

# Potential one-step strategy for PET degradation and PHB biosynthesis through co-cultivation of two engineered microorganisms

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## ABSTRACT

The management and recycling of plastic waste is a challenging global issue. Polyethylene terephthalate (PET), one of the most widely used synthetic plastics, can be hydrolyzed by a series of enzymes. However, upcycling the resulting monomers is also a problem. In this study, we designed a co-cultivation system, in which PET degradation was coupled with polyhydroxybutyrate (PHB) production. First, PETase from *Ideonalla sakaiensis* was expressed in *Yarrowia lipolytica* Polf with a signal peptide from lipase. The engineered PETase-producing *Y. lipolytica* was confirmed to hydrolyze bis(2-hydroxyethyl) terephthalate (BHET) and PET powder into the monomers terephthalate (TPA) and ethylene glycol (EG). Simultaneously, a TPA-degrading *Pseudomonas stutzeri* strain isolated from PET waste was transformed with a recombinant plasmid containing the *phbCAB* operon from *Ralstonia eutropha*, which encodes enzymes for the biosynthesis of PHB. The two co-cultivated engineered microbes could directly hydrolyze BHET to produce the bioplastic PHB in one fermentation step. During this process, 5.16 g/L BHET was hydrolyzed in 12 h, and 3.66 wt% PHB (3.54 g/L cell dry weight) accumulated in 54 h. A total of 0.31 g/L TPA was produced from the hydrolyzation of PET in 228 h. Although PHB could not be synthesized directly from PET because of the low hydrolyzing efficiency of PETase, this study provides a new strategy for the biodegradation and upcycling of PET waste by artificial microflora.

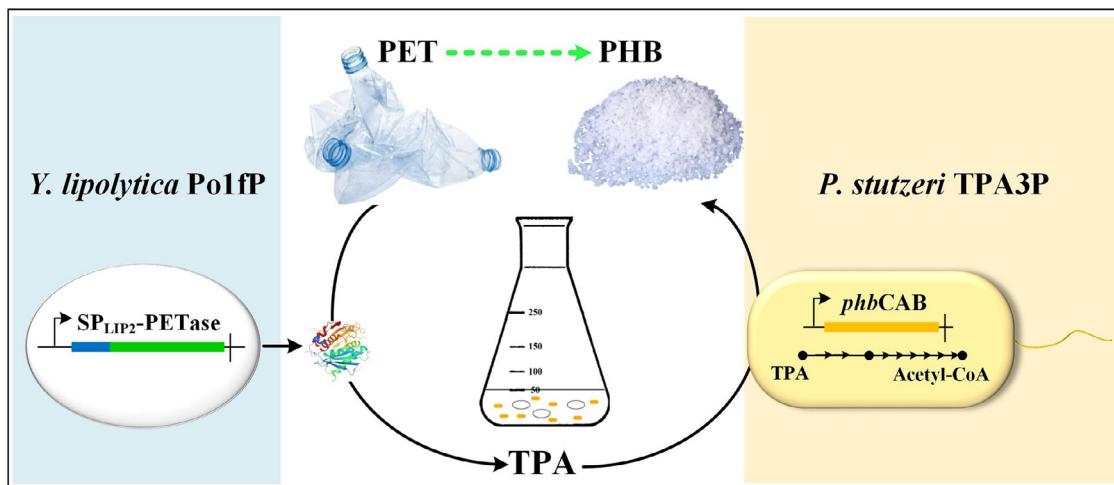
## 1. Introduction

The global annual production of polyethylene terephthalate (PET), one of the most commonly used plastics, has exceeded 30 million tons (Delle Chiaie et al., 2020). PET is mainly used to produce various single-use packaging materials, which become a major component of plastic waste after consumption (Prata et al., 2019). Traditional PET waste management methods, including mechanical recycling, landfilling, incineration, and chemical hydrolysis, have several disadvantages such as reducing mechanical properties, occupying land resources, producing hazardous substances, and high energy consumption (Rhodes, 2018). Therefore, it is necessary to develop innovative schemes for the upcycling of enormous quantities of plastic waste (Plastic upcycling, 2019). Many excellent applications inspired by natural ecosystems have been developed to date. Like the regenerative cycles for natural polymers and other waste streams, natural degradation and regeneration of plastic waste involve environmental weathering, microbial and enzymatic degradation, and the microbial digestion of degraded plastic constituents (Nikolaivits et al., 2021). Therefore, developing enhanced microorganisms to couple the depolymerization of plastic by secreted enzymes and biosynthesis of high-value products from monomers is a promising means to realize the circular economy of plastics.

Enzymatic degradation of PET has been well studied in recent years (Kawai, 2021). PET can be hydrolyzed by hydrolases such as cutinase, lipase, esterase, and carboxylesterase because of its hydrolysable chemical structure (Kawai et al., 2019; Carr et al., 2020). Some of the thermophilic cutinases show high degradation activity. For example, the cutinase from *Humicola insolens* (HiC) can hydrolyze low-crystallinity PET film, resulting in a 97% loss of weight within 96 h at 70 °C (Ronkvist et al., 2009). A leaf-branch compost cutinase (LCC) has a specific PET-degrading activity of 12 mg/h/mg enzyme at 50 °C (Sulaiman et al., 2012). The cutinase from *Thermobifida fusca* KW3 (TfCut2) can degrade semi-crystalline PET films resulting in a 12.9% loss of weight within 48 h at 65 °C in the presence of Ca<sup>2+</sup> and Mg<sup>2+</sup> (Then et al., 2015). In 2016, a mesophilic bacterium, *Ideonalla sakaiensis* was isolated from a PET-contaminated site by Yoshida et al. It can employ PETase and MHETase to depolymerize PET into terephthalate (TPA) and ethylene glycol (EG), which are further catabolized as carbon sources. PETase is more efficient than other enzymes in PET hydrolyzation at 30 °C, and its discovery is regarded as a milestone in PET biodegradation research (Yoshida et al., 2016; Joo et al., 2018; Palm et al., 2019; Yoshida et al., 2021). To increase the production of PETase, and thus improve the efficiency of converting PET into monomers, there have been many attempts to express and secrete PETase heterologously in different hosts. Huang et al. successfully engineered *Bacillus subtilis* 168 to produce functional PETase with its native signal peptide by inactivating the Tat components (Huang et al., 2018).

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**Fig. 1.** Schematic diagram of PET bioconversion through the co-cultivation of two engineered PETase- and PHB-producing microbes.

Seo et al. used *Escherichia coli* to express and secrete PETase with the Sec-dependent translocation signal peptide of maltoporin (Seo et al., 2019). Shi et al. engineered the signal peptide of pelB through random mutagenesis to improve the secretion efficiency of PETase in *E. coli* (Shi et al., 2021). However, the degradation of PET by these systems during fermentation was not studied.

In natural processes, plastic depolymerization products are reduced to CO<sub>2</sub> and water after microbial digestion, resulting in resource loss and carbon emissions. Therefore, as mentioned above, research in high-value product biosynthesis from monomers is essential to move the disposal of waste plastic toward a circular economy. It has been demonstrated that the PET monomer TPA is a suitable building block for the biosynthesis of several value-added compounds. For example, TPA from pyrolyzed or enzymatically degraded PET has been used as a feedstock for production of the bioplastic polyhydroxyalkanoate (PHA) by *Pseudomonas umerensis* GO16, which was isolated from soil exposed to PET granules (Kenny et al., 2008; Narancic et al., 2021; Tiso et al., 2021). *E. coli* also has been engineered to produce the value-added small molecule vanillin using TPA through enzymatic degradation of PET by LCC (Sadler and Wallace, 2021). These studies revealed a novel scheme for PET upcycling, which circumvents the costly purification of PET monomers. However, there have been no examples of performing the enzymatic hydrolysis of PET and the bioconversion of TPA simultaneously, which can further simplify the process and save costs.

*Yarrowia lipolytica*, one of the most extensively studied “non-conventional” and dimorphic yeasts, can naturally secrete several enzymes such as proteases, lipases, esterases, and RNases, and is currently used as a model for the study of protein secretion (Baghban et al., 2019; Park et al., 2019). Therefore, in this study we designed a co-cultivation system using *Y. lipolytica* to achieve the coupling of PET degradation and polyhydroxybutyrate (PHB) production (Fig. 1). Firstly, we engineered a PETase-expressing *Y. lipolytica* strain by fusing a PETase variant (Son et al., 2019) with the endogenous signal peptide of lipase 2. At the same time, a TPA-catabolizing strain, *Pseudomonas stutzeri*, isolated from PET waste was engineered to produce PHB from TPA. Finally, co-cultivation of the two engineered strains led to direct hydrolysis of bis(2-hydroxyethyl) terephthalate (BHET) to produce PHB. This study provides a full bioprocess strategy for the biodegradation and upcycling of PET waste.

## 2. Materials and methods

### 2.1. Strains and media

*E. coli* DH5α was cultured at 37 °C and used as the host to construct plasmids. *Y. lipolytica* Po1f was cultured at 30 °C and used as the host for

the secretory expression of PETase. *P. stutzeri* TPA3 is a TPA-catabolizing strain isolated from PET waste and was metabolically engineered to produce PHB from TPA in our previous study (Liu et al., 2021).

LB (5 g/L yeast extract, 10 g/L tryptone, and 10 g/L NaCl) was used for conventional cultivation of *E. coli* DH5α and *P. stutzeri* TPA3. YPD (10 g/L yeast extract, 20 g/L tryptone, and 20 g/L glucose) was used for conventional cultivation of *Y. lipolytica* Po1f, and Minimal SD Base (TaKaRa, China) with leucine dropout amino acid mixes was used for *Y. lipolytica* Po1f transformant selection. Mineral medium (0.85 g/L NaNO<sub>3</sub>, 0.66 g/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 5.37 g/L K<sub>2</sub>HPO<sub>4</sub>·3H<sub>2</sub>O, 2.26 g/L KH<sub>2</sub>PO<sub>4</sub>, 3.34 g/L Na<sub>2</sub>HPO<sub>4</sub>, 2.53 g/L NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O, 0.4 g/L MgSO<sub>4</sub>·7H<sub>2</sub>O, and 0.1% trace elements) with BHET and glucose was used for the co-cultivation of *P. stutzeri* TPA3P and *Y. lipolytica* Po1fP. The stock trace elements solution consisted of 5 g/L MnCl<sub>2</sub>·4H<sub>2</sub>O, 1.5 g/L CuSO<sub>4</sub>·5H<sub>2</sub>O, 50 g/L Na<sub>2</sub>EDTA, 1.0 g/L (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>·4H<sub>2</sub>O, 5.5 g/L CaCl<sub>2</sub>, 20 g/L ZnSO<sub>4</sub>·7H<sub>2</sub>O, 5.0 g/L FeSO<sub>4</sub>·7H<sub>2</sub>O, 1.61 g/L CoCl<sub>2</sub>·5H<sub>2</sub>O. If necessary, the appropriate antibiotics were added to the medium at the following concentrations: ampicillin, 100 mg/L; hygromycin (hyg) B, 1000 mg/L; and kanamycin 25 mg/L. The solid medium was supplemented with 20 g/L agar.

### 2.2. Plasmid construction and strain engineering

The DNA polymerases and restriction enzymes used in this work were purchased from TaKaRa (Beijing, China) and Thermo Fisher Scientific (Shanghai, China) respectively. Plasmids were assembled using MultiF Seamless Assembly Mix (ABclonal) according to the manufacturer's instructions. The codon optimized gene of the PETase variant (Son et al., 2019) was synthesized by GeneralBio, China. To randomly integrate the PETase gene into the *Y. lipolytica* Po1f genome (Cui et al., 2018), the target fragment was amplified using primers PETaseF and PETaseR (Table 1) which contain the homology regions with the vector pKI-hyg (Table 2). The constructed plasmid was digested with NotI and transformed into *Y. lipolytica* Po1f using the commonly used lithium acetate method (Cui et al., 2018). Transformants were selected on YPD-hyg plates.

To investigate the effect of signal peptides on the secretion of PETase, DNA fragments of SP<sub>LIP2</sub>, SP<sub>EXG1</sub>, SP<sub>XPR2</sub>, and SP<sub>RNY1</sub> (Table 3) were cloned in frame with the N-terminus of mature PETase using PCR extension with the corresponding primers (Darvishi et al., 2018). The episomal vector YLEP-leu was digested by Bsp119I and assembled with the target fragments flanked by the corresponding homologous arms. The constructed plasmids were then transformed into *Y. lipolytica* Po1f. Transformants were selected on leucine dropout plates.

**Table 1**

Primers used in this study.

Primers	Sequences (5'-3')	Purposes
PETaseF	AAGAACATTCAAAGGTTATGAACCTTCCCCGAGCCTCTGA	pKi-hyg-PETase construction
PETaseR	GCGTGACATAACTAATTACATGATTTAAGAGCAGTTGGCGGTTCGG	Reverse primer for the construction of PETase expression cassettes with different signal peptides
SP <sub>LIP2-1</sub>	TAAGAACATTCAAAGGTTATGAAGCTGTCTACTATCCG	YLEP-leu-SP <sub>LIP2</sub> -PETase construction
SP <sub>LIP2-2</sub>	ATGAAGCTGCTACTATCTGTTCACCGCCACTCTGGCCGCCCTCTGCCCTC	YLEP-leu-SP <sub>LIP2</sub> -PETase construction
SP <sub>LIP2-3</sub>	TGGCCGCCGCTCTGCCCTGCCCATCACCCCTTGAGGCTCAGACTAACCCCTACGCC	YLEP-leu-SP <sub>LIP2</sub> -PETase construction
SP <sub>EXG1-1</sub>	TAAGAACATTCAAAGGTTATGAAGCTGACTAAAGCTGG	YLEP-leu-SP <sub>EXG1</sub> -PETase construction
SP <sub>EXG1-2</sub>	ATGAAGCTGACTAAAGCTGGCCCTGGCTGAGGCTCAGACTAACCCCTACGCCAGA	YLEP-leu-SP <sub>EXG1</sub> -PETase construction
SP <sub>RNY1-1</sub>	AAGAACATTCAAAGGTTATGCAGTTCTCTGCCAAC	YLEP-leu-SP <sub>RNY1</sub> -PETase construction
SP <sub>RNY1-2</sub>	AGTTCTCTGGCCACCATCGTACCGTGGCCGCCGTCTGCCAGACTAACCCCTACGCCAGA	YLEP-leu-SP <sub>RNY1</sub> -PETase construction
SP <sub>XPR2-1</sub>	ATAAGAACATTCAAAGGTTATGAAGCTGGCTACCGC	YLEP-leu-SP <sub>XPR2</sub> -PETase construction
SP <sub>XPR2-2</sub>	ATGAAGCTGGCTACCGCTTCACCATTGACGCCGTGCTGCCAGACTAACCCCTACGCCAG	YLEP-leu-SP <sub>XPR2</sub> -PETase construction

**Table 2**

Plasmids used in this study.

Plasmids	Relevant properties	Sources
pKi-1	Integrative vector with LEU2 marker and UAS1B8-TEF promoter	Lab Stock (Cui et al., 2018)
pKi-hyg	Integrative vector with hyg marker and UAS1B8-TEF promoter	Lab Stock (Jiang et al., 2021)
YLEP-leu	Episomal vector with the LEU2 marker and UAS1B8-TEF promoter	Lab Stock (Cui et al., 2018)
pKi-hyg-PETase	pKi-hyg containing PETase with native signal peptide	This study
YLEP-leu-PETase	YLEP-leu containing PETase with native signal peptide	This study
YLEP-leu-SP <sub>HIC</sub> -PETase	YLEP-leu containing PETase with SP <sub>HIC</sub>	This study
YLEP-leu-SP <sub>LIP2</sub> -PETase	YLEP-leu containing PETase with SP <sub>LIP2</sub>	This study
YLEP-leu-SP <sub>EXG1</sub> -PETase	YLEP-leu containing PETase with SP <sub>EXG1</sub>	This study
YLEP-leu-SP <sub>RNY1</sub> -PETase	YLEP-leu containing PETase with SP <sub>RNY1</sub>	This study
YLEP-leu-SP <sub>XPR2</sub> -PETase	YLEP-leu containing PETase with SP <sub>XPR2</sub>	This study
pKi-hyg-SP <sub>LIP2</sub> -PETase	pKi-hyg containing PETase with SP <sub>LIP2</sub>	This study

**Table 3**

Amino acid sequences of the signal peptides.

Name	Protein	Source	Amino acid sequences
PETase	PETase	<i>I. sakaiensis</i>	MNFPRASRLMQAALVGLGLMAVSAAATA
LIP2	Lipase 2	<i>Y. lipolytica</i>	MKLSTILFTACATLAAALPSITPSEA
EXG1	Glucan 1,3-β-glucosidase	<i>Y. lipolytica</i>	MKLTKLVALAGAALA
RNY1	Ribonuclease T2-like	<i>Y. lipolytica</i>	MQFSLATIATVAAAVSA
XPR2	Alkaline extracellular protease	<i>Y. lipolytica</i>	MKLATAFTILTAVLA

### 2.3. Enzyme assays

*Y. lipolytica* Po1f-derived strains were cultured in YPD at 220 rpm and 30 °C for 72 h. The culture supernatants were harvested by centrifugation at 8500 rpm for 20 min at 4 °C. Before sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, the samples were concentrated 20-fold using a 10 K centrifugal filter at 4 °C. Enzyme assays for BHET were performed in 20 mM Tris-HCl (pH 7.0) with 4 mM BHET and a specific amount of crude enzyme supernatant, which corresponded to the same amount of biomass ( $V_{\text{supernatant}}/V_{\text{total}} = 1.295/\text{OD}_{600}$ ) at 30 °C. Two parallel experiments were carried out in each assay. To stop the reaction, an equal volume of acetonitrile was added to the sample. The samples were centrifuged and filtered with a 0.22 μm filter membrane, and then analyzed by high performance liquid chromatography (HPLC).

### 2.4. BHET plate transparent ring experiment

BHET powder sterilized by ultraviolet irradiation was added to high-temperature steam-sterilized mineral medium with 20 g/L glucose and the supplementary nutrients leucine and uracil, and stirred to prepare emulsified BHET medium. The medium was mixed with a melted sterile agar solution to prepare emulsified BHET plates. Strains were spread on the emulsified BHET plates for cultivation, and the formation of transparent rings was observed after 48 h.

### 2.5. PHB detection by Nile red staining

To detect the production of PHB, 0.2% (v/v) of 2 mM Nile red dye (BBI, China) dissolved in dimethyl sulfoxide was added to sterilized mineral medium containing 10 g/L TPA and 20 g/L agar (Spiekermann et al., 1999). Strains were spread on the plates for cultivation. Plates were photographed in a chemiluminescence imaging system (Clinx ChemiScope 6000 Pro). Colonies of PHB-accumulating strains fluoresce under a 530 nm fluorescent light source and 590 nm filter.

### 2.6. Production of PHB from BHET by co-cultivation of PETase and PHB-producing strains

Co-cultivation of PETase- and PHB-producing strains was carried out in mineral medium containing 10 g/L glucose, 5 g/L BHET, and the supplementary nutrients leucine and uracil at 30 °C and 220 rpm. The growth of the PETase-producing strains was characterized by counting the number of colonies on YPD plates containing ampicillin and that of the PHB-producing strains by counting the number of colonies on TPA plates without another carbon source. BHET, Mhet, and TPA were detected by HPLC, and PHB was quantified using gas chromatography (GC). Three parallel cultivations were carried out in the experiment.

### 2.7. The hydrolysis of PET by PETase-expressing *Y. lipolytica* Po1f

The hydrolysis of PET by PETase-expressing *Y. lipolytica* Po1f was carried out in mineral medium containing 20 g/L glucose and the sup-

plementary nutrients leucine and uracil at 30 °C and 220 rpm. Commercial PET powder (particle size: 300 µm, Alibaba) was sterilized by ultraviolet light for 12 h in a vertical clean bench and then added to the medium to a final concentration of approximately 10 g/L. Two parallel cultivations were carried out in the experiment.

### 2.8. Substrate and product analysis

BHET, MHET, and TPA were detected by an HPLC system equipped with an Agilent ZORBAX Extend-C18 column (4.6 × 150 mm). The mobile phase containing 0.1% (v/v) trifluoroacetic acid and 20% (v/v) acetonitrile was used as the mobile phase. The flow rate and column temperature were set to 0.8 mL/min and 40 °C, respectively. The effluent was monitored by a photodiode array detector at a wavelength of 240 nm.

When detecting EG, a Bio-Rad Aminex HPX-87H column (7.8 × 300 mm) and a refractive index detector were used. H<sub>2</sub>SO<sub>4</sub> (5 mM) was used as the mobile phase. The flow rate and column temperature were set to 0.6 mL/min and 65 °C, respectively.

PHB was detected by a GC system equipped with a Restek Rtx-5 column and an AOC-20i auto injector. The culture was centrifuged at 8000 rpm and 4 °C for 30 min to harvest the cells. The cells were weighed after lyophilization. Then, 150 µL H<sub>2</sub>SO<sub>4</sub>, 850 µL CH<sub>3</sub>OH, and 1 mL CHCl<sub>3</sub> were sequentially added to 15–20 mg cells and reacted at 100 °C for 1 h. Subsequently, the reaction flasks were removed from heat and 1 ml distilled water was added for stratification. The lower chloroform phase with the substance to be detected was taken for GC detection. The following detection procedure was used: (1) 80 °C for 1 min; (2) then ramped up from 80 °C to 120 °C at a rate of 10 °C/min, (3) then ramped up to 160 °C at a rate of 45 °C/min and held at a constant temperature for 5 min (Li et al., 2020).

## 3. Results

### 3.1. Engineering *Y. lipolytica* Po1f for the secretory expression of functional PETase

To achieve the degradation of PET, *Y. lipolytica* Po1f was used to express and secrete PETase from *I. sakaiensis* with its native signal peptide. The commercial dimer BHET, which contains two ester bonds, was selected as a representative substrate for the study of PETase activity. We observed that BHET emulsion became apparently transparent after incubating with the crude enzyme supernatant of PETase, but it was still turbid when incubated with the crude enzyme supernatant of wide-type *Y. lipolytica* Po1f or Tris-HCl buffer (Fig. 2A). This phenomenon indicated that the BHET was hydrolyzed by PETase secreted from the engineered *Y. lipolytica* Po1f. HPLC analysis showed obvious MHET formation in the reaction medium, while the BHET peak decreased, further demonstrated the hydrolysis of BHET occurred (Fig. 2B). To provide further proof for this conclusion, SDS-PAGE analysis of PETase in the culture supernatant was performed. Although *Y. lipolytica* produced many endogenous extracellular proteins, a darker band of the same size as PETase (approximately 28 kDa) was observed in the culture supernatant of the engineered *Y. lipolytica* Po1f, which indicated the secretory expression of PETase (Fig. 2C). Furthermore, when strains were cultured on emulsified BHET plates for 72 h, transparent rings formed around the colonies of PETase-expressing *Y. lipolytica* Po1f colonies, whereas there were no transparent rings around the colonies of the non-engineered strain (Fig. 2D). The results indicated that *Y. lipolytica* can express and secrete PETase, and it can hydrolyze BHET during cultivation at 30 °C.

### 3.2. Optimization of the secretory expression of PETase

To maximize the secretory expression of PETase, four endogenous *Y. lipolytica* signal peptides (SP<sub>LIP2</sub>, SP<sub>EXG1</sub>, SP<sub>XPR2</sub>, and SP<sub>RNY1</sub>) were individually fused to the N-terminus of mature PETase to mediate its

extracellular secretion. The native signal peptide SP<sub>PETase</sub> was used as a control. The amino acid sequences of the signal peptides are shown in Table 3. The episomal plasmid YLEP-leu was used for expression to reduce the impact of gene copy number. The effects of the signal peptides on the activity and secretion of PETase were evaluated. SP<sub>LIP2</sub> of the extracellular lipase encoded by the Lip2 gene resulted in the most secretion of PETase, followed by SP<sub>XPR2</sub>, SP<sub>RNY1</sub>, and SP<sub>EXG1</sub>. The native signal peptide SP<sub>PETase</sub> had the worst performance, resulting in the lowest BHET activity (Fig. 3ABC). Next, the SP<sub>LIP2</sub>-fused PETase gene was randomly integrated into the genome of *Y. lipolytica* Po1f in a homology-independent manner to generate variation in the chromosomal location and copy number, resulting in expression differences in the PETase gene. In this way, an optimized engineered strain with high extracellular PETase activity was obtained and named *Y. lipolytica* Po1fp (Fig. 3D). Interestingly, MHET produced from BHET was further decomposed to generate TPA during the enzyme assays (Fig. 3CD). However, previous studies showed that PETase has no catalytic activity on MHET (Yoshida et al., 2016; Knott et al., 2020).

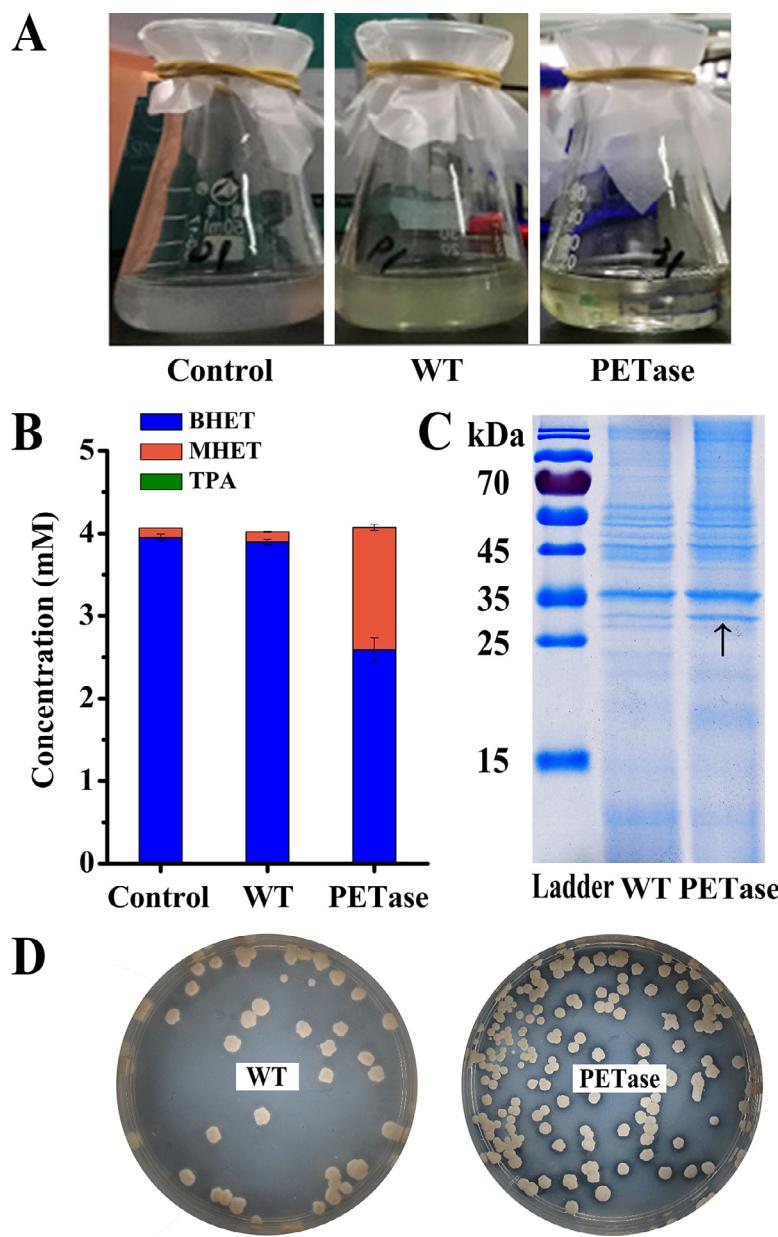
### 3.3. Production of PHB from the monomer TPA by *P. stutzeri* TPA3P

Although PETase-producing *Y. lipolytica* can hydrolyze BHET to produce TPA, TPA cannot be further metabolized by *Y. lipolytica* (Fig. S1AB). Therefore, an upcycling process should be incorporated. In our previous study, a TPA-catabolizing strain, *P. stutzeri* TPA3, was isolated from PET waste; however, this strain did not accumulate any type of PHA (Liu et al., 2021). Genomic analysis indicated that *P. stutzeri* TPA3 employs a dioxygenase (tphA<sub>2</sub>A<sub>3</sub>A<sub>1</sub>) and a dehydrogenase (tphB) to convert TPA to protocatechuate (PCA), which is subsequently metabolized to produce succinyl-CoA and acetyl-CoA through the PCA 3,4-cleavage pathway (Schlaflie et al., 1994; Choi et al., 2005; Sasoh et al., 2006). Therefore, it is possible to metabolically engineer *P. stutzeri* TPA3 to produce PHB from TPA through the heterogenous expression of *phbCAB* genes (Fig. 1). Therefore, we cloned the *phbCAB* operon from *Ralstonia eutropha* (Li et al., 2011; Kang et al., 2008). Nile red staining and GC analysis proved that the engineered strain *P. stutzeri* TPA3P can produce PHB (Fig. 4). In a shake flask, *P. stutzeri* TPA3P accumulated 11.56 wt% of cell dry weight PHB from 10 g/L TPA as the sole carbon source.

### 3.4. Co-cultivation of the two engineered strains for the production of PHB

To couple the generation of TPA from PET with PHB production, *Y. lipolytica* Po1fp was co-cultivated with *P. stutzeri* TPA3P using glucose and BHET as carbon sources. The glucose metabolism of *P. stutzeri* TPA3P was found to be weak in our trial experiments (Fig. S1F). As shown in Figure 5A, glucose was metabolized slowly during the first 12 h, and 10 g/L glucose was consumed by the two strains within 30 h. This is similar to the amount consumed when culturing *Y. lipolytica* Po1f alone (Fig. S1C). Therefore, *Y. lipolytica* Po1fp may utilize most of the glucose for growth and the secretory expression of PETase. The two strains were inoculated at an equal OD, and the initial OD value at 600 nm of the co-culture system was 0.1 for each strain. Colony count results showed that the two strains coexist well during co-cultivation (Fig. 5C). The secreted PETase completely hydrolyzed BHET to produce MHET within 12 h (Fig. 5B). Thereafter, MHET gradually decreased, while TPA and EG began to accumulate in the medium. The produced TPA provided a carbon source for the subsequent rapid growth of *P. stutzeri* TPA3P. However, the biomass of *P. stutzeri* TPA3P declined sharply in the final stage of cultivation, which may be due to the shortage of TPA and the cell sedimentation of unknown cause (Fig. 5BC). In terms of the accumulation of PHB, the cells dry weight was 5.60 g/L containing 0.22 wt% PHB at 29 h, while the values at 54 h were 3.54 g/L and 3.66 wt% (Fig. 5A). This indicated that PHB was produced from TPA by *P. stutzeri* TPA3P.

We also tested the direct degradation of PET by *Y. lipolytica* Po1fp. However, the hydrolysis efficiency was low. TPA was not detected in



**Fig. 2.** The secretory expression of functional PETase in *Y. lipolytica* Po1f. A, Observation of the hydrolysis of a BHET emulsion. B, The hydrolysates of BHET detected by HPLC. C, SDS-PAGE analysis of extracellular PETase. D, Observation of transparent rings on BHET plates. In the above experiments, Tris-HCl buffer was used as a control, WT refers to unengineered *Y. lipolytica* Po1f, and PETase refers to *Y. lipolytica* Po1f expressing PETase. Error bars in Fig. 2B indicate standard deviations from two parallel experiments.

the medium until 120 h, and the yields of TPA only reached 0.31 g/L in 228 h (Fig. 5D). No obvious accumulation of PHB was detected during the co-cultivation of *Y. lipolytica* Po1fP with *P. stutzeri* TPA3P, probably due to the low TPA accumulation.

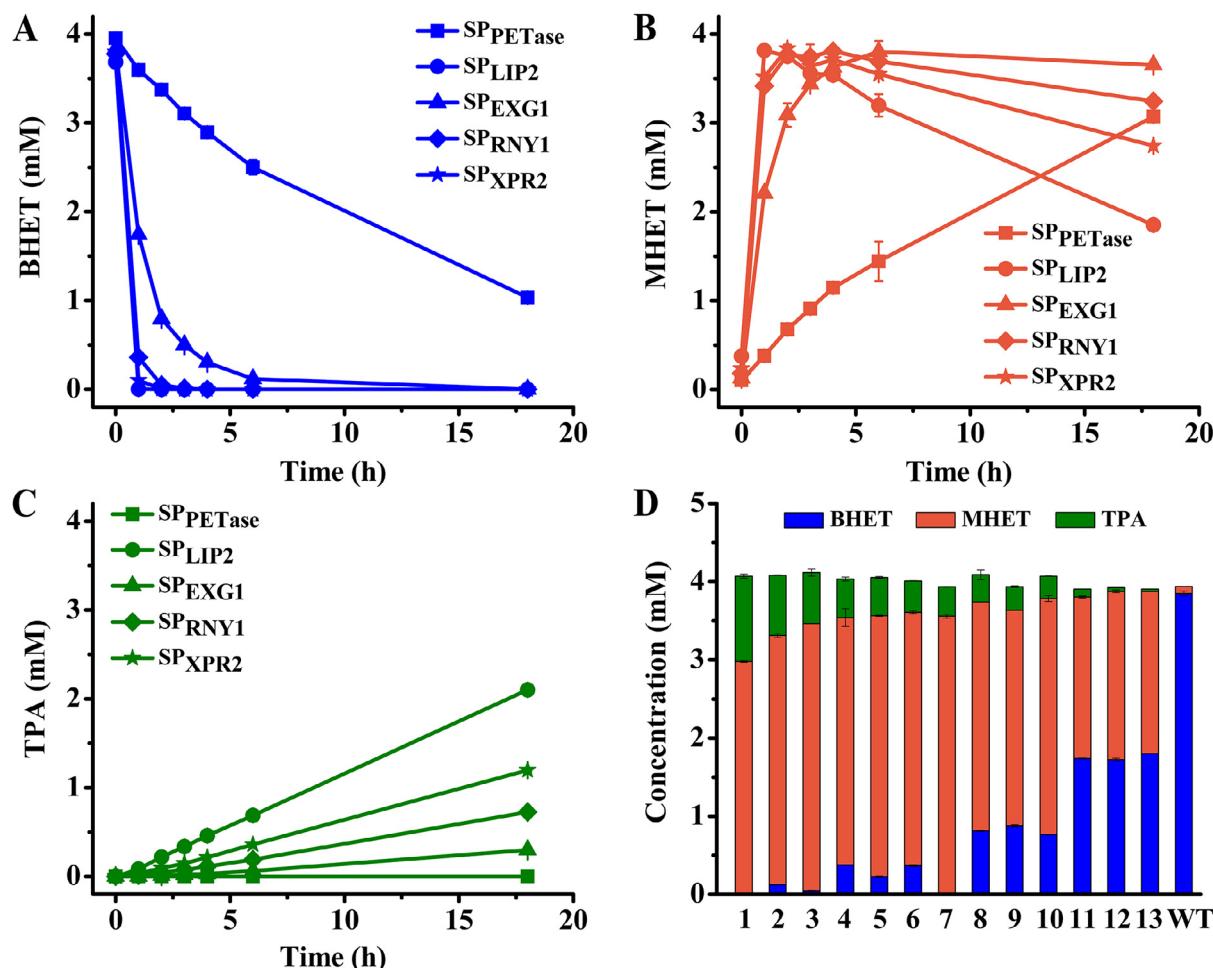
#### 4. Discussion

In this study, we successfully engineered an unconventional yeast, *Y. lipolytica*, to effectively express and secrete functional PETase. The signal peptide of an endogenous *Y. lipolytica* lipase (SP<sub>LIP2</sub>) was used to maximize protein secretion. In vitro, 3.68 mM BHET was hydrolyzed in 1 h using the crude enzyme supernatant of *Y. lipolytica* Po1f (YLEP-leu-SP<sub>LIP2</sub>PETase), while 1 mM BHET was hydrolyzed in 2 h by crude enzyme supernatant of a previously reported *E. coli* system (Shi et al., 2021). Moreover, unlike *E. coli*, the yeast expression system does not require the addition of inducers, which reduces the production cost of enzymes.

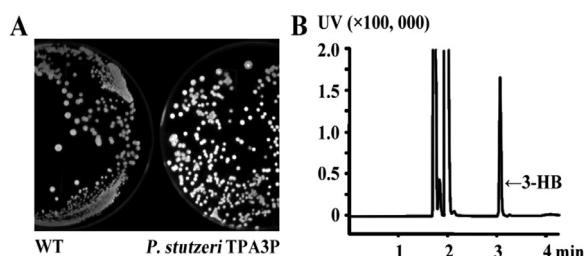
The degradation of BHET in the culture medium by the optimized strain *Y. lipolytica* Po1fP was also studied; this strain can hydrolyze

20 mM BHET within 12 h during cultivation. Interestingly, we also found that MHET, which was generated from the hydrolysis of BHET by PETase, can be further decomposed into the monomers TPA and EG. As it was previously reported that Lip7 has a higher substrate preference for shorter chain length esters (Fickers et al., 2011), we hypothesize that the hydrolysis of MHET may be due to the nonspecific reaction of endogenous extracellular lipases or esterases of *Y. lipolytica*. Thus, another advantage of using *Y. lipolytica* as a host for heterologous expression of PETase for the degradation of PET is that it does not require additional expression of MHETase.

To couple the generation of TPA with PHB production, *P. stutzeri* TPA3P, a TPA-degrading strain identified in our previous study, was engineered to produce PHB without induction and was co-cultured with PETase-expressing *Y. lipolytica*. The co-cultivation system was designed based on the fact that *Y. lipolytica* cannot utilize TPA, while *P. stutzeri* TPA3P has a very weak glucose utilization capability. Both engineered strains maintained high activity in the co-cultivation system. TPA generated from the hydrolysis of BHET by *Y. lipolytica* Po1fP was finally utilized by *P. stutzeri* TPA3P for growth and to synthesize PHB.



**Fig. 3.** Optimization of the secretory expression of PETase. A-C, Hydrolytic activity of PETase secreted by different signal peptides using BHET as a substrate. The change in the amounts of residual BHET (A) and the products MHET (B) and TPA (C) are shown over time. D, Screening for *Y. lipolytica* Po1f strains with high expression of PETase generated using homology-independent gene integration. Error bars indicate standard deviations from three independent experiments for signal peptide optimization and two independent experiments for strain screening.

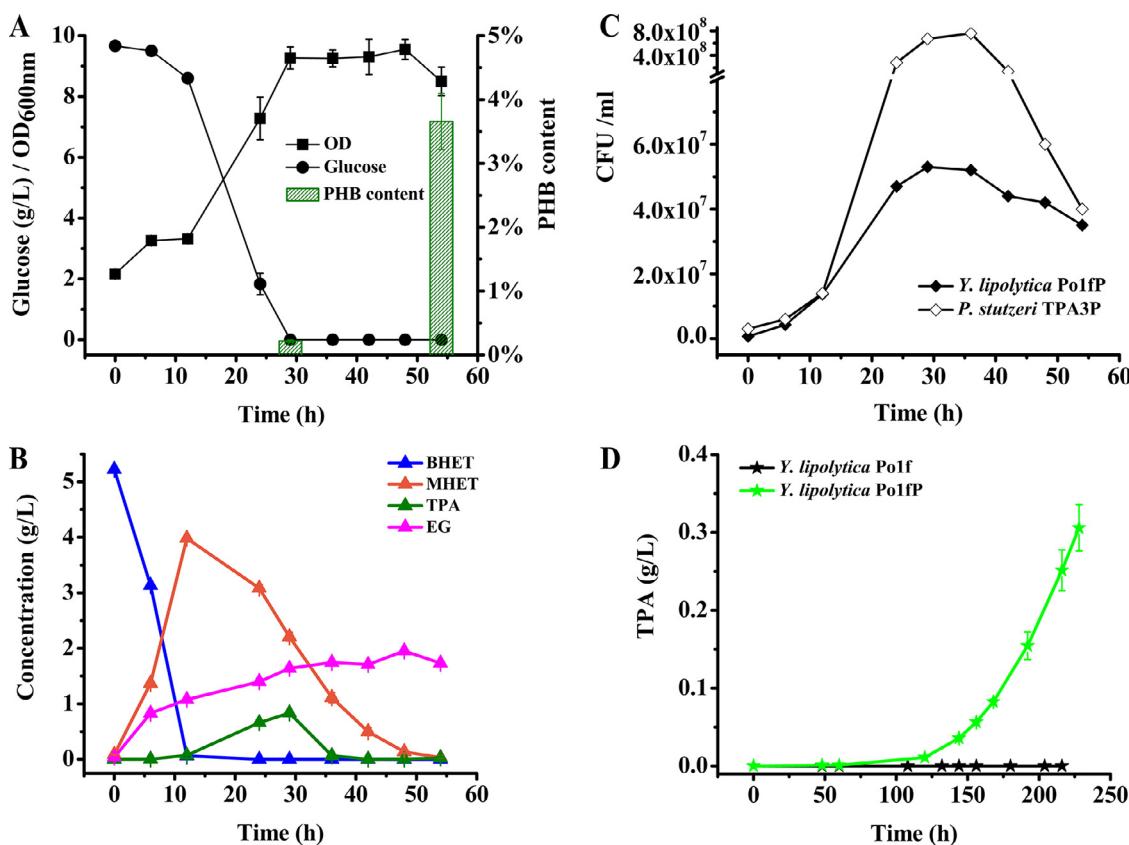


**Fig. 4.** Production of PHB from TPA by *P. stutzeri* TPA3P. A, Fluorescent Nile red staining of *P. stutzeri* TPA3P cells accumulating PHB. Cells were grown for 72 h on mineral medium (4  $\mu$ M Nile red) containing TPA. *P. stutzeri* TPA3P exhibited stronger fluorescence than the wild-type strain. B, PHB was quantified using gas chromatography. 3-HB, methyl-(R)-3-hydroxybutyrate.

However, the yield of PHB was not satisfactory. One reason is that in this proof-of-concept study, *P. stutzeri* TPA3P was not optimized with regards to PHB production. The vector pBBR1MCS-2, which was used for the heterologous expression of the *phbCAB* genes, is a medium-copy-number plasmid (Kovach et al., 1995; Perez-Pantano et al., 2000). This may limit the expression of the *phbCAB* genes, resulting in low capability of the strain to synthesize PHB. Another more important reason is that the hydrolysis of PET using PETase at room temperature is still

inefficient, resulting in TPA being produced in limited amounts that are not enough for PHB synthesis. Although many PETase mutants with improved properties have been reported, such as Ts-PETase (Zhong-Johnson et al., 2021), DuraPETase (Cui et al., 2021), and a double point mutant (Meng et al., 2021), the efficiency of PET degradation at room temperature remains the main limitation. Therefore, more work should be carried out to improve the activity, stability, and production of PETase. In addition, PET is easily hydrolyzed by enzymes at the glass transition temperature (Tournier et al., 2020; Kawai, 2021) because its amorphous region becomes flexible, which improves the accessibility of enzymes (Alves et al., 2002). Based on this consensus, many efforts have been devoted to improving the thermostability of PET hydrolases through protein engineering. One example of the great progress that has been made is that LCC variants with higher thermostability (improvement of melting temperatures ranging from 9.3 °C to 13.4 °C) depolymerized 90% of PET into monomers within 10 h at 72 °C (Tournier et al., 2020). Therefore, the development of thermophilic enzyme expression systems is also a feasible solution. For example, in 2020 Yan et al. genetically engineered *Clostridium thermocellum* to express the thermophilic cutinase LCC and degrade commercial PET films during a batch cultivation at 60 °C; more than 60% of the PET film was converted into soluble monomer feedstocks after 14 days (Yan et al., 2020).

In conclusion, we demonstrated the possibility of co-cultivating two engineered microbes to achieve the simultaneous degradation and upcy-



**Fig. 5.** Co-cultivation of *Y. lipolytica* Po1fP and *P. stutzeri* TPA3P. A, OD, glucose consumption, and PHB content. B, BHET hydrolysis curve. C, Curve showing strains growth characterized by plate colony-counting methods. D, Curve showing PET hydrolysis by *Y. lipolytica* Po1fP. Error bars indicate the standard deviations from three experiments for BHET hydrolysis and two independent experiments for PET hydrolysis.

pling of PET. Our findings indicate that synthetic biology and synthetic microflora may play an important role in the biodegradation of synthetic plastic waste in the future.

#### Declaration of Competing Interest

No conflict of interest exists in the submission of this manuscript, and manuscript is approved by all authors for publication.

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#### Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:[10.1016/j.engmic.2021.100003](https://doi.org/10.1016/j.engmic.2021.100003).

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