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Application of microbial consortium and recombinant microorganisms in polyethylene degradation

（微生物群集および組換え微生物を用いたポリエチレンの分解）

Hokkaido University Graduate School of Agriculture

Frontiers in Biosciences Doctor Course

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ABSTRACT

Application of microbial consortium and recombinant microorganisms in polyethylene degradation

(微生物群集および組換え微生物を用いたポリエチレンの分解)

Polyethylene (PE), a commonly used plastic, has become a major global pollutant. Although there have been reports of microorganisms and potential enzymes capable of degrading PE, the current understanding of the degradation process and the practical application is limited. A microbe possessing all the necessary PE-degrading enzymes and effectively degrading and mineralizing PE has yet to be isolated. Individual microbes, therefore, with limited metabolic functions, may be less efficient in breaking down PE when used alone. As a potential solution to this limitation, one approach involves creating a microbial consortium with a specialized member for each enzyme. Another approach entails genetically engineering a suitable host bacterium to express these target enzymes.

1. Consortium strategy for PE biodegradation

In this strategy, an artificial bacterial consortium was constructed to degrade PE. Bacterial strains for the consortium were selected based on their reported ability to degrade PE, as found in the literature. Enzyme activity of various PE-degrading enzymes, such as laccases, lipases, esterases, and alkane hydroxylases, with a focus on their extracellular expression, was assessed for these strains. Based on these results, an artificial bacterial consortium was constructed and used for PE degradation.

PE pellets were incubated with the consortium culture for 200 days. Scanning electron microscopy (SEM) of PE samples showed multilayered biofilms and surface-embedded bacteria. SEM images of PE samples after biofilm removal, revealed surface deterioration and pitting. Fourier-transform infrared spectroscopy (FTIR) analysis further supported biodegradation by revealing the production and consumption trends of PE oxidative products. Next-generation sequencing (NGS) analysis showed *Rhodococcus erythropolis* to be the dominant bacteria in both the culture broth and on the PE samples. Additionally, the colonization and biofilm formation of *R. erythropolis* was confirmed by SEM images of PE samples treated with its pure cultures.

The results of consortium study indicate the potential of an artificial microbial consortium for PE degradation and also demonstrate the suitability of *R. erythropolis* as a host for the genetic engineering strategy.

2. Genetic engineering strategy for PE biodegradation

In this strategy, for effective PE depolymerization, the constructed host must express the introduced enzymes extracellularly and be able to effectively colonize the PE surface to ensure efficient localized enzymatic degradation. Based on the consortium study *R. erythropolis* was chosen as the host due to its colonization ability. *R. erythropolis* was observed to produce esterase, lipase, and alkane hydroxylase efficiently, but its laccase production was relatively weak. Therefore, laccase was chosen for heterologous expression in this study.

Specifically, three extracellular putative laccase genes from *Streptomyces spp*. were selected and cloned from their genomes. These include *epoA* from *S. griseus* and two

multicopper oxidase family protein genes, *mco1* and *mco2* from *S. jumonjinensis*. The three putative laccase genes with their original native signal peptide were cloned into the expression vector. Additionally, to improve the recombinant protein expression in the host and to overcome any potential signal peptide (SP)-gene incompatibility issues, the native SP of the *Streptomyces* putative laccase genes was replaced with that of the *Rhodococcus* SP as necessary. Six modified *R. erythropolis* twin-arginine translocation (Tat) SP, linked to abundantly expressed proteins from the *R. erythropolis* proteomics study, were used. Furthermore, additional protein transporter elements were incorporated into the vector to balance the recombinant protein production and secretion in the host. These vectors were then introduced into the *Rhodococcus* host. Clones were assessed for extracellular enzyme expression using the laccase substrates, ABTS and guaiacol.

The three *Streptomyces* putative laccases were successfully expressed extracellularly in the *Rhodococcus* host. Enzyme expression levels differed among various SP-gene combinations, even when using the same SP. These results highlight the importance of SP-gene compatibility in heterologous protein secretion studies. Notably, the modified *Rhodococcus* SP improved the extracellular expression of the introduced genes in cases where the original SP-gene combinations did not perform well. Furthermore, a *Rhodococcus* recombinant was selected for each of the three putative laccases with the best performing SP-gene combination and they were utilized for PE degradation.

The results of the genetic engineering study demonstrate the potential of *R. erythropolis* as a host for expressing PE-degrading enzymes and the applicability of its recombinants in PE biodegradation.

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and her ability to juggle numerous experiments effortlessly is nothing short of a magic trick, and I aspire to be as skilled as her one day.

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CHAPTER I

1. General Introduction

1.1 Motivation & Background

Plastics, synthetic polymers made from petrochemicals, have become widespread in our society due to their versatility, durability, and inexpensiveness (Andrady & Neal, 2009). These synthetic polymers, third only to cement and steel for being the most widely produced materials globally, have become a marker for a new era of plastics. Their production, distribution, consumption and disposal trends have integrated them into Earth's natural cycles, birthing what many now call the Plasticene era (Rangel-Buitrago *et al.*, 2022).

From Bakelite, the first known synthetic plastic in 1907, we have come a long way in diversifying synthetic polymers (Thompson *et al.*, 2009). Plastic polymers can be classified based on various parameters, of which their structural classification stands out prominently.

Structurally, in terms of their polymer backbone, synthetic plastics can be divided into two categories: ones with a heteroatomic backbone, like polyethylene terephthalate (PET) and polyurethane (PUR), and ones with a carbon-carbon (C-C) backbone, like polypropylene (PP), polystyrene (PS), polyethylene (PE) and polyvinyl chloride (PVC) among others (Figure 1.1) (Tournier *et al.*, 2023; Wei & Zimmermann, 2017; Y. Zhang *et al.*, 2022).

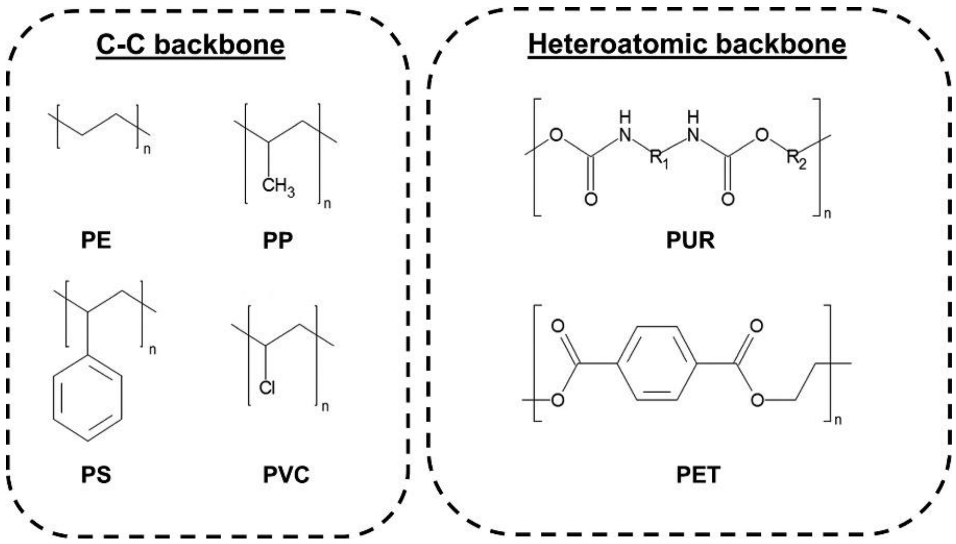


Figure 1.1. Classification of plastics by their backbone structure (adapted from Wei & Zimmermann, 2017)

Another way of categorizing polymers structurally in relation to their thermal behaviour is as thermoplastics and thermosetting plastics. Thermoplastics are made of long linear chains and soften when heated and harden when cooled and, therefore, can be reshaped many times, like polystyrene (PS), poly(vinyl chloride) (PVC), polyethylene terephthalate (PET), and polyethylene (PE). On the other hand, thermosetting plastics, as their name suggests, are permanently set as solids once heated and cooled. These plastics form an irreversible network of cross-linked bonds upon curing and, therefore, cannot be remoulded once set. A few examples of thermoset plastics are polyepoxides and formo-phenolics (Tournier *et al.*, 2023).

From the multitude of commercially available plastics, a select few plastics are produced in high volumes while costing a relatively low price in their production. These

are commodity plastics, and due to their easy availability and affordability, these plastics find widespread use in a variety of industries, particularly in the manufacturing of single-use plastic products. Commodity plastics typically include low-density polyethylene (LDPE), high-density polyethylene (HDPE), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), and polyethylene terephthalate (PET), among others (Andrady & Neal, 2009).

Plastics are a necessary evil. The durable properties that make plastics so resourceful are the very ones that make these synthetic materials ever so persistent in nature, making them a highly recalcitrant pollutant (Williams & Rangel-Buitrago, 2022). Plastic debris undergoes ageing due to interactions with different biotic and abiotic agents in the environment. These ageing processes, along with the intrinsic properties of the plastic polymer, determine the overall fate of plastics in nature. As plastic debris ages, it fragments into smaller pieces, such as microplastics (smaller than 5 mm) and nanoplastics (smaller than 0.1 μm). Additionally, microplastics can also originate from the leakage of manufactured microplastics (Andrady, 2011; Barnes *et al*., 2009; de Souza Machado *et al*., 2018).

In recent years, microplastics have gained significant attention due to their widespread contamination of biological systems. Microplastics and nanoplastics are particularly concerning because their small size allows them to be easily ingested. As bio-inert materials, their bioaccumulation poses significant risks, not only as physical hazards but also as chemotoxic hazards due to the chemical additives called plasticizers used in plastic production. Furthermore, microplastics can serve as carriers for pathogenic microorganisms. In terrestrial environments, they also contaminate soil and

freshwater systems (Andrady, 2011; Bowley *et al*., 2021; de Souza Machado *et al*., 2018; Rochman *et al*., 2013; Thompson *et al*., 2009).

The continuous churning out of more and more of these synthetic polymers, contrasted with an inadequate management and disposal system, paints a rather grim picture: exacerbating plastic pollution. To put into perspective, of the estimated 6300 million metric tonnes of globally generated plastic waste from 1950 to 2015, only around 9% was recycled, while a large portion, accounting for about 79%, ended up in landfills or the environment. Keeping with this production curve, 34 billion metric tonnes of plastic will have been produced by 2050, where 12,000 million metric tonnes of plastic will go to landfills or the natural environment (Figure 1.2) (Geyer *et al*., 2017).

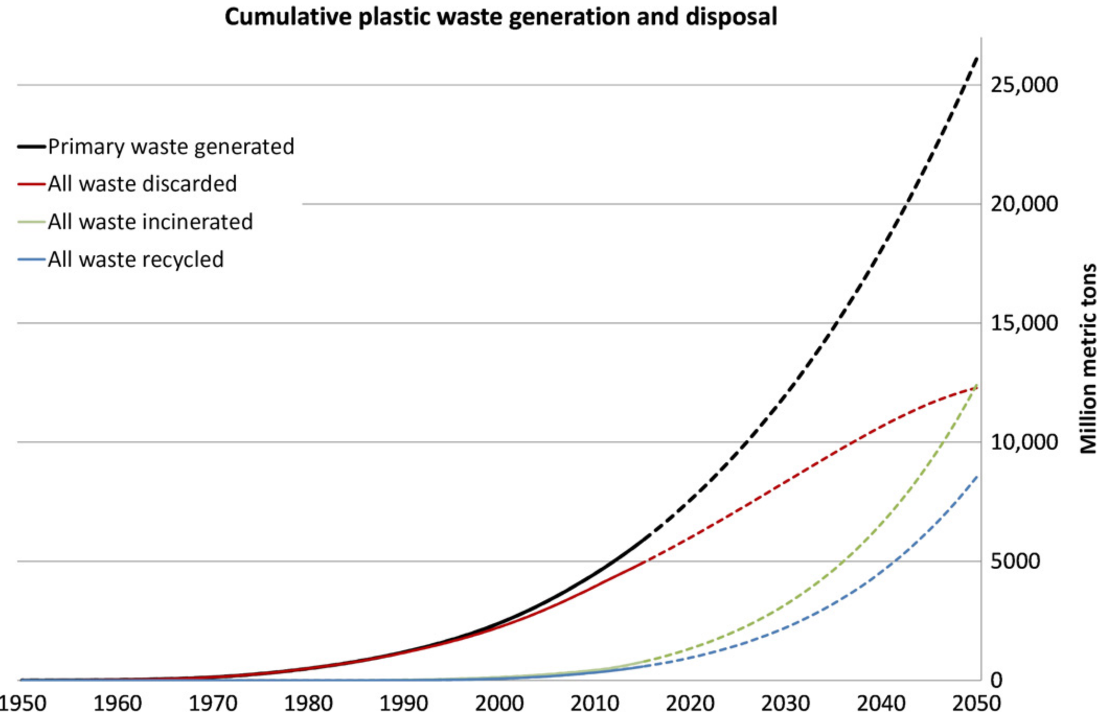


Figure 1.2. Plastic waste generation-disposal data and forecast predictions for 2050 (adapted from Geyer et al., 2017).

Current approaches for the disposal and management of plastic waste are not very sustainable. Plastic litter in landfills persists for a long time due to its non-biodegradable nature, with the resulting leachates having harmful environmental impacts. On the other hand, incineration of plastic litter contributes to air pollution and releases toxic gaseous pollutants, posing a risk to human health (Nanda *et al*., 2022; Shen *et al*., 2020). As a result, research efforts to develop eco-friendly alternatives, such as biopolymers, biodegradable plastics and to explore the biodegradation of plastics using biological systems are rapidly gaining momentum.

Bioplastics are plastics that are either bio-based i.e., derived wholly or partly from renewable resources like biomass, are biodegradable or a combination of these properties. Bioplastics are a family of plastics which can be broadly categorised as biobased biodegradable, biobased non-biodegradable and fossil-based biodegradable plastics (Figure 1.3) (European Bioplastics, 2016).

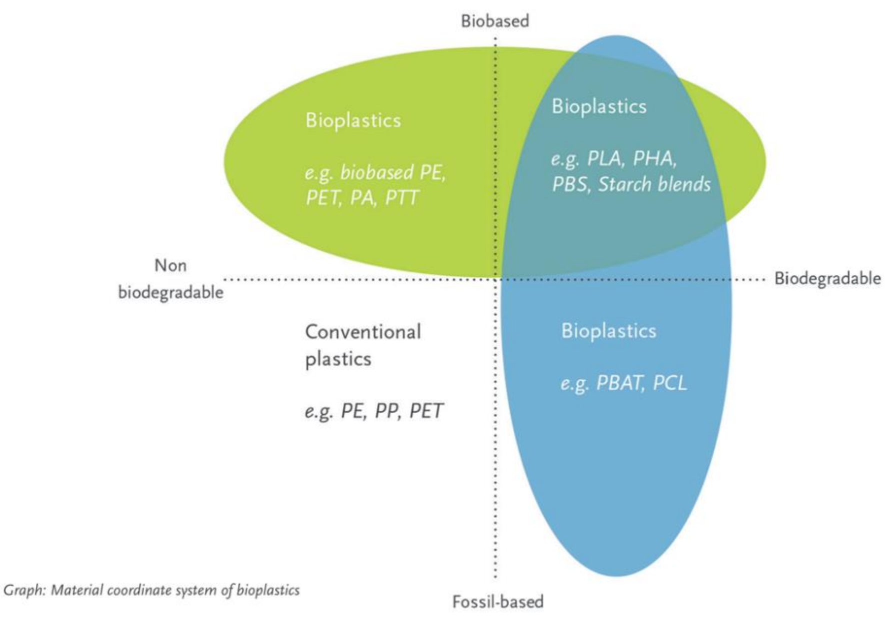


Figure 1.3. (continued on the next page)

Figure 1.3. (figure on the previous page) Biodegradability chart of different biobased and fossil-based plastic materials; PE, polyethylene; PET, polyethylene terephthalate; PA, polyamide; PTT, polytrimethylene terephthalate; PP, polypropylene; PLA, polylactic acid; PHA, polyhydroxyalkanoate; PBS, polybutylene succinate; PBAT, polybutylene adipate-*co*-terephthalate; PCL, polycaprolactone (adapted from European Bioplastics, 2016)

Some common commercial biodegradable plastics include polyhydroxyalkanoate (PHA), polyhydroxybutyrate (PHB), and polylactic acid (PLA), among others (Nanda *et al*., 2022). Although various bio-based alternatives to petroleum-based plastics exist, they are typically produced in small quantities and at higher costs (Rosenboom *et al*., 2022). Nevertheless, bioplastics offer a viable solution for reducing dependence on synthetic, non-biodegradable plastics.

Biological systems offer promising solutions to combat plastic pollution. Research on using insects, fungi, and bacteria for plastic biodegradation is expanding in scope. One such method is entomoremediation, where insect larvae like waxworms and mealworms ingest and degrade synthetic polymers in synergy with their gut microbiome (Bulak *et al*., 2021; J. Yang *et al*., 2014).

Microorganisms are another effective tool for plastic biodegradation. The microbial biodegradation of plastics begins with the colonization of plastics by microorganisms, establishing a "plastisphere" community where plastisphere refers to the diverse community of microorganisms present on the surface of plastics (Zettler *et al*., 2013; Y. Zhang *et al*., 2022). Microorganisms harbouring synthetic polymer-degrading enzymes

can be considered green tools for recycling plastics. Several bacteria and fungi have been reported to degrade different synthetic polymers through methods that are representative of assessing the changes in the polymer structure (like scanning electron microscopy (SEM), Fourier-transform infrared spectroscopy (FT-IR) and gel permeation chromatography (GPC)), assessing the physical loss of polymer mass (like evaluating CO_2 evolution), and assessing the generation of plastic metabolites (like gas chromatography-mass spectrometry (GC-MS)) (Lear *et al.*, 2021). A variety of polymers have been assessed in this regard: polystyrene degradation by *Bacillus*, *Pseudomonas* and *Acinetobacter* (Ganesh Kumar *et al.*, 2021; Kim *et al.*, 2021), polyurethane by *Penicillium* and *Moraxella* (Magnin *et al.*, 2019; Maheswaran *et al.*, 2024), polyvinyl chloride by *Vibrio, Altermonas and Cobetia* (Khandare *et al.*, 2021), and PET by *Ideonella sakaiensis* (Yoshida *et al.*, 2016). As for polyethylene (PE), it is the most widely used and produced plastic polymer and presents unique challenges in biodegradation (Geyer *et al.*, 2017). Microbial colonization poses a significant challenge because of the hydrophobic nature of PE. Microorganisms capable of producing metabolites, such as biosurfactants, or forming biofilms to overcome this hydrophobicity show promise as potential plastisphere inhabitants (Pathak & Navneet, 2017; Y. Zhang *et al.*, 2022). Furthermore, PE has additional limitations due to its high molecular weight and carbon (C-C) backbone. These factors significantly contribute to its high recalcitrance to biodegradation.

Chemical and biochemical processes that can reduce the molecular weight and induce polymer oxidation are crucial for making PE more accessible to microorganisms. These processes are often caused by the synergistic actions of abiotic (photo/thermal oxidation) and biotic factors (such as biosurfactants and exoenzymes) (Restrepo-Flórez

et al., 2014). The microbial colonization of PE initiates the biodegradation process, followed by the enzymatic breakdown of the polymer, involving both extracellular and intracellular enzymes. Extracellular enzymes are crucial for the depolymerization of PE via hydrolytic cleavage to generate smaller oligomers. These oligomers can then be taken up by microbial cells for further metabolism, facilitated by intracellular enzymes (Figure 1.4) (Cai *et al*., 2023; Ghatge *et al*., 2020).

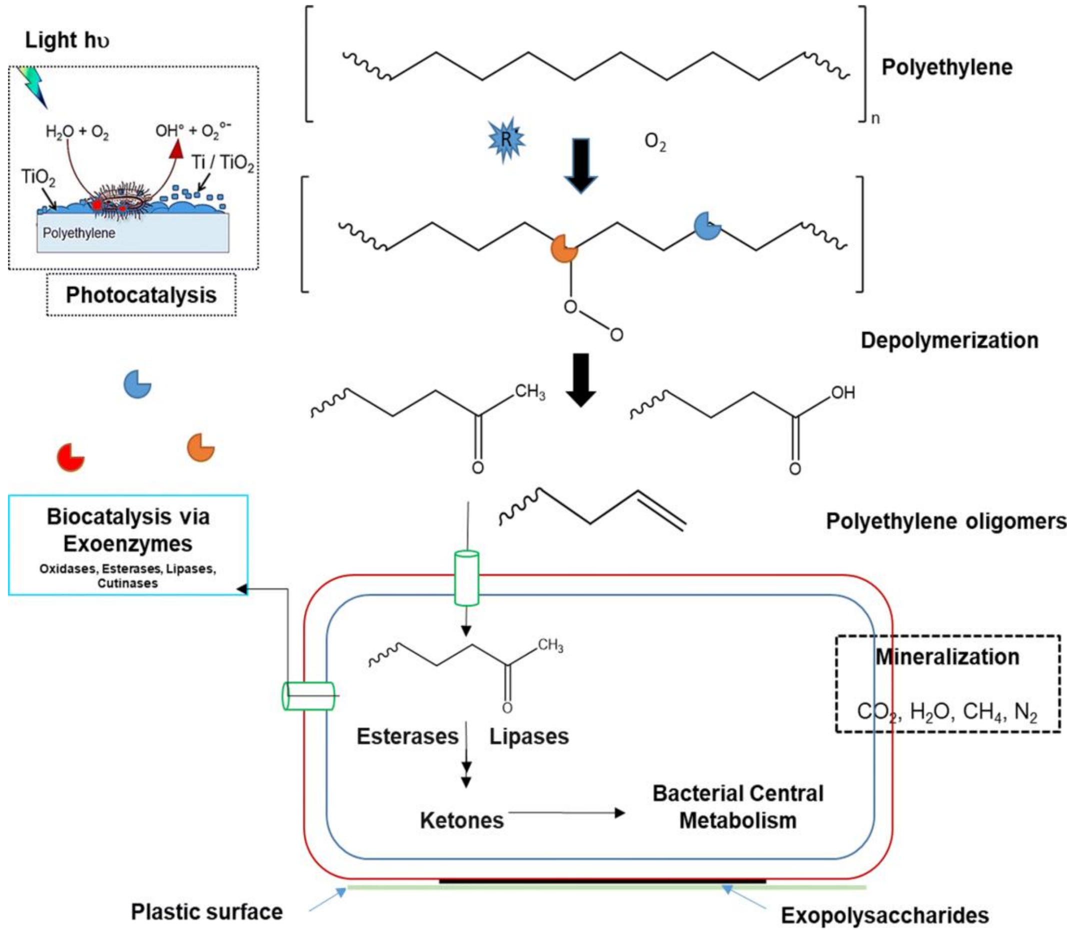


Figure 1.4. A hypothetical mechanism of polyethylene degradation mediated by abiotic (UV irradiation) and biotic factors (microorganisms) (adapted from Ghatge *et al*., 2020)

To date, the enzymes reported for PE degradation are usually associated with lignin or hydrocarbon degradation. Lignin-degrading enzymes, such as laccases, manganese peroxidases, and lignin peroxidases, and hydrocarbon-degrading enzymes, such as the AlkB family of alkane hydroxylases (AHs), have been associated with PE degradation (Iiyoshi *et al*., 1998; Jeon & Kim, 2015; Tao *et al*., 2023; Zampolli *et al*., 2021, 2023).

Both enzyme classes are involved in the depolymerization of PE, with AHs being involved in terminal and subterminal oxidation (Wei & Zimmermann, 2017). Additionally, hydrolases, such as lipases and esterases, are surface-modifying enzymes that increase the surface hydrophilicity of plastic polymers, promoting microbial colonization and degradation (Cai *et al*., 2023; Tao *et al*., 2023).

The current literature postulates the role of enzymes like laccases and a few peroxidases in the depolymerization of the PE polymer. A molecular-docking study hypothesized a mechanism of PE- laccase reaction, where the active site of laccase interacts with PE, participating in a series of electron transfer reactions. As a result, more oxygen containing functional groups (like alcohols, aldehydes) are further introduced into the PE molecule, eventually resulting in depolymerization. The smaller oligomers thus generated are amenable for further metabolism. Additionally, enzymes like AHs oxidise *n*-alkanes into their corresponding alcohols and subsequently into their corresponding carboxylic acids, which are further metabolised by the β-oxidation pathway. In a similar fashion, the subterminal hydroxylation of alkanes produces esters, which are further hydrolyzed by esterases, eventually entering the β-oxidation pathway (Ghatge *et al*., 2020; Jin *et al*., 2023; Santacruz-Juárez *et al*., 2021). The collective action of these enzymes facilitates the hydrolytic cleavage of PE polymers/oligomers,

ultimately leading to the mineralization of PE (Amobonye *et al*., 2021; Ghatge *et al*., 2020).

A few bacterial and fungal species that express these enzymes, such as *Rhodococcus ruber* and *Phanerochaete chrysosporium*, have been shown to degrade PE to some extent (Gilan *et al*., 2004; Iiyoshi *et al*., 1998). Despite these findings, the practicality of using microbes and their corresponding genes and enzymes for PE biodegradation is severely lacking, and understanding of the underlying mechanisms remains incomplete. Moreover, a microbe possessing all the necessary PE-degrading enzymes and effectively degrading and mineralizing PE has yet to be isolated. This gap in research highlights the importance and relevance of further studies in this field.

Individual bacterial strains may have limited metabolic capabilities; therefore, they may not be fully effective in breaking down PE when used alone. Effective PE breakdown and utilization by a single microorganism has yet to be reported. One approach to address this limitation is the construction and application of a microbial consortium comprising a specialist microbe for each enzyme. Another approach is to genetically engineer a suitable host to express the target PE-degrading enzymes. These two strategies are central to this dissertation and are investigated experimentally in the upcoming chapters.

1.2 Aim & Objectives

This dissertation aims to explore microbial biodegradation of PE plastic and is divided into two experimental studies:

Study I: The objective is to test an artificial bacterial consortium for PE biodegradation. Additionally, from this study, a suitable host is selected from among the consortium bacteria for exploring a genetic engineering strategy to break down PE.

Study II: The objective is to employ genetic engineering to modify a suitable host bacterium to express polyethylene degrading enzymes for its application in PE biodegradation.

1.3 Dissertation outline

In the current chapter, I have presented the motivation behind the research theme, briefly touching upon plastic pollution and its global impact. Additionally, I have highlighted the scope and lack of research in the field of microbial biodegradation of plastics, which is a potential green alternative for addressing plastic pollution.

The subsequent chapters are organized as follows: Chapters II and III provide a descriptive account of Study I and Study II, respectively, with each chapter subdivided into systematized individual sections. Chapter IV concludes the research study and presents a few prospects for future experimentations.

CHAPTER II

STUDY I

2. Consortium strategy for PE biodegradation

2.1 Introduction

In natural environments, the microbial degradation of plastic occurs through the collective efforts of different diverse microbial communities. Many plastisphere studies have revealed the complexity of multi-microbial biofilms that naturally form on plastic debris (Delacuvellerie *et al*., 2022; I. V. Kirstein *et al*., 2019; Zettler *et al*., 2013).

Microbial consortia, which are combinations of different microorganisms, show enhanced biodegradation potential for various polymers compared to pure cultures (Skariyachan *et al*., 2022). For instance, separate polymer degradation studies by Montazer *et al*. and Skariyachan *et al*. reported a higher polymer weight loss by bacterial strains when used as a consortium over individual bacterial treatments (Montazer *et al*., 2021; Skariyachan *et al*., 2018). Another study by Hu *et al*. reported the synergistic biodegradation of *n*-alkane by a two-member bacterial community (Hu *et al*., 2020).

Effective polyethylene (PE) degradation requires the collective action of several different enzymes. In a harmonious consortium, the metabolic limitations of individual bacteria can be overcome as different strains contribute different enzymes and metabolize the various byproducts resulting from PE degradation. Moreover, in such a consortium setting, any taxing metabolic load can be shared among the members, reducing the burden on any single strain (Jin *et al*., 2023; Qian *et al*., 2020).

Additionally, artificial consortia offer a more controlled environment, making it easier to investigate degradation processes. The functioning of artificial consortia can be enhanced by adding specific microorganisms or simplifying the consortium to study the genetic and metabolic basis (Cao *et al*., 2022). Therefore, consortia of microorganisms can be applied to effectively degrade complex compounds over using pure cultures.

In Study I of this dissertation, the microbial consortium approach is explored. Bacterial strains for the consortium were selected based on the PE degradation reports from the literature, where the same bacterial strains or closely related strains from these reports were taken into account. The enzyme activity of various PE-specific enzymes, including laccases, lipases, esterases, and AHs, with respect to their extracellular expression was assessed for these bacterial strains. Finally, the selected bacterial strains were assembled into a consortium to utilize their combined potential for PE biodegradation. In addition, this study also aimed to select a suitable consortium strain to serve as a versatile host for the genetic engineering strategy. The assessment of consortium strains was based on their ability to colonize PE, their long-term survival on PE, and their possession of PE degradation-related enzyme genes in order to select the most suitable candidate host.

2.2 Materials and Methods

Chemicals and media

Guaiacol, Tween-20, and polyethylene (SIGMA 428043-250G; low density, pellet form, diameter 3–4 mm) were purchased from Sigma-Aldrich. *n*-Hexadecane and *n*-

eicosane were purchased from Wako Pure Chemical Industries, *n*-dotriacontane was purchased from TCI Chemicals, and Tween-80 was purchased from MP Biomedicals. Luria–Bertani (LB) and W-minimal media (Kitagawa *et al*., 2018) were used as cultivation media. Their specific compositions are as follows: LB medium (per L of medium): 10 g tryptone, 5 g yeast extract, 5 g NaCl, and 15 g agar; W-minimal medium: 1000× buffer stock solution (per L of medium): 10.75 g MgO, 2 g $CaCO_3$, 4.5 g $FeSO_4{\cdot}7H_2O$, 1.44 g $ZnSO_4{\cdot}7H_2O$, 1.12 g $MnSO_4{\cdot}4H_2O$, 0.25 g $CuSO_4{\cdot}5H_2O$, 0.28 g $CuSO_4{\cdot}7H_2O$, 0.06 g H_3BO_3, 51.3 mL conc. HCl; 100× buffer stock solution (1 L): 10 g $MgSO_4{\cdot}7H_2O$, 0.5 g $FeSO_4{\cdot}7H_2O$, 100 mL 1000× buffer; 1× buffer (990 mL): 0.85 g KH_2PO_4, 4.9 g Na_2HPO_4, 0.5 g $(NH_4)_2SO_4$; final W-minimal medium (1 L): 990 mL 1× buffer, 10 mL 100× buffer.

Microbe selection

To include the necessary PE-degrading enzymes, bacteria for the consortium were selected based on the literature on microbial PE degradation. To date, several bacteria have been reported to exhibit PE degradation activities, including *Rhodococcus* (Bonhomme *et al*., 2003; Gibu *et al*., 2019; Gilan *et al*., 2004; Laczi *et al*., 2015; Pi *et al*., 2017; Sauvageau *et al*., 2009; Zampolli *et al*., 2022), *Brevibacillus* (Hadad *et al*., 2005), *Enterobacter* (J. Yang *et al*., 2014), *Bacillus* (Z. Yao *et al*., 2022), *Phormidium* (Sarmah & Rout, 2018), *Hyphomonas* (Zettler *et al*., 2013), *Alcanivorax* (Delacuvellerie *et al*., 2019), *Pseudomonas* (Mukherjee *et al*., 2018), and *Streptomyces* (El-Shafei *et al*., 1998).

Based on these previous reports, and the availability of genome information, the same strain or closely related members from the reported literature were selected.

Strains used in this study are listed in Table 2.1, and the reference strains are listed in Table 2.2. In addition, an *E. coli* strain was also included. *E. coli* was reported to form biofilm on plastic; it was used as the enzyme assay control (Ganesan *et al*., 2022).

Table 2.1. Bacterial strains used in this study (Study I)

Bacterial strain	Relative growth*	Reference Sequence Accession No.
Hyphomonas neptunium NBRC 14232	S	NC_008358.1
Hyphomonas polymorpha NBRC 102482	S	ARYM00000000.1
Brevibacillus borstelensis NBRC 15714	S	BJOK00000000.1
Alcanivorax sp. NBRC 101098	I	AP014613.1
Alcanivorax profundi JCM 31866	I	QYYA00000000.1
Enterobacter asburiae NBRC 109912	S	CP011863.1
Bacillus subtilis JCM 1465T	S	AP019714.1
Pseudomonas fluorescens JCM 5963T	I	VFEP00000000.1
E. coli K-12	S	NC_000913.3
Streptomyces griseus NBRC 13350	S	AP009493.1
Rhodococcus ruber JCM 3205T	VF	BCXE00000000.1
Rhodococcus rhodochrous JCM 3202T	F	LT906450.1
Rhodococcus jostii RHA1	VF	NC_008268.1
Rhodococcus erythropolis NBRC 100887	F	AP008957.1
Rhodococcus zopfii DSM 44189	F	WBMO00000000.1
Phormidium sp. NBRC 102691 (green)	-	NA
Phormidium sp. NBRC 102724 (yellow)	-	NA

NBRC: Biological Resource Center, NITE (Japan)

JCM: Japan Collection of Microorganisms

DSM: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

NA: Not available

*Relative growth in W medium + 0.5% hexadecane. The incubation time required to reach an OD_{600} of 2 is <24 hrs, <28 hrs, <30 hrs, and >30 hrs for VF (very fast), F (fast), I (intermediate), and S (slow), respectively.

Table 2.2. Characteristics of reference stains from literature

Reference strains from literature	Substrate	References	Relation to the strain used in this study
Hyphomonas	Plastic marine debris bacteria	Zettler *et al.* 2013	Same genera
Brevibacillus borstelensis strain 707	LDPE degradation	Hadad *et al.* 2005	Same species
Alcanivorax borkumensis	LDPE degradation	Delacuvellerie *et al.* 2019	Same genera
Enterobacter asburiae YT1	LDPE degradation	Yang *et al.* 2014	Same species
Bacillus subtilis ATCC 6051	LDPE degradation	Yao *et al.* 2022	Same strain (JCM 1465T = ATCC 6051)
Pseudomonas fluorescens ATCC 13525	LDPE degradation	Mukherjee *et al.* 2018	Same strain (JCM 5963T = ATCC 13525)
E. coli K-12	Biofilm formation on PE and other plastics	Ganesan *et al.* 2022	Same strain
Streptomyces sp.	PE (containing 6% starch) degradation	El-Shafei *et al.* 1998	Same genera
Rhodococcus ruber C208*	LDPE degradation	Gilan *et al.* 2004	Same species
Rhodococcus rhodochrous ATCC 13808*	Plasticizer degradation	Sauvageau *et al*. 2009	Same strain (JCM 3202T = ATCC 13808)
Rhodococcus rhodochrous ATCC 29672*	PE (containing TDPA) degradation	Bonhomme *et al*. 2003	Same species
Rhodococcus jostii RHA1*	*n*-alkane degradation	Gibu *et al.* 2019	Same strain
Rhodococcus erythropolis PR4 NBRC 100887*	Hydrocarbon degradation	Laczi *et al.* 2015	Same strain
*Rhodococcus zopfii**	Crude oil degradation	Pi *et al.* 2017	Same species
Phormidium lucidum	LDPE degradation	Sarmah & Rout 2018	Same genera

**Rhodococcus* plastic-degrading enzymes genomic study (Zampolli *et al*. 2022)

Extracellular enzyme assay and alkane utilization assay on agar plates

The following four enzymes are important for microbial PE degradation: laccase, esterase, lipase, and AH (Ghatge *et al*., 2020; Restrepo-Flórez *et al*., 2014). Plate assays were performed to determine whether the investigated strains have PE degradation/utilization enzyme activities, especially extracellular activities. Among the strains listed in Table 2.1, two *Phormidium* strains were not used for the plate assay because they did not grow on the solid agar media.

Laccase plate assay

LB agar plates supplemented with 0.02% guaiacol and 0.5 or 1 mM $CuSO_4$ were used to screen for laccase activity. Bacterial strains were inoculated by streaking or spotting and incubated at 28°C or 37°C. The oxidation of guaiacol by the laccase enzyme is indicated by the formation of a reddish-brown product. Extracellular laccase activity is observed as a reddish-brown halo around the bacterial colony, whereas intracellular laccase activity is seen when the cells alone turn reddish-brown (Kiiskinen *et al*., 2004).

Lipase/esterase plate assay

Tween-20 and Tween-80 agar plates were used for this assay. They were prepared as follows: (amount per L); 10 g peptone, 5 g NaCl, 0.1 g $CaCl_2 \cdot 2H_2O$, 20 g agar, and 10 mL (v/v) Tween-20/-80 (Kumar *et al*., 2012). The bacterial strains were spotted onto these plates and incubated at 28°C or 37°C. Tween-80 was used to detect lipase activity, as it is primarily hydrolyzed by lipases owing to its oleic acid esters, whereas Tween-20 was used to detect esterase activity, as it contains esters of short-chain fatty acids.

Lipase and esterase activities were indicated by the appearance of a calcium salt precipitate and/or the clearing of such a precipitate resulting from the complete degradation of the salt of the fatty acid around the bacterial colony (Kumar *et al*., 2012; Sierra, 1957).

Alkane utilization plate assay

Alkanes of varying carbon chain lengths were used to assess alkane utilization by the selected bacterial strains. Specifically, *n*-hexadecane (C16, oil phase at ambient temperature), *n*-eicosane (C20, solid phase), and *n*-dotriacontane (C32, solid phase) were used in this study. Alkane test plates were prepared as follows. For *n*-hexadecane, 0.2% volume of the substrate was added to W-minimal medium agar and solidified. For *n*-eicosane and *n*-dotriacontane, the substrates were first dissolved in diethyl ether at a concentration of 5 mg/mL, and then the liquid was sprayed onto the surface of solidified W-minimal media agar (approximately 1 mL/plate). In this strategy, a thin layer of *n*-eicosane or *n*-dotriacontane was formed on the plates. After the complete evaporation of the solvent, the plates were inoculated with the bacteria. Control plates were prepared without alkane supplementation. The bacterial strains were spotted onto these plates and incubated at 28°C or 37°C. Alkane utilization activity was indicated by the growth of the test strain on alkane-containing plates, and extracellular enzyme activity was evaluated by the formation of a clear zone around the colony on the *n*-eicosane- and *n*-dotriacontane-supplemented plates.

Consortium construction and cultivation

Liquid hydrocarbons such as *n*-hexadecane have been reported to improve the accessibility of PE to bacteria when added to the culture broth (Montazer *et al*., 2020).

Moreover, a study by Gilan *et al*. found that adding mineral oil to the culture medium improved bacterial colonization and PE biodegradation (Gilan *et al*., 2004). Hence, *n*-hexadecane was added to the culture broth used in this study. All the consortium strains were initially pre-cultured individually in W-minimal medium containing 0.5% *n*-hexadecane (*Bacillus subtilis* and *Streptomyces griseus* were pre-cultured in LB, as their growth in W-minimal medium was very slow). The pre-culture broth (OD_{600} = ~2–3) was used as the seed inocula at the following volumes: 100 µL of very fast growers in W-minimal medium (*R. ruber* and *R. jostii* RHA1), 150 µL of fast growers (*R. rhodochrous*, *R. zopfii*, and *R. erythropolis*), 500 µL of the inocula of bacteria with intermediate growth (*Alcanivorax* strains and *Pseudomonas fluorescens*), and 1 mL of slow growers (*Hyphomonas* strains, *Brevibacillus borstelensis*, *Enterobacter asburiae*, *B. subtilis*, *Escherichia coli* K-12, *S. griseus*, and *Phormidium* strains) (Table 2.1). Independent seeds were mixed and introduced into a final volume of 100 mL of W-minimal medium broth supplemented with 0.1% *n*-hexadecane. PE pellets were treated in a 70% ethanol bath for 15 min and dried before adding them to the consortium. Thirty PE pellets were added per flask. The consortium culture was performed in triplicates (three flasks: R1, R2, and R3), and a flask without any bacterial inoculum was used as the control. The flasks were maintained at 28°C on a rotary shaker at 90 rpm. Additionally, after 60 days, half of the spent media in the culture flasks was replaced with fresh W medium.

Sampling, DNA extraction, and next-generation sequencing of 16S rRNA genes

Bacterial samples from the consortium culture broth were collected on days 1, 10, 25, 40, 60, and 120 for DNA extraction and community analysis. PE samples were

collected for plastisphere DNA extraction on day 120. DNA was extracted using the conventional phenol-chloroform extraction method (Sambrook et al., 1989). Using the extracted DNA as the template, the bacterial V4 hypervariable region of the small-subunit rRNA (SSU rRNA) gene was amplified for all samples (consortium culture broth and plastisphere) using the primers 515F (5′-GTGYCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). Amplicon PCR and follow-up index PCR were performed according to the Illumina 16S Metagenomic Sequencing Library Preparation Guide (Part # 15044223 Rev. B; Illumina, San Diego, USA). PCR was performed using a KAPA HiFi HotStart Ready Mix (Kapa Biosystems, Wilmington, USA). The final DNA libraries were prepared according to the manufacturer's instructions and ran on an Illumina iSeq100 Sequencing System using the iSeq 100 i1 Reagent v2 (300-cycle) kit (Illumina), with the run set for paired-end sequencing.

Community analysis

Downstream data analysis for the FASTQ output from the Illumina iSeq100 sequencing step was performed using Quantitative Insights into Microbial Ecology 2 (QIIME 2) version 2019.10 (Bolyen *et al.*, 2019). The DADA2 plugin of QIIME2 was used for denoising, merging, and chimera removal and to construct an amplicon sequence variant (ASV) table (Callahan *et al.*, 2016). For sequence reference and taxonomic annotations, Silva 138 SSURef NR99 was used to train the classifier before annotating the reads (Quast *et al.*, 2013). Subsequent analyses were performed using the marker data profiling module of MicrobiomeAnalyst (a web-based platform) (Chong *et al.*, 2020; Dhariwal *et al.*, 2017; Lu *et al.*, 2023). In the Data Filtering step, the following filters were applied: in the low count filter, the minimum count was set to 10,

with prevalence in samples set to 10%. In the Data Normalization step, total sum scaling (TSS) was applied. Taxonomy bar plots and pie charts of the culture consortium and PE plastisphere communities were visualized with MicrobiomeAnalyst.

Scanning electron microscopy (SEM)

The PE samples were collected on days 10, 60, 120, and 200 for SEM observation. For SEM visualization and imaging, PE samples with biofilms, PE samples after biofilm removal, and respective control samples were obtained. For biofilm removal, the PE samples were treated with a lysozyme solution (2 mg/mL) and proteinase K (final concentration, 1 mg/mL) in STE buffer (0.1 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0), followed by treatment with 2% SDS for 3–4 hours at 37°C. Afterward, the samples were washed thrice with warm, sterile distilled water. SEM samples were fixed with 2% (w/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 1 h at room temperature. Stepwise dehydration was performed using a graded ethanol series (50%, 75%, 90%, 95%, and 100%) for 15 min each, followed by substitution with dehydrated tert-butyl alcohol. The samples were then freeze-dried using a VFD-21S t-BuOH freeze dryer (Vacuum Device Co., Ltd., Japan). Next, the prepared SEM samples were mounted on aluminium stubs using carbon paste (Pelco Colloidal Graphite, Ted Pella, Inc.), coated with gold using a DII-29010SCTR Smart Coater, and then visualized and imaged using a JSM-6010PLUS/LV SEM at 15 kV.

Fourier transform infrared spectroscopy (FTIR)

For FTIR analysis, the PE samples were collected after biofilm removal, and the respective control samples were also collected. For the biofilm removal, the PE samples were treated with proteinase K (final concentration, 1 mg/mL) then with 2% SDS for 3–

4 hours at 37°C. Afterward, the samples were washed thrice with warm, sterile distilled water, followed by a 15-min wash in 70% ethanol. FTIR spectra were obtained in the transmission mode using an ATR PRO ONE FT/IR-6600 (JASCO). Spectra were recorded at a resolution of 4 cm^{-1} in the wavenumber range of 4000–400 cm^{-1}. (The FTIR analysis was carried out at the Chitose Institute of Science and Technology, Advanced Research Infrastructure for Materials and Nanotechnology of Japan).

2.3 Results & Discussion

Extracellular enzyme assays and alkane utilization assays on agar plates

Laccase plate assay

Of the strains tested, only *S. griseus* displayed a reddish-brown halo around the colony, indicating extracellular laccase activity (Figure 2.1b). Most of the other strains displayed reddish-brown colonies with no halo, suggesting intracellular laccase activity, as seen in the *Hyphomonas* strains (Figure 2.1d) and *P. fluorescens* and *Rhodococcus* strains (Table 2.3). No laccase activity was observed in *B. subtilis*. The two *Alcanivorax* strains did not grow on the test plates, probably because of their high sensitivity to copper, which is required for laccase enzyme activity (Table 2.3). Based on the genomic information available, putative laccase genes were detected in all strains, with some even having multiple genes. However, only intracellular laccase activity was detected in several strains. For example, *R. erythropolis* has seven copies of a putative laccase in its genome (Ausec *et al.*, 2011), and the presence of a signal peptide was predicted in all of them. Laccases are thought to play an important role in PE degradation by cleaving its

backbone. Therefore, the fact that many microbial strains do not exhibit extracellular laccase activity is an important issue for their potential application. In contrast, the laccase EpoA was previously reported in *S. griseus* (Endo *et al*., 2002), the only species where extracellular activity was observed. The PE degradation activity of *S. griseus* observed in this study is presumed to be caused by this enzyme.

Esterase/lipase plate assay

Esterase activity was assessed using Tween-20. Nearly all tested strains displayed Ca salt precipitation, indicating positive esterase activity (Figure 2.1e–f). *Brevibacillus borstelensis* and the two *Alcanivorax* strains displayed no growth, indicating their sensitivity to Tween-20. In the lipase assay, all tested strains produced Ca salt precipitates on the Tween-80 agar plates, confirming their lipase activity (Figure 2.1g and Table 2.3). These results indicate that extracellular esterase and lipase activities are ubiquitous, unlike laccase activity.

Alkane utilization plate assay

All the five *Rhodococcus* exhibited proper growth on all three *n*-alkanes (Table 2.3). *Hyphomonas polymorpha* and *E. asburiae* grew in *n*-hexadecane-containing media. Other strains showed either faint growth or no growth. Among these strains, *B. subtilis*, *E. coli*, and *S. griseus* grew on both control and alkane-supplemented agar plates, with slightly improved growth observed on the alkane-supplemented agar plates. Therefore, these strains were marked as faint growth (fG) in Table 2.3. Because PE is a long alkane, microorganisms that can assimilate long alkanes are advantageous for the decomposition of PE. *Rhodococcus* has been previously reported to utilize alkanes with a >C20 chain length in the liquid phase (Whyte *et al*., 1998; Zampolli *et al*., 2014).

These results confirmed their ability to grow on agar, and they may be promising members for this application. Notably, only *Rhodococcus* strains produced clear zones on *n*-eicosane-supplemented agar plates (Figure 2.1i). To my best knowledge, this is the first report of a microorganism that produces a clear zone on solidified *n*-eicosane. AlkB, a well-known AH, also widely distributed among genus *Rhodococcus*, is a membrane-integrated enzyme that requires an electron transport protein to function (Takei *et al.*, 2008; Vomberg & Klinner, 2000; Xiang *et al.*, 2023). Another known AH, CYP153 (cytochrome P450), is a cytoplasmic enzyme that also requires an electron transport protein (Ji *et al.*, 2013; Van Beilen & Funhoff, 2007). As these enzymes are membrane-bound or cytoplasmic, they are active intracellularly and therefore, it is unlikely that these enzymes function outside the cell to form the clear zone. There may be other unknown extracellular alkane-degrading enzymes produced by these rhodococci. For instance, extracellular AHs were reported to be produced by thermophilic bacteria which were involved in crude oil degradation (Yusoff *et al.*, 2020). Alternatively, the biosurfactant produced increased alkane solubility, turning it invisible on the plates. Nonetheless, both cases offer significant advantages for alkane and PE degradation.

The plate assays for enzymes, including laccases, esterases, lipases, and AHs revealed that none of the individual bacterial strains expressed all four target enzymes extracellularly (Table 2.3). Moreover, the degree of enzyme production varied among the different bacterial strains. Hence, utilizing these strains as a consortium could help compensate for these discrepancies, not only in terms of enzyme profiles but also in the production of other beneficial metabolites, such as biosurfactants (Mukherjee *et al.*, 2016). Each strain expressed at least one of the target enzymes extracellularly, so they were included in the consortium.

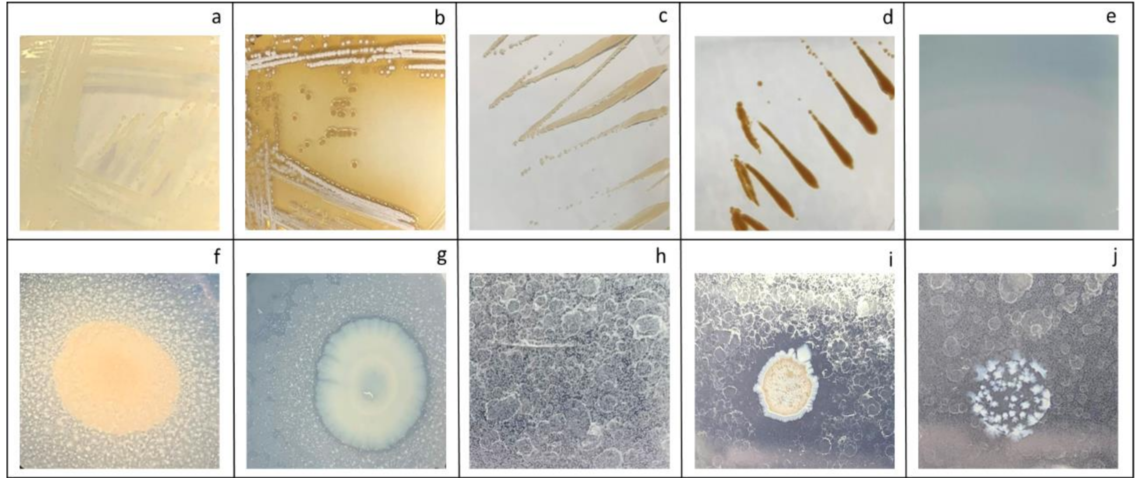


Figure 2.1. Enzyme plate assays. **Laccase plate assay (a–d)**, (a) *S. griseus* on control plate (without substrate), (b) *S. griseus* on test plate (with substrate) showing extracellular laccase activity, (c) *H. polymorph*a on control plate, (d) *H. polymorpha* on test plate showing intracellular laccase activity, **Esterase plate assay (e–f)**, (e) appearance of Tween-20/-80 test plate without bacteria, (f) *R. erythropolis* on Tween-20 test plate showing precipitation (extracellular esterase activity), **Lipase plate assay:** (g) *P. fluorescence* on Tween-80 test plate showing precipitation (extracellular lipase activity), **Alkane utilization assay (h–j)**, (h) *n*-eicosane-sprayed agar test plate showing surface-dried solid *n*-eicosane, (i) *R. rhodochrous* on test plate showing growth and a clear zone, (j) *B. borstelensis* on test plate showing growth.

Table 2.3. Extracellular enzyme activity and alkane utilization on agar plate

Bacterial strain	Laccase	Esterase	Lipase	Alkane utilization		
				hexadecane	eicosane	dotriacontane
Hyphomonas neptunium NBRC 14232	-, (int+)	+	+	fG	ng	ng
Hyphomonas polymorpha NBRC 102482	-, (int+)	+	+	GR	ng	ng
Brevibacillus borstelensis NBRC 15714	-, (int+)	ng	+	fG	fG	fG
Alcanivorax sp. NBRC 101098	ng	ng	+	ng	ng	ng
Alcanivorax profundi JCM 31866	ng	ng	+	ng	ng	ng
Enterobacter asburiae NBRC 109912	-, (int+)	+	+	GR	fG	fG
Bacillus subtilis JCM 1465T	-, (int-)	+	+	fG	fG	fG
Pseudomonas fluorescens JCM 5963T	-, (int+)	+	+	fG	fG	fG
E. coli K-12	-, (int+)	+	+	fG	fG	fG
Streptomyces griseus NBRC 13350	+	+	+	fG	fG	fG
Rhodococcus ruber JCM 3205T	-, (int+)	+	+	GR	GR, CL	GR
Rhodococcus rhodochrous JCM 3202T	-, (int+)	+	+	GR	GR, CL	GR
Rhodococcus jostii RHA1	-, (int+)	+	+	GR	GR, CL	GR
Rhodococcus erythropolis NBRC 100887	-, (int+)	+	+	GR	GR, CL	GR
Rhodococcus zopfii DSM 44189	-, (int+)	+	+	GR	GR, CL	GR

+: extracellular activity positive, –: extracellular activity negative

(int+): intracellular activity positive, (int-): intracellular activity negative

GR: Growth, ng: no growth, fG: faint growth, CL: clear zone observed

Consortium culture with PE and consortium community analysis

Most consortium studies have used microbial isolates from enriched or natural plastisphere sources. For example, Joshi *et al*. constructed a bacterial consortium with four bacterial isolates from marine plastic debris (Joshi *et al*., 2022), Han *et al*. built a consortium of *Arthrobacter sp*. and *Streptomyces sp*. isolated from agricultural soils (Han *et al*., 2020), and D'Souza *et al*. formulated a fungal consortium from pure cultures of three *Aspergillus spp*. (DSouza *et al*., 2021) for PE degradation.

In this study, an artificial bacterial consortium was constructed where all the constituent members were tested for enzymatic activity related to PE degradation, for the first time. The resulting bacterial consortium was employed for the biodegradation of PE.

Culture broth community analysis

The taxonomy bar plots in Figure 2.2 depict the dynamics of the culture broth community over 120 days, displaying the top 10 abundant genera at each sampling day. The culture broth community drastically changed from day 1 to day 10 and then gradually became a mostly stable community afterward. There was a significant shift in the relative abundances of the consortium members in the first few days (days 1–10), characterized by a marked growth in *Rhodococcus* and a substantial drop in the abundance of *Bacillus*. The initial fluctuations due to microbe-microbe interactions (such as competition) subsided, and subsequently, the culture broth community gradually changed and reached a more stable state. The community analysis, being DNA-based, might also include DNA from dead cells, which means that the stability of the consortium does not necessarily imply that all members are actively breaking down

PE. In this context, consortium stability can be interpreted as a state where these bacteria can coexist, where some contribute directly to PE degradation while others do not, but play a supportive role.

Notably, a stable community composition was evident from days 40 to 120. The dominant taxa observed after day 40 were *Rhodococcus*, *Enterobacter*, *Marinobacter*, and *Hyphomonas*.

Interestingly, certain bacteria, such as *Marinobacter* and *Pelagibacterium*, were observed in the culture broth, although they were not part of the initial consortium community. This unexpected finding can be attributed to the non-axenic cultures of *Phormidium* strains harboring hydrocarbonoclastic contaminants that thrive under the culture conditions. The association of these bacteria with *Phormidium* was confirmed via 16S rRNA sequence analysis of the original stock of two *Phormidium* cultures from a bioresource organization. The culture collection note clearly stated that these stocks were a mixture of multiple strains with a strong symbiotic relationship; therefore, contamination did not change the outcomes of the experiment disturb this experiment. The presence of *n*-hexadecane in the consortium culture likely played a role in facilitating the growth of these bacteria. It is worth noting that *Pelagibacterium* is associated with oil-contaminated soils, while *Marinobacter* is a well-known hydrocarbonoclastic bacterium (Huang *et al*., 2021). Furthermore, a study conducted by Sun *et al*. reported that *Marinobacter* strains exhibited robust growth with *n*-hexadecane as the sole carbon source. Additionally, these strains express several AH genes (Sun *et al*., 2018). The *n*-hexadecane added at the start of the culture was expected to be consumed quickly by *Rhodococcus* and other consortium bacteria. However, the

continuous presence of these contaminant hydrocarbonoclastic bacteria over a long period might have also contributed to PE decomposition.

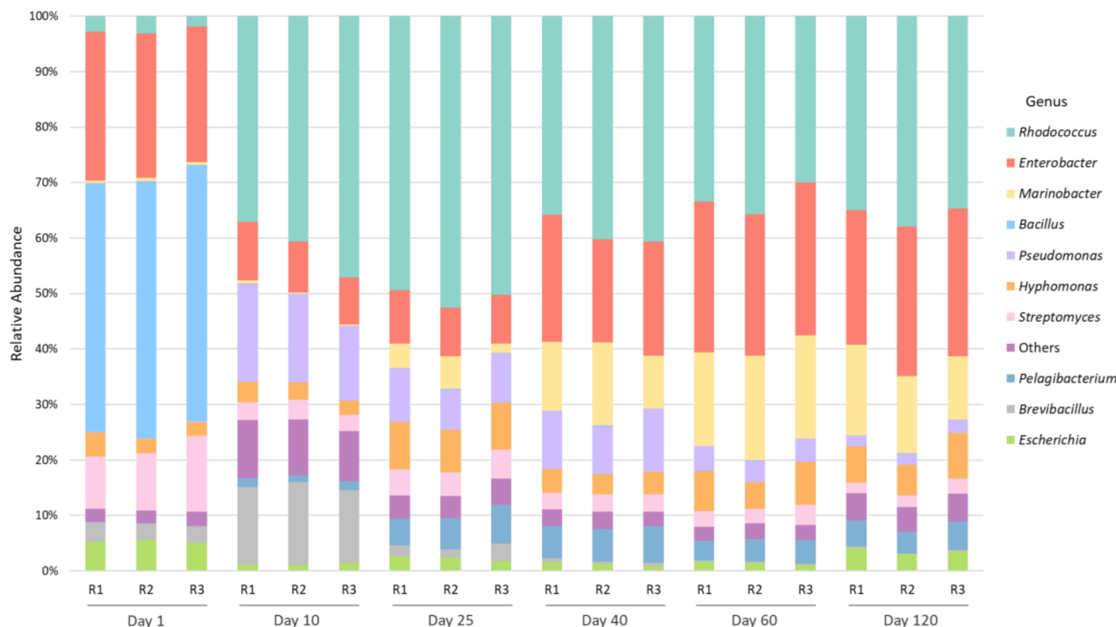


Figure 2.2. Taxonomy bar plots illustrating the culture broth community dynamics over a 120-day period, showcasing the top 10 abundant genera at each sampling day (R1, R2, R3 denote the triplicate flasks)

Plastisphere community analysis

The taxonomy bar plots in Figure 2.3 show the plastisphere communities in the PE samples at day 120. Compared to its relative abundance in the culture broth (36%), *Rhodococcus* overwhelmingly dominated the plastisphere community, accounting for approximately 78% of the bacteria (Figure 2.4).

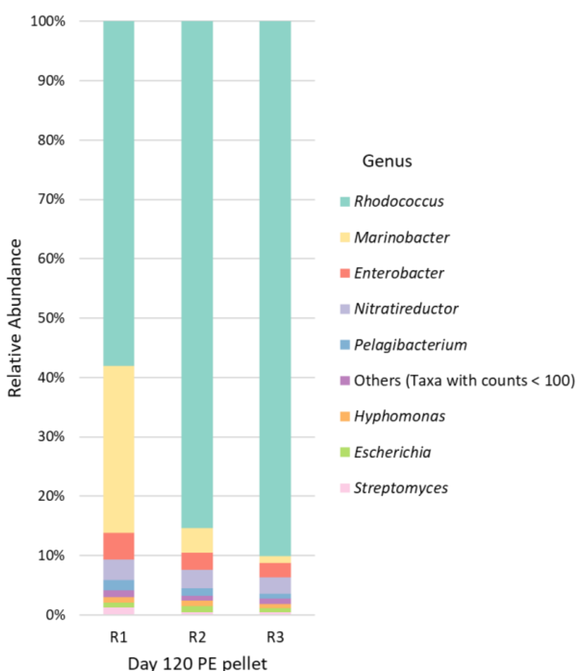


Figure 2.3. Taxonomy bar plots depicting the plastisphere community (Day 120) (R1, R2, R3 denote the triplicate flasks)

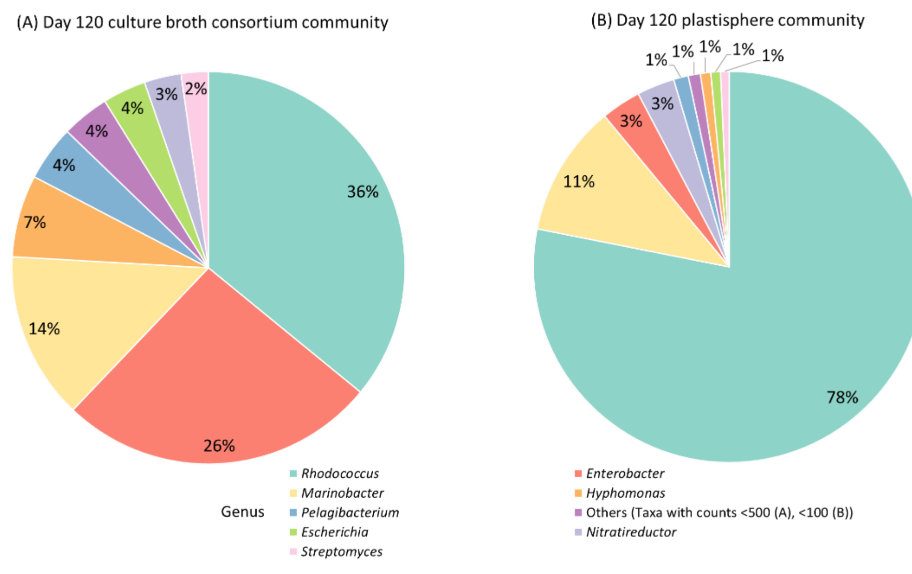


Figure 2.4. Pie charts of Day 120 consortium community in the culture broth vs. plastisphere (average value of triplicates)

The high relative abundance of *Rhodococcus* in the plastisphere suggests its potential role as a primary colonizer that establishes itself early and produces robust long-term biofilms on the PE surface. Studies have reported that many established plastisphere microorganisms inherently produce biofilms; *Rhodococcus* naturally forms biofilms (Amaral-Zettler *et al*., 2020; Pátek *et al*., 2021).

The presence of *Rhodococcus* in plastispheres has been previously reported: Wang *et al*. suggested the potential role of *R. erythropolis* as a microplastic degrader in a mangrove microplastic plastisphere study (Wang *et al*., 2024), Ya *et al*. reported the enrichment of *Rhodococcus* in PE microplastics (Ya *et al*., 2022) and Rüthi *et al*. reported that *Rhodococcus* is a plastisphere bacterium found in Alpine and Arctic soils (Rüthi *et al*., 2020).

The early establishment of *Rhodococcus* might have enhanced its competitive advantage in colonizing PE pellets, potentially hindering the proliferation of other strains (Amaral-Zettler *et al*., 2020). Additionally, *Rhodococcus* produces biosurfactants in response to hydrocarbons. The presence of *n*-hexadecane in the culture broth may have induced biosurfactant production in the *Rhodococcus* strains, thereby improving colonization. Biosurfactants specifically localized on the outer cell surface of *Rhodococcus* cells increase the hydrophobicity of their cell surface and facilitate bacterial attachment to the hydrophobic PE surface (Cappelletti *et al*., 2020). *Rhodococcus* sp. usually produce trehalose-based glycolipid biosurfactants (Cappelletti *et al*., 2020). For instance, *R. qingshengii* was reported to carry genes involved in the production of trehalose-based glycolipid biosurfactants like *otsA*, *otsB*, *treY* and *treZ*

(Markova *et al*., 2023). The genome information of *R. erythropolis* and *R. rhodochrous* used in this study also revealed the presence of these biosurfactant genes.

Rhodococcus ASVs obtained from the NGS data analysis were further analysed to distinguish the five *Rhodococcus* strains used in the consortium. *R. erythropolis* is found to be the sole prominent species within the entire *Rhodococcus* community, establishing its dominance in both the culture broth and the plastisphere. *R. erythropolis* outperformed the other *Rhodococcus* strains in terms of growth and colonization of PE. These findings regarding *R. erythropolis* are reported for the first time.

Other plastisphere bacteria, including *Enterobacter*, *Nitratireductor*, *Pelagibacterium*, *Hyphomonas*, and *Marinobacter*, only constituted a small fraction. *Nitratireductor* and *Marinobacter*, which are deep-sea hydrocarbonoclastic bacteria, are contaminants in the *Phormidium* cultures. Similar to *Marinobacter*, *Nitratireductor* has been shown to exhibit robust growth on *n*-hexadecane as its sole carbon source and possesses hydrocarbon-degrading genes such as alkane monooxygenase (Sun *et al*., 2018).

PE biodegradation assessment

SEM imaging of consortium culture

SEM analysis was performed on PE samples to assess both bacterial colonization and surface degradation. SEM images obtained from samples on day 10 showed dense, multilayered biofilms on the PE surface, along with some planktonic cells and no obvious surface damage was observed (Figures 2.5a–c). Day 60 PE pellets show three-dimensional biofilm clusters, also seen are bacteria growing in and accentuating PE

pellet surface irregularities. Pits and cavities due to embedded bacteria are seen after biofilm removal (Figures 2.5d–f). In the later samples (days 120 and 200), bacterial biofilm structures were a mix of cells and debris, and showed bacteria embedded as pits on the PE surface and growing in the surface crevices of the PE pellet; irregularities on the PE surface became more prominent at this stage (Figures 2.5g–l). After the removal of the bacterial biofilms, the PE pellets from days 60, 120 and 200 revealed pits and cavities and showed flaking and surface damage on the treatment PE pellets due to bacterial colonization and degradation. This degradation results from the collective action of the bacterial consortium community.

SEM imaging of R. erythropolis pure culture

Decomposition of the PE surface was confirmed by SEM, and consortium community analysis showed that *R. erythropolis* was the predominant strain on the PE surface, so this strain was selected and cultured with PE to evaluate its individual colonization and biodegradation ability. Figures 2.5m–o show SEM micrographs of day 60 PE pellets incubated with *R. erythropolis* alone. Multilayered biofilms were observed on the PE pellet, as observed in the consortium study, however, consortium biofilms were more extensive. Surface erosion was also observed as illustrated in Figure 2.5f. The appearance of the PE surface treated with *R. erythropolis* is similar to that of the consortium study (Figure 2.5o). However, the erosion efficiency cannot be compared since their initial biomass was not normalized.

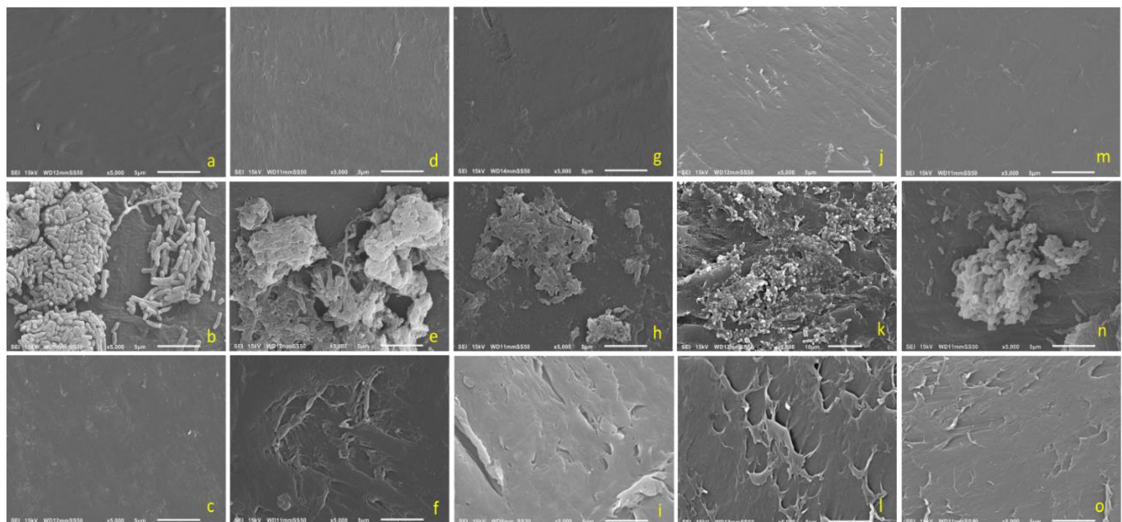


Figure 2.5. SEM micrographs (a–l: consortium samples). a; Day 10 PE pellet control. b; Day 10 PE pellet biofilm showing multi-layered structures. c; Day 10 PE pellet after biofilm removal. d; Day 60 PE pellet control. e; Day 60 PE pellet showing thick biofilm clusters of consortium bacteria. f; Day 60 PE pellet after biofilm removal showing surface damage. g; Day 120 PE pellet control. h; Day 120 PE pellet showing biofilm. i; Day 120 PE pellet after biofilm removal showing surface damage. j; Day 200 PE pellet control. k; Day 200 PE pellet biofilm showing bacteria embedded in valleys and surface damage. l; Day 200 PE pellet after biofilm removal showing surface damage. (m–o: *R. erythropolis* pure culture samples). m; Day 60 PE pellet control. n; Day 60 PE pellet showing *R. erythropolis* biofilm. o; Day 60 PE pellet after *R. erythropolis* biofilm removal showing surface damage.

FTIR analysis of consortium treated PE

It has been previously reported that, upon reacting with microbial exoenzymes (such as laccases and AHs), the PE polymer can be broken down into smaller oligomers, which are taken up by bacterial cells where they are further metabolized via the β-oxidation pathway and mineralized to CO_2 and H_2O (Ghatge *et al.*, 2020; Tao *et al.*, 2023). Moreover, the microbe-mediated hydroxylation of *n*-alkanes produces their corresponding alcohols, which are then oxidized to aldehydes, ketones, and carboxylic acids (Eubeler *et al.*, 2010).

The PE samples used in this study were pellets 3–4 mm in diameter, which were difficult to prepare for FTIR analysis. The obtained FTIR spectra were not sufficiently strong, although several signals supported the biodegradation of PE.

FTIR peaks shows the creation of PE oxidation products by the bacterial enzymes in the initial stages. Day 25 PE treatment samples show peaks corresponding to carbonyl groups (-C=O) with a strong peak at 1647 cm^{-1}; also seen are terminal double bonds in the region of 915-905 cm^{-1} with a peak at 908 cm^{-1} and at 842 cm^{-1} (Figure 2.6). The appearance of carbonyl groups and terminal double bonds (915-905 cm^{-1}) have been reported to be a result of biotical degradation of PE (Adithama *et al.*, 2023; Albertsson *et al.*, 1987; J. Zhang *et al.*, 2020). Also observed are peaks corresponding to O-H stretching (hydroperoxide and alcohol) at 3182 cm^{-1} and 3394 cm^{-1} in Day 25 PE treatment samples (Figure 6) (Joshi *et al.*, 2022; Li *et al.*, 2020; S. S. Yang *et al.*, 2022). Peaks in the 1100 – 1400 cm^{-1} region corresponding to ethers and other –C-O-C groups are also seen in Day 25 PE treatment samples at 1120 cm^{-1},1216 cm^{-1} and 1245 cm^{-1} (Figure 2.6) (Biki *et al.*, 2021; Hou *et al.*, 2022; Kowalczyk *et al.*, 2016). Additionally,

peaks at 1080, 1081 cm^{-1} are seen in Day 25 and Day 200 PE treatment samples, respectively, which correspond to ethers (Biki *et al*., 2021). Peaks observed at Day 25 (842 cm^{-1}, 908 cm^{-1}, 1120 cm^{-1}, 1216 cm^{-1}, 1245 cm^{-1}, 1647 cm^{-1}, 3182 cm^{-1} and 3394 cm^{-1}) have disappeared or shrunk in the later part (Day 200). These PE degradation products (carbonyls, ethers, hydroperoxides and other alcohols) brought about by the bacterial enzymes, being short-chained and amenable for further enzymatic breakdown, are consumed and hence disappear in the later stages.

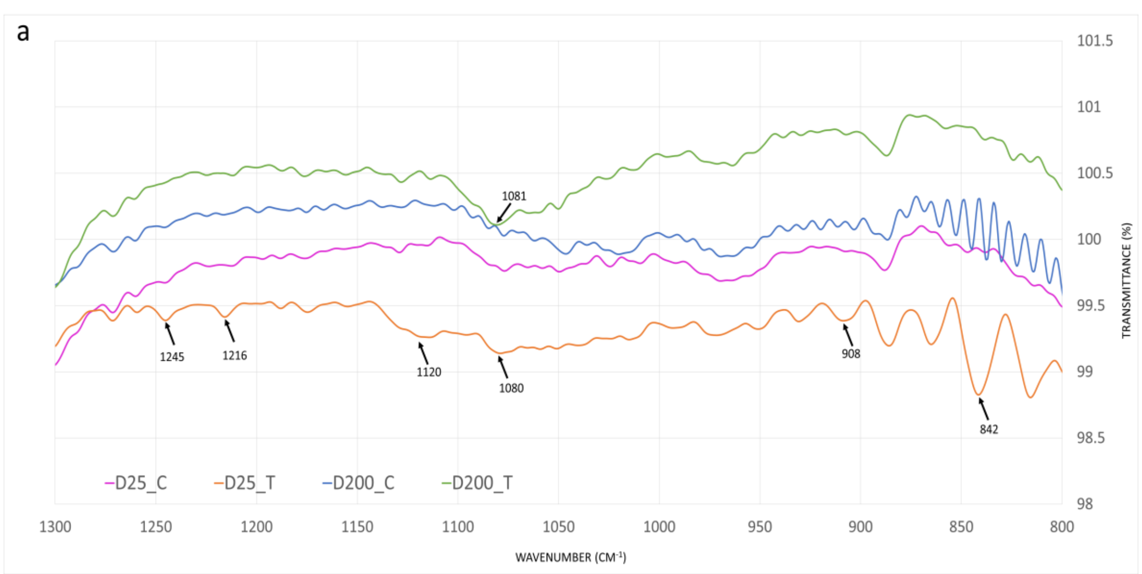
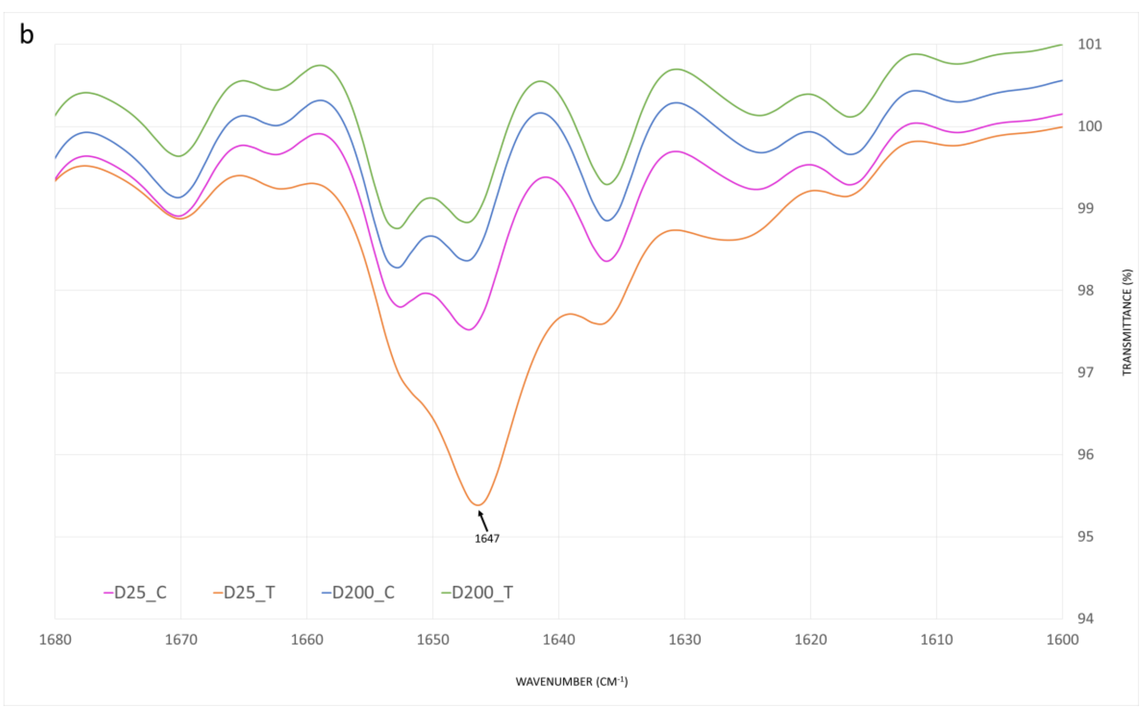


Figure 2.6. FTIR spectra of Day 25 and Day 200 PE treatment samples and their respective controls. Regions shown: (a)1300-800, (b)1680-1600, (c)3600-3100.

Figure 2.6. ***continued***



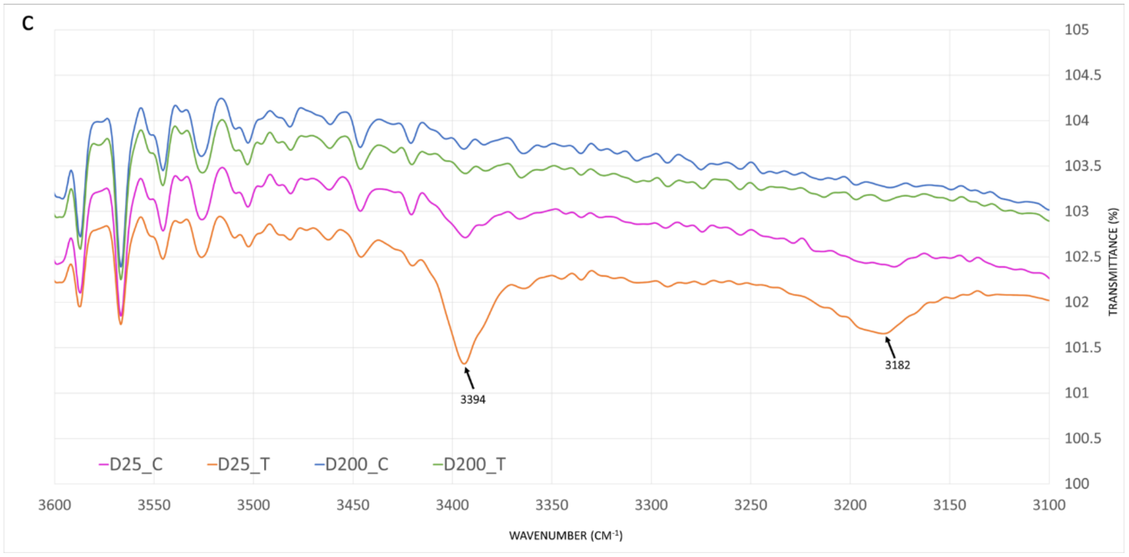


Figure 2.6. FTIR spectra of Day 25 and Day 200 PE treatment samples and their respective controls. Regions shown: (a)1300-800, (b)1680-1600, (c)3600-3100.

2.4 Conclusion

In study I, the consortium strategy was investigated for its applicability in PE degradation. The bacterial consortium community dynamics of both the culture broth and the plastisphere were monitored with NGS analysis. SEM and FTIR were employed to assess the changes in the polymer structure.

R. erythropolis was demonstrated to be a competitive plastisphere species and likely played a major role in the consortium. Although further research is needed to determine the individual contribution of other consortium strains, *S. griseus* which was the only strain that showed extracellular laccase activity, might be important in the overall PE deterioration.

This study highlights the potential applicability of artificial microbial consortia for plastic degradation. A variety of consortium cocktails can be further explored, where bacteria, fungi or a combination of both can be tailored to degrade specific types of plastic polymers. Additionally, this study underscores the potential of the robust PE colonizer, *R. erythropolis*, as a versatile host for genetic engineering studies aimed at PE degradation.

CHAPTER III

STUDY II

3. Genetic engineering strategy for PE biodegradation

3.1 Introduction

Genetic engineering is another approach to overcoming individual bacterial metabolic limitations, and it involves genetically modifying a suitable host bacterium to express all PE-degrading enzymes heterologously. Through this approach, target enzyme expression can be better regulated, and unwanted isozyme expression is less likely, as is often seen in wild-type strains (Arregui *et al*., 2019).

Heterologous expression of potential PE-degrading enzymes in bacterial/fungal hosts has been reported in the literature. For example, Gu *et al.* expressed two fungal laccase isoenzymes in the yeast host *Pichia pastoris* (Gu *et al*., 2014), Mo *et al*. reported the expression and secretion of three laccases in *E. coli* (Mo *et al*., 2022), Whyte *et al*. cloned AH genes from *Rhodococcus* in *E. coli* and *Pseudomonas* (Whyte *et al*., 2002), while Zadjelovic *et al*. expressed an *Alcanivorax* esterase gene in *E. coli* (Zadjelovic *et al*., 2020). *E. coli* is commonly used as the bacterial host in most protein expression studies. In the consortium experiment, however, *Rhodococcus* was found to perform better as a plastisphere bacteria than *E. coli*. *Rhodococcus* has been well-studied for its ability to strongly degrade aromatic and linear hydrocarbons, including polychlorinated biphenyls and dioxins (Kitagawa *et al*., 2001, 2004). In addition to its hydrocarbon degradative ability, it has also been reported to produce secondary metabolites, such as biosurfactants and antibiotics (Inaba *et al*., 2013; Kitagawa &

Tamura, 2008; Philp *et al*., 2002). *Rhodococcus* is easy to culture and grows relatively quickly, and genetic tools, such as plasmid vectors, gene disruption methods, and genome manipulation methods, have been well-studied making *Rhodococcus* an excellent host for gene expression (Kitagawa *et al*., 2013; Kitagawa & Hata, 2023; Saito *et al*., 2019).

In Study II of this dissertation, the genetic engineering approach is explored, considering two key factors: (1) a host capable of effectively colonizing PE and (2) ample extracellular expression of the introduced enzymes by the host. These factors are crucial for enabling effective PE depolymerization by ensuring efficient localized enzymatic degradation.

In the consortium study of this dissertation, *R. erythropolis* exhibited robust colonization of PE, fulfilling criterion (1). Additionally, *R. erythropolis* was observed to produce esterase, lipase, and AH efficiently, though its laccase production was relatively weak. Therefore, laccase was selected as the candidate enzyme for heterologous expression in study II with the goal of meeting criterion (2). The resultant successful *Rhodococcus* laccase recombinants were then used to degrade PE.

3.2 Materials and Methods

Chemicals and media

Guaiacol and commercial *Trametes versicolor* laccase were purchased from Sigma-Aldrich. *n*-Hexadecane, 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic Acid) Diammonium Salt (ABTS) and 2,2,6,6-Tetramethyl-1-piperidinyloxy, Radical

(TEMPO) were purchased from Wako Pure Chemical Industries. Luria–Bertani (LB) medium, W-medium and W-minimal medium (Kitagawa *et al*., 2018) were used as cultivation media. Their specific compositions are as follows: LB medium (per L of medium): 10 g tryptone, 5 g yeast extract, 5 g NaCl, and 15 g agar; W-medium: 1000× buffer stock solution (per L of medium): 10.75 g MgO, 2 g $CaCO_3$, 4.5 g $FeSO_4 \cdot 7H_2O$, 1.44 g $ZnSO_4 \cdot 7H_2O$, 1.12 g $MnSO_4 \cdot 4H_2O$, 0.25 g $CuSO_4 \cdot 5H_2O$, 0.28 g $CuSO_4 \cdot 7H_2O$, 0.06 g H_3BO_3, 51.3 mL conc. HCl; 100× buffer stock solution (1 L): 10 g $MgSO_4 \cdot 7H_2O$, 0.5 g $FeSO_4 \cdot 7H_2O$, 100 mL 1000× buffer; 1× buffer (990 mL): 0.85 g KH_2PO_4, 4.9 g Na_2HPO_4, 0.5 g $(NH_4)_2SO_4$; final W-medium (1 L): 990 mL 1× buffer, 10 mL 100× buffer, supplemented with succinate, sucrose and casamino acids (0.2% w/v each), and thiamine (0.002% w/v). W-minimal medium is W-medium without succinate, sucrose, casamino acids and thiamine.

Extracellular laccase screening in bacteria

Over sixty laboratory bacterial strains were screened for extracellular laccase activity. Test plates were LB agar supplemented with 0.02% guaiacol and $CuSO_4$ at concentrations of 0.1 mM, 0.5 mM, or 1 mM. Bacterial strains were streaked onto the agar plates and incubated at 28°C or 37°C. Laccase oxidizes guaiacol, leading to the creation of a reddish-brown product. A reddish-brown halo around the bacterial colony is indicative of extracellular laccase activity, while intracellular laccase activity is characterized by the cells alone turning reddish-brown (Kiiskinen *et al*., 2004).

Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 3.1. *E. coli* DH5α was used for general cloning and was cultivated in LB medium at 37°C.

Table 3.1. Bacterial strains and plasmids used in this study (Study II)

Strain or plasmid	Relevant characteristic(s)	Reference or origin
Strains		
E. coli DH5α	General cloning	Takara Bio Inc.
Rhodococcus erythropolis JCM 3201	Wild type	JCM
R. erythropolis JCM 3201 strain L-88	Expression host; lysozyme sensitive mutant of strain *R. erythropolis* JCM 3201	Mitani *et al.* 2005
Plasmids		
pNit-QC2	Expression vector for *Rhodococcus sp*., nit promoter (constitutive), Cm^r, Ap^r * (*E. coli-Rhodococcus* shuttle vector)	Nakashima & Tamura 2004
pNit-QC2-EpoA	pNit-QC2 harbouring e*poA*	This study
pNit-QC2-MCO1	pNit-QC2 harbouring *mco1*	This study
pNit-QC2-MCO2	pNit-QC2 harbouring *mco2*	This study
pNit-QC2-TatAC	pNit-QC2 harbouring *tatA*, *tatC*	This study
pNit-QC2-TatAC-T1-EpoA	pNit-QC2-TatAC harbouring *epoA* with C2sig-T1 signal peptide	This study
pNit-QC2-TatAC-T2-EpoA	pNit-QC2-TatAC harbouring *epoA* with C2sig-T2 signal peptide	This study
pNit-QC2-TatAC-T3-EpoA	pNit-QC2-TatAC harbouring *epoA* with C2sig-T3 signal peptide	This study
pNit-QC2-TatAC-T4-EpoA	pNit-QC2-TatAC harbouring *epoA* with C2sig-T4 signal peptide	This study
pNit-QC2-TatAC-T5-EpoA	pNit-QC2-TatAC harbouring *epoA* with C2sig-T5 signal peptide	This study
pNit-QC2-TatAC-T6-EpoA	pNit-QC2-TatAC harbouring *epoA* with C2sig-T6 signal peptide	This study
pNit-QC2-TatAC-T1-MCO1	pNit-QC2-TatAC harbouring *mco1* with C2sig-T1 signal peptide	This study
pNit-QC2-TatAC-T2-MCO1	pNit-QC2-TatAC harbouring *mco1* with C2sig-T2 signal peptide	This study
pNit-QC2-TatAC-T3-MCO1	pNit-QC2-TatAC harbouring *mco1* with C2sig-T3 signal peptide	This study
pNit-QC2-TatAC-T4-MCO1	pNit-QC2-TatAC harbouring *mco1* with C2sig-T4 signal peptide	This study
pNit-QC2-TatAC-T5-MCO1	pNit-QC2-TatAC harbouring *mco1* with C2sig-T5 signal peptide	This study
pNit-QC2-TatAC-T6-MCO1	pNit-QC2-TatAC harbouring *mco1* with C2sig-T6 signal peptide	This study

JCM: Japan Collection of Microorganisms

*Ap^r: ampicillin resistance; Cm^r: chloramphenicol resistance

R. erythropolis L-88 was used as the expression host strain and was cultivated in W-medium at 24°C or 28°C. The antibiotics used for the transformant selection were ampicillin and chloramphenicol for *E. coli* and *R. erythropolis*, respectively. Ampicillin was supplemented to the culture media at the following concentrations: 100 µg/ml for solid media and 50 µg/ml for liquid media. Chloramphenicol was supplemented to the culture media at the following concentrations: 25 µg/ml for solid media and 17 µg/ml for liquid media.

Cloning genes, signal peptides and expression vector

Based on the results of extracellular laccase screening in bacteria, candidate laccase genes were selected for heterologous expression in the *Rhodococcus* host. The selected laccase genes were PCR amplified from the genomes of their respective bacteria and initially cloned with their original signal peptides into the constitutive pNit-QC2 expression vector (Nakashima & Tamura, 2004). These selected laccase genes were cloned into the NdeI and XhoI sites of pNit-QC2, which had been double-digested with NdeI and XhoI restriction enzymes. Additionally, for cases where the recombinant enzyme expression with the original signal peptide was deficient, two optimization steps were performed to enhance overall protein expression: (1) optimizing the signal peptide (SP) and (2) improving protein expression/ secretion capacity. SP optimization: to address potential SP-gene incompatibility issues and improve secretory expression in the host, six modified SP from *R. erythropolis*, linked to abundantly expressed proteins from its proteomics study, were prepared. These six SP, which were also codon optimized for improved recombinant protein expression, were used in this study (Table 3.2) (Saito *et al*., 2019).

Table 3.2. Sequence of *R. erythropolis* signal peptides (in bold) used in this study (Study II) along with their associated protein

Signal peptide	ID	Amino acid sequence
C2sig-T1	RE2895_04660	**MVHQAERKAEHSSREDGVSRRGFLAASVGAAALAGISWAPAGA**VPWGSASTIAPPPGFPSDIPLSQQAYANWS REIMLESVWTATARHSDDVVTLANWAFDNGYTVRAKGTMHGWSPLTVVPGAPSDRVLLVDTMANLNSVVVQHG TPATVTAGAGASIEAILTALEREGLGWANAPAIGELSIAGALAIGAHGATYPAVGETITPGQSYGSLSNLITEITVVAW DEGSNRYALKTFHRSDDEITAFLTHLGRTFVTSVTLQAGENYRLRCQSFTDIPWQELFAAPDSAGRTYESFVEKSGR VEAIWFPFTQTPWLKVWTPTPVKPPESREVSGPYNYFFSDAIPEEVTTPLGMVAQGLQAATPLFGASQFGAVAAGL ALTDTDDLWGWSKDVLFYLRHTTLRVVAGGGAVITKRSNIGRVVHEMTSWLNERMTHYASLGQYPVNMPFEVRL CGVDDSGEVLVDSAGVPDLSAVRPRADRQDWDTAIWMNVVSIPGTAGLPAFLREMERWMVANYSGDYATFRPEW SKGWAFTDQAAYQDDEFLTSTVPATFRAGGDGNWDFALATLDEHDPHRVFSNTFIDRVLPPS
C2sig-T2	RE2895_07550	**MTAQDEKFRLSRRGFMAAGAGAVAATAFAGWTPAYA**VPAGSSGSAGGPVSTLTPPPAFPEGIALYQQAYQNWS KEIMLDAIWTCSPKTPEDVVRLANWGHANGYTIRPRGAMHGWTPLTIVNGAPVDKVILADTTVHLTGVSVNAGG SPATVTAGPGATLDAITTALQAQGLGFANLPAPGVLTIAGCLAVDAHGAALPAEGEAHVPGQTFGSLSNLVTSLTAV VWNGSEYALETYARSDAAIKPLLTHLGRTFLTSVTLQAAPNYRMRCVSHTDIGWQELFGARGASGRTFEKFVREN GRAEAIWYPFTERPWMKVWSLAPTKPPFSREVTGPYNYIFSDNLPEPVTDMIGQINAGNPGIAPAFGQIMYATTVA GLAATFSNDLWGWSKDVQFYIRATTLRLTEGGGAVITSRANIGQVIHDFTQWFNGRMEYYRSIGQFPLNGPVEIRC CGLDQPSDVEVDSAGAPTISAMRPRPDHPEWDTAIWLNVLGVPGTPGMFAFYREMEQWMRNHYNNNDATFRPE WSKGWAFGPDKPYTDAPIITQGLPQTYRDGVPSSDNWDTANAAYNALDPHKVFSNTFLDQLLP
C2sig-T3	RE2895_18810	**MTNINRRSFLTFAGLGAVGAVSLGKPWGLQYAGA**STIPTSGPGTTLESVSTPVSSSGYTRLTAGPGWANIVRSEL AEPKSGREDRRTALASLVQLTDVHIVDAQSPMRFEYVHQITGSAFRPQETLTAHGLISLVRRVNSIGSGPHTSRPFDA VVTTGDNTDNKEFAELDWFLTSLNGGTVVANTGAKDRYEGVQNSGADLYWNPESPMLDMYKKAGFPEIPDFFGA AFTPVSSPGLNTPWYCVFGNHDDSVSGTVPSGIPPLEAMYTGSLKFEVPGSPEQAKQIDIATKFDPSAIPGILSAFTT PPRQVTPDPSRAPFTPRQFIAAHLDPAHTGPGPVGHGFAPDAGETGIGYYSFQIAPGVVGISMDSTNRAGLVDGSLG AAQFQWIENTLRAGSSTYYDAAGSRVTEPRSDTYFVLFSHHTSDTMDNLIPDPENVLEPRLRGSQLLDLLHRFPNV LAWVNGHTHENKITPWPGATPEQGFWEINTASHIDYPQLGRIIEIADNHDGTVSLLATLFEAESPNSVEYSDKSAAG LASLYRELSFNDIHRDPKLTGTGVDQNVELLVHTGR

C2sig-T4	RE2895_30060	**MQRRDMQRRDFFKAAGAGAAVAAATPLMSTSTSHA**TPAPLRALPMGADHYRALVPELFAPSPTPPEHSEAIVIG SGFGASATALRLAQSGTQVTILERGLRWPHDPQREIHTSDMLADGRGVFRRTSFTNLTGLPVACDYFSGVLDATDY QHISVWRGAAVGGGSIIFTGVMIAPERRFFDAVFGNSLDYDEMASTWYPKVRQMLRLSPLPEDIYQTPNFGHSRR WDQDARRAGFDPQRIDGIWNWDVVRSELDGRTRASATVGDSNMGNSNGAKFDLTQNYIPAAEATGRATVCYGH QVLAISRERDGRYVVDVESTDPTGGVLARKTLTCDRLFLGAGSIGTSELLVRAQATGALPNLNEHVGKGWGTNGD AGMVRSFGFSDGTAQAAPSASRIVDESGMPLSLENWYVPGLPLNIGMLGTLGMTLDSQRADFAYDGGSDRVVLN WPKNGNDATVEALRAVQNKMAFAGTTLPSALPFAKDVNSSFTAHPLGGAVLGKATDGYGRVKGYDGLYVMDGA AIPGSTGTVNPSLTITALAERNIAQIIKSGR
C2sig-T5	RE2895_38460	**MEYTLARNAAEDLTKGLGRRGFMRAVAALGAGVGVAGVASG**CAHSNASPAGSSSSALPFADGIPILQPGSGNV SGDHYLSSDPSDVMWGYVPNIHTREVMRMKSGQTVTIDALSHEGILEDQGRNPLEYFGSKGVSEKDVLEDAIAVA AEYNRTERNFDKDGPHVVTGPVFVEGAQPGDVLKIETLEAIPRVPYGVVSSRHGKGALAVTADGTAPAGITLDEV MPPVATDGRATKDPLKYGGVSTFTAIEDGKGMMPFGQSKVRFPLRPFMGMMGVAYSSDADPTSPSSNSIPPTLGGG NIDIRHLGVGSTFYLPVFAEGALFYVGDPHMAMGDGEAALTAMEGSLRGTFRLSVCKKDSGDAPSVAFGYPFAET EEAWIPIGLSDPNGSVDGQNSDLNGAMRRAVVNALDFLEHDRGMDRATAYAYLSAAADFTVSQVVDRTVGVHGQ IVKSHFE
C2sig-T6	RE2895_48670	**MSIEVSRRSVLIGGSVVAGTALLGTPARA**SVPTSAGIPDLDVHVIVVDGMRPDELRSELTPTLTGLAAGGIHYPDA SAITIAETLPNHTAMMTGVLPARSGVPANSVYDPAIDKKRDLDRPSDLQASTVLDRVRTELGLTTASVLSKRYLHGL FGDRASLVWDPQPLVPGTEHAPDNFTIDALIRIVGGNSPRLSFTNLGDVDRVGHLDLSGPSIRIARTAALQNTDNQV RRFVDFLHDTGRWNRSILIVLADHSMDWSEPARLIGLDRPLNADPLLAGKIRIAQNGGADLIYFIGPDGERAEAVSR IQQIVDGVDGVESSHLPAEFDLGTNAGDVVAFCAQGWRFSDPTPLSNPIPGNHGHFVTLPIPFFLSGGHPALDGGRK IDTQARTIDVAPTVAALLGLGAPAGGWDGVARTTGVSLNV

The six *Rhodococcus* SP were PCR amplified from the genome of *R. erythropolis* and were prepared as the cassette. The SP forward primers contained the overhang sequences of the cloning vector, while the SP reverse primers contained a short linker sequence. This linker sequence, which corresponds to Ser-Ser-Gly-Gly tetrapeptide, was added between the SP and the laccase gene to facilitate the preparation of the SP-gene library for the various SP-gene combinations.

The laccase genes (without their original native SP) were PCR amplified with a new set of primers to facilitate ligation with the signal peptide cassette. For the second set of laccase gene amplification, the forward primers contained the linker sequence, and the reverse primers contained overhang sequences of the cloning vector.

Improving protein expression/ secretion capacity: the expression vector was modified to include additional protein transporter elements to balance the protein production and secretion rate. Based on the study by Xie *et al*., which demonstrated that additional protein transporter components improved protein overexpression, a similar modification was implemented in this study (Xie *et al*., 2019).

The protein transporter genes *tatA* (RE2895_30310) and *tatC* (RE2895_30300) were PCR amplified from *R. erythropolis* along with their RBS sequences and cloned into the XhoI and SpeI sites of pNit-QC2, yielding pNit-QC2-TatAC. Subsequently, the C2sig-T1-T6 SP-laccase fragments were cloned into the NdeI and XhoI sites of pNit-QC2-TatAC, which had been double-digested with NdeI and XhoI restriction enzymes.

The PCR primers used in this study are listed in Table 3.3.

Table 3.3. Primers used in this study (Study II)

Origin	Gene ID	Forward primer	Reverse primer	Feature amplified
S. griseus NBRC 13350	SGR_RS04945	AAGGAGATATACATATGGACCGAAGGACCTTCAGC	GATGGTGATGCTCGAGTCAGTGCTGGTGCTCCGC	(gene) *epoA* (with OGSP*)
S. jumonjinensis JCM 4947	FF041_32455	AAGGAGATATACATATGCTGGACAGACGACGCATG	GATGGTGATGCTCGAGCTATGGAACGATCTCCATC	(gene) *mco1* (with OGSP)
S. jumonjinensis JCM 4947	FF041_27680	AAGGAGATATACATATGCGTACGCACATCACACGC	GATGGTGATGCTCGAGCTACTTCCGATAGCCGAG	(gene) *mco2* (with OGSP)
S. griseus NBRC 13350	SGR_RS04945	TCGAGCGGTGGAGCCGAGAATCCGCCGCGTAC	CTGAACGTGACTCGAGTCAGTGCTCGTGTTCGTGTG	(gene) *epoA* + SSGG linker
S. jumonjinensis JCM 4947	FF041_32455	TCGAGCGGTGGAGGGGGCTCCGGGAACACCGC	CTGAACGTGACTCGAGCTATGGAACGATCTCCATCT	(gene) *mco1* + SSGG linker
R. erythropolis JCM 2895	RE2895_04660	AAGGAGATATACATATGGTGCACCAGGCGGAGCGGAAGGCGGAACACAGCTCGAGAGAAGACGGTG	TCCACCGCTCGAGCCCCAGGGAACAGCTCCCG	(SP) C2sig-T1 + SSGG linker
R. erythropolis JCM 2895	RE2895_07550	AAGGAGATATACATATGACCGCCCAAGACGAGAAGTTCCGCCTGTCCCGACGAGGTTTCATGGCCG	TCCACCGCTCGAGCCGGCGGGGACGGCGTAGG	(SP) C2sig-T2 + SSGG linker
R. erythropolis JCM 2895	RE2895_18810	AAGGAGATATACATATGACCAACATCAACCGCCGCAGCTTCCTCACCTTCGCCGGACTCGGCGCCG	TCCACCGCTCGACGGAATGGTCGACGCGCCTG	(SP) C2sig-T3 + SSGG linker
R. erythropolis JCM 2895	RE2895_30060	AAGGAGATATACATATGCAGCGGAGGGACATGCAGCGGCGCGACTTCTTCAAAGCCGCCGGGGCGG	TCCACCGCTCGATGGTGCGGGGGTGGCGTGGC	(SP) C2sig-T4 + SSGG linker
R. erythropolis JCM 2895	RE2895_38460	AAGGAGATATACATATGGAGTACACCCTCGCCCGCAACGCCGCCGAAGACCTGACGAAAGGTCTTG	TCCACCGCTCGATCCAGCCGGTGACGCGTTCG	(SP) C2sig-T5 + SSGG linker
R. erythropolis JCM 2895	RE2895_48670	AAGGAGATATACATATGAGCATCGAAGTCAGCCGCCGGTCCGTCCTGATCGGTGGAAGTGTTGTCG	TCCACCGCTCGAGGTGGGAACACTCGCGCGAG	(SP) C2sig-T6 + SSGG linker
R. erythropolis JCM 2895	RE2895_30310	GCTTAGATCTCTCGAGTCACGTTCAGTGAGTTCGC	-	(gene) *tatA*, *tatC* with RBS
R. erythropolis JCM 2896	RE2895_30300	-	CGGTGGGTCGACTAGTAGGGTTTCACACGCCGCC	(gene) *tatA*, *tatC* with RBS

*OGSP = original native *Streptomyces* signal peptide

The In-Fusion HD cloning kit (Takara, Japan) was used to construct the final ligated vectors with *E. coli*. Plasmids of the various SP-gene combinations were extracted from their respective *E. coli* clones, and their size and sequence were verified. The verified plasmids were then introduced into the *Rhodococcus* host via electroporation using the Gene Pulser Xcell electroporation system (Bio-Rad).

Expression of the recombinant laccases in *R. erythropolis*

The recombinant *R. erythropolis* clones were screened for secretory laccase activity on W-medium agar test plates supplemented with 0.02% guaiacol and 0.5 mM $CuSO_4$. The clones were streaked onto the agar plates and incubated at 28°C.

Laccase enzyme assay

The successful recombinant clones for each SP-putative laccase gene combination were cultured in 10 mL W-medium containing 17 µg/mL chloramphenicol at 28°C and 150 rpm. A blank vector clone served as the control. The bacteria were cultured until they reached an OD_{600} of 3.5-4. At this point, 0.5 mM $CuSO_4$ was added, and cultivation was continued under the same conditions until an OD_{600} of approximately 5 was reached. The cells were then centrifuged at 3000 rpm for 20 minutes at 4°C and the recovered supernatant was filtered through a 0.2 µm Minisart filter (Sartorius) and then concentrated using 10 kDa MWCO Amicon Ultra filters (Merck Millipore) to achieve a 90-100x concentrated crude supernatant. The protein content of the concentrated crude supernatant was measured using the Qubit protein assay on a Qubit 4 fluorometer (Thermo Fisher Scientific).

Laccase activity was determined using a modified version of a previously described method (Shin & Lee, 2000). The assay involved monitoring the oxidation of ABTS by the enzyme at 420 nm using a UV-vis spectrophotometer (BioSpectrometer basic, Eppendorf). For the assay, 0.03 μg/uL of the concentrated crude supernatant was used. The reaction mixture consisted of 100 mM sodium acetate buffer (pH 5.2) and 3 mM ABTS (final conc.), to which an equal volume of the concentrated crude supernatant was then added. The reaction was carried out at 28°C. Laccase activity was calculated using the formula defined by E. Baltierra-Trejo *et al*. One unit of enzyme activity is defined as the amount of enzyme that oxidizes 1 μmol of the substrate ABTS per minute, using a molar extinction coefficient (ε) of 36,000 $M^{-1}cm^{-1}$ (Baltierra-Trejo *et al*., 2015).

SDS-PAGE

Selected recombinant clones and a blank vector clone were precultured in 10 mL W-medium containing 17 μg/mL chloramphenicol at 28°C and 150 rpm overnight. 10 mL precultures were transferred to 100 mL of the same fresh media containing 0.5 mM $CuSO_4$ and cultivated at same culture conditions for 30 hours. Cell mass from 10 ml volumes of the grown cultures was used to extract total and soluble/ insoluble protein fractions each. In brief, for total protein extraction, cell pellets were resuspended in 100 mM sodium phosphate buffer containing 8 M urea. For soluble protein extraction, cell pellets were resuspended in 50 mM sodium phosphate buffer containing 300 mM NaCl. Cell disruption was performed using lysing matrix B tubes (MP Biomedicals) with the Multi-beads Shocker instrument (Yasui Kikai, Japan). The lysed cells were then centrifuged at 15,000 rpm for 15 minutes to recover the supernatant, with total protein extraction performed at 20°C and soluble protein extraction at 4°C. To obtain the

insoluble protein fraction, the cell pellets remaining after soluble protein extraction were resuspended in the urea buffer, mixed thoroughly, and centrifuged again at 15,000 rpm for 15 minutes at 20°C. 100 mL filtered culture supernatant was concentrated using 10 kDa MWCO Amicon Ultra filters (Merck Millipore) to a final volume of 150 µL.

The protein content of both the total cellular protein fraction and the concentrated crude supernatant fraction was measured using the Qubit protein assay on a Qubit 4 fluorometer (Thermo Fisher Scientific).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed to visualize the recombinant proteins in the different protein fractions. 10 µg/µL of total cellular protein fraction and 15 µg/uL of concentrated crude supernatant fractions were loaded onto a 5-20 % m-PAGEL gel (ATTO) and visualised by Coomassie Brilliant Blue staining.

Application of *R. erythropolis* laccase recombinants in PE biodegradation

Selected recombinant laccase clones and a blank vector clone were precultured in 12 mL W-minimal medium containing 17 µg/mL chloramphenicol and 0.1% *n*-hexadecane and cultivated at 28°C and 150 rpm until OD_{600} of 2-3 was reached. Subsequently, 12 mL of blank vector preculture and 12 mL of a mixture of recombinant laccase clones precultures (made by mixing equal volumes of the selected recombinant laccase clones) were each transferred to fresh 100 mL of W-minimal medium containing 17 µg/mL chloramphenicol, 0.05% *n*-hexadecane and 0.002% w/v thiamine. Liquid hydrocarbons like *n*-hexadecane can enhance bacterial accessibility to PE and also boost bacterial colonization. Therefore, *n*-hexadecane was added to the culture media (Gilan *et al*., 2004; Montazer *et al*., 2020).

PE pellets were treated in a 70% ethanol bath for 15 min and dried before adding them to the culture flasks. Twenty PE pellets were added per flask. The recombinant laccase clones treatment (i.e., mixture of selected recombinant laccase clones) was conducted in triplicate (three flasks: R1, R2, and R3), alongside a blank vector clone treatment. Additionally, a flask without any bacterial inoculum was used as a control. The flasks were maintained at 28°C on a rotary shaker at 90 rpm for 60 days. After 10 and 30 days, 17 μg/mL chloramphenicol, 0.25 mM $CuSO_4$ and 100 μM of the laccase mediator, TEMPO, were added to the flasks. Additionally, after 30 days, half of the spent medium in each culture flask was replaced with fresh W-minimal medium.

Scanning electron microscopy (SEM)

The PE samples for SEM observation were collected on days 10, 30, and 60 from the recombinant laccase clones treatment flasks, blank vector clone treatment flask and control flask to assess the bacterial colonization and surface degradation. For SEM visualization and imaging, PE samples with biofilms, PE samples after biofilm removal, and respective control samples were obtained. For biofilm removal, the PE samples were treated with a lysozyme solution (2 mg/mL) and proteinase K (final concentration, 1 mg/mL) in STE buffer (0.1 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0), followed by treatment with 2% SDS for 3–4 hours at 37°C. Afterward, the samples were washed thrice with warm, sterile distilled water. SEM samples were fixed with 2% (w/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 1 h at room temperature. Stepwise dehydration was performed using a graded ethanol series (50%, 75%, 90%, 95%, and 100%) for 15 min each, followed by substitution with dehydrated tert-butyl alcohol. The samples were then freeze-dried using a VFD-21S t-BuOH freeze

dryer (Vacuum Device Co., Ltd., Japan). Next, the prepared SEM samples were mounted on aluminium stubs using carbon paste (Pelco Colloidal Graphite, Ted Pella, Inc.), coated with gold using a DII-29010SCTR Smart Coater, and then visualized and imaged using a JSM-6010PLUS/LV SEM at 15 kV.

3.3 Results & Discussion

Extracellular laccase screening in bacteria

Secretory laccase production in the selected host, *R. erythropolis*, was poor despite its genome information showing the presence of a laccase domain protein and two multicopper oxidase proteins. Therefore, the enzyme laccase was selected for heterologous expression to enhance the PE degradation ability of the *Rhodococcus* host by compensating for its inadequate laccase activity.

Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2), a type of multicopper oxidase (MCO), are enzymes known for oxidizing a wide range of substrates using oxygen as an electron acceptor. Also referred to as polyphenol oxidases, urushiol oxidases, or p-diphenol oxidases, laccases play essential roles in various biological processes (Arregui *et al*., 2019; Janusz *et al*., 2020).

Copper-containing proteins are prevalent in nature and are vital for dioxygen transport and activation, and electron transfers (Crichton & Pierre, 2001). Among these proteins, MCOs are particularly notable for oxidizing substrates by reducing O_2 to H_2O, without the release of harmful reactive oxygen species (ROS) (Komori & Higuchi, 2015). As members of the MCO family, laccases are highly versatile enzymes that can

oxidize a wide range of phenolic and non-phenolic molecules due to their broad substrate specificity (Yaropolov *et al*., 1994). These enzymes are widely distributed across nature and can be found in fungi, bacteria, plants, and insects (Arora & Gill, 2000; Bai *et al*., 2023; Endo *et al*., 2002; Hattori *et al*., 2005).

The broad substrate range of laccases makes them promising candidates for several applications, including industrial effluent treatment, bio-bleaching, and bioremediation (Fillat *et al*., 2010; Sondhi *et al*., 2018). Notably, laccases are considered essential enzymes for the depolymerization of PE, highlighting their importance in PE degradation. Due to the high molecular weight and size of the PE polymer, it is inaccessible for bacteria to use as a carbon source in its polymer form. Therefore, PE depolymerizing enzymes, like laccases, are essential for breaking down the large PE polymer into smaller oligomeric fragments, which can then be taken up and metabolized by the microbial cells (Amobonye *et al*., 2021; Cai *et al*., 2023; Montazer *et al*., 2020).

To select for the candidate laccase genes for introducing into *Rhodococcus*, several bacteria were screened on guaiacol supplemented agar plates for extracellular laccase activity, the results of which are shown in Table 3.4.

Table 3.4. Bacterial laccase plate assay screening results

Bacterial strain	Laccase plate assay result
Actinomadura atramentaria NBRC 14695T	-, (int-)
Actinomadura harenae JCM 32659T	-, (int+)
Actinoplanes missouriensis NBRC 102363	-, (int+)
Actinosynnema mirum NBRC 14064T	-, (int+)
Allokutzneria albata JCM 9917T	-, (int+)
Amycolatopsis alba NBRC 15602T	-, (int+)
Amycolatopsis mediterranei JCM 4789T	-, (int+)

Amycolatopsis methanolica NBRC 15065T	-, (int+)
Amycolicicoccus subflavus NBRC 109087	-, (int+)
Arthrobacter crystallopoietes JCM 2522T	-, (int+)
Bacillus licheniformis JCM 2505T	-, (int-)
Bacillus megaterium NBRC 15308T	-, (int-)
Bacillus subtilis JCM 1465T	-, (int-)
Bacillus subtilis subsp. subtilis NBRC 111470	-, (int-)
Bacillus vallismortis JCM 12234T	-, (int+)
Brevibacillus borstelensis NBRC 15714	-, (int+)
Caulobacter segnis JCM 7823T	-, (int-)
Comamonas testosteroni NBRC 109938	-, (int+)
Corynebacterium halotolerans JCM 12676T	-, (int+)
Cupriavidus necator JCM 20644	-, (int+)
Deinococcus grandis JCM 6269T	-, (int-)
Deinococcus proteolyticus NBRC 101906	-, (int+)
E. coli K-12	-, (int+)
Enterobacter asburiae NBRC 109912	-, (int+)
Hoyosella subflava JCM 17490T	-, (int+)
Hyphomonas neptunium NBRC 14232	-, (int+)
Hyphomonas polymorpha NBRC 102482	-, (int+)
Kitasatospora setae NBRC 14216	-, (int-)
Kribbella flavida NBRC 14399	-, (int+)
Kutzneria albida NBRC 13901	-, (int+)
Methylobacterium currus JCM 32670T	-, (int-)
Methylobacterium frigidaeris JCM 32048T	-, (int-)
Methylobacterium platani JCM 14648T	-, (int-)
Methylobacterium radiotolerans NBRC 15690	-, (int+)
Mycolicibacterium aurum JCM 6366T	-, (int+)
Mycolicibacterium austroafricanum JCM 13017	-, (int+)
Mycolicibacterium chitae JCM 12403T	-, (int+)
Mycolicibacterium thermoresistibile JCM 6362T	+
Nakamurella multipartita NBRC 105858	-, (int+)
Nocardiopsis dassonvillei subsp. dassonvillei NBRC 14626	-, (int+)
Novosphingobium tardaugens JCM 11434T	-, (int-)
Paenibacillus chitinolyticus JCM 12162T	-, (int-)
Paenibacillus thiaminolyticus JCM 8360T	-, (int-)
Pseudomonas fluorescens JCM 5963T	-, (int+)
Pseudomonas mandelii JCM 21619T	-, (int+)
Pseudomonas putida JCM6157	-, (int+)
Pseudonocardia autotrophica JCM 4348T	-, (int+)
Pseudonocardia dioxanivorans JCM 13855T	-, (int+)
Rhodococcus erythropolis PR4 NBRC 100887	-, (int+)
Rhodococcus jostii RHA1	-, (int+)
Rhodococcus opacus NBRC 108011 strain B4	-, (int+)
Rhodococcus rhodochrous JCM 3202T	-, (int+)

Rhodococcus ruber JCM 3205T	-, (int+)
Rhodococcus wratislaviensis NBRC 100605T	-, (int+)
Rhodococcus zopfii DSM 44189	-, (int+)
Saccharothrix espanaensis NBRC 15066	-, (int+)
Sphingomonas paucimobilis NBRC 13935	-, (int+)
Sphingomonas wittichii JCM 15750T	-, (int+)
Sphingopyxis macrogoltabida JCM 10192T	-, (int+)
Streptomyces albofaciens JCM 4342T	-, (int+)
Streptomyces albulus JCM 5054	-, (int+)
Streptomyces griseus NBRC 13350	+
Streptomyces jumonjinensis JCM 4947	+
Streptomyces noursei JCM 4922T	-, (int+)
Streptomyces rimosus JCM 4073T	-, (int+)
Streptosporangium roseum NBRC 3776	-, (int+)
Tsukamurella paurometabola NBRC 16120	-, (int+)

JCM: Japan Collection of Microorganisms

NBRC: Biological Resource Center, NITE (Japan)

DSM: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

+: extracellular activity positive, –: extracellular activity negative

(int+): intracellular activity positive, (int-): intracellular activity negative

Of the bacterial strains screened for secretory laccases, only three *Actinobacterial* strains, *Mycolicibacterium thermoresistibile* (JCM 6362T), *Streptomyces griseus* (NBRC 13350) and *Streptomyces jumonjinensis* (JCM 4947) produced a reddish-brown halo around the colony, indicating extracellular laccase activity. Most of the tested bacterial strains displayed reddish-brown colonies with no halo, suggesting intracellular laccase activity, and a few showed no activity at all (Table 3.4).

Studies on bacterial laccases are not as extensive as those on fungal laccases but have recently gained momentum due to their advantageous biochemical properties, such as having a higher thermostability when compared to fungal laccases. While most

fungal laccases are secretory, most bacterial laccases are typically localised intracellularly (Guan *et al*., 2018; Janusz *et al*., 2020). This intracellular localization of laccase enzyme was demonstrated through the plate assay results in this study, where most of the test strains showed intracellular laccase activity. For the bacterial strains possessing laccase genes that showed no laccase activity, neither secretory nor intracellular, on the test plates, it may be possible that the substrate specificity and inducing conditions may dictate the enzyme expression. Nonetheless, only three of the tested strains, *M. thermoresistibile*, *S. griseus* and *S. jumonjinensis,* exhibited clear secretory activity, which was visibly evident on the test plates.

The genome information of these three *Actinobacteria* was available, and it revealed the putative laccase genes possessed by these strains. The genome of *S. griseus* (NBRC 13350) has three genes encoding a laccase (SGR_RS02120), an EpoA copper oxidase (SGR_RS04945) and a multicopper oxidase family protein (SGR_RS26230); *S. jumonjinensis* (JCM 4947) has two multicopper oxidase family proteins (FF041_32455 and FF041_27680) and, *M. thermoresistibile* (JCM 6362T) has a multi-copper oxidoreductase (SAMEA4412656_01888).

Heterologous expression of candidate laccase genes in *R. erythropolis*

Based on the laccase plate assay results, three extracellular putative laccase genes from *Streptomyces spp.* were selected: *epoA* (SGR_RS04945) from *S. griseus* and two multicopper oxidase family protein genes, designated in this study as *mco1* (FF041_32455) and *mco2* (FF041_27680) from *S. jumonjinensis*.

These three putative laccase genes with their original native *Streptomyces* SP were initially cloned from their respective genomes and introduced into the pNit-QC2 expression vector (Figure 3.1).

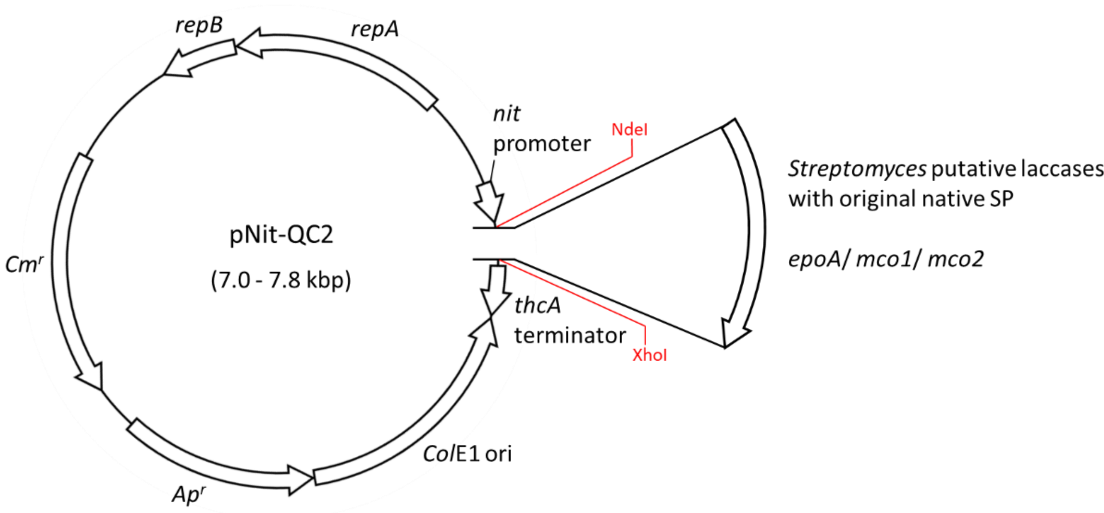


Figure 3.1. Schematic map of pNit-QC2 vector with the *Streptomyces* putative laccases, EpoA, MCO1 and MCO2, cloned with their original native signal peptides

According to the web prediction tool SignalP 6.0, all three selected *Streptomyces* putative laccase genes were found to have a twin-arginine translocation (Tat) SP (Teufel *et al*., 2022). Since these putative laccase genes were associated with the Tat protein transport system in their native bacteria, the *Rhodococcus* SP selected as replacements were also of the Tat type. Moreover, these *Rhodococcus* SP were codon optimized for improving the recombinant protein expression as reported by Saito *et al*., where protein expression was enhanced by producing synonymous mutations in the 33 head

nucleotides (i.e. 11 codons) with replacement of rare codons so as to have weaker mRNA secondary structures in the head nucleotides to have improved translational efficiency (Saito *et al*., 2019). Furthermore, the additional protein transporter elements added to the vector, *tatA* and *tatC*, also belonged to the Tat secretion system of *R.* erythropolis. The expression vector pNit-QC2-TatAC was thus assembled, and the various SP-gene combinations were prepared using the *Rhodococcus* C2sig T1-T6 SP and the *Streptomyces* putative laccases EpoA and MCO1 (Figure 3.2).

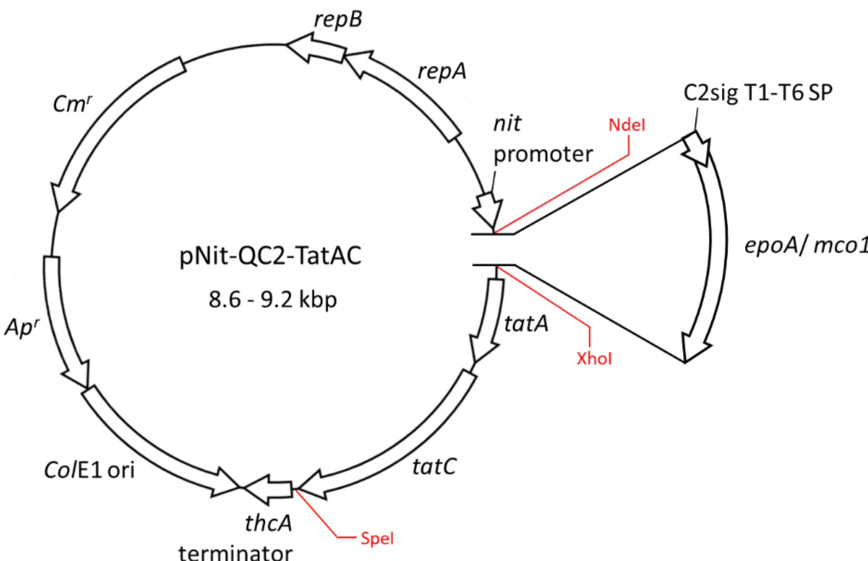


Figure 3.2. Schematic map of pNit-QC2-TatAC vector with the *Rhodococcus* C2sig T1-T6 signal peptides conjugated to the *Streptomyces* putative laccases EpoA and MCO1

The laccase plate assay results for the recombinant clones revealed variations in the levels of secretory protein expression among different SP-gene combinations (Figure

3.3). The same SP showed differences in protein secretion levels when conjugated to the different laccase genes. The initial laccase plate assays of the recombinants with the original *Streptomyces* SP (OGSP) showed that only the OGSP MCO2 clones exhibited good secretory recombinant laccase activity (Figure 3.3).

Gene / Signal peptide	OGSP	C2sig-T1	C2sig-T2	C2sig-T3	C2sig-T4	C2sig-T5	C2sig-T6
EpoA		NS		NS			
MCO1			NS				
MCO2		(-)	(-)	(-)	(-)	(-)	(-)
Blank vector							

Figure 3.3. Laccase plate assay showing the recombinant putative laccase activity of the different SP-gene combination *Rhodococcus* recombinant clones, along with the blank vector control clone. (OGSP: original native *Streptomyces* SP, C2sig-T1-T6: *Rhodococcus* SP, NS: not successful in cloning)

The secretion of EpoA and MCO1 was subsequently improved using the *Rhodococcus* SP in the modified TatAC vector (Figure 3.3). Notably, for the EpoA and

MCO1 recombinant clones, having the modified *Rhodococcus* SP-gene with the *tatA-tatC* added vector enhanced the secretory expression of these enzymes compared to their original SP-gene combinations for most of the SP-gene combinations. Among the EpoA recombinants, clones with C2sig-T4 and C2sig-T6 SP demonstrated strong guaiacol oxidation on the test agar plates. Among the MCO1 recombinants, clones with C2sig-T3 and C2sig-T5 SP showed strong secretory recombinant laccase activity. In some clones such as C2sig-T5 EpoA, OGSP MCO1 and C2sig-T4 MCO1, the recombinant protein secretion was not clearly visualized on the test agar plates. However, their activity was confirmed in the liquid media. Some SP-gene combinations like C2sig-T6 MCO1 did not show any secretory recombinant laccase activity, while some combinations like C2sig-T1, T3 EpoA and C2sig-T2 MCO1 performed poorly to produce any viable recombinants.

The significance of SP-gene compatibility has been deemed essential in the literature. Studies have demonstrated improved protein expression by modifying certain genetic elements such as signal peptides (SP), expression vectors, and cultivation conditions. For instance, a study by Mo *et al*. employed Novel Signal Peptide 4 (NSP4) in combination with optimized culture conditions to enhance recombinant protein expression in *E. coli* (Mo *et al*., 2022). Similarly, Xie *et al*. manipulated the expression system at various levels of the protein secretion process, including transcriptional, translational, secretory, and protein folding levels, to overexpress the recombinant enzyme in their host bacterium (Xie *et al*., 2019). Saito *et al.* reported a codon optimization method to enhance the recombinant protein expression (Saito *et al*., 2019).

Gu *et al*. reported the successful expression of two laccase isoenzymes in yeast by attaching an additional ten-amino-acid tag at the N-terminus (Gu *et al*., 2014).

Laccase enzyme assay

ABTS was used as the substrate to measure the secreted recombinant laccases in the culture supernatant of the different SP-gene clones. In the presence of oxygen, laccase oxidizes the colourless ABTS to its green-coloured ABTS radicals (More *et al*., 2011). Figure 3.4 shows the extracellular recombinant putative laccase activity for the various SP-gene *Rhodococcus* recombinant clones.

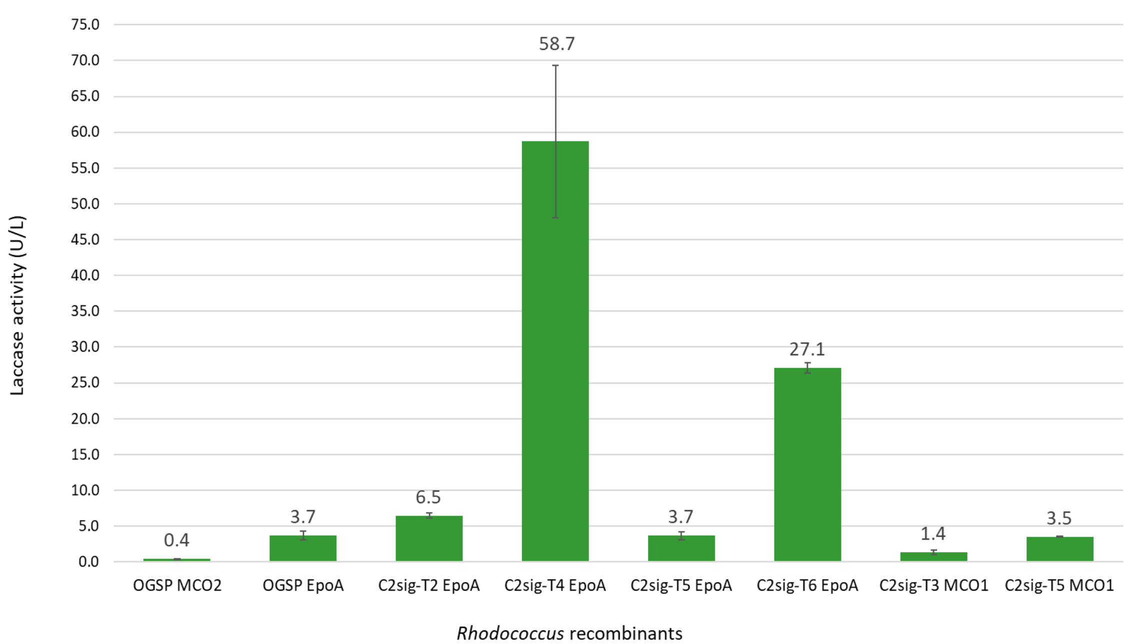


Figure 3.4. Laccase enzyme activity assay of the concentrated crude supernatants from the different SP-gene combination *Rhodococcus* recombinant clones. The assays were performed in 100 mM sodium acetate buffer (pH 5.2) containing 3 mM ABTS. (Assay was performed in triplicates; error bars represent the standard deviation of the mean)

Among all the recombinants, the C2sig-T4 EpoA clone exhibited the highest secretory recombinant enzyme activity at 58.7 U/L with ABTS. (Recombinants such as OGSP MCO1, C2sig-T1 MCO1, C2sig-T4 MCO1) showed very weak activity with ABTS, hence not included in the graph) (Figure 3.4).

EpoA laccase was observed to have a stronger preference for ABTS compared to MCO1 and MCO2 laccases, as EpoA was able to readily oxidase ABTS, producing an intense green colour. *S. jumonjinensis* putative laccases, MCO1 and MCO2 did not show activity as rapidly or intensely as EpoA with ABTS. However, they showed intense reddish-brown discolouration with guaiacol, as observed in the plate assays (Figure 3.3). Substrate specificity, therefore, plays a role here. For instance, Zampolli *et al*. demonstrated the substrate specificity of two laccase-like multicopper oxidases (LMCOs) that showed the highest activity with the laccase substrate 2,6-dimethoxyphenol (2,6-DMP) in comparison to ABTS and guaiacol (Zampolli *et al*., 2023).

The enzyme-substrate preferences are therefore to be considered, and further characterization of the *Streptomyces* EpoA, MCO1 and MCO2 putative laccases regarding their substrate specificity with different compounds needs to be explored.

In conclusion, all three putative laccases, EpoA, MCO1 and MCO2, were able to oxidize the laccase substrates guaiacol and ABTS, with EpoA showing the highest activity and preference with the non-phenolic laccase substrate, ABTS. Among the different SP-gene combinations, the recombinant clones OGSP MCO2, C2sig-T5 MCO1 and C2Sig-T4 EpoA demonstrated the best secretory recombinant laccase activity.

SDS-PAGE

Figure 3.5 shows the SDS-PAGE analysis of the total, supernatant, soluble and insoluble protein fractions of the *Rhodococcus* recombinant laccase clones: OGSP MCO2, C2sig-T4 EpoA, C2sig-T5 MCO1 along with the blank vector clone.

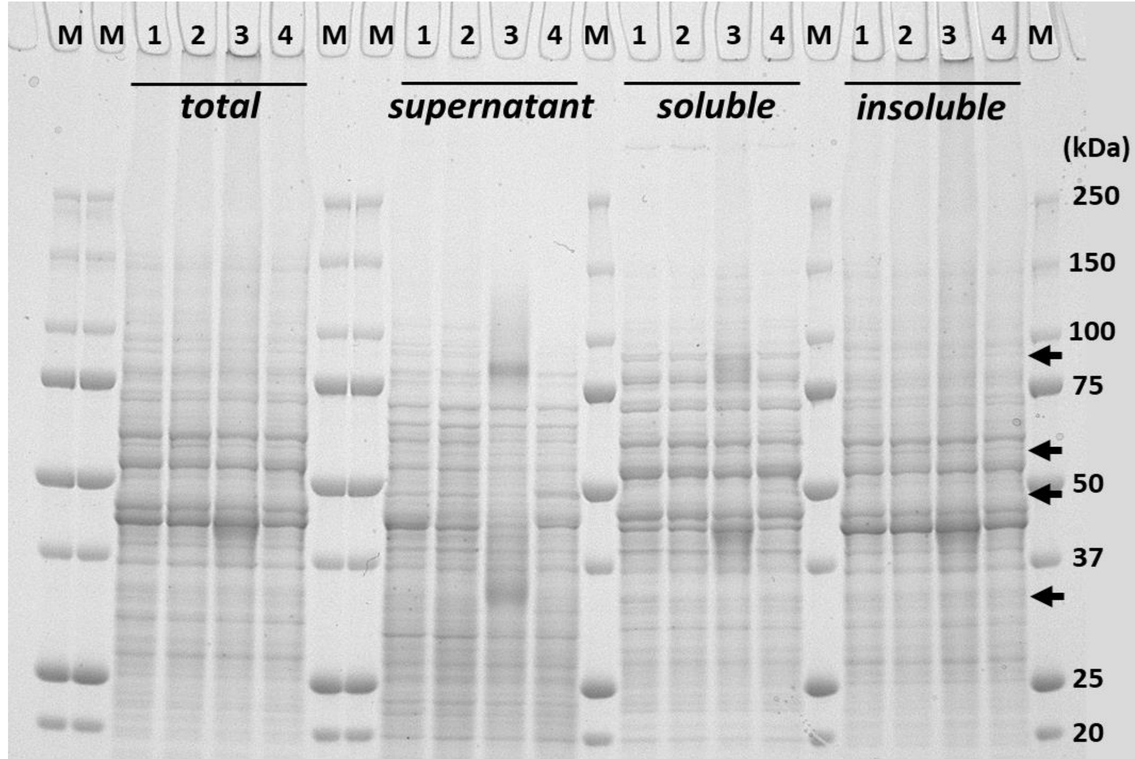


Figure 3.5. SDS-PAGE analysis of total, supernatant, soluble and insoluble protein fractions from *Rhodococcus* recombinant laccase clones. Lanes: 1: blank vector, 2: OGSP MCO2, 3: C2sig-T4 EpoA, 4: C2sig-T5 MCO1, M: protein marker

The molecular mass of the mature MCO2 laccase, inferred from its primary sequence, is around 55 kDa. Although the corresponding protein bands were not clearly

visualised, the recombinant putative laccase activity of OGSP MCO2 clone with the laccase substrates guaiacol and ABTS was demonstrated. The molecular mass of the mature EpoA laccase, inferred from its primary sequence, is around 34 kDa. However, for the C2sig-T4 EpoA clone, two recombinant protein bands were observed: one around 34 kDa in the supernatant fraction and another, slightly smaller than 100 kDa, in both the supernatant and soluble protein fractions (Figure 3.5). A similar size of approximately 100 kDa for active EpoA was reported by Endo *et al.*, who first reported EpoA in *S. griseus*. The EpoA protein band reported by Endo *et al.* was also three times higher than the mature protein, which the authors suggested was due to active EpoA existing as a homotrimer (Endo *et al.*, 2002). The molecular mass of the mature MCO1 laccase, inferred from its primary sequence, is around 51 kDa. Recombinant protein bands corresponding to 51 kDa were observed in the supernatant and soluble protein fractions for the C2sig-T5 MCO1 clone.

Application of *R. erythropolis* recombinants in PE biodegradation

The *Rhodococcus* recombinant laccase clones C2Sig-T4 EpoA, C2sig-T5 MCO1 and OGSP MCO2 demonstrated the best secretory recombinant laccase activity among all other SP-gene combinations for each laccase. Therefore, a mixture of these laccase recombinant clones was utilized for PE degradation.

During culturing, copper supplementation was done as copper is an essential ion for laccase; it regulates and enhances the activity of laccase (Endo *et al.*, 2002; Xie *et al.*, 2019; Zampolli *et al.*, 2023). Additionally, a laccase mediator, TEMPO, was added as such mediator compounds can improve the effectiveness of laccase-catalysed reactions. In a laccase-mediator system, the laccase enzyme first oxidizes the mediator, which in

turn oxidizes the substrate. The redox potential of laccase limits its activity for a few substrates, but using mediator compounds that, on oxidation by laccase, create high redox potential intermediates allows laccase to indirectly oxidize substrates with high redox potentials that were originally inaccessible to laccase (C. Yao *et al*., 2022). TEMPO was selected as the mediator compound owing to its activity and stability, where it could remain active over time, which was preferred in the culturing conditions (Kurniawati & Nicell, 2007; C. Yao *et al*., 2022).

PE biodegradation assessment

SEM analysis

SEM imaging was performed for PE pellets sampled from the recombinant laccase clones treatment flasks, blank vector clone treatment flask and control flask to assess the bacterial colonization and surface degradation. SEM images obtained from PE samples on day 10 showed dense biofilms on the PE surface, along with planktonic cells. Slight surface flaking was observed in the biofilm-removed samples for both the recombinant laccase clones and blank vector clone treatments (Figures 3.6a–e). Both day 30 and day 60 PE pellets exhibited three-dimensional cell clusters along with planktonic cells on the PE surface. Additionally, embedded cell clusters and bacteria growing and accentuating preexisting surface irregularities were observed. Flaky surface cavities brought about by bacterial colonization are seen in biofilm-removed samples for both the recombinant laccase clones and blank vector clone treatment samples (Figures 3.6f–j, k-o). By day 60, a more pronounced flaking and surface erosion of the PE pellets from both treatments were observed.

Visually, the surface erosion of PE could not be well-differentiated between the recombinant laccase clones treatment and blank vector clone treatment. Therefore, further confirmatory analyses, such as Fourier-transform infrared spectroscopy (FTIR) and gas chromatography-mass spectrometry (GC-MS), are necessary to evaluate the efficiency of the laccase recombinants in PE degradation.

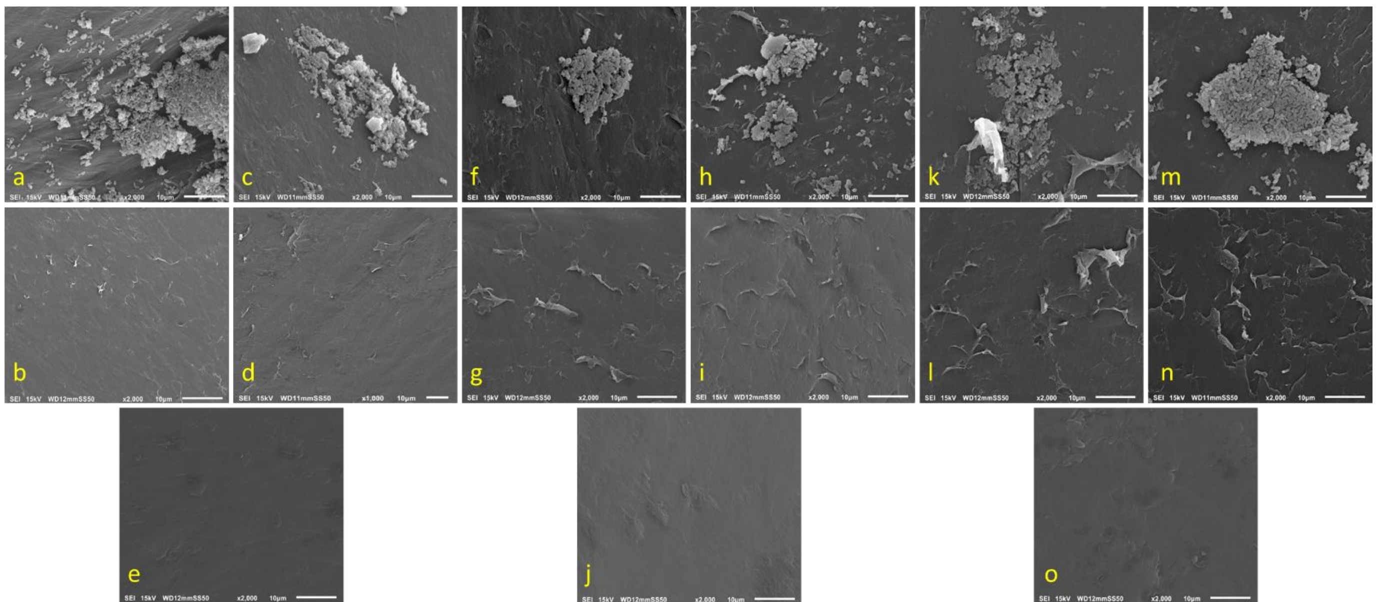


Figure 3.6. SEM micrographs of PE pellets from the blank vector clone treatment (BCT), recombinant laccase clones treatment (RCT) and control. (a–e: Day 10 PE samples) a; BCT-showing initial multi-layered biofilms. b; BCT-PE surface after biofilm removal. c; RCT-biofilm clusters of recombinant clones. d; RCT-PE surface after biofilm removal. e; PE pellet control. (f–j: Day 30 PE samples) f; BCT-showing bacterial biofilm. g; BCT-PE surface after biofilm removal showing a flaky surface. h; RCT-biofilm clusters on PE surface. i; RCT-PE surface after biofilm removal showing surface flaking. j; PE pellet control. (k–o: Day 60 PE samples) k; BCT-showing bacterial biofilm. l; BCT-PE surface after biofilm removal with pronounced surface flaking. m; RCT-showing multi-layered biofilm. n; RCT-PE surface after biofilm removal with pronounced surface flaking. o; PE pellet control.

3.4 Conclusion

In study II, the genetic engineering approach was employed to enhance the PE degradation ability of the *Rhodococcus* host. To address the inadequate secretory laccase activity of this host, three *Streptomyces* putative laccases, namely, EpoA, MCO1 and MCO2 were heterologously expressed in the host. Extracellular expression of the introduced genes was improved by implementing strategies like replacing the native SP of *Streptomyces* putative laccase genes with that of the *Rhodococcus* host SP and by incorporating additional protein transporter elements in the vector to balance recombinant protein production and secretion in the host.

The recombinant enzyme expression was observed to vary among the different SP-gene combinations, even when using the same SP. Using the *Rhodococcus* SP in combination with the additional protein transporters, enhanced the extracellular expression of the recombinant putative laccase in the host. These findings highlight the significance of SP-gene compatibility in heterologous protein secretion studies.

The three introduced putative laccases were successfully expressed extracellularly in the *Rhodococcus* host. Additionally, for PE degradation, a *Rhodococcus* recombinant was selected for each of the three putative laccase genes with the best performing SP-gene combination. Resultantly, a mixture of the *Rhodococcus* recombinant clones: C2Sig-T4 EpoA, C2sig-T5 MCO1 and OGSP MCO2 was used to degrade PE. The initial SEM analysis showed colonization and surface erosion by the recombinant laccase clones treatment. However, as visually, the surface erosion of PE could not be clearly differentiated between the recombinant laccase clones treatment and blank

vector clone treatment, further analyses such as FTIR and GC-MS are necessary to confirm the effectiveness of the laccase recombinants in PE degradation.

CHAPTER IV

4. General Conclusion

Microbes hold significant promise as innovative eco-friendly tools for remediating plastic pollution (Wei & Zimmermann, 2017; Williams & Rangel-Buitrago, 2022). Various strategies can be employed to enhance the efficiency of microorganisms for such applications. This dissertation explored two distinct approaches. Study I investigated the application of different bacterial strains as a consortium to degrade PE, while Study II investigated the enhancement of the target enzyme expression of a selected host bacterium to break down PE more effectively.

In Study I, the consortium strategy for PE degradation was evaluated. NGS analysis monitored the consortium community dynamics in the culture broth and plastisphere, while SEM and FTIR assessed the polymer structural changes brought about by the collective efforts of the consortium members. Study I demonstrated the potential of an artificial bacterial consortium for PE degradation. Moreover, here, *R. erythropolis* emerged as a competitive plastisphere species showing robust colonization on PE, highlighting its potential as a host for the genetic engineering strategy.

Study II focused on enhancing the PE degradation ability of the selected host *R. erythropolis* by genetic engineering. Three *Streptomyces* putative laccases (EpoA, MCO1 and MCO2) were heterologously expressed in the *Rhodococcus* host. Strategies such as replacing the native *Streptomyces* SP with that of optimised *Rhodococcus* SP and incorporating additional protein transporter elements improved the extracellular enzyme expression. Furthermore, the best-performing recombinant clones from the

different SP-gene combinations for each of the three putative laccase recombinants were selected and were used as a mixed culture to degrade PE.

Future prospects for consortium-based studies include the development of tailored microbial consortia designed to target specific polymers. Studies on naturally formed plastisphere communities can improve our understanding of how these communities assemble and function. Additionally, through these studies, we can identify the primary colonizers and generalist core plastisphere species (Dang *et al.*, 2008; I. V. Kirstein *et al.*, 2018; Zettler *et al.*, 2013). The recurrence of such generalist taxa in diverse plastispheres signals their important ecological role in such communities, including their contribution to the overall polymer degradation. Moreover, through metagenomic studies of the plastisphere community genome, we can further explore microbial diversity and enzymes, and elucidate the functional pathways involved in plastic degradation (Shilpa *et al.*, 2022). These insights from studying natural plastispheres can help create artificial consortia that could be better manipulated and made more efficient in breaking down synthetic polymers.

In the case of genetic engineering, future efforts could focus on the heterologous functional expression of putative plastic-degrading enzymes sourced from other bacteria, fungi, plants, and other organisms. Additionally, enzyme manipulation strategies, like directed evolution, could be employed to enhance enzyme properties like optimal pH, substrate specificity and thermostability (Gupta *et al.*, 2010; Liu *et al.*, 2011; Torres-Salas *et al.*, 2013). Other areas for exploration include diversifying plastic-specific chassis strains to include thermophiles or halophiles, which are favourable for industrial

applications (Ko *et al*., 2020). Integrating these strategies with genetic engineering can help create versatile live whole-cell systems for efficient plastic degradation.

Additionally, experimenting with different culture media and using various supplementary agents, such as enzyme-specific cofactors and mediators, could enhance the efficiency of microbial plastic degradation systems (Jin *et al*., 2023).

In conclusion, both approaches, consortium-based and genetic enhancement of versatile hosts, show considerable potential for innovative developments in targeting plastic degradation and provide the option of customization to target specific polymers.

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