Supporting Information (SI)

Polymorphism Control of Polyethylene Terephthalate (PET) Degradation Product via Mechanochemistry Leads to Accelerated Microbial Degradation

Deepika Shingwekar,[a] Nicholas Lutz,[a] Delbert S. Botes,[c] Elani J. Cabrera-Vega,[d] Gonzalo Campillo-Alvarado,[a,*] Jay L. Mellies,[b,*] Jesus Daniel Loya[a,*]

[a] Department of Chemistry, Reed College, Portland, OR 97202-8199 USA
[b] Department of Biology, Reed College, Portland, OR 97202-8199 USA
[c] Department of Chemistry, University of Missouri, Columbia, MO 65211, USA
[d] Department of Chemistry, Portland State University, Portland, OR, 97201, USA

Jesus Daniel Loya, E-mail: jdloya@reed.edu
Jay L. Mellies, E-mail: mmelliesj@reed.edu
Gonzalo Campillo-Alvarado, E-mail: gcampillo@reed.edu

Supplementary Information

S1. Experimental Methods

Materials:
Bis(2-hydroxyethyl) terephthalate (**BHET**) and solvents used for crystallization were purchased from Sigma-Aldrich. All reagents and solvents were used as received.

Synthesis of α and δ form via slow evaporation
Slow evaporation was utilized to synthesize the **α** and **δ** forms from commercially available **BHET** and experimentally synthesized **BHET** mixture from the glycolysis reaction (**Table S1**). General procedure for the different polymorphs synthesis is as follows: **BHET** (20 mg, 0.0787 mmol) was dissolved in 2 mL of solvent in a 20 mL scintillation vial, and the solvent was allowed to evaporate at room temperature or at 4°C. After 7 days, thin flat crystals suitable to screen with SCXRD formed to determine polymorphic form.

Table S1. Solvent slow evaporation experiments for **BHET** polymorph synthesis

Trial	Starting Material	Solvent	Temperature (ºC)	Result
1	Commercial **BHET**	Chloroform	25	**α** form
2	Commercial **BHET**	Methanol	25	**α** form
3	Commercial **BHET**	Acetone	25	**α** form
4	Commercial **BHET**	DCM	25	**α** form
5	Commercial **BHET**	Acetonitrile	25	**α** form
6	Commercial **BHET**	Methanol	4	**δ** form
7	**BHET** Glycolysis Product	Chloroform	25	**α** form
8	**BHET** Glycolysis Product	Methanol	4	**δ** form

Neat Milling Experiments
Experiments were conducted using a Retsch Mixer Mill MM 400 ball mill, in 10 mL stainless steel milling jars with two 7 mm diameter stainless steel milling balls acquired from Retch. 500 mg of the **BHET** mixture glycolysis product or commercial **BHET** was added to the milling jar. The samples were milled at 1500 rpm for periods ranging from 15 to 90 minutes. Experimental conditions with results are reported in **Table S2**.

Liquid Assisted Grinding (LAG) Experiments

Interconversion between **BHET** polymorphs was achieved through mechanochemistry. Experiments were conducted in 10 mL stainless steel milling jars with two 7 mm diameter stainless steel milling balls acquired from Retch. 500 mg of the **BHET** mixture glycolysis product or commercial **BHET** and 150 µL of solvent were added into the milling jar. The mixtures were milled at 1500 rpm for periods ranging from 5 to 210 minutes. Experimental conditions with results are reported in **Table S2**.

Table S2. Mechanochemical Experiments for **BHET** Polymorph Synthesis and Interconversion.

Starting Material	Reaction Conditions	Result
BHET Glycolysis Product	No solvent, 1500 RPM, 15 minutes	**α** and **δ** forms
BHET Glycolysis Product	No solvent, 1500 RPM, 60 minutes	**α** and **δ** forms
BHET Glycolysis Product	No solvent, 1500 RPM, 90 minutes	**α** form
BHET Glycolysis Product	Chloroform, 1500 RPM, 5 minutes	**α** and **δ** forms
BHET Glycolysis Product	Chloroform, 1500 RPM, 10 minutes	**α** and **δ** forms
BHET Glycolysis Product	Chloroform, 1500 RPM, 15 minutes	**α** and **δ** forms
BHET Glycolysis Product	Chloroform, 1500 RPM, 30 minutes	**α** and **δ** forms
BHET Glycolysis Product	Chloroform, 1500 RPM, 60 minutes	**α** form
BHET Glycolysis Product	MeOH, 1500 RPM, 15 minutes	**α** and **δ** forms
BHET Glycolysis Product	MeOH, 1500 RPM, 90 minutes	**δ** form
BHET Glycolysis Product	MeOH, 1500 RPM, 120 minutes	**δ** form
δ form powder	Chloroform, 1500 RPM, 60 minutes	**α** form
α form powder	MeOH, 1500 RPM, 210 minutes	**δ** form
Commercial **BHET**	No solvent, 1500 RPM, 90 minutes	**α** form
Commercial **BHET**	MeOH, 1500 RPM, 210 minutes	**δ** form

Heating Stage Interconversion

A Linkam LTS420 heating stage was utilized to determine if there was a solid-state transition between the two polymorphs. **δ** form powder (20 mg) was added to a glass microscope slide, loaded into a heating stage, and heated to 107ºC at a rate of 20ºC/min. The sample was left at 107ºC for 30 minutes, before cooling by leaving the sample on the bench for 12 hours at 25ºC. The sample was analyzed using PXRD.

Instrumentation

Fourier transform infrared spectroscopy (FTIR) spectroscopy

FTIR spectra were obtained using a Thermo iS5 FT-IR Spectrometer with an iD7 attenuated total reflectance (ATR) accessory. Samples were ground with a mortar and pestle before analysis, if not already milled using a ball mill. Data were visualized using Graph Pad Prism version 10.1.1.

Dynamic Light Scattering (DLS)

Size distributions of **BHET** polymorphs were determined by dynamic light scattering (DLS) using a Malvern Nano ZSP (Malvern Panalytical, Malvern, UK). Measurements (10 scans) were performed from each sample (**α** or **δ** form) suspended in methanol (3 mg of solid in 1 mL).

Biodegradation Methods:

BHET biodegradation was conducted using a consortium of 5 bacterial strains first reported by León-Zayas et al., 2019, including two *Bacillus* and three *Pseudomonas* spp.[1] Bacterial cultures of the full consortium consisted of 0.6% (w/v) either the **α** or **δ** form of **BHET**, 0.05% (w/v) yeast extract, and 10 mL Liquid Carbon-Free Basal Media (LCFBM) (0.1% (w/v) $FeSO_4 \cdot 7\ H_2O$, 0.1% (w/v) $MgSO_4 \cdot 7\ H_2O$, 0.01% (w/v) $CuSO_4 \cdot 5\ H_2O$, 0.01% (w/v) $MnSO_4 \cdot 5\ H_2O$, and 0.01% (w/v) $ZnSO_4 \cdot 7\ H_2O$, 0.2% (w/v) $(NH_4)_2SO_4$, 1.38% (w/v) NaH_2OPO_4, pH 7.4) in 18 mm by 150 mm glass bacterial culture tubes. The experimental cultures were inoculated with 1% (v/v) of normalized OD_{600} consortium initially and reinoculated with the same amount of the normalized consortium at the 3-week time point. The consortium contained equal quantities of each of the five bacterial strains. Experimental samples were inoculated with the consortium created in triplicate, along with an equal number of negative controls containing no bacteria. Cultures were incubated at 30 °C standing in the dark. Three experimental replicates and three negative controls were removed from incubation each week, over a total of 4 weeks for testing with High-Performance Liquid Chromatography (HPLC) quantitative analysis.

Statistical Analysis

Statistical analyses were performed using RStudio analytical software to determine if there is a significant difference in the level of biodegradation and hydrolysis of **BHET** between the alpha and delta polymorphs by the bacterial consortium. Following **BHET** concentration calculations completed using Equation (4), concentration values for all experimental replicates were normalized against the **BHET** concentration from negative control samples. Each control (3 total) was subtracted from each biological replicate (3 total per polymorph) to generate 9 total normalized values per polymorph per time point. After both polymorph biodegradation measurements were normalized, a Dunnett's test was performed to determine the presence or absence of statistical significance of biodegradation and hydrolysis levels between the **α** and **δ** polymorphs. P-values were recorded for later analysis, utilizing a 95% confidence interval.

S2. NMR spectra of polymorphs

1-D ^{1}H-NMR experiments were recorded using a 400 MHz Bruker Avance II spectrometer. Approximately 25 mg of product was dissolved in 600 µL of DMSO-d6 and filtered through a 0.22 µm PES syringe filter to create each sample. The chemical shifts were compared to the BHET reference spectra found in Ghaemy et al., 2005.[2]

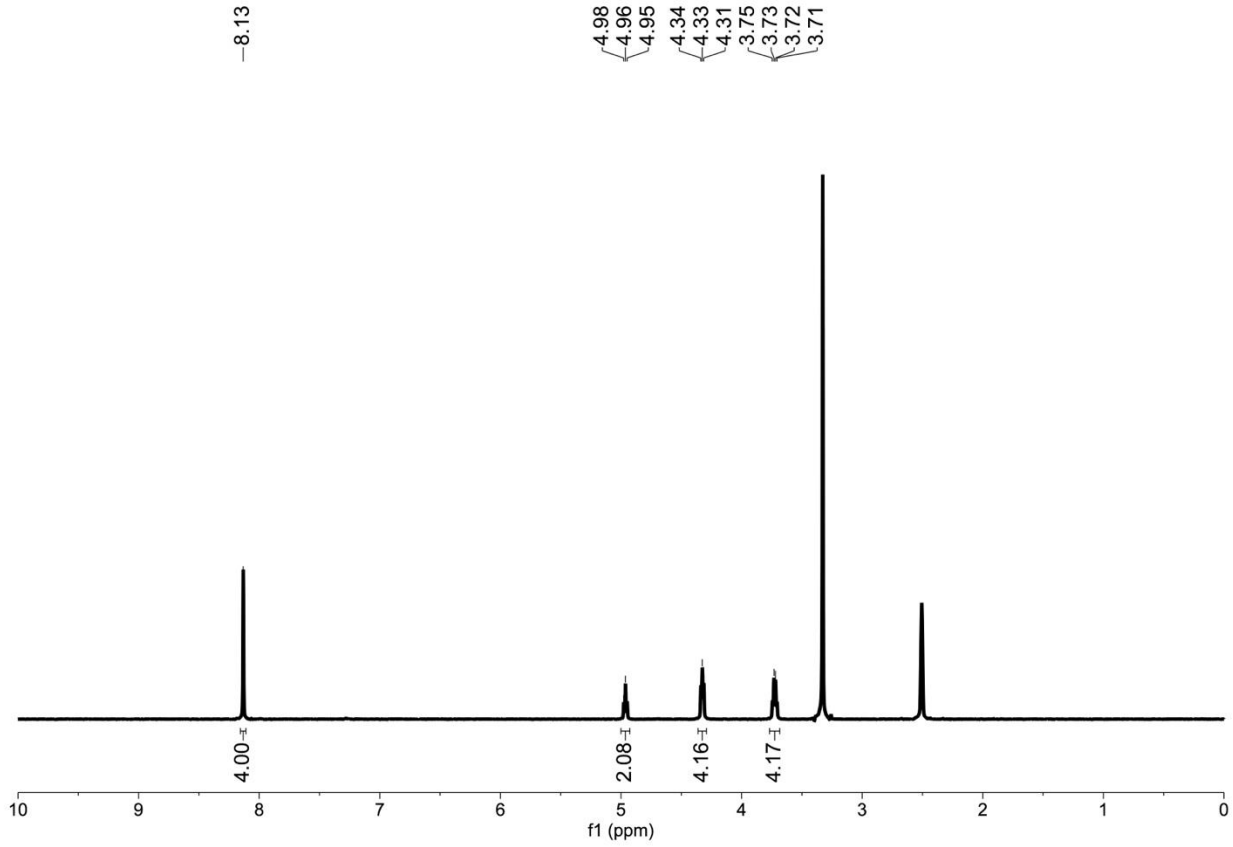

Figure S1. ^{1}H NMR full spectrum from 10 to 0 ppm of **α** form of **BHET**. The spectrum is referenced to DMSO-d6. A water impurity can be seen at 3.4 ppm.

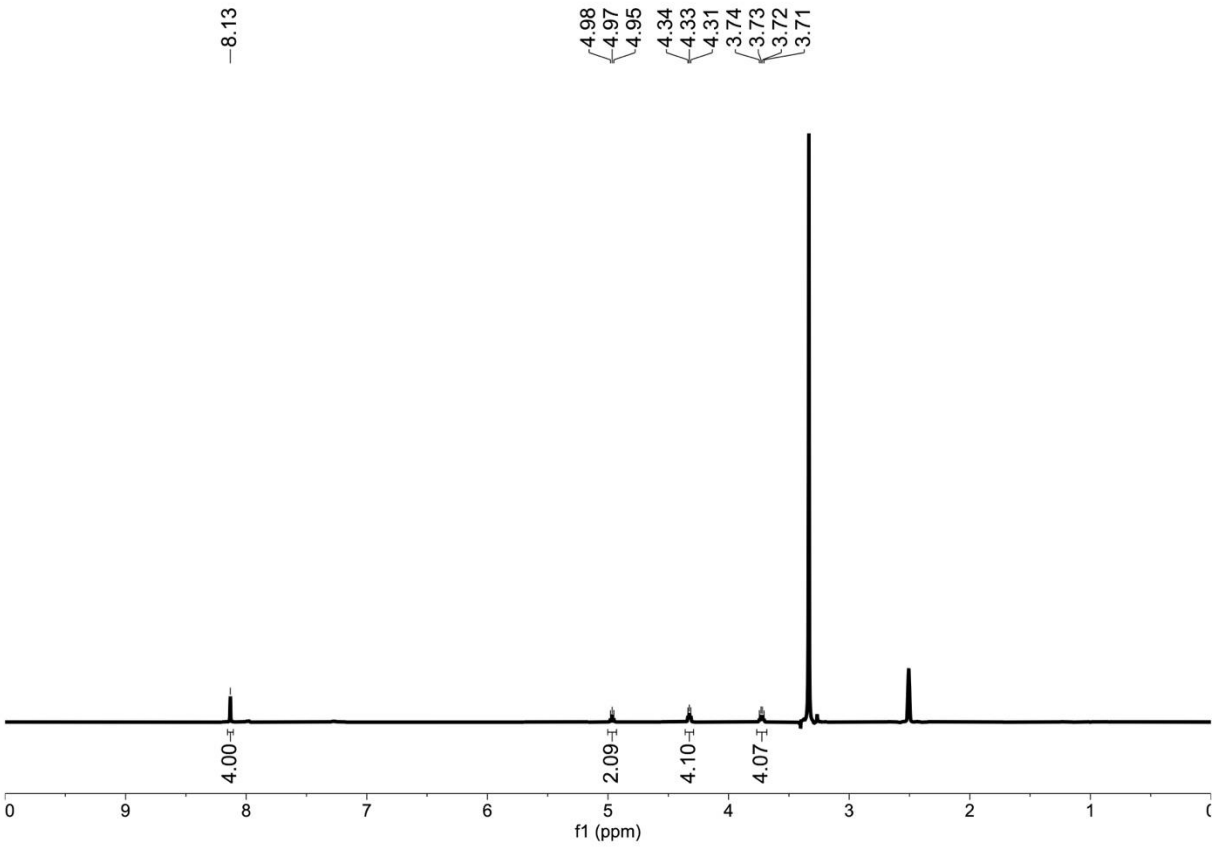

Figure S2. ^{1}H NMR full spectrum from 10 to 0 ppm of **δ** form of **BHET**. The spectrum is referenced to DMSO-d6. A water impurity can be seen at 3.4 ppm.

S3. Powder X-ray Diffraction (PXRD) Data

Powder X-ray diffraction was used to determine the composition of the **BHET** polymorphs. PXRD spectra were obtained with a Scintag XDS-2000 powder X-ray diffractometer using Cu Kα irradiation (λ = 0.154 nm) operated at -40kV and 30 mA in rt conditions. Triplicate patterns of each sample were taken and averaged to reduce signal noise. Data analysis was performed on the Match! software version 14.1.25024 and visualized using GraphPad Prism 10.1.1.[3]

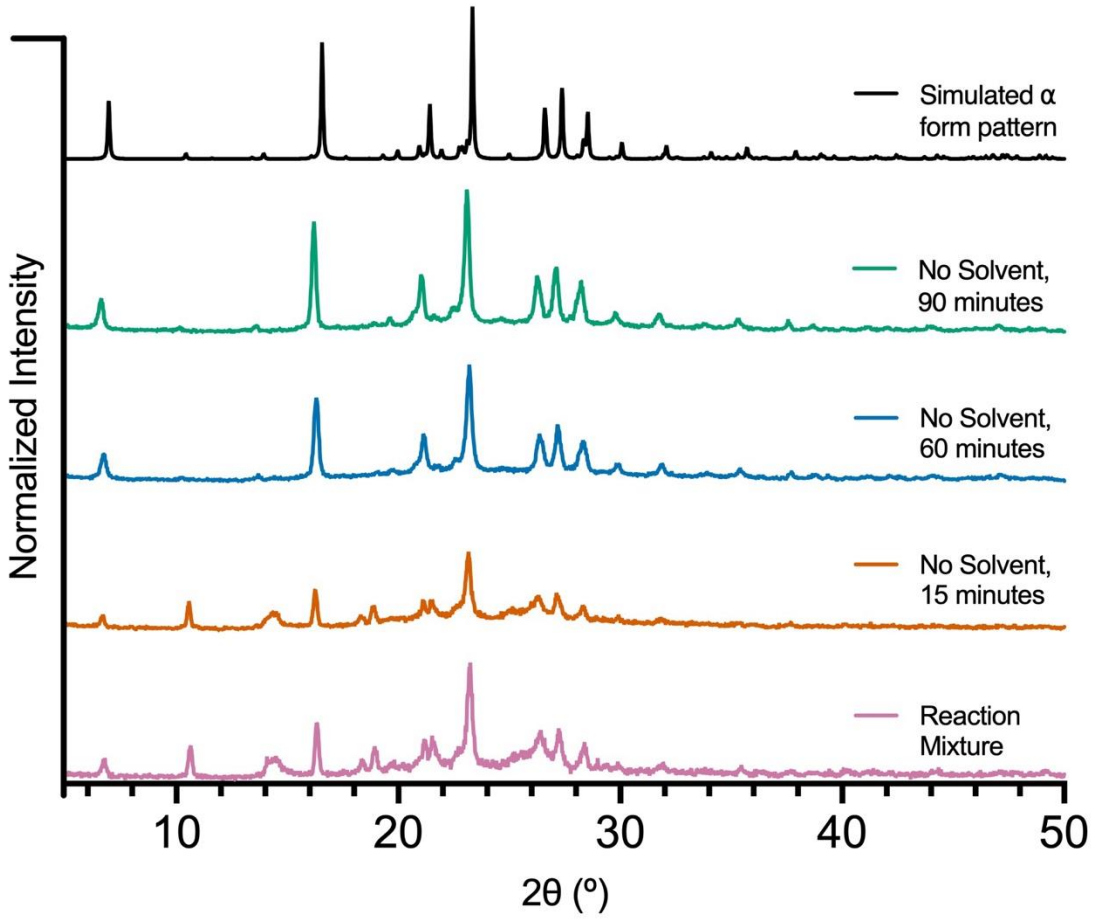

Figure S3. Powder X-ray diffraction patterns of neat milling experiments. All experiments used the **BHET** glycolysis product, a mixture of both **α** and **δ** polymorphs, as the starting material.

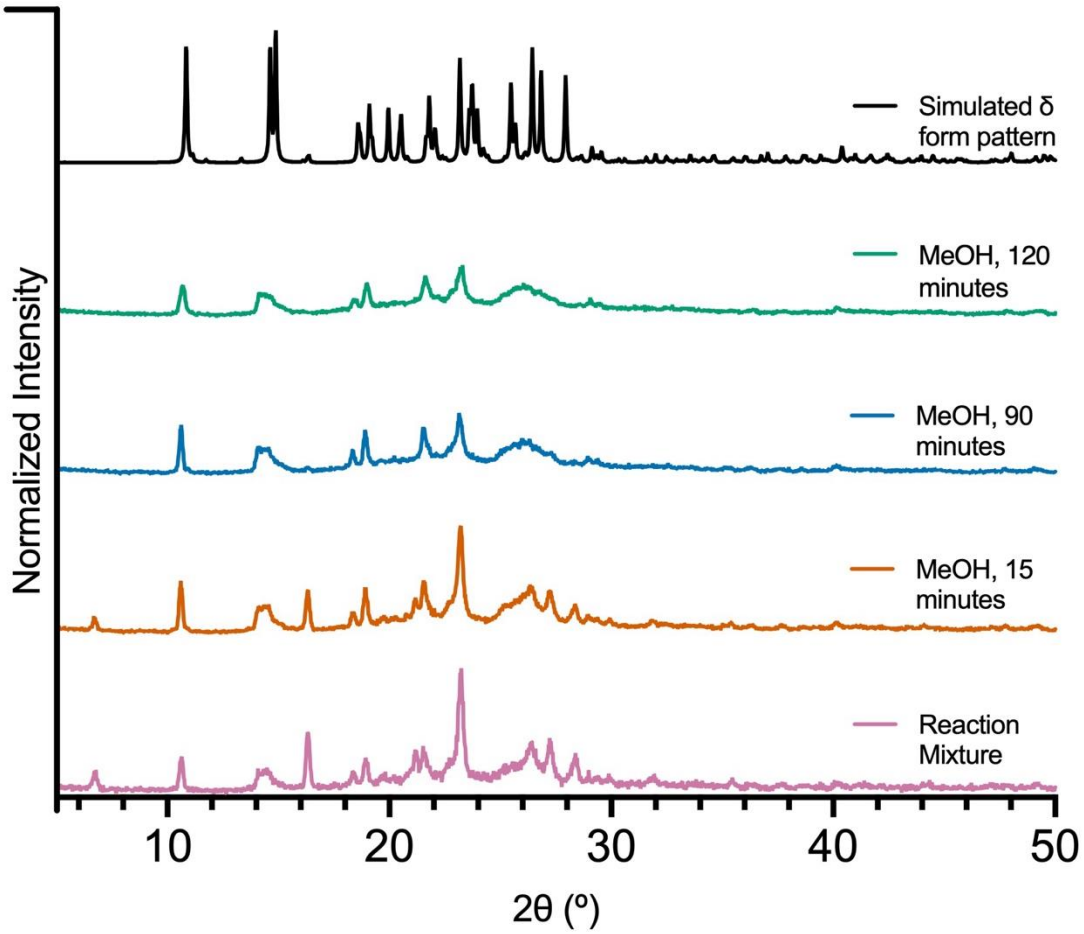

Figure S4. Powder X-ray diffraction patterns of LAG mechanochemistry experiments using MeOH as the solvent. All experiments used the **BHET** glycolysis product, a mixture of both **α** and **δ** polymorphs, as the starting material.

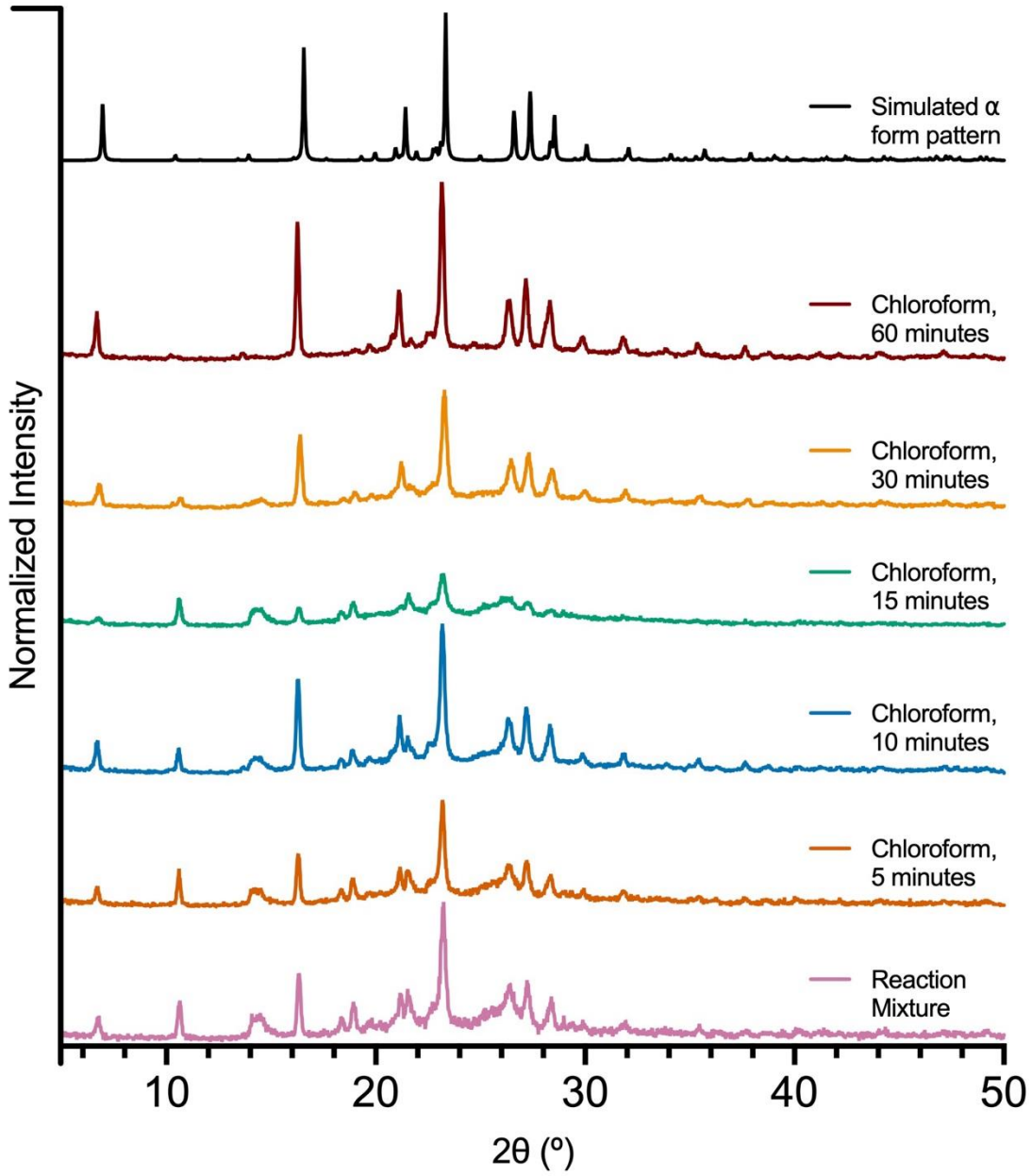

Figure S5. Powder X-ray diffraction patterns of LAG mechanochemistry experiments using chloroform. All experiments used the **BHET** glycolysis product, a mixture of both **α** and **δ** polymorphs, as the starting material.

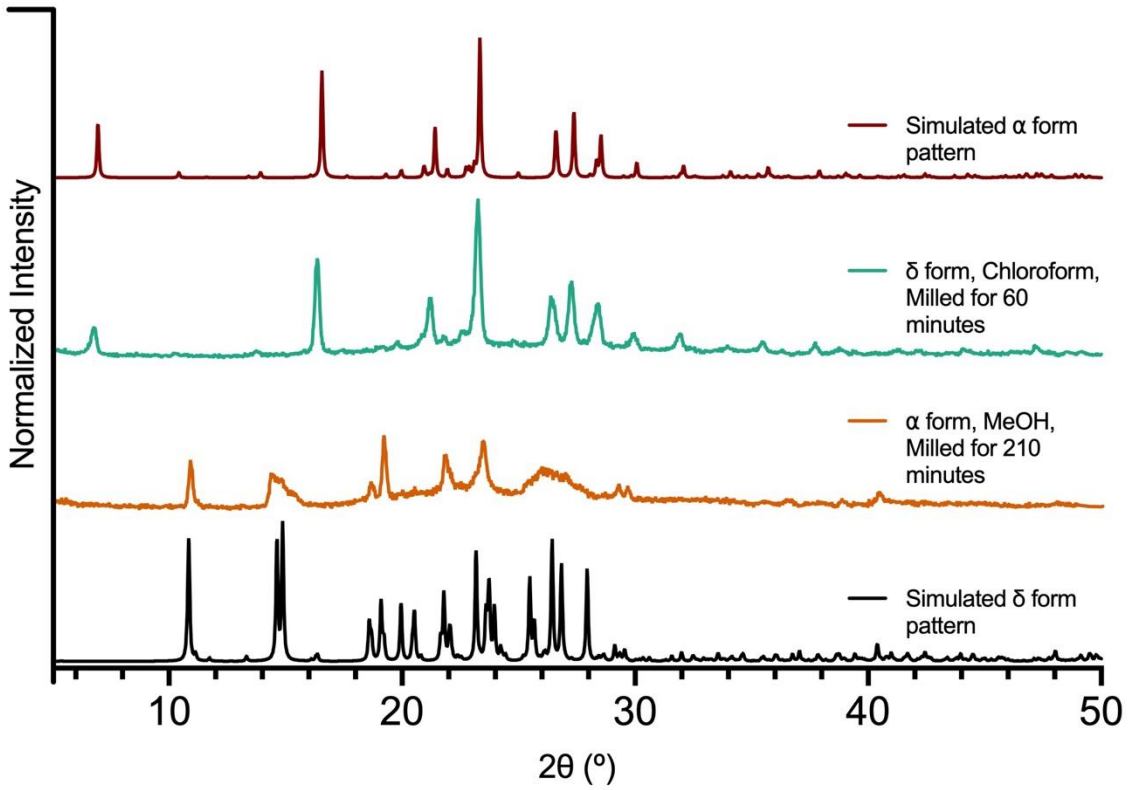

Figure S6. Powder X-ray diffraction patterns of mechanochemical polymorph interconversion.

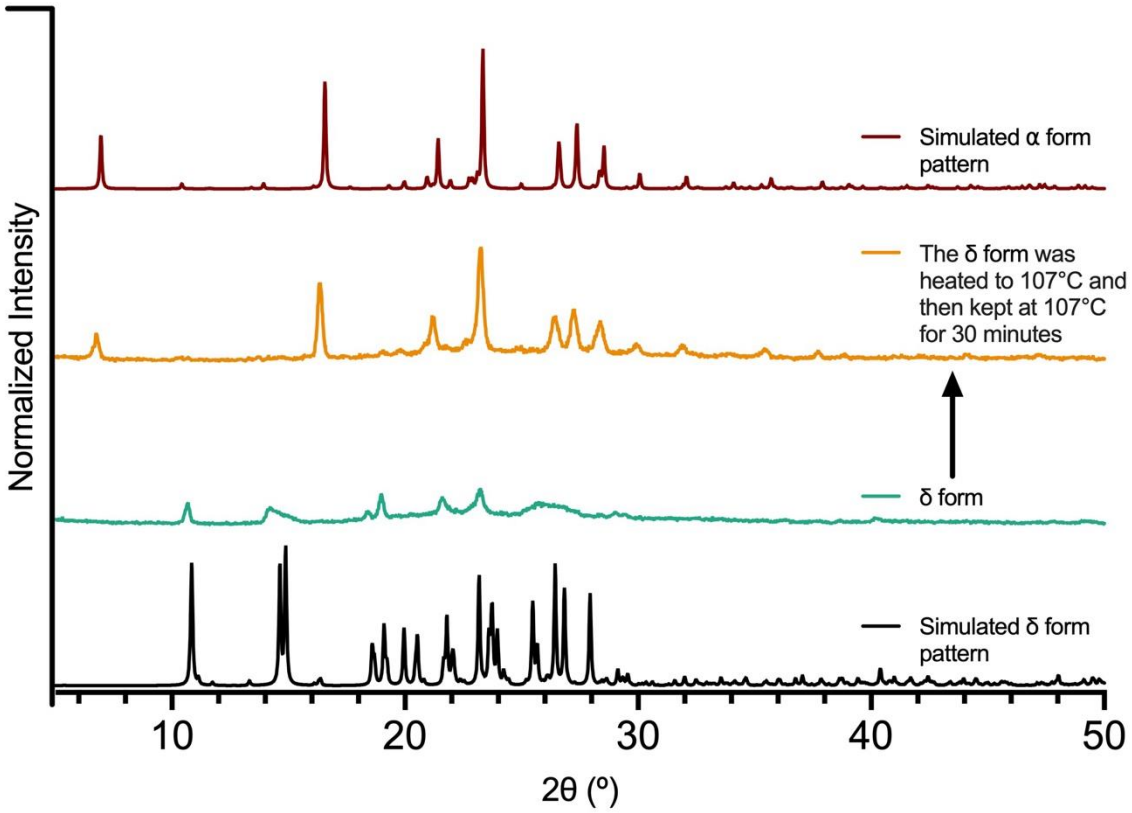

Figure S7. Powder X-ray diffraction patterns of temperature-dependent polymorph interconversion.

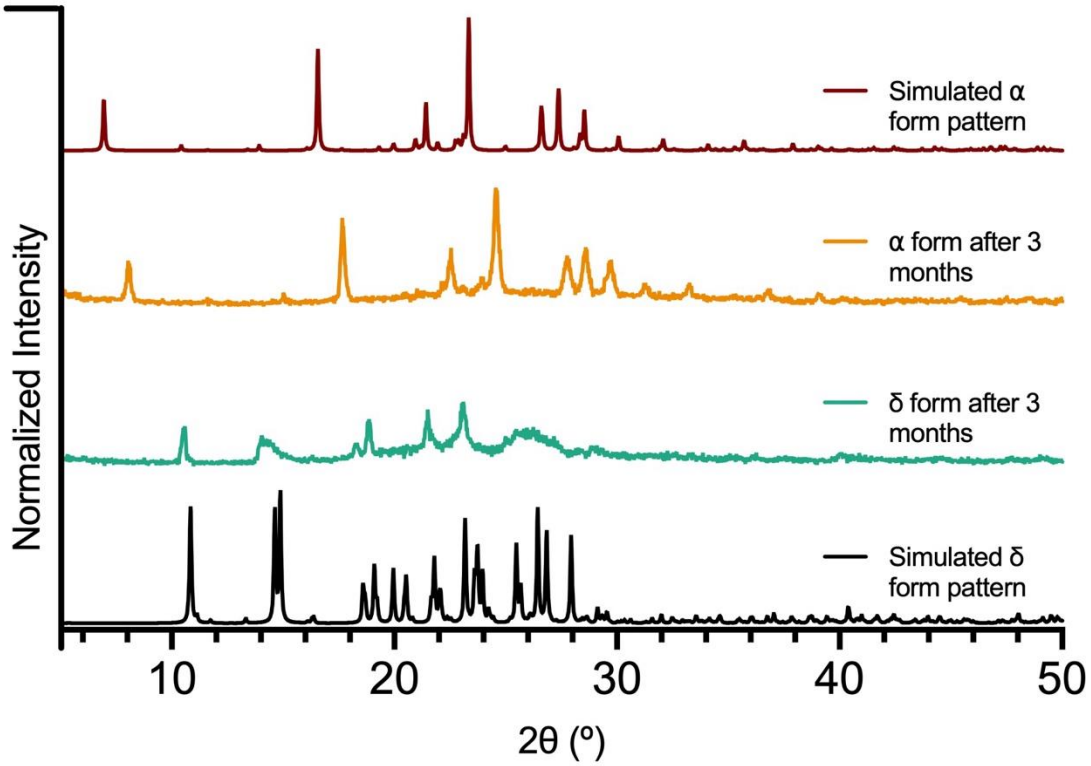

Figure S8. Powder X-ray diffraction patterns of **BHET** polymorphs after sitting at 25ºC for 3 months.

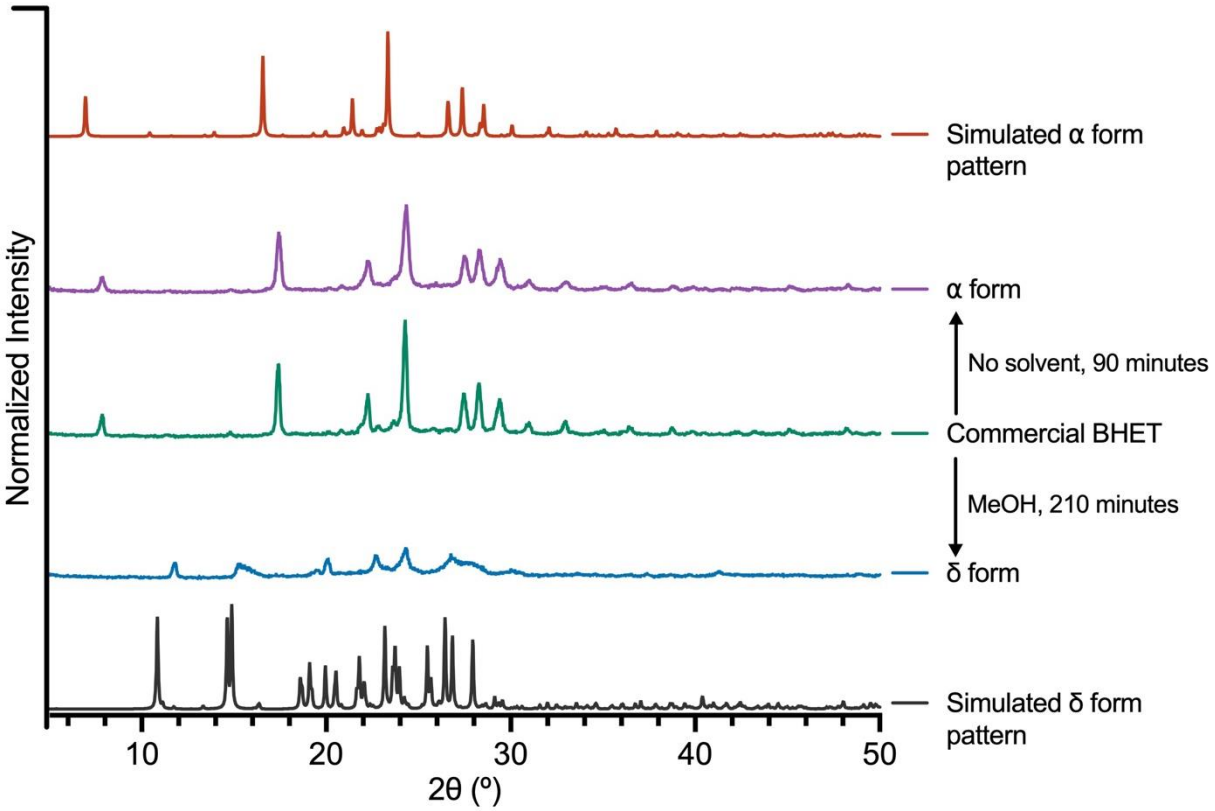

Figure S9. Powder X-ray diffraction patterns of mechanochemistry experiments using commercial **BHET** as the starting material.

S4. DSC and TGA

DSC experiments were performed on a TA Instrument DSC250 equipped with an RCS 90 refrigerated cooling system and under a nitrogen atmosphere (50 mL/min). Samples were run at 10°C/min in aluminum pans sealed with hermetic lids. Thermogravimetric analysis was performed on a TA Instrument TGA Q50 operating under a nitrogen atmosphere (40 mL/min) with a platinum pan at a rate of 10°C/min.

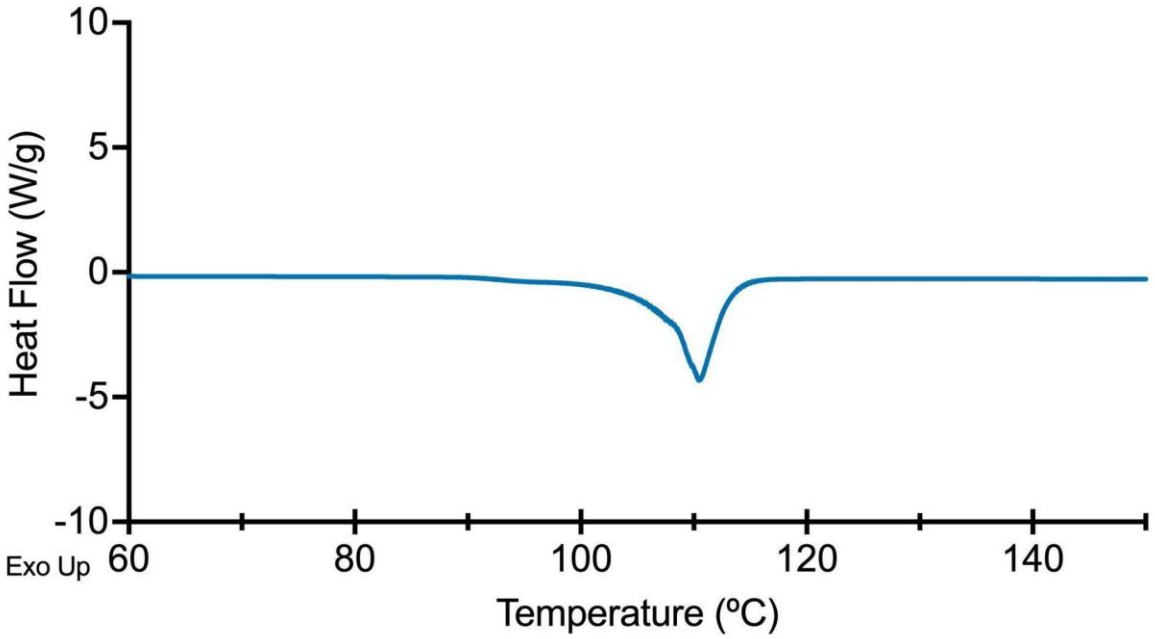

Figure S10. DSC curve of α form.

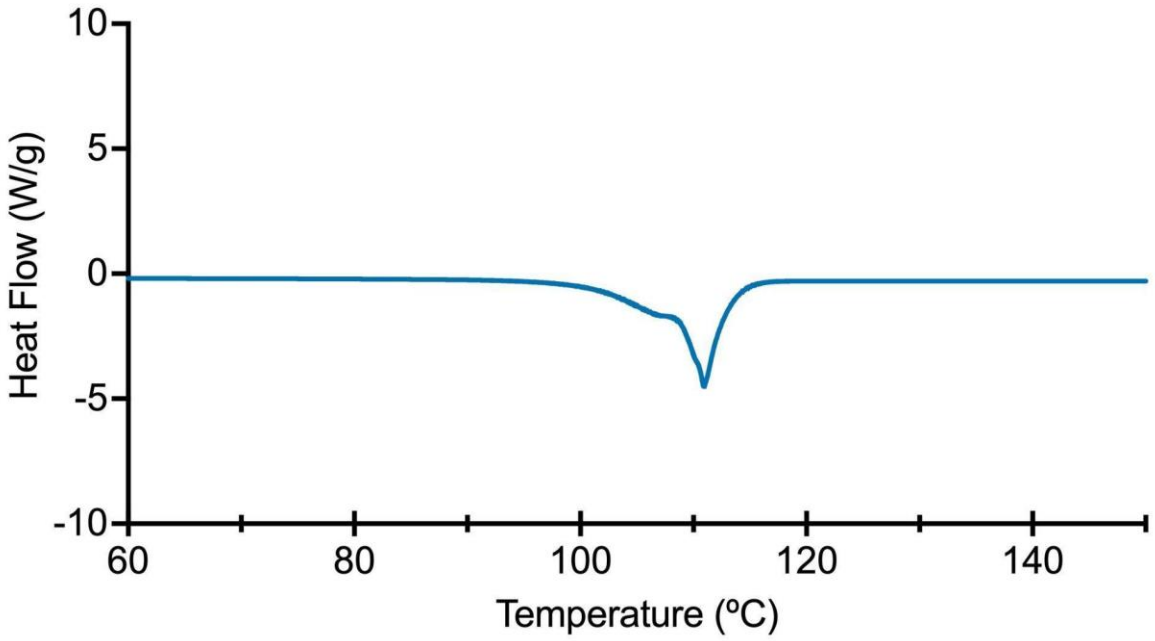

Figure S11. DSC curve of **δ** form.

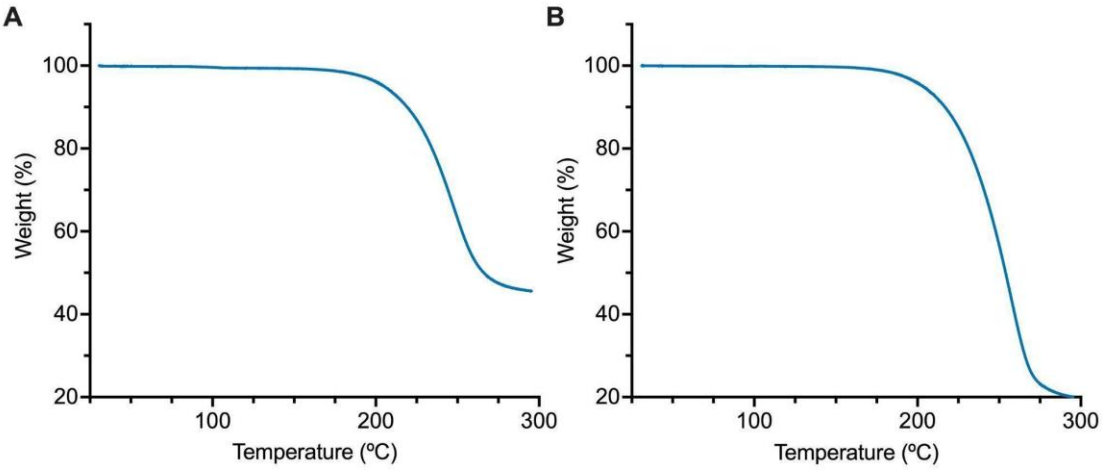

Figure S12. TGA curves of A) **α** form and B) **δ** form.

S5. HPLC Chromatograms

High-Performance Liquid Chromatography for BHET Degradation Analysis

BHET degradation was quantified within each culture using methods adapted from Shingwekar et al., 2023, Edwards et al., 2022 and Furukawa et al., 2018.[4–6] Briefly, 3.75 mL of HPLC-grade DMSO was added to whole bacterial cultures and mixed thoroughly to solubilize any remaining **BHET** in the culture media. The culture and DMSO mixture was further diluted with DMSO in a 1:4 ratio to generate 1 mL samples and filtered through a 0.22 μm PES syringe filter to sterilize each sample and remove any remaining large particles before HPLC analysis.

The analysis of glycolysis products was conducted using an Agilent Technologies 1100 series HPLC equipped with a ZORBAX Eclipse Plus C18 (Rapid Resolution, 4.6 × 100 mm 3.5 Micron) column (Agilent Technologies, Santa Clara, CA, USA). The mobile phase consisted of 70% MilliQ water, 20% acetonitrile, and 10% formic acid, and moved through the column at a flow rate of 1.0 mL min^{-1} at 40°C.

A 5 mM Monomethyl Terephthalate (MMT) internal standard was added to each sample for quantitative analysis, and internal response control factors (IRFs) were used to determine the **BHET** concentration in each microbial culture. IRFs were created using a known amount of MMT and BHET (5 mM MMT, 2 mM **BHET** in 1 mL DMSO), and the internal response factor was calculated using Equation (3):

$$Internal\ Response\ Factor\ (IRF) = \frac{[S_{IS}] \times [C_{BHET}]}{[S_{BHET}] \times [C_{IS}]}$$

where S_{IS}, S_{BHET}, C_{IS}, and C_{BHET} refer to the signal area of the internal standard, the signal area of BHET, the concentration of the internal standard (mM), and the concentration of BHET (mM), respectively. The concentration of BHET was then calculated using Equation (4):

$$BHET\ concentration\ (mM) = \frac{[C_{IS}] \times [S_{BHET}] \times [IRF]}{[S_{IS}]}$$

where IRF is the value calculated in Equation (3). Experimentally, MMT, **BHET**, **MHET**, and TPA peaks were observed around 3.4, 1.9, 1.6, and 1.3 min, respectively.

Degradation was calculated through three measures: the hydrolysis level (H), biodegradation level (B), and the total degradation level (T). All measures use **BHET** concentration (denoted as C_i, where i = week X inoculated sample, week X uninoculated sample, or week 0 uninoculated sample **BHET** concentration, and X = some number of weeks greater than 0) for calculations. Since cultures were grown in liquid media primarily composed of water, some **BHET** hydrolysis also naturally occurred. The hydrolysis level (H) measures **BHET** degradation that was independent of the bacteria, and compares the concentrations of the negative controls over time using equation (5):

$$H(\%) = (1 - \frac{C_{Week\ X\ uninoculated\ sample}}{C_{Week\ 0\ uninoculated\ sample}}) \times 100$$

The biodegradation level (B) includes the **BHET** degradation from the bacterial consortium, and compares the concentration of the experimental, inoculated samples to the negative controls which were tested at the same time point. This biodegradation level was calculated using equation (6):

$$B(\%) = (1 - \frac{C_{Week\ X\ inoculated\ sample}}{C_{Week\ X\ uninoculated\ sample}}) \times 100$$

The total degradation level (T) combines degradation done by hydrolysis and the consortium, and can be calculated using equation (7):

$$T(\%) = B + H$$

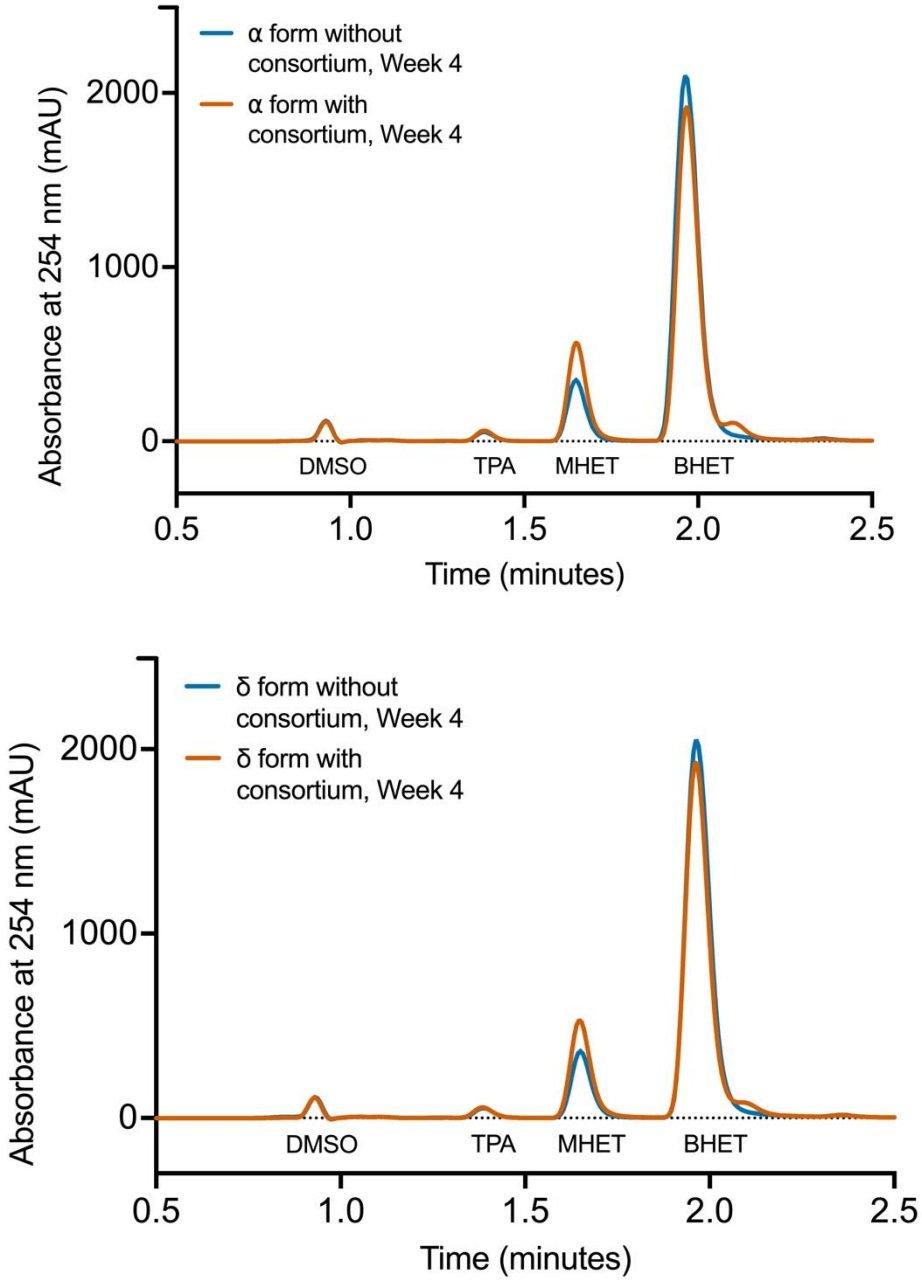

Figure S13. HPLC chromatogram overlay of BHET degradation. HPLC overlaid chromatograms of **α** form (top) and **δ** form (bottom) biodegradation. The dotted line indicates zero absorbance. A 5 mM MMT internal standard was added to each sample, observed at 4.4 min (not pictured), and **BHET** was observed at 1.9 min.

Table S3. 4-week biodegradation, hydrolysis, and total degradation levels for the **α** and **δ** forms of **BHET**.

Name	Biodegradation level (%)[1]	Hydrolysis level (%)[1]	Total Degradation level (%)[1]
α form	11.58	13.35	24.93
δ form	8.33	13.77	22.10

[1]The hydrolysis, biodegradation, and total degradation levels were calculated using equations 5, 6, and 7, respectively.

S6. Crystallographic Data for BHET Polymorphs

Table S4. Crystallographic data of reported **BHET** Polymorphs.

Name	**α** form	**β** form	**δ** form
CSD-Refcode	HETPAL	HETPAL01	HETPAL02
Crystal System	Monoclinic	orthorhombic	triclinic
Space Group	$P2_1/a$	$P2_12_12_1$	P-1
a/Å	25.72(2)	7.644(3)	8.2252(3)
b/Å	5.47(1)	5.692(2)	9.3639(9)
c/Å	8.59(2)	27.136(5)	16.5008(12)
α/°	90	90	87.306(7)
β/°	98.9 (2)	90	84.160(5)
γ/°	90	90	80.338(6)
Z	4	4	4
Z’	1	1	2
Reference	Kashino and Haisa, 1975.[7]	McDonald et al., 1983.[8]	Scé et al., 2019.[9]

S7. Dynamic Light Scattering data

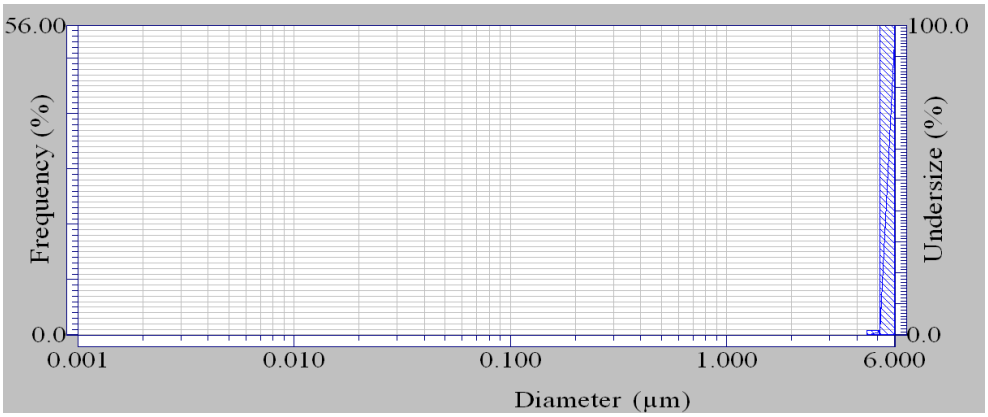

Figure S14. Averaged DLS measurement for the **α** form in methanol.

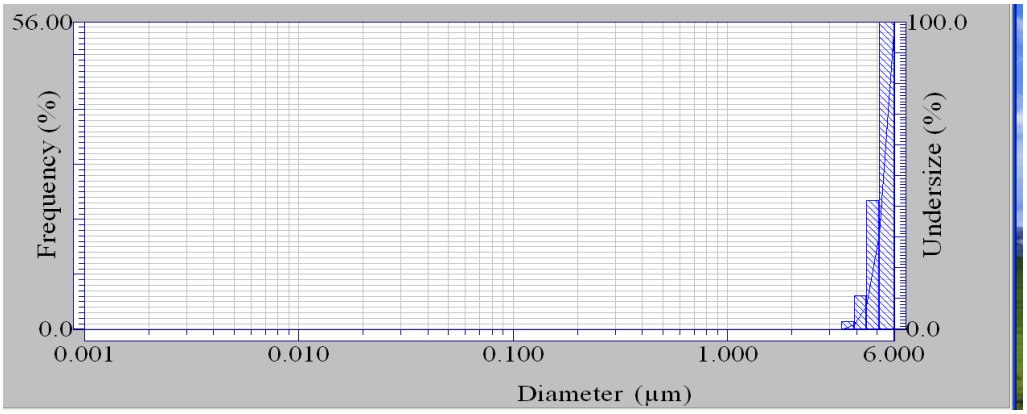

Figure S15. Averaged DLS measurement for the **δ** form in methanol.

S8. References

1. R. León-Zayas, C. Roberts, M. Vague and J. L. Mellies, *Microbiol Resour Announc*, DOI:10.1128/MRA.00237-19.
2. M. Ghaemy and K. Mossaddegh, *Polymer Degradation and Stability*, 2005, **90**, 570–576.
3. H. Putz and K. Brandenburg GbR, Match! - Phase Analysis using Powder Diffraction (version Version 3) Crystal Impact, Kreuzherrenstr. 102 53227 Bonn, Germany.
4. D. Shingwekar, H. Laster, H. Kemp and J. L. Mellies, *Bioengineering*, 2023, **10**, 1253.
5. S. Edwards, R. León-Zayas, R. Ditter, H. Laster, G. Sheehan, O. Anderson, T. Beattie and J. L. Mellies, *Int J Mol Sci*, 2022, **23**, 5612.
6. M. Furukawa, N. Kawakami, K. Oda and K. Miyamoto, *ChemSusChem*, 2018, **11**, 4018–4025.
7. S. Kashino and M. Haisa, *Acta Crystallogr B Struct Sci*, 1975, **31**, 1819–1822.
8. W. S. McDonald, E. L. V. Lewis and D. I. Bower, *Acta Crystallogr C Cryst Struct Commun*, 1983, **39**, 410–412.
9. F. Scé, I. Cano, C. Martin, G. Beobide, Ó. Castillo and I. De Pedro, *New J. Chem.*, 2019, **43**, 3476–3485.