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Metabolic engineering of *Pseudomonas putida* KT2440 for valorization of terephthalic acid to produce catechol and muconic acid

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ABSTRACT

Polyethylene terephthalate (PET) is one of the most widely used synthetic plastics, yet its environmental persistence necessitates sustainable end-of-life management strategies. Microbial upcycling of PET-derived monomers, particularly terephthalic acid (TPA), into high-value chemicals represents a promising alternative to conventional recycling. In this study, *Pseudomonas putida* KT2440 was engineered as a modular bioconversion platform for efficient TPA valorization into aromatic products, including catechol and muconic acid (MA). Introduction of the *Comamonas* sp. E6 *tphBAabc* operon combined with deletion of *pcaHG* in the chromosome of *P. putida* enabled conversion of TPA into protocatechuate (PCA). However, limited native TPA uptake was found. To overcome this bottleneck, nine TPA transporters were screened, identifying *Acinetobacter baylyi* ADP1 MucK^{mut} and *Rhodococcus opacus* TpaK as superior transporters that enhanced TPA assimilation. Using these transporters, catechol biosynthesis was enabled by plasmid-based expression of *Enterobacter cloacae aroY* and chromosomal deletion of *catA1* and *catA2*. Notably, the *tpaK*-expressing strain achieved catechol production of 8.51 mM with an 86.0% molar yield. Extension of the pathway toward MA through plasmid-based expression of *aroY, catA1* and *catA2*, and chromosomal deletion of *catB* and *catC* enabled efficient MA production, reaching 8.45 mM MA with a 93.1% molar yield. In addition, TPA derived from chemically depolymerized post-consumer PET was effectively utilized for catechol production. Furthermore, fed-batch fermentation validated process scalability, achieving 15.04 mM catechol from 16.76 mM TPA. Collectively, this work demonstrates that recombinant *P. putida* KT2440 can be constructed as a robust chassis for sustainable PET upcycling through implementation of an efficient TPA transporter.

1. Introduction

Synthetic nondegradable polymers such as polyethylene terephthalate (PET), polyethylene (PE), and polypropylene (PP) have become indispensable in modern society. Among them, PET—composed of terephthalic acid (TPA) and ethylene glycol (EG) as monomers—is one of the most widely consumed synthetic polymers due to its excellent mechanical strength, chemical stability, and low cost, supporting its extensive use in beverage bottles, packaging materials, and textile fibers [1]. However, improper management of post-consumer PET has led to its accumulation in terrestrial and marine ecosystems, where fragmentation generates microplastics that pose serious ecological and human health risks [2,3]. Accordingly, the need for sustainable strategies to mitigate the environmental impact of PET waste has become increasingly urgent.

To tackle these challenges, upcycling via open-loop recycling—the conversion of plastic waste into higher-value products—has emerged as a promising strategy to alleviate the growing plastic pollution crisis and

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to foster a circular plastics economy [4,5]. Among various upcycling approaches, biological upcycling offers an environmentally friendly alternative by harnessing engineered microbes to transform plastic-derived monomers into value-added products [6,7]. A critical first step in biological PET upcycling is the depolymerization of PET into its monomers, TPA and EG. Several depolymerization strategies have been developed, including glycolysis, chemical hydrolysis, enzymatic hydrolysis using PET hydrolases such as cutinases and PETases, methanolysis, and thermal depolymerization [8,9]. These processes generate renewable feedstocks that can subsequently be funneled into microbial metabolism for the production of high-value materials.

TPA is of particular interest because its catabolism proceeds through protocatechuate (PCA), a central intermediate of aerobic aromatic metabolism in *Pseudomonas putida*. PCA can be further converted through ring-cleavage reactions into intermediates of the β-ketoadipate pathway, enabling subsequent funneling to central carbon metabolism and thereby providing access to a broad spectrum of downstream products. As a result, microbial production of a variety of high-value chemicals has been achieved using these depolymerized PET monomers as renewable feedstocks [10], including polyhydroxyalkanoates (PHA), 2-pyrone-4,6-dicarboxylic acid (PDC), gallic acid (GA), vanillic acid (VA), and pyrogallol in various host strains [11–13]. One study demonstrated PET upcycling in *Escherichia coli* by converting TPA, into β-ketoadipic acid (βKA), a building block for nylon-6,6. By introducing TPA degradation (*tphAabc* and *tphB*) and βKA synthesis modules (*aroY, catBC* and *pcaD*) along with optimization of fermentation conditions, the final engineered *E. coli* strain produced 13.61 mM βKA with 96% conversion efficiency, demonstrating the feasibility of *E. coli* as a microbial platform for PET upcycling [13]. Another study demonstrated the biological upcycling potential of PET monomers using metabolically engineered *E. coli* [11]. In this work, chemically depolymerized PET hydrolysate was separated into its two constituent monomers, TPA and EG, and each monomer was funneled into distinct biochemical routes. Recombinant *E. coli* harboring TPA degradation pathways converted PET-derived TPA into multiple higher-value aromatic products, including GA, pyrogallol, catechol, muconic acid (MA), and VA via PCA, through combined reactions of hydroxylation, decarboxylation, oxidative ring cleavage, and methylation, achieving relatively high molar conversion yields ranging from 32.7% to 92.5%. In parallel, the other PET-derived monomer, EG, was biologically valorized by the EG-oxidizing strain *Gluconobacter oxydans*, producing glycolic acid (GLA) with an exceptionally high molar yield of 98.6%.

Considering that *P. putida* exhibits metabolic versatility, innate robustness and high tolerance to aromatic compounds, and possesses a well-established synthetic biology toolkit, subsequent studies in *P. putida* further advanced biological PET valorization [14]. One study engineered *P. putida* KT2440 to produce βKA from bis(2-hydroxyethyl) terephthalate (BHET) [15]. In this work, the *tphA2A3BA1* operon from *Comamonas* sp. E6, encoding the TPA 1,2-deoxygenase (TphA) and the DCD dehydrogenase (TphB) that convert TPA into PCA, was introduced. This operon was combined with the high-affinity TPA transporter TpaK from *Rhodococcus jostii*, enabling more effective substrate uptake. In addition, PETase and MHETase from *Ideonella sakaiensis* were employed to hydrolyze PET into TPA *in vivo*. To redirect aromatic flux toward βKA, the *pcaIJ* genes, encoding the 3-oxoadipate CoA-transferase, were further deleted. As a result, final engineered *P. putida* strain produced 15.1 g/L βKA at a 76% molar yield from 31.5 g/L of BHET under fed-batch conditions. Another study developed a multifunctional *P. putida* strain that integrated extracellular PET depolymerization module with intracellular MA biosynthetic module [16]. In this system, *P. putida* was first engineered to secrete the PET hydrolase, leaf-branch compost cutinase (LCC), while TPA metabolism was enabled by introducing a *tph* cluster—comprising *tphR* (transcriptional regulator), *tpaK* (TPA transporter), and the catabolic genes *tphA* and *tphB*—into the *pcaHG* locus to block PCA degradation to increase the intracellular pool of key precursor. Downstream conversion was constructed by introducing *aroY* from *Enterobacter cloacae* together with its accessory gene *ecdB* to decarboxylate PCA into catechol, followed by deletion of the *catRBC* operon and strong overexpression of *catA* to channel flux into MA. To further support cell growth based on PET-derived EG, repression on glyoxylate metabolism was relieved via transcriptional repressor *gclR* deletion and overexpression of *glcDEF* encoding glycolate oxidase. With this integrated strategy, the final engineered strain achieved up to 0.50 g MA / g PET hydrolysate in flask cultivation.

Collectively, these findings suggest that efficient TPA valorization requires the establishment of robust catabolic pathways encompassing both high-affinity transporters for TPA uptake and key intracellular enzymes for its conversion to central metabolites. Recognizing the importance of identifying and implementing novel transporters and catabolic enzymes, we sought to expand the metabolic repertoire of TPA assimilation in *P. putida*. Therefore, in this study, we developed a microbial platform by screening and introducing TPA transporters into *P. putida* KT2440 to enhance the TPA uptake efficiency. Subsequently, downstream metabolic pathways were further introduced for the efficient conversion of purified TPA into high-value aromatic chemicals such as catechol and MA. To further demonstrate the practical allocability of our system, waste PET-derived TPA was evaluated alongside commercial TPA.

2. Materials and methods

2.1. Plasmids and construction of bacterial strains

All the bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* XL1-Blue (Stratagene, La Jolla, CA, USA) was used for general gene cloning. All DNA manipulations were performed by following standard procedures [17]. *P. putida* KT2440 and its mutants were used as host strains for catechol and MA production.

The polymerase chain reactions (PCRs) were performed using a T100 Thermal Cycler (Bio-Rad, USA). Primers synthesized by Bioneer (Daejeon, Korea) for gene cloning are listed in Table S1. To construct plasmids for expression of TPA transporters, five transporter genes (PP2040 from *P. putida*, PcaK from *P. putida*, TphCII-TpiBA from *Comamonas* sp. E6, MucK and MucK^{mut} from *Acinetobacter baylyi* ADP1) were PCR-amplified from the corresponding genomic DNA using the primers listed in Table S1. In addition, four transporter genes (PcaK2 from *Paraburkholderia* sp. PGU19, J4H94_21245 from *Pseudomonas stutzeri*, K1T34_04620 from *Amycolatopsis* sp. DSM110486 and TpaK from *Rhodococcus opacus* were commercially synthesized (Cosmogenetech, Seoul, Korea). Each transporter gene was then cloned into the pSEVA234-MCS under the trc promoter, generating a series of pSEVA234 derivatives. For catechol and MA biosynthesis pathway construction, the *aroY*, *catA1*, and *catA2* genes were sequentially assembled into pSEVA631pt, while the selected transporters (MucK^{mut} or TpaK) were cloned into pSEVA221pt under the tac promoter. The final plasmids were introduced into engineered *P. putida* strains via electroporation for subsequent bioconversion experiments. All the restriction enzymes used for DNA manipulation were purchased from New England Biolabs (Massachusetts, USA).

Chromosomal gene modifications in *P. putida* KT2440 were carried out using plasmids derived from pK19mobsacB, which carries a non-replicative origin in *P. putida* [18]. First, 500 bp fragments homologous to the upstream and downstream regions of the target gene were amplified by PCR and ligated into the *Hin*dIII and *Eco*RI sites of pK19mobsacB, generating chromosomal deletion plasmids. Second, for gene integration, a pathway gene cassette was inserted between the two homology arms previously cloned into pK19mobsacB, resulting in the construction of integration plasmids. Then, all pK19mobsacB-based plasmids were introduced into *P. putida* KT2440 via conjugation using *E. coli* S-17–1 as the donor strain [18]. Trans conjugates were selected on LB agar plates supplemented with kanamycin. Finally, integration of corresponding genes into chromosome was confirmed by *SacB*-mediated

Table 1
Strains and plasmids used in this study.

Strains and plasmids	Relevant Characteristics	Reference or Source
Strains		
E. coli XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^R)]	Stratagene
E. coli S17–1	*hsdR* pro *recA* carrying RP4–2-Tc::Mu in the chromosome Conjugative transfer of pK19mobSacB derivative plasmids into *P. putida* for gene replacement	[18]
P. putida KT2440	Wild type	ATCC 47054
P. putida TphBAabc	*P. putida* KT2440 Δ*endA1*::Ptac::*tphAabc* Δ*endA2*::Ptac::*tphB*	This study
P. putida PCA	*P. putida* KT2440 Δ*endA1*::Ptac::*tphAabc* Δ*endA2*::tac::*tphB* Δ*pcaHG*	This study
P. putida PCA-01	*P. putida* PCA harboring pSEVA234 PP2040	This study
P. putida PCA-02	*P. putida* PCA harboring pSEVA234 Pcak	This study
P. putida PCA-03	*P. putida* PCA harboring pSEVA234 TphCII TpiBA	This study
P. putida PCA-04	*P. putida* PCA harboring pSEVA234 MucK	This study
P. putida PCA-05	*P. putida* PCA harboring pSEVA234 MucK^{mut}	This study
P. putida PCA-06	*P. putida* PCA harboring pSEVA234 Pcak2	This study
P. putida PCA-07	*P. putida* PCA harboring pSEVA234 J4H94_21245	This study
P. putida PCA-08	*P. putida* PCA harboring pSEVA234 K1T34_04620	This study
P. putida PCA-09	*P. putida* PCA harboring pSEVA234 TpaK	This study
P. putida CA	*P. putida* KT2440 Δ*endA1*::Ptac::*tphAabc* Δ*endA2*::Ptac::*tphB* Δ*pcaHG* Δ*catA1/catA2*	This study
P. putida CA-01	*P. putida* CA harboring pSEVA631pt AroY and pSEVA221pt MucK^{mut}	This study
P. putida CA-02	*P. putida* CA harboring pSEVA631pt AroY and pSEVA221pt TpaK	This study
P. putida MA	*P. putida* KT2440 Δ*endA1*::Ptac::*tphAabc* Δ*endA2*::Ptac::*tphB* Δ*pcaHG* Δ*catBC*	This study
P. putida MA-01	*P. putida* MA harboring pSEVA631pt AroY-CatA1/CatA2 and pSEVA221pt MucK^{mut}	This study
P. putida MA-02	*P. putida* MA harboring pSEVA631pt AroY-CatA1/CatA2 and pSEVA221pt TpaK	This study
Plasmids		
pSEVA234-MCS	Expression vector, *ori*V(pBBR1) *lacI*^{q} P$_{trc}$ *aphA,* Km^R	[20]
pSEVA234 PP2040	pSEVA234 derivative; P$_{trc}$, *P. putida* PP2040; Km^R	This study
pSEVA234 PcaK	pSEVA234 derivative; P$_{trc}$, *P. putida pcaK*; Km^R	This study
pSEVA234 TphCII TpiBA	pSEVA234 derivative; P$_{trc}$, *Comamonas* sp. E6 *tphCII tpiBA*; Km^R	This study
pSEVA234 MucK	pSEVA234 derivative; P$_{trc}$, *Acinetobacter baylyi* ADP1 *mucK*; Km^R	This study
pSEVA234 MucK^{mut}	pSEVA234 derivative; P$_{trc}$, *Acinetobacter baylyi* ADP1 *mucK* M34L T342I; Km^R	This study
pSEVA234 PcaK2	pSEVA234 derivative; P$_{trc}$, *Paraburkholderia* sp. PGU19 *pcaK2*; Km^R	This study
pSEVA234 J4H94_21245	pSEVA234 derivative; P$_{trc}$, *Pseudomonas stutzeri* J4H94_21245; Km^R	This study
pSEVA234 K1T34_04620	pSEVA234 derivative; P$_{trc}$, *Amycolatopsis* sp. DSM110486 K1T34_04620; Km^R	This study
pSEVA234 TpaK	pSEVA234 derivative; P$_{trc}$, *Rhodococcus opacus tpaK*; Km^R	This study
pK19mobsacB	Suicide vector; *sacB* gene; Km^R	[19]
pK19mobsacB EndA1	derived from pK19mobsacB by inserting the segment *endA1* (up + down); *endA1* deletion vector	This study
pK19mobsacB EndA2	derived from pK19mobsacB by inserting the segment *endA2* (up + down); *endA2* deletion vector	This study
pK19mobsacB PcaHG	derived from pK19mobsacB by inserting the segment *pcaHG* (up + down); *pcaHG* deletion vector	This study
pK19mobsacB CatA1	derived from pK19mobsacB by inserting the segment *catA1* (up + down); *catA1* deletion vector	This study
pK19mobsacB CatA2	derived from pK19mobsacB by inserting the segment *catA2* (up + down); *catA2* deletion vector	This study
pK19mobsacB CatBC	derived from pK19mobsacB by inserting the segment *catBC* (up + down); *catBC* deletion vector	This study
pSEVA631pt-MCS	Expression vector, *ori*V(pBBR1), *lacI*^{q} P$_{tac}$ *aacC1,* Gm^R	Lab stock
pSEVA631pt AroY	pSEVA631pt derivative; P$_{tac}$, *Enterobacter cloacae aroY*; Gm^R	This study
pSEVA631pt AroY-CatA1/CatA2	pSEVA631pt derivative; P$_{tac}$, *Enterobacter cloacae aroY* and *Pseudomonas putida catA1/catA2*; Gm^R	This study
pSEVA221pt-MCS	Expression vector, *ori*V(IncP/RK2), *lacI*^{q} P$_{tac}$ *aphA,* Km^R	Lab stock
pSEVA221pt MucK^{mut}	pSEVA221pt derivative; P$_{tac}$, *Acinetobacter baylyi* ADP1 *mucK* M34L T342I; Km^R	This study
pSEVA221pt TpaK	pSEVA221pt derivative; P$_{tac}$, *Rhodococcus opacus tpaK*; Km^R	This study

Km^R, kanamycin resistance; Gm^R, gentamicin resistance; ATCC, American Type Culture Collection

counter selection on sucrose-containing medium [19].

2.2. Culture conditions

For DNA manipulations, a Luria-Bertani (LB) medium (containing 10 g/L of tryptone, 5 g/L of yeast extract, and 10 g/L of NaCl) was used for cultivation of *E. coli* XL1-Blue, in which kanamycin (Km, 40 μg/mL) and gentamicin (Gm, 30 μg/mL) were added to the medium based on the resistant markers of the plasmids.

A chemically defined MR medium was employed to utilize TPA and glycerol as a carbon source in recombinant *P. putida* KT2440 strains. The MR medium (pH 7.0) contained (per liter) 6.67 g KH_2PO_4, 4 g $(NH_4)_2HPO_4$, 0.8 g $MgSO_4{\cdot}7\ H_2O$, 0.8 g citric acid, and 5 mL trace metal solution. The trace metal solution contained (per liter) 10 g $FeSO_4{\cdot}7\ H_2O$, 2 g $CaCl_2$, 2.2 g $ZnSO_4{\cdot}7\ H_2O$, 0.5 g $MnSO_4{\cdot}4\ H_2O$, 1 g $CuSO_4{\cdot}5\ H_2O$, 0.1 g $(NH_4)_6Mo_7O_{24}{\cdot}4\ H_2O$, and 0.02 g $Na_2B_4O_7{\cdot}10\ H_2O$ and 0.5 M HCl. Solutions of glycerol and $MgSO_4{\cdot}7\ H_2O$ were sterilized separately. For preparation of TPA stock solutions, disodium commercial TPA and PET-derived TPA were prepared as separate stock solutions, each dissolved in distilled water with mild heating (50–60 °C) and stirring until fully solubilized. PET-derived TPA was prepared from post-consumer PET beverage cups according to a previously reported method [11]. After dissolution, the pH of each solution was adjusted to 7.0, followed by sterile filtration through a 0.22-μm membrane filter. The resulting TPA solutions were used for subsequent flask and fed-batch fermentation experiments.

Seed cultures of *P. putida* strains were prepared in 50 mL round-bottom tubes containing 10 mL of the LB medium and were incubated overnight at 30 °C in a rotary shaker at 230 rpm. Subsequently, 1 mL of the overnight seed culture was inoculated into 250 mL baffled flasks containing 100 mL of the MR medium, before being cultured for 96 *h* at 30 °C and 250 rpm. Glycerol, TPA, appropriate antibiotics (Kanamycin and Gentamicin) were added to the medium. All the flask cultures were performed in triplicate.

Fed-batch fermentations were conducted in a 5 L stirred-tank bioreactor (BioCNS, Korea) containing 2 L of a MR medium at 30 °C. Agitation was initially set to 500 rpm and subsequently adjusted to maintain the dissolved oxygen (DO) level above 40% air saturation. The culture pH was controlled at 7.0 through automatic addition of 5.6% (v/v) ammonia water. Antifoam was supplied as required using polypropylene glycol (Sigma-Aldrich USA). Appropriate antibiotics were included in the medium during the entire fermentation process.

After inoculation, the culture was grown until reaching an OD_{600} of

approximately 3–4, during which the residual glycerol concentration decreased to 3–5 g/L. At this stage, TPA feeding was initiated by adding an initial pulse of 5 mM TPA. This feeding strategy was designed to ensure sufficient biomass accumulation prior to substrate conversion and to minimize potential inhibitory effects of TPA during the early cultivation stage. Two feeding solutions were used during the fermentation: Feeding solution A contained (per liter) 200 g glycerol, 4 g $MgSO_4{\cdot}7H_2O$, 40 mg kanamycin, and 30 mg gentamicin; Feeding solution B consisted of 400 mM TPA.

Glycerol was intermittently fed to maintain the glycerol concentration above 10 g/L in the culture medium. TPA was added to 5 mM increments whenever the residual TPA concentration approached 2 mM, based on periodic sampling and offline HPLC analysis. These feeding strategies ensured that the culture did not experience substrate depletion or inhibitory spikes during fermentation.

2.3. Analytical methods

The cell growth was monitored by measuring absorbance at 600 nm (OD_{600}) using a UV-1800 spectrophotometer (Shimadzu, Japan). TPA and the products converted from TPA were analyzed using high-performance liquid chromatography (HPLC; Agilent 1100, Agilent Technologies, Santa Clara, CA, USA) equipped with a variable wavelength detector (VWD) and a C18 column (Rstech, Daejeon, Korea) operated at 30 °C with a flow rate of 1.0 mL/min. The mobile phase (A) was composed of acetonitrile and 0.1% trifluoracetic acid (v/v), and (B) was made up of water and 0.1% trifluoroacetic acid (v/v) (A:B=1:9). The injection volume was 1 μL, and UV detection was performed at 254 nm.

The concentration of glycerol was measured by HPLC equipped with a refractive index (RID) detector and a Bio-RAD Aminex HPX-87H column (Bio-Rad) at 50 °C with a mobile phase of 5 mM H_2SO_4 at a flow rate of 0.8 mL/min.

3. Results and discussion

3.1. Engineering of P. putida KT2440 for TPA catabolism

In *Comamonas* sp. E6, TPA catabolism is mediated by the *tph* operon, which encodes the multicomponent TPA 1,2-dioxygenase (*tphAabc*) and the associated dehydrogenase (*tphB*). Together, these enzymes convert TPA into PCA. Based on these findings, the *tphAabc* and *tphB* genes were introduced into *P. putida* KT2440 to enable TPA assimilation by chromosomal integration deleting *endA* gene. In parallel, the *pcaHG* genes encoding PCA 3,4-dioxygenase were deleted in the chromosome to prevent PCA degradation and secure it as a key intermediate for downstream metabolic valorization.

To examine the TPA utilization capability of engineered *P. putida* strains, flask cultivations were conducted in MR medium containing 5 mM TPA and 10 g/L glycerol at 30 °C for 96 h. As shown in Fig. 1, the wild-type strain consumed only 0.42 mM TPA, indicating that the native metabolic capacity is insufficient for effective TPA assimilation. Consistent with this limited uptake, no detectable production of PCA was observed in the wild-type strain. In contrast, the engineered strain harboring the TPA degradation pathway (*tphAabc* and *tphB* genes) together with *pcaHG* deletion (*P. putida* PCA) consumed 2.49 mM TPA and produced 0.43 mM PCA under the same cultivation conditions (Fig. 1). These results demonstrate that introduction of *tphBAabc* enables TPA catabolism and deletion of *pcaHG* is needed for securing PCA as a metabolic intermediate. Nevertheless, the overall level of TPA consumption is still limited, suggesting that further metabolic engineering strategies in substrate uptake—particularly through the introduction of high-affinity TPA transporters—should be implemented to establish an efficient TPA assimilation pathway in *P. putida*.

3.2. Screening of transporters for TPA uptake

Although pathway engineering improved TPA assimilation in *P. putida* through the introduction of the *tphBAabc* operon and chromosomal deletion of *pcaHG*, the overall TPA uptake capacity remained insufficient, resulting in a limited intracellular PCA pool for downstream valorization. To enhance TPA utilization and increase precursor availability, we explored whether heterologous TPA transporters can further improve TPA assimilation.

Nine potential TPA transporters from diverse microorganisms were selected and examined for their ability to enhance TPA uptake in *P. putida*. Five previously reported transporters, PP2040 and PcaK from *P. putida*, TphcII-TpiBA from *Comamonas* sp. E6 [21], MucK from *Acinetobacter baylyi* ADP1 [22], and its engineered variant MucK M34L T342I ($MucK^{mut}$), were included based on earlier studies indicating their contribution to TPA uptake. In addition, four putative candidates—PcaK2 from *Paraburkholderia* sp. PGU19, J4H94_21245 from *Pseudomonas stutzeri*, K1T34_04620 from *Amycolatopsis* sp. DSM110486 and TpaK from *Rhodococcus opacus*—were further screened through Basic Local Alignment Search Tool (BLAST) using known TPA transporters as queries. Each gene was cloned into a pSEVA234-MCS plasmid and individually introduced into the *P. putida* PCA to assess their TPA transport activity. The transporters used in this study are listed in Table S1.

To evaluate the performance of the introduced transporters, nine recombinant strains (*P. putida* PCA-01 to PCA-09) were cultivated in MR medium containing 5 mM TPA and 10 g/L glycerol at 30 °C for 96 h. The initial TPA concentration for transporter screening was set to 5 mM based on preliminary experiments using the *P. putida* PCA strain, which consumed approximately 2.49 mM TPA under these conditions. This concentration allowed reliable monitoring of TPA uptake and PCA

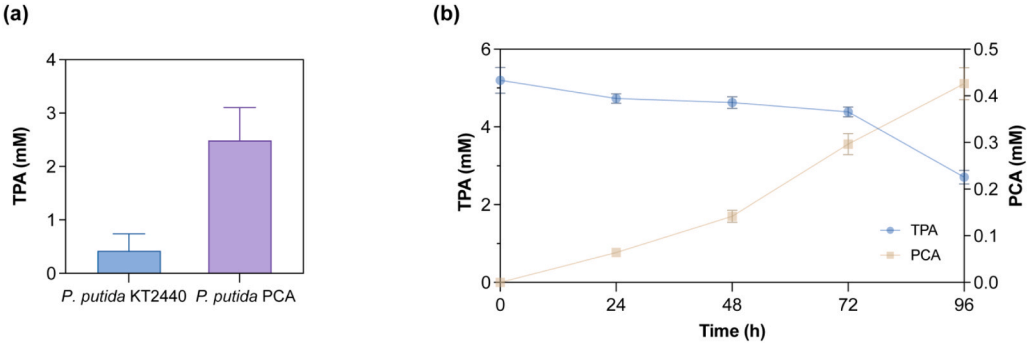


Fig. 1. (a) TPA consumption of *P. putida* KT2440 and *P. putida* PCA strain in flask culture containing 5 mM TPA and 10 g/L glycerol. (b) Time-course profiles of TPA consumption and PCA accumulation of *P. putida* PCA in flask culture containing 5 mM TPA and 10 g/L glycerol. The genotype of the *P. putida* PCA strain is described in Table 1. All flask cultivations were performed in triplicate. Data are presented as mean ± standard deviation.

formation during early-stage pathway evaluation. Substantial variation in TPA consumption was observed among the nine recombinant strains (Fig. 2). Whereas the control strain, *P. putida* PCA, consumed 2.49 mM TPA and produced 0.43 mM PCA, among the transporter-expressing strains, *P. putida* PCA-01 to PCA-03 consumed 2.91, 1.49 and 1.91 mM TPA, respectively, accompanied by PCA production of 0.22, 0.13 and 0.16 mM. Likewise, *P. putida* PCA-06 to PCA-08 exhibited relatively low TPA utilization, consuming 2.25, 0.82 and 2.17 mM TPA, with corresponding PCA production levels of 0.18, 0.04 and 0.29 mM. In contrast, markedly enhanced TPA uptakes were observed in *P. putida* PCA-04 and PCA-05. The PCA-04 strain consumed 3.92 mM TPA and produced 0.69 mM PCA, whereas *P. putida* PCA-05 completely utilized the supplied TPA, consuming 5.30 mM and producing 0.72 mM PCA. A comparable performance was observed for *P. putida* PCA-09, which also achieved complete TPA consumption (5.33 mM) along with the highest PCA production of 1.11 mM.

To further assess whether the most effective transporters identified in the initial screen could maintain its enhanced activity under higher substrate availability, three transporters—MucK, MucK^{mut} and TpaK—were selected for additional evaluation. Flask cultivations were conducted in MR medium containing 10 mM TPA and 10 g/L glycerol at 30 °C for 96 h. Under these conditions, *P. putida* PCA-04 strain consumed 3.85 mM TPA and produced 0. 77 mM PCA, whereas *P. putida* PCA-05 strain consumed 7.74 mM TPA with 1.77 mM PCA production. *P. putida* PCA-09 strain exhibited the highest activity, consuming 8.24 mM TPA and producing 1.81 mM PCA (Fig. 3). These results reaffirmed that MucK^{mut} and TpaK exhibit superior transport activities under elevated TPA availability and thus represent the most effective transporters for enhancing both TPA uptake and precursor (PCA) formation. Accordingly, MucK^{mut} and TpaK were selected as the top-performing transporters for subsequent biochemical synthesis.

More broadly, inefficient substrate uptake is often a major bottleneck in the microbial conversion of aromatic compounds. Therefore, systematic transporter screening and engineering, as demonstrated in this study, may represent a broadly applicable strategy to enhance the assimilation of other aromatic substrates in Pseudomonas putida and related microbial hosts.

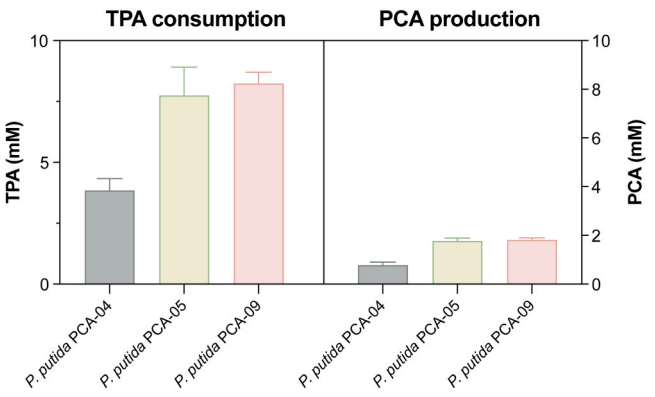


Fig. 3. Comparison of TPA consumption and PCA production by engineered *P. putida* PCA strains under flask cultivation conditions. TPA consumption and PCA accumulation were measured in flask cultures containing 10 mM TPA and 10 g/L glycerol. All flask cultivations were performed in triplicate. Data are presented as mean ± standard deviation.

3.3. Versatile bioconversion of synthetic and post-consumer PET–derived TPAs to catechol

Building upon the enhanced TPA uptake achieved through transporter introduction, we next sought to demonstrate the applicability of our platform for the production of value-added aromatic chemicals. As a first proof-of-concept, catechol was selected as a representative product to establish a biological funneling strategy using our developed engineered *P. putida* strains equipped with improved TPA assimilation capacity.

For this purpose, the two most effective transporters, MucK^{mut} and TpaK, were introduced and a catechol biosynthetic pathway was constructed in *P. putida* PCA. Catechol production pathway was constructed by plasmid-based expression of the *E. cloacae aroY* gene, which mediates the decarboxylation of PCA to catechol, together with deletion of the native *catA1* and *catA2* genes in the chromosome of *P. putida* PCA to prevent catechol degradation (Fig. 4, *P. putida* CA). When *P. putida* CA was cultivated in MR medium containing 10 mM TPA and 10 g/L glycerol at 30 °C for 96 h, the strain expressing *mucK*^{mut} (*P. putida* CA-01) consumed 5.93 mM TPA and produced 5.32 mM catechol,

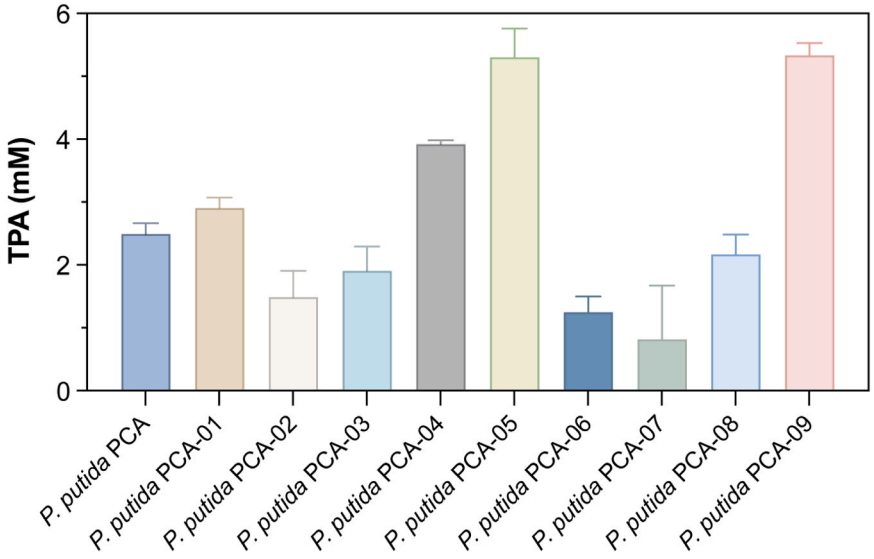


Fig. 2. Screening of TPA transporters for enhanced TPA consumption in *P. putida* PCA strains. *P. putida* PCA-01 to PCA-09 represent PCA strains expressing individual TPA transporters, as listed in Table 1. TPA consumption was measured in flask cultures containing 5 mM TPA and 10 g/L glycerol. The initial TPA concentration was nominally set to 5 mM; however, the concentrations shown in the figure are based on experimentally measured values determined by HPLC analysis. All flask cultivations were performed in triplicate. Data are presented as mean ± standard deviation.

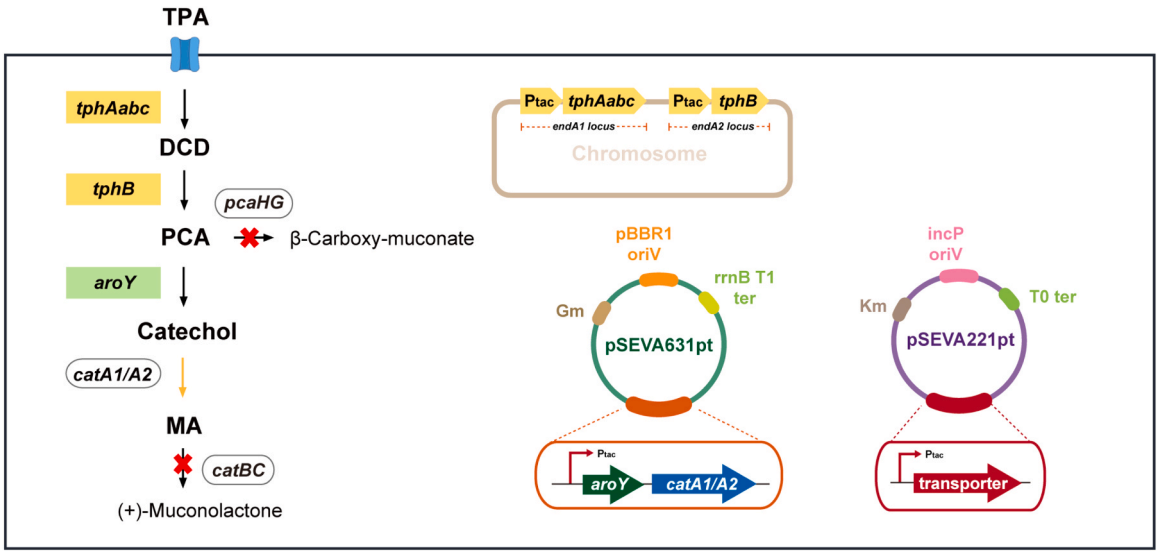


Fig. 4. Schematic overview of the engineered metabolic pathway from TPA to MA in *P. putida* KT2440. Chromosomally integrated genes are shown in yellow rectangular boxes, with their genomic insertion loci indicated separately. Heterologous genes expressed from plasmids are shown in green rectangular boxes, and plasmid constructs are illustrated alongside the pathway. Native *P. putida* genes are indicated by rounded rectangles, and deleted genes are marked with crosses. For catechol production, *aroY* expressed pSEVA631pt, whereas for pathway extension toward MA production, *catA1* and *catA2* were additionally cloned into pSEVA631pt to enable overexpression. DCD, 1,2-dihydroxy-3,5-cyclohexadiene-1,4-dicarboxylate.

corresponding to a molar conversion yield of 89.9% based on the consumed TPA. In contrast, the strain expressing *tpaK* (*P. putida* CA-02) completely consumed the supplied TPA (9.89 mM) and produced 8.51 mM catechol, resulting in a molar yield of 86.0%, and demonstrating markedly improved substrate utilization capability and target product formation. These results indicate that coupling high-affinity TPA transporters with the catechol biosynthetic module can significantly enhance TPA-to-catechol conversion, with the TpaK transporter demonstrates its superior performance (Fig. 5).

To further assess the practical applicability of this system, we next evaluated whether TPA obtained from post-consumer PET can serve as a viable substrate. Waste PET was chemically depolymerized to TPA following a previously reported method [11], and the resulting PET-derived TPA was subsequently purified prior to use. The purified PET-derived TPA was then supplied under the same flask cultivation conditions (10 mM PET-derived TPA, 10 g/L glycerol, 30 °C, 96 h). Under these conditions, *P. putida* CA-02 consumed 10.37 mM of the 10.9 mM PET-derived TPA supplied and produced 8.34 mM catechol, corresponding to a molar yield of 80.4% based on consumed TPA. This performance was comparable to that obtained using commercial TPA (Fig. 5), demonstrating that PET-derived TPA can be effectively utilized without compromising catechol biosynthesis efficiency. These results underscore the robustness of the engineered strain and its suitability for practical PET waste upcycling scenarios.

3.4. *Versatile bioconversion of synthetic and post-consumer PET–derived TPA to MA*

The successful establishment of catechol biosynthesis confirmed that TPA catabolism in *P. putida* can be extended toward the production of downstream aromatic metabolites. To further broaden the utility of *P. putida* as a microbial chassis for PET upcycling, we next focused on the production of MA, a key platform chemical and precursor for nylon and resins. MA biosynthesis requires an extended pathway beyond PCA and catechol, providing a useful framework to assess whether the engineered strains can sustain additional metabolic valorization steps.

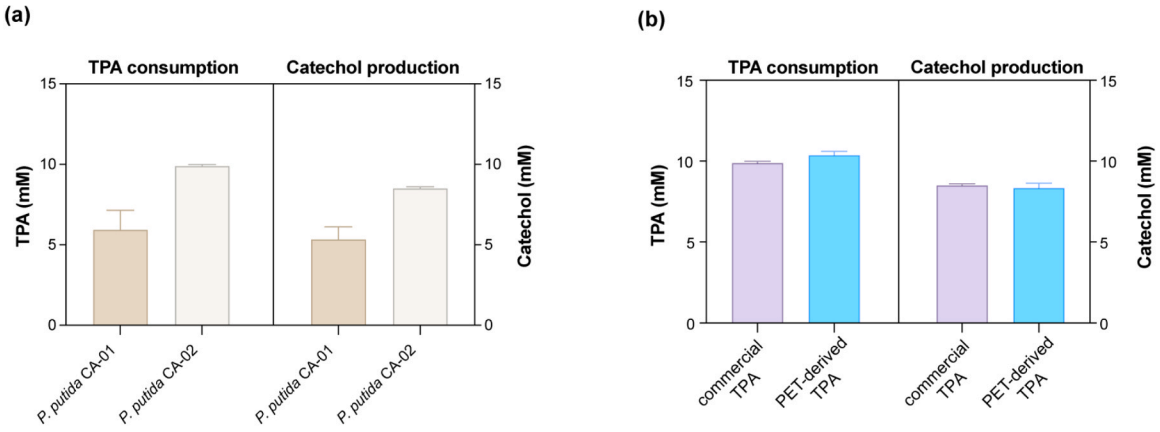


Fig. 5. Comparison of TPA consumption and catechol production by engineered *P. putida* CA strains under flask cultivation conditions. The genotype of the *P. putida* CA strain is described in Table 1. (a) TPA consumption and catechol production by *P. putida* CA-01 (expressing *mucK*^{mut}) and *P. putida* CA-02 (expressing *tpaK*) in flask cultures containing 10 mM TPA and 10 g/L glycerol. (b) TPA consumption and catechol production by *P. putida* CA-02 using commercial TPA or PET-derived TPA as the substrate under the same cultivation conditions. All flask cultivations were performed in triplicate. Data are presented as mean ± standard deviation.

For MA production, the *P. putida* PCA strain was used as the base strain to make *P. putida* MA strain. To enable MA accumulation, the *catB* and *catC* genes, encoding muconate cycloisomerase and muconolactone D-isomerase, were deleted in the chromosome of *P. putida* PCA (Fig. 4). In addition, to strengthen flux from PCA through catechol and subsequently to MA, the *E. cloacae aroY* gene together with *catA1* and *catA2* were expressed in pSEVA631pt-MCS. Also, plasmid-based expression of the selected transporters $MucK^{mut}$ and TpaK was employed to ensure efficient TPA uptake, just as previously employed for catechol production. Flask cultivations were performed in MR medium containing 10 mM TPA and 10 g/L glycerol at 30 °C for 96 h.

Time-profile analysis revealed distinct production trends depending on the employed transporter. In *P. putida* MA-01 expressing $mucK^{mut}$, TPA was gradually consumed throughout the cultivation period. Correspondingly, MA production increased only modestly over time, showing a slow but steady rise between 48 and 96 h. By the end of cultivation, *P. putida* MA-01 produced 5.90 mM MA, corresponding to an 84.8% molar yield, with 6.96 mM TPA consumed, reflecting relatively limited flux from TPA toward the extended biosynthetic pathway. By contrast, *P. putida* MA-02 expressing *tpaK* displayed accelerated substrate assimilation, utilizing TPA at a much earlier stage. This rapid uptake was accompanied by an earlier and more pronounced initiation of MA formation, with MA levels increasing sharply after the midpoint of cultivation. As a result, *P. putida* MA-02 achieved higher titers, reaching 8.45 mM MA with a 93.1% molar yield, consuming 9.08 mM TPA after 96 h (Fig. 6). The overall production profile indicated that the enhanced transport capacity of the TpaK transporter more effectively supported the extended pathway flux, thereby enabling superior MA biosynthesis.

Following the successful validation of MA production from commercial TPA, we next examined whether this performance could be maintained when PET-derived TPA was used as the substrate. Flask cultivation was conducted with PET-derived TPA under the same cultivation conditions (10 mM PET-derived TPA, 10 g/L glycerol, 30 °C, 96 h). Under these conditions, *P. putida* MA-02 consumed 9.76 mM TPA and produced 7.62 mM MA, corresponding to a 78.1% molar yield (Fig. 6). This performance was comparable to that obtained using commercial TPA, confirming that PET-derived TPA can be effectively utilized without compromising MA biosynthesis efficiency. Collectively, these results indicate that the *P. putida* MA strain exhibits consistent performance regardless of TPA source.

Consistent with observations from catechol production, integrating an effective TPA transporter with targeted metabolic rewiring substantially improved pathway flux. This convergence of transporter engineering and pathway optimization underscores the robustness and versatility of the proposed strategy, enabling the extension of TPA bioconversion toward higher-value aromatic chemicals and reinforcing its potential for practical and industrial PET waste upcycling.

3.5. Enhanced TPA utilization in fed-batch fermentation of recombinant *P. putida* KT2440

To further assess the scalability and bioprocess robustness of the engineered strain, a fed-batch fermentation was carried out using recombinant *P. putida* CA-02 under controlled bioreactor conditions.

Throughout the fed-batch cultivation, *P. putida* CA-02 efficiently utilized both carbon and aromatic substrates, consuming a total of 30 g/L glycerol and 16.76 mM TPA. Correspondingly, catechol was produced progressively over the course of the fermentation. By the end of the process, the strain produced 15.04 mM catechol, corresponding to a molar conversion yield of 89.7%, indicating efficient channeling of TPA toward the engineered catechol biosynthetic pathway. In addition, time-course profiles indicated that catechol production closely followed TPA consumption, highlighting the tight coupling between substrate transport, PCA processing, and downstream conversion under scale-up processes (Fig. 7).

Taken together, these results confirm that the engineered strain, when integrated with an optimized fed-batch strategy, is capable of robust catechol production from TPA under scale-up conditions, demonstrating the practical applicability of the system for industrial bioconversion of PET-derived feedstocks.

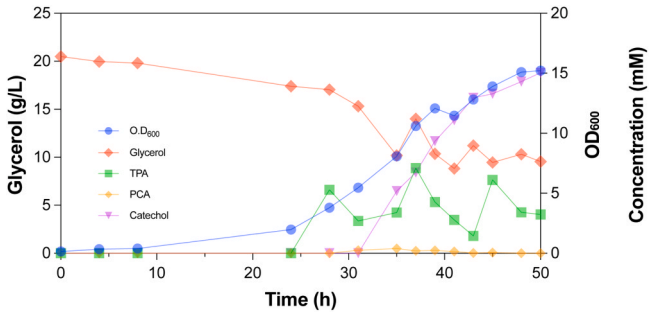


Fig. 7. Time-course profiles of cell growth, substrate concentrations, and catechol production by *P. putida* CA-02 during fed-batch fermentation. Glycerol concentration (g/L) is shown on the left y-axis, while Cell growth (OD_{600}), TPA, PCA and catechol concentrations (mM) are shown on the right y-axis.

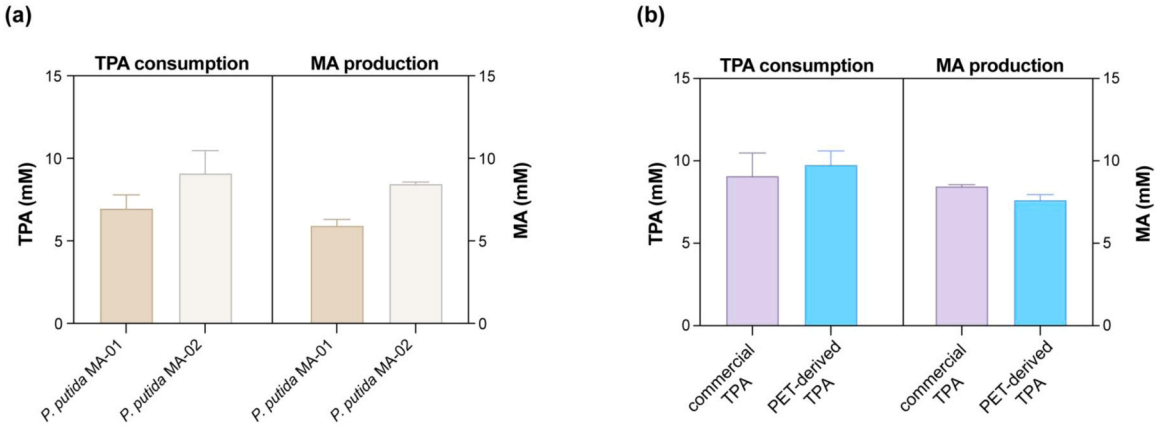


Fig. 6. Comparison of TPA consumption and MA production by engineered *P. putida* MA strains under flask cultivation conditions. The genotype of the *P. putida* MA strain is described in Table 1. (a) TPA consumption and catechol production by *P. putida* MA-01 (expressing $mucK^{mut}$) and *P. putida* MA-02 (expressing *tpaK*) in flask cultures containing 10 mM TPA and 10 g/L glycerol. (b) TPA consumption and MA production by *P. putida* MA-02 using commercial TPA or PET-derived TPA as the substrate under the same cultivation conditions. All flask cultivations were performed in triplicate. Data are presented as mean ± standard deviation.

4. Conclusion

In this study, we established a modular bioconversion platform in *P. putida* KT2440 for the efficient valorization of TPA into value-added aromatic chemicals. Introduction of the heterologous *tphBAbac* genes together with deletion of *pcaHG* in the chromosome enabled TPA conversion to PCA, while systematic screening of TPA transporters identified $MucK^{mut}$ and TpaK as key determinants for overcoming the intrinsic limitation in TPA uptake.

Integration of these transporters enabled efficient downstream biosynthesis of catechol and MA. In particular, the *tpaK*-expressing strain achieved complete TPA consumption and produced 8.51 mM catechol with an 86.0% molar yield, while extension of the pathway toward MA resulted in 8.45 mM MA at a 93.1% molar yield. Importantly, comparable conversion efficiencies were also maintained when PET-derived TPA was used as the substrate, demonstrating the robustness of the engineered strains toward waste-derived feedstocks. Furthermore, fed-batch fermentation validated the scalability of the platform, achieving 15.04 mM catechol from 16.76 mM TPA under controlled bioreactor conditions.

Overall, this work highlights transporter engineering as a pivotal strategy for enhancing TPA assimilation and demonstrates that coupling optimized uptake systems with modular metabolic pathways enables scalable and waste-compatible PET upcycling. The platform presented here provides a solid foundation for future development of sustainable bioprocesses converting plastic waste into renewable aromatic chemicals. Future studies may further improve TPA utilization at higher substrate concentrations through strategies such as enhancing cellular tolerance to aromatic compounds and optimizing substrate feeding strategies.

CRediT authorship contribution statement

Jina Son: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Eun Seo Lee:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Si Jae Park:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Jeong Chan Joo:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Ki Jun Jeong:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Younghoon Kim:** Methodology, Investigation, Data curation. **Hyun Gil Cha:** Methodology, Investigation, Data curation. **Byoung Wook Jeon:** Methodology, Investigation, Data curation. **Hye Jin Lim:** Methodology, Investigation, Data curation. **Hye Min Song:** Validation, Methodology, Investigation.

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2026.03.009.

Data availability

Data will be made available on request.

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