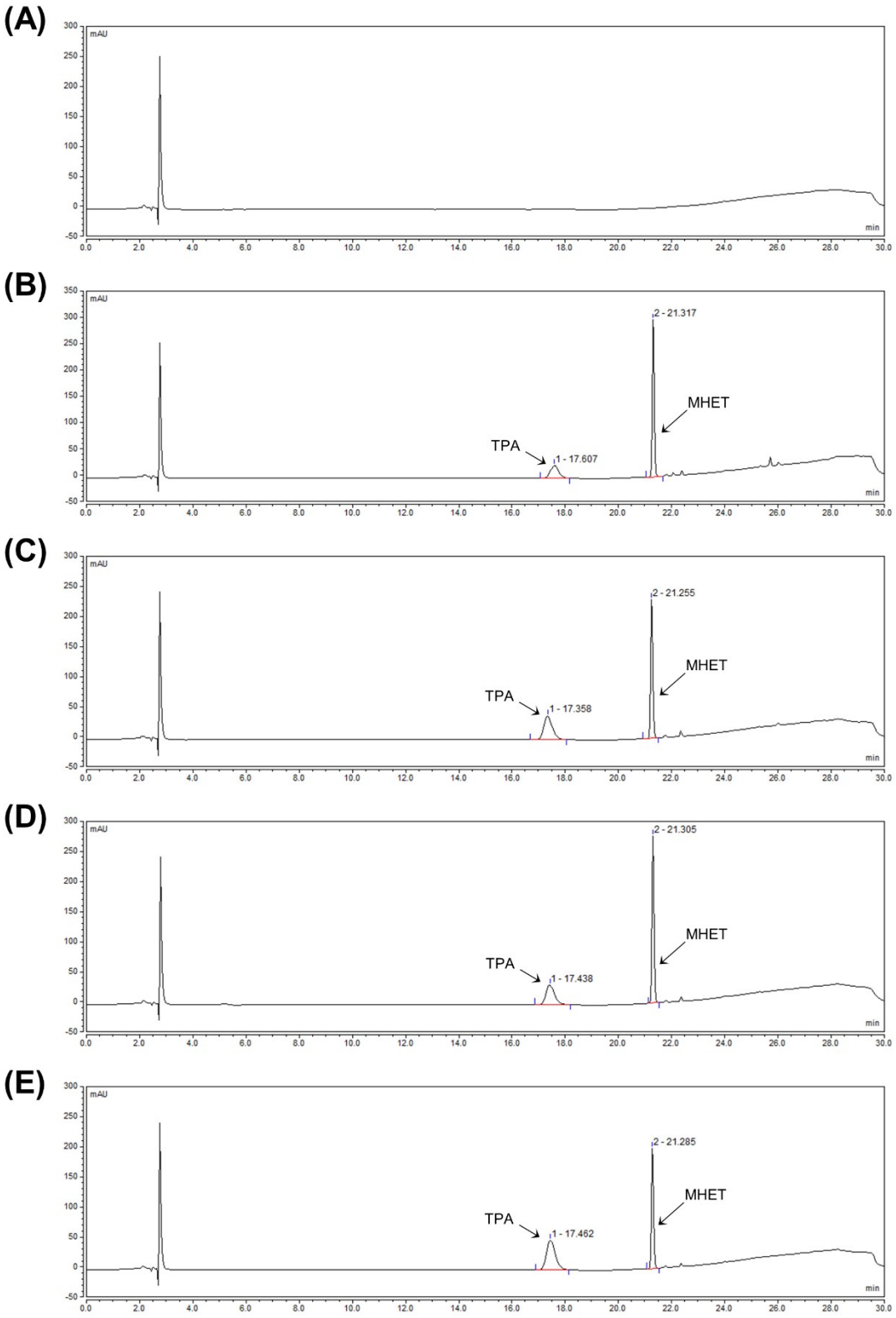

Fig. S3 HPLC spectrums of the hydrolyzing products of PET-NPs. The group (A) without enzyme, (B) containing wild-type ThcCut1, (C) containing ThcCut1-G63A, (D) containing ThcCut1-F210I, and (E) containing ThcCut1-G63A/F210I.

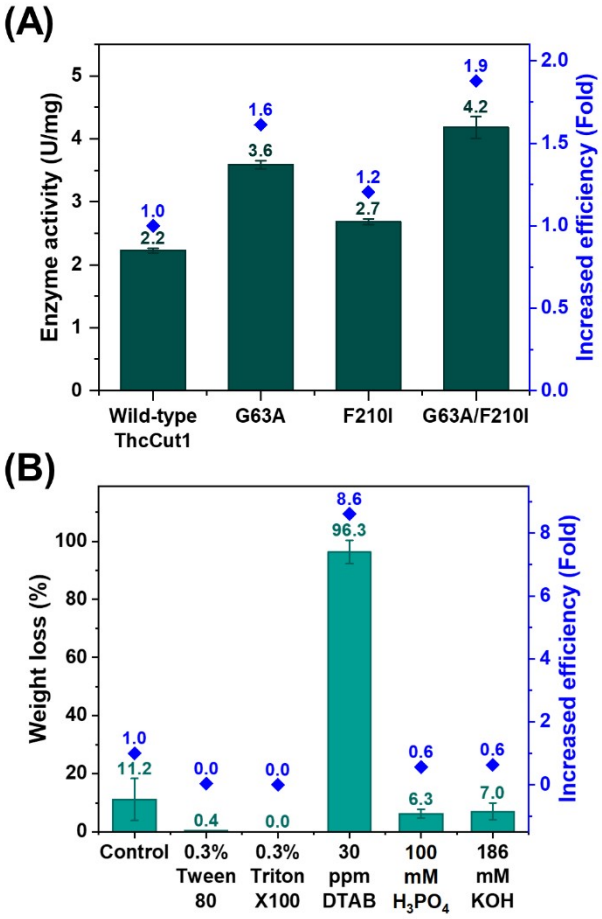

Fig. S4 The activity analysis of ThcCut1 variants and effects of different additives on AGfF-PET enzymatic hydrolysis. (A) The activity of ThcCut1 variants using PET-NPs hydrolysis. The temperature was maintained at 60 °C. (B) Weight loss of the pretreated AGfF-PET (0.9 × 0.9 cm^2, ~25 mg) by ThcCut1-G63A variant hydrolysis after 192 h of reaction.

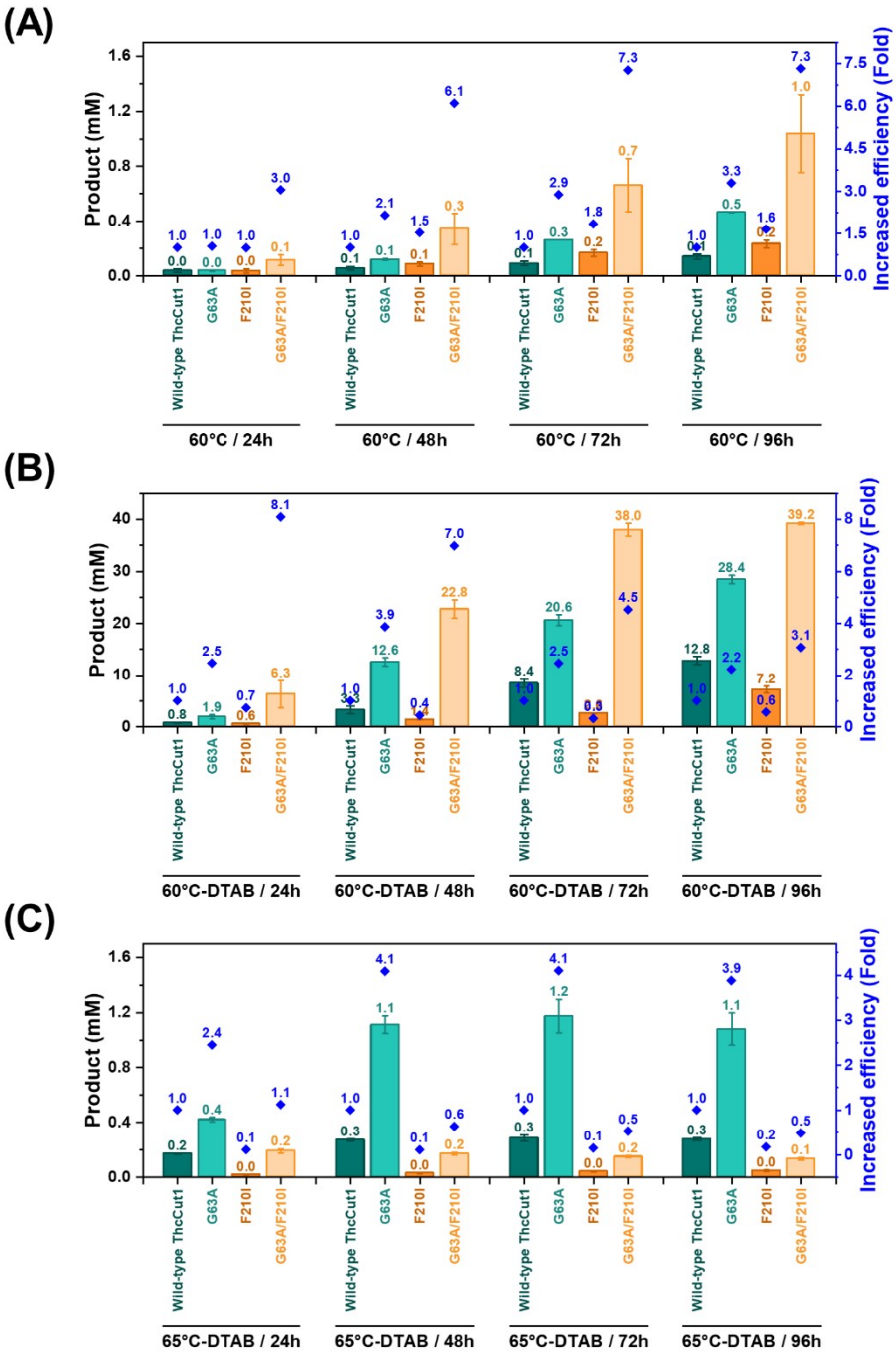

Fig. S5 Hydrolysis of AGfF-PET (0.9 × 0.9 cm^2, ~25 mg) by ThcCut1 and its variants under (A) 60 °C without surfactant, (B) 60 °C with DTAB, and (C) 65 °C with DTAB. The products were the sum of MHET and TPA.

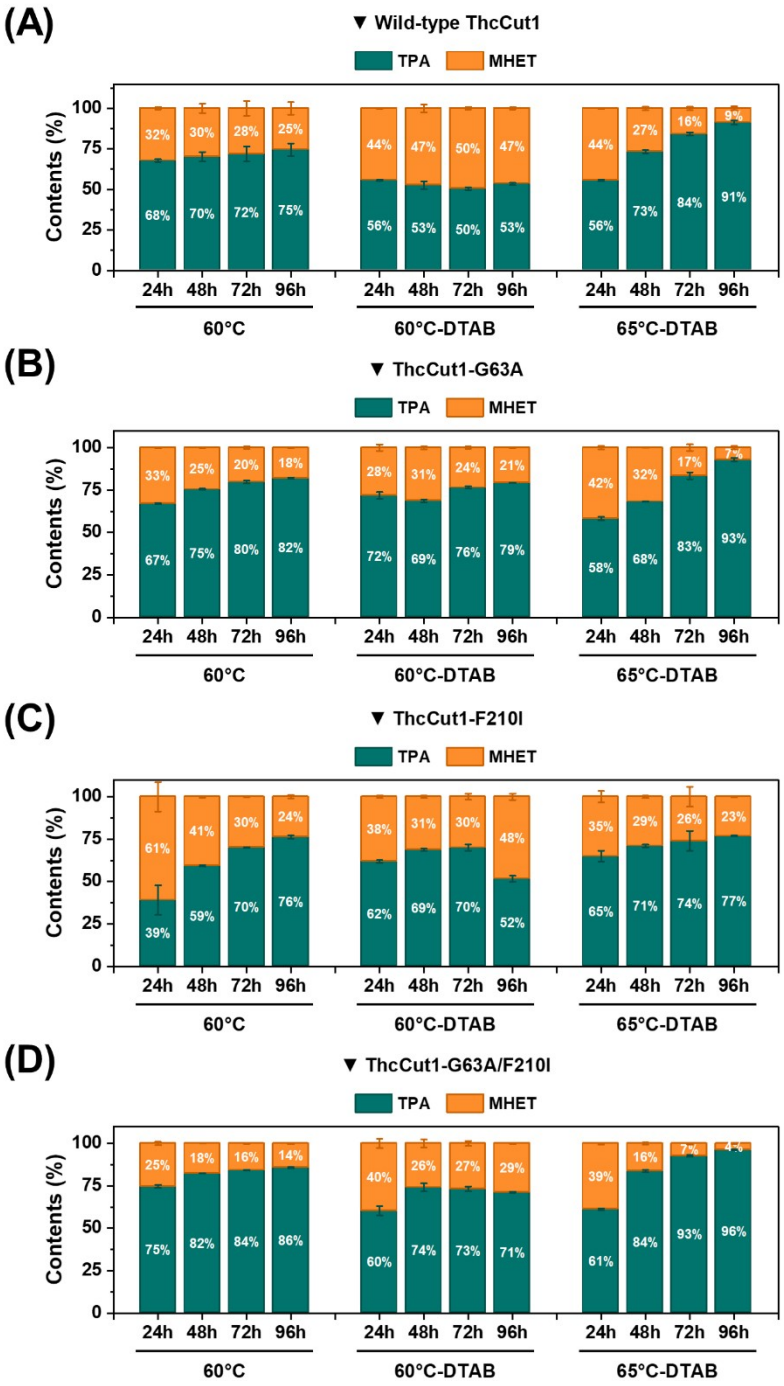

Fig. S6 Constitution of chemical components in the hydrolysate of the AGfF-PET (0.9 × 0.9 cm^2, ~25 mg) using (A) wild-type ThcCut1, (B) ThcCut1-G63A, (C) ThcCut1-F210I, and (D) ThcCut1-G63A/F210I.

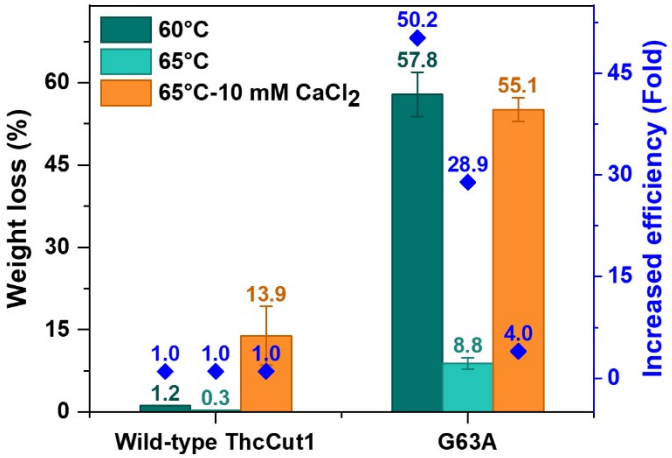

Fig. S7 Comparison of the hydrolysis of AGfF-PET (0.9 × 0.9 cm^2, ~25 mg) by wild-type ThcCut1 and the G63A variant. Samples were taken after 192 h of reaction.

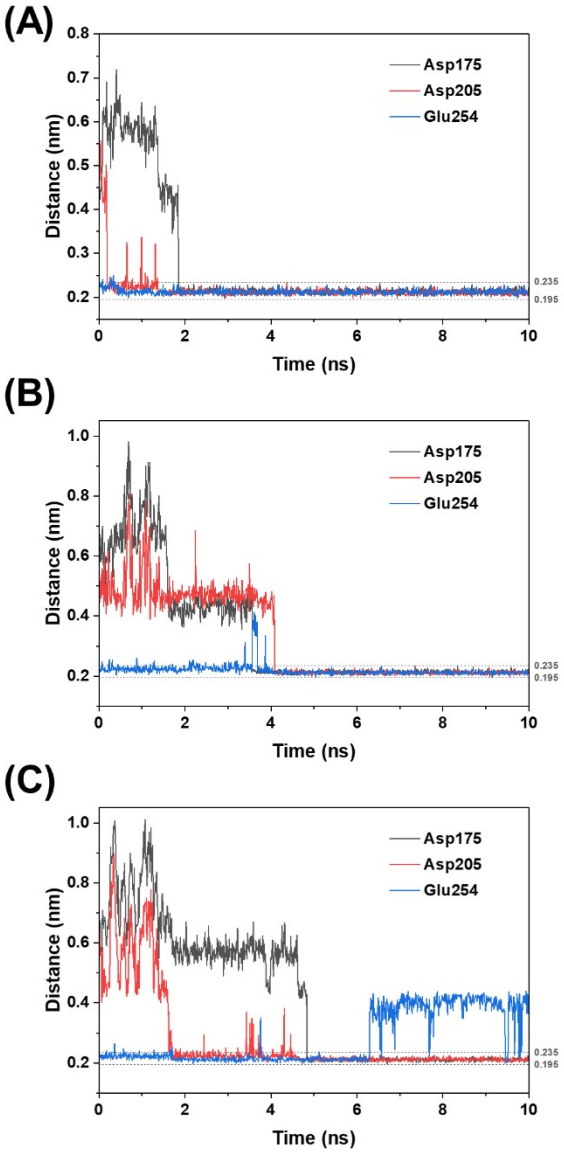

Fig. S8 The distance between the calcium ion and the carboxyl oxygen atom of the amino acid in ThcCut1 at 300 K by molecular dynamics (MD) simulation.

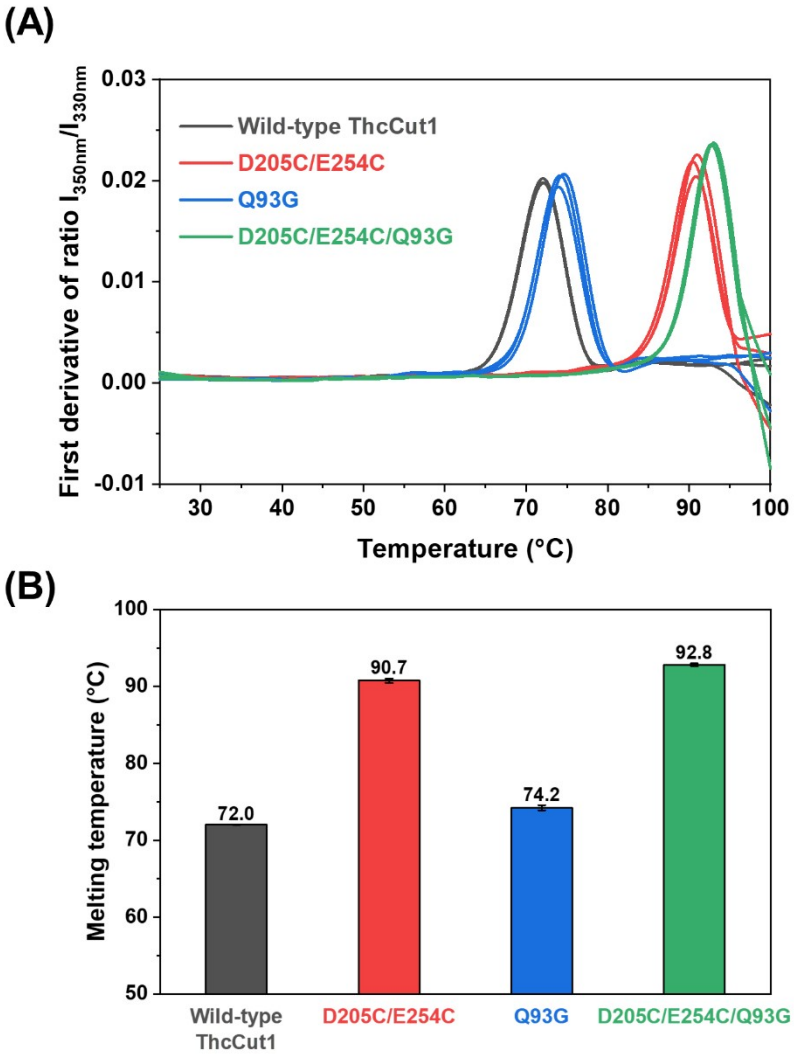

Fig. S9 Analysis of thermal stability of ThcCut1 variants. (A) Thermal stability measurements of ThcCut1 variants through differential scanning fluorimetry (DSF). (B) Comparison of melting temperature (T_m) of ThcCut1 variants.

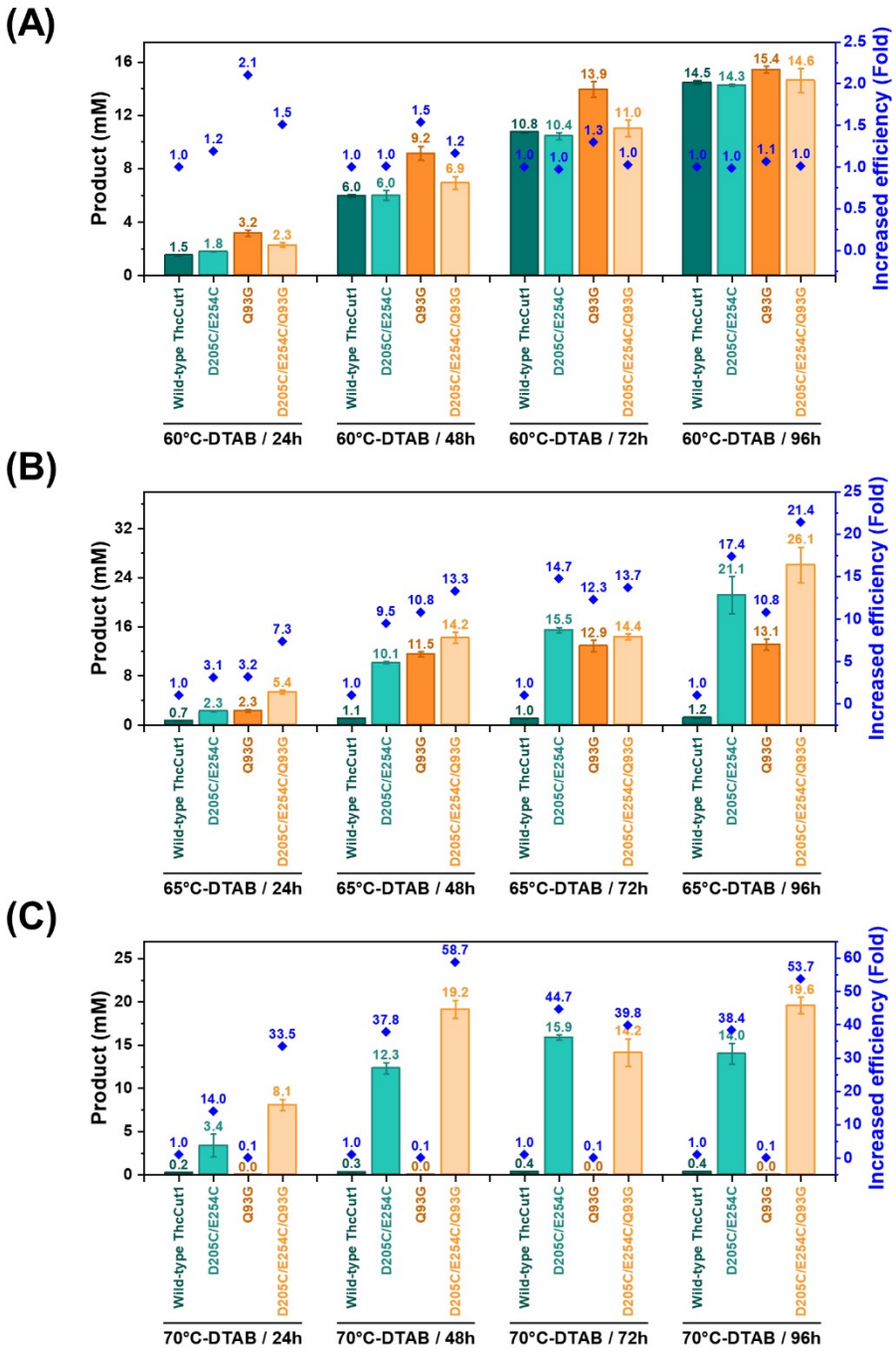

Fig. S10 Hydrolysis of AGfF-PET (0.9 × 0.9 cm^2, ~25 mg) by ThcCut1 variants under (A) 60 °C, (B) 65 °C, and (C) 70 °C. DTAB was added into the medium as surfactant. The products were the sum of MHET and TPA.

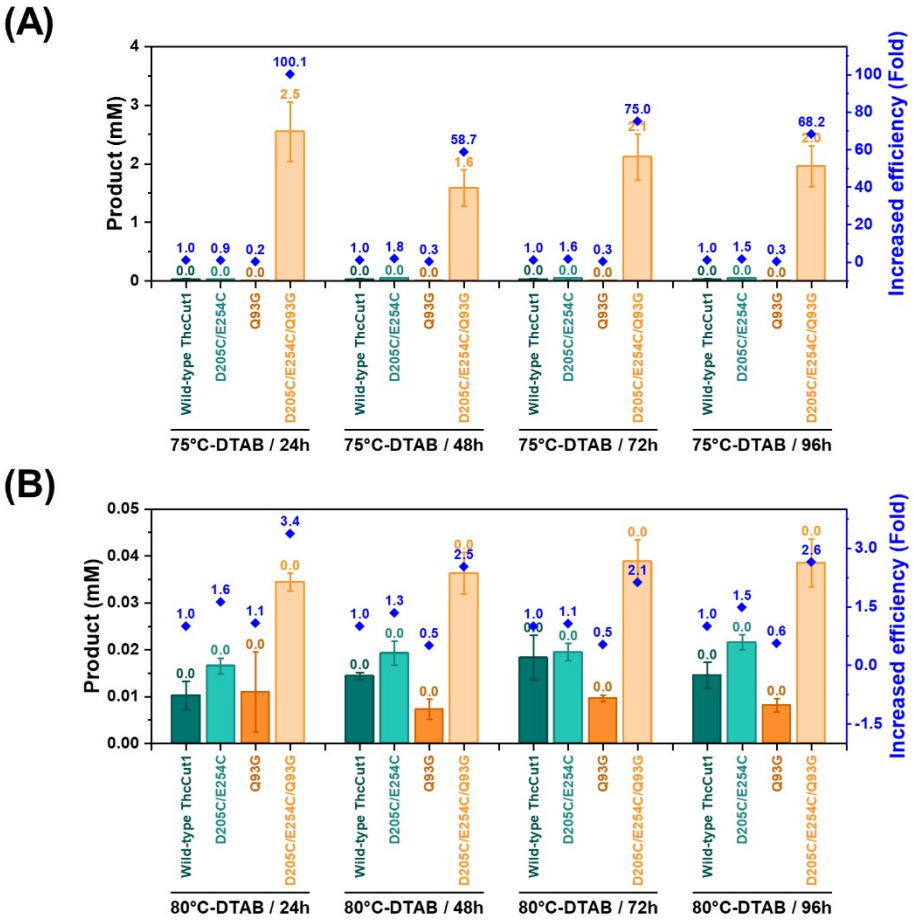

Fig. S11 Hydrolysis of AGfF-PET (0.9×0.9 cm^2, ~25 mg) by ThcCut1 variants under (A) 75 °C, and (B) 80 °C. DTAB was added into the medium as surfactant. The products were the sum of MHET and TPA.

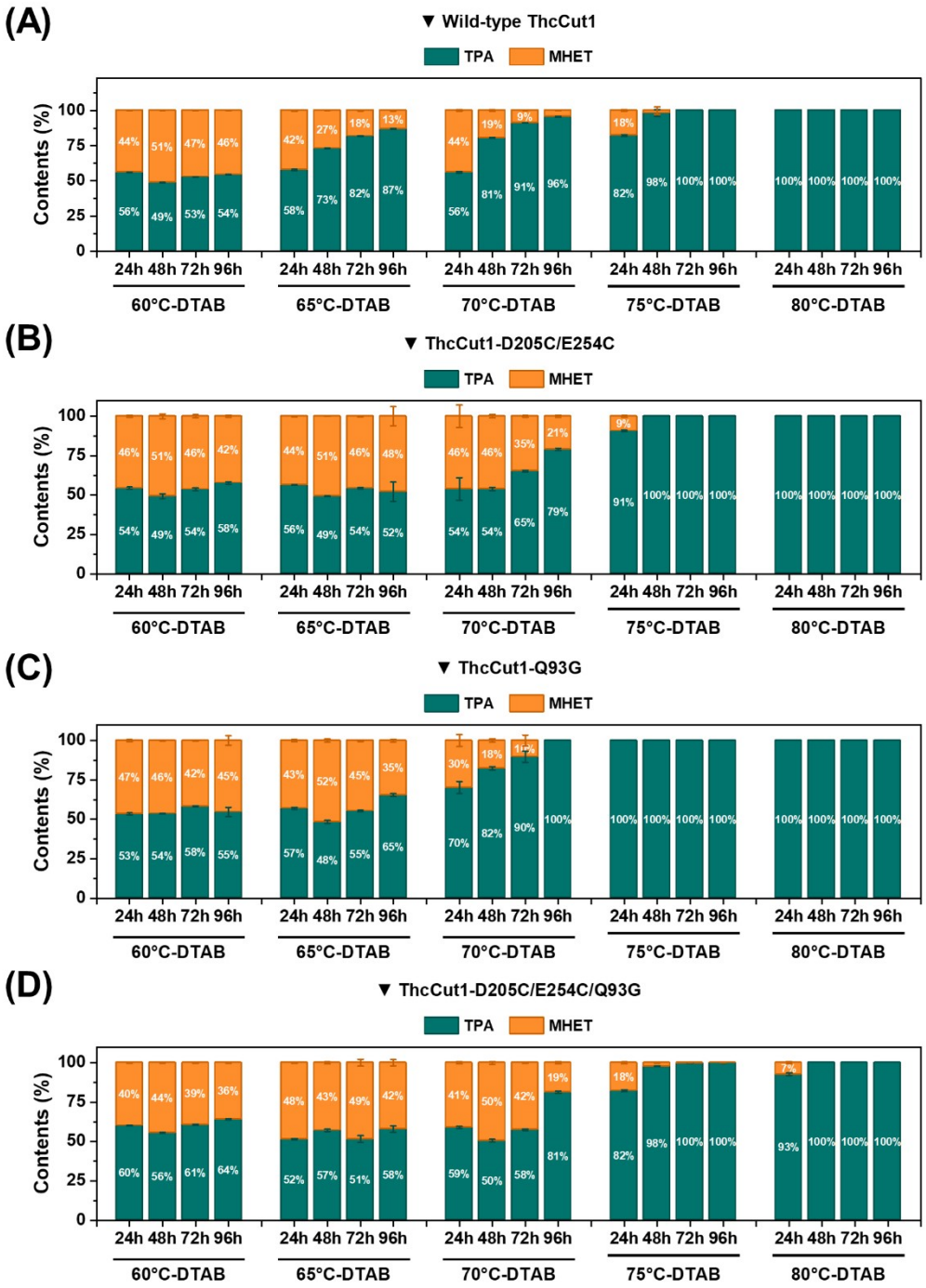

Fig. S12 Constitution of chemical components in the hydrolysate of AGfF-PET (0.9 × 0.9 cm^2, ~25 mg) using (A) wild-type ThcCut1, (B) ThcCut1-D205C/E254C, (C) ThcCut1-Q93G, and (D) ThcCut1-D205C/E254C/Q93G.

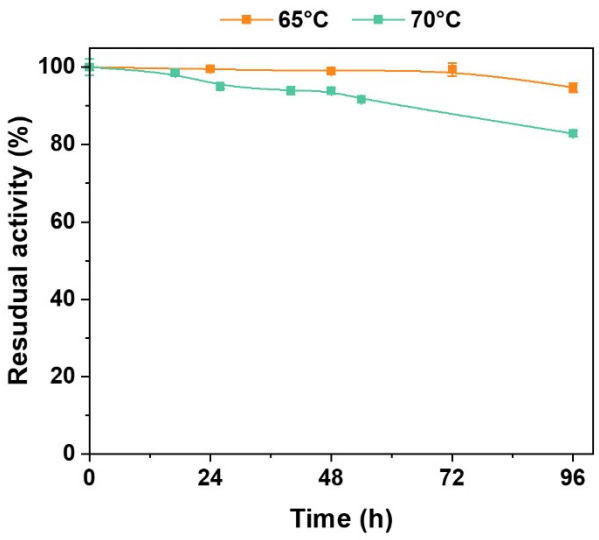

Fig. S13 Thermal residual enzyme activity of ThcCut1-D205C/E254C/Q93G.

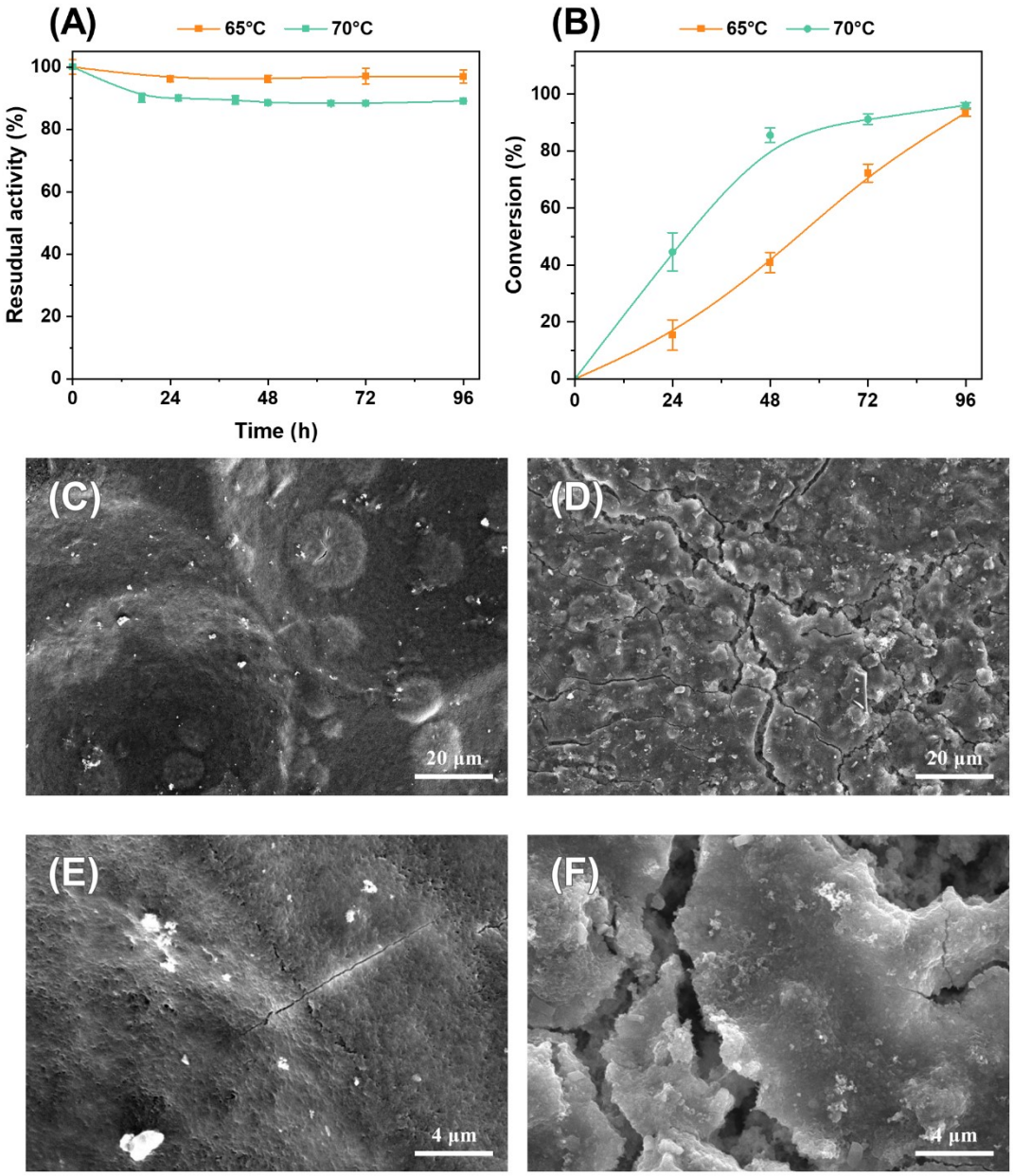

Fig. S14 ThcCut1-G63A/F210I/D205C/E254C/Q93G variant at different temperatures for (A) thermal residual enzyme activity, and (B) the reaction process of hydrolysis with AGfF-PET (0.9 × 0.9 cm^2, ~25 mg). SEM image of the residual AGfF-PET hydrolyzed by ThcCut1-G63A/F210I/D205C/E254C/Q93G under (C) 65 °C and 20 μm scale bar, (D) 70 °C and 20 μm scale bar, (E) 65 °C and 4 μm scale bar, and (F) 70 °C and 4 μm scale bar.

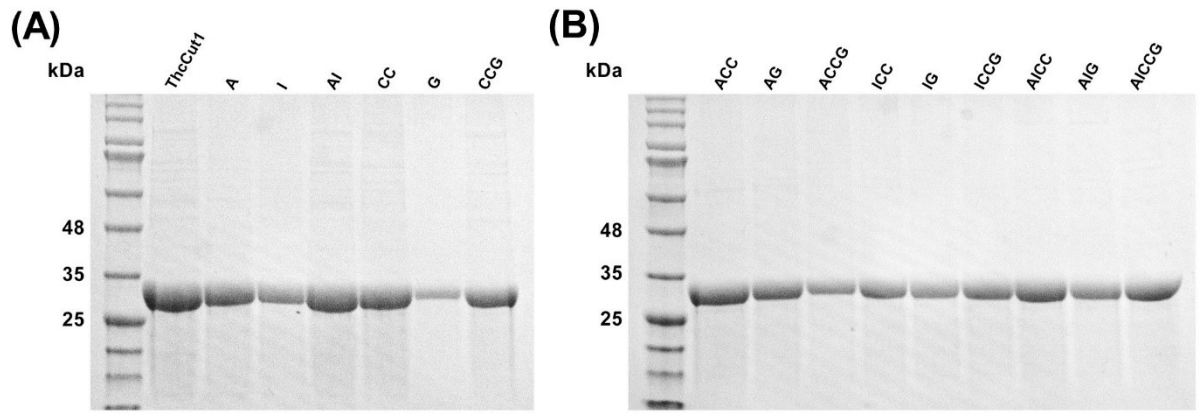

Fig. S15 SDS-PAGE analysis of wild-type ThcCut1 and its variants.

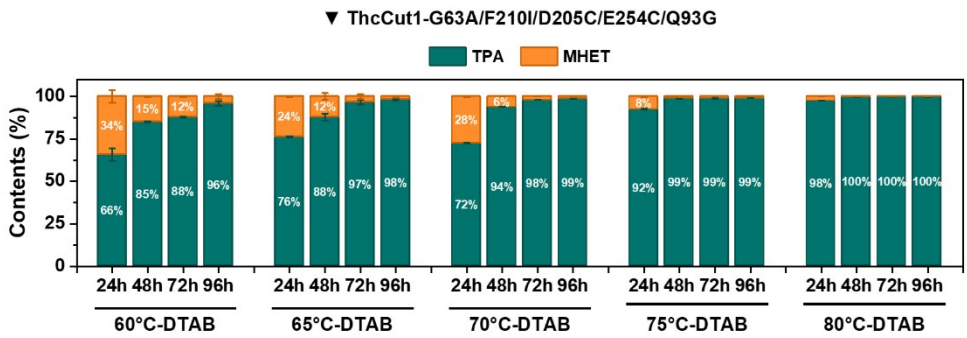

Fig. S16 Constitution of chemical components in the hydrolysate of AGfF-PET (0.9 × 0.9 cm^2, ~25 mg) using ThcCut1-G63A/F210I/D205C/E254C/Q93G under different temperatures.

4. Supporting Nucleotide Sequences

4.1 Wild-type ThcCut1

CATATGGCGAATCCGTATGAACGCGGCCCGAATCCGACCGATGCGCTGTTAGAA

GCGAGCAGCGGTCCATTTAGCGTGAGCGAAGAAAATGTGAGCCGCCTGAGCGCG

AGCGGCTTTGGTGGTGGTACCATTTATTATCCGCGCGAAAATAATACCTACGGCG

CGGTGGCGATTAGCCCGGGTTATACCGGTACCGAAGCGAGCATTGCGTGGCTGG

GTGAACGCATTGCGAGCCATGGTTTTGTGGTGATTACCATTGATACCATTACCAC

CCTGGATCAGCCGGATAGCCGCGCGGAACAATTAAATGCGGCGTTAAATCACAT

GATTAACCGCGCGAGCAGCACCGTGCGCAGTAGAATTGATAGCAGCCGTCTGGC

GGTTATGGGCCATAGCATGGGCGGTGGTGGTACCTTACGTTTGGCGAGCCAACG

CCCAGATTTAAAAGCGGCGATTCCGTTGACCCCATGGCATCTGAATAAAAATTGG

AGCAGCGTGACCGTGCCGACCCTGATTATTGGCGCGGATCTGGATACCATTGCGC

CGGTGGCGACTCATGCGAAACCATTTTATAATAGCCTGCCGAGCAGCATTAGCA

AAGCGTATCTGGAACTGGATGGCGCGACCCATTTTGCGCCGAATATTCCGAATAA

AATTATCGGCAAATACAGCGTGGCGTGGCTGAAACGCTTTGTGGATAATGATAC

CCGCTATACCCAGTTTCTGTGCCCGGGCCCACGTGATGGCTTATTTGGTGAAGTT

GAAGAATATCGCAGCACCTGCCCGTTTCTCGAG

4.2 ThcCut1-G63A

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AGCGGCTTTGGTGGTGGTACCATTTATTATCCGCGCGAAAATAATACCTACGGCG

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GTGAACGCATTGCGAGCCATGGTTTTGTGGTGATTACCATTGATACCATTACCAC

CCTGGATCAGCCGGATAGCCGCGCGGAACAATTAAATGCGGCGTTAAATCACAT

GATTAACCGCGCGAGCAGCACCGTGCGCAGTAGAATTGATAGCAGCCGTCTGGC

GGTTATGGGCCATAGCATGGGCGGTGGTGGTACCTTACGTTTGGCGAGCCAACG

CCCAGATTTAAAAGCGGCGATTCCGTTGACCCCATGGCATCTGAATAAAAATTGG

AGCAGCGTGACCGTGCCGACCCTGATTATTGGCGCGGATCTGGATACCATTGCGC

CGGTGGCGACTCATGCGAAACCATTTTATAATAGCCTGCCGAGCAGCATTAGCA

AAGCGTATCTGGAACTGGATGGCGCGACCCATTTTGCGCCGAATATTCCGAATAA

AATTATCGGCAAATACAGCGTGGCGTGGCTGAAACGCTTTGTGGATAATGATAC

CCGCTATACCCAGTTTCTGTGCCCGGGCCCACGTGATGGCTTATTTGGTGAAGTT

GAAGAATATCGCAGCACCTGCCCGTTTCTCGAG

4.3 ThcCut1-F210I

CATATGGCGAATCCGTATGAACGCGGCCCGAATCCGACCGATGCGCTGTTAGAA

GCGAGCAGCGGTCCATTTAGCGTGAGCGAAGAAAATGTGAGCCGCCTGAGCGCG

AGCGGCTTTGGTGGTGGTACCATTTATTATCCGCGCGAAAATAATACCTACGGCG

CGGTGGCGATTAGCCCGGGTTATACCGGTACCGAAGCGAGCATTGCGTGGCTGG

GTGAACGCATTGCGAGCCATGGTTTTGTGGTGATTACCATTGATACCATTACCAC

CCTGGATCAGCCGGATAGCCGCGCGGAACAATTAAATGCGGCGTTAAATCACAT

GATTAACCGCGCGAGCAGCACCGTGCGCAGTAGAATTGATAGCAGCCGTCTGGC

GGTTATGGGCCATAGCATGGGCGGTGGTGGTACCTTACGTTTGGCGAGCCAACG

CCCAGATTTAAAAGCGGCGATTCCGTTGACCCCATGGCATCTGAATAAAAATTGG

AGCAGCGTGACCGTGCCGACCCTGATTATTGGCGCGGATCTGGATACCATTGCGC

CGGTGGCGACTCATGCGAAACCATTTTATAATAGCCTGCCGAGCAGCATTAGCA

AAGCGTATCTGGAACTGGATGGCGCGACCCATATAGCGCCGAATATTCCGAATA

AAATTATCGGCAAATACAGCGTGGCGTGGCTGAAACGCTTTGTGGATAATGATA

CCCGCTATACCCAGTTTCTGTGCCCGGGCCCACGTGATGGCTTATTTGGTGAAGT

TGAAGAATATCGCAGCACCTGCCCGTTTCTCGAG

4.4 ThcCut1-G63A/F210I

CATATGGCGAATCCGTATGAACGCGGCCCGAATCCGACCGATGCGCTGTTAGAA

GCGAGCAGCGGTCCATTTAGCGTGAGCGAAGAAAATGTGAGCCGCCTGAGCGCG

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GTGAACGCATTGCGAGCCATGGTTTTGTGGTGATTACCATTGATACCATTACCAC

CCTGGATCAGCCGGATAGCCGCGCGGAACAATTAAATGCGGCGTTAAATCACAT

GATTAACCGCGCGAGCAGCACCGTGCGCAGTAGAATTGATAGCAGCCGTCTGGC

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TGAAGAATATCGCAGCACCTGCCCGTTTCTCGAG

4.5 ThcCut1-D205C/E254C

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GTGAACGCATTGCGAGCCATGGTTTTGTGGTGATTACCATTGATACCATTACCAC

CCTGGATCAGCCGGATAGCCGCGCGGAACAATTAAATGCGGCGTTAAATCACAT

GATTAACCGCGCGAGCAGCACCGTGCGCAGTAGAATTGATAGCAGCCGTCTGGC

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CCGCTATACCCAGTTTCTGTGCCCGGGCCCACGTGATGGCTTATTTGGTGAAGTT

TGCGAATATCGCAGCACCTGCCCGTTTCTCGAG

4.6 ThcCut1-Q93G

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4.7 ThcCut1-D205C/E254C/Q93G

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4.8 ThcCut1-G63A/D205C/E254C

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GTGAACGCATTGCGAGCCATGGTTTTGTGGTGATTACCATTGATACCATTACCAC

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4.9 ThcCut1-G63A/Q93G

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4.10 ThcCut1-G63A/D205C/E254C/Q93G

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TTGCGAATATCGCAGCACCTGCCCGTTTCTCGAG

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