

1 Engineering surface electrostatics affords control over morphological 2 preference, synergy, and activity in polymer degrading enzymes

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9 Abstract

10 The biocatalytic recycling of plastics, such as polyethylene terephthalate (PET), promises a sustainable
11 alternative to our present open-loop cycles. Engineering of PET-hydrolases for this purpose has focused on
12 improving activity near the glass-transition temperature of the polymer by increasing their thermostability,
13 neglecting other features of the protein-polymer system that affect enzymatic activity. Here, we isolate the effect
14 of electrostatics on the activity of a thermophilic PETase by rationally redesigning its surface charge, while
15 preserving its thermodynamic properties. The enzyme variant, *Sfnv*, shows orders of magnitude improvements
16 in binding affinity and in activity towards untreated plastic films, with inverted morphological preference. When
17 combined, the wildtype enzyme and *Sfnv* act synergistically, revealing an entirely new mechanism for
18 cooperative activities driven by complimentary electrostatic interactions at the PET surface. These findings
19 highlight unexplored avenues in improving PETase function through the control of morphological preference or
20 introduction of protein cooperativity by exploiting protein electrostatics.

21 Introduction

22 Using enzymes for biocatalytic depolymerisation of plastics provides a low-energy and low-resource alternative to
23 the current open-loop recycling of common consumer materials¹. In the case of polyethylene terephthalate
24 (PET), this process allows for the nearly quantitative recovery of the constituent monomers terephthalic acid
25 (TPA) and ethylene glycol (EG), making new routes to (bio)-chemical valorisation and repolymerisation
26 accessible^{2,3}. The range of PETases, enzymes capable of carrying out this conversion, has also been expanded in
27 recent years through discovery and further engineering⁴⁻⁸. Most of these research efforts have targeted large-
28 scale bio-recycling through increases in enzymatic thermostability, allowing for improved reaction rates and
29 depolymerisation extents by taking advantage of higher polymer chain mobility near the bulk PET glass transition
30 temperature (T_g)⁹⁻¹⁴. This primary focus on thermostability has deepened our understanding of
31 thermodynamically important structural features of PET-degrading enzymes;^{10,14} however, it has left obscure
32 much of the fundamental nature of the interactions between the proteins and the polymer surface, which
33 ultimately governs enzymatic selectivity, optimal conditions and industrial performance.

34 Plastic-degrading enzymes face a multitude of challenges associated with the physical-chemical properties of
35 their solid, hydrophobic substrate beyond polymer chain dynamics, which cannot be overcome by enhanced
36 enzymatic thermostability and higher reaction temperatures. On solid substrates, catalytic activities can be
37 influenced by restricted diffusion, variations in the density of surface charge, degree of crystallinity, and protein-
38 protein interactions¹⁵⁻¹⁹. These complications have been shown to underlie some of the more counter intuitive
39 behaviours we observe in natural PETases, such as diminished activities at elevated enzyme
40 concentrations,^{17,20,21} which prevent some members of the enzyme family from being used at an industrial scale.
41 Studying such attributes can therefore clarify fundamental enzyme-polymer interactions underlying catalytic
42 activities, allowing us to better engineer biocatalysts towards industrial recycling applications.

43 In this study we examine *SfCut*, a highly active, thermostable PETase from *Saccharopolyspora flava* most closely
44 related to PHL7⁸ (Figure 1), previously reported⁴ to have an exceptional preference for micronized PET powder
45 over amorphous PET film. We sought to understand the molecular basis for this morphological preference,
46 hoping to gain insight into how substrate specificity is controlled. Through focused structural analysis, we
47 identified that the principal feature differentiating *SfCut* from other PETases was its markedly negative charge; to
48 explore the implications of this property, we used a rational protein design approach that maintained the
49 thermodynamic stability of *SfCut* while inverting its overall charge. This allowed us to clarify the role of protein
50 electrostatics in substrate morphological preference and provided new insights into how we can use protein

51 charge to exploit protein-protein interactions at the plastic surface. By connecting structural analysis with careful
52 protein design, this study contributes to advancing the development of more efficient targeted strategies for
53 improving enzymes for plastic biodegradation.

54 **Results and Discussion**

55 **Structural analysis of *SfCut* and surface charge redesign**

56 To understand the parameters that influence the substrate morphological preference of *SfCut*, we carefully
57 examined its structure. Like other reported PETases, *SfCut* (PDB: 7QJP) adopts a typical alpha-beta hydrolase
58 fold, with a conserved disulfide bond between Cys243 and Cys260. The active site matches that of a typical PET-
59 degrading serine hydrolase, composed of a catalytic triad (Ser132, Asp178 and His210), a conserved tryptophan
60 (Trp157), an oxyanion hole (Phe64 and Met133) and a lipase box. Despite these features, *SfCut* is strikingly
61 different to other highly active PETases owing to its negative surface charge and total charge density of -0.55
62 per kDa at neutral pH. These charges are mostly found on the surface opposite to the active site, away from
63 residues expected to directly interact with the plastic (Figure 2a/c). This pronounced charge appears to modulate
64 the morphological selectivity of *SfCut*, causing its activity to drop considerably between pH 6.0 and pH 7.5 on
65 films while it increases on amorphous powders⁴. As PET has a stable, negative zeta potential independent of pH
66 at these conditions²², the changes in depolymerisation rates implicate the involvement of both protein and
67 polymer electrostatics as significant factors in these properties.

68 We aimed to investigate how protein charge influences substrate morphology preference of *SfCut* by extensively
69 redesigning its surface. To ensure clarity in our analysis, we sought to isolate the effects of charge substitutions
70 from inadvertent changes to enzyme activity caused by variations in protein thermostability. To avoid this, we
71 selected a manual design approach over computational methods, as the later have been constructed in a way
72 that produces stabilised proteins as outputs. For design purposes, all charged residues in the crystallographic
73 structure, including histidines involved in salt bridges, were identified and counted towards the overall protein
74 charge (-16). Owing to the lack of observed crystallographic density, we did not count the first and last glutamic
75 acid residues in the sequence.

76 To minimise disruption of the protein-polymer interface and the active site, only residues at a distance greater
77 than 10 Å from the catalytic triad were considered for mutation. For the purposes of design, we ranked residues
78 based on how amenable to change they might be, excluding those that were buried, involved in critical polar
79 contacts, stabilising secondary structural elements, or providing an ambiguous structural role. Our choices of
80 sites for mutation were supported by referencing the position-specific scoring matrix (PSSM) of *SfCut*
81 (Supplementary Spreadsheet 1) excluding residues at highly conserved sites. As a priority for mutation, we
82 focused on changing isolated negatively charged residues on *SfCut*'s surface, considering structural context to
83 minimise steric clashes and preserve native contacts. Once all the free residues amenable to change were
84 exhausted, we proceeded to consider mutations within larger salt-bridge clusters followed by neutral surface
85 residues. In the interest of investigating solely the effect of the surface charge, we did not intentionally introduce
86 mutations that could create new stabilising interactions.

87 The redesigned variant, here referred to as *SfInverse* (*SfInv*), incorporates 24 mutations (Supplementary Table
88 2; Supplementary Figure 2) at sites with an average information content of 0.41, resulting in an overall charge of
89 +16 (Δ_{charge} of +32) and a charge density of + 0.54 per kDa at neutral pH. Structural predictions using
90 ColabFold^{23,24} and ESMfold²⁵ (pLDDT > 0.9, Supplementary Figure 3) closely align with the crystal structure of
91 *SfCut*, having C_α RMSD values of 0.38 Å and 0.52 Å, respectively. These results suggested that the redesign was
92 likely to preserve the protein fold, without introducing structural changes that could impact enzymatic function.

93 **Biophysical characterisation of *SfInv***

94 We sought to confirm that the extensive surface modifications introduced to *SfInv* did not impact its biophysical
95 properties or its ability to hydrolyse PET plastic. *SfInv* expressed in *E. coli* at comparable levels to the wildtype
96 enzyme, and its identity was confirmed by mass spectrometry after purification (Supplementary Spreadsheet 2).
97 Both *SfInv* and *SfCut* showed similar melting temperatures and thermo-kinetic profiles when analysed by DSC.
98 The apparent T_m were 73 °C for *SfInv* and 76 °C for *SfCut* (Figure 2d, Supplementary Figure 4, Supplementary
99 Table 3), with both enzymes unfolding in a single irreversible step therefore realising our design ambition of
100 minimising the impact on thermodynamic stability.

101 The protein fold and success of the design were further confirmed through circular dichroism (CD) and structure
102 determination by X-ray crystallography. *SfInv* readily crystallised under several conditions, and its structure was
103 solved to a final resolution of 1.17 Å (Supplementary Table 4). The asymmetric unit in the structure contained

104 two chains, which aligned with a C_{α} RMSD 0.24 Å, and the more complete chain B was used for structural
105 analysis. The solved crystallographic structure of *Sfnv* (PDB: 9EWR) confirmed that the enzyme retained its
106 alpha-beta hydrolase fold (Figure 2b) and exhibited the expected positively charged surface (Figure 2c).
107 Structural alignment with the wildtype protein yielded a C_{α} RMSD 0.36 Å and a TM-score²⁶ of 0.99 (Figure 2b),
108 demonstrating that the overall fold and structure were preserved, despite extensive modification. We were able
109 to confirm that no new stabilising interactions were introduced, and that the backbone conformation, disulfide
110 bond, and salt-bridges remained largely intact, with the only exceptions being due to crystallographic contacts
111 spanning symmetry related monomers (Supplementary Figure 5). CD also revealed that both proteins remain
112 well folded under the reported optimum reaction temperature of *SfCut* (50 °C) (Figure 2e).

113 With confidence in the achievement of our structural design goals, we explored the activity of *Sfnv* on
114 amorphous PET powder. *Sfnv* exhibited an optimum reaction temperature at 50 °C, similar to that of the
115 wildtype enzyme (Figure 2d), but demonstrated a higher pH optimum (pH 9) compared to *SfCut* (pH 7.5). This
116 shift to a higher pH optimum aligns with the activity profiles of positively charged PETases, which demonstrate
117 pH optima above pH 8^{4,27}. We anticipated that the changes in the surface charge between *SfCut* and *Sfnv* could
118 lead to substantial differences in their binding affinity to PET. Indeed, despite its relatively high activity, *SfCut*
119 showed no appreciable binding to the amorphous PET powder under tested conditions (Figure 3b,
120 Supplementary Figure 6). This stands in clear contrast to *Sfnv*, which demonstrated a K_d of less than 4.5 nM
121 with a surface coverage (Γ_{max}) of 10-16.5 nmol g^{-1} PET, consistent with characteristics measured with other
122 highly active PETases²⁸. These results confirmed the success of our design efforts, and provided a basis for us to
123 examine the isolated effects of protein charge on PET-degrading activities and morphological preference.

124 **The role of surface charge on protein-polymer interactions**

125 To explore how their significant difference in protein surface charge manifested in terms of depolymerase activity
126 across PET morphologies, we examined *SfCut* and *Sfnv* on three PET substrates: amorphous powder (aPow),
127 amorphous film (aFilm) and semi-crystalline powder (cPow) (Figure 3a, Supplementary Figure 7). *Sfnv*
128 demonstrated higher activity than the wildtype enzyme across all substrates tested, with broader pH and
129 temperature optima, as well as three-fold and six-fold more product release on semi-crystalline powders and
130 amorphous powders respectively. Our results confirmed previous observations that *SfCut* has a considerably
131 lower activity on films compared to powders, independent of substrate crystallinity. In contrast, *Sfnv* showed an
132 inverted selectivity: its activity on films was enhanced, approximately 2-fold higher than on powders,
133 demonstrating that enzyme specificity for different substrate morphologies is a genetically encoded, engineerable
134 property. This corresponds to over 200-fold enhancement in the activity on amorphous PET films compared to
135 *SfCut*, effected simply by surface charge inversion. Despite the substantial difference in evident binding affinity
136 to amorphous powders, both enzymes show activity saturation on amorphous films above 250 nM, confirming
137 that binding alone is insufficient to explain their catalytic properties (Supplementary Figure 8). Although not an
138 intended outcome of our design process, *Sfnv* demonstrated a similar level of conversion as the most promising
139 industrialised enzyme, LCC^{ICCG},¹⁰ at their respective optima (Figure 3a). In a direct comparison at 50 °C, *Sfnv*
140 outperformed LCC^{ICCG} at laboratory scale (Supplementary Figure 9).

141 Although the greatly enhanced binding affinity observed with *Sfnv* can partially explain its higher enzymatic
142 activity, differences in enzymatic morphological preference must be caused by a change in how the differently
143 charged enzymes interact with the polymer. Electrostatic interactions between the polymer surface and the
144 enzymes can be mediated by both unspecific and specific interactions with ions in solution, therefore we
145 assessed the activities of both enzymes at a range of ionic strengths, using both monovalent and divalent salts.
146 The negatively charged wildtype enzyme, *SfCut*, showed a monotonic rise in activity on amorphous films as ionic
147 strength increased from 0 M to 4 M, with a relative activity enhancement of nearly 600-fold (Figure 3c, &
148 Supplementary Figure 10a). This behaviour contrasts with that of some of the best-performing PETases, such as
149 LCC^{ICCG}, where the depolymerase activity is not affected by the ionic strength of the solution (Figure 3c &
150 Supplementary Figure 10b). For *Sfnv* the effect of ionic strength was less pronounced (Figure 3c), with activity
151 peaking at 250 mM ionic strength at approximately five-fold higher than in buffer with 0 M salt, before declining
152 to approximately 7% of its maximal value at 2 M (Supplementary Figure 10a). These results on amorphous films
153 were consistent across different salts (Supplementary Figure 10), indicating that the selectivity observed in *SfCut*
154 and *Sfnv* is driven by electrostatic interactions between the enzyme and the polymer surface, rather than by
155 specific interactions mediated by salts.

156 Interestingly, the effect of ionic strength on the enzyme activity was distinct on amorphous powders. *Sfnv*
157 showed the same monotonic rise in activity as *SfCut*, although less pronounced (Supplementary Figure 10c), with
158 both enzymes seeing no benefit to activity above an ionic strength of 1 M. Overall, positively charged *Sfnv* sees
159 relatively moderate benefits of increased ionic strength, with a greater effect observed on powders than films,

160 whereas *SfCut* shows substantial improvements in activity with salt, benefitting more on films than powders. This
161 demonstrates that the interplay between the electrostatic fields of the polymer and the protein varies between
162 polymer morphologies, influencing the observed selectivity of the two enzymes, with notably more dominant
163 effects on PET film digestions.

164 Despite the benefit of ionic strength on the activity of *SfCut*, it is important to note that the total product
165 released by *SfCut* did not surpass that of *SfInv* at its maximum under the conditions tested. Additionally, while
166 increased salt concentration did have an effect on the apparent T_m of *SfCut*, causing an increase of 4 °C at 2 M
167 sodium chloride, no such change was observed for *SfInv* (Supplementary Figure 11). This suggests that some of
168 the activity improvement in *SfCut* with increasing ionic strength may be a result of enhanced thermostability, an
169 effect that is absent in *SfInv*.

170 ***SfInv* shows greatly enhanced PET-degrading activity at pilot scale and on post-consumer waste**

171 The improved selectivity of *SfInv* towards amorphous PET films under analytical conditions suggested a potential
172 for digesting unmodified amorphous films at pilot scale, pH-controlled experiments. Under these conditions (1
173 mg_{enzyme} g_{PET}⁻¹ with high solids loading of 20% w/v)^{1,29}, the wildtype enzyme (*SfCut*) demonstrates limited
174 activity, achieving less than 1% substrate conversion and a monomeric product yield of 0.15 g/L as measured by
175 HPLC. In contrast, the engineered *SfInv* exhibited significantly higher efficiency, achieving approximately 8%
176 substrate conversion within 24 hours (Figure 4a), with a monomer yield of 18.6 g/L. Strikingly, at substantially
177 lower enzyme loading (1 μM, ~0.15 mg_{enzyme} g_{PET}⁻¹), *SfInv* achieved the same high levels of conversion within the
178 same time frame, suggesting there is substantial potential for reaction condition optimisation to maximise the
179 digestion of amorphous PET films while reducing resource requirements (Supplementary Figure 12).

180 Comparable results were also found to be true on untreated post-consumer waste (PCW) in the form of PET-film
181 sandwich trays (Figure 4b, Supplementary Table 5, Supplementary Figures 13 and 14). *SfInv* efficiently digested
182 over 10% of the waste within 24 hours, achieving a measured yield of 19.25 g/L, while *SfCut* yielded negligible
183 amounts of product (Figure 4b). This further highlights that it may be possible to engineer enzymes specifically
184 targeting the processing of complex post-consumer waste streams of PET with limited pre-treatments, upstream
185 of current methods reliant on resource-intensive preparation of micronized powders.²⁹

186 ***SfCut* and *SfInv* act synergistically in degrading aPET films**

187 Building on the evident enhancement in activity and change in selectivity of *SfInv* over *SfCut* on PET substrates,
188 we sought to understand whether the enzymes act at distinct sites on the PET surfaces with differing geometries
189 or electrostatic charge, which could account for the observed differences in morphological preferences and allow
190 for the enzymes to work synergistically in degrading PET. Specifically, we hypothesised that the amount of
191 product released from a mixture of the two enzymes could exceed the sum of the product release for each
192 enzyme individually at its respective concentration. To quantify this, we define synergy as:

193 (1)
$$\text{Synergy} \equiv \frac{PR_{\text{mix}}}{PR_{SfCut} + PR_{SfInv}} > 1$$

194 where PR_{SfCut} , PR_{SfInv} , and PR_{mix} are the product released by each enzyme at specified concentrations, and their
195 mixture, respectively. When *SfCut* and *SfInv* were mixed at different concentrations and ratios, and applied to
196 PET films, we observed clear evidence of synergistic activity (Figure 5a). The effect is most notable at lower
197 concentrations of *SfInv*, with a synergy value above 2 (Figure 5c). However, at elevated concentrations of *SfInv*,
198 the synergistic benefit is lost, possibly owing to surface crowding^{17,20} or competition for binding sites on the
199 plastic substrate. In contrast, increasing *SfCut* does not eliminate the observed synergy, suggesting that each
200 enzyme plays a distinct role when acting in concert on the film surface.

201 To further clarify the mechanism of the synergistic activity, we explored potential causes involving the differing
202 enzyme charges. One possibility was that the enzymes might assemble in solution in a way that enhances their
203 stability and activity. To test this, we incubated the enzymes together at elevated concentrations (~ 34 μM,
204 compared to the 0.05-1 μM enzyme concentrations used in polymer digestions) and evaluated whether they form
205 complexes by size exclusion chromatography on the mixture (Figure 5e); no shift was observed in the elution
206 profile, suggesting that the enzymes do not appreciably bind to each other in solution. To exclude the possibility
207 that the enzymes are targeting different sites on the polymer chain and therefore modifying the plastic surface
208 such that it becomes a better substrate for the oppositely charged enzyme, we examined whether the enzymes
209 showed a stepwise synergy. The activities of the enzymes were evaluated on amorphous PET film coupons that
210 had been pre-treated with either *SfCut* or *SfInv* (Figure 5d), with no significant difference in activity observed on
211 films pre-treated with either enzyme, showing that the surface targets for both enzymes are largely the same.

212 Given these findings, and that *S/Cut* alone shows negligible binding to PET, it is likely that the synergy observed
213 at principally low concentrations of *S/Inv* is a result of charge masking, similar to that of the ionic strength
214 experiments. In this case, *S/Inv* binds to the plastic surface masking the negative charges of the polymer and
215 any bound *S/Cut*, which in turn allows more *S/Cut* to bind productively. Similar effects have been previously
216 observed with the use of synthetic surfactants³⁰, where anionic detergents were used to increase the negative
217 surface charge of the polymer, therefore attracting the cationic *IsPETase*³¹ to the surface, accelerating enzymatic
218 degradation. In this case, however, the enzymes themselves are modulating the surface charge.

219 The difference in how the film surface is modified by the enzymes acting individually or as a mixture can be
220 visualised after partial digestion of PET coupons using SEM (Figure 5b). *S/Cut* increases the roughness of the
221 surface leaving what appears to be thread-like crystalline regions behind, whilst *S/Inv* shows broad pitting, a
222 surface modification commonly observed with other highly active PETases^{18,31}. However, with the enzyme
223 mixture, an unusual double-pitting effect is observed. Broad pits similar in dimensions to those formed by *S/Inv*
224 contain deeper pits within them, perhaps from *S/Cut* being 'funnelled' into the centre of areas where the
225 positively charged *S/Inv* masks the negative charge on the surface of the film.

226 As far as we are aware, this is the first time that synergy has been observed in enzymes directly modifying
227 plastic surfaces. These results reveal a previously unexplored mechanism by which enzyme mixtures can
228 enhance the degradation of plastic through beneficial electrostatic interactions, offering new opportunities for
229 improving bio-recycling through synergistic enzymatic activity.

230 **Conclusions**

231 Prior to this study the most significant changes in the activity of PET degrading enzymes were realised by
232 increasing the performance of the biocatalysts at elevated temperatures, where the polymer is more dynamic.
233 This focus on thermostability has obfuscated how other physical-chemical properties of the polymer surface
234 influence the activity of PETases, limiting our understanding on the enzyme-substrate interactions at play.

235 Here, by rational redesign of the surface of a highly active PETase, *S/Cut*, we were able to investigate the impact
236 of electrostatics on enzymatic activity in the absence of confounding factors like thermodynamic stability or
237 changes in features near the active site^{13,30,32}. Through carefully crafted studies on the effects of ionic strength
238 on product released from different substrates, we demonstrate that substrate selectivity is driven by polymer
239 morphologies having distinct electrostatic profiles, and therefore influencing enzyme-surface interactions in
240 contrasting manners. Measured zeta potentials are known to have smaller magnitudes on rough surfaces than on
241 smooth surfaces^{33,34}, and the impacts of this are likely manifesting here. Within the diffuse layer around the
242 neutrally charged polymer surface, the anionic *S/Cut* experiences repulsion while the cationic *S/Inv* experiences
243 attraction, with these interactions being more pronounced on smooth films than on rough powders.

244 By focusing on electrostatic interactions governing the activity of PET degrading enzymes at the polymer surface,
245 we demonstrated that it is possible to not only tune the selectivity of PETases for substrates of differing
246 morphologies, but also increase their binding affinity through the rational engineering of surface charge away
247 from the active site. The process of introducing large scale changes in protein surface electrostatic potential in
248 *S/Inv* also afforded substantially improved activities on all PET substrates tested, exposing the benefits of using
249 an electrostatically-oriented approach to engineering plastic depolymerases for improved activities at the low salt
250 concentrations relevant for industrial processes. As we demonstrate through the preservation of the
251 thermodynamic stability of *S/Inv*, this approach is complementary to established methods designed to enhance
252 the thermostability of enzymes and can be introduced in the engineering process towards better industrial PET
253 depolymerases.

254 We also found that synergistic activity between PETases can be realised through the applications of
255 complementarily charged proteins. While the enhancement in activity we observed on films is relatively modest,
256 just over two-fold, this advance is the first method for building synergy into enzymes acting at plastic surfaces
257 and opens the door to engineering campaigns specifically tailored towards improving it. In contrast to synergies
258 between enzyme pairs acting at different chemical sites on a substrate, the mechanism of synergy in this case
259 appears to depend upon the electrostatic masking effect of the positively charged *S/Inv* when bound to PET,
260 which in turn promotes the activity of the negatively charged *S/Cut*. This mechanism is likely generalisable and
261 opens the possibility of exploiting this discovery to reduce the enzyme loading needed for industrial processes or
262 to create dynamic mixtures of enzymes capable of accommodating variations in surface electrostatics across
263 substrates or over the course of depolymerisations.

264 Through isolating the effect of electrostatics on *SfCut*'s activity on PET, we have been able to establish an
265 engineering approach that allows tuning of binding affinity, improvement of enzymatic turnover, control of
266 substrate morphological preference, and introduction of functional synergy in PETases. These insights not only
267 expand the repertoire of established features under the control of rational protein design for plastic
268 depolymerases, but are also likely not limited to PET. As such, we expect these results to be translatable to
269 enzymes capable of digesting a broad range of synthetic polymers, and that the considerations established here
270 will prove crucial when finding and engineering enzymes to tackle plastics found in complex industrial and post-
271 consumer waste streams.

272 **Materials and Methods**

273 Amorphous PET film (ES30-FM-000145) and semi-crystalline PET powder (ES30-PD-006031) were purchased
274 from Goodfellow. Post-consumer plastic waste was obtained from PET sandwich packaging. All reagents for
275 molecular biology and strains were purchased from New England Biolabs. All other reagents and buffer
276 components were acquired from Fisher Scientific or Merck, unless stated otherwise.

277 **Phylogenetic and sequence analysis:** Protein sequences of reported PETases were aligned using
278 ClustalOmega³⁵ with default settings. A phylogenetic tree was built from the sequence alignment using IQtree³⁶
279 with 100,000 UltraFast bootstraps³⁷, nearest neighbour interchange (NNI) search, automatic model selection³⁸,
280 and 100,000 cycles of single branch testing (SH-aLRT)³⁹. Sequence identities were found using protein BLAST⁴⁰
281 with default settings. The position specific scoring matrix was calculated in POSSUM⁴¹ using Uniref50 as the
282 database, with 3 iterations and an E-value threshold of 0.001.

283 **Manual surface redesign:** The surface redesign was done manually using the crystal structure of *SfCut* (611,
284 PDB: 7QJP). All charged residues with crystallographic densities were identified, including histidines involved in
285 salt bridges or catalytic contacts; those that were not interacting were counted as non-charged. Redesign
286 focused on charged surface residues more than 10 Å away from the active site not involved in any polar or
287 evident structural contacts, when these were exhausted mutations within larger salt-bridges and to uninvolved
288 surface neutral residues were considered. No mutations were added to purposefully increase or impair enzymatic
289 stability and activity. The overall charge was changed from -16 to +16, by mutating negatively charged as well
290 as neutral surface residues mostly not involved in stabilisation of the protein structure by visual inspection. The
291 final *SfInv* sequence was modelled using ColabFold/AlphaFold2^{23,24} and ESMFold²⁵ to confirm that no disruption
292 to the protein structure by the design was predicted.

293 **Plasmid construction:** Genes for *SfCut* and *SfInv* were synthesised by Twist Bioscience. *SfCut* was cloned by
294 Twist directly into pET21b(+), *SfInv* was synthesised as a gene fragment and cloned into pET28b(+) using
295 Gibson assembly. The assembly mixture was transformed into NEB5 α competent cells, DNA purified (Qiagen
296 miniprep kit), and sequence confirmed by Sanger sequencing (Eurofins Genomics). Both constructs include a C-
297 terminal His-tag, and were sequence optimised for *Escherichia coli*.

298 **Protein expression and purification:** Proteins were expressed using BL21(DE3) *E. coli* strain. Cells were
299 grown in terrific broth with the selection antibiotic at 37 °C, in 4.5 L cultures in bioreactors (Eppendorf BioFlo
300 120w) with pH and air flow control (Biocommand Bioprocessing Software). Protein expression was induced at an
301 OD₆₀₀ of 1.2 for 18 hours at 20 °C, using a final concentration of 1 mM IPTG. Harvested cells were resuspended
302 in HisTrap binding buffer (20 mM Tris-HCl pH 8.0, 300 mM sodium chloride, 40 mM imidazole) with nuclease
303 (expressed in house) and 25mM of magnesium added. The resuspended cells were then homogenised, sonicated
304 (Amplitude 40, 3 sec ON, 9 sec OFF for a total processing time of 6-10mins), and clarified by centrifugation at
305 55,000 x g. Clarified lysate was filtered through a 0.45 μ m MCE filter and purified by affinity chromatography on
306 a HisTrap FF (5 mL) column, eluted over a gradient up to 500 mM imidazole. The protein peak was further
307 purified by size exclusion chromatography using a Superdex 16/600 HiLoad 75pg equilibrated with 50 mM
308 sodium phosphate pH 7.5, and 100 mM sodium chloride. SDS-PAGE was run to assess purity.

309 **Differential scanning calorimetry:** Apparent melting temperature (T_m) values for the purified proteins were
310 determined using a MicroCal PEAQ-DSC with automated sampler (Malvern Panalytical), using a buffer matched to
311 that of the size exclusion chromatography step as reference. The analyses were performed using 1 mg/mL of
312 protein, at a temperature range of 30-100 °C, using low feedback, at 192 °C/ hour, 96 °C/ hour, 90 °C/ hour, 48
313 °C/ hour, 24 °C/ hour, 12 °C/ hour and 6 °C/ hour. Baseline subtraction was performed using the instrument's
314 data analysis software. Calfitter 2.0⁴² was used to derive the activation energy (E_{act}), the heat capacity change
315 (ΔC_p), the activation enthalpy change (ΔH^\ddagger), and the reference temperature of the irreversible melting step (T_{act})
316 using the thermal denaturation model with the lowest SSR value.

317 **Structure determination by X-ray crystallography:** *SflnV* was concentrated to 10 mg/mL and crystallised
318 by the sitting drop vapour diffusion method using a Mosquito crystallisation robot (SPT Labtech) and SWISSCI 3-
319 lens low profile plates in condition C8 of the SaltRx screen (Hampton Research): 0.1 M Tris pH 8.5 and 3.5 M
320 sodium formate. Crystals were cryo-protected with 20% glycerol before flash-freezing in liquid nitrogen.
321 Diffraction data were collected at the Diamond Light Source (Didcot, UK) at beamline I03 and automatically
322 processed with the AutoPROC+STARANISO^{43,44} pipeline on ISPyB. The structure was solved by molecular
323 replacement on CCP4 Cloud using Molrep⁴⁵ and an AlphaFold2-model²³. Coot was used for model building,
324 followed by model refinement using Refmac⁴⁶. The final structure was evaluated with MolProbity⁴⁷, and the
325 structure has been visualised in VMD⁴⁸ and ChimeraX⁴⁹. The structure was deposited in the PDB with code 9EWR.
326 Data and refinement statistics can be found in Supplementary Table 4.

327 **Structural characterisation by circular dichroism:** Spectra were collected on a PiStar-180 (Applied
328 Photophysics) with water bath temperature control. Protein samples were analysed at a concentration of 0.1
329 mg/mL in 10 mM sodium phosphate pH 7.5 with 20 mM sodium chloride at 50 °C, in a 1 mm stoppered quartz
330 cuvette. Data collected at wavelengths between 200 and 260 nm, with half bandwidth of 1.5 nm and a
331 wavelength interval of 0.5 nm (1 sec per point, 5 repeats), were averaged and baseline subtracted using a
332 matched buffer blank. Raw ellipticity data was converted to mean residue ellipticity by dividing by the path
333 length, concentration, molecular weight and number of residues.

334 **Amorphous PET powder production and analysis:** Sheets of amorphous PET film were cut into strips,
335 immersed in liquid nitrogen and cryo-milled at 2,400 rpm in a SM300 cutting mill (Retsch), with a bottom sieve
336 with 4 mm square holes. Subsequently, this product was reduced in size further by immersing in liquid nitrogen
337 and cryo-milling at 18,000 rpm in a ZM200 centrifugal mill, with a 0.12 mm ring sieve with trapezoidal holes. The
338 particle size and crystallinity of the cryo-milled amorphous PET powder was compared to that of the purchased
339 semi-crystalline PET powder, as well as the PET film, using a CAMSIZER X2 (Microtrac MRB) and Differential
340 Scanning Calorimetry (Supplementary Table 5, Supplementary Figures 13 and 15).

341 **Reaction quenching and product quantification by HPLC:** All reactions were quenched by addition of
342 equal volume of HPLC-grade methanol, and PET solids removed. Samples were centrifuged at 10,000 x g, using
343 a table-top centrifuge, prior to analyte quantification by HPLC. For samples with subsequent polymer analysis,
344 the PET was washed by rinsing three times with a 1% (w/v) SDS solution, followed by multiple rinses with
345 distilled water. The partially digested polymer substrates were then dried at room temperature under vacuum
346 before analysis. The HPLC analysis was adapted from a reported UPLC method¹⁴ to allow for HPLC pressures as
347 previously described⁵⁰. Samples were evaluated on a pre-equilibrated C18 Kinetex LC column (00B-4605-AN)
348 with a guard, at 1.1 mL min⁻¹ with 0.1% formic acid and acetonitrile as the stationary and mobile phases
349 respectively. Samples were prepared with a known dilution to an absorbance at 240 nm of around 1.0 before 10
350 µL were loaded onto the column using an automatic sampler (Agilent). Samples were eluted with an isocratic
351 elution at 13% mobile phase for 0.87 minutes, followed by a step to 95% mobile phase for 1.12 minutes and a
352 re-equilibration at 13% mobile phase until a total time of 3.6 minutes. Peaks were integrated using Agilent's
353 OpenLab software and the product quantification was performed against calibration curves of known standards
354 (TPA, MHET, BHET). An example of the HPLC trace and elution times is provided in Supplementary Figure 16.

355 **PET degradation assays at small scale:** Unless stated otherwise, small scale assays were set up in 1.5 mL
356 tubes with 11 mg of PET substrate, and incubated in triplicate 500 µL reactions for 24 hours at 300 rpm on
357 thermomixers. A final concentration of 100 nM enzyme and 100 mM sodium chloride was used, in 50 mM sodium
358 phosphate pH 7.5, or 50 mM glycine pH 9.0 for *SfCut* and *SflnV* respectively. In the case of the ionic strength
359 tests 50 mM HEPES pH 7.5 and 50 mM CHES pH 9.0 were used instead, with varying amounts of sodium
360 chloride, magnesium chloride and sodium sulphate. These buffers were chosen in order to minimise their
361 contribution towards the ionic strength. Nevertheless, the buffer component was fully accounted for in the ionic
362 strength calculations by considering their pK_as, and calculating the concentration and charges of their respective
363 ionic species. The ionic strength of the solution was calculated by using the formula $I = \frac{1}{2} n \sum_i (C_i Z_i^2)$, where I
364 represents the ionic strength, n is the number of ions in solution, C_i is the concentration of a specific ion in moles
365 per litre, and Z_i the valence of the particular ion species. For the temperature and pH optima experiments, the
366 reactions were performed at three temperatures (40 °C, 50 °C and 60 °C) and using three different buffers (50
367 mM MES pH 6.0, 50 mM sodium phosphate pH 7.5, and 50 mM glycine pH 9.0) with 100 mM sodium chloride. To
368 determine the concentration dependency for each of the enzymes, both were tested individually, and together at
369 equimolar ratios, to a final enzyme concentration ranging from 0 - 1 µM.

370 **Binding isotherms:** Binding affinities to the plastic were measure in two ways as described previously²⁸. All
371 samples were incubated for 1 hour at 4 °C with rolling in low-binding 1.5 mL microcentrifuge tubes (Eppendorf)
372 to prevent loss from non-specific binding. After centrifugation, free enzyme concentrations were determined

373 using a Micro BCA protein assay kit (Thermo Scientific) with a calibration curve derived for the respective
374 enzyme. 150 μ L samples were mixed with the Micro BCA Working Reagent as per the kit's instructions, and
375 incubated for 1 hour at 50 °C in a covered microtiter plate mixing at 300 rpm. 200 μ L samples were measured at
376 562 nm on a plate reader. **Substrate saturation:** Amorphous PET powder at a fixed substrate loading (100 g/L for
377 *SfCut* or 50 g/L for *Sfinv*) was incubated with 0.05 - 1.5 μ M enzyme. The substrate coverage was calculated
378 using the equation $\Gamma = \frac{E_{tot} - E_{free}}{L_{PET}}$, and the K_d obtained by fitting the data to the Langmuir adsorption isotherm
379 equation $\Gamma = \frac{\Gamma_{max} E_{free}}{E_{free} + K_d}$, where Γ_{max} is the substrate coverage at surface saturation. **Enzyme saturation:** 1 μ M
380 enzyme was incubated with substrate loads ranging from 0 – 200 g/L. The bound fraction was calculated from
381 the difference between total and free enzyme concentrations. K_d and Γ_{max} were derived from fitting the data to
382 the equation²⁸ $\theta = \frac{1}{2} \left[\left(1 + \frac{K_d + \Gamma_{max} L_{PET}}{E_{tot}} \right) - \sqrt{\left(1 + \frac{K_d + \Gamma_{max} L_{PET}}{E_{tot}} \right)^2 - 4 \left(\frac{\Gamma_{max} L_{PET}}{E_{tot}} \right)} \right]$, where the fraction bound $\theta =$
383 $\frac{E_{tot} - E_{free}}{E_{tot}}$, and L_{PET} is the experimental solids loading.

384 **PET degradation assays – pilot scale and post-consumer waste:** Duplicate reactions were undertaken in
385 250mL MiniBio Reactors (Applikon Biotechnology) to a total reaction volume of 100 mL. Reactions were carried
386 out at 20% (w/v) solids loading and a final enzyme concentration of 1 mg of enzyme per gram of PET. Reactions
387 were incubated at 50 °C, stirring at 200 rpm for 24 hours. Substrates used in the reactions were amorphous PET
388 film or washed and dried post-consumer plastic film waste, roughly cut into 1 cm x 1 cm squares. All pH probes
389 were calibrated immediately prior to analysis, pH changes were followed and maintained by the automatic
390 addition of 1 M (*SfCut*) or 5 M (*Sfinv*) freshly prepared sodium hydroxide via the system pump, which was
391 calibrated prior to the experiment. The base addition was followed as a function of time by the Lucullus® Process
392 Information Management System software and converted to percentage hydrolysis by calculating the moles of
393 TPA neutralised. At the end of the incubation, samples were taken and quenched as described previously using
394 an equal volume of methanol for HPLC analysis. The PET film was recovered by filtration, rinsed thoroughly with
395 deionised water, and allowed to dry before weighing to confirm depolymerisation extent.

396 **Polymer analysis and characterisation:** Polymer DSC was performed on a Netzsch DSC 214 Polyma,
397 equipped with aluminium crucibles and lids. Approximately 10 mg of samples was heated from 25 to 300 °C at a
398 rate of 10 °C per minute in a nitrogen atmosphere. Measurements were performed in triplicate, and sample
399 crystallinity was calculated using the equation % Crystallinity = $\frac{\Delta H_m - \Delta H_c}{\Delta H_m}$, where ΔH_m is the enthalpy of melting
400 of the sample ΔH_c is the enthalpy of crystallisation of the sample and ΔH_m° is the enthalpy of melting for a
401 theoretical 100 % crystalline sample (taken as 140.1 Jg⁻¹).

402 **PET degradation assays for investigating synergy:** To quantitate synergy across enzyme concentrations
403 and ratios, amorphous PET coupons were incubated in 50 mM sodium phosphate pH 7.5, 100 mM sodium
404 chloride, for 24 hours, with various enzyme ratios and concentrations ranging from 0 – 200 nM, as per Figure 5.
405 To investigate whether the synergy was caused by opposite charge effects between the enzymes, size exclusion
406 chromatography was performed with 1 mg of total enzyme ($\sim 34 \mu$ M) on its own or at equimolar ratio using a
407 Superdex 75 equilibrated with 50 mM sodium phosphate pH 7.5, and 100 mM sodium chloride. For the stepwise
408 synergy studies, a final concentration of 100 nM enzyme was used. Films were pre-treated with the selected
409 enzyme for 24 hours, reactions quenched and the coupons washed and dried as described above. Coupons were
410 then incubated a further 24 hours with the second selected enzyme before the reactions were quenched one
411 final time prior to product analysis by HPLC.

412 **Scanning electron microscopy:** Samples were incubated with 100 nM of the respective enzyme for 24 hours
413 as described above in PET degradation assays for investigating synergy. For the mixed enzyme samples, a total
414 of 200 nM enzyme at an equimolar ratio was used instead. Samples were quenched, washed and dried as
415 described above before analysis by SEM. PET film samples were mounted onto aluminium stubs using carbon
416 adhesive tabs, and sputter coated with Au/Pd under argon using a Quorum Q150RES (Quorum Technologies
417 Ltd). Samples were imaged using a MIRA3 FEG-SEM Microscope (TESCAN) operated at 3 kV.

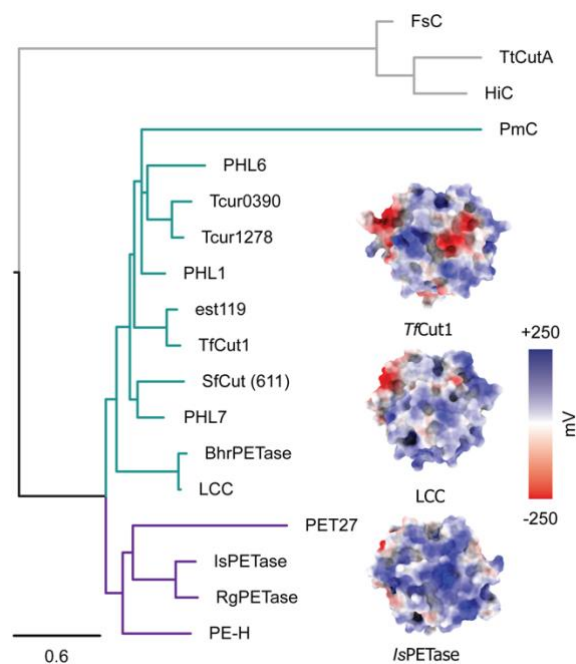
418 **Author contributions**

419 BRL conceptualised and supervised the project. The manuscript was written by LO and BRL, and reviewed by all
420 authors. VB collected polymer DSC data. Enzymes were expressed by EMR. KRG collected crystals for structure
421 determination of *S₁nv*, and MZ solved the crystal structure. All other data was collected, processed and analysed
422 by LO. ARP and BRL acquired funding.

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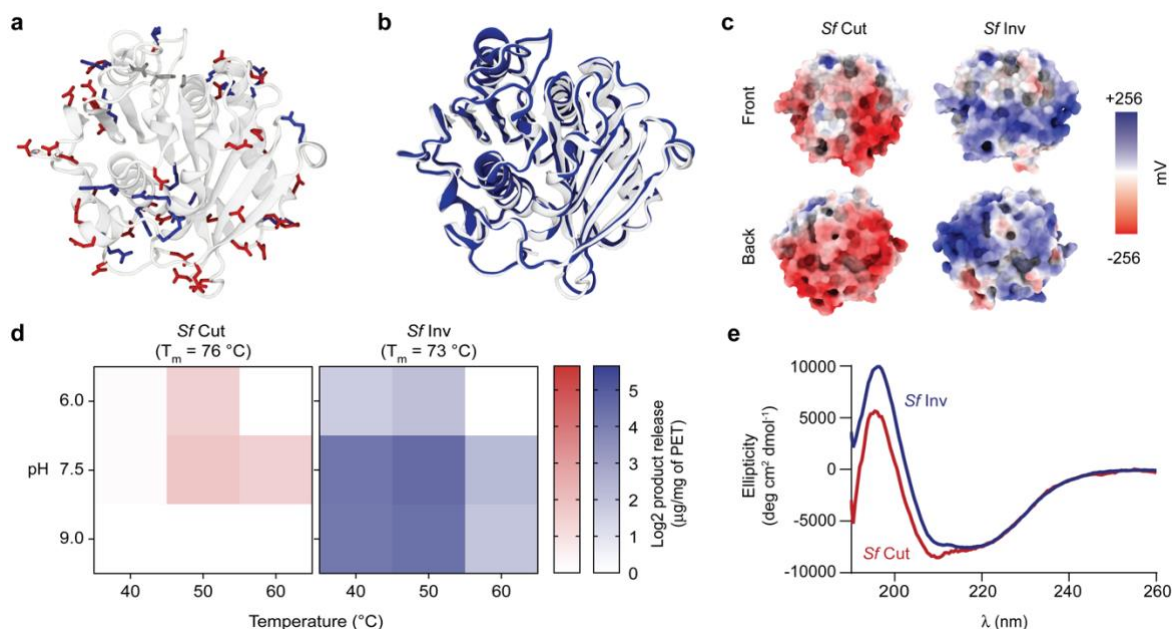
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430 **Figures**



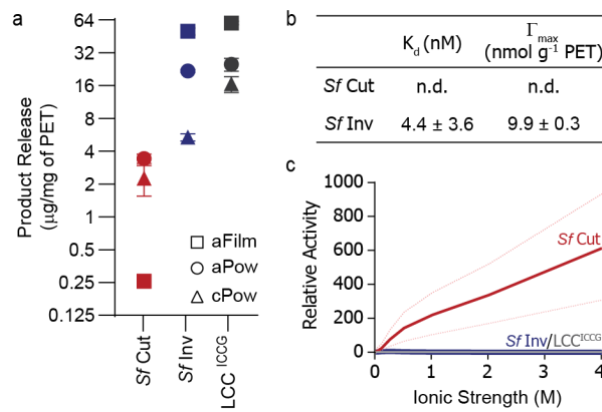
431

432 **Figure 1. Phylogenetic tree showing relationship between *SfCut* and other previously reported PETases.** The
 433 structure of three well characterised PETases are represented as surfaces coloured by their surface potential at pH 7.5, as
 434 calculated by APBS⁵². *SfCut* falls within the same clade as the thermotolerant type I PETases (green). Type II bacterial PETases
 435 are shown in purple and those of fungal origin are shown as the grey outgroup. Accession codes for the PETases used can be
 436 found in Supplementary Table 1 and the alignment used to build the tree in Supplementary Figure 1.



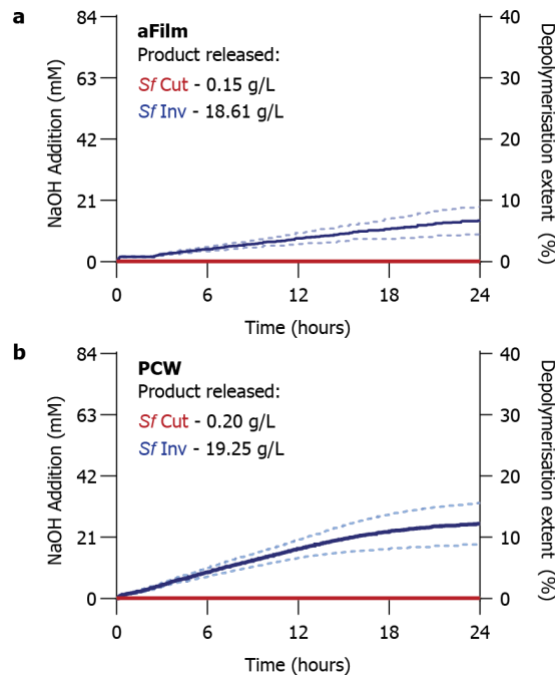
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438 **Figure 2. Biophysical characterisation of *SfCut* and *SfInv*.** **a** Cartoon representation of *SfCut*, with positively and negatively
 439 charged residues represented as sticks. Positive charges are shown as blue, negative charges as red. Active site residues are
 440 shown in grey at the top of the structure. **b** Structural alignment between *SfCut* (white, PDB: 7QJP) and the crystal structure of
 441 *SfInv* (blue, PDB: 9EWR) confirms the adoption of an alpha-beta hydrolase fold and the success of the design with a C_{α} RMSD
 442 of 0.36 and a TM-score of 0.99. **c** Surface representation of the potential surfaces of *SfCut* and *SfInv* at pH 7.5, as calculated by
 443 APBS. **d** Heatmap for activity optima of *SfCut* and *SfInv* at 100 nM on amorphous PET powders across temperatures and pH.
 444 Apparent T_m at a scan rate of 90 °C/hour for the respective enzymes is indicated. Additional data for PET films and semi-crystalline
 445 powders can be found in Supplementary Figure 7. **e** Circular dichroism spectra at 50 °C shows both enzymes remain well
 446 structured at their reaction optima.



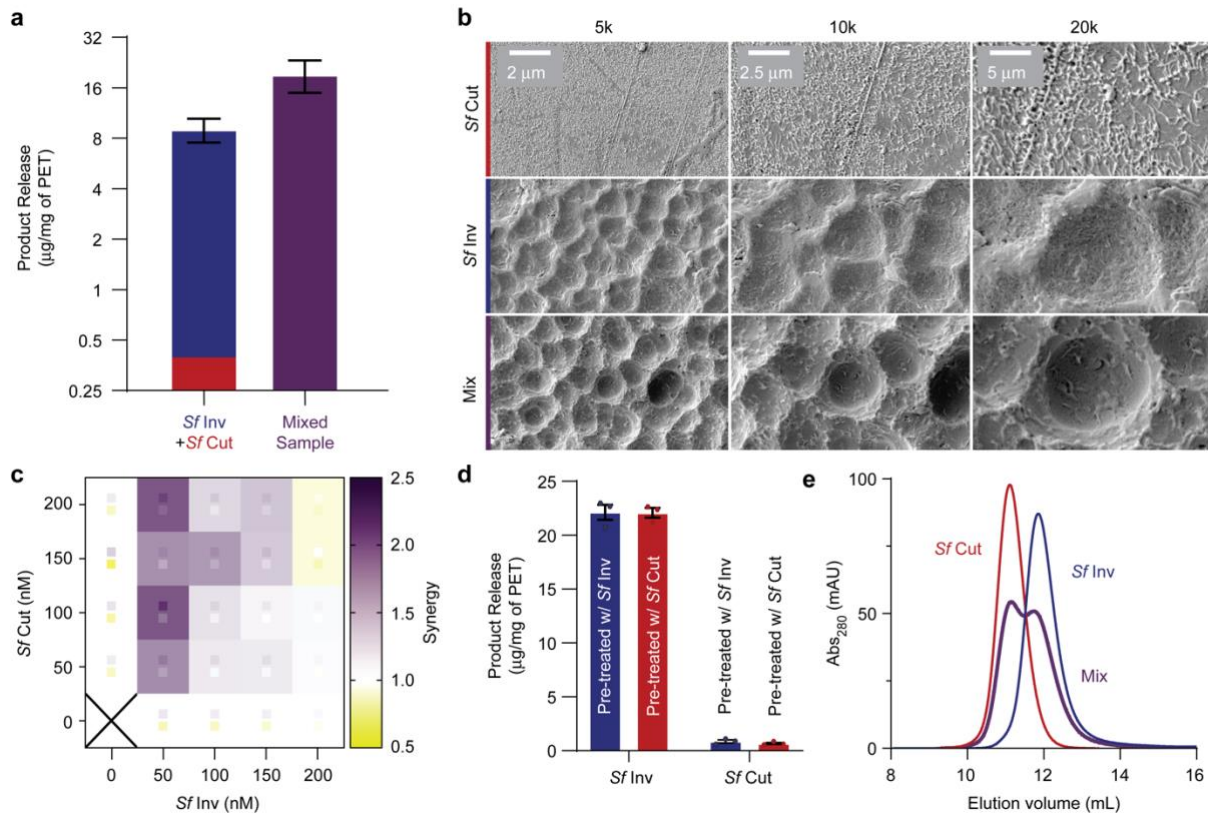
447

448 **Figure 3. Investigating how ionic strength plays a role in protein-polymer interactions.** **a** Substrate
 449 preference for *SfCut*, *SfInv*, and LCC^{ICCG} on a Log₂ scale. Experiments were carried out at the optimum temperature
 450 and pH for the individual enzymes at 100 nM, with 100 mM sodium chloride. **b** *SfInv* has a measurable affinity to
 451 PET powders, while *SfCut* interacts too weakly to measure (binding curves and full statistics are detailed in
 452 Supplementary Figure 4). **c** Ionic strength affects the relative activities of *SfCut* and *SfInv* on aFilms in different
 453 ways: *SfCut* benefits from the increased ionic strength, whilst *SfInv*, like LCC^{ICCG}, is largely not affected. Plot is
 454 shown as relative activity with respect to the activity of each enzyme in buffer without added salt. Lighter dotted
 455 lines represent standard deviations calculated with propagated error.



456

457 **Figure 4. Depolymerisation of PET at pilot scale by *SfCut* and *SfInv*.** Enzymes were incubated with 20%
 458 (w/v) PET substrate, at an enzyme loading of 1 mg per g of PET. *SfCut* is shown in red, whereas *SfInv* is shown
 459 in blue. Figure illustrates depolymerisation overtime of amorphous PET film (**a**) and post-consumer PET waste (**b**).
 460 Average trace showed as a solid line, each of the replicates are represented as dotted lines in the corresponding
 461 colour. Product release as determined by HPLC shown in each panel.



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Figure 5. Investigating the synergy between *SfCut* and *SfInv* on aPET film. **a** Difference in total product release by pure enzymes and their mixture, at the condition with highest synergy (100 nM:50 nM of *SfCut* to *SfInv*). Pure enzymes are represented as a stacked plot, where red represents *SfCut* and blue *SfInv*. The enzyme mixture is represented as a purple bar. Product release is shown as a Log₂ scale for clarity. Error bars represent 1 SD. **b** Scanning Electron Microscopy (SEM) images of amorphous PET film after incubation with enzyme. The top two panels show the film after incubation with *SfCut* or *SfInv* individually, whilst the bottom panel shows the film after incubation with the enzyme mix. **c** Heatmap of the synergy ratios across different concentrations of *SfCut* and *SfInv*, with the purple as productive synergy and yellow as a decrease in total product release. Standard deviation is shown as smaller squares in the same colour scheme. **d** Total product release for the stepwise synergy, where the pure enzymes were incubated with an amorphous film coupon pre-digested by either *SfInv* (blue) or *SfCut* (red). Error bars represent 1 SD. **e** Chromatogram from Size Exclusion Chromatography (SEC) for *SfCut* (red), *SfInv* (blue) and a mixed sample of both enzymes at equimolar ratios (purple).

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