Supporting Information

Accelerated biodegradation of polyurethanes through embedded cutinases

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Detailed Experimental Procedures

Material

Reagents were purchased in analytical grade from Merck (Darmstadt, Germany), Fisher Scientific (Schwerte, Germany), Acros Organics (Geel, Belgium) or Carl Roth (Karlsruhe, Germany) unless otherwise stated. The TPU samples were sourced from Covestro Deutschland AG (Leverkusen, Germany).

Strains and cutinases

The two *Escherichia coli* (*E. coli*) strains DH5α and BL21(DE3) were acquired from Fisher Scientific (Schwerte, Germany). The cutinase Novozym® 51032, reported as cutinase from *Humicola insolens* (HiC),[1] was obtained from Strem Chemicals (Bischheim, France).

Preparation of HiC lyophilisate

HiC was purchased as stock solution. For incorporation and heat tests, buffer exchange was conducted via crossflow filtration using a Vivaflow® 50 cassette (Sartorius, Göttingen, Germany) with a 10 kDa MWCO and 20 mM ammonium acetate buffer (pH 8.0). The gained cutinase solution was frozen at -80 °C and subsequently freeze-dried for 3 days at -68 °C and 0.1 mbar (Alpha 1-2 LDplus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). The obtained cutinase lyophilisate powder was stored at 4 °C in a sealed tube (Labcon™ SuperClear™, Petaluma, USA) for further use.

Plasmid construction

The genes encoding the BhrPETase, DuraPETase^{N233K}, and SvCut190*SS were synthesized by Eurofins Genomics (Ebersberg, Germany) and subsequently cloned into the multiple cloning site of the pET26b(+) vector from Merck (Darmstadt, Germany) using restriction cloning techniques. The same cloning method and vector were employed for the genes encoding the LCC, LCC-ICCG, LCC-WCCG, and ThcCut1. For the PES-H1 gene, the vector was switched to pET28a(+) from Merck (Darmstadt, Germany), while the TfCut2 gene was cloned into the pBAD expression vector from Invitrogen life technologies/Thermo Fisher Scientific (Darmstadt, Germany). The gene for ThcCut1-ACCG[2] was derived from the wild-type ThcCut1 gene through mutation polymerase chain reaction (Biometra TAdvanced 96 SG, 230 V, Göttingen, Germany) and subsequently cloned into the pET26b(+) vector. Chemically competent *E. coli* DH5α cells were transformed for plasmid amplification. Single colonies were selected and used to inoculate 4.5 ml of Lysoegny broth (LB) supplemented with either 50 µg/ml kanamycin or 100 µg/ml ampicillin depending on resistance cassette on the plasmid vector. For working stocks, the resulting *E. coli* cultures were preserved with 20 % (v/v) glycerol and stored at -80 °C. For sequencing at GeneWiz (Leipzig, Germany) plasmids were isolated from grown liquid *E. coli* cultures using the GeneJET Plasmid Miniprep kit from Thermo Fisher Scientific (Darmstadt, Germany) and prepared according to the manufacturer's protocols.

Heterologous protein expression of cutinases

For the heterologous expression of cutinases, the *E. coli* strain BL21(DE3) was transformed with the respective plasmid constructs and used as expression host. Pre-cultures were prepared in a volume of 4.5 ml LB medium supplemented with 1 % glucose and either 50 µg/ml kanamycin or 100 µg/ml ampicillin, depending on the plasmid's resistance cassette. The LB medium was inoculated directly from each respective glycerol stock and incubated overnight at 37 °C and 200 rpm agitation (ISF1-X, Adolf Kühner AG, Birsfelden, Switzerland). For the main culture, 200 ml of ZYP-5052[3] autoinduction medium with the appropriate antibiotic (50 µg/ml kanamycin or 100 µg/ml ampicillin) was used (Table S1). For the pBAD expression system, 200 ml of LB medium with 100 µg/ml ampicillin was utilized. The main culture was inoculated with 0.2 % (v/v) of the overnight culture and incubated at 37 °C and 200 rpm for 4.5 h (ISF1-X). In the pBAD containing culture, expression was induced by the addition of 0.2 % (v/v) L-arabinose to the LB medium. Both autoinduction and arabinose-induced expression were carried out for 24 h at 20 °C with agitation at 200 rpm (ISF1-X). After the total incubation time, the cells were harvested by centrifugation at 4 °C for 20 minutes at 8,000 x g (Multifuge X4R Pro, Thermo Fisher Scientific, Waltham, USA).

Table S1 Cultivation and expression media used in this work.

Medium name and components	**Ingredients**
Lysogeny broth medium	10 g/l tryptone, 10 g/l NaCl, 5 g/l yeast extract
ZYP-5052 autoinduction medium (1,000 ml)	
ZY-Basis	928 ml
20 x P	50 ml
50 x 5052	20 ml
500 x $MgSO_4$	2 ml
1,000 x Trace elements	0.2 ml
ZY-Basis	10 g/l tryptone, 5 g/l yeast extract
20 x P	268 g/l Na_2HPO_4, 136 g/l KH_2PO_4, 66 g/l $(NH_4)SO_4$
50 x 5052	250 g/l glycerol, 25 g/l glucose, 100 g/l α-lactose
500 x $MgSO_4$	246.5 g/l $MgSO_4$
1,000 x Trace elements	50 mM $FeCl_3$, 20 mM $CaCl_2$, 10 mM $MnCl_2$, 10 mM $ZnSO_4$, 2 mM $CoCl_2$, 2 mM $CuCl_2$, 2 mM $NiCl_2$, 2 mM $NaMoO_4$, 2 mM Na_2SeO_3, 2 mM H_3BO_3

Cell lysis after protein expression

Harvested cells expressing cutinases not intended for purification via affinity chromatography were resuspended in 10 ml 20 mM KPi buffer at pH 7.5. Harvested cells expressing cutinases intended for purification via affinity chromatography were resuspended in 10 ml of imidazole-containing lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole, pH 8.0). Cell lysis was performed using three sonication cycles (Bandelin Sonoplus HD, sonotrode MS73, Berlin, Germany), each lasting 2 minutes with an amplitude of 50 %. The cells were under constant cooling in an ice bath during sonication. To separate cell debris from soluble cutinases, the lysed cells

were centrifuged at 10,000 x g and 4 °C for 20 min (Multifuge X4R Pro), and the supernatant was subsequently filtered using a 0.2 µm sterile PES filter (Fisherbrand™, Thermo Fisher Scientific, Waltham, USA).

Lyophilisation of cell lysates after protein expression

All supernatants obtained after cell lysis, centrifugation, and sterile-filtration, which contained the soluble fraction of the expressed but non-purified cutinases BhrPETase, DuraPETase^{N233K}, SvCut190*SS, ThcCut1, ThcCut1-ACCG, LCC, LCC-ICCG, LCC-WCCG, TfCut2, and PES-H1, were frozen at -80 °C. Subsequently, all frozen supernatants were freeze-dried for 3 days at -68 °C and 0.1 mbar (Alpha 1-2 LDplus). The produced lyophilised lysates were stored at 4 °C until further use.

Cutinase purification via immobilized metal affinity chromatography (IMAC)

The SvCut190*SS was expressed both without and with a His-tag to enable purification via IMAC. The purification columns (Thermo Scientific™ Pierce™, Thermo Fisher Scientific, Rockford, USA) were prepared by loading them with the appropriate volume of Ni-NTA resin (HisPur™ Ni-NTA Resin, Fisher Scientific, Rockford, USA). The Ni-NTA resin was then equilibrated with 8 column volumes (CV) of lysis buffer. After equilibration, the filtered lysate containing the soluble fraction of SvCut190*SS was applied to the column. Following lysate application, the column was washed with 10 CV of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole). Elution of the SvCut190*SS was done with 4 CV of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole, pH 8). The pooled elution fractions were then concentrated with Amicon® Ultra centrifugal filters (10 kDa MWCO). Buffer exchange and desalting of the concentrated SvCut190*SS solution was performed using the gravity protocol for PD-10 columns according to the manufacturer's instructions. The SvCut190*SS was stored in 20 mM potassium phosphate buffer at pH 7.5. For lyophilisation the solution was frozen at -80 °C. Freeze-drying was done overnight at -68 °C and 0.1 mbar (Alpha 1-2 LDplus). The produced lyophilisate was stored at 4 °C until further use.

Determination of protein concentration

Protein concentration was quantified with the Bradford assay and a bovine serum albumin (BSA) standard curve, in the concentration range from 125 to 1,000 µg/ml. Cutinase samples were prepared by dissolving the dry lyophilisate in water for at least 2 h at room temperature, followed by sterile filtration. To determine the cutinase concentration during cutinase purification, the liquid elution fractions were used for the Bradford assay. If necessary, the cutinase samples were diluted. For the measurement, 5 µl of BSA or cutinase solution was used, to which 250 µl of Quick Start Bradford Reagent 1x (Pierce™ Bradford Plus Protein Assay Reagent, Thermo Scientific, Rockford, USA) was added. Following a 5-minute incubation at 30 °C, the absorbance was measured at 595 nm with a microplate reader (Varioskan LUX, Thermo Fisher Scientific, Waltham, USA). All measurements were performed in triplicates or duplicates.

Quantification of cutinase activity with *p*-nitrophenyl butyrate (pNPB)

The hydrolytic activity of cutinases was quantified by monitoring the release of *p*-nitrophenol (pNP) from the hydrolysis of pNPB at the maximum absorbance peak of pNP at 410 nm with a microplate reader (Varioskan

LUX). For the quantification of pNP release, the absorbance of a pNP standard curve was measured in the concentration range from 0.001 to 0.05 mg/ml. The extinction coefficient was determined with the pNP absorbance values at different concentrations and Lambert Beer's law. For the determination of the volumetric cutinase activity in U/ml, sequential dilutions of the cutinase solution were prepared in 100 mM KPi buffer, pH 7.5. For the assay, 20 µl of cutinase solution and 180 µl pNPB substrate solution (0.5 mg/ml diluted in 100 mM KPi buffer, pH 7.5) were used and transferred to a 96-well microplate (Fisherbrand™ 235200, Thermo Fisher Scientific, Waltham, USA). The increase of pNP was measured at 30 °C in a kinetic measurement every 15 seconds over a period of 15 minutes. All measurements were performed in triplicates or duplicates. The volumetric activity was calculated with the following equation:

$$\text{Volumetric activity } [\frac{U}{ml}] = \frac{\Delta OD_{410\ nm} * V_M * V_F}{d * V_e * \varepsilon}$$

where: $\Delta OD_{410\ nm}$ = kinetic slope in linear range at 410 nm [absorbance/min], V_M = reaction volume [ml], V_F = dilution factor, d = thickness of microplate wall [cm], ε = molar extinction coefficient of pNP [l/(mol*cm)], V_e = sample volume [ml]

The specific activity was calculated by taking the protein concentration in mg into account. One cutinase unit (U) is defined as the amount of cutinase in mg releasing one µmol of pNP per minute under the tested conditions.

Heat tolerance tests with simultaneously incorporation into polyester polyol

The thermotolerance of the cutinases between 100 °C and 200 °C was assayed using a low melting polyester polyol prepared from adipic acid, monoethylene glycol and butane diol, to combine heated incubation and incorporation. For the heat test at 100 °C, 700 µl of molten polyester-polyol were transferred to a preheated 1.5 ml tube (Fisherbrand™ 509-GRD-PFB, Thermo Fisher Scientific, Waltham, USA) and maintained at 65 °C in a heating block (ThermoMixer® C, Eppendorf SE, Hamburg Germany). Cutinase lyophilisate was then added to each corresponding tube in an amount of 0.5 wt% relative to the polymer weight. An additional 700 µl of molten polyester polyol was added to each tube, and the mixture was stirred with a toothpick and a shaker (Vortex-Genie®, Scientific Industries, New York, United States). During the preparation, all tubes were kept at 65 °C in the heating block (ThermoMixer® C) to maintain the polyester polyol in a molten state. Subsequently, all tubes were placed in a preheated heating block (ThermoMixer® C) set to 100 °C. The samples were incubated at 100 °C with agitation at 1500 rpm for 60 minutes. The start sample was taken as soon as the polymer temperature reached 100 °C. To monitor the polymer's temperature, a reference sample without cutinase lyophilisate was treated the same way as the tubes containing cutinase by first placing it in the 65 °C heating block (ThermoMixer® C) and then moving it to the 100 °C heating block with a thermometer (IKA® ETS-D5, IKA-Werke, Staufen im Breisgau, Germany) placed inside the polymer. Incubation samples were taken by transferring 850 µl of the molten polymer to a metal U disk and allowed to cool down and harden at room temperature. For the heat test >100 °C 50 g of the polyester polyol was weighed into a round bottom flask with a total volume of 250 ml. The flask was placed in an oil bath (Korasilon M100) to keep the polymer liquid at approximately 65 °C. Next, 0.1 wt% of cutinase lyophilisate was weighed and ground into a fine powder. The cutinase lyophilisate was then added to the molten polyester polyol. The mixture of polymer and lyophilisate was well combined using a magnetic stirrer (IKA® RCT

basic S000, IKA-Werke, Staufen im Breisgau, Germany). The temperature was gradually increased and polymer samples of 750 µl were taken every 10 °C increase in temperature and transferred to a metal U disk for cooling.

Phenol red assay

The produced platelets from the incubation and incorporation of cutinase lyophilisate in polyester polyol at 100 °C were applied in a qualitatively cutinase activity study with the pH indicator phenol red. For this, 50-60 mg of samples with incorporated cutinase lyophilisate were weighed into a 2 ml tube (Fisherbrand™ 509-GRD-PFB). To each tube containing the polymer samples with cutinase, as well as to the negative controls without cutinase, 1,000 µl of KPi buffer (100 mM, pH 7.5, 50 µg/ml kanamycin and 100 µg/ml ampicillin) was added. For polymer samples tubes where the cutinase was to be added externally as positive control (0.9-1.8 wt% relative to polymer weight) 900 µl of the same KPi buffer was added. Subsequently, 50 µl of the phenol red stock solution (1 mg/ml phenol red in 20 % v/v ethanol) was added to each tube. The samples were incubated as single samples in a heating block (ThermoMixer® C) at 30 °C for 3 h and at 40 °C for 15 h at a rotation speed of 900 rpm. The release of acid, indicated by a yellow colour change, was documented photographically.

Quantification of cutinase activity with pH stat system

To monitor the remaining ester hydrolysis activity of cutinases incubated in the range of 100-200 °C in molten polyester polyol, the samples were assayed by a pH-stat pre-screening assay to measure sodium hydroxide consumption over time during the hydrolysis of ester bonds. For the screening assay, the BioLector® Pro micro-fermentation system (Beckman Coulter Life Sciences, Brea California, United states), in combination with the microfluidic microplates (FlowerPlate®, Beckman Coulter Life Sciences), was used to maintain constant reaction conditions. The measurement principle relies on acid release from the test polymer due to enzymatic ester hydrolysis. To maintain a constant reaction pH, the BioLector® Pro (Beckman Coulter Life Sciences) system pumps a controlled dosage of sodium hydroxide (NaOH, 3 M) from the reservoir wells into the culture wells of the Flower Plate® (Beckman Coulter Life Sciences) where the polymer samples are incubated in aqueous buffer (KPi, 100 mM, pH 7.0, 50 µg/ml kanamycin). The total amount of consumed base over the reaction time serves as a measure for enzymatic degradation activity. For the screening assay, approximately 50 mg of polymer sample, with or without incorporated cutinase, was weighed into each culture well of the microplate, ensuring equal particle size. 1,000 µl of the reaction buffer was added to samples with incorporated cutinases, and 950 µl buffer was added to samples without incorporated cutinases. For the samples without incorporated cutinases, dissolved cutinase lyophilisate was added externally as positive control at a final concentration of 0.1 wt% relative to the polymer weight. Control samples without any cutinase additions were tested to determine auto-hydrolysis. All reactions were performed as single measurements. The samples were incubated for a maximum of 94 h (HiC only 45 h) at 30 °C, pH set at 7.0, with a rotation speed of 600 rpm under 85 % humidity in the BioLector® Pro, monitoring the base consumption over time.

Enzymatic degradation studies of thermoplastic polyurethanes (TPUs) under laboratory conditions

TPU samples were provided as granule or stripe format. 200 mg of each TPU sample (Table S2), previously dried at 70 °C for 24 h (FD 115, Binder GmbH, Tuttlingen, Germany), was placed in a 15 ml tube with sealed lid (Labcon™ SuperClear™). KPi buffer (200 mM, pH 8.0, 50 µg/ml kanamycin, 100 µg/ml ampicillin, and 300 µg/ml hygromycin B) was added to each sample tube, followed by the addition of 5 % (v/v) of sterile-filtered HiC to a final volume of 10 ml. For the extrusion pre-tests, only TPU 4 and 5 were used in the degradation study. Again, 200 mg of each previously dried TPU sample was placed in a 15 ml tube with sealed lid (Labcon™ SuperClear™). KPi buffer was added to each sample tube, followed by the cutinase addition a final volume of 5 ml. For TPU 4, cutinase concentrations of 0.1, 0.3, 0.5, 0.8 and 1 wt% relative to the polymer were tested. For TPU 5, only 0.3 and 0.5 wt% of cutinase addition was tested. Stock solutions of HiC, ThcCut1, ThcCut1-ACCG, SvCut190*SS, LCC-ICCG, and the empty vector control pET26b_EV were prepared by dissolving cutinase lyophilisate in demineralised water followed by sterile filtration. Control samples without cutinase addition were tested as well, to determine auto-hydrolysis. Reactions were set up as duplicates in the degradation test with 5 % (v/v) HiC addition and as single reactions for all other degradation tests. Samples were incubated in a heating block (ThermoMixer® C) at 37 °C and 200 rpm for 32 or 33 days. For adipic acid quantification, sterile samples were taken from the supernatant and prepared for high performance liquid chromatography (HPLC) analysis (Agilent 1260 Infinity II system, Santa Clara, USA). For weight loss measurements samples were washed two times with demineralised water and dried at 70 °C for 24 h. Samples were weight with a microbalance (Cubis®-1442AC MSA225S-110-DI, Sartorius, Göttingen, Germany).

Preparation of cutinase-embedded TPUs via melt extrusion

For the incorporation of cutinases into TPUs, a twin-screw micro-compounder (Xplore MC 15, Xplore Instruments BV, Sittard, Netherlands), was used. For TPU 4 the polymer granules were milled under liquid nitrogen flow (MM 500 Nano, Retsch GmbH, Haan, Germany) to a particle size of ≤ 1 mm, while TPU 5 was used unground with a particle size of approximately 3-5 mm. In both cases, the material was dried for 48 h at 70 °C (FD 115, Binder GmbH) before use in the extrusion process. Cutinases to be incorporated were prepared as non-purified cell free lyophilised extracts from cutinase expression (crude lyophilised clarified lysate). Only HiC and SvCut190*SS were incorporated as purified cutinases, while for the SvCut190*SS also the non-purified cutinase lyophilisate was used as comparison. For a homogeneous cutinase distribution, a premix of TPU and cutinase lyophilisate was prepared, with the cutinase amounts listed in Table S3, relative to the TPU weight. For the purified SvCut190*SS the cutinase load was reduced to 0.18 wt% due to a higher specific activity compared to the non-purified cutinase, so that purified and non-purified cutinases were incorporated with the same pNPB Units per mg TPU. HiC, was incorporated three times: i) 0.3 wt% HiC, ii) 0.3 wt% NHS-labelled HiC (only at 150 °C), and iii) 0.033 wt%, where the last concentration was to compare the cutinase activity to that of the lowest used cutinase activity represented by LCC-ICCG with 0.023 pNPB U/mg TPU.

To avoid contamination between sample batches and cutinase dilution, the micro-compounder (Xplore MC 15) was flushed with TPU followed by one premix of TPU/cutinase before each new sample batch was added to the device. The filling of the compounder took ≤ 1 minute. After the entire sample was filled into the mixing area, compounding was carried out for 1.5 minutes at the set melt temperature (Table S2), with a rotation speed of the

two screws of 50 rpm before the TPU exited the compounder in the form of a strand. Samples were cooled down at room temperature and dried at 70 °C for 24 h before degradation studies were conducted.

Tension rods were only prepared with HiC and ThcCut1-ACCG, both with a concentration of 0.3 wt% relative to the TPU weight. For TPU 4 210 °C and for TPU 5 180 °C was used as processing temperature. The molten TPU extrudate was injected into a micro -injection moulding machine (Xplore 12cc, Xplore Instruments BV, Sittard, Netherlands), to achieve the specific dumb-bell test specimen shape of type 5A.

Table S2 Melt extrusion conditions for cutinase/protein incorporation into TPU 4 and TPU 5. Different TPU/protein combinations and corresponding incorporation temperatures were used, as indicated below. Only HiC was embedded in both, TPU 4 and TPU 5. BSA**:** bovine serum albumin.

	HiC	**SvCut190*SS non-purified[b]**	**SvCut190*SS purified[b]**	**ThcCut1-ACCG[b]**	**LCC-ICCG[b]**	**Empty vector[b]**	**BSA[b]**
Incorporation temperature (°C)	210[a]						
	200	200	200	200	200		
	190	190	190	190	190		
	180[b]	180	180	180	180		
	170[b]	170	170	170	170		
	160[b]	160	160	160	160		
	150[b]	150	150	150	150	150	150
Concentration (wt%)	0.3	0.3	0.18	0.3	0.3	0.3	0.3
	0.033						
Specific activity (pNPB Units/g TPU)	197	93	96	37	23	No activity	No activity
	23						

[a] only for TPU 4 [b] only for TPU 5

Enzymatic degradation studies of extruded TPU granules with embedded cutinases under laboratory conditions

Degradation studies with cutinase-embedded TPU samples were conducted in deep-well plates (Axygen™ P-DW-10ML-24-C-S, Corning, New York, USA). 200 mg of each previously at 70 °C for 24 h dried (FD 115, Binder GmbH), TPU sample was weighed (Cubis®-1442AC MSA225S-110-DI, Sartorius in triplicates into each corresponding well. 5 ml of KPi buffer (200 mM, pH 8.0, 50 µg/ml kanamycin, 100 µg/ml ampicillin, and 300 µg/ml hygromycin B) was added to each sample with embedded cutinases as well as to the negative controls without cutinase addition. The same KPi buffer was added to the positive controls with external cutinase addition to a final volume of 5 ml, taking the volume of externally added cutinase solution into account, so that a final cutinase concentration of 0.3 wt% relative to the TPU weight was achieved. For the positive controls, cutinase stocks were prepared by dissolving cutinase lyophilisate in demineralised water followed by sterile filtration. Each plate was sealed with an aluminium self-adhesive foil (Nunc™, Thermo Fisher Scientific, Waltham, USA) and incubated at 37 °C, 150 rpm for 21 days (ISF1-X). Sterile samples for adipic acid quantification were taken from

the supernatant at specific timepoints and prepared for HPLC analysis (Agilent 1260 Infinity II system). After the total incubation period, solid samples were washed two times with demineralised water, dried at 70 °C for 24 h (FD 115), and stored at room temperature for further analyses.

HPLC measurements

Samples for HPLC measurements were filtered through a 0.2 µm PVDF filter and the sample pH was adjusted by the addition of phosphoric acid in a final concentration of 1 % (v/v). An Agilent 1260 Infinity II system equipped with a multisampler (G7167A), a diode array detector for UV and visible light range and a Zorbax Eclipse Plus C18 column (4.6 mm × 150 mm, 5 µm, Agilent, Santa Clara, USA), was used for the liquid chromatography. Adipic acid quantification was conducted with a gradient method, which involved an aqueous mobile phase (0.085 % v/v phosphoric acid in demineralised water) and 99.8 % acetonitrile (Table S3) at a column temperature of 35 °C. The mobile phase flow rate was set at 1.5 ml/min, and 5 µl of the sample was injected. Data analysis was performed using the Chromeleon Chromatography Data System 7.3.1 software (Thermo Fisher Scientific, Waltham, USA).

Table S3 HPLC gradient method used in this study for adipic acid measurements. For target elution a mixture of acetonitrile and water was used.

Time (min)	0.085 % phosphoric acid (%)	99.8 % Acetonitrile (%)
0	95	5
0.5	95	5
4.5	75	25
8.5	5	95
10.5	5	95
11.5	95	5
14	95	5

Light and scanning electron microscopy

Washed and dried TPU samples after the degradation tests were investigated via light and scanning electron microscopy (SEM). For light microscopy a DM2700 M microscope (Leica Microsystems, Wetzlar, Germany) was used. The samples' surfaces were investigated as whole granule as well as the interior by cutting the granules with a scalpel. The SEM pictures were made with a TM4000 Plus tabletop device (Hitachi, Tokyo, Japan) at 15 kv acceleration voltage. The samples were investigated under medium vacuum using either the signal from secondary or backscatter electrons.

HiC staining and fluorescent microscopy

HiC labelling with NHS-fluorescein (5/6-carboxyfluorescein succinimidyl ester) was done according to Huang *et al.* (2023)[4]. Briefly, 80.66 mg HiC lyophilisate was dissolved in 14.4 ml demineralised water. 13.87 mg NHS-fluorescein was dissolved in 1.6 ml dimethyl sulfoxide. Both solutions were combined and incubated for 4 h under slight agitation, at room temperature in the dark. Excess dye was removed via buffer exchange (20 mM KPi, pH 7.5) with a PD-10 column. The resulting solution was frozen at -80 °C and subsequently freeze-dried for 3 days

at -65 °C and 0.1 mbar (Alpha 1-2 LDplus). The lyophilisate was used in the melt extrusion-based incorporation at 150 °C into TPU 5.

For fluorescent microscopy, microtome cuts were produced with a thickness of 20 µm using a rotary microtome (HM 355 S, Microm International GmbH, Dreieich, Germany). The samples were inspected using a Leica DM6000 microscope (Leica Microsystems, Wetzlar, Germany) with fluorescence mode (with a band-pass filter of 420-490 nm for the excitation and a long-pass filter of 515 nm for the emission).

Atomic force microscopy (AFM) and infrared photo-induced force microscopy (PiFM)

For AFM and PiFM TPU samples before degradation studies were used. Sample preparation was done by the preparation of even block faces in transverse direction using cryo cutting on a Leica EM UC7/FC7 ultramicrotome (Leica Microsystems, Wetzlar, Germany) with diamond knives (knife angle 45°, clearance angle 6°). The knife temperature was set at -30 °C, with the specimen and cryochamber at -130 °C. The cutting speed was 1 mm/s.

The TPU microtome cut was placed on an AFM sample stub and immobilized on the AFM stage. AFM images were obtained under ambient conditions in tapping mode using a Dimension Icon multimode atomic force microscope (Bruker Nano Surfaces, Madison, United States) using silicon cantilevers with resonance frequencies of 300-400 kHz (model: TESP, Bruker Nano surfaces).

PiFM was carried out using a Vista 75 microscope (Molecular Vista, Inc., San Jose, United States) to obtain height and mechanical phase information, as well as the near field optical response. Laser wavelength was tuned from 800 to 1800 cm^{-1} (spectral line width 1 cm^{-1}) using the MIRcat™ mid-IR quantum cascade laser (QCL). A platinum-iridium coated AFM cantilever (NCH-300 kHz, Nanosensors Inc., Neuchatel, Switzerland) was used and oscillated at the second mechanical resonance frequency (f1) to detect surface topography and at the first mechanical resonance frequency (f0) to detect photo-induced force, while the laser was modulated at the difference frequency (f1-f0) with an average power of 1 mW. All PiFM images were recorded at 256 x 256 pixels using a scan rate of 0.5 Hz with a setpoint of 75 % and amplitude of 2 nm. PiF spectra were acquired using acquisition time of 26 s/spectrum over the set spectral range and were laser power normalized and smoothened. For data evaluation an infrared (IR) spectrum of HiC lyophilisate was measured using a Tensor II spectrometer (Bruker Nano surfaces) equipped with an attenuated total reflection unit. The spectrum was recorded between 400-4,000 cm^{-1} in absorbance mode. A total of 16 scans were performed to obtain the final spectrum.

Mechanical studies

Tensile tests with TPUs with and without incorporated HiC or ThcCut1-ACCG before degradation study were performed using a Zwick testing machine (ZwickRoell GmbH & Co. KG, Ulm, Germany) according to ISO 527-01. Samples were measured at room temperature with a tensile speed of 200 mm/min. Dumb-bell test specimen type 5A with dimension of 75 mm x 4 mm x 2 mm were used. Measurements were conducted with at least two test specimens per TPU.

Solution viscosity determination

The solution viscosity of samples before degradation study was determined using a Stabinger viscometer (SVM3000/G2, Anton Paar, Graz, Austria). Sample solutions (0.4 wt%) were prepared by dissolving the TPU in

N-methylpyrrolidone (NMP) at approximately 70 °C. Solutions were equilibrated overnight before measurement. The kinematic viscosity (ν) was measured at 25 °C, with blank measurements (pure solvent) performed at the beginning and end of each measurement series. The relative viscosity was calculated according to the following equation:

$$\text{Relative viscosity } [\frac{mm^2}{s}] = \frac{\text{Ø } \nu \text{ of sample solutions}}{\text{Ø } \nu \text{ of blanks}}$$

Differential scanning calorimetry (DSC) analysis

DSC measurements with samples after degradation studies were performed with a Netzsch Polyma 214 DSC device (NETZSCH-Gerätebau GmbH, Selb, Germany) using sample weights of 10 mg. Measurements were performed with three cycles starting with a heating cycle from -75 °C to 260 °C, followed by cooling to -75 °C, and finally another heating to 260 °C with a heating rate of 20 K/min. From the DSC analysis, the glass transition temperature (T_g), melting temperature (T_m), and the crystallisation and melting enthalpies were determined.

Biodegradation studies of extruded TPU granules with embedded cutinases

Biodegradation tests with activated sludge were conducted according to the manometric respirometry method 301 F specified by the OECD (Organisation for Economic Co-operation and Development) guideline for testing chemicals for their ready biodegradability, using the BD600 system (Tintometer GmbH, Lovibond Water Testing, Dortmund, Germany).
The required amount of the polymer test substance/sodium acetate control/cellulose control was calculated based on the theoretical oxygen demand (ThOD):

$$\text{ThOD } \left(\frac{\text{mg } O_2}{\text{mg polymer}}\right) = \frac{16\,[2C + 0.5\,(H - Cl - 3N) + 3S + 2.5P + 0.5Na - O]}{MW}$$

$$\text{mg polymer} = \frac{400 \text{ mg } O_2}{1 \text{ l water}} * 0.157 \text{ l water} * \frac{1 \text{ mg polymer}}{\text{ThOD mg } O_2}$$

The calculation implies that carbon is mineralized to CO_2, hydrogen to H_2O, phosphorus to P_2O_2, and sodium to Na_2O. The halogen is eliminated as hydrogen halide and nitrogen as ammonia. For the calculation, the volume of the test medium (157 ml) and the maximum biochemical oxygen demand (BOD) value in the measurement range of the equipment (400 mg/l) were considered as well. The composition of 1 l test medium was as follows: 25 ml activated sludge, bubbled with compressed air at a constant temperature of 23 °C for at least 24 h beforehand, 970 g demineralised water, and 1 ml of the salt and trace element solutions A-D specified in OECD 301 A, section 5 (phosphate buffer, $CaCl_2$, $MgSO_4$, and $FeCl_3$). The test medium was saturated at 20 °C by aeration with clean compressed air for 20 minutes while stirring. The biodegradability studies were conducted in duplicates, with sodium acetate as a positive control ensuring proper equipment function and microbial activity, and a blank sample

without test substance monitoring all other microbial processes. The test substance was placed in each respective glass bottle, whereafter 157 ml of test medium was added together with 5 drops of allylthiourea as nitrification inhibitor to prevent bacteria from producing NOx and ensure all nitrogen is eliminated as NH_3. A seal gasket was inserted into the bottle neck, and 4 drops of 45 % KOH solution were added to capture generated CO_2 leading to a pressure drop proportional to the consumed oxygen, representing the BOD. The BOD sensors were secured onto the bottles which were afterwards positioned on stirring plates within a 20 °C climate chamber and incubated in the dark for 90 days. To determine the biodegradability of the test substance, the BOD for complete biodegradation was calculated and compared to the measured BOD value:

$$\text{BOD for complete biodegradation } \left(\frac{\text{mg } O_2}{\text{l}}\right) = \text{mg polymer} * \frac{\text{ThOD mg } O_2}{\text{1 mg polymer}} * \frac{1}{0.157 \text{ l water}}$$

$$\text{Biodegradability (\%)} = \frac{\text{BOD measured } (\frac{\text{mg } O_2}{\text{l}})}{\text{BOD for complete biodegrdation } (\frac{\text{mg } O_2}{\text{l}})} * 100$$

Biodegradation of the positive reference sodium acetate reached more than 60 % biodegradation in 14 days, as required by the OECD 301 F protocol. Cellulose was used as additional control.

Supporting Results

Table S4 List of cutinases used in this study.

Cutinase name	Species	T_m [°C]
HiC	*Thermomyces (Humicola) insolens*	80 [1]
BhrPETase	HR29 (*Chloroflexi*)	101 [5]
LCC	Metagenomic DNA	84.7 [6]
LCC-ICCG	Metagenomic DNA	94 [6]
LCC-WCCG	Metagenomic DNA	98 [6]
DuraPETase^{N233K}	*Idonella sakaiensis*	83.5 [7]
TfCut2	*Thermobifida fusca*	71.2 [8]
ThcCut1	*Thermobifida cellulosilytica*	72 [2]
ThcCut1-ACCG	*Thermobifida cellulosilytica*	93.2 [2]
SvCut190*SS	*Saccharomonospora viridis*	85.7 [9]
PES-H1	Metagenomic DNA	77.1[10]

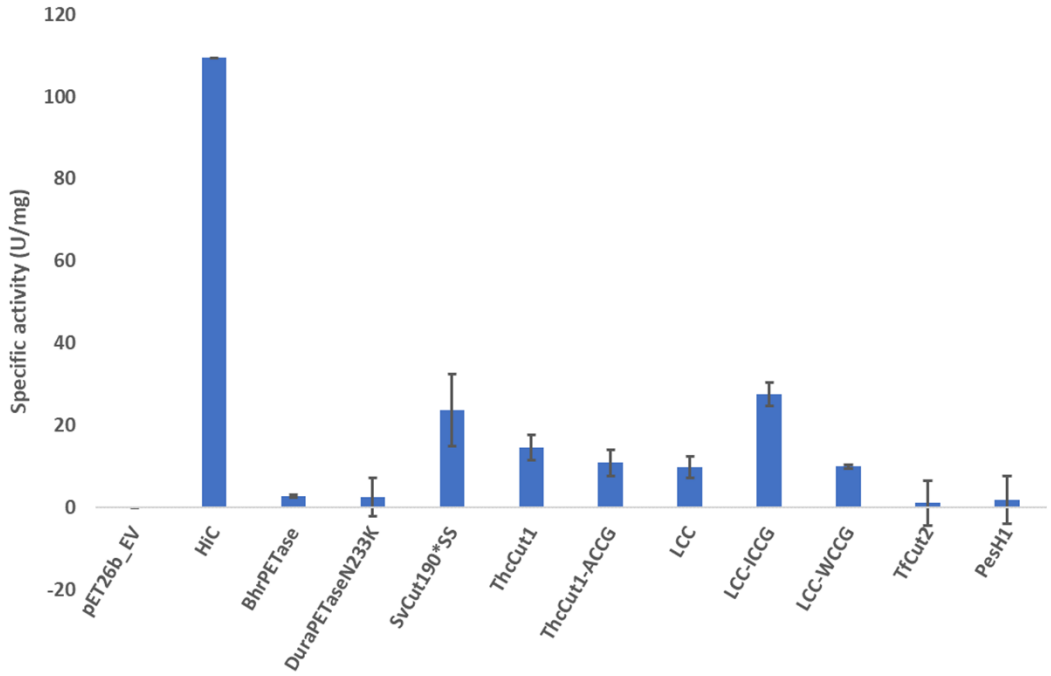

Fig. S1 Specific activity [U/mg] of cutinase lyophilisates used for heat tolerance tests with polyester polyol at 100-200 °C. The specific activity was measured using pNPB as substrate. The increase of pNP upon ester hydrolysis of pNPB was measured at 30 °C in a kinetic measurement every 15 seconds over a period of 15 minutes at 410 nm. The initial slope was used to calculate cutinase activity. The shown data represent the means and standard deviations of duplicate measurements.

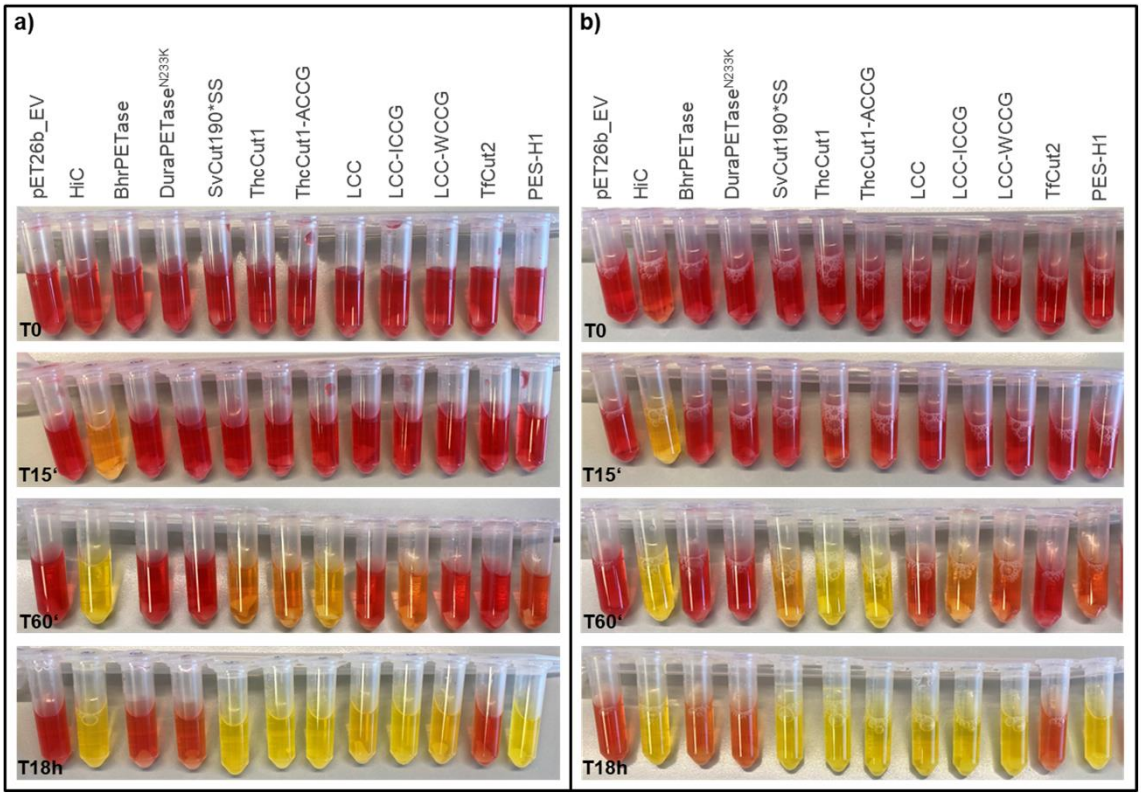

Fig. S2 Phenol red assay with polyester polyol with a) 0.5 wt% incorporated cutinase preparation incubated at 100 °C for 60 minutes and b) 0.9-1.8 wt% external added cutinases. In both cases, 50-60 mg of the solid polymer was used for the phenol red assay in a buffered aqueous system (KPi, 100 mM, pH 7.5, 50 µg/ml kanamycin and 100 µg/ml ampicillin). The reactions contained 50 µl of the phenol red stock solution (1 mg/ml phenol red in 20 % v/v ethanol). Samples were incubated at 30 °C for 3 h and subsequently at 40 °C for 15 h. Acid release upon enzymatic ester hydrolysis was monitored by color change from red over orange to yellow.

Table S5 Weight loss and adipic acid release of TPU 1-5 after 33 days of incubation at 37 °C in buffer (200 mM KPi, pH 8.0) with external addition of 5 % (v/v) HiC or without HiC addition. Samples were washed with distilled water and dried at 70 °C for 24 h before weight measurement was performed with a microbalance. The data with HiC addition is given as the average of duplicate reactions while the degradation of TPU without HiC treatment was measured in single setups.

TPU	HiC addition	Weight loss (%)	Adipic acid release (%)
1	HiC	0.06	0.47 ± 0.04
1	-	0.04	0.65
2	HiC	0.43	1.1 ± 0.1
2	-	0.1	0.69
3	HiC	0.98	2.06 ± 0.08
3	-	0.38	0.99
4	HiC	11.8	22.75 ± 0.14
4	-	0.32	0.98
5	HiC	37.78	46.28 ± 2.13
5	-	0.64	1.32

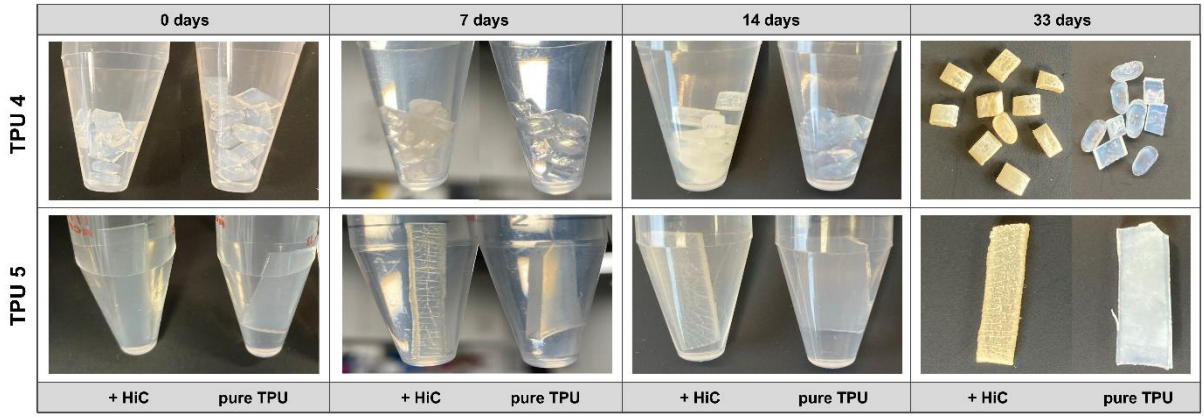

Fig. S3 Images of HiC treated and non-treated TPU 4 and 5. The samples were incubated in KPi (200 mM, pH 8.0) for 33 days at 37 °C with or without the addition of 5 % (v/v) HiC solution.

Table S6 Weight loss and adipic acid release (%) from TPU 4 after 33 days of incubation at 37 °C in buffer (200 mM KPi, pH 8.0) with external addition of 0.1-1 wt% of HiC, ThcCut1, ThcCut1-ACCG, SvCut190*SS, LCC-ICCG, and the empty vector control pET26b or without cutinase addition. Samples were washed with distilled water and dried at 70 °C for 24 h before weight measurement was performed with a microbalance. All values are corrected by the corresponding control: empty vector for lysates and the negative control without cutinase addition for HiC.

Cutinase addition	Cutinase concentration (wt%)	Weight loss (%)	Adipic acid release (%)
HiC	0.1	1.56	1.61
ThcCut1		1.56	1.69
ThcCut1-ACCG		1.50	1.69
SvCut190*SS		1.48	1.38
LCC-ICCG		1.59	0.78
pET26b_EV		0.56	0.29
HiC	0.3	2.18	2.39
ThcCut1		2.18	2.63
ThcCut1-ACCG		1.93	2.13
SvCut190*SS		2.45	2.30
LCC-ICCG		2.00	1.47
pET26b_EV		0.66	0.29
HiC	0.5	2.55	3.07
ThcCut1		2.40	3.14
ThcCut1-ACCG		2.38	2.72
SvCut190*SS		3.53	3.53
LCC-ICCG		2.26	1.97
pET26b_EV		0.81	0.34
HiC	0.8	3.05	4.14
ThcCut1		3.63	4.34
ThcCut1-ACCG		2.93	3.40
SvCut190*SS		4.34	4.86
LCC-ICCG		2.86	2.47
pET26b_EV		0.92	0.43
HiC	1	3.95	4.98
ThcCut1		4.44	5.45
ThcCut1-ACCG		3.08	3.88
SvCut190*SS		5.47	6.18
LCC-ICCG		2.9	2.81
pET26b_EV		0.49	0.25
No addition	-	0.29	0.53

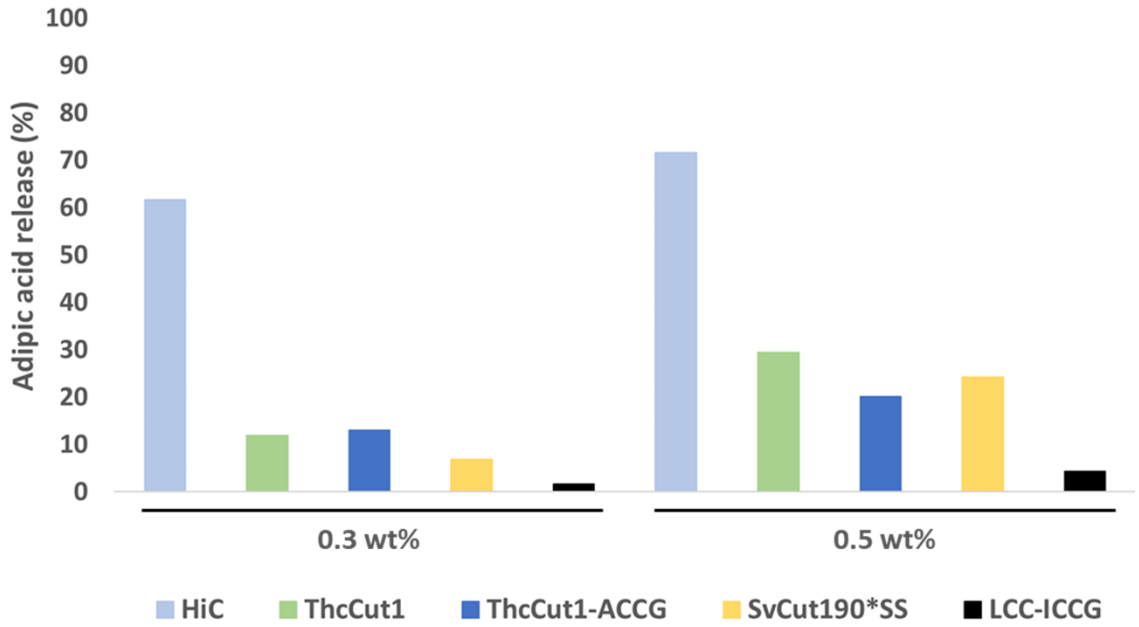

Fig. S4 Adipic acid release from TPU 5 after 32 days of incubation at 37 °C with different cutinases, the empty vector control or no cutinase addition. The degradation study was performed in a buffered system using KPi (200 mM, pH 8.0). Each reaction contained 0.3 or 0.5 wt% external added cutinase or empty vector lyophilisate relative to the TPU weight. As negative control, TPU was incubated without cutinase treatment. Adipic acid was quantified via HPLC. Values were corrected by the negative controls. For the empty vector control, no adipic acid release could be detected.

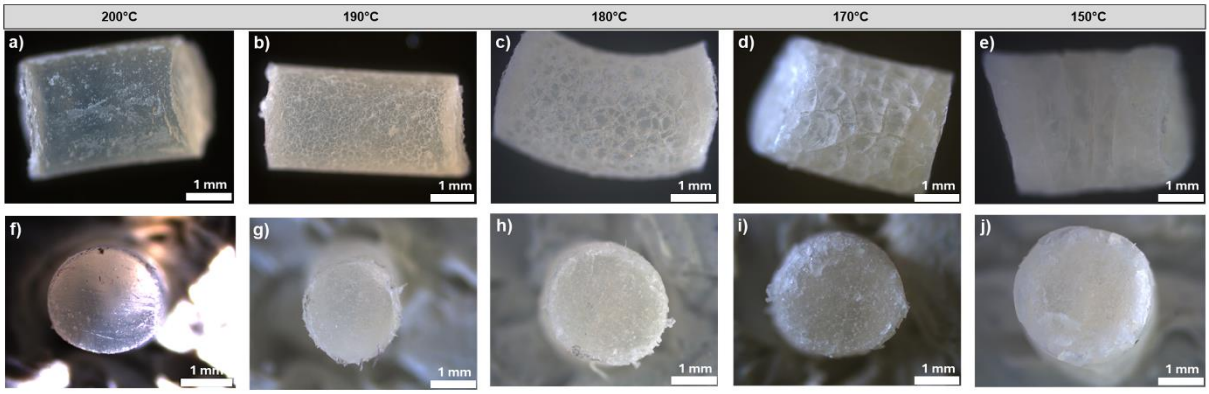

Fig. S5 Light microscopy images of TPU 5 after 21 days incubation at 37 °C in KPi (200 mM, pH 8.0) with incorporated HiC (0.3 wt%). Incorporation was done via melt extrusion at the indicated temperatures. a-e) TPU granule surface, f-j) TPU granule cross-section. All images were taken in darkfield at 25x magnification.

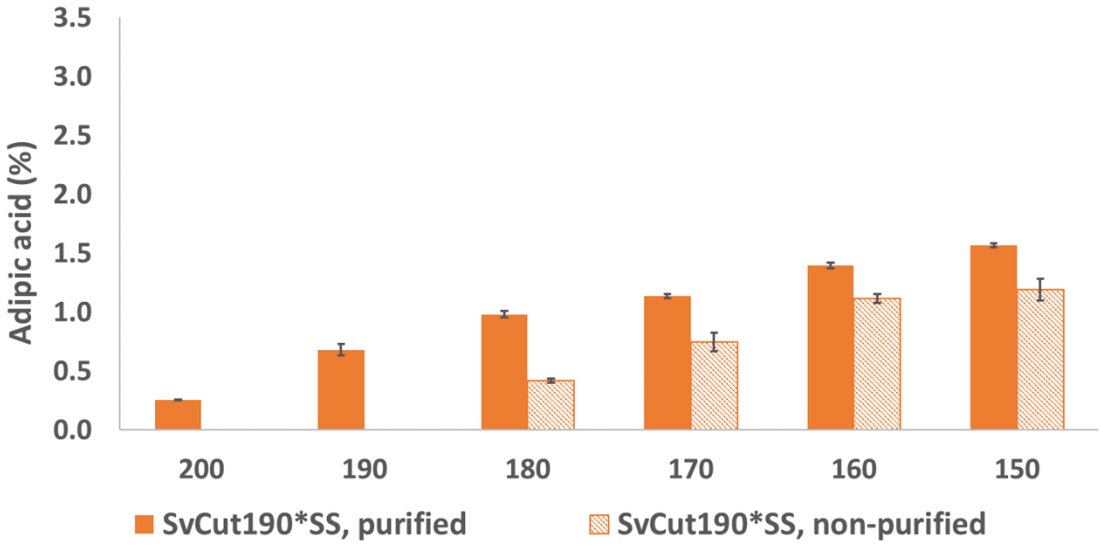

Fig. S6 Adipic acid release from TPU 5 with 0.18 wt% (96 pNPB U/g TPU) purified and 0.3 wt% (93 pNPB U/g TPU) non purified (lyophilised clarified lysate) SvCut190*SS. Cutinase incorporation was done via melt extrusion. For the degradation study the TPU samples were incubated in aqueous buffer (KPi, 200 mM, pH 8.0). The data is given as the means ± standard deviations of triplicate reactions, corrected by the values measured in the empty vector (for lysate) and negative control (autohydrolysis for purified SvCut190*SS). Data shown here was measured after 12 days of incubation.

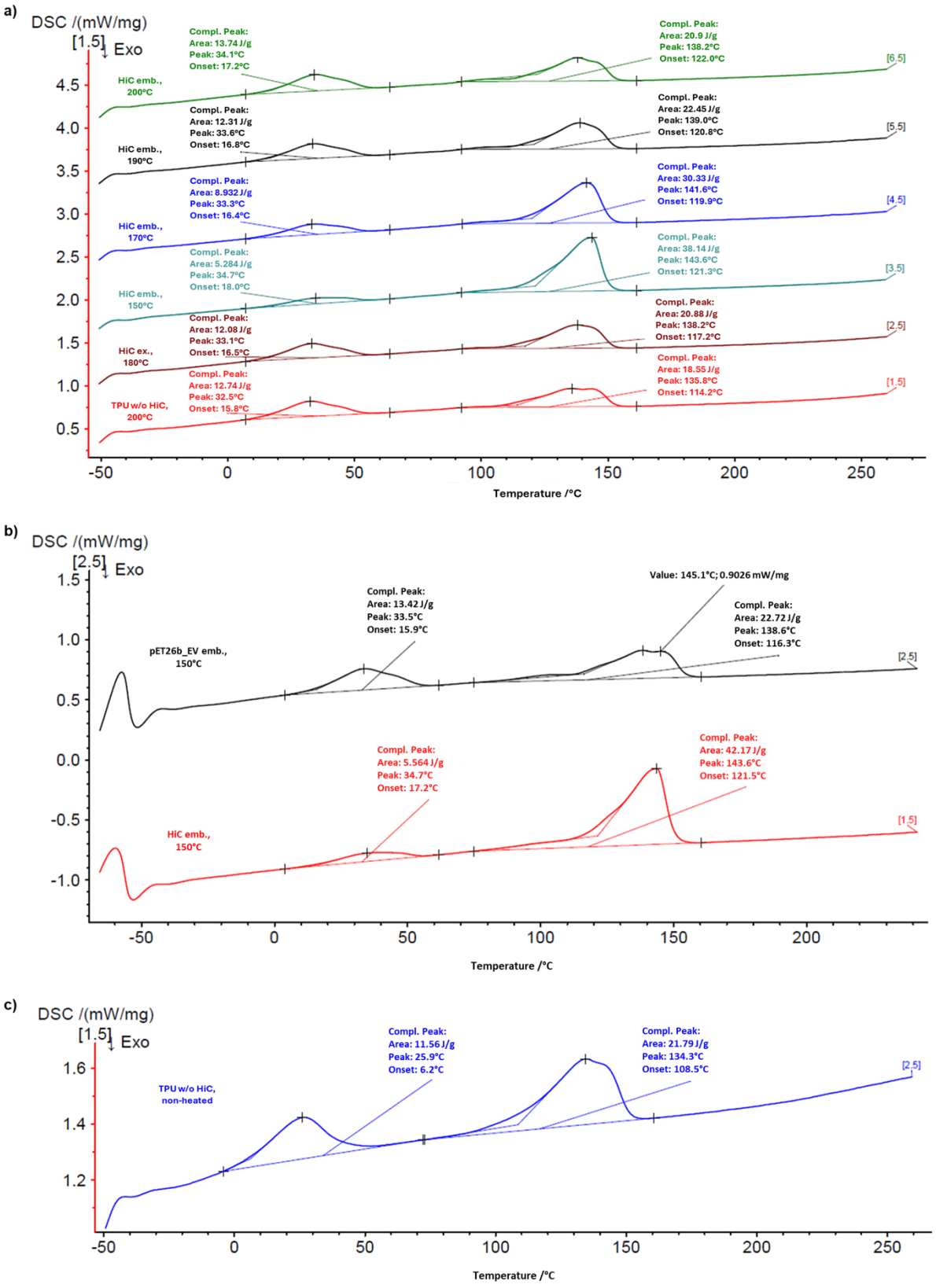

Fig. S7 DSC measurements of TPU 5 during the final heating cycle from -75 °C to 260 °C with a temperature increase of 20 K/min. a) TPU 5 samples after an incubation for 21 days at 37 °C with embedded (emb.) and externally added (ex.) HiC, as well as TPU 5without HiC treatment, b) incorporated pET26b_EV and HiC. c) original TPU 5 before extrusion and no degradation study (w/o: without).

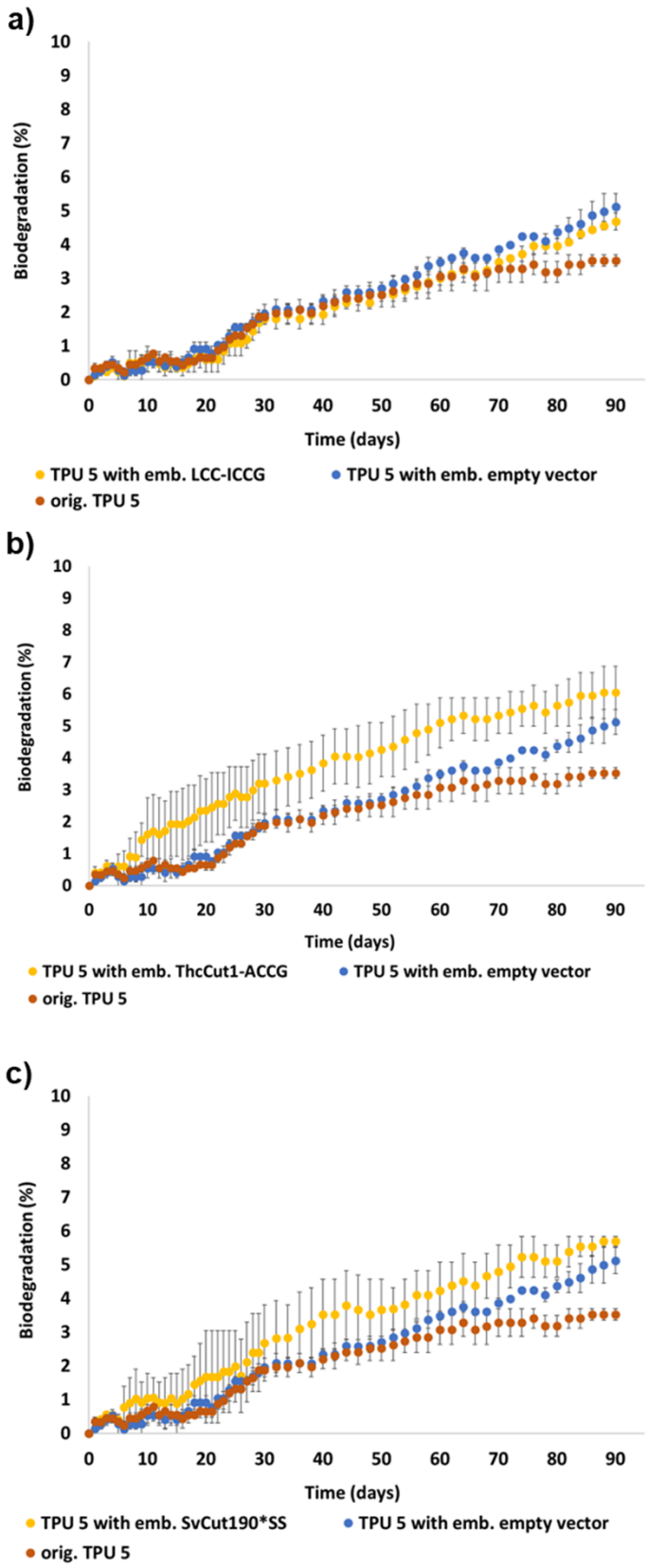

Fig. S8 Biodegradation for cutinase-containing TPU 5, processed at 150 °C. Degradation studies were conducted according to OECD 301 F with activated sludge. TPU 5 with 0.3 wt% embedded (emb.) a) LCC-ICCG lysate, b) ThcCut1-ACCG lysate, and c) SvCut190*SS lysate. Original (orig.) TPU 5 before melt extrusion and TPU 5 with 0.3 wt% incorporated empty vector lysate served as negative controls. Biodegradation was monitored by the microbial oxygen demand over a period of 90 days at 20 °C.

Table S7 Adipic acid release and biodegradation of TPU 5 with embedded ThcCut1-ACCG after eight months of storage. Cutinase embedment was done via melt extrusion at a processing temperature of 170 °C with a cutinase concentration of 0.3 wt%. TPU samples were stored under ambient humidity and temperature (30-40 %; 21-23 °C). Adipic acid releases were measured after 21 days of TPU 5 incubation at 37 °C in buffer (200 mM KPi, pH 8.0). The data is given as the means of triplicate reactions, corrected by the values measured in the empty vector. Biodegradation studies were conducted according to OECD 301 F with activated sludge. Biodegradation was monitored by the microbial oxygen demand over a period of 90 days at 20 °C. The data is given as the means of duplicates.

	Adipic acid release (%)	**Biodegradation (%)**
T0	0.48 ± 0.07	10.82 ± 1.12
8 months	0.68 ± 0.16	9.13 ± 0.61

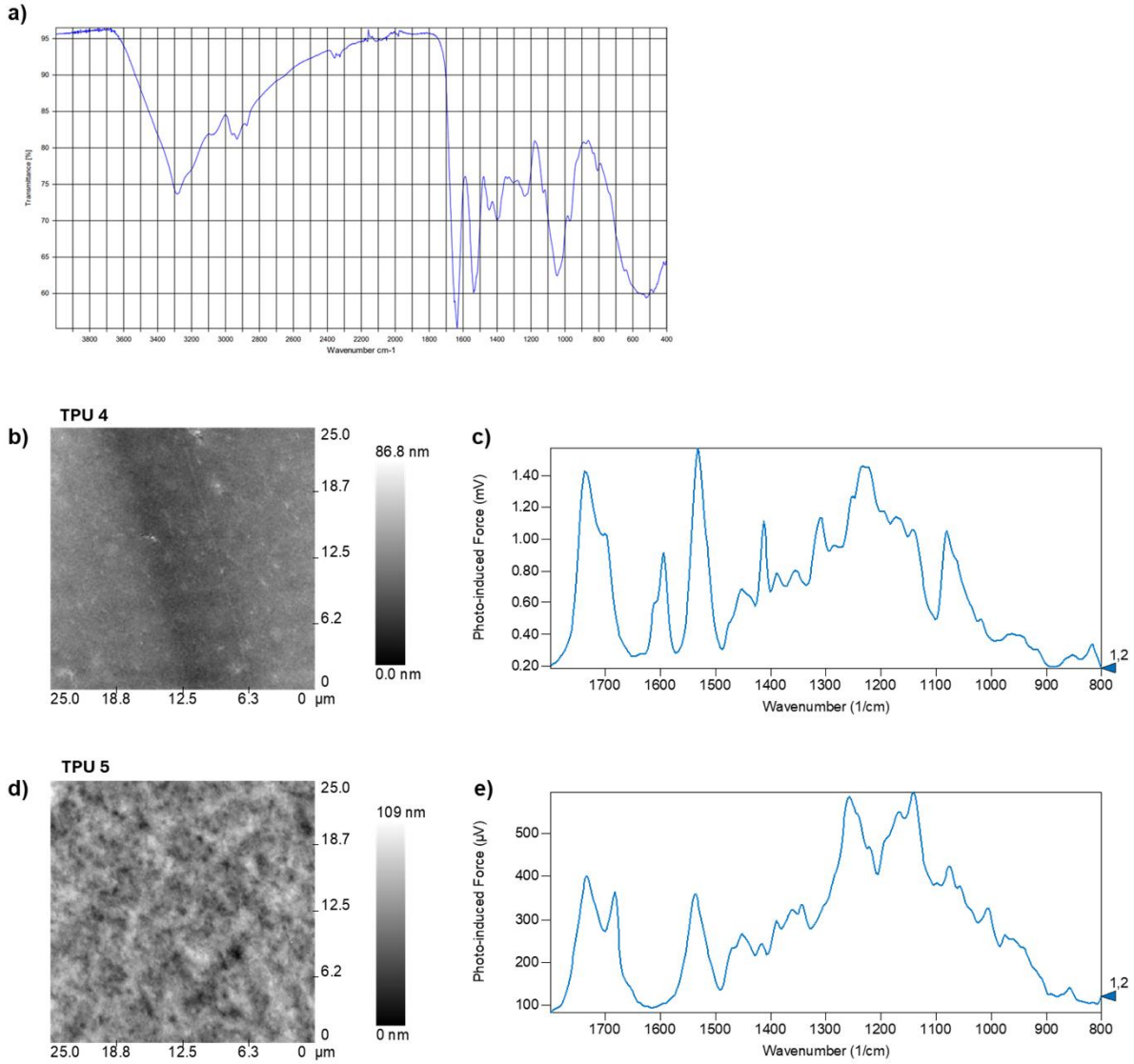

Fig. S9 Topographic PiFM images of TPU 4 and 5 processed at 200 °C and 160 °C respectively. a) FT-IR spectrum of HiC, with characteristic amide I stretching (1640 cm-1). Topographic surface image of b) TPU 4 and d) TPU5. PiFM spectra of c) TPU 4 and e) TPU 5 both without amide I stretching.

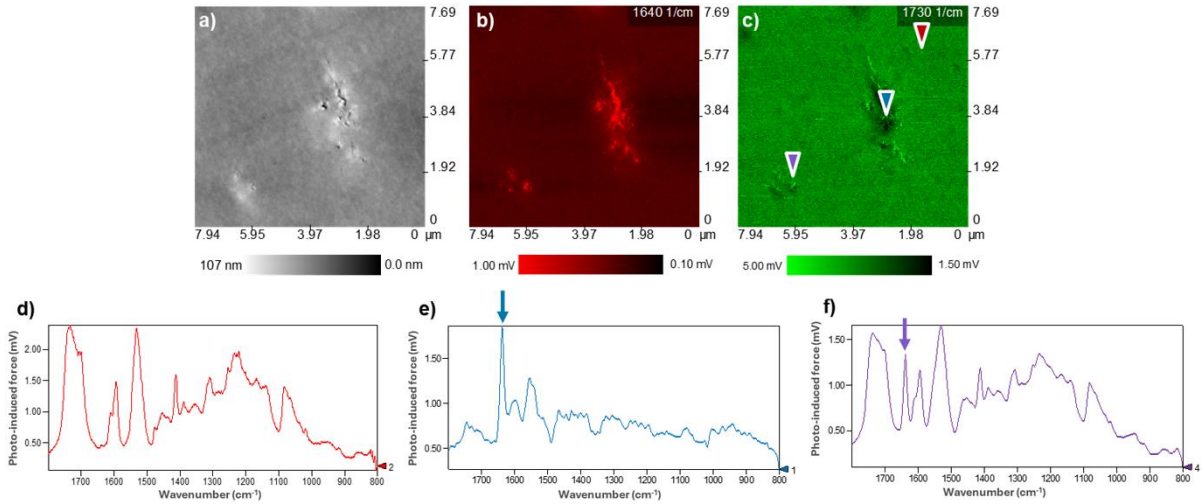

Fig. S10 PiFM images and spectra of TPU 4 with incorporated SvCut190*SS. Cutinase incorporation was done at 195 °C at a concentration of 0.3 wt%. a) Topographic image, and optical image b,c) at a wavenumber of b) 1640 cm-1 (amide I stretching), and c) 1730 cm-1 (carbonyl stretching, characteristic for polyester) were recorded. Brighter areas in the topographic image indicate an elevation on the surface. In the optical images, brighter areas indicate higher and darker areas indicate lower absorption for the specific molecular composition, at each wavelength. PiFM spectra are displayed for d) red arrow location from c) without amide I vibration, e) blue and f) purple arrow location from c) both with amide I vibration at 1640 cm-1, indicating protein presence.

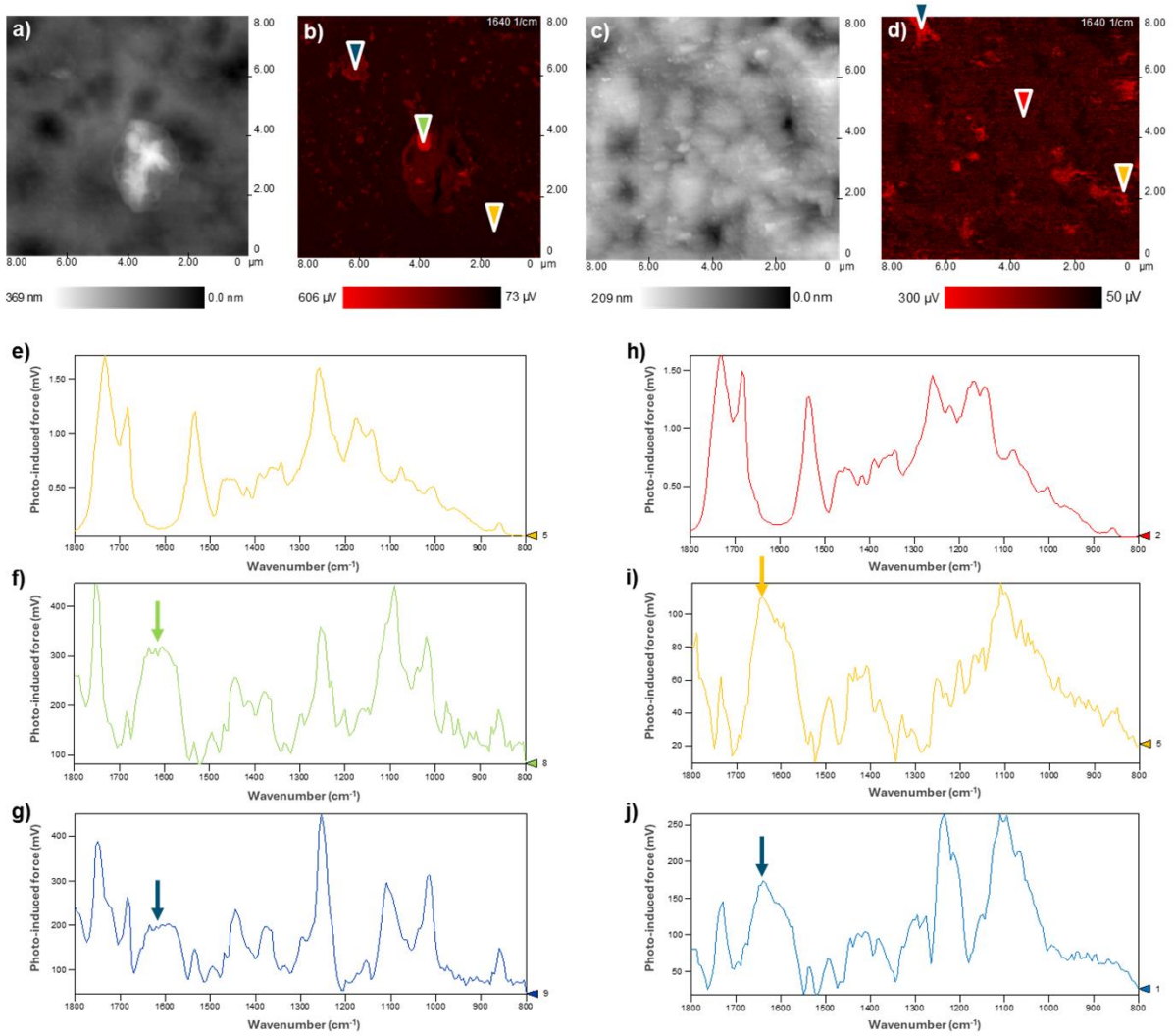

Fig. S11 PiFM images and spectra of TPU 5 with incorporated HiC or SvCut190*SS. Cutinase incorporation was done at 200 °C at a concentration of 0.3 wt%. Topographic image of a) TPU 5 with HiC, and c) TPU 5 with SvCut190*SS. Optical image of b) TPU 5 with HiC, and d) TPU 5 with SvCut190*SS excited with a wavenumber of 1640 cm^{-1} (amide I stretching). Brighter areas in the topographic image indicate an elevation on the surface. In the optical images brighter areas indicate higher absorption for the specific molecular composition, at 1640 cm^{-1}. PiFM spectra for HiC-containing TPU 5 are displayed for e) yellow arrow location from b) without amide I vibration, f) green and g) blue arrow location from b), both with amide I vibration at 1640 cm^{-1}, indicating protein presence. Corresponding spectra for SvCut190*SS-containing TPU 5 are displayed as h) for red arrow location from b) without amide I vibration, i) yellow arrow and j) blue arrow location from d) with amide I vibration at 1640 cm^{-1}.

Protein Sequences

BhrPETase

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TLRISEQIPTLKAGVPLTPWHTDKTFNTPVPQLIVGAEADTVAPVSQHAIPFYQNLPSTTPKVYVELDNA
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DuraPETase^{N233K}

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SvCut190*SS

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ThcCut1-ACCG

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LCC

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LCC-ICCG

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TfCut2

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PesH1

MANPYERGPDPTESSIEAVRGPFAVAQTTVSRLQADGFGGGTIYYPTDTSQGTFGAVAISPGFTAGQESI
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