Supporting information for "Polyethylene degradation by a *Rhodococcous* strain isolated from naturally weathered plastic waste enrichment"

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Supplementary Texts

Materials and Methods

Text S1. Establishment of enrichment cultures, bacterial strain isolation, morphological and physiological characterization. Naturally weathered plastic waste was harvested from a lakeside environment in Norman, OK. The plastic was cut into pieces (about 0.5 inch × 1inch) using a sterile scissor and incubated in 100 ml carbon-free basal medium (CFBM) in 300 ml flasks to enrich plastic degrading microorganisms. The enrichment cultures were kept at 30^{o}C with shaking (100 rpm). After 12 transfers of the enrichment cultures, a few small pieces of plastics with visually observable biofilms were transferred into disposable culture tubes with 5 ml CFBM + 0.01% yeast extract to promote microbial growth. After 35 days of incubation, the culture was vortexed and diluted (1 to 100) with CFBM and plated (50 µl per plate) onto ½ LB plates for bacteria strain isolation. Single colonies were streaked two rounds on ½ LB plates to get pure cultures. Overnight cell cultures were used for Gram staining following standard protocol. Cell morphology was observed using scanning electron microscope (SEM). Fresh overnight cell cultures were fixed using 4% gluaraldehyde in 0.1 M phosphate buffer (pH 7.4) for overnight at 4^{o}C. After fixation, the samples were washed three times using 0.1 M phosphate buffer for 10 min each followed by three washes with distilled water for 10 min each. Dehydration with ethanol was achieved by 10 min each at 25%, 50%, 70%, 85%, and 95% concentration. The final dehydration with 100% ethanol was conducted three times with 10 min each. The dehydrated cell samples were critical point-dried and metal coated for SEM imaging. Removal of buffer or supernatant in all steps was achieved by centrifuging the samples at 5,000 rpm for 5 min.

Text S2. Lipase activity assay. The qualitative lipase activity assay was conducted by using agar plates supplemented with olive oil as a source of long-chain fatty acids and Rhodamine B as a

flurosence dye binding to the cleaved fatty acids. The agar plates containing nutrient broth (8 g/liter), sodium chloride (4 g/liter), and agar (10 g/liter) were prepared. Olive oil (31.25 ml) and Rhodamine B (10 ml of 1 mg/ml stock) were then added into one-liter hot medium (about 60^{o}C) and stirred vigorously before pouring into plates. Bore holes (~6 mm diameter) on solidified plates were made using the wide end of the sterile pipette. 30 µl cell cultures were added into each bore hole and the plates were incubated at 30^{o}C for 48 hours. The lipase activity was examined by irradiating the plates with UV light at 350 nm using the Azure Biosystems 400 imaging system (Dublin, CA, USA).

Text S3. 16S rRNA gene amplicon sequencing and whole genome sequencing. Genomic DNA was isolated from enrichment culture or pure cell culture using GenElute™ Bacterial Genomic DNA Kits (Sigma, Cat # NA2110) and 16S rRNA was amplified by using primers 27F and 1492R following the standard protocol[1]. Sanger sequencing of the purified PCR fragments were performed at Oklahoma Medical Research Facility (OMRF, Oklahoma City, OK, USA). For the whole genome sequencing, 1 µg genomic DNA was used for construction of the sequencing libraries with KAPA Hyper Prep Kit (KR0961-v2.15, Kapabiosystems). DNA was fragmented (~ 300 bp) with a Covaris M220 focused-ultrasonicator (Covaris, Woburn, MA) and applied to ligation, amplification, and purification following the standard protocol. The sequencing was conducted with Hiseq3000 PE150 platform at OMRF. Sequence adaptors and reads with low-quality scores were removed using "BBDuk" in the BBTools package[2]. Next, error correction and normalization of the remaining reads were performed using "BBnorm" in the BBTools package. Finally, the high-quality reads were assembled into contigs using SPAdes v.3.15.2 and the contigs were annotated using the PATRIC platform[3].

Text S4. PE powder degradation test. PE powder (Sigma-Aldrich, Cat:427772-250G, 0.5 g) were weighed using a fine scale and transferred to a 50 ml centrifuge tube. 20 ml 70% ethanol was added into the tube and rotated for 30 min at room temperature. Sterilized PE powder was collected by centrifuging the tubes at 12,000×g for 30 min with slow acceleration and deceleration. After removal of the supernatant, PE powder was air-dried overnight under a biosafety hood. Three groups including C1 to C4 for PE powder only, C5-C8 for bacteria only, and T1-T4 for bacteria and PE powder were set up and incubated for 30 days at 30°C with shaking (200 rpm).

Text S5. Characterization of PE physical and chemical changes and potential degradation products.

After 30 days of incubation, PE powder was harvested using a 0.22 μm filter unit (150 ml) (VWR, Cat #10040-460) and the flow through was collected for GC-MS analysis of the potential degradation products. PE powder on the membrane was washed off multiple times using 2% SDS and collected in 50 ml centrifuge tube to a final volume of about 30 ml per tube. The tubes were rotated (80 rpm) overnight at room temperature. The washed powder was collected using a filtration unit (VWR, Cat #10040-460, 0.45μm) and air dried for overnight under a biosafety hood. Dried PE powder was mounted to SEM tubes followed by metal coating for SEM imaging (Samuel Roberts Noble Microscopy Laboratory, University of Oklahoma), or used for HT-GPC analysis (10 mg per sample, the Soft Matter Facility at Texas A&M University) to determine the molecular weight and FTIR (IRPrestige-21, Shimadzu) analysis to determine the functional group changes. FTIR measurements were performed using smartsingle-bounce ATR with a diamond tip in the 4000-400 cm^{-1} range. The IR spectrum table & chart from Sigma-Aldrich was used as reference

for peak alignments (https://www.sigmaaldrich.com/US/en/technical-documents/technical-article/analytical-chemistry/photometry-and-reflectometry/ir-spectrum-table). For GC-MS analysis of the supernatant, 3 ml of each supernatant sample was extracted with 1v/v of dichloromethane. The organic layer was separated and concentrated into 500 µl of dichloromethane. 1µl of each sample was used for GC-MS analysis on Ultra GC/DSQ (ThermoElectron; Waltham, MA). DB-5MS was used as a gas chromatographic column with dimensions of 30 m length, 0.25 mm i.d., and 0.25 µm film thickness (Agilent Technologies; Santa Clara, CA). Helium (>99.999%) was used as a carrier gas at constant flow of 1.2 ml/min through the column. The inlet temperature was held at 225^{o}C and was used in splitless mode. The column temperature was maintained at 50°C for 3 min and raised to 300°C at a rate of 6°C/min, and then held for 3 min. Transfer line and ion source were held at 250°C. Electron impact at 70eV was used for ionization.

Text S6. Time series proteomics analysis of A34 using PE powder as the sole carbon source.

The A34 strain was revived in small volume TSB (4 ml per tube) by incubating at 30^{o}C overnight. The cell cultures were inoculated into bigger volume TSB (100 ml) and grew to late log-phase. Collect time zero cell samples for preparation of intracellular protein samples grown in TSB by centrifuging at 8,000g for 10 min at 4^{o}C. Supernatant were obtained by filtering the cell culture using 0.2 µm filter unit and concentrated using Vivaspin® 5 kDa MWCO Concentrator (Sigma-Aldrich, catalogue #: Z614009). After that, cell cultures were washed with CFBM twice and resuspended in CFBM (1 to 10 dilution), and time zero sample was collected for the time-series proteomics. Four replicates test flasks were prepared with 100 ml cell culture and 0.5 g PE powder per flask. The cell cultures were incubated at 30^{o}C with shaking (200 rpm). Samples were collected

at 3, 6, 12, 24, or 48 hours. Supernatant samples were collected at 48 hours and concentrated as described above. All samples were kept at -80^{o}C before protein extraction. Protein extraction, proteomics sample preparation and digestion were conducted following our previous protocol[4] with minor modifications. Briefly, cell pellets were re-suspended in lysis buffer (50 mM Tris-HCl pH 8.0, 2% SDS, and 0.1 M dithiothreitol) followed by disruption using a bead-beating method (MP Biomedicals, Irvine, CA). The supernatant was collected by centrifugation at 15,000 × g for 10 min at 4^{o}C. Proteins were precipitated overnight using trichloroacetic acid (25% v/v) at 4°C and collected by centrifugation at 20,800 × g for 20 min at 4^{o}C. The protein pellets were washed three times with ice-cold acetone and resuspended in guanidine buffer (6 M Guanidine HCl, 10 mM DTT in Tris CaCl2 buffer (50 mM Tris, 10 mM $CaCl_2$, pH 7.6)). Protein concentrations were quantified using the Thermo Scientific™ Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). To prepare samples for proteomics assay, 20 mg protein was processed using the filter-aided sample preparation method[4] and digested with a trypsin-LysC mixture (Promega, Madison, WI). The resulting peptide samples were purified using Pierce C18 Spin Columns (Thermo Fisher Scientific, Waltham, MA) and eluted in 20 µl elution buffer.

Tryptic peptides were separated by reverse phase XSelect CSH C18 2.5 µm resin (Waters) on an in-line 150 x 0.075 mm column using an UltiMate 3000 RSLCnano system (Thermo). Peptides were eluted using a 120 min gradient from 98:2 to 65:35 buffer A:B ratio (Buffer A, 0.1% formic acid, 0.5% acetonitrile; Buffer B, 0.1% formic acid, 99.9% acetonitrile). Eluted peptides were ionized by electrospray (2.4 kV) followed by mass spectrometric analysis on an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA). MS data were acquired using a Fourier transform MS (FTMS) analyzer in profile mode at a resolution of 240,000 over a range of 375 to 1500 m/z. Following high-energy collisional dissociation (HCD) activation,

MS/MS data were acquired using the ion trap analyzer in centroid mode and normal mass range with normalized collision energy of 28-31% depending on charge state and precursor selection range.

Mass spectrometry spectra were searched using Sipros Ensemble against a matched protein database constructed from the genome sequencing[5]. Raw search results were filtered to achieve a 1% false-discovery rate (FDR) at the peptide level. Peptide identifications were assigned to proteins or protein groups following the parsimonious rule. A minimum of one unique peptide was required for each identified protein or protein group. Intensity-based label-free quantification of protein was performed using ProRata[6]. The protein abundance was represented by the total peak height of all quantified unique peptides from one sample and was normalized by the mean across the entire dataset. Normalized proteomics data were further analyzed by protein function and corresponding pathways. Proteins detected in two or more samples out of four replicates were kept for analysis. The signal intensity for each protein was the average of the replicates and log2 transformed value for the convenience of comparison.

Text S7. Heterologous protein expression and crude enzyme incubation experiment. Coding sequences of a multicopper oxidase peg1726 and a putative esterase peg6607 were cloned into protein expression vector pET-28A using primers (1726-FP: TAACTTTAAGAAGGAGATATACATGGACGAGTCACACAAGATCC; 1726-RP : ATCTCAGTGGTGGTGGTGGTGGTGCGTCACCACCCGATATCGCA; 6607-FP: TAACTTTAAGAAGGAGATATACCATGCGTGCTCGAATTTTCAAGC; 6607-RP: TCAGTGGTGGTGGTGGTGGTGGACGCCCAGGGAGTGAGC). After sequencing verification, the *E. coli* clones harboring the correct protein expression vectors were grown in LB

and induced by 0.3 mM IPTG following standard protocol. The supernatant protein containing the target protein was quantified using Pierce BCA protein assay kit (ThermoFisher, cat #: 23225). The incubation volume in each tube (50 ml Falcon tube) was 10 ml lysis buffer (20 mM Tris-HCl pH8.0, 150 mM NaCl, and 10 mM imidazole) containing about 8µg of crude enzyme and 0.5g ethanol-sterilized PE powder and incubated at 30^{o}C with shaking (120 rpm) for one week. Five sample groups with three replicates each were set up: PE powder only, PE powder + uninduced supernatant, PE powder + induced peg1726 supernatant, PE powder + induced peg6607 supernatant, PE powder + induced peg1726 supernatant + induced peg6607 supernatant. After incubation, the PE powder was washed with 2% SDS and dried before FTIR assay as described above.

Reference:

(1) Frank, J. A.; Reich, C. I.; Sharma, S.; Weisbaum, J. S.; Wilson, B. A.; Olsen, G. J. Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. *Appl Environ Microbiol* **2008**, *74* (8), 2461-2470. DOI: 10.1128/aem.02272-07 From NLM.

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(5) Guo, X.; Li, Z.; Yao, Q.; Mueller, R. S.; Eng, J. K.; Tabb, D. L.; Hervey, W. J., IV; Pan, C. Sipros Ensemble improves database searching and filtering for complex metaproteomics. *Bioinformatics* **2017**, *34* (5), 795-802. DOI: 10.1093/bioinformatics/btx601 (acccessed 3/20/2023).

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Supplementary Figures

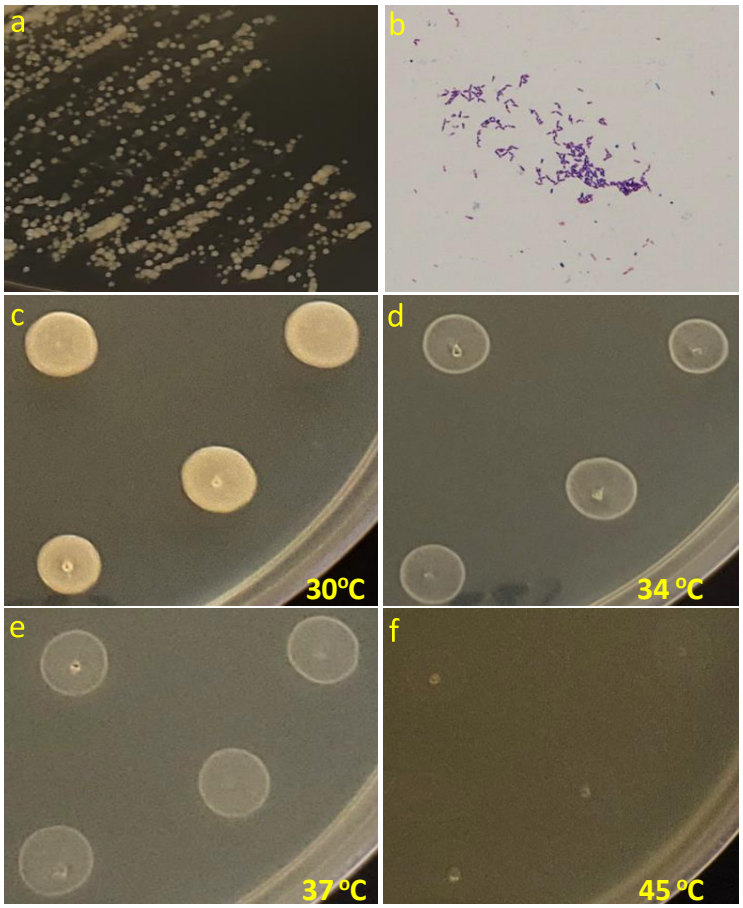


Figure S1. Morphology, gram staining, and optimal growth temperature of *Rhodococcus* strain A34. a. Morphology of strain A 34 on TSA plate. b. Gram-staining of strain A34. c~f. Optimal growth temperature tested on TSA plates. Best growth was observed at 30°C. As the temperature increased, the growth was inhibited, no growth was observed at 45°C.

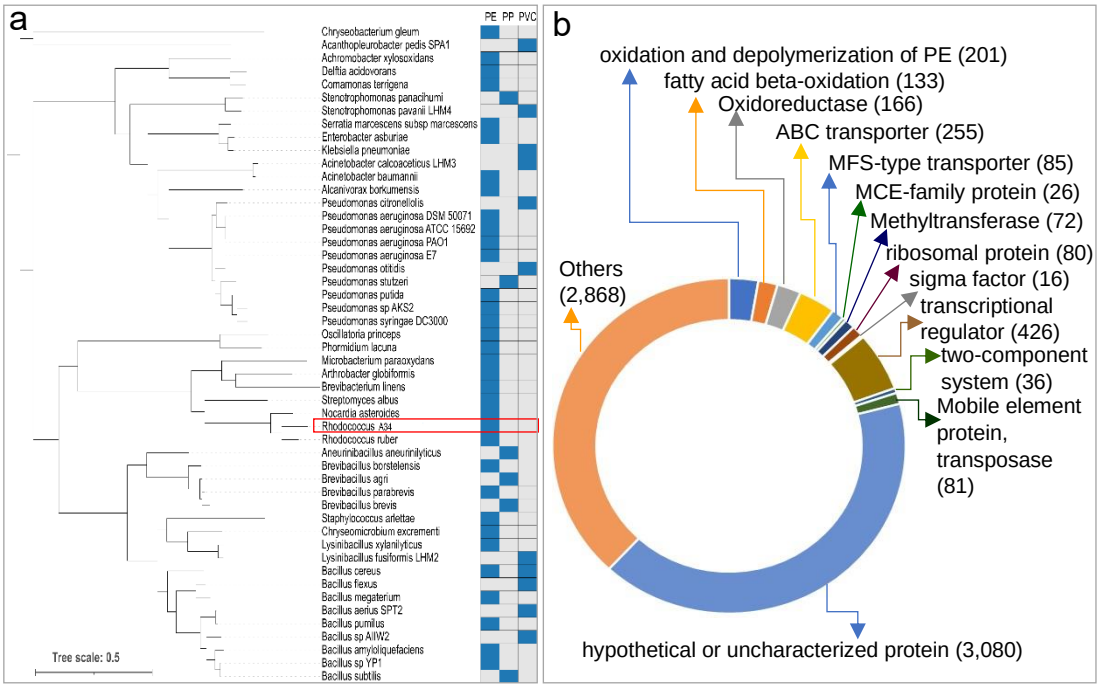


Figure S2. Genome-level phylogeny of strain A34 and genetic potential of A34 genome. a. The phylogenetic tree that was built using the reference genomes from GTDBtk. b. Classification of genes annotated in A34 genome.

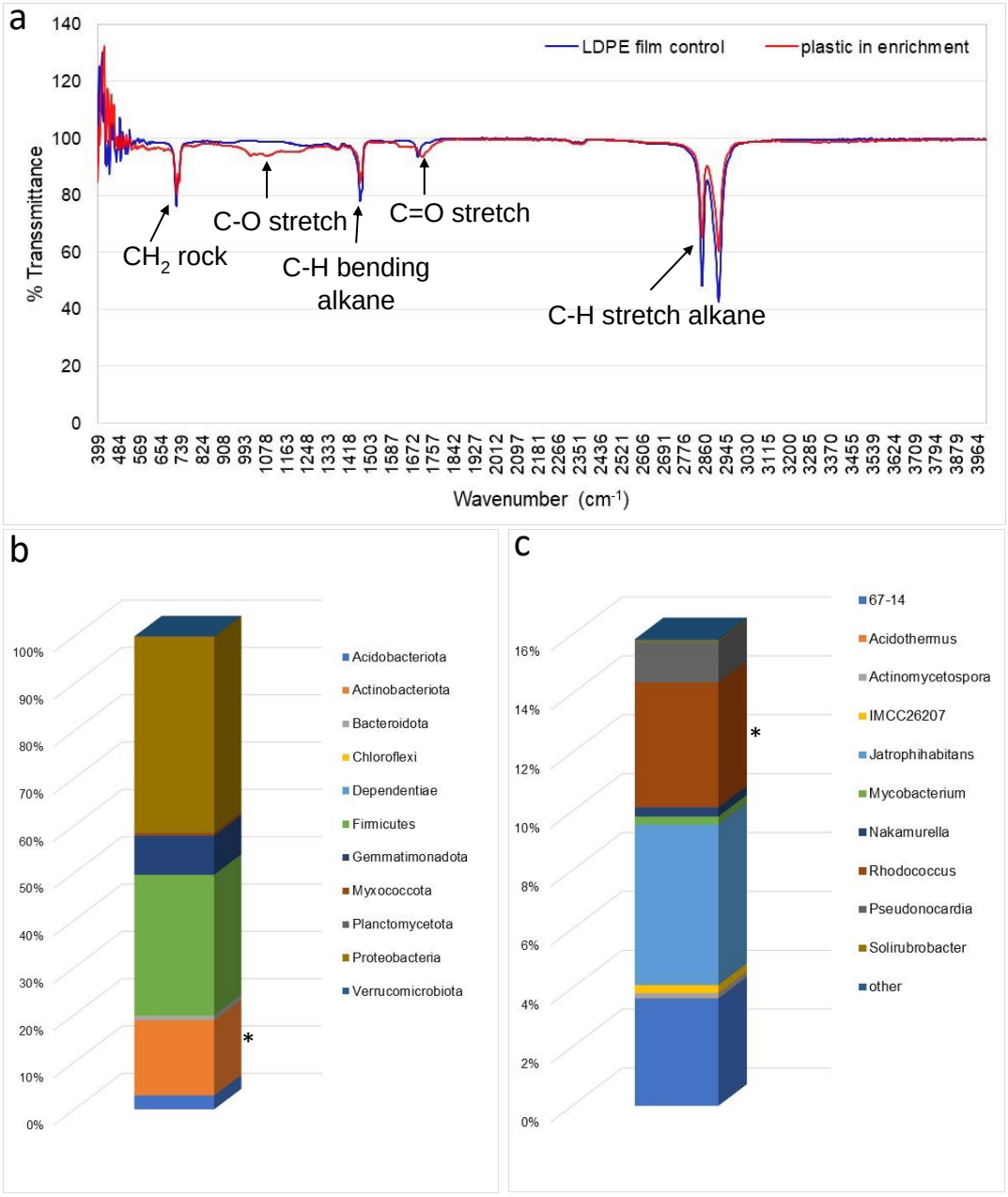


Figure S3. Type of plastic and the microbial community compositions in the enrichment cultures. a. FTIR spectrum of the plastic in enrichment # 5 where *Rhodococcus* strain A34 was isolated from. LDPE film (GoodFellow, product number: 311301) was used as control. b. The bacterial community composition in enrichment #5 at phylum level. c. The genus level composition of Actinobacteriota. *: the phylum or the genus A34 belong to.

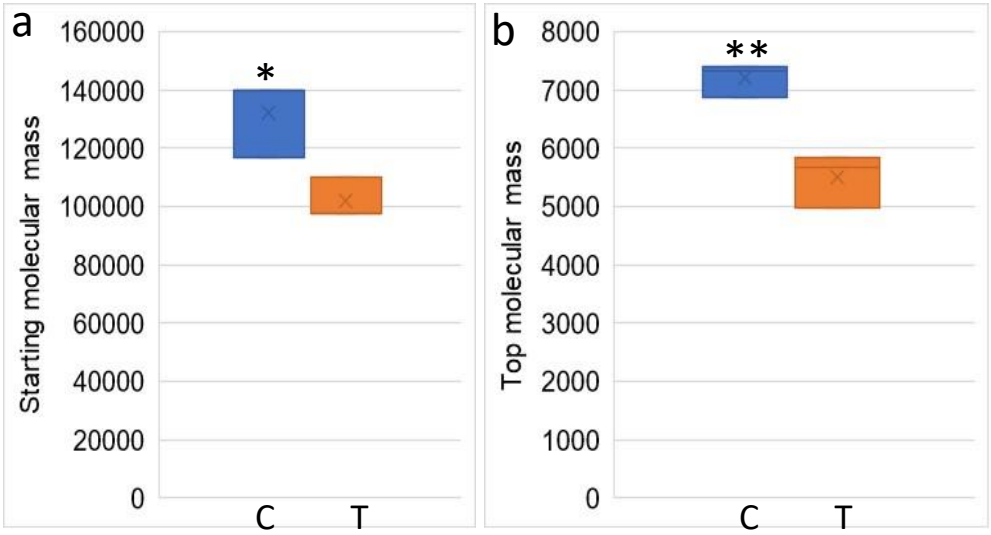


Figure S4. The size distribution of PE powder significantly decreased in samples incubated with A34 (T) compared to samples without A34 (C) for 30 days. a. starting molecular mass (p = 0.025). b. top molecular mass (p = 0.005). Unpaired two-tailed *t* test, *$p < 0.05$, **$p < 0.01$.

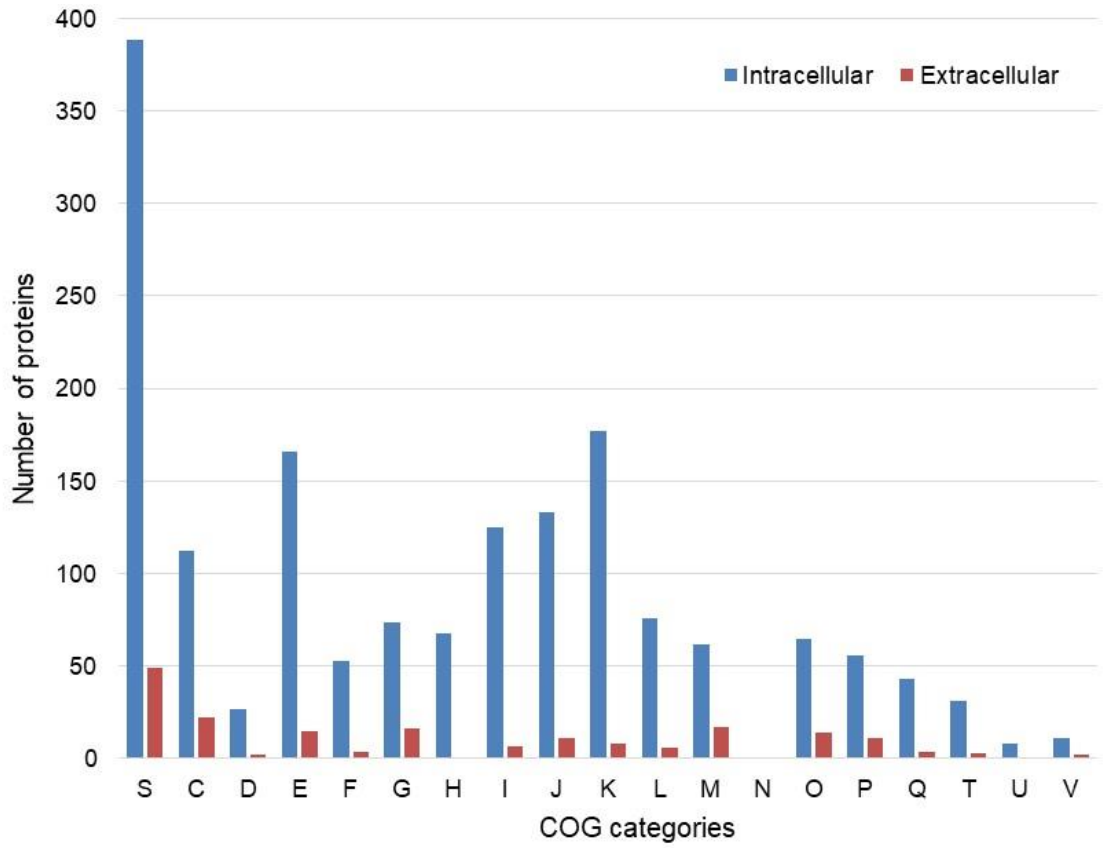
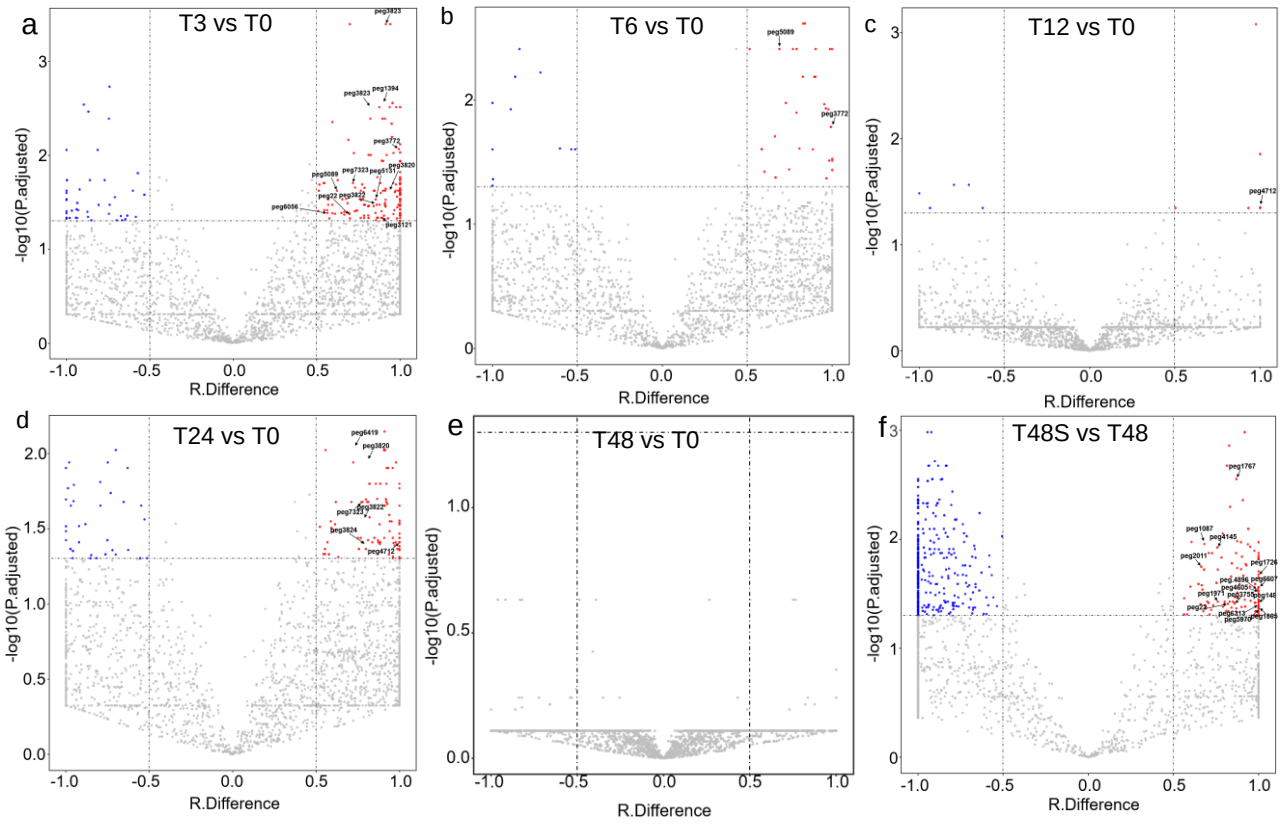


Figure S5. Proteins detected in time zero samples of *Rhodococcus* strain A34 grown in rich medium TSB. COG categories were used for classification of protein. Intracellular: proteins extracted from cell pellet. Extracellular: proteins extracted from supernatant. S, function unknown; C, energy production and conversion; D, cell cycle control, cell division, chromosome partitioning; E, amino acid transport and metabolism; F, nucleotide transport and metabolism; G, carbohydrate transport and metabolism; H, coenzyme transport and metabolism; I, lipid transport and metabolism; J, translation, ribosomal structure and biogenesis; K, transcription; L, replication, recombination and repair; M, cell wall/membrane/envelop biogenesis; N, cell motility; O, post-translational modification, protein turnover, chaperones; P, inorganic ion transport and metabolism; Q, secondary metabolites biosynthesis, transport and metabolism; T, signal transduction; U, intracellular trafficking and secretion; V, defense mechanisms.



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239 **Figure S6.** Volcano maps showing proteins with significant abundance changes at each timepoint
240 compared to time zero (a to e) or extracellular vs intracellular (f, T48S vs T48). red dots, increased
241 abundance; blue dots, decreased abundance. Key enzymes involved in PE oxidation,
242 depolymerization and fatty acid beat oxidations were labelled.

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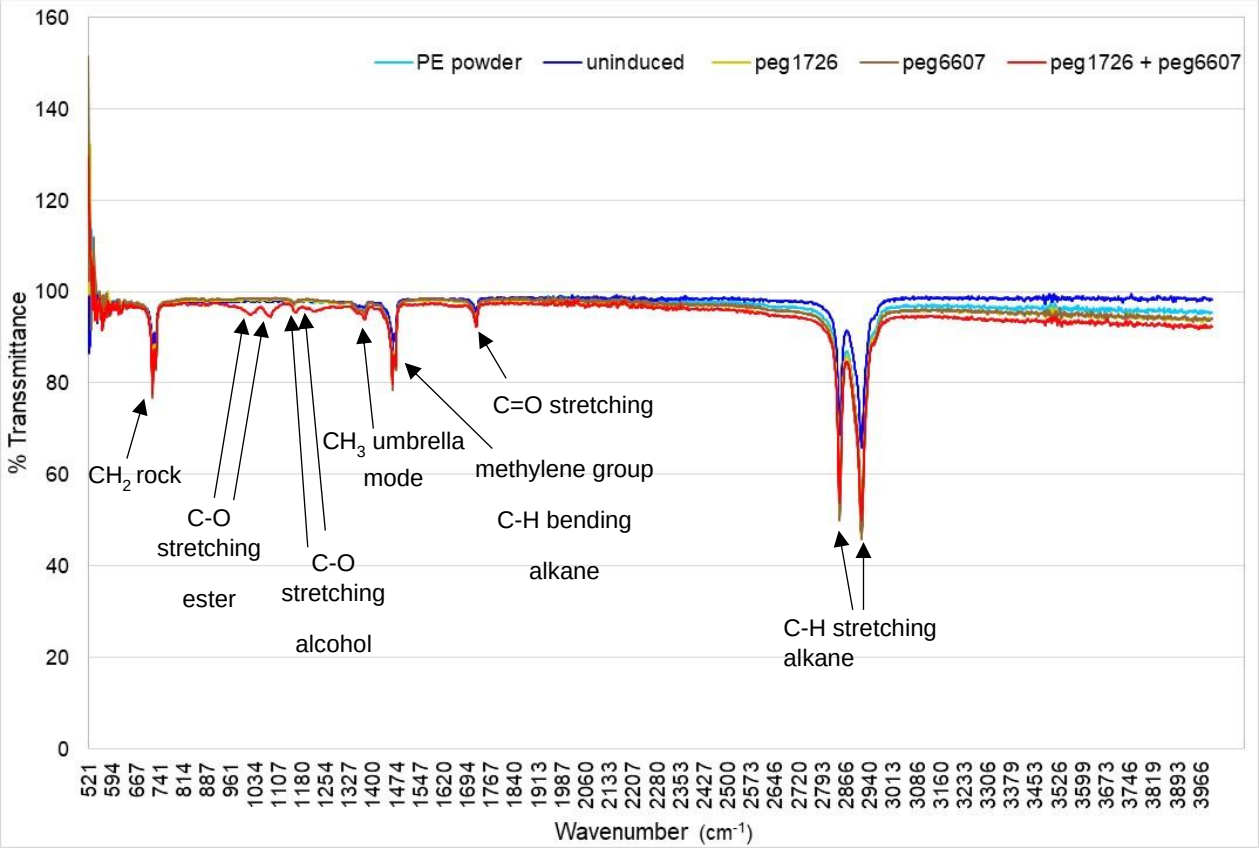

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245 **Figure S7.** PE functional group changes after incubation with heterologous expressed crude

246 enzymes. peg1726, a multicopper oxidase. peg6607, a putative esterase.

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