Electronic Supplementary Material (ESI) for Faraday Discussions.

Supplementary Information

Investigating the effect of fusion partners on the enzymatic activity and thermodynamic stability of poly(ethylene terephthalate) degrading enzymes

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Table of Contents

SI.1. Gibson Assembly Protocol

- Materials:

  Reaction buffers and master mixes stored at -70 °C.

  5x Isothermal Reaction Buffer Mix

  - 25% PEG-8000
  - 500 mM Tris-HCl pH 7.5
  - 50 mM $MgCl_2$
  - 50mM DTT
  - 5mM NAD
  - 1mM each of the four dNTPs

  1.33x Gibson Master Mix

  - Taq ligase (40 U/ul): 50 ul
  - 5x Isothermal reaction buffer: 100 ul
  - T5 exonuclease (1 U/ul): 2 ul
  - Phusion polymerase (2 U/ul): 6.25 ul
  - Nuclease-free water: 216.75 ul

- Protocol[1]:
  1. PCR amplify vector and insert fragments, extract single bands from agarose gel using standard kit protocols
  2. Add 5ul of purified PCR products to single aliquot of Gibson Master Mix.
     - Recommended: 100 ng of vector with 2x molar of insert (5x if insert < 200bp)
  3. Incubate reaction mix at 50 °C for 15 minutes in thermocycler
  4. Transform immediately into NEB 5-alpha chemical competent cells or store in the freezer.

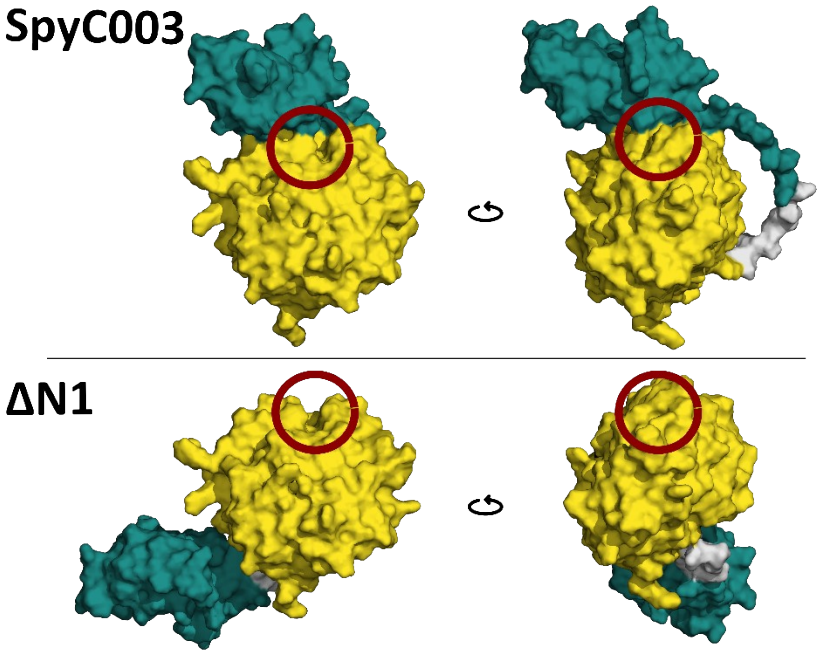
SI.2. Construct Summary

Construct	Vector	Expressed Protein Sequence (ORF)
MBP-SpyTag	pMAL-p4x	MKTEEGKLVIWINGDKGYNGLAEVGKKFEKDTGIKVTVEHPDKLEEKF PQVAATGDGPDIIFWAHDRFGGYAQSGLLAEITPDKAFQDKLYPFTW DAVRYNGKLIAYPIAVEALSLIYNKDLLPNPPKTWEEIPALDKELKAKGK SALMFNLQEPYFTWPLIAADGGYAFKYENGKYDIKDVGVDNAGAKAG LTFLVDLIKNKHMNADTDYSIAEAAFNKGETAMTINGPWAWSNIDTS KVNYGVTVLPTFKGQPSKPFVGVLSAGINAASPNKELAKEFLENYLLTD EGLEAVNKDKPLGAVALKSYEEELAKDPRIAATMENAQKGEIMPNIPQ MSAFWYAVRTAVINAASGRQTVDEALKDAQTNSSSNNNNNNNNNN NLGIEGRISEFGSHHHHHHHHHGNGENLYFQGSGRGVPHIVMVDAYKR YK*
Spycatcher003 ΔN1	pET29a(+)	MDSATHIKFSKRDEDGRELAGATMELRDSSGKTISTWISDGHVKDFYLY PGKYTFVETAAPDGYEVATPIEFTVNEDGQVTVDGEATEGDAHTLEHH HHHH*
*Is*PETase	pET21b(+)	MNFPRASRLMQAAVLGGLMAVSAAATAQTNPYARGPNPTAASLEAS AGPFTVRSFTVSRPSGYGAGTVYYPTNAGGTVGAIAIVPGYTARQSSIK WWGPRLASHGFVVITIDTNSTLDQPSSRSSQQMAALRQVASLNGTSS SPIYGKVDTARMGVMGWSMGGGGSLISAANNPSLKAAAPQAPWDS STNFSSVTVPTLIFACENDSIAPVNSSALPIYDSMSRNAKQFLEINGGSH SCANSGNSNQALIGKKGVAWMKRFMDNDTRYSTFACENPNSTRVSD FRTANCSLEHHHHH*
*Tf*Cut1	pET21b(+)	MANPYERGPNPTDALLEARSGPFSVSEENVSRLSASGFGGGTIYYPREN NTYGAVAISPGYTGTEASIAWLGERIASHGFVVITIDTITTLDQPDSRAE QLNAALNHMINRASSTVRSRIDSSRLAVMGHSMGGGGSLRLASQRPD LKAAIPLTPWHLNKNWSSVRVPTLIIGADLDTIAPVLTHARPFYNSLPTS ISKAYLELDGATHFAPNIPNKIIGKYSVAWLKRFVDNDTRYTQFLCPGP RDGLFGEVEEYRSTCPFLEHHHHHH*
LCCG^{ICCG}	pET29a(+)	MSNPYQRGPNPTRSALTADGPFSVATYTVSRLSVSGFGGGVIYYPTGTS LTFGGIAMSPGYTADASSLAWLGRRLASHGFVVLVINTNSRFDGPDSR ASQLSAALNYLRTSSPSAVRARLDANRLAVAGHSMGGGGTLRIAEQNP SLKAAVPLTPWHTDKTFNTSVPVLIVGAEADTVAPVSQHAIPFYQNLP STTPKVYVELCNASHIAPNSNNAAISVYTISWMKLWVDNDTRYRQFLC NVNDPALCDFRTNNRHCQLEHHHHHH*

*Is*PETase-SC ΔN1	pET29a(+)	MNFPRASRLMQAAVLGGLMAVSAAATAQTNPYARGPNPTAASLEAS AGPFTVRSFTVSRPSGYGAGTVYYPTNAGGTVGAIAIVPGYTARQSSIK WWGPRLASHGFVVITIDTNSTLDQPSSRSSQQMAALRQVASLNGTSS SPIYGKVDTARMGVMGWSMGGGGSLISAANNPSLKAAAPQAPWDS STNFSSVTVPTLIFACENDSIAPVNSSALPIYDSMSRNAKQFLEINGGSH SCANSGNSNQALIGKKGVAWMKRFMDNDTRYSTFACENPNSTRVSD FRTANCSGSGESGSGDSATHIKFSKRDEDGRELAGATMELRDSSGKTIS TWISDGHVKDFYLYPGKYTFVETAAPDGYEVATPIEFTVNEDGQVTVD GEATEGDAHTLEHHHHHH*
*Tf*Cut1-SC ΔN1	pET29a(+)	MANPYERGPNPTDALLEARSGPFSVSEENVSRLSASGFGGGTIYYPREN NTYGAVAISPGYTGTEASIAWLGERIASHGFVVITIDTITTLDQPDSRAE QLNAALNHMINRASSTVRSRIDSSRLAVMGHSMGGGGSLRLASQRPD LKAAIPLTPWHLNKNWSSVRVPTLIIGADLDTIAPVLTHARPFYNSLPTS ISKAYLELDGATHFAPNIPNKIIGKYSVAWLKRFVDNDTRYTQFLCPGPR DGLFGEVEEYRSTCPFGSGESGSGDSATHIKFSKRDEDGRELAGATMEL RDSSGKTISTWISDGHVKDFYLYPGKYTFVETAAPDGYEVATPIEFTVNE DGQVTVDGEATEGDAHTLEHHHHHH*
LCC^{ICCG}-SC ΔN1	pET29a(+)	MSNPYQRGPNPTRSALTADGPFSVATYTVSRLSVSGFGGGVIYYPTGTS LTFGGIAMSPGYTADASSLAWLGRRLASHGFVVLVINTNSRFDGPDSR ASQLSAALNYLRTSSPSAVRARLDANRLAVAGHSMGGGGTLRIAEQNP SLKAAVPLTPWHTDKTFNTSVPVLIVGAEADTVAPVSQHAIPFYQNLP STTPKVYVELCNASHIAPNSNNAAISVYTISWMKLWVDNDTRYRQFLC NVNDPALCDFRTNNRHCQGSGESGSGDSATHIKFSKRDEDGRELAGA TMELRDSSGKTISTWISDGHVKDFYLYPGKYTFVETAAPDGYEVATPIEF TVNEDGQVTVDGEATEGDAHTLEHHHHHH*

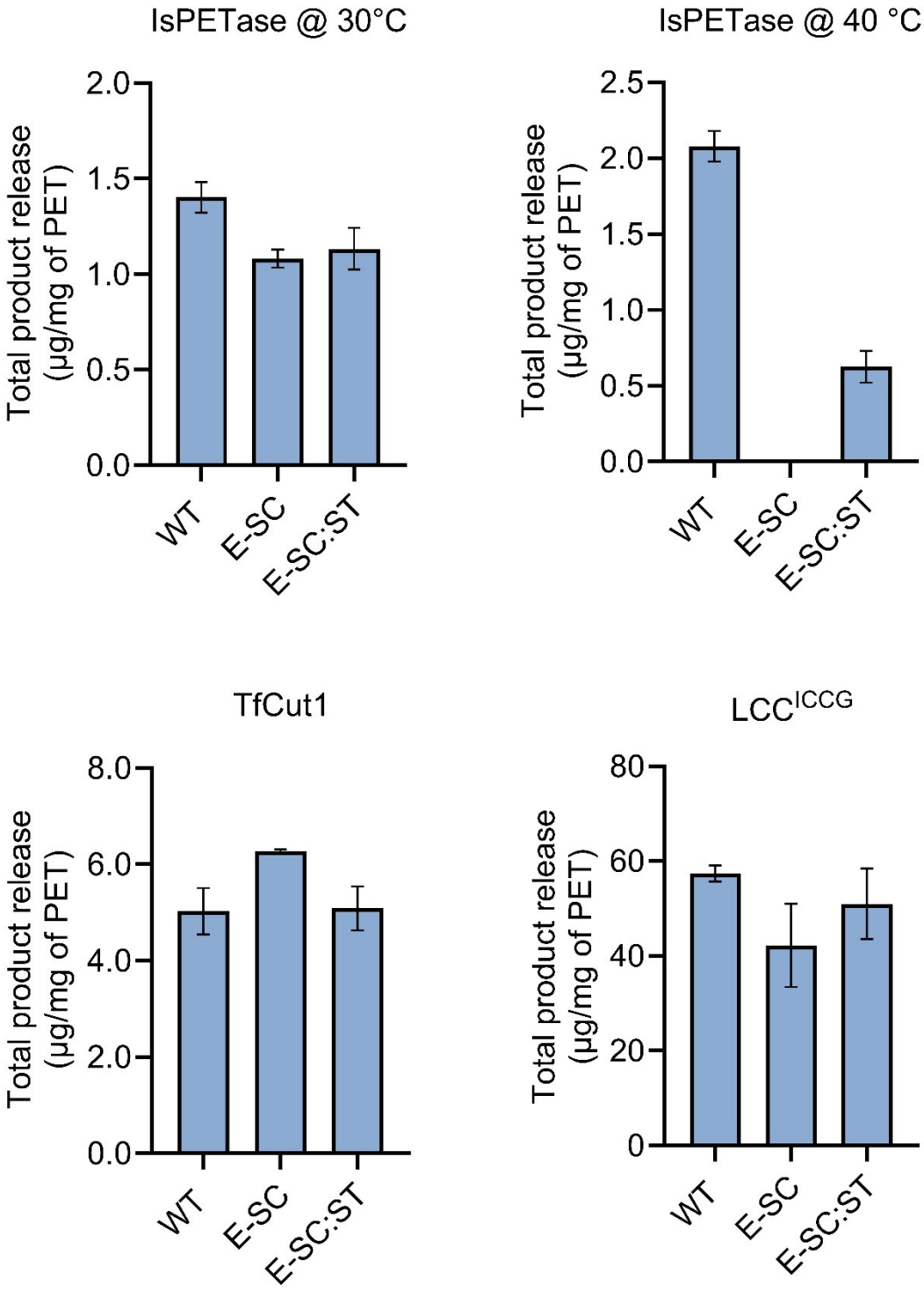
* TEV site highlighted in red, and native signal sequence in grey

SI.3. Structural model of Enzyme-SpyCatcher003 construct



Full length SpyCatcher003 interferes with both expression and activity of PET hydrolase fusions. AlphaFold[2,3] models of TfCut1-SpyCatcher003, show the full length SpyCatcher003 domain (teal), with an extended N-terminal loop that wraps around the globular PETase (yellow) blocking the active site (red circle) (SpyC003, top). In the ΔN1 variation, the active site in unperturbed with SpyCatcher located on the opposite face of the active site cleft.

SI.4. PET-Degrading Activity Data: Singular Plots



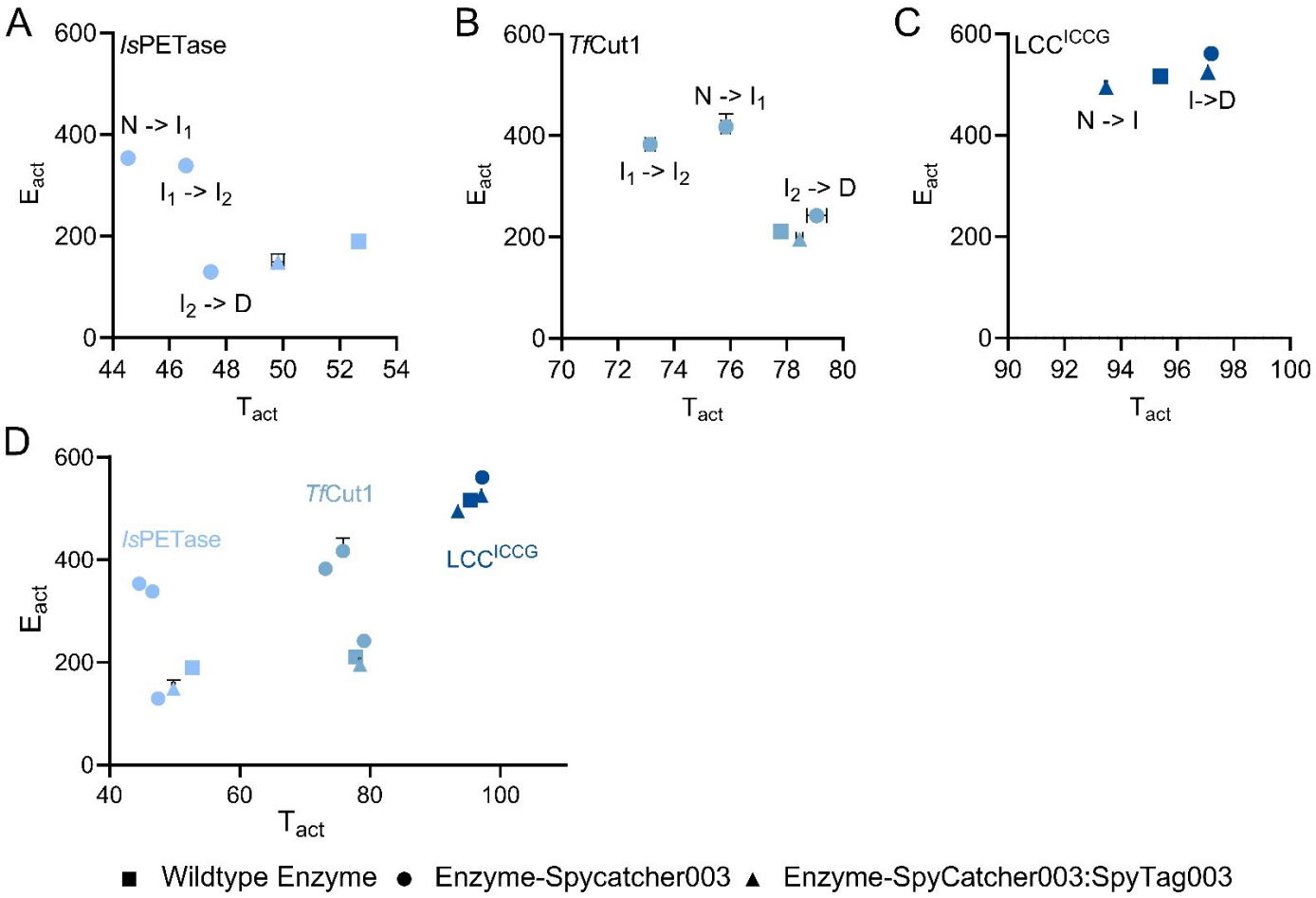
Total product released by enzymes acting on amorphous PET films. Error bars represent standard deviation. Reactions performed at 100 nM enzyme, for 25 hrs at 300 rpm and 100 mM NaCl. *Is*PETase was tested in 50 mM glycine pH 9.0 (both at 30 °C and 40 °C), *Tf*Cut1 and LCC^{ICCG} in 50 mM sodium phosphate pH 7.5 at 60 °C and 70 °C, respectively.

SI.5. DSC Data Extended: Fits and Statistics

Table with extended model fits and associated statistics. Models were chosen based on F-ratios comparing the degrees of freedom (DOF) and sum of squares due to regression (SSR) of simpler models to more complex, as recommended for the Calfitter[4] server. F ratios close to 1 imply the simpler model is a better match to the data. Error bars represent 95% confidence intervals.

Protein	Model	T_m/T_{act}	E_{act}	SSR	DOF	Chosen Model
SpyCatcher003	N = D (Van't Hoff's)	52.11 ± 0.02	N/A	0.07214	14228	*
SpyCatcher003:SpyTag003	N = D (Van't Hoff's)	97.74 ± 0.03	N/A	0.04513	4526	*
IsPETase	N -> D	52.67 ± 0.04	189.45 ± 2.21	0.03446	5813	*
IsPETase-SC	N - > D	48.58 ± 0.04	289.48 ± 3.52	0.15052	4660	
IsPETase-SC:ST	N -> D	49.82 ± 0.21	149.13 ± 16.14	0.08245	2518	*
TfCut1	N -> D	77.79 ± 0.07	210.81 ± 4.01	0.11687	10365	*
TfCut1-SC	N -> D	76.74 ± 0.14	237.79 ± 8.2	0.29323	3017	
TfCut1-SC:ST	N -> D	78.46 ± 0.11	195.97 ± 5.13	0.07377	1553	*
ICCG	N -> D	95.40 ± 0.01	516.35 ± 3.56	0.02153	2285	*
ICCG-SC	N -> D	97.2 ± 0.02	561.15 ± 5.16	0.11353	4200	*
ICCG-SC:ST	N -> D	97.04 ± 0.01	537.29 ± 2.63	0.04563	6283	
IsPETase-SC	N = I -> D	38.62 ± 1.00 48.82 ± 0.05	N/A 223.17 ± 2.85	0.10078	4658	
TfCut1	N = I -> D	66.33 ± 0.14 77.75 ± 0.05	N/A 234.38 ± 2.43	0.08928	14733	
IsPETase-SC	N -> I -> D	46.93 ± 0.05 242.24 ± 785.31	359.55 ± 6.03 0.00 ± 14.8	0.07378	4657	
TfCut1	N -> I -> D	69.96 ± 0.58 76.50 ± 0.11	331.22 ± 19.53 69.96 ± 0.58	0.27457	3014	
TfCut1-SC:ST	N -> I -> D	75.07 ± 0.13 73.08 ± 4.82	296.41 ± 7.88 24.43 ± 16.97	0.03417	1550	
ICCG-SC:ST	N -> I -> D	93.47 ± 0.05 97.08 ± 0.01	494.76 ± 7.62 524.91 ± 1.24	0.0238	6280	*
IsPETase-SC	N -> I_1 -> I_2 -> D	44.54 ± 0.01 46.58 ± 0.05 47.45 ± 0.02	353.54 ± 5.39 338.61 ± 2.28 129.63 ± 7.84	0.3989	4654	*
TfCut1-SC	N -> I_1 -> I_2 -> D	75.84 ± 0.16 73.15 ± 0.17 79.07 ± 0.35	417.10 ± 25.82 382.81 ± 7.27 242.05 ± 10.61	0.27001	3013	*

SI.6. DSC Fits: E_{act} vs T_{act} Plots



Comparison of T_{act} and E_{act} for enzymes and their fusions derived from the best fitting model for thermal denaturation in VSR-DSC. Wildtype enzymes are depicted as squares, enzyme-Spycatcher003 fusions as circles, and enzyme-SpyCatcher003:SpyTag003 as triangles. *Is*PETase (A) is coloured light-blue, *Tf*Cut1 (B) blue, and LCC^{ICCG} (C) dark blue. D shows a comparison between the three PETases and their fusions. Multistage unfolding mechanisms are indicated by N -> I (native to intermediate), I -> I (intermediate to intermediate), and I -> D (intermediate to denatured). Horizontal and vertical error bars show 95% confidence intervals.

SI.7 References

1 D. G. Gibson, L. Young, R. Y. Chuang, J. C. Venter, C. A. Hutchison and H. O. Smith, *Nat Methods*, 2009, **6**, 343–345.

2 J. Jumper, R. Evans, A. Pritzel, T. Green, M. Figurnov, O. Ronneberger, K. Tunyasuvunakool, R. Bates, A. Žídek, A. Potapenko, A. Bridgland, C. Meyer, S. A. A. Kohl, A. J. Ballard, A. Cowie, B. Romera-Paredes, S. Nikolov, R. Jain, J. Adler, T. Back, S. Petersen, D. Reiman, E. Clancy, M. Zielinski, M. Steinegger, M. Pacholska, T. Berghammer, S. Bodenstein, D. Silver, O. Vinyals, A. W. Senior, K. Kavukcuoglu, P. Kohli and D. Hassabis, *Nature*, 2021, **596**, 583–589.

3 M. Mirdita, K. Schütze, Y. Moriwaki, L. Heo, S. Ovchinnikov and M. Steinegger, *Nat Methods*, 2022, **19**, 679–682.

4 S. Mazurenko, J. Stourac, A. Kunka, S. Nedeljković, D. Bednar, Z. Prokop and J. Damborsky, *Nucleic Acids Res*, 2018, **46**, W344–W349.