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Research Paper

Upcycling of PET oligomers from chemical recycling processes to PHA by microbial co-cultivation

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ABSTRACT

Polyethylene terephthalate (PET) is the most widely consumed polyester plastic and can be recycled by many chemical processes, of which glycolysis is most cost-effective and commercially viable. However, PET glycolysis produces oligomers due to incomplete depolymerization, which are undesirable by-products and require proper disposal. In this study, the PET oligomers from chemical recycling processes were completely bio-depolymerized into monomers and then used for the biosynthesis of biodegradable plastics polyhydroxyalkanoates (PHA) by co-cultivation of two engineered microorganisms *Escherichia coli* BL21 (DE3)-LCC^{ICCG} and *Pseudomonas putida* KT2440-ΔRDt-ΔZP46C-M. *E. coli* BL21 (DE3)-LCC^{ICCG} was used to secrete the PET hydrolase LCC^{ICCG} into the medium to directly depolymerize PET oligomers. *P. putida* KT2440-ΔRDt-ΔZP46C-M that mastered the metabolism of aromatic compounds was engineered to accelerate the hydrolysis of intermediate products mono-2-(hydroxyethyl) terephthalate (MHET) by expressing *Is*MHETase, and biosynthesize PHA using ultimate products terephthalate and ethylene glycol depolymerized from the PET oligomers. The population ratios of the two microorganisms during the co-cultivation were characterized by fluorescent reporter system, and revealed the collaboration of the two microorganisms to bio-depolymerize and bioconversion of PET oligomers in a single process. This study provides a biological strategy for the upcycling of PET oligomers and promotes the plastic circular economy.

1. Introduction

Polyethylene terephthalate (PET) is a thermoplastic polymer produced by the polymerization of terephthalate (TPA) and ethylene glycol (EG) (Tsironi et al., 2022). Benefiting from the excellent properties of light weight, high tensile strength, wear resistance and hardness, PET is widely used in many important fields such as textiles, packaging and biomedicine (Ji, 2013). In 2021, the global consumption of PET reached 89.3 million tons, making it one of the main consumed plastic worldwide (Li et al., 2022). However, 95% of PET products have an average lifespan of only one year (Martín et al., 2021). Disposal of post-consumer PET plastic waste has received urgent global attention.

Recycling of plastic waste, also known as plastic circular economy, is essential to improve resource utilization and alleviate plastic pollution (Zhao et al., 2022). PET is recognized as the recyclable plastic. However, the most prevalent mechanical recycling of PET is usually used in lower-grade applications because of unavoidable quality reduction (Sardon & Li, 2020). Depolymerization of PET into monomers to synthesize new plastics or high-value compounds can avoid the aforementioned problems and achieve closed-loop recycling of PET plastic. PET can be depolymerized by various chemical methods such as glycolysis, methanolysis, hydrolysis, aminolysis and ammonolysis (Han, 2019; Suhaimi et al., 2022). Glycolysis is the most cost-effective and commercially viable process, which uses excess glycol, commonly EG, to depolymerize PET into the bis-2-(hydroxyethyl) terephthalate (BHET) and PET oligomers with various molecular weights (n = 3–8), at temperatures ranging from 170 to 300 °C (Barnard et al., 2021; Chen et al., 2021; Karayannidis et al., 2002; Stoski et al., 2016). PET glycolysis has been proposed over 50 years and has been constantly updated with the goal of completely depolymerizing PET into pure BHET for subsequent use as a raw material to produce new polymer (Barnard et al., 2021). However, in this process, PET is gradually decomposed into lower fractions, first as PET oligomers, then BHET dimers, and finally BHET (Stoski et al., 2016). The formation of incompletely depolymerized oligomers is unavoidable, often considered as a by-product and discarded during the separation and purification steps (Barnard et al., 2021). As a commercial PET recycling process, more international enterprises, such as DuPont, Goodyear, Shell Polyester, Zimmer, and Eastman Kodak, have set up

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glycolysis-based commercial or demonstration units (George & Kurian, 2014). The treatment of massive oligomer by-products has become a new issue that needs to be addressed (Barnard et al., 2021).

In recent years, biotechnology has played an increasingly important role in the degradation and recycling of PET waste (Jimenez et al., 2022; Mudondo et al., 2023). PET can be completely depolymerized into TPA and EG under green and mild conditions by PET hydrolases such as *Is*PETase and LCC (Lee et al., 2023). In particular, an improved leaf-branch compost cutinase variant LCC^{ICCG} can depolymerize 90% PET into monomers within 10 h at 72 ℃ (Tournier et al., 2020). In addition, the PET hydrolysis products, TPA and EG can be further biologically converted into more diverse high-value chemicals by the engineered microorganisms (Ningthoujam, 2022). For example, Kim et al. employed *Escherichia coli* to convert TPA into five valuable products, gallic acid, pyrogallol, catechol, muconic acid, and vanillic acid, by heterologously expressing metabolic genes required for the biosynthesis pathway (Kim et al., 2019). Sadler and Wallace expressed genes of the vanillin synthesis pathway in engineered *E. coli*, converting TPA into an important bulk chemical vanillin (Sadler & Wallace, 2021). In fact, the monomer TPA can be converted into various products through the following pathways. TPA can be converted into protocatechuate by some microorganisms (Choi et al., 2005; Narancic et al., 2021; Sasoh et al., 2006) through dioxygenase TphA and dehydrogenase TphB, and then used to synthesize 2-pyrone-4,6-dicarboxylate, vanillic acid, vanillin pyrogallol, muconic acid and β-ketoadipic acid (Kang et al., 2020; Kim et al., 2019; Liu et al., 2022b; Sadler & Wallace, 2021; Werner et al., 2021). An engineered multifunctional *Pseudomonas putida* KT2440 has been reported to depolymerize PET and biosynthesize muconic acid (Liu et al., 2022b). However, enzymatic depolymerization of the post-consumer PET waste requires melting pretreatment to reduce crystallinity (Singh et al., 2021), and high reaction temperatures (72 ℃) (Tournier et al., 2020). In comparison, the PET oligomers produced by chemical depolymerization of PET waste have low molecular weight and low crystallinity, therefore can be decomposed directly by PET hydrolases under more moderate conditions (Quartinello et al., 2017; Ribitsch et al., 2011). Thus, PET oligomers waste derived from incomplete depolymerization during the PET chemical recycling processes is very promising to be directly bio-depolymerized and bio-converted into high value chemicals through well-designed microorganisms by full biological methods.

Microorganisms such as *P. putida* KT2440 can naturally produce polyhydroxyalkanoates (PHA) from intracellular metabolites acetyl-coenzyme A thioesters of various *(R)*-3-hydroxy fatty acids employing PHA synthases (PhaC) for carbon and energy storage when there is a surplus of carbon and a limitation of an essential nutrient (Mitra et al., 2022). This natural polymer that accumulates in microbial cells is an extremely alternative to petroleum-based plastics due to its superior properties, such as biocompatibility, thermostability, and resilience (Muiruri et al., 2022). Most importantly, as a biodegradable plastic, PHA can be easily degraded by microorganisms thus avoiding environmental pollution.

Therefore, this study aims to develop a one-step biological process for upcycling PET oligomers to PHA by the co-cultivation of the two engineered microorganisms. To achieve this purpose, the most commonly used prokaryotic protein-expressing host *E. coli* BL21 (DE3) was used to express and secrete the excellent PET hydrolase variant LCC^{ICCG} into the medium. The continuous depolymerization of PET oligomers by LCC^{ICCG} was evaluated during the fermentation process. Subsequently, *Is*MHETase was expressed in the *P. putida* KT2440 to accelerate the hydrolysis of intermediate products mono-2-(hydroxyethyl) terephthalate (MHET). By unlocking the catabolic pathway of TPA and EG, overexpressing the PHA synthase PhaC1, the engineered strain *P. putida* KT2440-ΔRDt-ΔZP46C-M was constructed to biosynthesize PHA using TPA and EG, the depolymerization products of PET oligomers, as the carbon source. Finally, fluorescent protein labeling was used to dynamic characterize the two microbial population ratios during the co-cultivation. This study demonstrated that PET oligomers can be effectively depolymerized by microorganisms and used for the biosynthesis of PHA in one-step fermentation process. We hypothesized that the present study may provide a new strategy for upcycling of PET oligomers.

2. Materials and methods

2.1. Strains and cultivation

E. coli DH5α, *E. coli* BL21 (DE3) and derived strains were conventionally cultivated at 37 ℃ with shaking at 220 rpm in LB medium (5 g/L yeast extract, 10 g/L tryptone, and 10 g/L NaCl). *P. putida* KT2440 and derived strains were conventionally cultivated at 30 ℃ with shaking at 220 rpm in LB medium. For growth characterization, PET depolymerization and monomers bioconversion, the cultivation was performed in mineral medium (17.11 g/L $Na_2HPO_4{\cdot}12H_2O$, 3 g/L KH_2PO_4, 1 g/L NH_4Cl, 0.5 g/L NaCl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 1 mL/L trace elements) supplemented with carbon sources. The stock trace elements solution consisted of 50 g/L Na_2EDTA, 20 g/L $ZnSO_4{\cdot}7H_2O$, 1.61 g/L $CoCl_2{\cdot}5H_2O$, 5 g/L $MnCl_2{\cdot}4H_2O$, 5.5 g/L $CaCl_2$, 1.0 g/L $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 5.0 g/L $FeSO_4{\cdot}7H_2O$, 1.5 g/L $CuSO_4{\cdot}5H_2O$. The solid medium was supplemented with 20 g/L agar. If necessary, the appropriate antibiotics (50 μg/mL kanamycin or 100 μg/L ampicillin) were supplemented to the medium for plasmid maintenance.

2.2. Construction of plasmids and strains

The plasmids and strains used in this study can be found in Table S1. Oligonucleotide primers were synthesized by TsingKe, China, and were shown in Table S2. DNA amplifications for plasmid constructions were performed via polymerase chain reaction (PCR) with Phanta Max Super-Fidelity DNA Polymerase (Vazyme, China). Plasmids were assembled with MultiF Seamless Assembly Mix (ABclonal, China) according to the manufacturer's instructions and directly transformed into *E. coli* DH5α chemically competent cell (TsingKe, China) for plasmid maintenance. DNA fragments, plasmids and colonies were confirmed by Sanger sequencing (TsingKe, China).

For PET hydrolases expression, codon-optimized LCC^{ICCG}, FAST-PETase, *Is*MHETase genes were synthesized by GeneralBio, China. LCC^{ICCG} was expressed and secreted in *E. coli* BL21 (DE3) under the control of T7 promoter by pET26b without a need of signal peptide. FAST-PETase was expressed and secreted in *E. coli* BL21 (DE3) under the control of T7 promoter and PelB signal peptide by pET22b. *Is*MHETase was expressed and secreted in *P. putida* KT2440 derived strains by pBBR1MCS-2 with a P_{lac} promoter and the native signal peptide. The C-terminus of LCC^{ICCG} was linked with 6 × His tags for the next protein purification by nickel column chromatography. Green fluorescent protein (GFP) and red fluorescent protein (mCherry) (Jiang et al., 2022) were constitutively expressed by the expression cassettes concatenated behind LCC^{ICCG} and *Is*MHETase, respectively. For construction of the *P. putida* KT2440 derived strains producing PHA from TPA and EG, gene insertion and deletion were performed via two-step recombination using the vector pK18mobsacB as described in previous work (Liu et al., 2022b).

2.3. Expression, purification and concentration determination of extracellular LCC^{ICCG}

E. coli BL21(DE3)-LCC^{ICCG} was grown at 37 ℃ in LB medium with appropriate antibiotics to an optical density of 0.8 at 600 nm. IPTG was added at a concentration of 0.2 mM to induce the expression. After cultivation for another 20 h at 37 ℃, culture supernatant was harvested by centrifugation at 12,000 rpm for 30 min. The his-tagged proteins in the supernatant were purified using Ni-NTA Agarose (Qiagen, Hilden, Germany) by gravity-flow Ni-affinity chromatography. The NTA–Ni

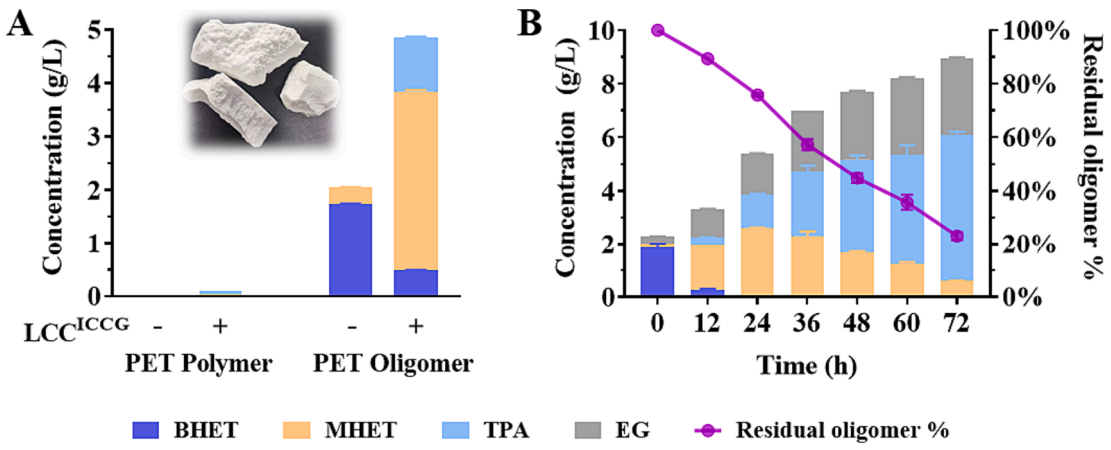

Fig. 1. Bio-depolymerization of PET oligomers. A, Hydrolysis of PET oligomers and PET polymers by purified LCC^{ICCG} at 30 °C. The reaction was performed for 12 h in 100 mM phosphate buffer with a PET content of 10 g/L and 3 mg_{enzyme}/g_{PET}. Error bars represent the standard deviation of two independent experiments. B, Depolymerization of PET oligomers by *E. coli* BL21 (DE3)-LCC^{ICCG} during cultivation at 30 ℃. The reaction was carried out at a PET oligomer content of 10 g/L. Error bars represent the standard deviation of two independent experiments.

agarose column was equilibrated with the binding buffer (20 mM Tris-HCl, pH 8.0, 300 mM NaCl), non-target proteins were washed off with the washing buffer (20 mM Tris-HCl, pH 8.0, 300 mM NaCl, 20 mM imidazole), target proteins were eluted with elution buffer (20 mM Tris-HCl, pH 8.0, 300 mM NaCl, 250 mM imidazole). The protein concentration was determined by Enhanced BCA Protein Assay kit (Biyuntian, Beijing, China) according to the instructions.

2.4. Enzymatic depolymerization of PET polymer and PET oligomer by purified LCC^{ICCG}

Amorphous PET polymer film was purchased from Goodfellow (Bad Nauheim, Germany) and PET oligomer waste was provided by Shuye Environmental Protection Technology Co., Ltd. (Guangzhou, China). Amorphous PET polymer film and PET oligomers were micronized and screened with a 35-mesh sieve prior to the enzymatic depolymerization. Enzyme assays were performed in 1 mL 100 mM phosphate buffer (pH 8.0) containing 10 mg PET powder and 30 μg purified LCC^{ICCG} at 30 ℃ for 12 h. The reaction was stopped by mixed with an equal volume of acetonitrile.

2.5. PET oligomer depolymerization in culture system

For the depolymerization of PET oligomer by individual cultivation of *E. coli* BL21 (DE3)-LCC^{ICCG} either co-cultivation of *E. coli* BL21 (DE3)-LCC^{ICCG} and *P. putida* KT2440-ΔRDt-ΔZP_{46}C-M, the cultivation was performed in 50 mL mineral medium containing 10 g/L glycerol and 10 g/L PET oligomers at 30 °C and 200 rpm. Both strains, *E. coli* BL21 (DE3)-LCC^{ICCG} and *P. putida* KT2440-ΔRDt-ΔZP_{46}C-M, were inoculated at the beginning of cultivation with an OD_{600} of 0.2. Regularly sampling to detect the concentration of glycerol and PET hydrolysis products.

2.6. PET hydrolysates and glycerol determination

EG and glycerol were determined by HPLC (LC-20AD, Shimadzu, Japan) equipped with a refractive index detector and a Bio-Rad Aminex HPX-87H column (7.8 × 300 mm) as described in previous work (Liu et al., 2022b). Bis-2-(hydroxyethyl) terephthalate (BHET), mono-2-(hydroxyethyl) terephthalate (MHET) and TPA were determined by HPLC equipped with a photodiode array detector at 240 nm and an Agilent ZORBAX Extend-C18 column (4.6 × 150 mm) as described in previous work (Liu et al., 2022b). In the calculation of the residual PET oligomers, BHET and MHET were considered oligomer components. The total amount of samples added initially (10 g/L) minus the mass of TPA and EG was the initial oligomer content and was considered as a residual ratio of 100%. Subsequently, PET oligomers were hydrolyzed to produce TPA and EG. However, the reduction in PET oligomers should deduct the mass of H_2O. Wherein, the molecular weight of OH^- was deducted from the molecule of TPA, and the molecular weight of H^+ was deducted from the molecule of EG. Thus, the formula for calculating the percentage of residual PET oligomers (P_n) was as follows:

$$P_n(\%) = 1 - \frac{(TPA_n - TPA_0) \times \frac{M_{TPA}-M_{OH}}{M_{TPA}} + (EG_n - EG_0) \times \frac{M_{EG}-M_H}{M_{EG}}}{10 - TPA_0 - EG_0}$$

where TPA_0 and EG_0 refer to the initial concentration of TPA and EG at 0 h; TPA_n and EG_n refer to the concentration of TPA and EG measured after enzymatic hydrolysis; M_{TPA}, M_{EG}, M_{OH} and M_H are the molecular weights of TPA (166.13 g/mol), EG (62.07 g/mol), OH (17 g/mol) and H (1 g/mol), respectively.

2.7. PHA production and determination

For PHA production, the cultivation was performed in mineral medium (as mentioned above) supplemented with carbon sources in condition of 30 ℃ and 220 rpm. To test the modified *P. putida* KT2440 strains with increased PHA-production, equimolar TPA (~16.61 g/L) and EG (~6.03 g/L) was supplemented as the carbon sources. For the depolymerization of PET oligomer to produce PHA in culture system, 10 g/L glycerol and 10 g/L PET oligomers were used as the carbon sources. When the TPA and EG supplemented or produced from PET oligomer depolymerization were completely consumed, the cells were collected by centrifuging at 8000 rpm and 4 ℃ for 30 min and then lyophilized in vacuum to extract and analyze PHA. Approximately 15–20 mg lyophilized cells were subsequently treated with 150 μL of H_2SO_4, 850 μL of CH_3OH, and 1 mL of $CHCl_3$ at 100 ℃ for 1 h. Then 1 mL ddH_2O was added, mixed and stratified, the $CHCl_3$ layer was analyzed by Gas Chromatography (GC) system (Shimadzu, Japan) equipped with a Restek Rtx-5 column and an AOC-20i auto injector as described in previous work (Liu et al., 2021). The produced monomers were further identified by comparing their GC spectra with that of the authentic standards in GC analysis.

2.8. Fluorescence characterization

For fluorescence intensity of the cell population, the culture of strains expressing fluorescent protein was centrifugated and re-suspended with mineral medium and then added to a 96-well plate. The cell density (OD_{600}), green fluorescence (excitation at 485 nm and

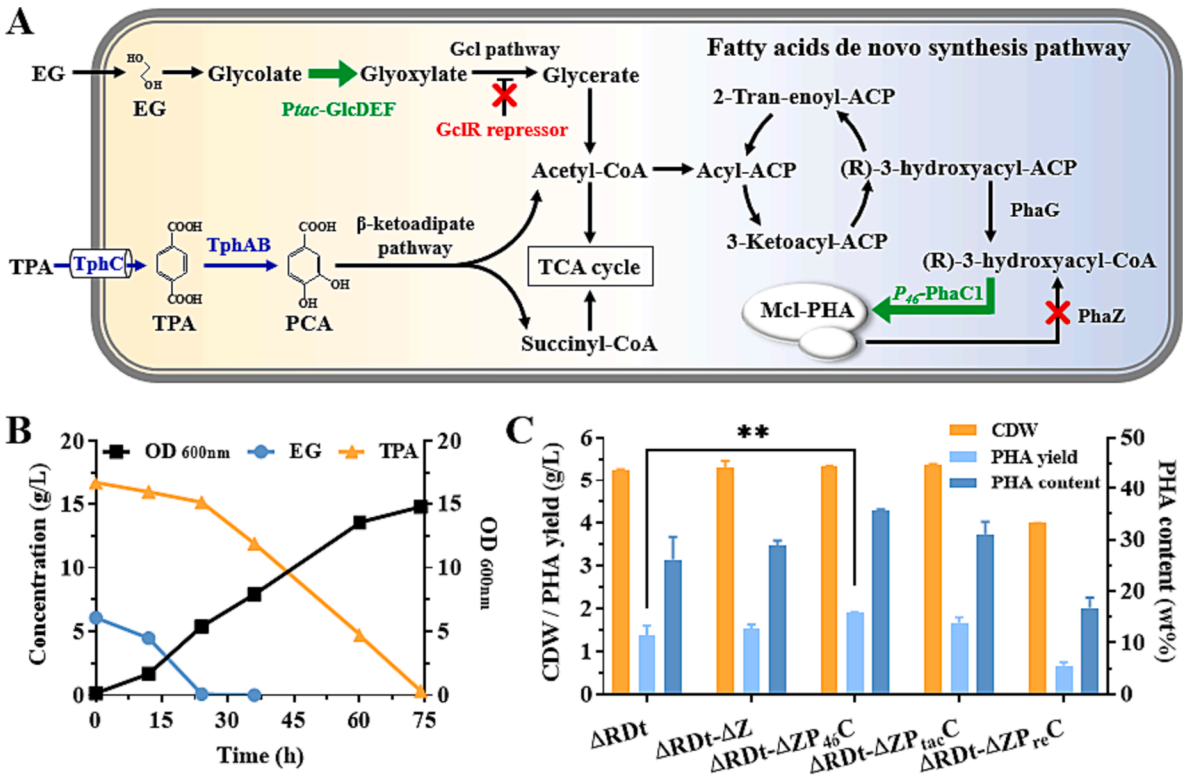

Fig. 2. Engineering of *P. putida* KT2440 to synthesize PHA from TPA and EG. A, Design of the metabolic pathway for the synthesis of PHA from TPA and EG. GlcDEF, glycolate oxidase; GclR, repressor of *gcl* operon; TphA, TPA 1,2-dioxygenase; TphB, 1,2-dihydroxy-3,5-cyclohexadiene-1,4-dicarboxylate dehydrogenase; PhaG, 3-hydroxyacyl-ACP:CoA transacylase; PhaZ, depolymerase; PhaC1, PHA synthase. Dark-green arrows represent the overexpression of endogenous genes; Light-blue arrows represent the expression of heterologous genes; Red arrows represent gene knockout. B, Growth curves of *P. putida* KT2440-ΔRDt in mineral medium with 16.67 g/L TPA and 6.08 g/L EG as the only carbon source at 30 °C, and 200 rpm. Error bars represent the standard deviation of three independent experiments. C, PHA accumulation by *P. putida* KT2440-ΔRDt and the derivatives after 72 h cultivation. ΔRDt-ΔZ, Deletion of *pha*Z in ΔRDt; ΔRDt-ΔZP_{46}C, Overexpression of *pha*C1 using P_{46} promoter in ΔRDt-ΔZ; ΔRDt-ΔZP_{tac}C, Overexpression of *pha*C1 using P_{tac} promoter in ΔRDt-ΔZ; ΔRDt-ΔZP_{re}C, Overexpression of *pha*C1 using P_{re} promoter in ΔRDt-ΔZ. Growth conditions: Strains were cultured in 50 mL mineral medium containing 100 mM TPA and EG in 300 mL shake flask, at 30 °C, and 200 rpm. Error bars represent the standard deviation of three independent experiments. ** represents $p < 0.01$ using two-tailed *t* test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

emission at 528 nm) and red fluorescence (excitation at 585 nm and emission at 640 nm) were determined using a Multi-Detection Microplate Reader (Synergy HT, Biotek) (Gu et al., 2020).

For fluorescence microscope observation, the strains expressing fluorescent protein were centrifugated and re-suspended with mineral medium, then observed using a positive fluorescence microscope (Eclipse 80i, Nikon Corp., Tokyo, Japan) at a magnification of × 100 (Gu et al., 2023).

3. Results and discussion

3.1. Engineering *E. coli* BL21 (DE3) to secrete PET hydrolase and depolymerize PET oligomers

We found LCC^{ICCG} can be efficiently transferred from cytoplasm to the medium without any signal peptide sequence, which is highly facilitated for the direct depolymerization of PET oligomers in the fermentation broth. This phenomenon is most likely attributed to its phospholipid hydrolysis activity, which led to membrane permeation (Shirke et al., 2018). Therefore, LCC^{ICCG} was expressed in *E. coli* BL21 (DE3) under the control of P_{T7} promoter. Protein purification analysis showed that the concentration of PET hydrolase LCC^{ICCG} in the fermentation broth reached around 50 mg/L after 16 h of expression.

The bio-depolymerization of PET oligomer and polymer was firstly compared using purified LCC^{ICCG}. It can be observed that the original white and brittle PET oligomer solid gradually reduced as the reaction processes, indicating the rapid depolymerization by LCC^{ICCG} (Fig. 1A). After 12 h of reaction, the content of MHET and TPA in the hydrolysate increased from the original 0.12, 0.03 to 3.36, 1.01 g/L (Fig. 1A). The total amount of PET oligomer hydrolysis products (the sum of BHET, MHET and TPA) increased from the initial 2.06 to 4.85 g/L (Fig. 1A). Whereas there was almost no increase in the hydrolysis products from PET oligomers in the control group without the addition of LCC^{ICCG}.

Moreover, the amorphous PET polymers with high molecular weight were hardly decomposable under the same reaction conditions. The total amount of aromatic products from PET polymer hydrolysis in the same condition was only 0.10 g/L (Fig. 1A). Similar to the previously reported results (Lu et al., 2022), PET polymers can only be effectively depolymerized by PET hydrolases after decrystallization pretreatment and under the reaction temperature above 50 ℃ (Lu et al., 2022), which exceed the cultivation temperature of most microorganisms. In contrast, we found that PET oligomers were highly susceptible to enzymatic degradation at room temperature even without any pretreatment, providing the potential to directly decompose in the medium in parallel with the secretion of PET hydrolase. Andressa et al. added PET oligomers or BHET to the culture medium to induce the expression of lipase in *Yarrowia lipolytica* and then depolymerize these substrates. 200 g/L BHET was completely degraded within 32 h, generating MHET and TPA (da Costa et al., 2020).

We also determined the depolymerization of PET oligomers by *E. coli* BL21(DE3)-LCC^{ICCG} in a culture system. As the culturing time increased, the PET oligomers were hydrolyzed and the depolymerization products gradually accumulated (Fig. 1B). Meanwhile, the proportion of terminal depolymerization products TPA and EG constantly increased, indicating

that the PET oligomers tended to be completely depolymerized (Fig. 1B). After 72 h of cultivation at 30 ℃, 76.9% of the PET oligomers were depolymerized and yielded 0.61 g/L MHET, 5.48 g/L TPA and 2.87 g/L EG (Fig. 1B). During this process, BHET was almost fully hydrolyzed within 24 h, and no longer accumulated. The terminal depolymerization products TPA and EG yielded a total of 8.35 g/L, accounting for 93.2% of the total hydrolysis products from 10 g/L PET oligomers. A variant of *Is*PETase, FAST-PETase, outperforming various PET hydrolases at mild temperatures was also used in an engineered coculture system. However, due to its low production (~4.2 mg/L) when expressing by *E. coli* BL21, its performance in direct degrading PET oligomers in culture is inferior to that of LCC^{ICCG} (Fig S1).

3.2. Engineering *P. putida* KT2440 to biosynthesize PHA from the PET monomers

P. putida KT2440 was selected as the chassis to synthesize biopolyester PHA from the PET monomers TPA and EG. Firstly, the native EG metabolism of *P. putida* KT2440 was enhanced by deletion of *gcl*R gene coding a transcriptional repressor protein against the *gcl* gene cluster responsible for glyoxylate metabolism, and overexpression of glycolate oxidase coding gene *glc*DEF by a constitutive P_{tac} promoter as described in previous study (Fig. 2A) (Liu et al., 2022b). Furthermore, TPA metabolism of *P. putida* KT2440 was achieved by chromosomal insertion of *tph* gene cluster responsible for the conversion of TPA to protocatechuate (Fig. 2A) (Elmore et al., 2017). The final engineered strain *P. putida* KT2440-ΔRDt can grow rapidly in mineral medium containing TPA and EG as the only carbon sources, and the OD_{600} reached 14.77 within 74 h, whereas the wild-type *P. putida* KT2440 was not able to grow with TPA or EG as the sole carbon source (Fig. 2B, Fig S2). 6.08 g/L EG and 16.67 g/L TPA were completely consumed within 24 h and 74 h, respectively (Fig. 2B). It is worth mentioning that, the TPA catabolic rate of *P. putida* KT2440-ΔRDt reached 0.22 g/(L·h), 14.40% higher than that of *P. putida* TDM461 (Werner et al., 2021), which represented the fastest metabolic rate of TPA among the reported *P. putida* KT2440 derived strains.

The engineered strain *P. putida* KT2440-ΔRDt accumulated 1.37 g/L PHA from 16.67 g/L TPA and 6.08 g/L EG (approximately an equal molar quantity of 100 mM, simulating PET hydrolysis products), the PHA content was 26.10% of CDW (Fig. 2C). Furthermore, *P. putida* KT2440 contains PHA depolymerase, which can cause the degradation of PHA and reduce the production (Cai et al., 2009). After deletion of the endogenous PHA depolymerase gene *pha*Z, the accumulation of PHA reached 28.85% of CDW and 1.53 g/L, increasing by 11.68% and 10.54% respectively (Fig. 2C). In addition to blocking the PHA depolymerase, overexpression of PHA synthase is also believed to increase PHA production (Zhang et al., 2021). Therefore, the PHA synthase gene *pha*C1 from *P. putida* KT2440 was overexpressed by replacing its promoter with a commonly used strong promoter P_{tac}, an endogenous promoter P_{46} (Liu et al., 2022a), and a heterologous promoter P_{re} of *phb*CAB gene from *Ralstonia eutropha*, respectively. Among the derived strains, *P. putida* KT2440-ΔRDt-ΔZP_{46}C with the endogenous promoter P_{46} can accumulate 35.64% of CDW and 1.90 g/L PHA, which increased by 38.69% and 36.55% compared with the original strain *P. putida* KT2440-ΔRDt (Fig. 2C). We optimized the concentration of NH_4Cl and culture temperature to maximize the PHA production, the initial condition of 1 g/L NH_4Cl and 30 °C was proven to be most favorable for PHA production (Fig S3).

3.3. Bio-depolymerize PET oligomers to synthesize PHA by co-cultivation

To direct synthesize PHA from PET oligomers in one pot, we attempted to co-culture the two engineered microorganisms. The growth of *E. coli* BL21 (DE3) and *P. putida* KT2440 using different carbon sources (glucose, glycerol, xylose, citrate, lactate, and acetate) was firstly evaluated in 24-well plates at 30 ℃ (Fig S4). *E. coli* BL21 (DE3) can rapidly grow with glycerol, whereas *P. putida* KT2440 had a delayed growth (Fig S4). Therefore, glycerol was selected as the co-feeding carbon source to support the production of LCC^{ICCG} by *E. coli* BL21 (DE3)-LCC^{ICCG}, and *P. putida* KT2440 would not competitively consume glycerol in the preliminary period of culture (Fig S4).

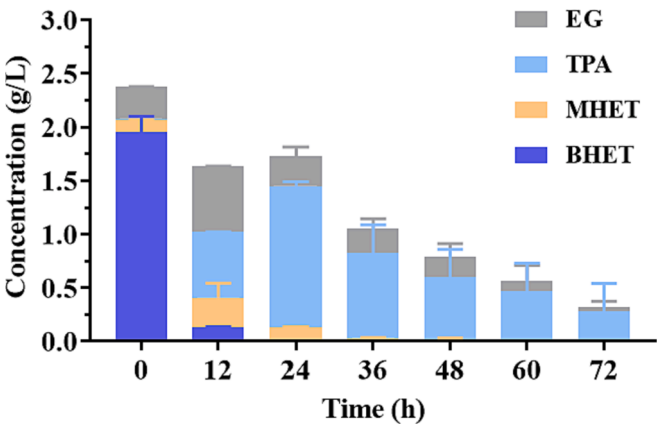

Fig. 3. The concentration of PET oligomer hydrolysis products (BHET, MHET, TPA and EG) during co-cultivation of the two engineered microorganisms. The cultivation was performed in 50 mL mineral medium containing 10 g/L glycerol and 10 g/L PET oligomers at 30 °C and 200 rpm. Both strains, *E. coli* BL21 (DE3)-LCC^{ICCG} and *P. putida* KT2440-ΔRDt-ΔZP_{46}C-M, were inoculated at the beginning of cultivation with an OD_{600} of 0.2.

In the PET oligomer depolymerization experiment, we found that the accumulation of MHET, an intermediate of PET depolymerization, reached 2.62 g/L at 24 h, even higher than that of TPA (Fig. 1B). Yoshida et al. have identified an *Is*MHETase from *Ideonella sakaiensis* that specialized in hydrolyzing MHET to TPA and EG (Yoshida et al., 2016). Since MHET was proven as an inhibitor for LCC in the PET depolymerization (Barth et al., 2016), the helper enzyme *Is*MHETase was expressed in a second chassis *P. putida* KT2440-ΔRDt-ΔZP_{46}C to facilitate the hydrolysis of MHET. During the co-cultivation of the two engineered microorganisms in 10 g/L PET oligomers and glycerol, we monitored the depolymerization products from PET oligomers in the medium. When individual cultivation of *E. coli* BL21 (DE3)-LCC^{ICCG} and co-cultivation *E. coli* BL21 (DE3)-LCC^{ICCG} with *P. putida* KT2440-ΔRDt-ΔZP46C-R harboring empty vector pBBR1MCS-2, the amount of MHET accumulate obviously and reached a maximum of 2.62 g/L and 2.86 g/L at 24 h, respectively. While, the amount of MHET maintained at a low level and the maximum is only 0.28 g/L at 12 h during the co-cultivation of *E. coli* BL21 (DE3)-LCC^{ICCG} and *P. putida* KT2440-ΔRDt-ΔZP46C expressing *Is*MHETase by pBBR-*Is*MHETase (Fig S5). The results indicated that expression of *Is*MHETase in *P. putida* KT2440-ΔRDt-ΔZP_{46}C-M accelerated the hydrolysis of MHET. The hydrolysis of PET oligomer may be further enhanced by optimizing the enzyme-substrate interfacial interactions, such as fusing the carbohydrate-binding module on the LCC (Chen et al., 2023). In addition, the total amount of PET oligomer hydrolysis products also remained at a low level (even less than the initial 2.5 g/L). Whereas the individual cultivation of *E. coli* BL21 (DE3)-LCC^{ICCG} constantly accumulated to 8.35 g/L, suggesting that depolymerization products from PET oligomers were directly assimilated by the second engineered strain *P. putida* KT2440-ΔRDt-ΔZP_{46}C-M (Fig. 3).

The yield of PHA reached 1.10 g/L without the optimization of fermentation conditions, accounting for 22.66% of the dry weight of the mixed cells from the two microorganisms (Table 1). Gas chromatography analysis showed that the monomers composition of PHA produced from the PET oligomers was mainly 3-hydroxydecanoate and 3-hydroxyoctanoate, accounting for 60.88% and 23.67%, respectively (Table 1). The high proportion of these medium-chain length monomers in the produced biopolymer provides higher breaking elongation, which is suitable for advanced applications such as elastomers (Vermeer et al.,

Table 1
Biosynthesis of PHA by individual cultivation and co-cultivation.

Cultivation mode	Individual cultivation	Co-cultivation
Strains	*P. putida* KT2440-ΔRDt-ΔZP$_{46}$C-M	*E. coli* BL21 (DE3)-LCC^{ICCG} and *P. putida* KT2440-ΔRDt-ΔZP$_{46}$C-M
Carbon sources	16.61 g/L TPA + 6.21 g/L EG	10 g/L PET oligomers
CDW (g/L)	5.34 ± 0.02	4.87 ± 0.05
PHA content (wt%)	35.64 ± 3.05%	22.66 ± 0.28%
PHA yield (g/L)	1.90 ± 0.16	1.10 ± 0.02
Conversion rate (g/g)	0.08	0.11
PHA composition (mol%)		
3-Hydroxybutyrate	0.78 ± 0.00%	0.54 ± 0.04%
3-Hydroxyhexanoate	3.94 ± 0.08%	6.47 ± 0.11%
3-Hydroxyoctanoate	25.32 ± 0.48%	23.67 ± 0.03%
3-Hydroxydecanoate	59.42 ± 0.14%	60.88 ± 0.31%
3-Hydroxydodecanoate	8.48 ± 0.30%	7.09 ± 0.17%
3-Hydroxytetradecanoate	2.06 ± 0.12%	1.34 ± 0.05%

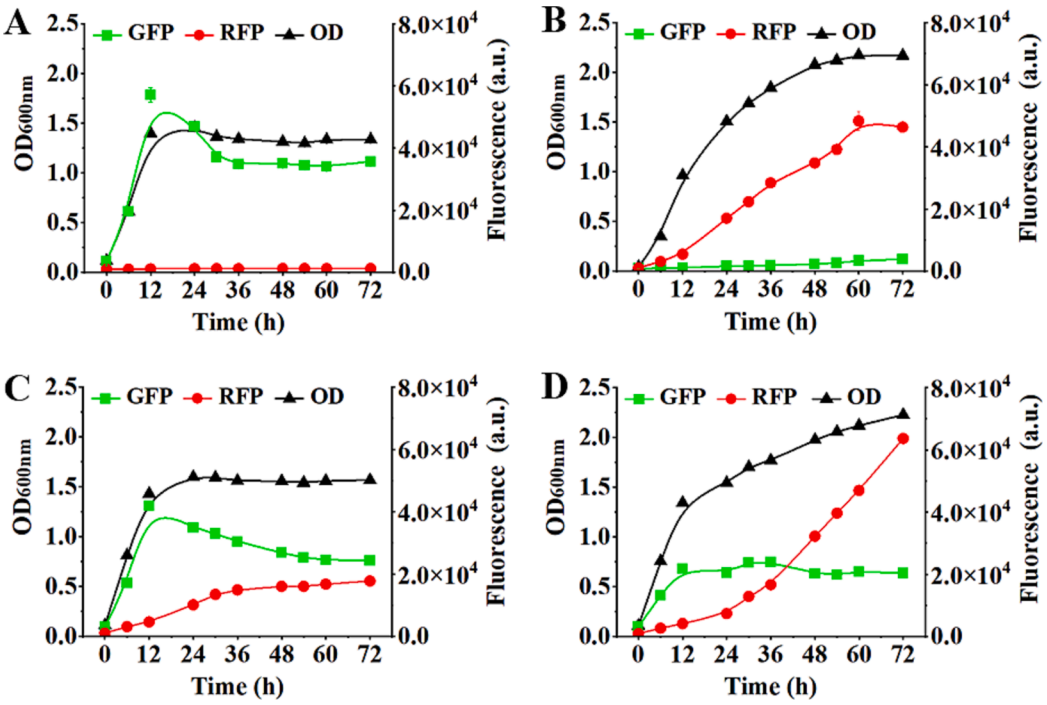

Fig. 4. The expression of fluorescent proteins by the engineered strains. A, Individual cultivation of *E. coli* BL21 (DE3)-LCC^{ICCG}-GFP using glycerol as the carbon source; B, Individual cultivation of *P. putida* KT2440-ΔRDt-ΔZP46C-M-mCherry using TPA and EG as the carbon sources; C, Co-cultivation of the two engineered strains using glycerol as the carbon source; D, Co-cultivation of the two engineered strains using glycerol, TPA and EG as the carbon sources. Error bars represent the standard deviation of two independent experiments.

2023). The yield and composition of PHA produced from co-cultivation were both similar to that of individual cultivation of *P. putida* KT2440-ΔRDt-ΔZP$_{46}$C using TPA and EG as carbon sources (Table 1), indicating that co-cultivation does not affect the PHA production. Several strains have been used to synthesize PHA from PET or PET monomers. Kenny et al. employed *P. putida* GO16 to produce PHA from PET pyrolysis products, the yield reached 0.06 g_{PHA}/g_{TPA}, corresponding to 0.03 g_{PHA}/g_{PET} (Kenny et al., 2008). An engineered *P. putida* KT2440 can synthesize 0.06 g mcl-PHA from per g of EG (Franden et al., 2018). In our previous study, 0.02 g PHB was produced from 1 g BHET through the co-cultivation of engineered *Yarrowia lipolytica* and *Pseudomonas stutzeri* (Liu et al., 2021). In this study, the conversion rate reached 0.11 $g_{PHA}/g_{oligomer}$, which is the currently highest level for PHA biosynthesis using PET or PET monomers as substrate.

3.4. Characterization of the dynamic population ratios during the co-cultivation

In order to characterize the dynamic population ratios of the two engineered strains during co-cultivation, the green fluorescent protein (GFP) and red fluorescent protein (mCherry) were respectively expressed in the engineered *E. coli* and *P. putida*. As a result, the biomass of the two strains can be assessed by the intensity of the corresponding fluorescent proteins.

In individual cultivation, the green fluorescence signal was consistent with the growth trend of *E. coli* BL21 (DE3)-LCC^{ICCG}-GFP using glycerol as the carbon source (Fig. 4A), and the red fluorescence signal was consistent with the growth trend of *P. putida* KT2440 ΔRDt-ΔZP46C-M-mCherry using TPA and EG as the carbon sources (Fig. 4B). Meanwhile, the low background fluorescence of the two strains enabled no crosstalk between the two fluorescent proteins, indicating that the biomass changes of the two microorganisms during co-cultivation could be evaluated by the intensity of the different fluorescence signals (Fig. 4A and 4B). When co-cultivation of the two microorganisms using glycerol as the sole carbon source, the green fluorescence increased rapidly, and the red fluorescence increased slowly, indicating that glycerol was mainly used for the growth of *E. coli* BL21 (DE3)-LCC^{ICCG}-GFP (Fig. 4C). However, when TPA and EG were added into the medium, the red fluorescence increased rapidly at a later stage, indicating that TPA and EG supported the growth of *P. putida* KT2440 ΔRDt-ΔZP46C-M-mCherry (Fig. 4D).

Then, the fluorescent reporter system was used to characterize the dynamic population ratios of two microorganisms during co-cultivation for PHA biosynthesis using PET oligomers as substrates. It was shown

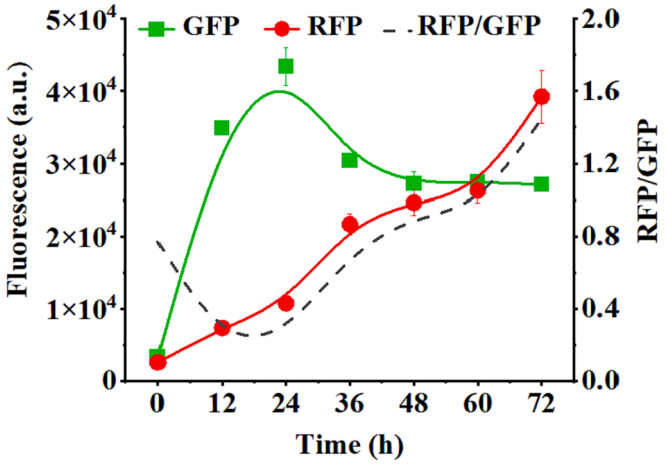

Fig. 5. Characterization of the dynamic population ratios during the synergistic depolymerization of PET oligomers to synthesize PHA by the two strains (*E. coli* BL21 (DE3)-LCC^{ICCG}-GFP and *P. putida* KT2440-ΔRDt-ΔZP46C-M-mCherry). Error bars represent the standard deviation of two independent experiments.

that green fluorescence expressed by the *E. coli* increased rapidly in the first 24 h, and then decreased to a constant level. Whereas the intensity of red fluorescence from *P. putida* KT2440-ΔRDt-ΔZP46C-M-mCherry was obviously lower than that of green fluorescence at the initial 24 h of co-cultivation, but could be constantly increased until the hydrolysates of PET oligomers were completely consumed (Fig. 5). The ratio of red fluorescence to green fluorescence decreased first and then increased around 24 h (Fig. 5), indicating that glycerol was mainly used by *E. coli* BL21 (DE3)-LCC^{ICCG}-GFP, while *P. putida* KT2440-ΔRDt-ΔZP46C-M-mCherry grew slowly with glycerol, and continued to grow later supported by PET hydrolysates TPA and EG.

The fluorescent protein signals have been frequently used to assess the population ratios during co-cultivation (Jiang et al., 2022), but the native differences among various fluorescent proteins, such as maturation time and expression intensity, may influence the results. We also observed the population ratios of the two strains in different periods of co-cultivation at individual level by fluorescence microscopy. Cells with green and red fluorescence could be clearly distinguished under the fluorescence microscope, and the most of cells showed green fluorescence at the beginning, whereas the number of red fluorescence cells increased with the duration of co-cultivation (Fig. 6). This trend was consistent with the fluorescence signal change at the population level. The dynamic population ratios were consistent with our design that *E. coli* BL21 (DE3)-LCC^{ICCG}-GFP preferentially uses glycerol to grow and secrete PET hydrolase LCC^{ICCG} to depolymerize PET oligomers, and then *P. putida* KT2440-ΔRDt-ΔZP46C-M-mCherry uses the depolymerization products TPA and EG to grow and biosynthesis of PHA.

4. Conclusion

This study constructed two engineered microorganisms and designed a co-cultivation scheme for upcycling PET oligomers to synthesize biopolyester PHA. The bio-depolymerization of PET oligomers and biosynthesis of PHA were achieved simultaneously in a one-step biological process with the cooperation of the two artificial microorganisms. The present work demonstrates that PET oligomers can be effectively depolymerized by microorganisms and used for the biosynthesis of high-value compounds, which will contribute to the circular economy of PET plastics. It can be expected that the yield of PHA will be further increased by subsequent fermentation process optimization. Furthermore, when implement it in real operation, a scale-up experiment using a reactor is necessary.

CRediT authorship contribution statement

Pan Liu: Conceptualization, Methodology, Resources, Data curation, Writing – original draft, Writing – review & editing. **Yi Zheng:** Resources, Data curation. **Yingbo Yuan:** Validation, Visualization. **Yuanfei Han:** Validation, Visualization. **Tianyuan Su:** Data curation, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition. **Qingsheng Qi:** Writing – original draft, Writing –

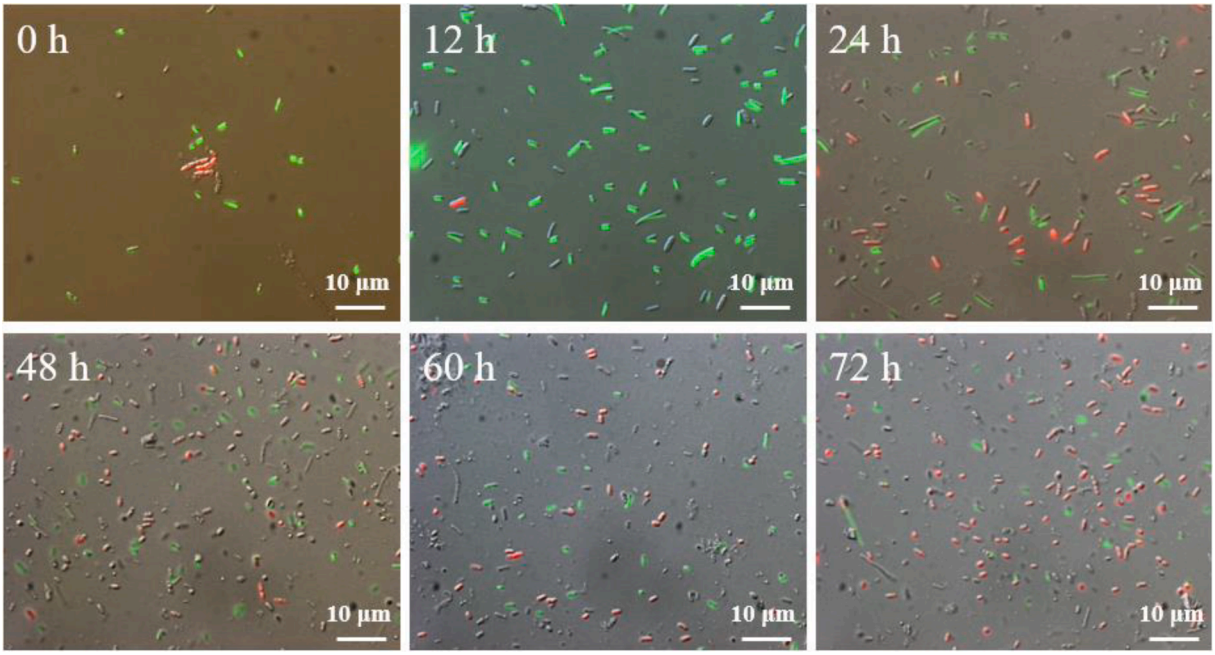

Fig. 6. Population ratios of the two strains at different periods of co-cultivation. The samples were appropriately diluted and observed under fluorescence microscope. Cells with green fluorescence were *E. coli* BL21 (DE3)-LCC^{ICCG}-GFP. Cells with red fluorescence were *P. putida* KT2440-ΔRDt-ΔZP46C-M-mCherry. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.wasman.2023.08.048.

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