Engineering a Synthetic Bacterial Consortium of *Escherichia coli* and *Pseudomonas putida* for Mixed Plastic Monomer Bioprocessing

by

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Author's Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Statement of Contributions

Some sections of this thesis contain work that have been published or are under consideration for publication as follows:

1. Dharmasiddhi, I. P. W., Chen, J., Arab, B., Lan, C., Euler, C., Chou, C. P., & Liu, Y. (2025). Engineering a Cross-Feeding Synthetic Bacterial Consortium for Degrading Mixed PET and Nylon Monomers. *Processes*, *13*(2). https://doi.org/10.3390/pr13020375
    a. This publication corresponds to Chapter 3 and Chapter 4 of my thesis. My contribution to the work involved methodology, software, formal analysis, investigation, writing, and visualization.
    b. Conceptualization, Y.L.; methodology, I.P.W.D., B.A. and Y.L.; software, I.P.W.D. and C.E.; formal analysis, I.P.W.D. and J.C.; investigation, I.P.W.D., B.A. and C.L.; writing—original draft preparation, I.P.W.D.; writing—review and editing, I.P.W.D., J.C., C.L., C.P.C. and Y.L.; visualization, I.P.W.D. and J.C.; supervision, Y.L.; funding acquisition, Y.L., C.P.C. and C.E. All authors have read and agreed to the published version of the manuscript.

I confirm that, except where due acknowledgement is made, the work presented in this thesis is my own and has not been submitted for any other degree of qualification at this or any other institution.

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 constraint-based modeling, high-performance liquid chromatography (HPLC), and comprehensive 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.

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Dedication

I dedicate this thesis to my beloved grandfather, Dr. Ida Pedanda Gede Nyoman Jelantik Oka, whose wisdom has always been a constant source of inspiration. His legacy of service, both as a veteran and a scientist, will forever live on in my heart and in the work I do.

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List of Abbreviations

Abbreviation	Meaning
ALE	Adaptive laboratory evolution
COBRA	Constraint-based reconstruction and analysis
COMETS	Computation of microbial ecosystems in time and space
dFBA	Dynamic flux balance analysis
EcEG1	*Escherichia coli* Nissle 1917 after stage 1 ALE
EcEG2	*Escherichia coli* Nissle 1917 after stage 2 ALE
EcWT	*Escherichia coli* Nissle 1917
EG	Ethylene glycol
FBA	Flux balance analysis
GEM	Genome-scale metabolic model
GPR	Gene-protein-reaction associations
HD	Hexamethylenediamine
HPLC	High-performance liquid chromatography
LLDPE	Linear low-density polyethylene
M9EG	Modified M9 media containing 10 g/L EG
M9EG/HD	Modified M9 media containing 10 g/L EG and 3 g/L HD
M9G	Modified M9 media containing 5 g/L glucose
M9GA	Modified M9 media containing 3 g/L glycolate and 1 g/L ammonium chloride
M9HD	Modified M9 media containing 3 g/L HD
M9NH	Modified M9 media containing 1 g/L ammonium chloride
MA	Cis-cis muconate
mcl-PHA	Medium-chain length polyhydroxyalkanoates
MHET	Mono-(2-hydroxyethyl) terephthalic acid/terephthalate
MNP	Micro and nano plastics
OD_{600}	Optical density at 600 nm
ODE	Ordinary differential equation
PAH	Polyaromatic hydrocarbons
PCR	Polymerase chain reaction

PpHD1	*Pseudomonas putida* KT2440 after stage 1 ALE
PpHD2	*Pseudomonas putida* KT2440 after stage 2 ALE
PpWT	*Pseudomonas putida* KT2440
PVC	Polyvinyl chloride
sgRNA	Single guide RNA
SBC	Synthetic bacterial consortium
SBC1	Synthetic bacterial consortium after stage 1 ALE
SBC2	Synthetic bacterial consortium after stage 2 ALE
TPA	Terephthalic acid

Chapter 1 – Introduction and Literature Review

1.1 The Power and Peril of Plastics

Plastics have become an essential commodity to everyday life. Their versatility, durability, and low costs have driven their widespread use in almost every field, such as packaging, construction, automobiles, medicine, and electronics. As a result, plastic consumption has increased approximately 180-fold between 1980 and 2018 (Nayanathara Thathsarani Pilapitiya & Ratnayake, 2024). However, there are severe and long-lasting environmental repercussions associated with their end-of-life fate (Geyer et al., 2017).

Conventional plastics are persistent in the environment, and do not easily degrade due to their high molecular weight and absence of natural analogs (Worm et al., 2025). Instead, they undergo fragmentation into smaller particles, known as micro- and nano-plastics (MNPs) (Kumar et al., 2023; K. Zhang et al., 2021). Mechanical forces, such as sediment abrasion and wave action, break plastics into smaller pieces, changing their size distribution without reducing overall mass. On the other hand, slow degradation of plastics can occur via photo-oxidation or microorganisms. However, these degradation processes are highly inefficient and can take decades to centuries (Chamas et al., 2020; Kubowicz & Booth, 2017)

As plastic waste continues to accumulate in landfills, oceans, and ecosystems, its impacts have attracted concern. It is predicted that around 9-23 million metric tons of plastic waste accumulate in oceans, lakes, and rivers, while another 13-25 million metric tons are deposited on land annually (MacLeod et al., 2021). Oftentimes as MNPs, these pollutants are pervasive, and are found in extremely remote locations, from mountain peaks to the depths of the arctic ocean (Barnes et al., 2010; Bergmann et al., 2022; Materić et al., 2022; Napper & Thompson, 2024).

The presence of plastic particles in ecosystems leads to extensive environmental consequences. Plastics act as carriers for chemical pollutants, such as persistent organic pollutants, heavy metals, and metalloids, adsorbed from the environment (Okoye et al., 2022). Ultimately, they accumulate in food chains and pose risks to wildlife and human health. Additionally, biodiversity is severely impacted, as wildlife suffers from ingestion, entanglement, and habitat disruption caused by plastic waste (Garcês & Pires, 2024). Economically, plastic pollution affects a wide range of industries

such as fisheries, aquaculture, tourism, recreation, and agriculture, leading to financial losses (Beaumont et al., 2019; Diggle & Walker, 2022).

1.2 Conventional Plastic Waste Management

Given the significant environmental repercussions, the need for efficient management strategies is evident. Conventional methods for plastic waste management include landfilling, incineration, pyrolysis, recycling and bioremediation (Evode et al., 2021).

1.2.1 Landfilling

Landfilling refers to the disposing of plastic waste and other materials in designated areas where they accumulate and are eventually buried (Evode et al., 2021). Out of all the waste management strategies, landfilling is the most common in many countries due to its low cost and technical requirement (Geyer et al., 2017; Vaverková, 2019). In the landfill, organic molecules undergo degradation and decomposition, which can take up to decades or centuries, depending on the material. Additionally, landfills pose the risk of MNPs leaching into the surrounding soil and water through landfill leachate, potentially constituting a threat to the environment, and to human health (Huang et al., 2022; Manikanda Bharath et al., 2023; Sun et al., 2021).

1.2.2 Incineration

Incineration is one of the two common thermal treatment methods for plastic waste. It involves the combustion of waste in the presence of oxygen (Evode et al., 2021). In addition to effectively managing large-scale plastic waste, incineration can recover thermal energy and maximize the resource utilization potential of plastic waste (Pan et al., 2020). Generally, byproducts of incineration include carbon dioxide and water. However, variations in plastic composition and incineration conditions can lead to the formation of toxic substances, such as polycyclic aromatic hydrocarbons (PAHs) and carbon monoxides. Additionally, recent reports have shown that incineration may release MNPs into the environment through the produced bottom ashes (Shen et al., 2021; Yang et al., 2021).

1.2.3 Pyrolysis

Pyrolysis is the breakdown of organic materials via intense heat, and in the absence of oxygen (Evode et al., 2021). The output of pyrolysis is dependent on the input, as the acquired plastic waste oftentimes constitutes a variety of plastic polymers. Consequently, the output of pyrolysis is

a mixture of hydrocarbons of varying chain lengths, in oil and wax form, which can be further refined into fuels or chemicals (Qureshi et al., 2020). It can deal with a wide variety of plastic waste simultaneously (Anuar Sharuddin et al., 2016). However, feedstock selection is a crucial factor in pyrolysis. Halogens, such as chlorine from polyvinyl chloride (PVC), can form corrosive byproducts, such as HCl, which damage reactor components (Giglio et al., 2023). They also contaminate pyrolysis oil and wax, reducing its usability, and can lead to the formation of hazardous pollutants like dioxins and furans.

1.2.4 Recycling

Recycling is the process of collecting and processing plastic waste to convert it into reusable materials or new products (Evode et al., 2021). Plastic recycling provides numerous advantages, such as reducing pollution, conserving natural resources, and decreasing energy consumption. It also minimizes harmful emissions from incineration and preserves valuable landfill space. Furthermore, recycling supports economic growth and encourages a more sustainable way of life. However, one of the greatest challenges associated with plastic recycling is the need for a sophisticated sorting process (Larder & Hatton, 2023; Lubongo et al., 2024; Pandey et al., 2015; Taneepanichskul et al., 2022). Sorting is essential because cross-contamination can interfere with recycling, therefore reducing the quality of the product. In fact, plastics only endure a finite number of recycling cycles, with around 10% going through multiple rounds (Singh & Walker, 2024). To preserve their properties, they are frequently mixed with virgin materials (Garcia & Robertson, 2017). Although recycling helps with recovery rates, the end products often have lower quality than those made from new plastics, which limits their applications and reduces their value (Alassali et al., 2021; Ragaert et al., 2017).

1.2.5 Bioremediation

Bioremediation is the use of biological agents to remove harmful substances from the environment (Evode et al., 2021; Kulshreshtha et al., 2014). One of the advantages pertaining to bioremediation is that it offers a green solution to plastic waste management (Zhou et al., 2022). It is a cost-effective approach that can selectively convert toxic substances into water, carbon dioxide, and other less harmful compounds (Ayilara & Babalola, 2023). Additionally, numerous species of bacteria, ubiquitously occurring in nature, have been reported to degrade various plastics (Asiandu et al., 2020; Taghavi et al., 2021; Urbanek et al., 2018). However, there are several

challenges to the bioremediation of plastic waste, including the low efficiency in degradation, scarcity of effective degradation enzymes, and obstacles in using some of the resulting breakdown products (Kubowicz & Booth, 2017; Weldon et al., 2025; Wu et al., 2025).

To address these challenges, synthetic biology technologies can offer innovative solutions to enhance the bioremediation of plastic waste (Wu et al., 2025). Through protein engineering, enzymes can be optimized for improved stability, catalytic activity, and substrate specificity (Y. Liu et al., 2022; Zhu et al., 2022). On the other hand, metabolic engineering can be employed to develop efficient pathways for plastic degradation (Franden et al., 2018; Werner et al., 2025). Plastic degradation products can also be redirected into valorized biochemicals, promoting a circular economy (Klauer et al., 2024; Tiso et al., 2021). Finally, the construction of a synthetic microbial consortia will allow different species to collaborate to degrade plastics through complementary enzymatic pathways (Bao et al., 2023; Qi et al., 2021; Salinas et al., 2024).

Adaptive laboratory evolution (ALE) is another technique that can be used to enhance efficiency of degradation. Here, microbes are grown under controlled conditions for extended durations, typically from weeks to years, facilitating the selection of improved phenotypes. Cells more suited to the conditions described are more likely to proliferate (Dragosits & Mattanovich, 2013). Numerous studies have demonstrated that ALE can enhance the tolerance or efficiency of a microorganism, or a consortium towards a particular substrate (Baek et al., 2016; R. Liu et al., 2019; X. Zhang & Reed, 2014).

1.3 Microbial Consortia for Plastic Waste Treatment

Microbial consortia are communities composed of multiple microbial populations that coexist in various natural environments. They play essential roles in processes beneficial to humans, including food digestion, wastewater treatment, and bioremediation (Brenner et al., 2008; Duncker et al., 2021). By functioning synergistically, consortia can perform more complex tasks compared to conventional monoculture, such as the degradation of plastic waste.

The advantages of microbial consortia are often attributed to the division of labor, with each strain specializing in performing specific sub-tasks within the broader process. This specialization enhances efficiency in completing the overall process (Brenner et al., 2008; Duncker et al., 2021; Qian et al., 2020; Roell et al., 2019). The division of labor is often complemented by cross-feeding,

where metabolic by-products or intermediates produced by one species serve as nutrients or energy sources for others (Culp & Goodman, 2023). Additionally, this may prevent the accumulation of inhibitory intermediates (Qi et al., 2021). Cooperative interactions within the consortia allow them to adapt to a variety of environmental hurdles.

Naturally occurring plastic-degrading microbial consortia have been extensively documented in previous literature (Gao & Sun, 2021; Roberts et al., 2020; Salinas et al., 2023; J. Zhang et al., 2024). Generally, they are isolated from environments that have been contaminated with plastics or hydrocarbons. For example, in the study by Roberts et al. (2020), a consortium that can degrade polyethylene terephthalate (PET) was isolated from petroleum-polluted soils. They screened for lipase activity, which is associated with PET degradation. Similarly, Salinas et al. (2023) uncovered a microbial consortium that could degrade linear low-density polyethylene (LLDPE). This was isolated from a soil sample that was contaminated with LLDPE.

1.3.1 Integrating Synthetic Biology and Microbial Consortia

Considering the ubiquitous nature of microbial consortia and the advancements of synthetic biology, combining these approaches can enable the development of tailored communities designed to perform more specific tasks with greater efficiency. (Qian et al., 2020; Roell et al., 2019; Wu et al., 2025). Two studies did this by carefully engineering the division of labor (Bao et al., 2023; Qi et al., 2021). In Bao et al. (2023), two engineered strains of *Pseudomonas putida* were designed to metabolize PET monomers, ethylene glycol (EG) and terephthalic acid (TPA), and upcycle it into medium chain length polyhydroxyalkanoates (mcl-PHA) and cis-cis muconate (MA), the value-added products. One strain focused on EG, while the other was tasked with consuming TPA, clearly indicating the division of labor. This study shows how synthetic biology can be used to integrate upcycling pathways, enabling the generation of valorized bioproducts alongside plastic degradation, which supports a circular economy (Klauer et al., 2024). On the other hand, Qi et al. (2021) engineered two *Bacillus subtilis* strains to secrete PETase and MHETase to hydrolyze PET. Following that, they incorporated *Rhodococcus jostii* and *P. putida* to consume the resulting PET monomers and mitigate their toxicity. This second study demonstrates how engineered consortia can leverage cross-feeding interactions to reduce toxicity, enhancing the efficiency of plastic degradation.

1.3.2 Challenges

Although it is clear how microbial consortia can be employed to manage plastic waste, one of the most significant hurdles is to maintain the stability and coexistence of multiple populations (Lin et al., 2024; Said & Or, 2017). Stability is crucial to the intended function of the microbial consortia (Roell et al., 2019). This challenge can be associated with the unpredictable long-term behavior and genetic stability of organisms within the consortia. Over time, factors such as mutations, selective pressures, and competitive interactions may lead to genetic drift, altering the composition and functionality of the community (Brenner et al., 2008; Duncker et al., 2021).

These challenges can be addressed through the strategic division of labor within microbial consortia like those described in previous works (Bao et al., 2023; Qi et al., 2021). Developing strains with interdependent metabolic pathways promote stability by ensuring mutual reliance (Duncker et al., 2021). For instance, engineering sequential pathways allows one strain to break down complex substrates into smaller, fermentable molecules that another species can utilize for further conversion (Lin et al., 2024). Additionally, interacting populations can be specifically designed to detoxify inhibitory substances, preventing the accumulation of toxic intermediates and maintaining a stable environment (Roell et al., 2019).

Another strategy to overcome the challenges is the use of programmed population control within the microbial consortia (Duncker et al., 2021). One example is the implementation of a predator-prey relationship, where one microbial strain acts as the predator, while the other serves as the prey (Balagaddé et al., 2008). The predator consumes or inhibits the prey population, preventing it from overgrowing. Meanwhile, the prey population provides nutrients or other benefits that support the growth of the predator population, ensuring their co-existence. Similarly, another 2019 study describes a “rock-paper-scissors” dynamic in a consortium to increase stability of gene circuits, where three strains of *Escherichia coli* were used (Liao et al., 2019). Here, each strain dominates one competitor but is susceptible to another, preventing any single strain from taking over the community. When mutations reduce the function of one strain, the second strain that carries the desired circuit kills the mutated strain, restoring the balance of the system.

Chapter 2 – Thesis Scope and Objectives

2.1 Problem Statement and Hypothesis

Engineered bacterial consortia for bioremediation and plastic waste management presents a promising alternative to traditional methods. Plastic waste often exists as mixed polymers. By tackling monomers from two different plastic polymers, this project aims to overcome the challenges of mixed plastic waste by eliminating the need for sorting. That being said, the overall objective of this thesis is to develop a synthetic bacterial consortium consisting of engineered *E. coli* and *P. putida* for the degradation of EG and hexamethylenediamine (HD) monomers of PET and nylon-6,6, respectively. The central hypothesis is that *E. coli* would assimilate carbon from EG and provide a usable carbon source for the *E. coli-P. putida* community. Conversely, *P. putida* would assimilate nitrogen from HD, and supply nitrogen for the consortium.

2.2 Research Scope and Objectives

Objective 1: *Conduct ALE on the bacterial consortium to enable it more effectively use EG and HD as the sole carbon and nitrogen sources, respectively.*

E. coli EG1 (EcEG1) and *P. putida* HD1 (PpHD1) was developed by direction evolution, integrated a CRISPR pool library and ALE, described in a previous study (Arab et al., 2024; Dharmasiddhi et al., 2025). The subsequent second-stage ALE of the two engineered strains will be conducted in a chemostat to ensure a constant supply of modified M9 media, with EG and HD as the sole carbon and nitrogen sources, respectively. The process will be carried out as close to steady-state conditions as possible. If a rise in optical density at 600 nm (OD_{600}) is detected, the inlet flowrate, equal to the outlet flowrate, will be increased accordingly. Conversely, if OD_{600} decreases, the flowrate will be reduced. An increase in OD_{600} indicates adaptation, necessitating a higher flowrate with more nutrients to sustain steady-state conditions.

Objective 2: *Perform the characterization of the evolved bacterial consortium to show its increased effectiveness, and to explain any changes in dynamics.*

Characterization will be conducted by comparing the pre-adaptive evolution strain with the resulting strains, *E. coli* EG2 (EcEG2) and *P. putida* HD2 (PpHD2), in their utilization of EG and HD through growth assays, and high-performance liquid chromatography (HPLC). Any changes

in the roles within the bacterial consortium will be examined. Additionally, flux balance analysis modeling will be performed to assess the metabolic shifts and identify key pathways.

Chapter 3 – Design, Evolution and Characterization of a Cross-Feeding Synthetic Bacterial Consortium

3.1 Background Information

PET and nylon-6,6 are some of the most common plastic pollutants in the environment (Castelvetro et al., 2021). The former is often used in drink and food packaging, while the latter is found commonly in clothes. PET is particularly notorious in the form of single-use bottles and containers, which are challenging to manage through conventional recycling methods (Hamade et al., 2020; Kankanige & Babel, 2020). Meanwhile, nylon-6,6 may shed microfibers during washing, entering wastewater systems, and ultimately contaminating aquatic environments (Lim et al., 2022; Tiwari et al., 2022).

3.1.1 Monomers of Polyethylene Terephthalate and Nylon-6,6

Efforts to mitigate these pollutants have led to the discovery and development of novel enzymes that efficiently depolymerize these compounds. PETase, is a well-characterized enzyme produced by *Ideonella sakaiensis*, that depolymerizes PET into mono-(2-hydroxyethyl) terephthalic acid (MHET). Also found in *I. sakaiensis*, MHETase further catalyzes the hydrolysis of MHET into EG and TPA, thereby completing the degradation process (Palm et al., 2019; Yoshida et al., 2021). Figure 1A clearly illustrates the metabolic breakdown of PET into its monomers. Similarly, nylon hydrolase targets nylon-6,6 polymers, cleaving the amide bonds to yield HD and adipic acid (Figure 1B) (Nagai et al., 2014).

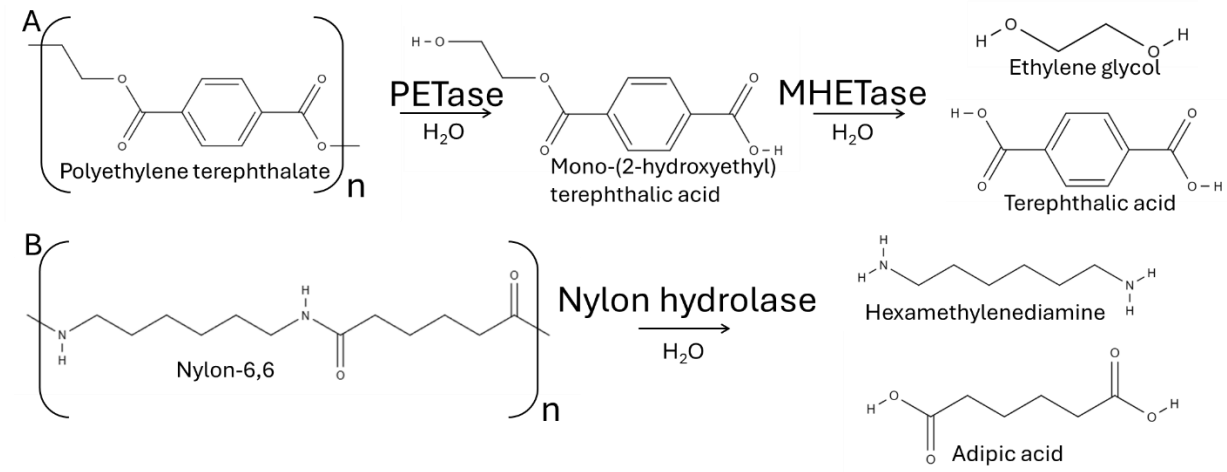


Figure 1. Mechanism of depolymerization for **(A)** PET and **(B)** nylon-6,6.

3.1.2 Consortium Design

To break down these monomers, and to meet the challenges of mixed plastics, we employ a synthetic bacterial consortium consisting of two engineered strains to consume two of the byproducts from the depolymerization of PET and nylon-6,6. The two genetic backbones for this project are *E. coli* Nissle 1917 (EcWT) and *P. putida* KT2440 (PpWT). Several past studies have demonstrated the use of *E. coli* to assimilate EG (Chi et al., 2024; Nemoto et al., 2024; Panda et al., 2021; Pandit et al., 2021; Yan et al., 2024). A common pathway in *E. coli* is via the *fucO* and *aldA* genes, which encode for 1,2-propanediol oxidoreductase and lactaldehyde dehydrogenase, respectively. Here, EG is directly converted into glycolaldehyde, and then into glycolate, which ultimately may enter the citric acid cycle (TCA) or glycolysis or be exported. Likewise, *P. putida* was selected for HD metabolism because of its remarkable metabolic versatility, and established role in bioremediation (Nogales et al., 2020; Weimer et al., 2020). HD metabolism results in the adipic acid being produced (Arab et al., 2024).

The consortium is designed to function symbiotically, with a distinct division of labor. *E. coli* facilitates EG assimilation, converting it into a carbon source for *P. putida*. In return, *P. putida* metabolizes HD, generating a nitrogen source that supports *E. coli* (Figure 2). This exchange is designed to be a mutually beneficial relationship. Engineering cross-feeding dependencies, such as this, can be an effective strategy to further enhance stability of the consortium. By promoting mutual reliance, this approach reduces the likelihood of dominance by one species, thereby maintaining population balance and ensuring long-term functionality (Duncker et al., 2021).

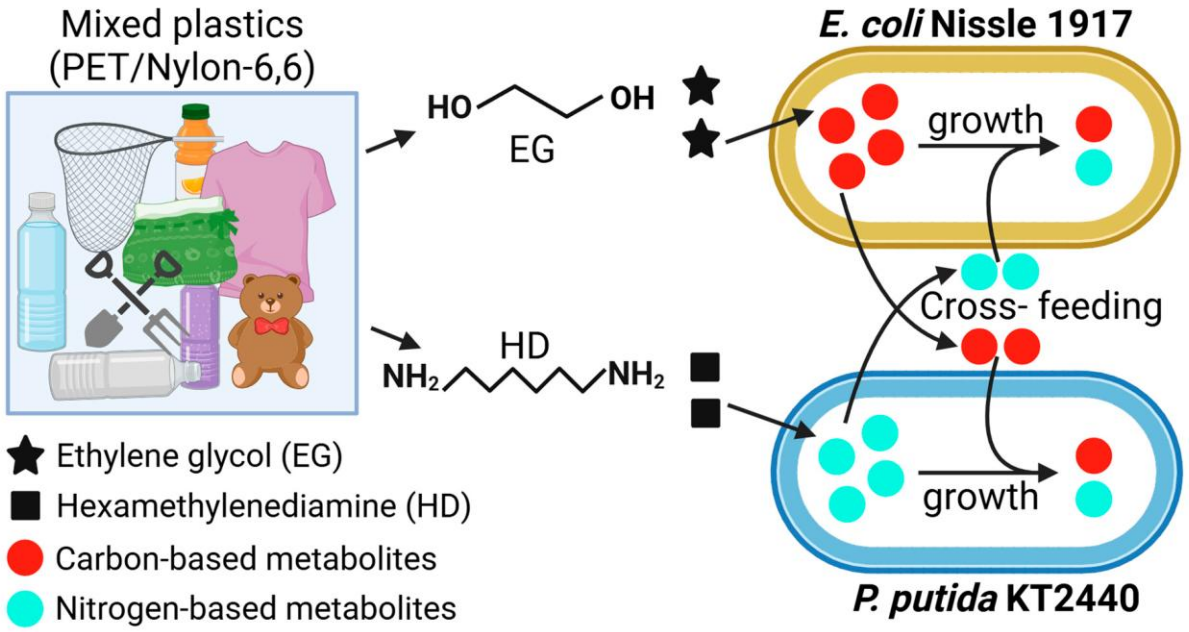


Figure 2. Design of a cross-feeding synthetic bacterial consortium for plastic monomers degradation. Cross-feeding strategy schematic: *E. coli* consuming EG (ethylene glycol) to provide a carbon source and *P. putida* utilizing HD (hexamethylenediamine) to provide a nitrogen source for the community. Taken from Dharmasiddhi et al. (2025)

The project employs a two-step ALE approach to optimize the strains for their respective nutrient sources and enhance their cooperative interactions. This strategy aims to improve their efficiency in degrading EG and HD, building on the successes of ALE observed in previous studies (Baek et al., 2016; Dragosits & Mattanovich, 2013; R. Liu et al., 2019; X. Zhang & Reed, 2014). The first stage was performed individually for *E. coli* and *P. putida* in test tubes by Dr. Jinjin Chen and Dr. Bahareh Arab, respectively. It involved a CRISPR pool containing a diverse library of single guide RNAs (sgRNAs) targeting various genes, while being exposed to gradually increasing amounts of EG or HD (Arab et al., 2024; Dharmasiddhi et al., 2025). Subsequently, the second stage of ALE on the resulting EcEG1 and PpHD1 involves a chemostat that is supplied with media containing EG and HD, where the flowrate is progressively increased.

3.2 Materials and Methods

3.2.1 Bacterial Strains and Media

The wild-type strains used in this study were *E. coli* Nissle 1917 and *P. putida* KT2440. Medium components were obtained from BioShop Canada Inc. (Burlington, ON, Canada), Bio Basics Inc. (Toronto, ON, Canada), or Sigma-Aldrich Co (Oakville, ON, Canada). The strains were stored in glycerol at -80 °C and streaked onto lysogeny broth (LB) agar plates (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) or M9 agar plates containing 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 $CoCl_2 \cdot 6\,H2O$, 0.1 g/L $CuCl_2$, 0.2 g/L $ZnCl_2 \cdot 4H_2O$, 0.2 g/L $NaMnO_4$, 0.05 g/L H_3BO_3 in 0.1 M HCl).

A wide range of modified M9 media were used to culture and evolve the strains, which include: M9G (M9 with 5 g/L glucose), M9EG (M9 with 10 g/L EG replacing glucose), M9NH (M9 with 1 g/L NH_4Cl), M9HD (M9 with 3 g/L HD replacing 1 g/L NH_4Cl), M9EG/HD (M9 with 10 g/L EG as the carbon source and 3 g/L HD as the nitrogen source), M9GA (M9 with 3 g/L replacing glucose), and M9AA (M9 with 3g/L replacing glucose). Culture samples were 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).

3.2.2 Adaptive Laboratory Evolution

PpWT and EcWT were progressively adapted to M9 media with increasing concentrations of HD and EG, respectively. PpWT was evolved into PpHD1 using a modified M9 medium, where the ratio of M9NH and M9HD was incrementally adjusted from 1:0, to 1:2, 1:5, 1:10, and finally 0:1, as presented in a previous study (Arab et al., 2024). On the other hand, EcWT was developed into EcEG1 with increasing ratios of M9G:M9EG from 1:0, 1:1, 1:3, 1:5, 1:7, to 0:1, respectively (Figure 3A) (Dharmasiddhi et al., 2025).

After the development of PpHD1 and EcEG1 in the first stage of ALE, they were combined in a 1:1 ratio. The resulting co-culture, SBC1, was grown at 30 °C for three days on a rotary shaker set at 220 rpm in a modified M9 medium containing 10 g/L EG and 3 g/L HD as the sole carbon and nitrogen source, respectively (M9EG/HD). Subsequently, 1 mL of the seed culture was

transferred into 50 mL of fresh M9EG/HD and grown overnight under similar conditions. The volume was then transferred into a chemostat to conduct ALE.

The chemostat, shown in Figure 3B, was operated at 30 °C while stirring at 1000 rpm for improved agitation. Additionally, it was fed with fresh M9EG/HD at a set flowrate. Steady-state conditions were maintained throughout ALE by regularly monitoring OD_{600} and maintaining it at approximately 0.4. The initial inlet flowrate was 0.1 mL/min and was modified accordingly to maintain steady-state conditions. After 25 days of ALE, glycerol stocks of the resulting strains SBC2 containing EcEG2 and PpHD2, were prepared for storage.

The resulting SBC2 was streaked on LB agar plates to obtain single colonies, and primers yltEcNF/yltEcNR (agtacatggcgtgaggctgat; cgtggcaggacagctacgc) and tGyqhdF2/tGyqhdR2 (tggcgaatctgcgtctgc; gagtgtccgggtctgttgtatg) were used to identify and to isolate for EcEG2 and PpHD2, respectively. Polymerase chain reaction (PCR) was performed using the KAPA2G Fast HotStart ReadyMix kit (Kapa Biosystems, Wilmington, MA, USA) following the manufacturer's cycling protocol.

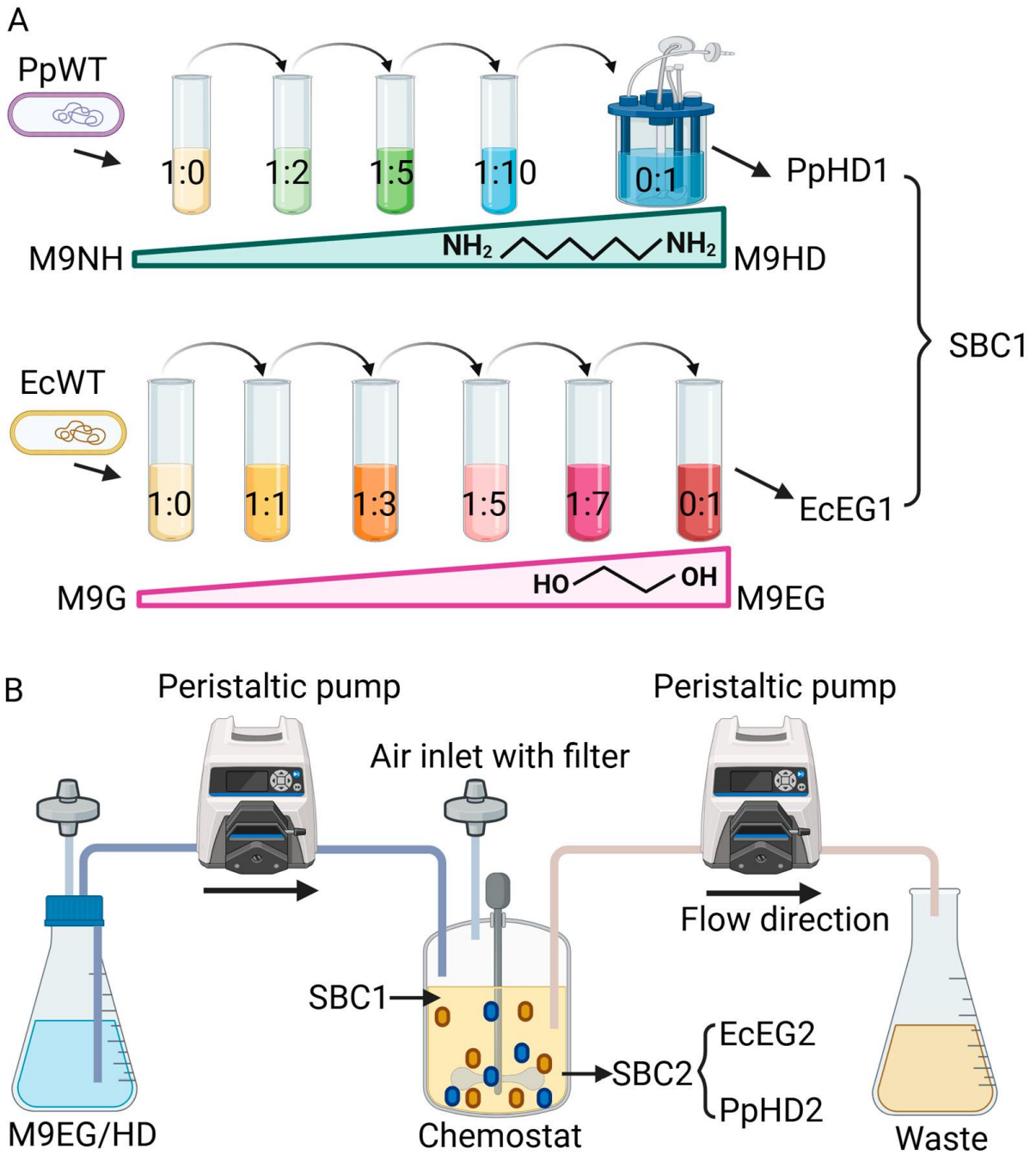


Figure 3. ALE of synthetic bacterial consortium for EG and HD degradation. **(A)** Adaptive evolution of PpWT (top) and EcWT (bottom) to obtain PpHD1 and EcEG1, separately. **(B)** Chemostat setup for consortium evolution: inflow of fresh M9EG/HD medium, agitation for mixing, and controlled outflow to maintain steady-state culture. Taken from Dharmasiddhi et al. (2025)

3.2.3 Endpoint Assay

Seed cultures were cultivated in LB if no growth was predicted during the assay, or in modified M9 medium with the appropriate carbon and nitrogen sources if growth was expected. Cultures were washed twice with the assay medium to remove any residual nutrients. The endpoint assays were conducted by inoculating cultures into assay tubes to achieve an initial OD_{600} of approximately 0.2. After incubation, final OD_{600} measurements were taken to evaluate the growth.

3.2.4 Growth Kinetics

Seed cultures were cultivated in the same method as those in the endpoint assay. Subsequently, they were inoculated into Costar 48-well plates with an initial OD_{600} of approximately 0.2, in a final volume of 500 µL per well. Measurements for OD_{600} were recorded every hour for a total of 72 hours using a BioTek Synergy4 plate reader (Agilent Technologies, Santa Clara, CA, USA), mixing every 15 minutes to ensure homogeneity.

3.2.5 Sampling and 16S rRNA Sequencing

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

3.2.6 High-Performance Liquid Chromatography

The conversion of EG into glycolate (GA) was analyzed using high-performance liquid chromatography (HPLC), following a previous study (Yan et al., 2024). Aliquots of 1 mL were taken from the cultures for each time point and were centrifuged at 12,000 rpm for 5 min. The supernatant was filtered through 0.22 µm syringe filters. The concentrations of EG and glycolic acid were quantified using an Aminex HPX-87H column (Bio-Rad, Mississauga, ON, Canada) operated at 60 °C. The mobile phase was 5 mM sulfuric acid, and the flow rate used was 0.6 mL/min.

3.3 Results

3.3.1 Chemostat Evolution of Strains

The first stage of ALE consisted of monoculture evolution in tubes, with PpWT adapted in M9HD and EcWT in M9EG. The second stage involved chemostat evolution in a bioreactor that was fed with M9EG/HD. The inlet flow rate data was continuously monitored to evaluate the metabolic adaptation of the consortium to EG and HD (Figure 4).

During the initial phase (days 0-4), the inlet flow rate was reduced to maintain steady-state conditions, reaching approximately 0.02-0.03 mL/min by days 3-4. This reduction was necessary due to the initially inefficient utilization of EG and HD by SBC1. This initial decrease in flow rate prevented the washout of the co-culture and allowed for the establishment of stable growth conditions. As the experiment progressed, gradual increases in the inlet flow rate were achieved. By day 10, the flow rate has increased to approximately 0.05 mL/min. This trend continued throughout the adaptation period, with more substantial increases achieved from day 11 onward. This progressive increase in the flowrate indicated improved substrate utilization by the evolved consortium.

By day 25, the inlet flow rate reached a final steady-state value of approximately 0.25 mL/min, which is nearly a tenfold increase from the initial steady-state level. This improvement reflects the successful adaptation of the consortium to metabolize EG and HD as the sole carbon and nitrogen sources, respectively. After the second stage of ALE, the evolved consortium was designated as SBC2. This was followed by the isolation, and characterization of PpHD2 and EcEG2, the designation of the individual strains post-ALE.

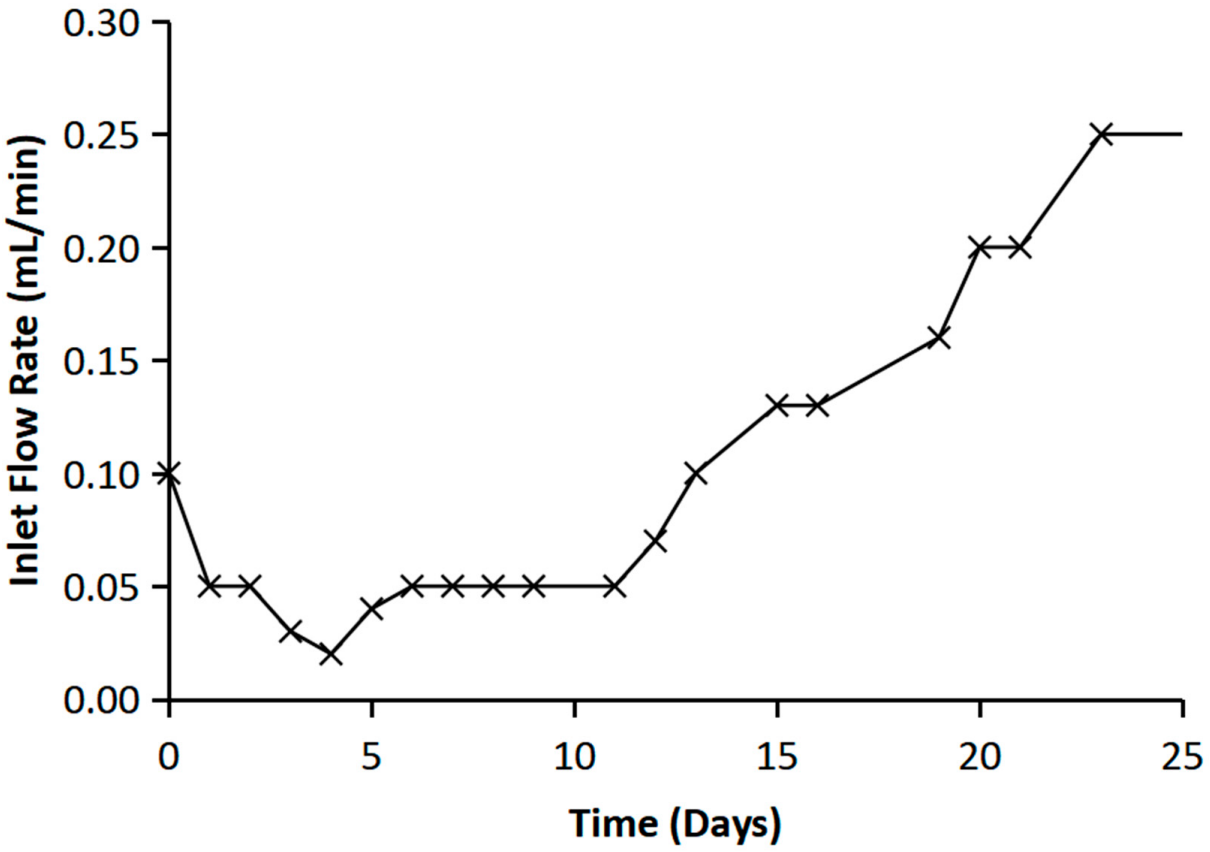


Figure 4. Inlet flow rate over time showcasing ALE for improved EG and HD utilization. Taken from Dharmasiddhi et al. (2025)

3.3.2 Characterization of *P. putida* Strains on Hexamethylenediamine Metabolism

The growth patterns of PpHD1 and PpHD2 attributed to using HD as the nitrogen source were assessed over 72 hours (Figure 5). In M9HD, which contains glucose and HD, both PpHD1 and PpHD2 displayed robust growth, reaching high OD_{600} values of approximately 1.0 within 72 hours (Figure 5A). This suggests that HD is an effective nitrogen source, provided that glucose is the carbon source. However, since the growth patterns are almost identical, there is limited evidence that PpHD2 more effectively utilizes HD when compared to PpHD1 after ALE.

When EG was used as the carbon source in the presence of HD (Figure 5B), PpHD2 exhibited slight but noticeable growth, whereas PpHD1 did not grow after 72 hours. The difference in growth patterns suggests that PpHD2 has evolved a limited capacity to metabolize EG as a carbon source during the second stage of ALE. To examine whether the observed growth was due

to adipate use, a by-product of HD metabolism, further studies were conducted with media lacking a carbon source or containing adipate as the carbon source (Arab et al., 2024).

In the absence of any additional carbon source (Figure 5C), neither PpHD1 nor PpHD2 grew. This rules out the possibility of using HD alone as both a carbon and nitrogen source. Similarly, when adipate was provided as the sole carbon source, along with ammonium chloride as the nitrogen source (Figure 5D), neither strain showed growth. These findings suggest that the growth of a PpHD2 monoculture in M9EGHD is attributed to its acquired ability to metabolize EG as the carbon source, rather than the use of HD as a carbon and nitrogen source.

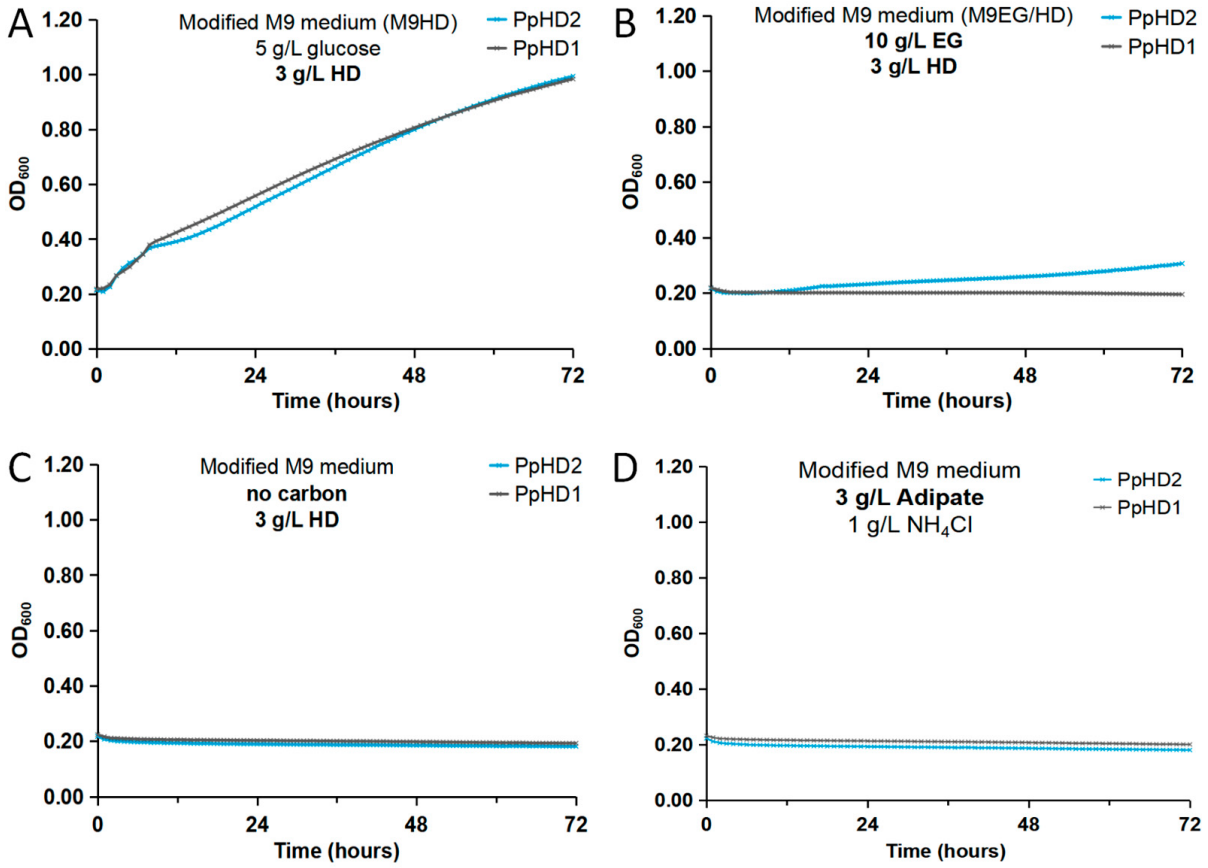


Figure 5. Growth of evolved *P. putida* strains in various modified M9 media formulations. **(A)** Growth of PpHD1 and PpHD2 strains in M9HD medium (n = 4). **(B)** Growth of PpHD1 and PpHD2 strains in M9EG/HD medium (n = 4). **(C)** Growth of PpHD1 and PpHD2 strains in modified M9 media with no added carbon source (n = 4). **(D)** Growth of PpHD1 and PpHD2 strains in modified M9 media with adipate (n = 4). Taken from Dharmasiddhi et al. (2025).

3.3.3 Characterization of Evolved Strains on Ethylene Glycol Metabolism

The ability of *E. coli* (EcWT, EcEG1, EcEG2) and *P. putida* (PpWT, PpHD1, PpHD2) to utilize EG as a carbon source as assessed using growth assays (Figure 6). On M9EG agar plates, EcEG1 exhibited prominent colony formation after 72 hours, highlighting its ability to use EG as the sole carbon source. In contrast, EcEG2 showed no growth, suggesting that there was a loss in ability to metabolize EG during the second stage of ALE (Figure 6A).

Further evaluation in liquid M9EG media over seven days (Figure 6B) reinforced these findings. EcEG1 achieved modest growth, which was reflected by an increase in OD_{600}, confirming its slow yet possible EG metabolism. On the other hand, EcEG2 exhibited negligible growth, like that of EcWT. This suggests a loss of its capacity to use EG.

Because of the observed growth patterns of PpHD2 in M9EG/HD, we wanted to further investigate whether its improved growth using EG and HD as the sole carbon and nitrogen sources, respectively, was attributed to a newfound ability of EG consumption. Among *P. putida* strains tested, only PpHD2 displayed notable growth in M9EG, reaching an OD_{600} of approximately 0.7 after 72 hours (Figure 6C). Neither PpWT nor PpHD1 strains exhibited significant growth, suggesting that PpHD2 has evolved the capability to use EG as the sole carbon source during the second stage of ALE.

The co-culture assays revealed complex interactions between strains. While the WT combination (SBC) and SBC1 showed minimal or no growth, SBC2 demonstrated significantly enhanced growth in M9EG, with an OD_{600} of approximately 1.0 after 72 hours (Figure 6D). This growth surpasses the growth observed by any individual monoculture. As a result, this indicates a synergistic interaction between PpHD2 and EcEG2, suggesting complementary metabolic cooperation or resource exchange that improves EG utilization efficiency within the consortium.

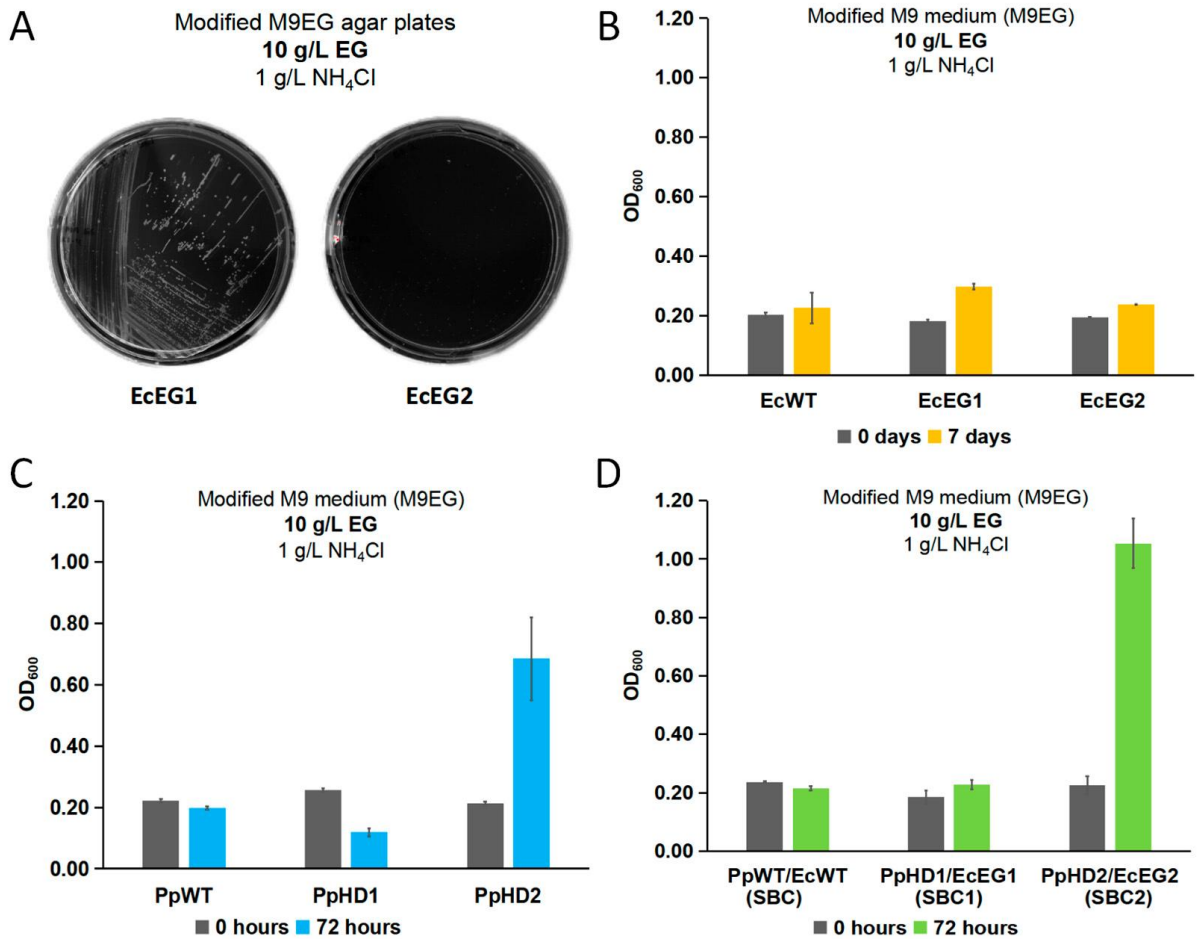


Figure 6. Growth of *E. coli*, *P. putida* and SBC strains in modified M9 media with EG as the carbon source. **(A)** Growth of EcEG1 and EcEG2 on M9EG agar plates. **(B)** Growth of *E. coli* strains in M9EG medium. Error bars indicate SD (n = 4). **(C)** Growth of *P. putida* strains in M9EG media. Error bars indicate SD (n = 4). **(D)** Growth in co-culture system. Error bars indicate SD (n = 4). Taken from Dharmasiddhi et al. (2025).

3.3.4 Shift in the Division of Labor

Due to the capacity of PpHD2 to metabolize both EG and HD, it is theoretically possible for it alone to grow in EG and HD as the sole carbon and nitrogen sources, respectively. However, the growth profiles (Figure 7A) indicate that the consortium benefits, even after the shift in the division of labor. The consortium reaches a maximum OD_{600} of approximately 1.0 after 72 hours, compared to the limited growth observed for the PpHD2 monoculture, which got to an OD_{600} of around 0.3.

The subpopulation composition analysis was based on 16S rRNA sequencing and was conducted by Hadi Dhiyebi at the Waterloo Genomics Surveillance Center. The results indicate that initially, EcEG2 dominated the population, comprising 61%, while PpHD2 represents 38% (Figure 7B). However, by 48 hours, this composition had shifted, with PpHD2 becoming the dominant strain at 55%. On the other hand, the population of EcEG2 declined to 44%. This reflects the growing advantage of PpHD2 in metabolizing both EG and HD. This change indicates a developing division of labor and metabolic cooperation within the microbial consortium. Moreover, the stabilization of population ratios over time reflects the establishment of a balanced and synergistic interaction between the two strains.

Glycolate is a known intermediate, and a possible export, in EG metabolism. Such metabolism has been extensively documented in both *E. coli* and *P. putida* (Chi et al., 2024; Franden et al., 2018; Mückschel et al., 2012; Panda et al., 2021; Pandit et al., 2021; Yan et al., 2024). That being said, HPLC measurements of glycolate provided further evidence of specialization in EG utilization within the consortium (Figure 7C). In the PpHD2 monoculture, glycolate accumulated significantly, reaching concentrations above 2.5 g/L by 72 hours. However, when grown as a co-culture, insignificant amounts of glycolate were detected. This suggests that while PpHD2 may grow using EG as the sole carbon source, it may have limited efficiency in metabolizing glycolate. Additionally, EcEG2 may have re-specialized in the role of by-product consumption of glycolate.

A previous study demonstrates that *E. coli* can grow effectively on glycolate as a carbon source (Höhmann et al., 2024). We further examined the capacity of the evolved strains to grow on glycolate as the sole carbon source (Figure 7D). This experiment was carried out by Ching Lan. The findings indicate that EcEG2 readily grew to an OD_{600} of approximately 1.0 after 48 hours. All in all, this highlights the synergistic interactions between PpHD2 and EcEG2 within the bacterial consortium. PpHD2 degrades EG to glycolate and consumes HD to provide a nitrogen source for the community. Then, EcEG2 efficiently degrades the resulting glycolate to further support the carbon needs of the community.

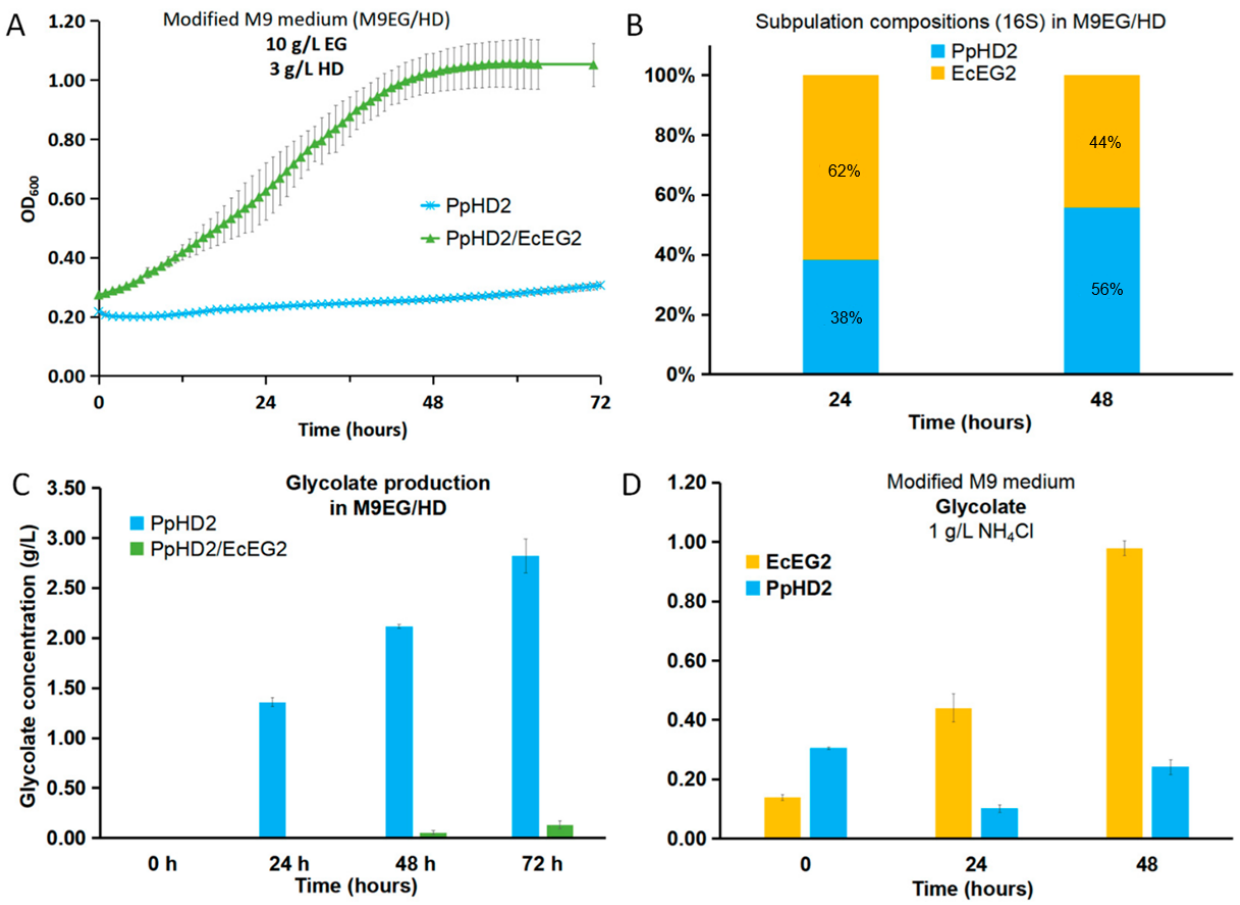


Figure 7. Growth dynamics and metabolic interactions of PpHD2 and EcEG2. **(A)** Growth curves of PpHD2 alone and in co-culture with EcEG2 in M9EG/HD. Error bars indicate SD (n = 4). **(B)** Subpopulation composition of the coculture in M9EG/HD determined by 16S rRNA sequencing. **(C)** Glycolate production of PpHD2 alone and in co-culture with EcEG2 in M9EG/HD measured via HPLC. Error bars indicate SD (n = 3). **(D)** Growth of PpHD2 and EcEG2 in M9 medium supplemented with glycolate as the sole carbon source. Error bars indicate SD (n = 4). Taken from Dharmasiddhi et al. (2025)

3.4 Discussion

Our study clearly highlights the potential of using synthetic bacterial consortia as a platform to address the challenges of mixed plastic waste. We showcase a consortium consisting of engineered *E. coli* Nissle 1917 and *P. putida* KT2440 to efficiently consume EG and HD as the sole carbon and nitrogen sources, respectively.

Previously, many studies have extensively examined the degradation of EG through metabolic rewiring or gene overexpression strategies (Chi et al., 2024; Yan et al., 2024). For example, the overexpression of *fucO* and *aldA* in *E. coli* is a common strategy for more efficient glycolate export, and EG consumption (Panda et al., 2021; Pandit et al., 2021). In *P. putida*, introducing the *glcDEF* gene cluster, which enables the conversion of glycolaldehyde into glycolate, was found to enhance EG metabolism (Franden et al., 2018).

On the other hand, our study employs ALE on the synthetic bacterial consortium to efficiently utilize both EG and HD. This process works on the principles of selective pressure, where cells more suited to EG and HD assimilation are more likely to proliferate and strengthen cooperative interactions (Dragosits & Mattanovich, 2013; X. Zhang & Reed, 2014). A study by Zhang & Reed (2014) demonstrated that the adaptive evolution of a synthetic cooperating community improved growth rates by approximately three-fold. The consortium consisted of leucine and lysine *E. coli* auxotrophs. Thus, they were only able to grow in glucose minimal media if the nutrient exchange of leucine and lysine occurred. Several other studies have shown the ability of ALE to enhance the tolerance or efficiency of a microorganism, or a consortium towards a particular substrate (Baek et al., 2016; R. Liu et al., 2019; Werner et al., 2025; X. Zhang & Reed, 2014).

Initially, the strains underwent monoculture adaptations, which resulted in PpHD1 and EcEG1, each with improved efficiency towards HD and EG metabolism, respectively. The subsequent second stage of ALE in a chemostat further optimized metabolic interactions, leading to the generation of SBC2, consisting of PpHD2 and EcEG2. The incremental increase in chemostat inlet flowrate clearly shows that the consortium has become more specialized to grow in M9EG/HD. Moreover, the results show a newly established division of labor between the members of the consortium.

While originally engineered to only consume HD, the resulting evolved ability of *P. putida* to assimilate both EG and HD play a key role in the function of the consortia. Past studies have shown the possibility of EG assimilation in *P. putida* KT2440 (Franden et al., 2018; Mückschel et al., 2012). Due to its metabolic versatility and adaptability, *P. putida* is a commonly used microbe in the realm of bioremediation (Nogales et al., 2020; Weimer et al., 2020). On the other hand, *E. coli*, while originally intended for EG assimilation, shifted towards a role of glycolate metabolism to

further contribute to the carbon needs of the consortium. *E. coli* is known to have a glycolate assimilation pathway, eventually integrating into glycolysis or TCA (Höhmann et al., 2024).

While *P. putida* can survive independently in M9EG/HD, it is evident that the presence of *E. coli* promotes a synergistic relationship. The limited growth of *P. putida* monocultures in M9EG/HD may be attributed to the accumulation of toxic intermediates such as glycolaldehyde and glyoxylate (Franden et al., 2018). Thus, *E. coli* potentially plays a crucial role in detoxification by preventing the accumulation of these harmful compounds. The balanced population ratios further illustrate this mutually beneficial interaction. By promoting mutual reliance, it reduces the likelihood of dominance by one species, thereby maintaining population balance and ensuring long-term functionality (Duncker et al., 2021).

Chapter 4 – Flux Balance Modeling of Role Redistribution

4.1 Background Information

In the last decade, constraint-based stoichiometric modeling has become an important tool in the field of systems biology (Maarleveld et al., 2013). This computational approach integrates genomic, biochemical and physiological data to systematically analyze metabolic networks by defining constraints, such as reaction stoichiometry. Specifically, in mixed culture systems, like that described in this thesis, constraint-based modeling can elucidate the inter-species interactions.

4.1.1 Genome-Scale Metabolic Models

Constraint-based modeling relies on comprehensive metabolic models. In 1999, the first genome-scale metabolic model (GEM) was described for *Haemophilus influenzae* RD (Edwards & Palsson, 1999). GEMs are comprehensive representations of cellular metabolism that combines genomic, biochemical, and physiological information in a framework. Since the first GEM, their development has become a fundamental modeling approach for investigating metabolism at the systems level (Gu et al., 2019). In 2015, researchers developed the Biochemical, Genetic, and Genomic knowledge base (BiGG) as a comprehensive database containing over 75 curated and standardized GEMs, integrated with genome annotations and external databases (King et al., 2016)

A GEM is typically built around a stoichiometric matrix, where rows represent metabolites and columns represent reactions (Tarzi et al., 2024). Each entry in this matrix indicates the stoichiometric coefficient of a metabolite within a particular reaction, therefore describing how reactants are transformed into products. Mass and charge balance for every reaction ensure the balance and stability of the metabolic network (Thiele & Palsson, 2010). Additionally, a GEM contains gene-protein-reaction (GPR) associations, which link specific genes to the enzymes responsible for catalyzing each reaction (Gu et al., 2019). These associations are hypothesized based on genomic and experimental data.

4.1.2 Flux Balance Analysis

One computational approach that heavily relies on the use of GEMs is flux balance analysis (FBA). It can be a powerful tool to study microbial metabolism by predicting the flow of metabolites through a GEM. FBA uses linear programming to solve reaction fluxes that satisfy

several constraints under steady state (Maarleveld et al., 2013). By optimizing a defined objective function, which is typically biomass production, FBA predicts how a living organism spends its metabolic resources in an environment, providing insights into growth, energy usage, metabolic capabilities, or the rate of production of a relevant metabolite (García Sánchez & Torres Sáez, 2014; Orth et al., 2010; Yilmaz & Walhout, 2017).

The optimization of an objective function is subject to several constraints that must be satisfied. The linear programming equation is given in Equation 1 (Maarleveld et al., 2013). In the objective, $c^T J$ is a dot product that results in a single scalar value, Z, representing the optimization objective. The coefficient vector c specifies the contribution of each reaction to the objective and J is the vector of fluxes through each reaction. Such constraints are expressed in two main forms, equations that balance reactions and inequalities that define bounds on the system (Orth et al., 2010). The former defines the mass balance constraint by enforcing that the total amount of metabolite produced must equal to the total amount consumed at steady-state. This is given by the expression $NJ = 0$, where N represents the stoichiometric matrix. On the other hand, the next constraint is applied to define the upper and lower bounds on each reaction flux, denoted by J^{max} and J^{min}, respectively. This reflects the limits of nutrient availability and reaction directionality, or other physiochemical limitations (Kauffman et al., 2003; van Pelt-KleinJan et al., 2021).

$$\text{Maximize of Minimize: } Z = c^T J,$$

Subject to:

$$NJ = 0$$

$$J^{min} \leq J \leq J^{max}$$

Equation 1. Core equations of traditional FBA, taken from Maarleveld et al. (2013)

Traditional FBA is built on several key assumptions that simplify the modeling of complex metabolic networks. The first is the steady-state assumption, where metabolite concentrations are said to be constant over time (Anand et al., 2020; Kauffman et al., 2003; Orth et al., 2010). Secondly, it assumes optimal metabolic behavior, in which cells optimize their metabolism towards the objective function (Lularevic et al., 2019; Schuster et al., 2008). Finally, fluxes are modeled as linear and are independent of kinetics, regulator effects, or any thermodynamic considerations

(Pozo et al., 2015). Despite the assumptions, FBA allows for a simplified and efficient computational prediction of cellular metabolism.

The purpose of FBA is to investigate how the post-adaptive evolution strain of *P. putida,* allocates its resources subject to varying amounts of EG and HD. This will provide insights into growth, metabolic capabilities, or the rate of production of relevant exports (García Sánchez & Torres Sáez, 2014; Orth et al., 2010; Yilmaz & Walhout, 2017). Additionally, FBA can pinpoint some of the drawbacks of using monoculture associated with stoichiometric balance in M9EG/HD, as opposed to our proposed consortium. For FBA, modeling will be done using a tool known Constraint-Based Reconstruction and Analysis (COBRA), which also has a Python interface (COBRApy) (Ebrahim et al., 2013). This is extensively used in the metabolic modeling of both prokaryotes and eukaryotes.

4.1.3 Dynamic Flux Balance Analysis

One of the bottlenecks of FBA is that it cannot model instances where the reprogramming of metabolic networks occurs, such as a response to changes in the environment. In 2002, one of the first extensions of flux balance analysis was reported to model diauxic growth in *E. coli* (Mahadevan et al., 2002). Dynamic flux balance analysis (dFBA) extends FBA by incorporating a time variable. While traditional FBA assumes a steady state where metabolite concentrations do not change, dFBA incorporates ordinary differential equations (ODEs) to model changes in extracellular metabolites and biomass over time (Antoniewicz, 2013; Henson & Hanly, 2014; Sen, 2022).

It works by solving a standard FBA problem based on the given conditions at each timestep by optimizing the objective function and satisfying the constraints. The resulting fluxes are then used in the ODEs to update the extracellular environment and biomass over time. This process is repeated iteratively for a set number of cycles, allowing for the modeling on how cells grow and interact with their environment dynamically (Henson & Hanly, 2014; Sen, 2022). Building on Equation 1, the extracellular environment is represented by the ODE, Equation 2 (Mahadevan et al., 2002). Here, S is the concentration of substrate i, and $X(t)$ is the biomass at time t. Likewise, Equation 3 represents the biomass production ODE, where μ is the growth rate.

$$\frac{dS_i}{dt} = (NJ)_i X(t)$$

Equation 2. Substrate consumption or production ODE. Adapted from Mahadevan et al. (2013)

$$\frac{dX}{dt} = \mu X(t)$$

Equation 3. Biomass production ODE. Adapted from Mahadevan et al. (2013)

The observed role re-distribution pertaining to EG consumption will be simulated using dFBA. This approach can effectively model cross-feeding interactions because dFBA is ideal for situations where there are changes in the extracellular environment time (Antoniewicz, 2013; Henson & Hanly, 2014; Mahadevan et al., 2002; Sen, 2022). Not only can this confirm the division of labor between strains, but also this would reveal how one strain supports the growth of the consortium by co-metabolism. Building on models and simulations developed in COBRA, this project employs the Computation of Microbial Ecosystems in Time and Space (COMETS) to perform dFBA simulations of the consortium (Dukovski et al., 2021). COMETS extends dFBA by allowing organisms to interact across space, by introducing grids, and time. This allows for the simulation of nutrient diffusion, localized cross-feeding, and population spreading.

4.2 Materials and Methods

4.2.1 Flux Balance Analysis

With code adapted from a prior study by Pandit et al. (2021), FBA was performed using the COBRApy package with Python 3.12.4 (Ebrahim et al., 2013). Acquired from the BiGG database, the base GEM used for FBA modeling was iJ1463, which corresponds to *P. putida* KT2440 (King et al., 2016; Nogales et al., 2020).

Most reactions, including glycolate assimilation, were already present in iJN1463. However, two additional reactions, EG uptake and its subsequent conversion into glycolaldehyde, were added as modifications to model iJN1463 to model EG metabolism (Franden et al., 2018; Mückschel et al., 2012; Pandit et al., 2021). The HD pathway was incorporated *de novo*, based on a previous study (Arab et al., 2024). The reaction mechanisms, which are required for FBA, were inferred based on the known functions of the genes *AlaC* and *FrmA*, as documented in the UniProt database (Bateman et al., 2023).

The conditions of M9EG/HD were simulated *in silico* by modifying several bounds. The objective function in this simulation is to maximize biomass. Firstly, the lower bounds of all

glucose and ammonium exchanges were set to 0, which blocks any uptake of those compounds. Subsequently, the carbon and nitrogen uptakes were restricted to varying amounts of EG and HD. The oxygen uptake bounds were kept as defined by Pandit et al. (2021), while the ATP maintenance was left unchanged.

4.2.2 Dynamic Flux Balance Analysis

COMETS was used to perform dFBA, with the following dependencies: Python 3.12.4, Gurobi 9.0.2, and Java 1.8.0 (Dukovski et al., 2021). The modified model for used for *P. putida* was carried over from traditional FBA with a redefined objective function of exporting glycolate (Franden et al., 2018; Mückschel et al., 2012; Nogales et al., 2020; Pandit et al., 2021). On the other hand, we used iHM1533 to represent *E. coli* EG2 (van 't Hof et al., 2022). No reactions were added, as glycolate uptake was already present in the model. The objective function set for *E. coli* was biomass production.

All simulations were conducted for a total of 50000 iterations with timesteps of 0.05. The maximum amount of biomass per unit space was kept open, and no death rate was set. The *in silico* media was designed to reflect M9EG with 100 mM EG. Other essential metabolites for growth were all set at an excess of 1000 mM. To represent a homogenous mixture, only 1 unit space was allowed. All bacteria were inoculated in the same unit space at an initial population of 5×10^{-6} grams of biomass. Initial bounds on the models are open and are dynamically adjusted by COMETS as the simulation progresses. The initial oxygen uptake for *P. putida* was set arbitrarily at (-0.1, 0). Finally, the ATP maintenance bounds on iJN1463 were changed to 6.82, identical to that of iHM1533, to ensure comparability between the two models.

4.3 Results

4.3.1 Monoculture FBA Modeling of *P. putida*

To better understand the metabolic advantages coming from the consortium to grow on M9EG/HD, we performed FBA on PpHD2. Simulations were carried out using M9EG/HD, with EG as the sole carbon source, and HD as the only nitrogen source. The objective was to assess whether the benefits of the consortium could be explained by stoichiometric imbalances in the metabolic network. For this, the model for *P. putida* KT2440, iJN1463, was adapted to include EG

uptake and its conversion into glycolate (Figure 8A), along with the HD degradation pathways (Figure 8B) (Arab et al., 2024; Nogales et al., 2020; Pandit et al., 2021).

Most of the EG pathway has already been included in the stock iJN1463 model. However, the added reactions for EG assimilation include EG uptake, and its subsequent conversion into glycolaldehyde by *PedE* and *PedH*, previously described in *P. putida* by a couple of past studies (Franden et al., 2018; Mückschel et al., 2012). On the other hand, it was found that *AraJ*, *AlaC* and *FrmA* are responsible for the assimilation of HD (Arab et al., 2024). Based on available data on UniProt, it is likely that *AlaC* is associated with the transamination reactions for HD (Bateman et al., 2023). Nitrogen is assimilated into amino acids, which can be metabolized further.

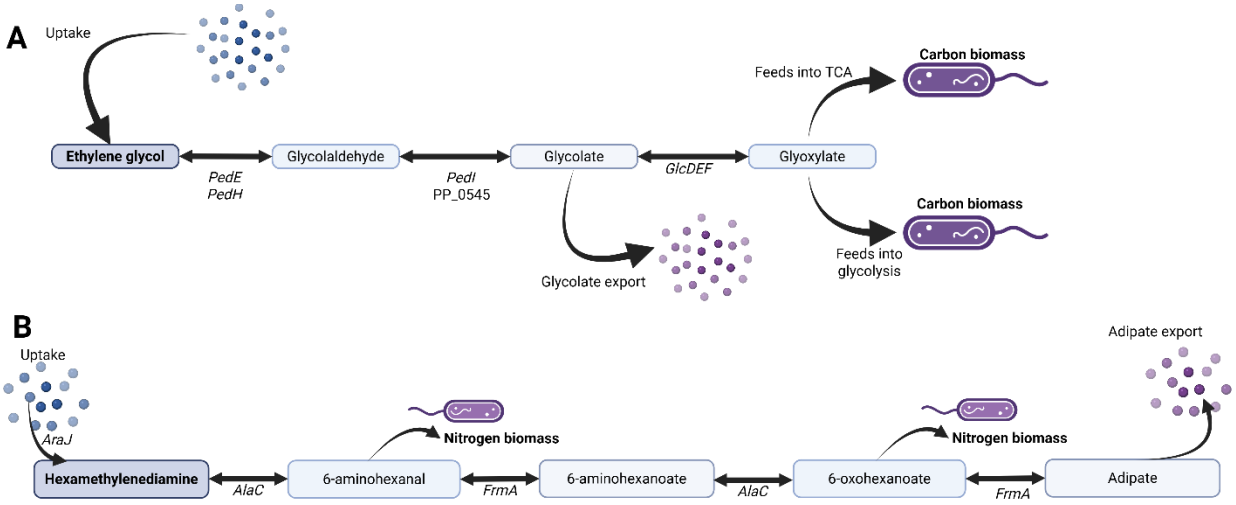


Figure 8. Pathway modifications for iJN63, which include **(A)** EG assimilation into biomass or glycolate export, and **(B)** HD metabolism to feed into biomass or export as adipate. Created using BioRender.com.

We set the objective to maximize biomass production. FBA simulations revealed that under conditions of high EG uptake and limited, non-zero HD uptake, glycolate export occurred. This indicates the possibility of a stoichiometric imbalance in the monoculture metabolism of PpHD2 (Figure 9A). While FBA assumes optimal metabolism, this can reflect PpHD2's limited ability to assimilate glycolate, as also observed experimentally. Although PpHD2 can metabolize EG and HD independently and sustain monoculture growth, the addition of EcEG2 offers an advantage of preventing glycolate build-up. Conversely, adipate exports are always present, if no bounds are set to zero. This must be true if only nitrogen can be assimilated from HD. Therefore, the carbon must

be secreted, as adipate, to satisfy the mass balance constraints (Figure 9B). This reflects the experimental observations regarding the cells not being able to grow using adipate or HD as the carbon source.

Additionally, the FBA results predicted that increasing the upper bounds of EG and HD uptake enhanced biomass production, though the benefit plateaued at higher uptake rates (Figure 9C). Notably, biomass output was more sensitive to changes in EG uptake than HD, emphasizing the importance of carbon assimilation from EG in overall growth.

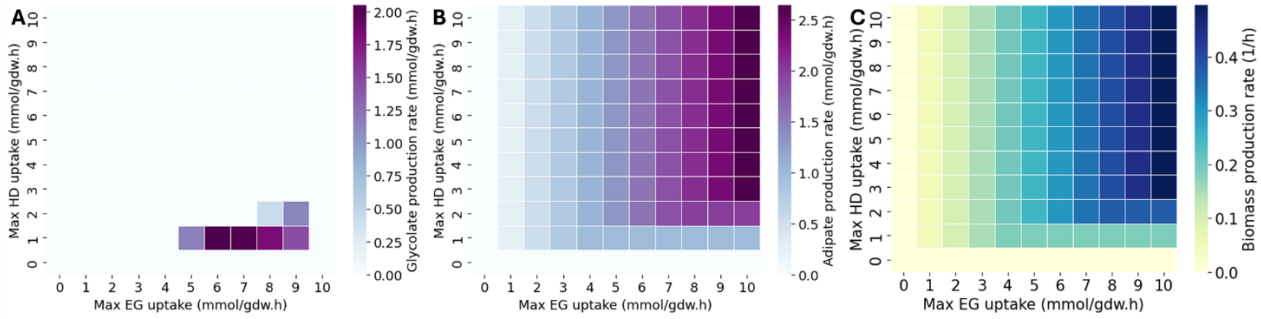


Figure 9. FBA simulation results for **(A)** glycolate export, **(B)** adipate export, and **(C)** biomass production under varying EG and HD upper bounds. Adapted from Dharmasiddhi et al. (2025)

4.3.2 Co-culture dFBA Modeling

Building on the results, we extended our computational analysis to co-culture modeling in M9EG. EG as a carbon source is of particular interest since the shift of consortium dynamics is attributed to it. The use of dFBA is more suitable for modeling a co-culture environment, especially where cross-feeding takes place, since it can account for the dynamics in the environment traditional FBA cannot capture (Antoniewicz, 2013; Henson & Hanly, 2014; Mahadevan et al., 2002; Sen, 2022). The objective was to simulate the cross-feeding dynamics within the consortium. To do that, we took the model for PpHD2 we developed for FBA and shifted its objective to force glycolate export. On the other hand, the other model iHM1533, stock for *E. coli* Nissle 1917, was adapted to represent EcEG2 with its objective function being biomass production (van 't Hof et al., 2022). Although this is the case, all biomass production in EcEG2 is dependent on the glycolate export from PpHD2, as the former can no longer metabolize EG.

dFBA was used to evaluate and compare the metabolic performances of the evolved consortium and PpHD in monoculture when grown in M9EG, with EG as the sole carbon source.

As shown in Figure 10A, both members of the synthetic bacterial consortium exhibit rapid exponential growth, reaching stationary phase by approximately cycle 1000. At this stage, PpHD2 achieves a biomass of around 2.5 g, accounting for about 60% of the total biomass, while EcEG2 constitutes the remaining 40%. This population ratio is consistent with the previously observed balance shown by 16S rRNA sequencing. The biomass profile is mirrored by the metabolite dynamics shown in Figure 10B, where EG is quickly consumed during the early growth phase. An increase in glycolate is observed, followed by the rapid depletion, highlighting the division of labor between the two strains: PpHD2 assimilates EG and secretes glycolate, while EcEG2 consumes the glycolate as a carbon source.

In contrast, the monoculture dFBA simulation of PpHD2 reveals the disadvantages of a single culture for EG assimilation. Although PpHD2 can grow on EG independently, it requires approximately 26000 cycles to reach stationary phase – a 26-fold increase in time compared to the consortium (Figure 10C). Despite the delay, PpHD2 reaches a higher final biomass of around 5 g. However, this result is likely influenced by model-specific stoichiometries and may not reflect the experimental reality.

Figure 10D shows that glycolate initially accumulates, as PpHD2 was set to optimize that function initially. As EG is gradually depleted, PpHD2 switches its metabolic mode, and consumes the glycolate it exported as a secondary carbon source to support continued growth. Glycolate transport is encoded in the model bidirectionally, and consumption becomes active once EG is exhausted.

Overall, the dFBA simulations show the efficiency and benefits of the synthetic cross-feeding interactions observed in the consortium. The co-culture leverages the division of labor to reduce the metabolic burden on a single strain. These findings are consistent with the previously described experimental observations and confirm that dFBA is a useful tool for capturing the dynamic and metabolic interdependencies in consortia.

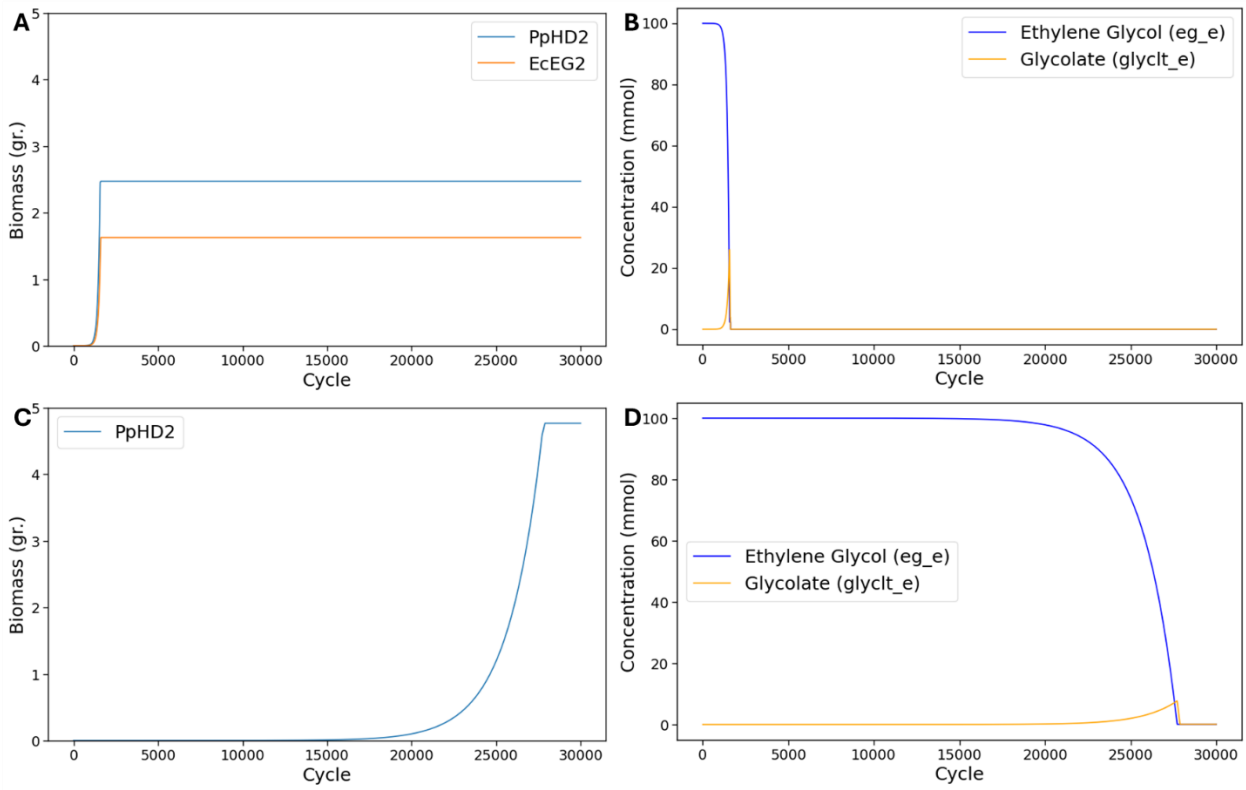


Figure 10. COMETS Simulation results. **(A)** Biomass and **(B)** EG and HD concentration time profile of the consortium in M9EG. **(C)** Biomass and **(D)** EG and HD concentration time profile of PpHD2 in M9EG.

4.4 Discussion

All in all, it can be said that conventional FBA provided a qualitative framework to examine the metabolic capacity on EG and HD (Ebrahim et al., 2013). By constraining EG as the sole carbon source, and HD as the sole nitrogen source in a genome-scale model, the FBA revealed stoichiometric bottlenecks associated with using a monoculture. Notably, when PpHD2 was simulated with high EG upper bounds, and low HD bounds, the model predicted the possible secretion of glycolate as an overflow metabolite. This occurs because carbon from EG cannot be completely assimilated into biomass when nitrogen is a limiting factor. Thus, the carbons must forcefully leave the system, as glycolate in this case, to satisfy mass balance constraints. These results are further validated by experimental data in chapter 4: In monoculture, PpHD2 accumulated glycolate when grown in M9EG/HD. Additionally, FBA confirmed that PpHD2, despite this imbalance, can sub-optimally use EG and HD alone to support biomass formation.

Optimizing the biomass objective in iJN1463 in M9EG/HD provided some insights into how cross-feeding could resolve any stoichiometric imbalances. In the presence of a co-culture, EcEG2 would consume the glycolate produced by PpHD2. This mitigates the carbon overflow problem as more could be channeled into biomass. Additionally, the FBA indicated that increasing availability of both EG and HD drives higher biomass production up to a point. Finally, the growth yield was more sensitive to EG input than to HD, suggesting that carbon catabolism is the limiting factor for PpDH2's growth. In iJN1463, more carbon is needed to sustain growth (Nogales et al., 2020).

FBA has highlighted the importance of efficient EG utilization. From this perspective, improvements in EG metabolic capacity should translate to greater biomass. The model can be used to suggest specific enzymatic steps as targets for enhancement, such as the downstream glycolate conversion steps, to mitigate the challenges of carbon overflow. A past study found that the overexpression of the glycolate oxidase operon, *GlcDEF*, improves overall EG consumption, consistent to the implications of FBA modeling (Franden et al., 2018). However, it should be noted that while traditional FBA offers as stoichiometric perspective on metabolism, it assumes an optimal steady-state and does not capture kinetic or regulatory effects. Therefore, these findings need to be evaluated in a dynamic context to better understand how the consortium behaves over time (Anand et al., 2020; Kauffman et al., 2003; Lularevic et al., 2019; Mahadevan et al., 2002; Pozo et al., 2015).

In the previous chapter, we mention that the shift in the division of labor within the consortium, as well as the increased growth in M9EG/HD is attributed to EG metabolism. For simplicity and to incorporate time and consortium interactions, we employed dFBA simulations for both the monoculture PpHD2 and the co-culture. COMETS integrates the stoichiometric models with ODEs to simulate the dynamic environment that occurs (Dukovski et al., 2021). This was ideal because we can expect changes to the extracellular environment in this consortium, such as the availability of glycolate as a carbon source as time passes.

The dFBA simulations reveal that the trend in EG and glycolate concentrations are very similar in both monoculture and co-culture: As EG is consumed, glycolate concentrations rise, and as EG is depleted completely, glycolate starts being consumed. However, this overall process occurs much faster in co-culture. Consequently, the co-culture grows to the stationary phase in a much shorter period too. This reflects the experimental data, highlighting the enhanced ability of the

consortium to degrade EG. Additionally, the dFBA co-culture simulation also captured the population dynamics that mirror real-life experimental observations. Firstly, EcEG2 only starts growing when PpHD2 grows. This is because EcEG2 is dependent on the carbon source supplied by PpHD2. Secondly, constituting approximately 60% of the total biomass, PpHD2 is slightly the most dominant population within the co-culture, possibly due to its ability to survive independently.

These results underscore the value of using constraint-based models in the analysis of synthetic microbial communities. By integrating FBA and dFBA into this study, we were able to showcase a mutualistic cross-feeding relationship into its fundamental metabolic transactions and subsequently rebuild it *in silico* to predict consortia behavior. There are a few important implications from this modeling framework for metabolic engineering and bioprocesses.

The predictive power of GEMs allows for the rapid *in silico* testing on how genetic modifications can improve the performance of a microbe or system, to carry out a task. In this project, the FBA results suggested that the growth PpHD2 could be improved by enhancing downstream glycolate conversion steps, as a previous study did (Franden et al., 2018). In the broader perspective, constraint-based modeling like FBA can be used to optimize microbial metabolism, or to guide metabolic engineering strategies, such as multiple pathway additions or deletions in a host organism to optimize the breakdown of compounds, including plastics (Anand et al., 2020; Gianchandani et al., 2010). Solutions from constraint-based modeling offer a powerful tool for scaling up from flask cultures into bioprocesses. By simulating the dynamic environment, such as how substrate concentrations, feeding rates, and community composition changes over time, dFBA can guide strategies to develop reactors and bioprocesses (Hanly & Henson, 2011; Henson & Hanly, 2014; Pandit et al., 2021; Vinestock et al., 2024).

Chapter 5 – Conclusion and Future Directions

This study demonstrates the successful development and systems-level characterization of a synthetic bacterial consortium composed of *P. putida* and *E. coli* for the simultaneous degradation of plastic derived monomers – EG and HD. The initial design was for *E. coli* to assimilate EG as the sole carbon source and supply it to the consortium. On the other hand, *P. putida* was to metabolize HD and supply a nitrogen source to the community. However, ALE has led to a shift in the division of labor, where *P. putida* is now responsible for both the assimilation of EG and HD. Through cross-feeding interactions, *E. coli* has transitioned to a role of glycolate metabolism, preventing carbon overflow, and oversaw a role of detoxification from harmful intermediates. Although *P. putida* can survive with EG and HD as the sole carbon and nitrogen sources, respectively, the use of the consortium has proven to be substantially more beneficial, reflected by enhanced growth, and the constraint-based modeling results.

Overall, these findings establish a strong proof-of concept for synthetic microbial consortia as a platform to overcome the challenge of complex plastic waste streams. However, several avenues can be pursued to further enhance the performance, scalability, and applicability of synthetic microbial consortia in plastic waste management.

Further metabolic engineering may be an imperative tool to transition from microbe-assisted plastic degradation into plastic upcycling. Traditional recycling often negatively impacts the quality of the products (Alassali et al., 2021; Ragaert et al., 2017). Conversely, plastic upcycling aims to upgrade the economic and functional value of plastic waste, thereby contributing towards a circular economy (Sohn et al., 2020). In addition to aligning with environmental sustainability, the use of microbes in this manner has attracted interest due to their specific and high yield potential (Weldon et al., 2025). A few recent studies have harnessed the potential of microbial consortia for plastic upcycling (Bao et al., 2023; Gregory et al., 2023). Following these efforts, future studies should focus on additional metabolic engineering to develop synthetic microbial consortia not only for degradation, but also for upcycling of plastic-derived waste. Additionally, the increase in monetary value in output may attract more development.

While many studies have demonstrated the use of microbial systems, not just consortia, to treat plastic waste, they have remained confined to proof-of-concepts (Bao et al., 2023; Chi et al., 2024; Gao & Sun, 2021; Klauer et al., 2024; Roberts et al., 2020; J. Zhang et al., 2024). Despite

the ongoing advancements, fully integrated platforms that combine feedstock pretreatment, microbial conversion, and product recovery for plastic waste upcycling remain limited or unavailable. A recent review identified the slow rate of microbial degradation as the primary bottleneck (Weldon et al., 2025). For such biological systems to achieve industrial relevance, there must be seamless coordination between upstream and downstream processes (Guajardo & Schrebler, 2024). To overcome this problem, future research may focus on the upscaling and applicability of microbial systems. Integrating constraint-based modeling with reactor-scale simulations may bridge this gap. Researchers can anticipate how microbial consortia will behave under varying feedstock compositions, dilution rates, and spatial constraints. This modeling-driven approach can inform the optimal design of bioprocess parameters, including feeding strategies, inoculation ratios, and residence times, to mitigate bottlenecks.

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