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Engineering a Cross-Feeding Synthetic Bacterial Consortium for Degrading Mixed PET and Nylon Monomers

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Abstract: Plastics are indispensable to modern life, but their widespread use has created an environmental crisis due to inefficient waste management. Mixed plastic waste, comprising diverse polymers, presents significant recycling challenges due to the high costs of sorting and processing, leading to ecosystem accumulation and harmful by-product generation. This study addresses this issue by engineering a synthetic bacterial consortium (SBC) designed to degrade mixed plastic monomers. The consortium pairs *Escherichia coli* Nissle 1917, which uses ethylene glycol (EG), a monomer derived from polyethylene terephthalate (PET), as a carbon source, with *Pseudomonas putida* KT2440, which metabolizes hexamethylenediamine (HD), a monomer from nylon-6,6, as a nitrogen source. Adaptive evolution of the SBC revealed a novel metabolic interaction where *P. putida* developed the ability to degrade both EG and HD, while *E. coli* played a critical role in degrading glycolate, mitigating its by-product toxicity. The evolved cross-feeding pattern enhanced biomass production, metabolic efficiency, and community stability compared to monocultures. The consortium's performance was validated through flux balance analysis (FBA), high-performance liquid chromatography (HPLC), and growth assays. These findings highlight the potential of cross-feeding SBCs in addressing complex plastic waste, offering a promising avenue for sustainable bioremediation and advancing future polymer degradation strategies.

Keywords: synthetic bacterial consortium; mixed plastic degradation; synthetic biology; bioremediation; adaptive evolution

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1. Introduction

The increasing accumulation of plastic waste poses a severe environmental challenge due to its resistance to degradation [1,2]. Plastic waste often consists of mixed polymers such as polyethylene terephthalate (PET), nylon-6,6, polypropylene (PP), and others, which complicates recycling and disposal processes. Classification of these mixed plastics into their individual types for recycling or biodegradation requires substantial energy, resources, and costs, making it an inefficient and unsustainable solution. The environmental consequences of this plastic pollution are profound, with an estimated 9–23 million metric tons of plastic entering aquatic ecosystems annually and 13–25 million metric tons accumulating on land [3]. These issues not only contribute to climate change, chemical contamination, and biodiversity loss but also result in significant economic impacts [4]. Despite advances in plastic waste management technologies, including mechanical recycling and incineration, existing methods fail to address the scale of the problem effectively, emphasizing the urgent need for alternative solutions [5,6].

Recent advances in plastic biodegradation have highlighted key enzymatic and microbial innovations, particularly for PET and nylon-6,6, two of the most prevalent synthetic polymers contributing to plastic pollution [7]. For PET, enzymes such as PETase and MHETase, derived from *Ideonella sakaiensis*, have demonstrated remarkable efficiency in depolymerizing PET into its monomers, terephthalic acid (TPA) and ethylene glycol (EG) [8,9]. Engineered variants of these enzymes have further improved their stability and catalytic activity, making them promising tools for biotechnological applications [10,11]. For nylon-6,6, extensive research has focused on discovering and engineering enzymes for efficient depolymerization of nylons to smaller metabolites. Among these efforts, the pioneering work by Kato et al. (2023) has greatly advanced our understanding of nylon degradation [12]. They identified and characterized key enzymes, including nylon hydrolases such as NylC, NylB, and NylA, which play crucial roles in degrading various nylon oligomers into smaller, more bioavailable intermediates [13]. However, the efficiency of these enzymes and microbes in both natural and engineered systems remains limited, and their potential for degrading mixed plastic waste is yet to be fully realized. Although these advancements lay the groundwork for addressing plastic waste, integrating enzymatic and microbial tools into systems capable of effectively managing mixed plastic waste streams remains a substantial challenge.

Microbial consortia offer a promising solution to these challenges by leveraging the distinct capabilities of multiple interacting microbial populations. Unlike single bacterial strains, which often face metabolic burdens and inefficiencies, consortia enable division of labor, where each species specializes in specific tasks within the degradation process [14]. This specialization enhances the overall efficiency of metabolizing complex feedstocks, such as mixed plastic waste. Additionally, advancements in adaptive evolution have demonstrated the ability to select for strains with enhanced metabolic capabilities [15], making it possible to develop robust microbial consortia tailored for specific bioremediation tasks. These consortia provide a scalable, natural solution for addressing the limitations of single-strain systems in biodegradation.

In this study, we developed a synthetic bacterial consortium (SBC) comprising *Escherichia coli* (*E. coli*) and *Pseudomonas putida* (*P. putida*), both enhanced through adaptive evolution, to degrade monomers derived from PET and nylon-6,6. The consortium was designed with a mutualistic division of labor: *E. coli* utilized ethylene glycol (EG) as a carbon source, producing carbon-based metabolites to support *P. putida*, while *P. putida* metabolized hexamethylenediamine (HD) as a nitrogen source, supplying essential nitrogen compounds to *E. coli*. Adaptive evolution further improved efficiency, enabling *P. putida* to metabolize both EG and HD, while *E. coli* focused on mitigating toxic by-products. These interactions enhanced resource utilization, reduced metabolic imbalances, and significantly increased biomass yield compared to monocultures. Depolymerizing plastic polymers through enzymes or bacteria remains a significant challenge for industrial applications. However, the degradation of these monomers can provide a driving force by coupling microbial growth to polymer breakdown, enhancing overall efficiency. This study demonstrates the potential of adaptive evolution and bacterial consortia as effective strategies, forming the basis for our broader goal of developing SBCs to bio-upcycle mixed plastic waste and drive sustainable plastic waste management.

2. Materials and Methods

2.1. Bacterial Strains and Media

The bacterial strains used in this study were wild-type *E. coli* Nissle 1917 (EcWT) and *P. putida* KT2440 (PpWT). All medium components were sourced from BioShop Canada Inc. (Burlington, ON, Canada), Bio Basic Inc. (Toronto, ON, Canada), or Sigma-Aldrich

Co (Oakville, ON, Canada). The strains were preserved as glycerol stocks at −80 °C and routinely streaked onto lysogeny broth (LB) agar plates (containing 10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) or M9 agar plates supplemented with the appropriate carbon or nitrogen sources. The M9 agar plates consisted of 15 g/L agar, 6.78 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.12 g/L $MgSO_4$, 0.5 mg/L thiamine, 0.011 g/L $CaCl_2$, 0.5 g/L NaCl, and 1 mL/L trace element solution (1.6 g/L $FeCl_3$, 0.2 g/L $CoCl_2 \cdot 6\ H_2O$, 0.1 g/L $CuCl_2$, 0.2 g/L $ZnCl_2 \cdot 4H_2O$, 0.2 g/L $NaMoO_4$, 0.05 g/L H_3BO_3 in 0.1 M HCl). To culture and evolve the strains, various modified M9 media were employed, including: M9G, M9 medium supplemented with 5 g/L glucose; M9EG, M9 medium with 10 g/L ethylene glycol (EG) replacing glucose; M9NH, M9 medium supplemented with 1 g/L NH_4Cl; M9HD, M9 medium with 3 g/L hexamethylenediamine (HD) replacing 1 g/L NH_4Cl; M9EG/HD, M9 medium with 10 g/L EG as carbon source and 3 g/L HD as nitrogen source. Culture samples were appropriately diluted using a 0.15 M saline solution to measure cell density (OD_{600}) with a spectrophotometer (GENESYS™ 40/50 Vis/UV-Vis, Thermo Fisher Scientific Inc., Sunnyvale, CA, USA).

2.2. Adaptive Evolution

In the first-stage evolution, PpWT and EcWT were gradually adapted in M9 media with increasing concentrations of HD and EG, respectively. PpWT was evolved to obtain PpHD1 in modified M9 media with the increasing ratio of M9NH:M9HD from 1:0, 1:2, 1:5, 1:10, to 0:1 that reported in our previous study [16,17], while adaptive evolution of EcWT was evolved to obtain EcEG1 in the modified M9 media with the increasing ratio of M9G:M9EG from 1:0, 1:1, 1:3, 1:5, 1:7, to 0:1, respectively (Figure 1A).

Following the development of PpHD1 and EcEG1, we combined them together with a ratio of 1:1 to obtain SBC1. Then, SBC1 was grown at 30 °C for three days on a rotary shaker set at 220 rpm in modified M9EG/HD (10 g/L EG and 3 g/L HD as the sole carbon and nitrogen sources, respectively). Following this, a 1 mL aliquot of the seed culture was inoculated into 50 mL of fresh M9EG/HD medium and grown overnight under identical conditions. The overnight culture was then transferred into a chemostat to initiate the adaptive evolution process shown in Figure 1B.

The chemostat system was maintained at 30 °C with continuous stirring at 1000 rpm to improve agitation, and it was supplied with fresh M9EG/HD medium. Steady-state conditions were sustained throughout the adaptive evolution process, with OD_{600} regularly monitored and maintained at approximately 0.4. The inlet flow rate was initially set at 0.1 mL/min and adjusted as needed to preserve steady-state conditions. After 25 days of adaptive evolution, glycerol stocks of the resulting strains SBC2 containing EcEG2 and PpHD2, were prepared for storage. Furthermore, the consortium was streaked on LB agar plates to obtain single colonies, and specific primers yltEcNF/yltEcNR (agtacatggcgtgaggctgat; cgtggcaggacagctacgc) and tGyqhdF2/tGyqhdR2 (tggcgaatctgcgtctgc; gagtgtccgggtctgttgtatg) were used to identify and to separate EcEG2 and PpHD2 for monoculture and co-culture conditions. Polymerase chain reaction (PCR) was performed using the KAPA2G Fast HotStart ReadyMix kit (Kapa Biosystems, Wilmington, MA, USA) following the manufacturer's cycling protocol.

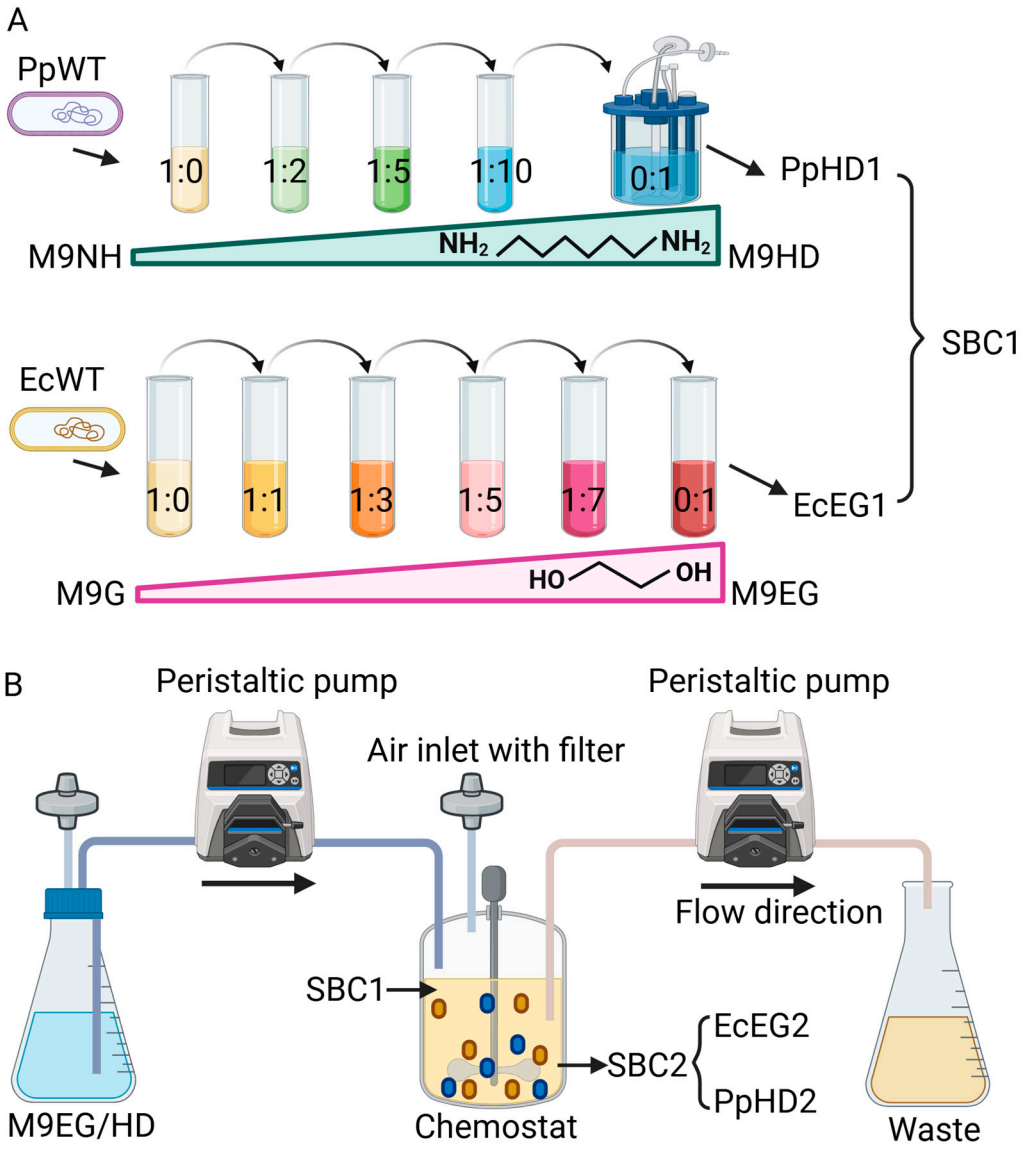

Figure 1. Adaptive evolution of the SBC for EG and HD degradation. (**A**) Adaptive evolution of PpWT (top) and EcWT (bottom) to obtain PpHD1 and EcEG1, separately. (**B**) Chemostat setup for SBC evolution: inflow of fresh M9EG/HD medium, agitation for mixing, and controlled outflow to maintain steady-state culture. Notes: M9G, M9-glucose; M9EG, M9-ethylene glycol; M9NH, M9-NH4Cl; M9HD, M9-HD; M9EG/HD, M9-EG and HD.

2.3. Endpoint Assay

Seed cultures were first cultivated in LB if no growth was anticipated during the assay, or in modified M9 medium if growth was expected. Cultures were washed twice with the assay medium to remove residual nutrients. Endpoint assays were initiated by inoculating cultures into assay tubes to achieve an initial OD_{600} of ~0.2. After the designated incubation period, final OD_{600} measurements were recorded to evaluate growth performance.

2.4. Growth Kinetics

Seed cultures were prepared as described for the endpoint assays and inoculated into Costar 48-well plates with an initial OD_{600} of ~0.2 in a final volume of 500 µL per well. OD_{600} measurements were recorded hourly for 72 h using a BioTek Synergy4 plate reader (Agilent Technologies, Santa Clara, CA, USA), with agitation every 15 min to ensure homogeneous mixing.

2.5. Sampling and 16S rRNA Sequencing

The strains PpHD2 and EcEG2 were inoculated at a 1:1 ratio into two culture tubes, each containing 4 mL of M9EG/HD media, and cultured at 30 °C with shaking at 220 rpm. Cells were harvested at 24 and 48 h, followed by genomic DNA extraction using a Genomic DNA preparation kit (QIAGEN, Toronto, ON, Canada). The 16S rRNA sequencing was performed and analyzed at the Waterloo Genomics Surveillance Centre.

2.6. Flux Balance Analysis

Flux balance analysis (FBA) was performed using COBRApy [18], with the simulation code adapted from Pandit et al. [19]. The genome-scale model of *P. putida* KT2440 was derived from the established BiGG database model iJN1463 [20,21]. The model was refined by integrating the proposed EG and HD metabolic pathways.

For the EG pathway, most reactions were already present in iJN1463, but additional reactions, including EG uptake and its conversion to glycolaldehyde, were incorporated [19]. The HD pathway was constructed de novo using data from a recent study [16]. Reaction mechanisms were informed by the known functions of AlaC and FrmA, as annotated in UniProt database [22].

To simulate conditions of the modified M9 medium, carbon and nitrogen uptake were restricted to EG and HD, respectively, with oxygen uptake rates kept as defined by Pandit et al. [19]. Default parameter values, including ATP maintenance, were retained. The biomass maximization objective was used to evaluate the growth potential of PpHD2 under these conditions.

2.7. High-Performance Liquid Chromatography

The conversion of EG into glycolate (GA) was analyzed using high-performance liquid chromatography (HPLC), following a previously established protocol [23]. Aliquots of 1 mL were taken from the cultures for each time point and were centrifuged at 12,000 rpm for 5 min. Subsequently, the supernatants were filtered through 0.22 µm syringe filters. EG and GA concentrations were quantified using an Aminex HPX-87H column (Bio-Rad, Mississauga, ON, Canada) operated at 60 °C. The mobile phase used was 5 mM sulfuric acid, delivered at a flow rate of 0.6 mL/min.

3. Results

3.1. Design of the Cross-Feeding SBC

Efficient biodegradation of mixed plastic polymers, such as PET and Nylon-6,6, remains a significant challenge despite decades of research [24]. A critical bottleneck is the complexity of breaking down these polymers and channeling their monomeric by-products into microbial metabolism. To lay the groundwork for future polymer degradation strategies, we focused on the assimilation of monomers—EG, derived from PET, and HD, derived from Nylon-6,6—as sole carbon and nitrogen sources, respectively. By coupling monomer degradation with bacterial growth, we aimed to establish a biological driving force that could be leveraged in subsequent efforts to enhance polymer breakdown.

Our strategy employs an SBC, as it offers distinct advantages over single-species approaches. Individual microbes often face limitations in processing complex substrates, whereas consortia leverage the complementary metabolic strengths of different species [14]. This division of labor facilitates robust and efficient substrate utilization. Here, we designed a cross-feeding system (Figure 2) where one bacterial strain metabolizes EG and the other assimilates HD, creating mutual metabolic interdependence to support consortium growth.

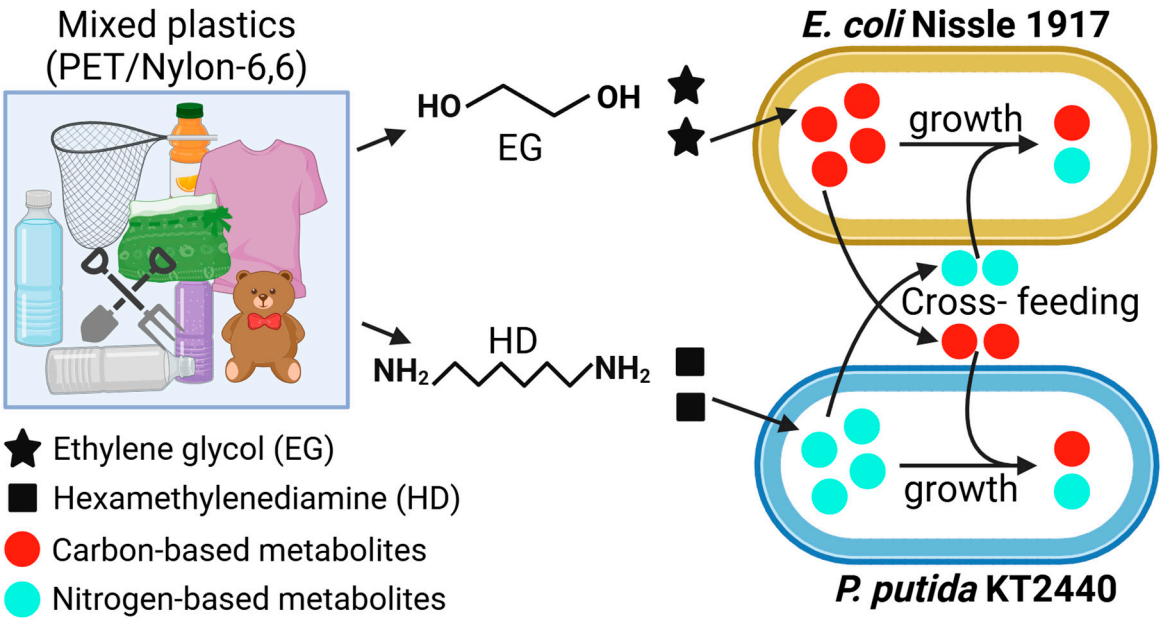

Figure 2. Design of a cross-feeding SBC for plastic monomers degradation. Cross-feeding strategy schematic: *E. coli* consuming EG (ethylene glycol) to provide carbon source and *P. putida* utilizing HD (hexamethylenediamine) to provide nitrogen source for the community.

We selected *E. coli* Nissle 1917 and *P. putida* KT2440 as the cellular chassis due to their complementary metabolic capabilities. *E. coli* was chosen for EG metabolism based on its genetic tractability, metabolic versatility, and prior demonstration of EG utilization as a feedstock. Notably, it possesses key enzymes such as FucO (CIW80_07675) and AldA (CIW80_00015), which convert EG into glycolate, a metabolite that can be further processed through the glycolate and glyoxylate degradation pathways, as verified by the BioCyc database "https://biocyc.org/" (accessed on 6 October 2024). Similarly, *P. putida* was chosen for HD metabolism due to its extensive metabolic versatility, well-documented role in bioremediation, and previous findings demonstrating its capability to utilize HD as a sole nitrogen source [16]. The cross-feeding strategy was designed to create synergy between these two species.

3.2. Chemostat Evolution of Strains

Although PpWT and EcWT theoretically possess the metabolic capability to utilize HD as a sole nitrogen source and EG as a sole carbon source, respectively, their growth under initial conditions was insignificant. To enhance their ability to metabolize HD and EG, a two-stage adaptive evolution process was conducted. The first stage involved monoculture evolution in tubes with gradually increasing concentrations of M9HD for PpWT and M9EG for EcWT, while the second stage consisted of chemostat evolution in a bioreactor (Figure 1).

The inlet flow rate data were collected during the 25-day chemostat evolution experiment (Figure 3). During the initial stage, the inlet flow rate had to be decreased to maintain steady-state growth of SBC2 due to their weak and slow ability to utilize HD and EG. Throughout the experiment, the inlet flow rate was adjusted to sustain steady-state conditions. Initially, it was reduced to establish the first steady state at approximately 0.02–0.03 mL/min on days 3–4. Over the course of adaptive evolution, the flow rate gradually increased, indicating that the SBC adapted to more effectively utilize EG and HD as sole carbon and nitrogen sources, respectively.

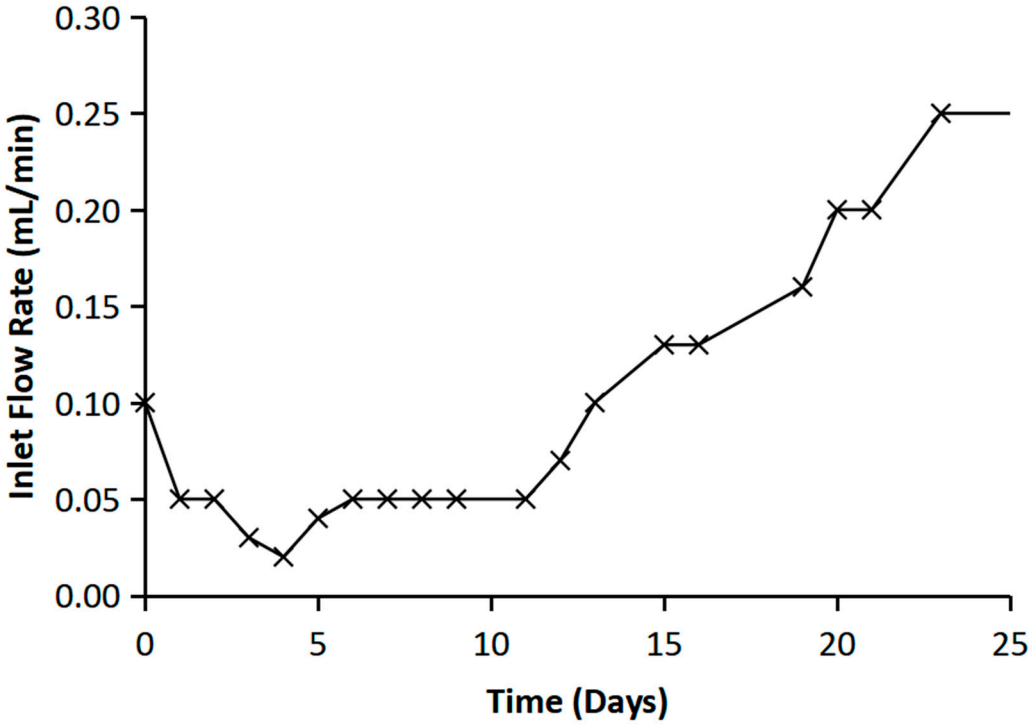

Figure 3. Inlet flow rate over time: adaptive evolution improved nutrient utilization.

By day 25, the flow rate had increased nearly tenfold, reaching a final steady-state rate of 0.25 mL/min. This significant improvement highlights the enhanced metabolic efficiency of the consortium. At the end of the adaptation period, the evolved strains were collected as SBC2 and further isolated into PpHD2 and EcEG2 for subsequent growth characterization.

3.3. Characterization of P. putida Strains on HD

The growth patterns of PpHD1 and PpHD2 using HD as the sole nitrogen source and various carbon sources were evaluated over 72 h, as shown in Figure 4. In M9HD supplemented with glucose (5 g/L) and HD (3 g/L) (Figure 4A), both strains exhibited efficient growth, indicating that HD can effectively serve as a nitrogen source when glucose is available as a carbon source. This result highlights the compatibility of HD with glucose for supporting bacterial growth. Furthermore, this demonstrates that our previous evolutionary engineering efforts [16] have significantly enhanced the ability of *P. putida* to utilize HD, showcasing its improved capacity to use HD efficiently in conjunction with a carbon source like glucose.

In contrast, when EG was used as the carbon source along with HD (Figure 4B), PpHD2 showed slight growth, while PpHD1 did not grow. This suggests that PpHD2 obtained a limited capacity to utilize EG as a carbon source during the second-stage coculture evolution. To investigate whether this slight growth might be attributed to the ability of PpHD2 to utilize adipate that generated from HD degradation, further experiments were conducted using media formulations shown in Figure 4C,D.

In Figure 4C, where no carbon source was added, neither strain exhibited growth, ruling out the possibility that HD alone could support bacterial growth as a source of both carbon and nitrogen. Similarly, in Figure 4D, where adipate was supplied as the sole carbon source along with ammonium chloride (NH_4Cl) as a nitrogen source, no growth was observed for either strain. These results confirm that PpHD2's slight growth in M9EG/HD (Figure 4B) is specifically due to its ability to degrade EG as a carbon source, albeit with limited efficiency, rather than any metabolic contribution from adipate that derived from

HD degradation. This highlights the restricted but notable metabolic capacity of PpHD2 to utilize EG as a carbon source.

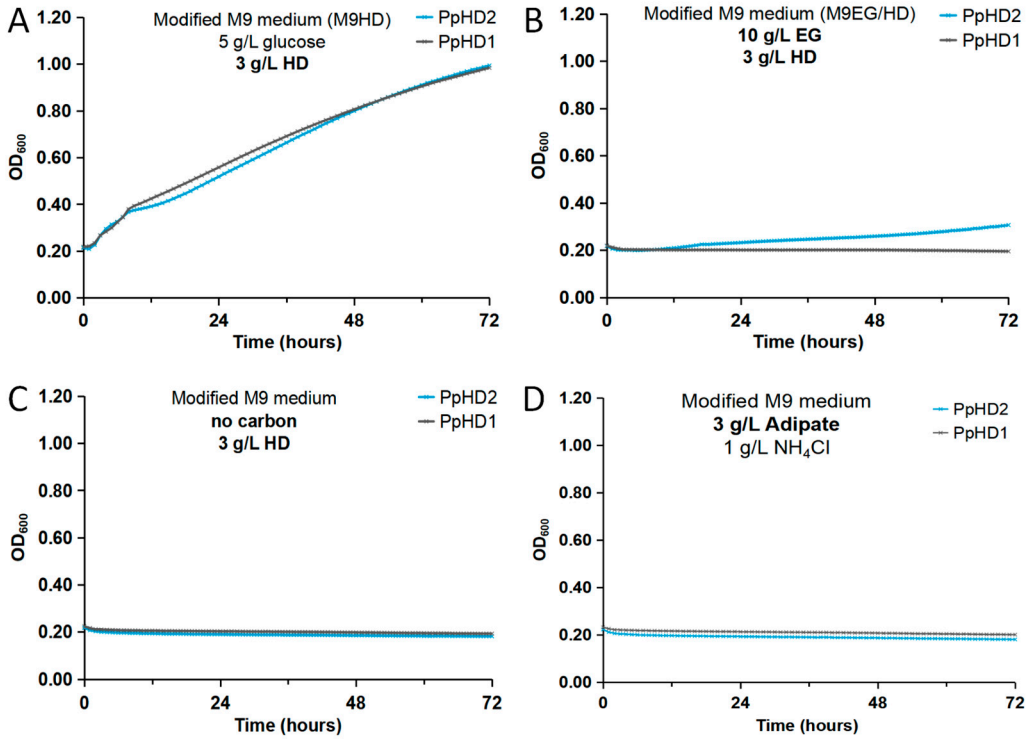

Figure 4. Growth of evolved *P. putida* strains in various modified M9 media formulations. (**A**) Growth of PpHD1 and PpHD2 strains in M9HD medium. (**B**) Growth of PpHD1 and PpHD2 strains in M9EG/HD medium. (**C**) Growth of PpHD1 and PpHD2 strains in modified M9 media with no added carbon source. (**D**) Growth of PpHD1 and PpHD2 strains in modified M9 media with adipate.

3.4. Characterization of Evolved Strains on EG

The ability of *E. coli* and *P. putida* strains to utilize EG as a carbon source was evaluated in isolation and co-culture. Figure 5A demonstrates the growth of EcEG1 and EcEG2 strains on modified M9EG agar plates containing 10 g/L EG and 1 g/L NH_4Cl after 72 h. While EcEG1 formed visible colonies, indicating its capacity to utilize EG as a carbon source after the first-stage evolutionary adaptation, EcEG2 failed to grow, with no colonies observed. This result suggests that EcEG2 lost its ability to metabolize EG as a carbon source during the second-stage coculture with *P. putida*. Figure 5B quantifies the growth of EcEG1 and EcEG2 in liquid M9EG media over 7 days, confirming that only EcEG1 retains the ability to slowly metabolize EG in monoculture, while EcEG2 shows no significant growth.

In contrast, Figure 5C shows that among the *P. putida* strains tested, PpHD2 demonstrated significant growth in M9EG media, while PpHD1 and the wild-type strain (PpWT) did not grow. This result highlights PpHD2's ability to utilize EG as a carbon source independently, supporting our observation in Figure 4B, where PpHD2 exhibited slight growth in M9EG/HD. However, when co-cultured with EcEG strains, a striking observation was made (Figure 5D): although EcEG strains initially adapted to EG, their ability to metabolize EG was suppressed when cultured with *P. putida*. Interestingly, the combination of PpHD2 and EcEG2 strains resulted in enhanced growth compared to PpHD2 alone. This synergistic growth suggests that while PpHD2 can use both HD as a nitrogen source and EG as a carbon source, the metabolic interaction with EcEG strains further boosts the efficiency of EG utilization in the co-culture system, likely due to complementary metabolic processes or resource sharing. This finding highlights the dynamic interactions between strains in the co-culture and their potential for enhanced substrate utilization.

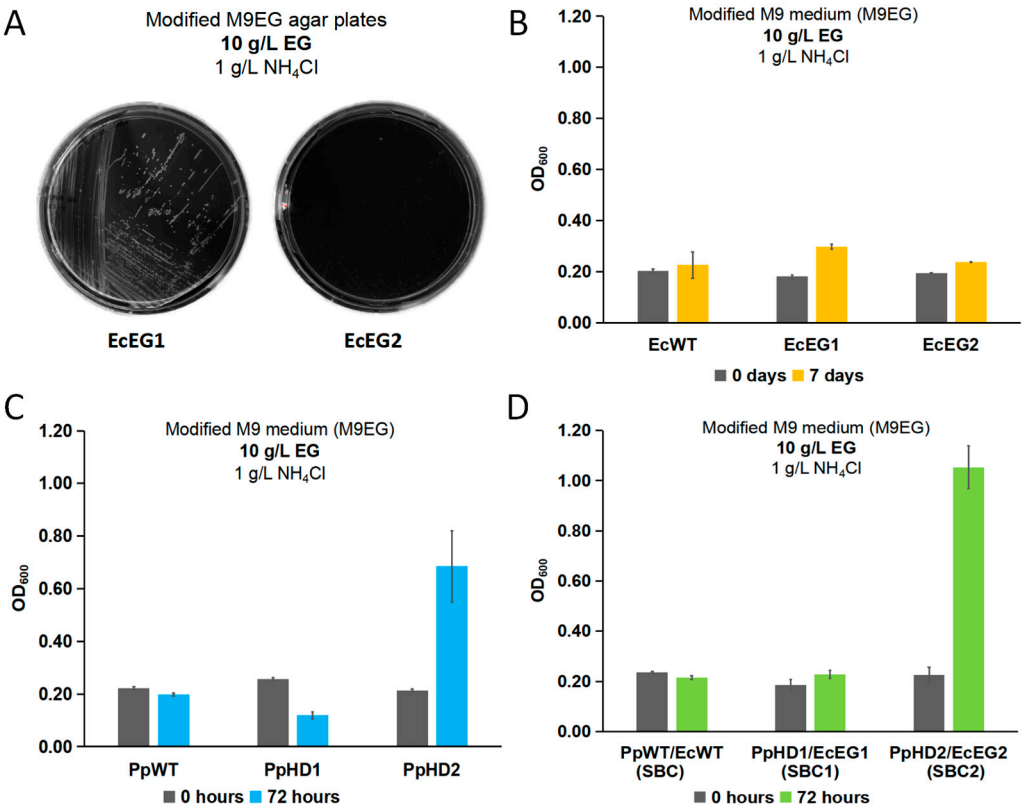

Figure 5. Growth of *E. coli*, *P. putida* and SBC strains in modified M9 media with EG as the carbon source. (**A**) Growth of EcEG1 and EcEG2 on M9EG agar plates. (**B**) Growth of *E. coli* strains in M9EG medium. (**C**) Growth of *P. putida* strains in M9EG media. (**D**) Growth in co-culture systems.

3.5. Analysis of Role Redistribution Between PpHD2 and EcEG2

Figure 6 illustrates the growth dynamics and metabolic interactions of the co-culture system in modified M9 medium. Figure 6A shows that the co-culture (PpHD2/EcEG2) exhibits significantly enhanced growth compared to PpHD2 alone, indicating that the presence of EcEG2 improves PpHD2′ utilization of HD and EG. Figure 6B provides insights into the subpopulation composition over time through 16S sequencing. Initially, PpHD2 and EcEG2 were inoculated at an approximately 1:1 ratio. After 24 h, EcEG2 dominates the population, constituting 61%, highlighting its early advantage. However, by 48 h, the balance shifts, with PpHD2 increasing its proportion to 55%, reflecting its growing advantage in metabolizing both HD and EG. This shift suggests an evolving division of labor and metabolic interactions within the consortium. The stabilization of the population ratios over time indicates the establishment of a balanced and cooperative dynamic between the two strains.

Inspired by a previous study [25] demonstrating that *E. coli* efficiently grows on glycolate as a carbon source, we hypothesized that EG is initially metabolized by PpHD2 into glycolate. Given PpHD2's limited efficiency in utilizing glycolate, evident by the glycolate build-up observed from HPLC, EcEG2 assumes the role of degrading glycolate, thereby supporting the community's carbon source requirements. Figure 6C shows HPLC measurements of glycolate production in M9EG/HD medium, revealing that glycolate concentrations increase significantly during PpHD2 monoculture. In contrast, in the co-culture system, glycolate concentrations remain at low levels, suggesting that EcEG2 efficiently consumes glycolate, preventing its accumulation.

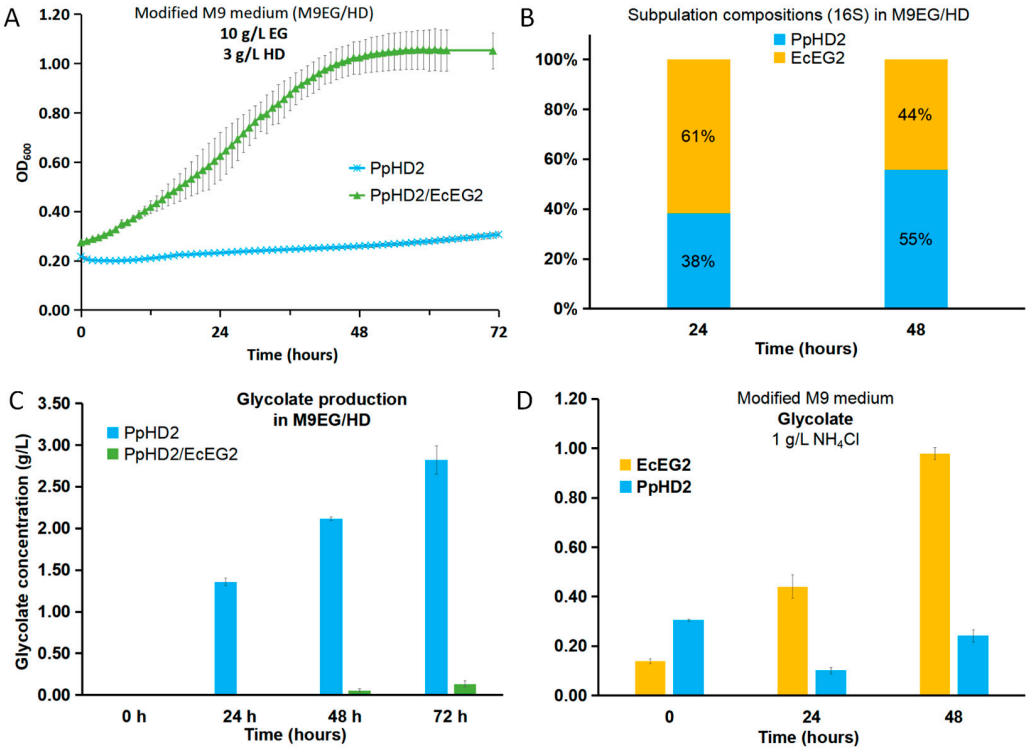

Figure 6. Growth dynamics and metabolic interactions of PpHD2 and EcEG2. (**A**) Growth curves of PpHD2 alone and in co-culture with EcEG2 in M9EG/HD. (**B**) Subpopulation composition of the coculture in M9EG/HD determined by 16S rRNA sequencing. (**C**) Glycolate production of PpHD2 alone and in co-culture with EcEG2 in M9EG/HD measured via HPLC. (**D**) Growth of PpHD2 and EcEG2 in M9 medium supplemented with glycolate as the sole carbon source.

To further confirm this hypothesis, we assessed the growth of EcEG2 and PpHD2 individually in modified M9 medium with glycolate as the sole carbon source (Figure 6D). The results show that EcEG2 grows well in the presence of glycolate, while PpHD2 exhibits a decrease in OD_{600}, possibly due to the accumulation of toxic intermediates such as glycolaldehyde and glyoxylate [26]. These findings highlight the complementary metabolic interactions between PpHD2 and EcEG2 in the co-culture system. PpHD2 degrades EG to glycolate and consumes HD to provide a nitrogen source for the community. Subsequently, EcEG2 takes over the role of efficiently degrading glycolate to support the community's carbon source needs. This division of metabolic labor promotes enhanced community growth, with PpHD2 contributing nitrogen availability and initial EG degradation, while EcEG2 ensures the efficient utilization of glycolate as a carbon source.

3.6. Metabolite Cross-Feeding and Flux Balance Analysis

We initially designed a cross-feeding system where *P. putida* would metabolize HD as a nitrogen source, and *E. coli* would degrade EG as the carbon source to complement community growth (Figure 1). However, the evolved system revealed a different dynamic (Figure 7A): PpHD2 primarily utilizes HD for nitrogen assimilation (upper) while degrading EG into glycolate (lower). The exported glycolate, which PpHD2 could not efficiently metabolize, was then consumed by EcEG2, enabling the latter to support the carbon requirements of the community. This new cross-feeding relationship demonstrates a division of labor distinct from the original design, emphasizing the evolved strains' ability to dynamically adjust their metabolic interactions to maximize the utilization of available resources in the co-culture. This adaptation highlights the potential of microbial

consortia to autonomously develop optimized cross-feeding mechanisms beyond initial experimental designs.

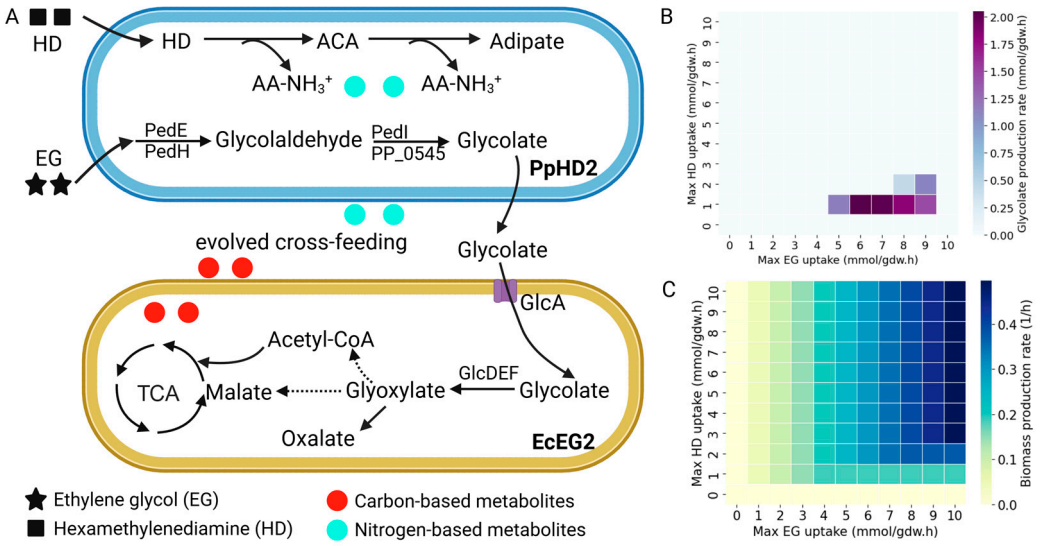

Figure 7. (**A**) The evolved cross-feeding module found in the SBC2. (**B**) Flux balance analysis (FBA) predicted the glycolate production rate (mmol/g DW h) in response to varying maximum uptake rates of EG and HD in modified metabolic conditions. (**C**) FBA predicted the biomass production rates (1/h) in response to varying maximum uptake rates of EG and HD in modified metabolic conditions.

To further explore the advantages of a co-culture, we conducted FBA on the post-adaptive evolution of *P. putida*. This analysis was performed in simulated media containing EG and HD as the sole carbon and nitrogen sources, respectively. The objective was to determine whether the observed benefits of the consortium could be attributed to stoichiometric imbalances in the metabolic pathways of PpHD2. The metabolic model of *P. putida* KT2440, iJN1463, was modified to include EG uptake and its subsequent conversion to glycolate and the HD pathway, which incorporates two key reactions contributing to nitrogen assimilation into biomass (Figure 7A).

The FBA simulations revealed that at high EG uptake rates with lower, non-zero HD uptake rates, glycolate export was observed, suggesting a stoichiometric imbalance in the PpHD2 monoculture (Figure 7B). This imbalance likely results from PpHD2's limited ability to consume glycolate efficiently. These results confirm that PpHD2 can independently use EG and HD as sole carbon and nitrogen sources, respectively, allowing it to survive in monoculture. However, the consortium with EcEG2 offers significant advantages through division of labor. Cross-feeding mitigates toxic by-products and enables the assimilation of glycolate, which would otherwise be exported by PpHD2. In the consortium, glycolate is efficiently consumed by EcEG2, contributing to enhanced biomass production within members of the consortium. This observation from FBA is consistent with findings from HPLC data and growth assays, which show glycolate accumulation in PpHD2 monoculture but not in the co-culture. FBA simulations also indicate that increasing the upper bounds of both EG and HD uptake resulted in higher biomass production rates, however, plateauing (Figure 7C). Biomass production was more sensitive to EG uptake than HD uptake, highlighting the critical role of EG metabolism in supporting overall growth. These results suggest that future efforts to engineer or overexpress key enzymes involved in EG uptake and degradation in PpHD2, such as PedE, PedH, PedI, and PP_0545, could further enhance the conversion of EG to glycolate and subsequently improve biomass production. While FBA provides a stoichiometric perspective of these metabolic interactions, it does not

account for thermodynamic constraints or kinetic parameters, underscoring the importance of experimental validation alongside computational models.

4. Discussion

The results of our study provide a compelling demonstration of the potential of SBC to address the challenge of mixed plastic waste degradation. By engineering a co-culture of *E. coli* Nissle 1917 and *P. putida* KT2440, we established a framework for efficiently degrading plastic-derived monomers such as ethylene glycol (EG) and hexamethylenediamine (HD). The observed metabolic interactions within the consortium, particularly the cross-feeding dynamics and adaptive ability of *P. putida* to degrade both EG and HD, are consistent with prior findings on microbial versatility in carbon and nitrogen assimilation.

Previous studies have extensively explored the utilization of EG by engineered *E. coli* and *P. putida*, often emphasizing metabolic rewiring by using gene overexpression strategies. Pandit et al. [19] engineered *E. coli* with aldA and fucO overexpression for efficient glycolate production, while Höhmann et al. [25] highlighted strain-specific regulatory adaptations for glycolate metabolism and found that overexpression of early degradation genes impairs growth on glycolate. Franden et al. [26] extensively engineered *P. putida* for EG utilization by introducing glcDEF genes to convert glycolaldehyde into glycolate. Additionally, overexpression of gcl, hyi, glxR, ttuD, and pykF enhanced glyoxylate consumption, directing it toward biomass production. In contrast, our study utilized adaptive evolution to enable an SBC for both HD and EG degradation efficiently. The role of *P. putida* in the SBC aligns with its known metabolic flexibility and robustness in utilizing diverse organic substrates [26–28]. The adaptive ability of *P. putida* to degrade EG, despite initially being optimized for HD metabolism, reflects a novel evolutionary trajectory induced by co-culture conditions. This evolutionary flexibility suggests that synthetic consortia can self-optimize to handle the heterogeneity of plastic waste, reducing the need for pre-sorting and enabling cost-effective bioremediation strategies.

While our study demonstrates the feasibility of SBCs for mixed monomer degradation, expanding their applicability to real-world plastic waste streams remains essential. Given the low efficiency of polymer biodegradation, an immediate strategy could involve combining microbial biodegradation with chemical and physical depolymerization techniques [29,30] to break down polymers into more accessible monomers. However, the ultimate goal should be to develop a more advanced and sustainable biological approach for comprehensive plastic waste recycling. Mimicking real-world waste conditions will be critical to refining the process, while computational modeling, systems synthetic biology, and metabolic engineering should work collaboratively to optimize bacterial consortium design strategies [31]. Growth-coupled strategies and cross-feeding interactions within the consortium can further enhance metabolic efficiency, stabilize community dynamics, and improve degradation performance. Incorporating additional bacterial species and polymer-degrading enzymes could enable comprehensive mixed-plastic degradation [32,33]. Long-term advancements could involve engineering pathways for producing valuable biochemicals, such as fatty alcohols [34], polyhydroxyalkanoates [35,36], valencene [37], or other high-value compounds, effectively transforming waste into resources and fostering a circular economy. Although adaptive evolution has enhanced consortium efficiency, elucidating the genetic and regulatory mechanisms through omics approaches will be crucial for further optimization [38–40]. In conclusion, this study demonstrates the feasibility of SBCs for mixed monomer degradation and marks an advancement toward our long-term vision of integrating evolved and engineered strains for the efficient breakdown of complex plastic waste and their conversion into valuable chemicals.

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