

Engineered Yeasts Displaying PETase and MHETase as Whole-Cell Biocatalysts for the Degradation of Polyethylene Terephthalate (PET)

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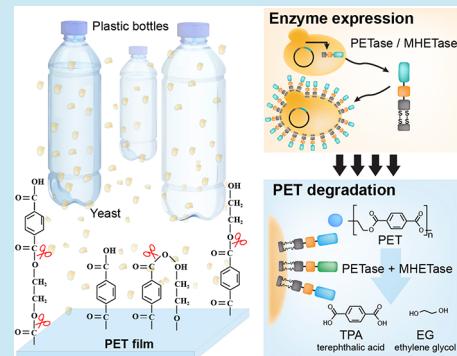
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ABSTRACT: Due to its low cost of manufacturing, poly(ethylene terephthalate) (PET, a polyester plastic) has been the most widely used plastic material for food packaging. However, PET is nonbiodegradable. It can take years to degrade when it is discarded into the environment. In recent years, plastic pollution has received much attention and has become a major environmental issue. In this study, we engineered yeast surfaces to display two PET-degrading enzymes (PETase and MHETase) to degrade PET plastics. The enzymes displayed on the yeast surface were characterized by using confocal microscopy and flow cytometry. The reaction conditions to degrade PET plastics using the engineered yeasts were optimal at pH 9 and 30 °C. In addition, the engineered yeasts showed great stability and reusability to degrade PET films. Furthermore, we demonstrated that the engineered yeasts as whole-cell catalysts can be used to degrade drinking water bottles into value-added products. This study provides a novel whole-cell biocatalyst using engineered yeasts to degrade plastic waste, offering a new strategy to solve plastic pollution and recycling challenges.

KEYWORDS: polyethylene terephthalate (PET), plastic waste, PETase, MHETase, whole-cell biocatalyst, yeast



1. INTRODUCTION

In the last century, the plastic industry has grown significantly. The total amount of plastic used exceeds 300 million tons every year, with an increase of 5% annually.^{1,2} However, less than half of these plastics are reused or recycled. As a result, tons of plastic are discarded into nature. In the environment, plastic can take years to degrade.³ Without full degradation, microplastics, defined as plastic pieces with a size of 1–5 mm, have been found everywhere around the world, including inside plants and humans.^{4–6} With an increasing demand for plastic items, plastic pollution is becoming a bigger problem year after year. Some strategies (e.g., prevention measures, cleanup activities, and awareness instruments) have been used to address this issue. For example, the ban on plastic bags has been successful in reducing the use of plastic shopping bags in supermarkets and shopping malls. Unfortunately, it is still not feasible to remove all discarded plastics from the environment and fix the existing widespread plastic pollution, especially the most widely used plastic, polyethylene terephthalate (PET).

For chemical recycling, PET plastics are broken down to their monomers of terephthalic acid (TPA) and ethylene glycol (EG) that can be refined or remanufactured into high-value products, including gallic acid, pyrogallol, catechol, muconic acid, vanillic acid, and glycolic acid.⁷ Due to the utilization of high pressure and temperature, hazardous organic solvents, and catalysts, chemical methods have several disadvantages, including high cost, complicated processing steps, and poor compatibility with

downstream purification processes.^{8,9} Biodegradation of PET plastics using microorganisms or enzymes has emerged as an effective alternative approach to address these issues.^{10,11} To perform environmentally friendly and green depolymerization of PET waste, researchers have discovered PET-degrading enzymes and improved their catalytic activities to green depolymerize PET waste. To date, about 50 PET-degrading enzymes (including lipases, cutinases, carboxylesterases, and esterases) from bacteria and fungi have been reported to degrade PET plastics.^{12–14} Although these enzymes can degrade PET waste, there is an urgent need to develop more practical technologies to recycle plastic waste.

In 2016, Yoshida et al. discovered a bacterium (*Ideonella sakaiensis*) that can secrete new types of PET-degrading enzymes to efficiently degrade and assimilate PET as a carbon and energy source, including PET hydrolase (PETase) and mono(2-hydroxyethyl) terephthalate (MHET) hydrolase (MHETase).¹⁵ Since then, the two PET-degrading enzymes have been widely studied as ideal enzymes to completely

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degrade PET waste. To date, numerous efforts have been made to improve the hydrolysis activities of PETase and MHETase to degrade PET waste. For example, chemical modification can improve enzymatic activity by providing PETase with a stable environment.¹⁶ Synthetic biology allows the fusion of multiple glutamic acid (Glu, E) and lysine (Lys, K) on the enzyme surfaces to protect the stability of PETase.^{17,18} In addition, PETase has been displayed on the surface of bacterial cells to increase the tolerance of pH and thermal variations.^{19–21} Although PETase and MHETase have gained significant attention as effective tools for reducing PET waste, the transition from theoretical to practical application faces significant challenges.

Cell surface display has become a highly promising strategy for expressing and engineering proteins inside and outside of the cell. Engineering cells to display a specific protein of interest on their cell surface, termed surface display systems, is attractive for many applications in microbiology and molecular biology.²² This strategy not only simplifies the synthesis process of the enzyme of interest, but also avoids the complex and costly processes of enzyme separation and purification that are generally necessary in alternative procedures.^{23,24} Cell surface display systems were initially developed for filamentous phage by fusing peptides on the surface of capsid proteins without affecting the phage's ability to infect bacteria.²⁵ However, it is not feasible to incorporate large polypeptides and proteins on phage surfaces. Alternatively, bacterial surfaces can be used to express large-sized proteins.^{26,27} Compared to bacterial systems, yeast offers structural robustness and several advantages for whole-cell biocatalysis, including higher resistance to fluctuations in pH and temperature, simplified genetic engineering, and greater stability across various bioprocessing environments.²⁸ Yeast cells can also be produced economically in large quantities and hold Generally Recognized As Safe (GRAS) status, further enhancing their suitability for environmental and industrial applications, particularly in scalable enzyme display and practical PET degradation.²⁹

In this study, we developed engineered yeasts with the surface display of PET-degrading enzymes as whole-cell biocatalysts to degrade PET plastics. Using molecular engineering tools, we displayed PETase and MHETase on yeast surfaces (*Saccharomyces cerevisiae EBY100*) that were confirmed by using confocal microscopy and a flow cytometer. In addition, we identified optimal reaction conditions (pH and temperature) of the yeast-displayed PETase and MHETase to improve the PET degradation efficiency. Furthermore, our developed engineered yeasts displaying PET-degrading enzymes were applied to commercial postconsumer drinking water bottles. The engineered whole-cell biocatalysts demonstrated a significant breakdown rate and hold promise for resolving the current crisis of PET pollution, potentially providing a financially feasible and environmentally friendly alternative to chemical recycling.

2. MATERIALS AND METHODS

2.1. Materials and Chemicals.

Bis(2-hydroxyethyl) terephthalate (BHET), terephthalic acid (TPA), ethylene glycol (EG), glycine, and dithiothreitol (DTT) were purchased from Sigma-Aldrich (Saint Louis, MO). Mono(2-hydroxyethyl) terephthalate (MHET) was purchased from Advanced Chem-Blocks, Inc. (Hayward, CA). Q5 High-Fidelity 2X Master Mix, NEBuilder HiFi DNA Assembly Master Mix, OneTaq 2X Master Mix with Standard Buffer, Monarch Plasmid Miniprep

Kit, Monarch DNA Gel Extraction Kit, and other reagents used in cloning work were purchased from New England BioLabs (Ipswich, MA). Commercial amorphous PET film (transparent, 0.25 mm thickness, catalog number ES30-FM-000145) was obtained from Goodfellow Corporation (Pittsburgh, PA). DyLight 488 Anti-HA tag antibodies and other chemicals were purchased from Thermo Fisher Scientific (Waltham, MA).

2.2. Strains, Plasmids, and Media. The *S. cerevisiae EBY100* (*S. cerevisiae EBY100*) was kindly provided by R. Clay Wright at Virginia Tech. The pCTcon2 plasmid (# 41843) was purchased from Addgene (Watertown, MA). Table S1 contains comprehensive information about the strains and plasmids used in this study. The primers (Table S2) were designed for Gibson Assembly and synthesized by Sigma-Aldrich (St. Louis, MO). The yeast-codon-optimized genes for PETase and MHETase are synthesized by Twist Bioscience (San Francisco, CA) and are available in Table S3.

Culture conditions: *Escherichia coli* (*E. coli*) Top 10 were cultured at 37 °C in LB media supplemented with 100 µg/mL ampicillin if needed. *S. cerevisiae EBY100* was cultured at 30 °C in YPAD media. Engineered yeasts were selected in SDCAA plates. The proteins displayed on yeast surfaces were induced in SGCAA medium with galactose as the inducer.

2.3. Construction and Expression of PETase and MHETase on Yeast Surfaces. The codon-optimized genes for PETase and MHETase were amplified using Polymerase Chain Reaction (PCR). The PCR products were purified using the DNA Gel Extraction Kit and assembled into pCTcon2 backbone using NEBuilder HiFi DNA Assembly Master Mix. The plasmids of pCTcon2-PETase and pCTcon2-MHETase were transformed into *E. coli* Top 10 competent cells for amplification.³⁰ The inserts were then confirmed using Sanger sequencing. The correct plasmids containing the genes coded for PETase and MHETase were then transformed into *S. cerevisiae EBY100* competent cells according to a previously reported method with minor modifications.³¹ The positive colonies on the SDCAA selective plates were confirmed by using yeast colony PCR.

To express PETase and MHETase on the yeast surface, a fresh colony was inoculated into SDCAA medium (5 mL) and incubated at 30 °C overnight. The expression of PETase and MHETase was induced by inoculating the overnight culture into the SGCAA medium ($OD_{600} = 0.5$) and incubated at 30 °C for 48 h. Engineered yeast with the pCTcon2 plasmid was used as a positive control. After incubation, the cells were harvested by centrifugation at 3000g for 10 min. They were then washed and resuspended in glycine-NaOH buffer (50 mM, pH 9) to obtain EY-pCTcon2, EY-PETase, and EY-MHETase. The wild-type yeasts as negative controls were cultured in YPAD media.

2.4. Characterization of PETase and MHETase on Yeast Surfaces. Anti-HA Tag antibodies were used to confirm the expression of PETase and MHETase on yeast surfaces following the procedure reported by Luo et al. with minor modifications.³² Wild-type, EY-pCTcon2, EY-PETase, and EY-MHETase (around 1×10^7 yeast cells) were centrifuged at 3000g for 2 min and resuspended in phosphate-buffered saline (PBS, 100 µL, pH 7.4, with 0.5% bovine serum albumin). Anti-HA Tag antibodies (1 µL) were added and gently rotated at room temperature for 1 h in the dark. Following the incubation, yeast cells were rinsed 3 times using PBS and subsequently resuspended in PBS (50 µL). The fluorescence immunostaining was characterized using a Confocal Laser Scanning Microscope

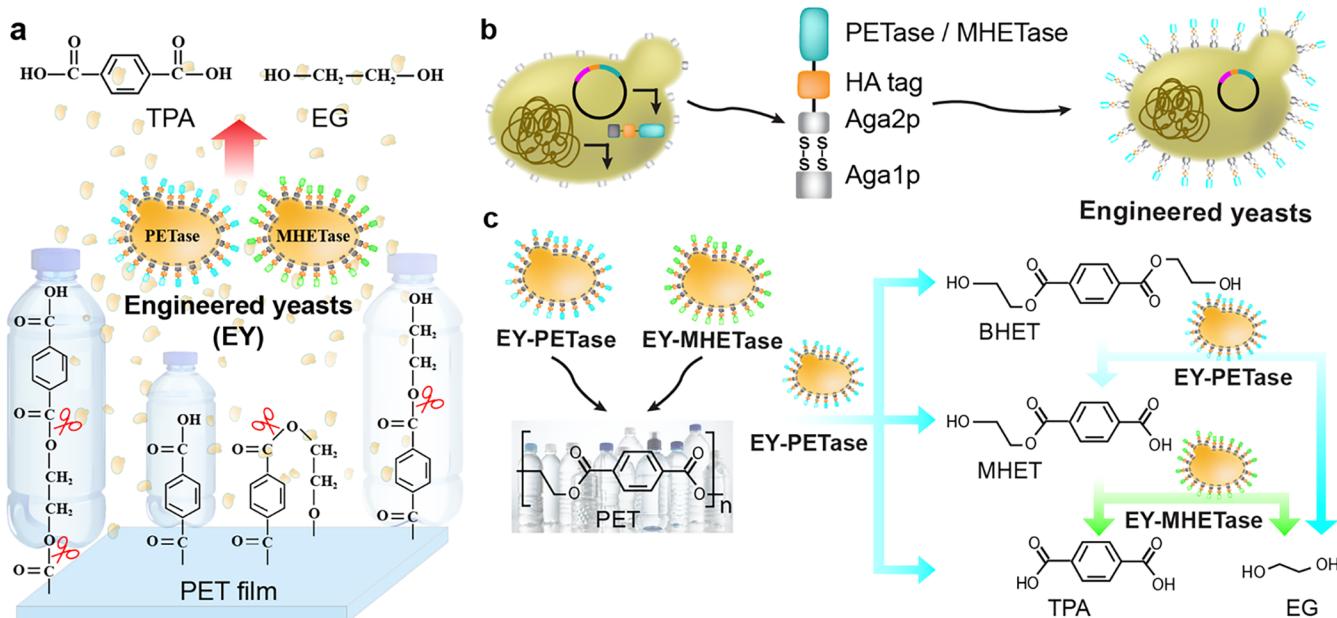


Figure 1. (a) Schematic illustration of whole-cell biocatalysts used to degrade drinking water bottles into value-added products (TPA and EG). (b) Expression of PET-degrading enzymes on yeast surfaces. (c) EY-PETase and EY-MHETase break down the PET plastic bottle into smaller components, including TPA and EG.

(Nikon, Melville, NY) and Attune NxT Acoustic Focusing Cytometer (ThermoFisher Scientific, Waltham, MA).

2.5. Degradation of PET Plastics Using Whole-Cell Biocatalysts. The PET films were first cut into circular forms with a diameter of 7 mm by using a perforating machine (hole punch). Each film was treated in solutions (1% sodium dodecyl sulfate, SDS), 20% ethanol, and distilled water for 1 h to guarantee complete cleaning. The films were then dried at 50 °C to remove any residues. Before degradation, PET films were preincubated in glycine-NaOH buffer (50 mM, pH 9, 0.025% SDS, and 10% DMSO) for at least 1 h to increase the film hydrophilicity. The pH and temperature for the degradation conditions were optimized. For pH, PET degradation was performed in PBS (pH 6, 7, and 8) and glycine-NaOH buffer (pH 9 and 10). Four temperatures (20, 30, 40, and 50 °C) were used to optimize the temperature.

For the PET degradation, engineered yeasts (4×10^7 yeast cells) were suspended in glycine-NaOH buffer (800 μ L). The pretreated PET films were added to the engineered yeast and incubated at 30 or 40 °C for 7 days. After the incubation, the reactions were stopped by adding an equal volume of 100% methanol (MeOH), followed by heating at 85 °C for 10 min. After centrifugation at 15,000g for 10 min, the degrading products (BHET, MHET, and TPA) in the supernatant were analyzed using high-performance liquid chromatography (HPLC). A Prominence LC-20 HPLC System (Shimadzu, Durham, NC) equipped with a ZORBAX Eclipse Plus C18 column (95Å, 5 μ m, 4.6 \times 150 mm) was employed to analyze BHET, MHET, and TPA.^{13,21,33} The mobile phase A consists of 0.1% formic acid, and the mobile B is composed of acetonitrile (v/v = 8:2) at a flow rate of 0.8 mL·min⁻¹. The injection volume was set to 5 μ L. For the scanning electron microscopy (SEM) image, the treated PET films underwent a cleaning procedure involving a sequential wash with 1% SDS, 20% ethanol, and distilled water. After air-drying, the films were imaged by using SEM.

To degrade postconsumer drinking water bottles, we selected two types of postconsumer PET bottles: the Great Value Purified Drinking Water bottle and the Deer Park Spring Water bottle. Four different parts of the bottles were cut into circles with a diameter of 7 mm by using a hole punch, including the shoulder, body, transition to base, and base.

2.6. Stability and Reusability of Whole-Cell Biocatalysts to Degrade PET Films. To evaluate the stability and reusability of the whole-cell biocatalysts, we conducted a series of successive degradation cycles on PET films using engineered yeasts. For each cycle, the engineered yeasts were incubated with pretreated PET films in glycine-NaOH buffer (50 mM, pH 9, 0.025% SDS, and 10% DMSO) at 30 °C for 3 days. After each degradation cycle, the engineered yeasts were separated from the reaction solution through centrifugation at 3000g for 5 min. They were subsequently rinsed with a glycine-NaOH buffer solution (50 mM, pH 9) to thoroughly remove any remaining substrate.

To obtain free PETase and MHETase fusions, the engineered yeasts (4×10^7 yeast cells) were subjected to centrifugation at 3000g for 5 min. Subsequently, they were washed and resuspended in glycine-NaOH buffer (50 mM, 800 μ L, pH 9, 0.025% SDS, and 10% DMSO) supplemented with DTT (100 μ M). The disulfide reduction reactions were gently shaken at 37 °C for 2 h to ensure the detachment of PETase and MHETase fusions from the cell walls. Afterward, the supernatant, obtained through centrifugation at 4000g for 10 min at 4 °C, was utilized for the degradation of PET films.³⁴

2.7. Quantitative Determination of PET Degradation Products. Standard curves for quantitative analysis were established using known concentrations of terephthalic acid (TPA), mono-(2-hydroxyethyl) terephthalate (MHET), and bis(2-hydroxyethyl) terephthalate (BHET). Each standard solution was injected in triplicate into the HPLC system to generate calibration curves by plotting peak area (in milliabsorbance units·seconds, mAU·s) against analyte concentration (μ M). Linear regression analysis was performed to

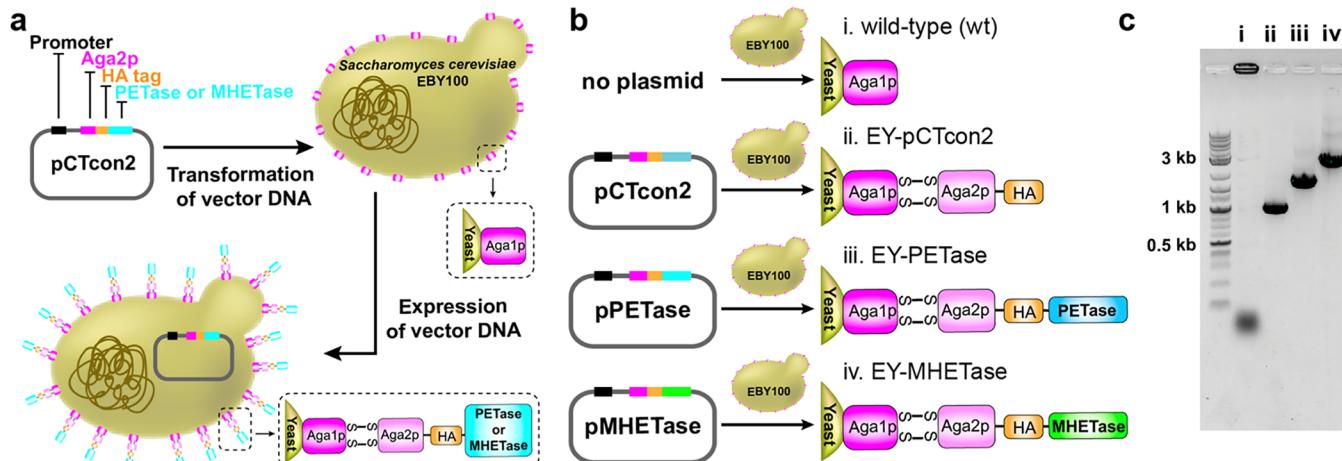


Figure 2. (a) Schematic illustration of a yeast surface to display PETase and MHETase using yeast display technology. (b) Diagrams of control plasmids (i. wild-type and ii. pCTcon2) and plastic-degrading enzyme expression plasmids (iii. pPETase and iv. pMHETase), and their corresponding displayed protein fusions. (c) Confirmation of the transformation of plastic-degrading enzyme expression and control plasmids into yeast *EBY100* using yeast colony PCR and agarose gel electrophoresis.

obtain the calibration equations for each compound. The concentrations of TPA, MHET, and BHET in experimental samples were calculated using their respective calibration equations:

$$\text{concentration } (\mu\text{M}) = \left[\frac{\text{peak area} - \text{intercept}}{\text{slope}} \right]$$

Here, the slope and intercept values were obtained from the linear regression analysis of each standard curve. All experiments were conducted with three replicates, and the results are presented as mean \pm standard deviation. Statistical analyses were carried out by using GraphPad Prism.

2.8. Determination of PET Crystallinity. The PET crystallinity of different parts from PET drinking water bottles was measured using a differential scanning calorimeter (DSC, TA-Q2000, New Castle, DE) equipped with a liquid nitrogen cooling system. The predried PET samples (around 5–6 mg) were placed in a Tzero aluminum pan, which was then sealed with a matched aluminum lid. The DSC analysis was performed by heating the sample from 35 to 300 °C at a rate of 5 °C·min⁻¹, followed by cooling from 300 to 35 °C at the same rate under a nitrogen atmosphere. The percentage of crystallinity was calculated on the first heat scan using the following equation:

$$\text{crystallinity } (\%) = \left[\frac{\Delta H_m - \Delta H_{cc}}{\Delta H_m^\circ} \right] \times 100$$

where ΔH_m is the enthalpy of melting the PET sample (J·g⁻¹), ΔH_{cc} is the enthalpy of cold crystallization (J·g⁻¹), and ΔH_m° is the enthalpy of melting 100% crystalline PET, which is 140 J·g⁻¹.^{35–37}

3. RESULTS AND DISCUSSION

3.1. Degradation of PET Plastics Using Engineered Yeasts as Whole-Cell Biocatalysts. Among several types of plastics, polyethylene terephthalate (PET) is the most popular plastic produced globally with an annual production of approximately 359 million tons in 2020.^{38,39} PET is a linear thermoplastic polyester made by a condensation reaction between two petroleum-derived small chemicals of terephthalic acid (TPA) and ethylene glycol (EG).^{40,41} Although PETase

and MHETase have been discovered to degrade PET, poor enzymatic activity limits their practical applications.

In this study, we genetically engineered yeast surfaces to display PETase and MHETase, which were then employed to degrade postconsumer drinking water bottles into high-value monomers, including TPA and EG (Figure 1a). Yeast cells were specifically selected as the host for surface display due to their distinct advantages as biocatalysts. Unlike bacterial systems, yeast possesses a mechanically robust cell wall that enhances structural stability and enables repeated use under harsh reaction conditions.⁴² This protective barrier allows yeast to tolerate a wide range of environmental stresses. Moreover, yeast offers efficient and flexible genetic engineering tools, making it well suited for constructing strains that express complex enzyme systems.⁴³

We expressed PETase and MHETase on the surface of separate yeast strains using the pCTCon2 plasmid encoding an Aga2 fusion of PETase or MHETase.⁴⁴ As shown in Figure 1b, *S. cerevisiae* EBY100 expresses Aga1 proteins (Aga1p) on the whole-cell surface, serving as anchors to link the Aga2 protein (Aga2p) coded from an expression plasmid (pCTcon2) through two disulfide bridges. The covalent bonds result in the tethering of PETase and MHETase on the yeast cell surface, generating EY-PETase and EY-MHETase.

The engineered yeasts displaying PETase and MHETase can break down PET plastics by breaking ester linkages, and the final products include TPA and EG. As shown in Figure 1c, EY-PETase can efficiently convert PET polymers into mono(2-hydroxyethyl) terephthalate (MHET), TPA, and a minor amount of bis(2-hydroxyethyl) terephthalate (BHET), and sequentially cleave BHET into MHET and EG.⁴⁵ The resulting MHET can be further degraded by EY-MHETase into TPA and EG.⁴⁵ Both PETase and MHETase act synergistically for the complete hydrolysis of PET plastics. Therefore, EY-PETase and EY-MHETase can work synergistically to recycle PET plastics into the smallest high-value units (TPA and EG).

3.2. Construction of PET-Degrading Enzyme Expression Plasmids. Due to the high surface expression and eukaryotic protein engineering mechanisms, yeast display is superior to the traditional bacterial or phage display platforms.⁴⁶ In addition, yeast can offer advantages in the low cost of

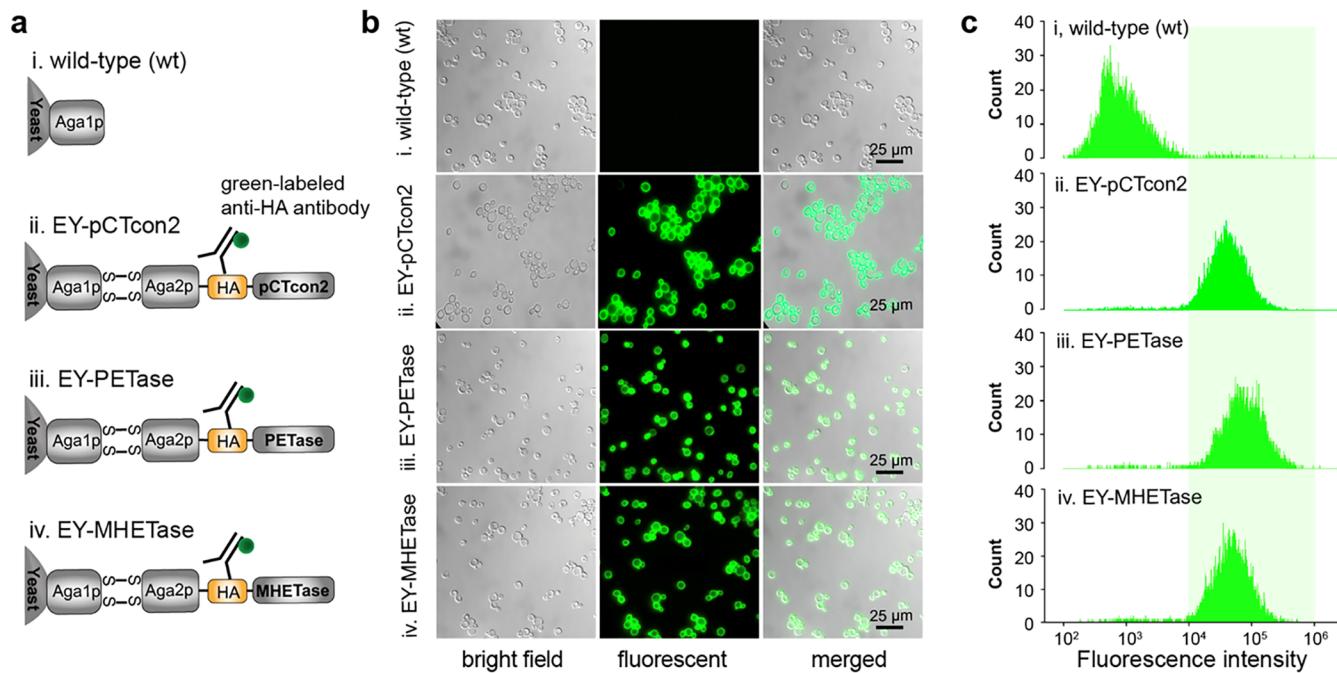


Figure 3. (a) Schematic illustration of the anti-HA Tag antibody binding assay on the yeast surface. (b, c) Confirmation of the PETase and MHETase fusion expressed on the yeast surface using confocal images and flow cytometric analysis. The fluorescence intensity was monitored at 520 nm upon excitation at 488 nm.

production as well as high stability during storage. Although different yeast strains and yeast surface proteins have been used to display heterologous proteins, yeast Aga2p of the mating protein a-agglutinin is the most commonly used.⁴⁷ As shown in Figure 2a, *S. cerevisiae* EBY100 expresses Aga1p on the whole-cell surface, serving as the anchor to link Aga2p through two disulfide bridges. The covalent binding results in the tethering of PETase and MHETase to the yeast cell surface. The gene encoding Aga1p is stably integrated into the yeast chromosome, while the gene encoding Aga2p is cloned into a yeast display plasmid vector (pCTcon2). The expression of Aga2p is controlled by a galactose-inducible promoter. To quantify and characterize the expressed PETase and MHETase on the yeast cell surface, a hemagglutinin (HA) tag is typically fused in-frame with the Aga2p coding sequence prior to either PETase or MHETase.

In previously reported studies, PETase and MHETase have shown the capability to degrade PET waste.^{15,48–52} To degrade plastics, we engineered yeast (*S. cerevisiae* EBY100) to display PETase and MHETase on yeast cell surfaces. As shown in Figure 2b, genes coding for these two PET-degrading enzymes (870 bp for PETase and 1809 bp for MHETase) were yeast-codon-optimized and then cloned into the pCTcon2 backbone in-frame after the Aga2p coding sequence and a hemagglutinin (HA) epitope tag, respectively. These plasmids were amplified in bacteria and confirmed by Sanger sequencing. The resulting PETase and MHETase expression plasmids were transformed into EBY100 competent cells and transformants were selected using SDCAA media (excluding tryptophan), generating EY-PETase and EY-MHETase.³¹ Yeast colony PCR was used to confirm the transformation of these plasmids. Wild-type yeast and the engineered yeast with the pCTcon2 plasmid (named EY-pCTcon2) were used as negative and positive controls. The expected bands for the protein fusions (976 bp for Aga2p-HA-pCTcon2, 1660 bp for Aga2p-HA-PETase, and 2599 bp for

Aga2p-HA-MHETase) were observed, and there was no band for the wild-type yeast (Figure 2c). All of these results indicated that we constructed the PET-degrading enzyme expression plasmids and transformed them into yeast cells.

3.3. Characterization of the PETase and MHETase Expression on Yeast Surfaces. Generally, there are approximately 5×10^4 copies of Aga1p on each yeast cell surface, but the covalent binding of Aga2p may be lower depending on the stability and solubility of PETase and MHETase.⁵³ To quantify and characterize the expressed ligands on the yeast cell surface, an HA tag is typically fused in-frame with the Aga2p coding sequence before PETase and MHETase. Employing confocal microscopy and flow cytometry, we specifically targeted the HA tag with fluorescently labeled anti-HA antibodies, allowing for the precise and comprehensive visualization of PETase and MHETase expression on yeast surfaces. Wild-type yeast and EY-pCTcon2 were, respectively, utilized as negative and positive controls.

To express PET-degrading enzymes on yeast surfaces, SGCAA medium was used, in which galactose induced the expression of Aga2p-HA-PETase/MHETase fusions. The expressed PET-degrading enzymes on the yeast surfaces were confirmed by labeling HA tags with fluorescently labeled (DyLight 488) anti-HA antibodies (Figure 3a). Compared to the wild-type yeast, all engineered yeasts (EY-pCTcon2, EY-PETase, and EY-MHETase) were stained green, indicating that the Aga2p-HA-pCTcon2/PETase/MHETase fusions successfully attached to the yeast surfaces (Figure 3b). Afterward, the fluorescence intensities on the yeast surfaces were analyzed using a flow cytometer. As shown in Figure 3c, the differences in fluorescence signal between wild-type yeast and engineered yeasts can be clearly observed, in which engineered yeasts have strong fluorescence intensities. All of these results indicated that PET-degrading enzymes (PETase and MHETase) have been successfully expressed on yeast surfaces.

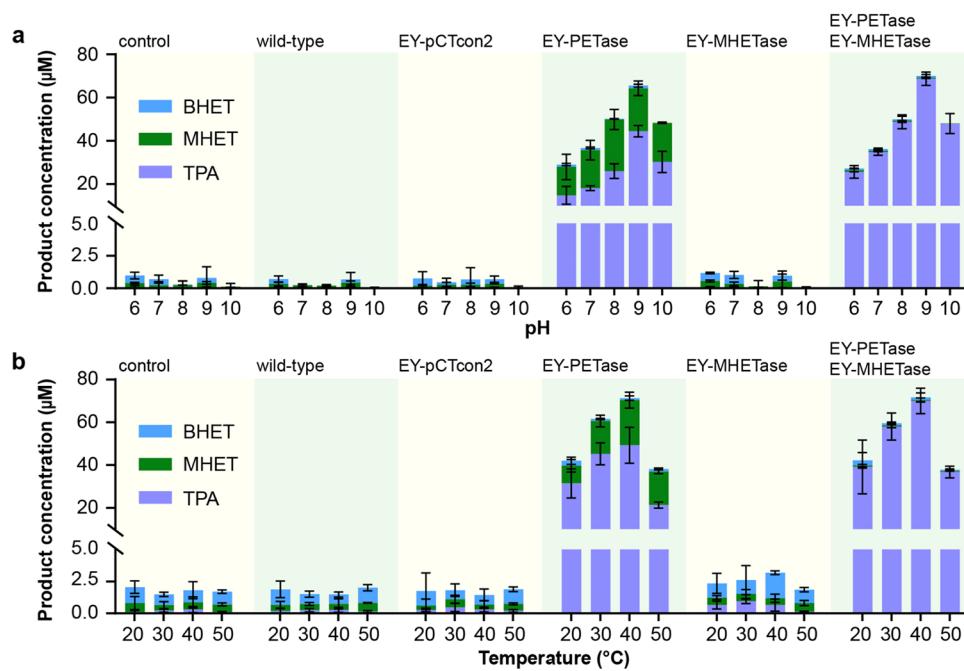


Figure 4. (a, b) Degradation of PET films occurred at different pH values (6, 7, 8, 9, and 10) and different temperatures (20, 30, 40, and 50 °C). Following hydrophilization, the film solutions were inoculated with different yeasts (no yeast control, wild-type, EY-pCTcon2, EY-PETase, EY-MHETase, and EY-PETase + EY-MHETase) at equal cell density.

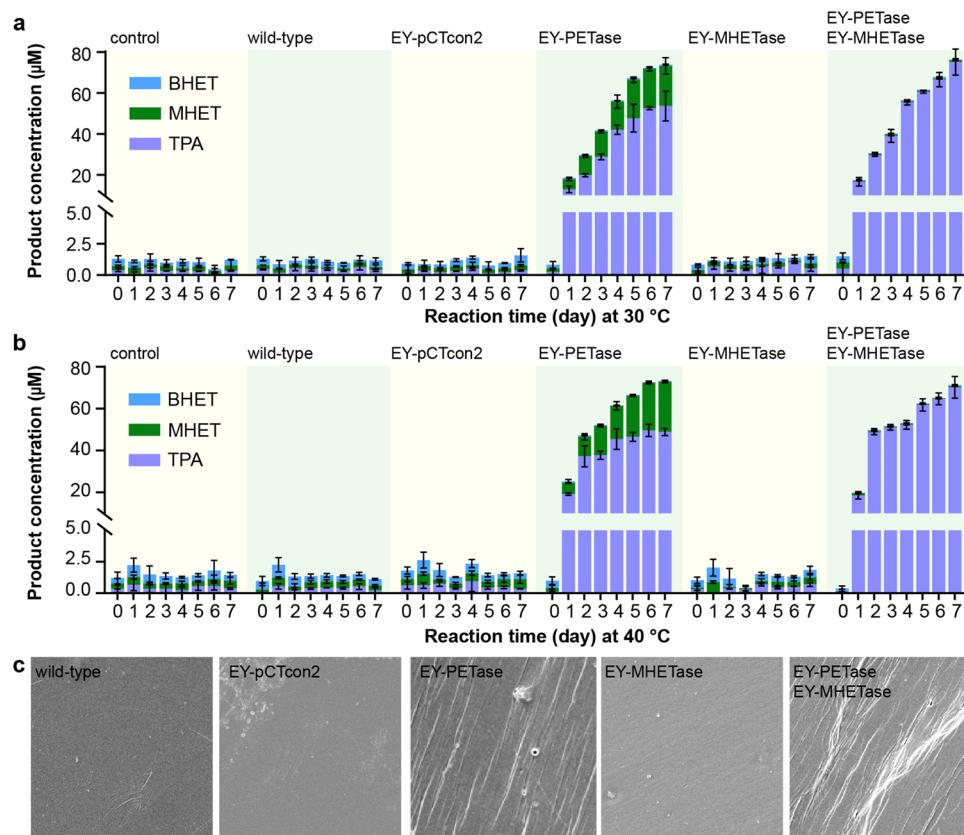


Figure 5. (a, b) Degradation of PET films using engineered yeasts was carried out for 7 days at 30 and 40 °C, including control, wild-type, EY-pCTcon2, EY-PETase, EY-MHETase, and EY-PETase + EY-MHETase. (c) The corresponding SEM images of the degraded PET films were obtained using engineered yeasts.

3.4. Optimization of the PET Degradation Conditions Using Whole-Cell Biocatalysts.

Enzymatic reactions are extremely sensitive to the conditions under which they take

place. In particular, pH and temperature play crucial roles in influencing the efficiency and rate of enzymatic activities. To maximize the efficiency of PET degradation, we identified the

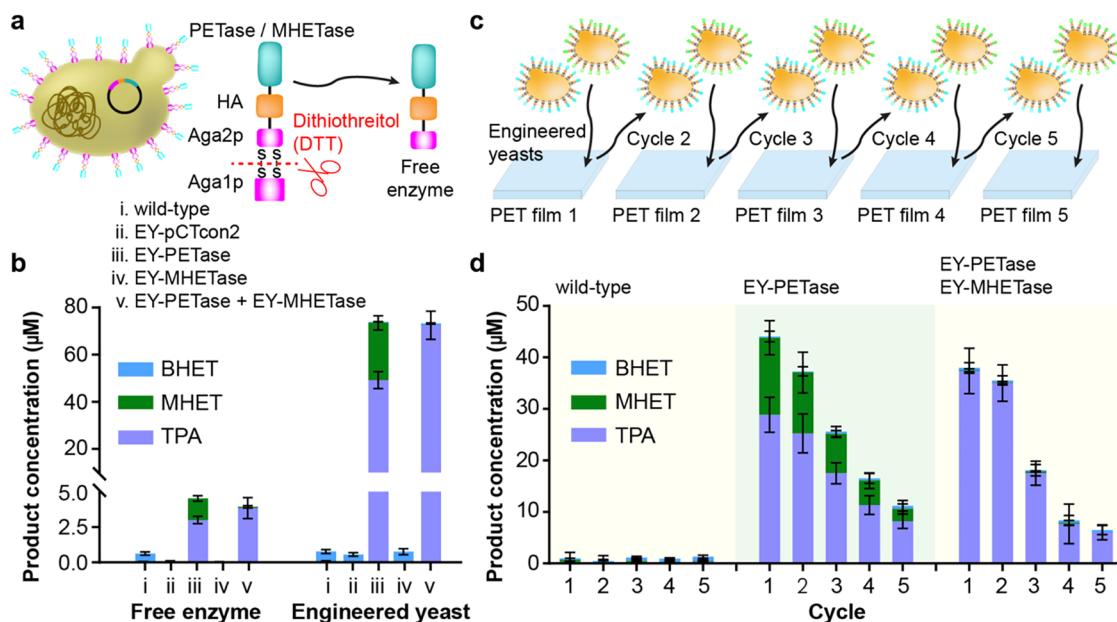


Figure 6. (a) Free PETase and MHETase were obtained using dithiothreitol (DTT) which can break the disulfide bond between Aga1p and Aga2p. (b) Comparison of the degradation of PET films between free enzymes and engineered yeasts displayed one, including control, wild-type, EY-PETase, EY-MHETase, and EY-PETase + EY-MHETase. (c) Schedule illustration of the recycling of engineered yeasts. (d) Effects of recycling times on the PET film degradation using engineered yeasts, including control, EY-PETase, and EY-PETase + EY-MHETase.

optimal pH and temperature conditions for enzymatic activities of engineered yeasts that display PET-degrading enzymes. The hydrophilization of the PET films also plays an important role in PET degradation. Following a previously reported method, the PET films were pretreated by soaking in 1% SDS, 20% ethanol, and distilled water, respectively. And then they were preincubated in glycine-NaOH buffer (50 mM, pH 9, 0.025% SDS, and 10% DMSO) for at least 1 h.

To optimize the pH, PET plastics were degraded at a range of pH values (from 6 to 10) using various engineered yeasts, including wild-type, EY-pCTcon2, EY-PETase, EY-MHETase, and EY-PETase + EY-MHETase. The reaction solution without any yeasts was used as a control. After incubating for 7 days, the resulting products (BHET, MHET, and TPA) were analyzed using HPLC. As shown in Figure 4a, a significant amount of degradation products was observed in groups containing EY-PETase (including EY-PETase and EY-PETase + EY-MHETase), whereas minimal degradation was detected in other groups. For the group of EY-PETase + EY-MHETase, less MHET was observed, as the EY-MHETase further degraded MHET into TPA and EG. The optimal pH for the degradation of plastics was 9. The optimal conditions observed for PET degradation at pH 9 align well with previous reports, indicating enhanced catalytic performance of PET-degrading enzymes such as PETase and MHETase under mildly alkaline conditions and moderate temperatures. At pH 9, catalytic residues within PETase and MHETase are likely in their optimal ionization states, facilitating efficient ester bond hydrolysis. This optimal ionization enhances the substrate binding affinity and catalytic turnover rates. Additionally, under slightly alkaline conditions, the PET surface tends to become more amorphous and hydrophilic, significantly improving enzyme accessibility to the ester linkages and thus enhancing degradation efficiency.^{35,54} Afterward, we investigated the effects of temperature on PET degradation, including 20, 30, 40, and 50 °C (Figure 4b). Similarly, we observed that the concentration of degraded

products increased from 20 to 40 °C, and declined at 50 °C. The degradation efficiency showed no significant difference between 30 and 40 °C for either EY-PETase or EY-PETase + EY-MHETase.

3.5. Degradation of PET Films Using Whole-Cell Biocatalysts.

Next, we applied the engineered yeasts to degrade PET films under optimal conditions. The pretreated PET films were incubated with the engineered yeasts at a pH of 9 and a temperature of 30 or 40 °C for 7 days. We collected samples daily throughout the reaction period and analyzed the degradation products (BHET, MHET, and TPA) using HPLC. As shown in Figure 5a,b, the EY-PETase and EY-PETase + EY-MHETase group generated more degradation products than the controls, indicating that the PETase displayed on yeast surfaces can degrade PET films. Compared to EY-PETase, less MHET was detected in the EY-PETase + EY-MHETase group, as MHET was further degraded into TPA and EG by EY-MHETase. In addition, the degradation products gradually increased as the number of days increased at 30 °C. However, there was a decrease in enzyme activity by the fourth day at 40 °C. Most products were generated in the first 2 days. This could be explained by the PETase and MHETase losing their activity with the increase of the reaction time, and the PETase and MHETase were more stable at 30 °C. The identified optimal temperature of 30 °C represents a compromise between catalytic activity and enzyme stability. While elevated temperatures generally increase reaction kinetics, they may simultaneously promote enzyme denaturation or negatively impact the stability of the expression system. Our experimental results indicate that EY-PETase and EY-MHETase maintain stable enzymatic activity at 30 °C, effectively degrading PET without notable thermal inactivation.¹⁵

The morphologies of PET films after degradation were characterized using scanning electron microscopy (SEM). Scratch-like linear structures were observed for the PET films when incubated with EY-PETase and EY-PETase + EY-

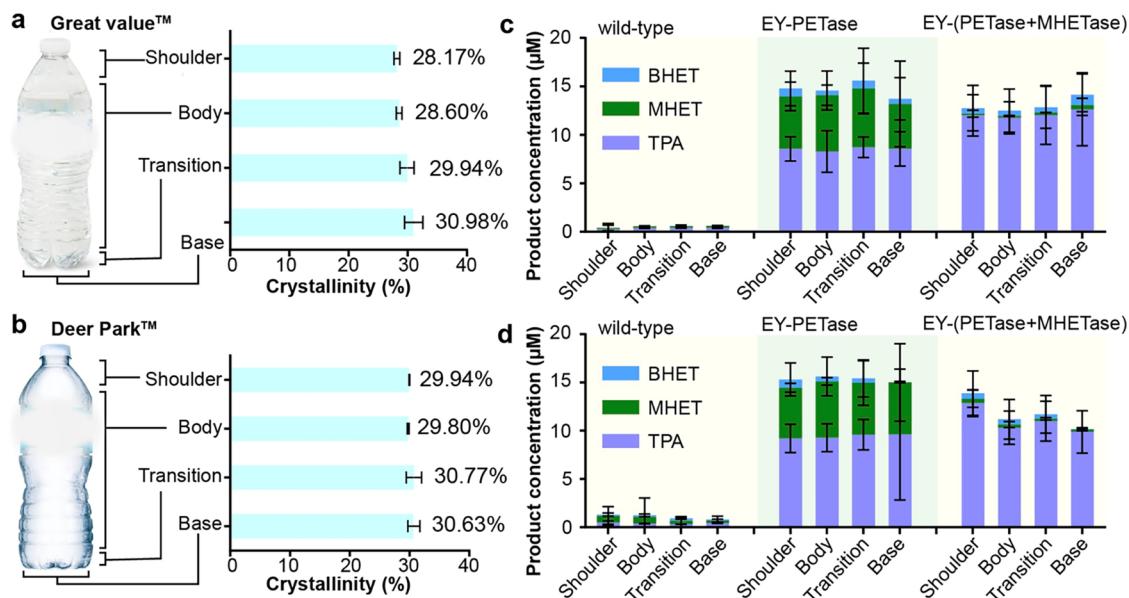


Figure 7. (a, b) Crystallinity (%) of the top-to-bottom sections of drinking water bottles (Great Value and Deer Park), including shoulder, body, transition, and base. (c, d) Degradation of drinking water bottles using engineered yeasts as whole-cell biocatalysts, including wild-type, EY-PETase, and EY-PETase + EY-MHETase.

MHETase, not in other groups (Figure 5c). This phenomenon may be the result of the processive degradation of aligned polymer fibers by EY-PETase in the surface region of the PET films. The initial cleavage resulted in the formation of shorter polymer fragments, which, in turn, increased the number of sites that could be attacked by EY-PETase and EY-MHETase. The results suggested that the engineered yeasts have the potential to degrade postconsumer PET plastics.

3.6. Reusability and Stability Assays of the Whole-Cell Biocatalysts. Enzyme immobilization is a strategy to improve the stability and efficiency of an enzyme. To test whether the enzymatic activities of PETase and MHETase displayed on yeast surfaces can be improved, we compared the performance of the PET plastic degradation between immobilized PETase and MHETase against free ones. As shown in Figure 6a, the free enzymes were obtained using dithiothreitol (DTT), which can break the disulfide bond between Aga1p and Aga2p. The same amount of PETase and MHETase from free enzymes or engineered yeasts was applied to degrade the pretreated PET films. After being incubated for 7 days under the optimal conditions, the resulting products were analyzed using HPLC (Figure 6b). Similarly, the groups containing PETase, either in free enzymes or on engineered yeast surfaces, were able to degrade PET films. In addition, the groups of EY-PETase and EY-PETase + EY-MHETase generated about 20 times more products than the free enzymes, indicating that the total activity of the immobilized enzymes on the engineered yeast surfaces has been significantly improved.

Moreover, the immobilized enzymes can be easily reused. We next investigated the durability and effectiveness of the engineered yeasts in degrading PET films. Two groups of EY-PETase and EY-PETase + EY-MHETase were tested for their reusability, and wild-type yeast was used as a control. As shown in Figure 6c, the engineered yeasts were recycled 4 times (a total of 5 cycles) after incubating with PET films for 3 days under the optimal reaction conditions. After each cycle, the products were analyzed using HPLC. The product concentrations showed a gradual downward trend through successive recycling processes

(Figure 6d). For EY-PETase, approximately 40% of the initial activity was retained by Cycle 5. For the EY-PETase + EY-MHETase group, the TPA concentration in Cycle 5 was less than 30% of the initial value. However, the overall decline in total degradation performance is likely overestimated if judged solely by MHET levels, as MHET was further converted to TPA and EG by MHETase. Therefore, the lower MHET levels in the EY-PETase + EY-MHETase group reflect enhanced degradation completeness rather than reduced enzymatic activity. These results collectively demonstrate that engineered yeast surface display enhances PETase and MHETase activity and enables enzyme reuse, making this system promising for large-scale PET biorecycling.

3.7. Degradation of PET Water Bottles Using Whole-Cell Biocatalysts. According to Euromonitor International (London, England), PET plastics for beverage bottles account for 67% of the market share, and 44.7% for single-serve bottles in the United States.⁴⁰ PET does not readily decompose when disposed of in the environment and is known for its semicrystalline property, which is distinguished by highly ordered and densely packed crystalline regions. This heightened degree of crystallinity leads to PET being extremely resistant to enzymatic degradation.^{36,55,56}

It is also important to investigate whether our engineered yeasts displaying PETase and MHETase can degrade semicrystalline postconsumer PET plastics, moving beyond amorphous films. Here, we applied our engineered yeasts to degrade two commercial drinking water bottles (Great Value and Deer Park). Because of the blow-mold expansion processing, drinking water bottles have high crystallinity, and are less susceptible to enzymatic degradation. We first measured the crystallinity of various sections of drinking water bottles, including shoulder, body, transition, and base. As shown in Figure 7a,b, all sections of the drinking water bottles have a high crystallinity (from 28 to 31%).

Next, we hole-punched the four sections of the drinking water bottles into small round punches with a diameter of 7 mm, respectively. As above, the punches were treated using 1% SDS,

20% ethanol, and distilled water and then preincubated in glycine-NaOH buffer (50 mM, pH 9, 0.025% SDS, and 10% DMSO) for at least 1 h. Under optimal conditions (a pH of 9 and a temperature of 30 °C), the pretreated punches were degraded using EY-PETase and EY-PETase + EY-MHETase for 7 days. In addition, wild-type yeasts were used as a negative control. After the incubation, the resulting monomers (BHET, MHET, and TPA) were measured using HPLC. The results showed that our engineered yeasts could degrade postconsumer PET drinking water bottles (Figure 7c,d). Although the high crystallinity of the drinking water bottles only generated TPA product at a concentration of less than 20 μM. Our engineered yeast cells successfully partially degraded two highly crystalline, postconsumer drinking water bottles into valuable monomers such as TPA and likely EG, demonstrating the practical potential of this method for recycling PET waste. This innovative approach presents a promising strategy for addressing the growing plastic waste pollution crisis and contributes to the advancement of sustainable waste management solutions.

4. CONCLUSIONS

In summary, we applied synthetic biology to genetically engineer yeast surfaces to display two PET-degrading enzymes (PETase and MHETase) that degrade PET plastics. The engineered yeasts displaying PETase and MHETase could be easily produced on a large scale through simple yeast cultivation. Afterward, the enzyme expression on the yeast surfaces was confirmed by using confocal microscopy and a flow cytometer. The optimized pH for the degradation of PET films is 9, and the temperature is 30 °C. Under the optimized degradation conditions, our developed engineered yeasts as whole-cell biocatalysts can degrade PET films at a rate more than 20 times that of free enzymes. In addition, the engineered yeasts can be reused and maintain 30% of the activity after 4 cycles to degrade PET films. Furthermore, two postconsumer drinking water bottles with high crystallinity levels were partially degraded into value-added TPA and, likely, EG monomer products using our engineered yeasts, opening a promising avenue to recycle PET plastic wastes. For real-world applications, practical strategies for recovering and reusing engineered yeast cells could include immobilization methods such as encapsulation or attachment to solid supports, facilitating easy separation and recovery from reaction mixtures. Further investigations could also explore the development of cost-effective recycling systems, such as continuous-flow bioreactors or filtration-based approaches, to maximize yeast reusability and reduce operational costs. This innovative approach offers a promising avenue for addressing the increasing challenges of plastic waste pollution and for advancing sustainable solutions.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acssynbio.5c00209>.

Strains and plasmids used in this study (Table S1); primers used in this study (Table S2); and codon-optimized DNA sequences for PETase and MHETase expression (Table S3) ([PDF](#))

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Author Contributions

J.C., C.J., and R.C.W. conceived the study and designed experiments. J.C., C.J., K.Z., and R.C.W. performed data acquisition and analysis. All authors drafted, revised, and approved the manuscript content.

Notes

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■ ABBREVIATIONS

PET	polyethylene terephthalate
PETase	polyethylene terephthalate hydrolases
MHETase	mono(2-hydroxyethyl) terephthalate hydrolase
EBY100	<i>Saccharomyces cerevisiae</i>
TPA	terephthalic acid
EG	ethylene glycol
MHET	mono(2-hydroxyethyl) terephthalate
BHET	bis(2-hydroxyethyl) terephthalate
Glu, E	glutamic acid
Lys, K	lysine
DTT	dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
PCR	polymerase chain reaction
PBS	phosphate-buffered saline
SDS	sodium dodecyl sulfate
DMSO	dimethyl sulfoxide
MeOH	methanol
HPLC	high-performance liquid chromatography
SEM	scanning electron microscopy
NaOH	sodium hydroxide
Aga1	aga1 proteins
Aga2	aga2 proteins
HA	tag hemagglutinin tag

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