

## Supplementary Data

### Development of a highly active engineered PETase enzyme for polyester degradation

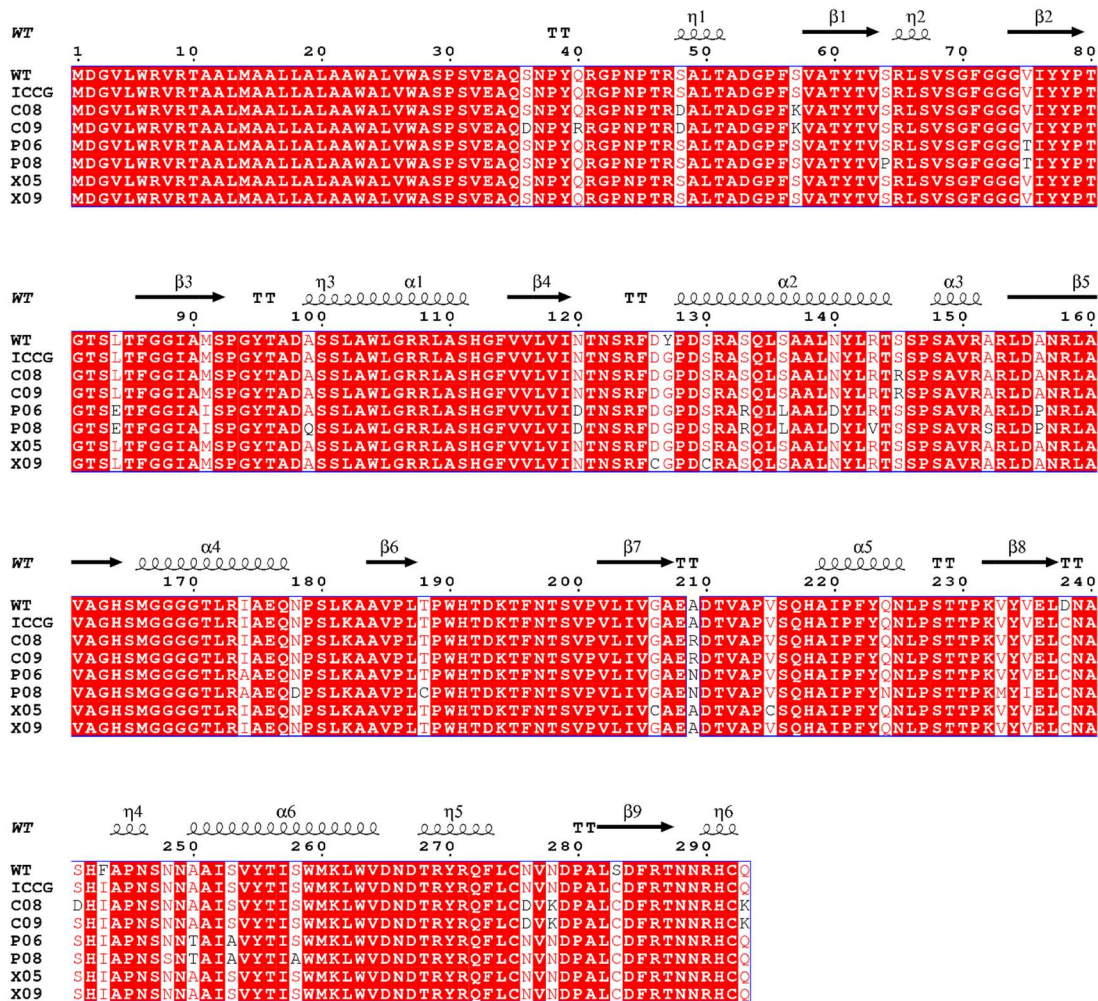
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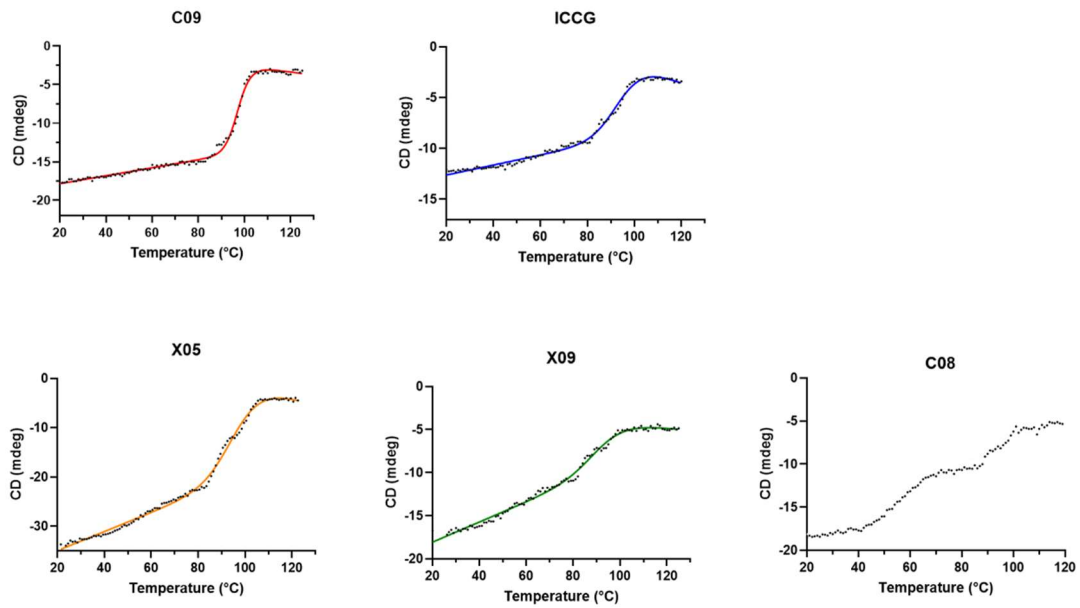
‡ These three authors contributed equally to this work

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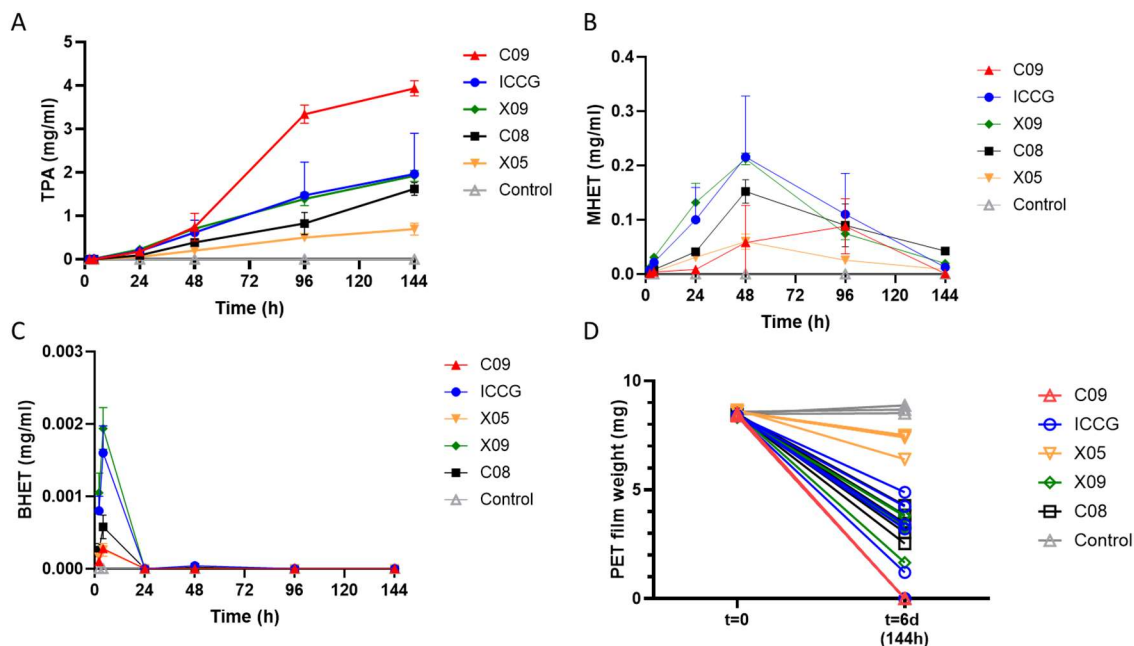


**Figure S1. Sequence alignment.** Sequence alignment of the wild type LCC enzyme (Uniprot ID G9BY57), the ICCG variant developed by Tournier et al., and the engineered enzymes developed in this work (C08, C09, P06, P08, X05, X09). Alignment is performed with Clustal Omega, while the graphical representation is obtained using ESPrit 3 web server.

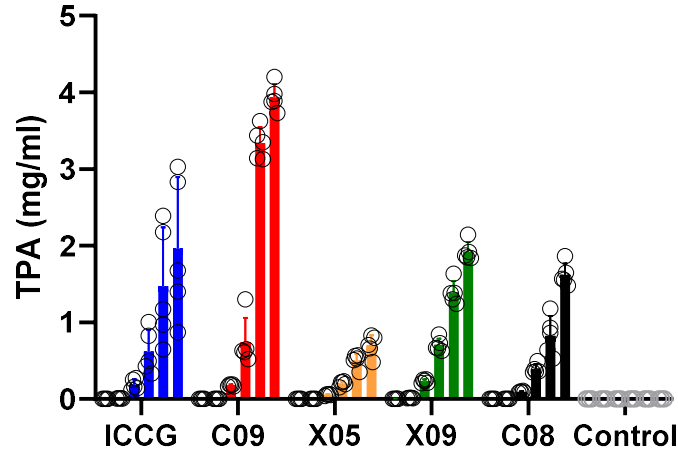
Circular dichroism curves corresponding to the denaturation temperature ( $T_m$ ) for the different mutants, measured from 20 °C to 120 °C at 222 nm.



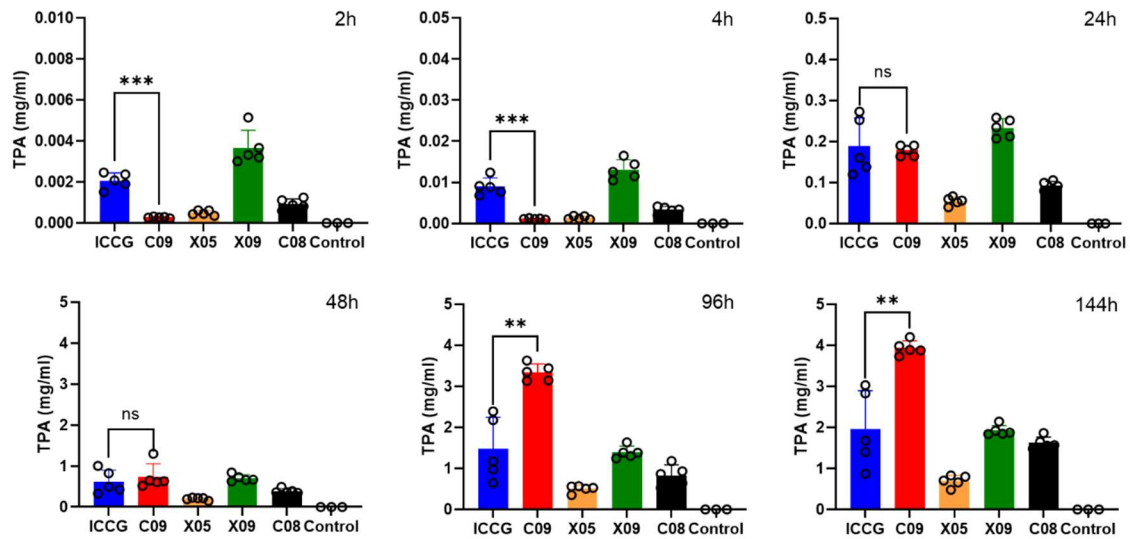
**Figure S2. Representative melting curves.** Each panel show a representative melting curve measured by circular dichroism at 222 nm from 20°C to 120°C for the corresponding sample; black dots represent experimental data points, and the solid line indicates the fitted curve. For sample C08, it was not possible to obtain a reliable fit.



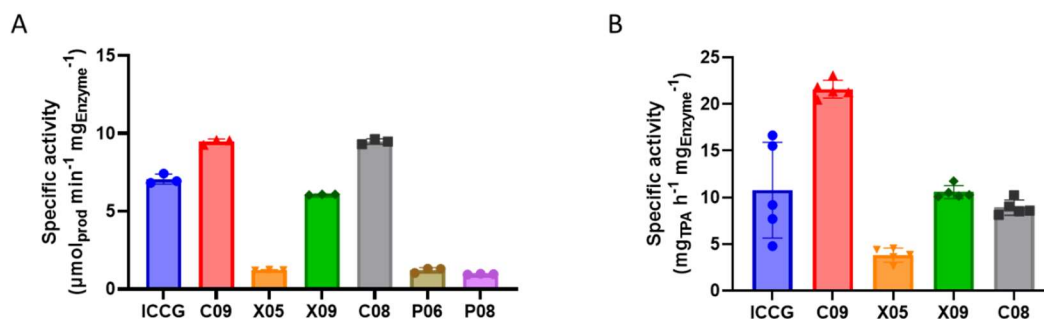
**Figure S3. Product formation and PET film degradation.** A) Terephthalic acid (TPA) production over time quantified by High Performance Liquid Chromatography (HPLC) analysis after reaction with 40 nM enzyme (ICCG – C09 – X05 – X09 – C08 (n=5) – no enzyme (control n=3)). The error bars denote standard deviation. Data points have been reused from Figure 2A B) Bis(2-hydroxyethyl) terephthalate (BHET) production over time quantified by HPLC analysis after reaction with 40 nM enzyme (ICCG – C09 – X05 – X09 – C08 (n=5) – no enzyme (control n=3)). The error bars denote standard deviation. C) MHET production over time quantified by HPLC analysis after reaction with 40 nM enzyme (ICCG – C09 – X05 – X09 – C08 (n=5) – no enzyme (control n=3)). The error bars denote standard deviation. D) PET film weight before (t=0) and after treatment (t=6d, 144h) with 40 nM enzyme (ICCG – C09 – X05 – X09 – C08 (n=5), no enzyme (control n=3)). Mean weight loss for ICCG at 6 days is 61%. Mean weight loss for C09 at 6 days is 100%.



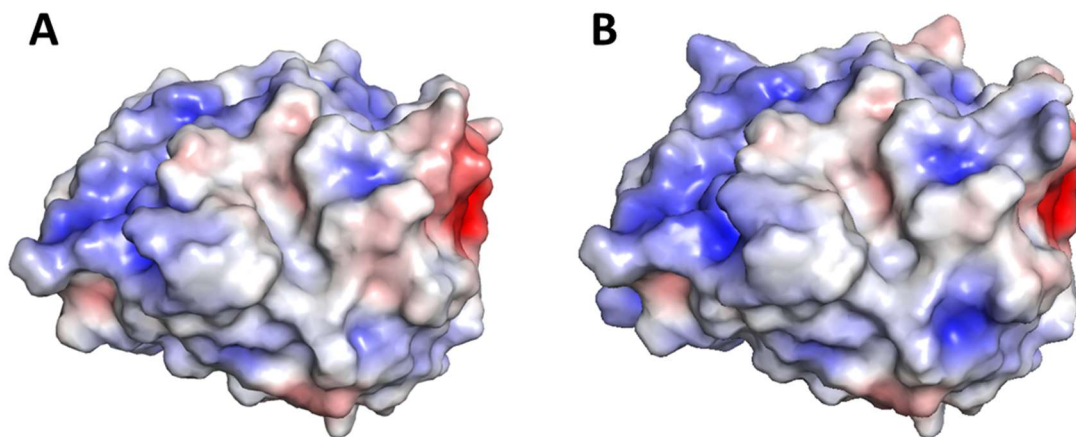
**Figure S4. Time course of the terephthalic acid (TPA) production from different enzymes.** Experiments performed using 40 nM enzyme at 68°C (n=5). Time points: 2h, 4h, 24h, 48h, 96h and 144h. The error bars denote standard deviation. Data points have been reused from Figure 2A.



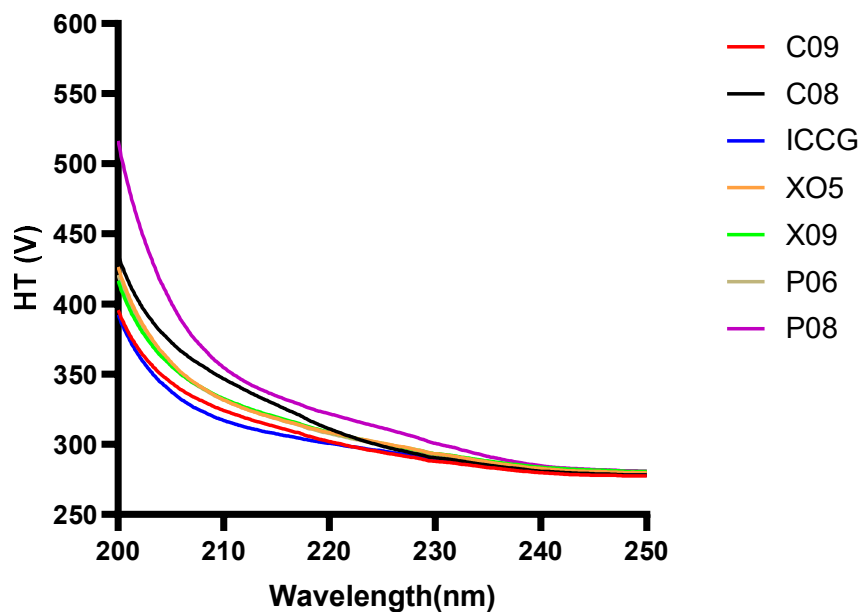
**Figure S5. Terephthalic acid (TPA) production from different enzymes (ICCG – C09 – X05 – X09 – C08 (n=5)) and control (no enzyme (n=3)) at 40 nM, 68°C and at different time points (2h, 4h, 24h, 48h, 96h and 144h).** ns (non significant); \*\*P<0.01; \*\*\*P<0.001 (one-tailed unpaired Welch's t-test). The error bars denote standard deviation. Data points have been reused from Figure 2A and Figure 2B.



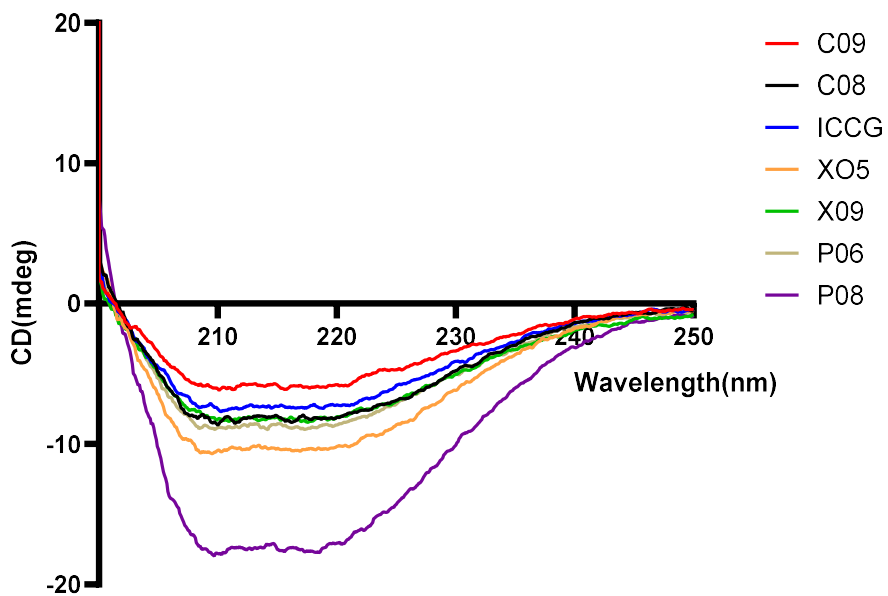
**Figure S6. Comparison of the specific activity of each enzyme variant against p-Nitrophenyl Acetate (pNPA) and polyethylene terephthalate (PET).** A) Esterase activity against pNPA was assessed measuring the absorbance at 405 nm, corresponding to the formation of p-nitrophenolate (prod) at the endpoint of 10.55 min. Reactions were performed in triplicates ( $n=3$ ) in a 96-well microplate at 25°C using 1 mM pNPA in a buffer containing 20 mM Tris-HCl (pH 8.0) and 300 mM NaCl with enzyme concentration in the range of 0.6-0.07  $\mu\text{g}/\text{ml}$  (ICCG = 0.12  $\mu\text{M}$ ; C09 = 0.12  $\mu\text{M}$ ; X05 = 1.06  $\mu\text{M}$ ; X09 = 0.2  $\mu\text{M}$ ; C08 = 0.11  $\mu\text{M}$ ; P06 = 1.04  $\mu\text{M}$ ; P08 = 0.90  $\mu\text{M}$ ). The specific activity was measured as the ratio of  $\mu\text{mol}$  of product produced over time and the mg of enzyme used in the reaction. The error bars denote standard deviation. B) PET hydrolysing specific activity against amorphous PET films. All enzyme reactions were performed at a concentration of 40 nM at 68°C in quintuplicates ( $n=5$ ) in 2.0 mL microcentrifuge tubes in the same reaction buffer (20 mM Tris-HCl pH 8.0, 300 mM NaCl). Amorphous PET films (6 mm circular disks of 8.4 mg weight) were then placed in 2 ml microcentrifuge tubes. The specific activity was measured as the ratio of mg of terephthalic acid (TPA) product produced (measured by High-Performance Liquid Chromatography) over time (endpoint of 144 h) and the mg of enzyme used in the reaction. The error bars denote standard deviation. Data points at 144h have been reused from Figure 2C.



**Figure S7. Electrostatic potential surfaces, calculated by the Adaptive Poisson-Boltzmann Solver module as implemented in PyMol for ICCG (A) and C09 (B).** Red spheres indicate negative charges, while blue spheres represent positive charges. The positively and negatively charged surfaces are colored blue and red, respectively, and the electro-neutral (or nonpolar/hydrophobic) surface is shown in white. The catalytic cleft is shown at the center of the two images. C09 features a more positively charged surface than ICCG. Figures are generated with PyMol.



**Figure S8. Secondary structure measurements by circular dichroism.** The plot shows voltage vs. wavelength for the different enzymes.



**Figure S9. Secondary structure measurements by circular dichroism.** The plot shows ellipticity vs. wavelength for the different enzymes.

**Table S1. Production yields of the recombinant enzyme variants.**

Enzyme	Production yield (mg/L)
C09	18
X05	20
X09	22
C08	18
P06	12
P08	15
ICCG	20

**Table S2. Experimental and predicted secondary structures for the C09 enzyme.**

Secondary Structures	Crystal structure (8CMV)	Circular Dichroism (Dichroweb)
Helices	0.38	0.34
Sheets	0.21	0.20
Loops	0.40	0.47

**Table S3. Terephthalic acid (TPA) production as measured by High-Performance Liquid Chromatography.** Quantification of TPA (mg/mL) in reaction supernatants represented in Figure 2A. Reactions were carried out using 40 nM enzyme at 68°C.

Enzyme (replicate #)	Time, h					
	2	4	24	48	96	144
<b>C09(1)</b>	0.00023	0.0009	0.16	0.52	3.14	3.73
<b>C09(2)</b>	0.00028	0.0011	0.16	1.30	3.63	3.88
<b>C09(3)</b>	0.00028	0.0011	0.18	0.65	3.35	4.20
<b>C09(4)</b>	0.00032	0.0013	0.19	0.63	3.44	3.98
<b>C09(5)</b>	0.00026	0.0012	0.19	0.61	3.13	3.89
<b>X05(1)</b>	0.00043	0.0011	0.060	0.23	0.57	0.83
<b>X05(2)</b>	0.00062	0.0019	0.056	0.22	0.56	0.80
<b>X05(3)</b>	0.00046	0.0012	0.052	0.20	0.50	0.66
<b>X05(4)</b>	0.00061	0.0018	0.066	0.22	0.53	0.71
<b>X05(5)</b>	0.00035	0.0010	0.040	0.15	0.35	0.48
<b>X09(1)</b>	0.0033	0.0125	0.26	0.84	1.64	2.14
<b>X09(2)</b>	0.0032	0.0115	0.21	0.66	1.38	1.84
<b>X09(3)</b>	0.0051	0.0165	0.25	0.73	1.24	1.92
<b>X09(4)</b>	0.0030	0.0105	0.21	0.63	1.30	1.85
<b>X09(5)</b>	0.0037	0.0144	0.23	0.67	1.38	1.87
<b>ICCG(1)</b>	0.0025	0.0124	0.25	0.83	2.18	2.83
<b>ICCG(2)</b>	0.0021	0.0091	0.27	1.01	2.39	3.03
<b>ICCG(3)</b>	0.0024	0.0089	0.16	0.50	1.17	1.68
<b>ICCG(4)</b>	0.0019	0.0077	0.12	0.33	0.65	0.87
<b>ICCG(5)</b>	0.0015	0.0068	0.14	0.43	0.97	1.40
<b>C08(1)</b>	0.0009	0.0020	0.079	0.35	1.18	1.87
<b>C08(2)</b>	0.0008	0.0034	0.084	0.36	0.93	1.57
<b>C08(3)</b>	0.0012	0.0042	0.091	0.50	0.86	1.48
<b>C08(4)</b>	0.0011	0.0039	0.106	0.39	0.64	1.65
<b>C08(5)</b>	0.0006	0.0032	0.099	0.36	0.53	1.56
<b>Control(1)</b>	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
<b>Control(2)</b>	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
<b>Control(3)</b>	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD

< LOQ - below the lowest analyte concentration that can be quantitatively detected with a stated accuracy and precision;

< LOD - no peak found

**Table S4. Mono(hydroxyethyl)terephthalate (MHET) production as measured by High-Performance Liquid Chromatography.** Quantification of MHET (mg/mL) in reaction supernatants. Reactions were carried out using 40 nM enzyme at 68°C.

Enzyme (replicate #)	Time, h					
	2	4	24	48	96	144
<b>C09(1)</b>	0.0011	0.0032	0.008	0.02	0.14	0.0005
<b>C09(2)</b>	0.0013	0.0037	0.008	0.18	0.01	0.0004
<b>C09(3)</b>	0.0014	0.0035	0.010	0.03	0.07	0.0003
<b>C09(4)</b>	0.0016	0.0041	0.009	0.03	0.12	0.0007
<b>C09(5)</b>	0.0014	0.0039	0.007	0.03	0.10	0.0024
<b>X05(1)</b>	0.0015	0.0034	0.035	0.08	0.033	0.011
<b>X05(2)</b>	0.0025	0.0055	0.030	0.06	0.029	0.010
<b>X05(3)</b>	0.0017	0.0034	0.032	0.06	0.026	0.008
<b>X05(4)</b>	0.0026	0.0056	0.035	0.06	0.026	0.008
<b>X05(5)</b>	0.0015	0.0034	0.022	0.04	0.014	0.005
<b>X09(1)</b>	0.011	0.030	0.19	0.22	0.093	0.023
<b>X09(2)</b>	0.011	0.028	0.11	0.22	0.074	0.019
<b>X09(3)</b>	0.017	0.037	0.14	0.22	0.063	0.018
<b>X09(4)</b>	0.010	0.025	0.10	0.20	0.073	0.020
<b>X09(5)</b>	0.012	0.034	0.12	0.20	0.070	0.017
<b>ICCG(1)</b>	0.0076	0.028	0.17	0.30	0.18	0.0101
<b>ICCG(2)</b>	0.0087	0.019	0.16	0.36	0.20	0.0082
<b>ICCG(3)</b>	0.0090	0.022	0.06	0.19	0.08	0.0203
<b>ICCG(4)</b>	0.0054	0.020	0.05	0.09	0.03	0.0063
<b>ICCG(5)</b>	0.0066	0.019	0.06	0.14	0.06	0.0184
<b>C08(1)</b>	0.0019	0.0050	0.044	0.14	0.15	0.0472
<b>C08(2)</b>	0.0037	0.0087	0.034	0.15	0.10	0.0429
<b>C08(3)</b>	0.0041	0.0101	0.039	0.19	0.09	0.0388
<b>C08(4)</b>	0.0029	0.0097	0.046	0.14	0.06	0.0434
<b>C08(5)</b>	0.0031	0.0081	0.043	0.14	0.05	0.0401
<b>Control(1)</b>	< LOD	<LOD	< LOD	<LOD	< LOD	<LOD
<b>Control(2)</b>	< LOD	<LOD	< LOD	<LOD	< LOD	<LOD
<b>Control(3)</b>	< LOD	<LOD	< LOD	<LOD	< LOD	<LOD

< LOQ - below the lowest analyte concentration that can be quantitatively detected with a stated accuracy and precision;

< LOD - no peak found

**Table S5. Bis(hydroxyethyl)terephthalate (BHET) production as measured by High-Performance Liquid Chromatography.** Quantification of BHET (mg/mL) in reaction supernatants. Reactions were carried out using 40 nM enzyme at 68°C.

Enzyme (replicate #)	Time, h					
	2	4	24	48	96	144
<b>C09(1)</b>	0.0001	0.0002	<LOD	<LOD	<LOQ	<LOD
<b>C09(2)</b>	0.0001	0.0003	<LOD	<LOQ	<LOD	<LOD
<b>C09(3)</b>	0.0001	0.0003	<LOD	<LOD	<LOD	<LOD
<b>C09(4)</b>	0.0001	0.0003	<LOD	<LOD	<LOD	<LOD
<b>C09(5)</b>	0.0001	0.0003	<LOD	<LOD	<LOD	<LOD
<b>X05(1)</b>	0.00011	0.0002	<LOQ	<LOQ	<LOQ	<LOQ
<b>X05(2)</b>	0.00020	0.0003	<LOQ	<LOQ	<LOQ	<LOD
<b>X05(3)</b>	0.00014	0.0002	<LOQ	<LOQ	<LOQ	<LOQ
<b>X05(4)</b>	0.00022	0.0004	<LOQ	<LOQ	<LOQ	<LOQ
<b>X05(5)</b>	0.00012	0.0002	<LOQ	<LOQ	<LOQ	<LOD
<b>X09(1)</b>	0.00092	0.0021	<LOQ	<LOQ	<LOQ	<LOD
<b>X09(2)</b>	0.00091	0.0018	<LOQ	<LOQ	<LOQ	<LOQ
<b>X09(3)</b>	0.00151	0.0022	<LOQ	<LOQ	<LOQ	<LOQ
<b>X09(4)</b>	0.00083	0.0015	<LOQ	<LOQ	<LOQ	<LOD
<b>X09(5)</b>	0.00107	0.0021	<LOQ	<LOQ	<LOQ	<LOD
<b>ICCG(1)</b>	0.00077	0.0020	<LOQ	0.0001	<LOQ	<LOD
<b>ICCG(2)</b>	0.00100	0.0010	<LOQ	<LOQ	<LOQ	<LOD
<b>ICCG(3)</b>	0.00096	0.0018	<LOQ	0.0001	<LOQ	<LOQ
<b>ICCG(4)</b>	0.00049	0.0016	<LOQ	<LOQ	<LOQ	<LOD
<b>ICCG(5)</b>	0.00078	0.0016	<LOQ	<LOQ	<LOQ	<LOD
<b>C08(1)</b>	0.00012	0.0003	<LOQ	<LOQ	<LOQ	<LOQ
<b>C08(2)</b>	0.00033	0.0006	<LOQ	<LOQ	<LOQ	<LOQ
<b>C08(3)</b>	0.00035	0.0007	<LOQ	0.0001	<LOQ	<LOQ
<b>C08(4)</b>	0.00024	0.0007	<LOQ	<LOQ	<LOQ	<LOQ
<b>C08(5)</b>	0.00025	0.0006	<LOQ	<LOQ	<LOQ	<LOQ
<b>Control(1)</b>	< LOD	<LOD	< LOD	<LOD	< LOD	<LOD
<b>Control(2)</b>	< LOD	<LOD	< LOD	<LOD	< LOD	<LOD
<b>Control(3)</b>	< LOD	<LOD	< LOD	<LOD	< LOD	<LOD

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 < LOD - no peak found

**Table S6. Diffraction data collection and refinement statistics.** Statistics for the highest-resolution shell are shown in parentheses.

<b>Structure</b>	<b>LCC-ICCG-C09</b>
<b>PDB code</b>	8CMV
<b>Wavelength (Å)</b>	0.7338
<b>Space group</b>	P6 <sub>3</sub>
<b>Unit cell axes (Å)</b>	a=108.876, b=108.876, c=35.402,
<b>Unit cell angles (°)</b>	$\alpha = 90.00, \beta = 90.00, \gamma = 120.00$
<b>Resolution range (Å)</b>	54.497-1.280 (1.302-1.280)
<b>Total reflections</b>	1293458(62613)
<b>Unique reflections</b>	62178(3088)
<b>Multiplicity</b>	20.8(20.3)
<b>Completeness (%)</b>	100.0 (100.0)
<b>mean(I) / sig(I)</b>	6.5 (0.3)
<b>Wilson B-factor</b>	15.27
<b>Rmerge</b>	0.294 (13.691)
<b>Rmeas</b>	0.302 (14.041)
<b>Rpim</b>	0.066 (3.103)
<b>CChalf</b>	0.998 (0.346)
<b>Reflections in refinement</b>	62017 (6053)
<b>Reflections in free set</b>	3153 (317)
<b>Rwork</b>	0.1634
<b>Rfree</b>	0.1871
<b>RMSD bonds (Å)</b>	0.012
<b>RMSD angles (°)</b>	1.77
<b>Ramachandran favoured (%)</b>	98.44
<b>Ramachandran allowed (%)</b>	1.56
<b>Ramachandran outliers (%)</b>	0
<b>Rotamer outliers (%)</b>	0.9
<b>Clash score</b>	5.65
<b>Overall number of atoms (non-H)</b>	2286
<b>in macromolecules</b>	2010
<b>in ligands</b>	28
<b>in solvent</b>	248
<b>Average B-factor (Å<sup>2</sup>)</b>	16.14
<b>for macromolecules</b>	14.3
<b>for ligands</b>	37.33
<b>for solvent</b>	28.7