聖若瑟大學
UNIVERSITY OF SAINT JOSEPH

CATÓLICA
FACULTY OF BIOTECHNOLOGY
PORTO

ISOLATION AND CHARACTERISATION OF PLASTIC POLYETHYLENE TEREPHTHALATE (PET) DEGRADING AND POLYHYDROXYALKANOATES (PHAs) PRODUCING BACTERIA FROM SOIL AND WATER

Thesis submitted to the academic faculty in partial fulfilment of the requirements for the degree of PhD in Science in the Institute of Science and Environment of the University of Saint Joseph, Macao

AND

Thesis submitted to the Universidade Católica Portuguesa to attain the degree of PhD in Biotechnology – with specialization in Environmental Science and Engineering

by

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July 2023

ENDORSEMENT

I certify that this report is solely my work and that it has never been previously submitted to any other higher education institution for any academic award.

Muhammad Bashir Saidu

We, the supervisors, believe that this Thesis is ready for assessment and reaches the accepted standard for the degree of PhD in Science in the Institute of Science and Environment of the University of Saint Joseph and PhD in Biotechnology – with specialization in Environmental Science and Engineering - in Universidade Católica Portuguesa.

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Sumário

A procura por plásticos tem gerado enormes quantidades destes resíduos no meio ambiente, os quais persistem e impactam negativamente os ecossistemas. O polietileno tereftalato (PET) é um dos polímeros termoplásticos mais comuns no mercado. A preocupação com os resíduos plásticos motiva o desenvolvimento de estratégias para aumentar a biodegradação e encontrar polímeros alternativos. Neste trabalho foi investigada a possibilidade de usar bactérias para degradar PET e para produzir bioplásticos (polihidroxialcanoatos, PHAs). Por fim, foi testada a integração dos dois processos. No geral, o trabalho teve como objetivo investigar o potencial de reciclar PET em bioplástico usando bactérias.

O potencial de consórcios bacterianos obtidos de várias amostras ambientais para degradar grânulos de PET em matriz líquida foi investigado. Os resultados revelaram degradação máxima de 1,1% por um dos consórcios testados. O intermediário de degradação do PET, ácido tereftálico (TPA), não foi detetado ao final de 55 dias. Os resultados da espectroscopia de infravermelho por transformada de Fourier (FTIR) mostraram grandes desvios e curvas espectrais na estrutura química do PET em comparação com o controlo não inoculado.

A biodegradação de filmes de PET enterrados no solo (A), e com plantas de mangal (B), e bioaumentados com um consórcio bacteriano (C) também foi investigada. Os ensaios foram conduzidos por 270 dias em condições ambientais. Os resultados não revelaram diferenças entre os tratamentos na degradação, com uma perda de peso máxima de 0,118% no tratamento com bioaumento. No entanto, os resultados do Microscópio Eletrônico de Varredura (SEM) e FTIR indicaram mudanças significativas na superfície, deslocamentos de pico espectrais e extensões nas estruturas químicas do PET. Consórcios bacterianos isolados do solo dos tratamentos experimentais foram avaliados quanto à degradação dos

monômeros de PET, TPA e monoetilenoglicol (MEG), e intermediário Bis (2-hidroxietil) tereftalato (BHET). Os consórcios foram inoculados em meio mínimo com 1000 mg/L TPA ou BHET ou 1113 mg/L MEG como fontes de carbono. Os resultados mostraram degradação completa de TPA e degradação significativa de BHET (96,09%) e MEG (83,65%) pelos consórcios.

Na segunda parte do estudo, foram isoladas bactérias de várias amostras ambientais e triadas quanto à produção de PHA usando a coloração Sudan Black B. Foram capturadas imagens com Microscópio Eletrônico de Transmissão para confirmar as inclusões de PHA intracelular. Um total de 35 isolados foram rastreados para PHA, e 22 mostraram coloração positiva. O isolado que apresentou maiores níveis de síntese de PHA (EC2-30-3) foi identificado com base na sequência do gene 16S rRNA como *Bacillus* sp. e selecionado para culturas de fermentação e degradação de monômeros de PET para produção de PHA. Foi cultivado em meio mínimo com 1000 mg/L TPA e 1113 mg/L MEG como fonte de carbono por oito dias. O isolado cresceu melhor em meio contendo MEG, que foi selecionado como modelo de substrato para fermentação de PHA. Para integrar a biodegradação dos monômeros de PET e a produção de PHA, o isolado foi cultivado em 0,2% de MEG. Um controlo com 0,2% de glicose foi preparado e as culturas foram incubadas por 96 horas. *Bacillus* sp. EC2-30-3 apresentou maior acumulação de PHA em meio com MEG (40,31%) do que glicose (25,53%). Este é o primeiro estudo a reportar que *Bacillus* sp. usa monômero de PET como carbono para produzir um biopolímero. Os resultados de FTIR do PHA extraído identificaram suas unidades funcionais como grupos C–H, CH3, C=O e C–O. As bandas de absorção obtidas estão intimamente relacionadas com a estrutura do PHB. O estudo confirmou, assim, a capacidade das bactérias isoladas em degradar monômeros de PET e produzir biopolímeros.

Os resultados deste trabalho abrem a possibilidade de aumentar o uso de bactérias para mitigar o impacto do PET no meio ambiente acoplada à produção de bioplásticos mais ecológicos.

Palavras-chave: Polietileno tereftalato, Polihidroxialcanoatos, mangal, biodegradação, bactérias.

Abstract

The demand for plastic has led to enormous plastic waste in the environment, which persist and negatively impact the ecosystems. Polyethylene terephthalate (PET) is one of the most common thermoplastic polymers available on the market. The concerns about plastic waste generated an interest in strategies to enhance its biodegradation and finding alternative polymers. In this work was investigated the possibility of using bacteria to degrade PET and to produce bioplastics (Polyhydroxyalkanoates, PHAs). Finally, the integration of the two processes was tested. Overall, the work aimed to investigate the potential to recycle PET into bioplastic using bacteria.

The potential of bacterial consortia from various environmental samples to degrade PET granules in liquid matrix was investigated. . The results revealed maximum PET granules degradation of 1.1 % by one of the tested consortia. PET degradation intermediate terephthalic acid (TPA) was not detected at the end of 55 days. Fourier-transform infrared spectroscopy (FTIR) results showed major spectral peak shifts and bends on PET chemical structure compared to non-inoculated control.

The biodegradation of PET films buried in the soil (A), with mangrove plants (B), and bioaugmented with a bacterial consortium (C) was also investigated. The experiments were conducted for 270 days at ambient conditions. The results revealed no difference between treatments in the degradation, with a maximum weight loss of 0.118 % in the bioaugmented treatment. Nevertheless, Scanning Electron Microscope (SEM) and FTIR results indicated significant surface changes, spectral peak shifts, and stretches in PET chemical structures. Bacterial consortia isolated from the soil of the experimental treatments were assessed for degradation of PET monomers, TPA and monoethylene glycol (MEG), and intermediate Bis(2-hydroxyethyl) terephthalate (BHET). The consortia were inoculated in flasks

containing minimal media with 1000 mg/L TPA or BHET or1113 mg/L MEG as the sole carbon source. Results showed complete degradation of TPA and significant degradation of BHET (96.09%), and MEG (83.65%) by the consortia.

In the second part of the study, bacteria were isolated from various environmental samples and screened for PHA production using Sudan Black B staining on colonies and smeared glass slides. Transmission Electron Microscope images were captured to confirm the intracellular PHA inclusions. A total of 35 isolates were screened for PHA, and 22 showed positive staining. The isolate showing higher levels of PHA synthesis (EC2-30-3) was identified based on 16S rRNA gene sequence as *Bacillus* sp. and selected for PET monomers degradation and fermentation cultures for PHA production. It was cultured in minimal (Moreira et al., 2013) media with 1000 mg/L TPA and 1113 mg/L MEG as the carbon source for eight days. The isolate grew better in media containing MEG, which was selected as a substrate model for PHA fermentation. To integrate PET monomers biodegradation and production of PHA, the isolate was cultured in 0.2 % MEG. A control with 0.2 % of glucose was prepared, and the cultures were incubated for 96 hours. *Bacillus* sp. EC2-30-3 showed higher PHA accumulation in media supplied with MEG (40.31%) than glucose (25.53%). This is the first report showing that *Bacillus* sp. uses PET monomer as carbon source to produce a biopolymer. FTIR results of the extracted PHA identified its functional units as C–H, CH3, C=O, and C–O groups. The absorption bands obtained are closely related to the structure of PHB. The study thus confirmed the ability of the isolated bacteria to degrade PET monomers and produce biopolymers.

The results of this work open the possibility for upscaling the use of bacteria to mitigate the impact of PET on the environment while producing environmentally friendly bioplastics.

Keywords: Polyethylene terephthalate, Polyhydroxyalkanoates, mangroves, biodegradation, bacteria.

Acknowledgements

I praise and glorify God Almighty for the gift of life, good health, and the precious opportunity and the strength and capability to proceed successfully.

Many people deserve my most profound appreciation because the PhD journey would not have succeeded without them. I apologise in advance in case somebody is missing from this list.

First, with deepest gratitude, I sincerely thank my esteemed and worthy supervisors, Professor David Manuel Flores Gonçalves, Professor Paula M. L. Castro, and Professor Irina Sousa Moreira, for their availability, patience, and wise advice. More specifically, I want to thank Professor David Manuel Flores Gonçalves for always being available to help and guide me in every matter related to my research and personal life. His immense support, unswerving patience, trusting my decisions and motivation fathered my work at every stage. More than a supervisor, he became a brother. I cannot thank him enough. I want to thank Professor Irina Sousa Moreira for always ensuring my research was progressing in the right direction, for the countless hours she spent listening and discussing topics related to my research and suggesting new paths and for her help and guidance during my journey and stay in Portugal. I am deeply indebted to Professor Paula M. L. Castro for her patience, guidance and wise pieces of advice and for ensuring the progress of my research, and for giving me the opportunity to conduct my PhD work in the Faculty of Biotechnology, Universidade Catolica Portuguesa, Portugal.

I want to express my gratitude to FDCT, Macao (Fundo para o Desenvolvimento das Ciências e da Tecnologia, Macau), Project 0004/2019/APJ, for financial support.

I would also like to thank the scientific collaboration of CBQF under the FCT project UIDB/50016/2020.

I want to express my special thanks to Karen for her outstanding support and caring and her encouragement and motivation for giving me a chance to always be part of her team members, which were crucial to my research and personal life.

I am very grateful to Sara Cardoso for giving her time and help during the preparation and work of molecular samples and DNA sequence analyses. I am also thankful to Deepa and Flora Gordillo for their friendship, encouragement, and availability to discuss topics related to my research.

I thank Professor James Ker-hei Fang, Dr WU Rongben, and Ho Yuen Wa (The Hong Kong Polytechnic University, Hong Kong) for their patience, collaboration, and help with sample analyses,

I thank Professor Peixin Du and Guo, MUST (Macao University of Science and Technology), who guided me through the FTIR analysis.

Many thanks to Anthony Okorocha (USJ) for his patience and always support with advice and to Mónica Coutinho at UCP, for their precious help on bureaucratic matters.

I also thank Dr. Keren, HKU (The University of Hong Kong), who guided me through the TEM and SEM imaging.

I thank all the ISE (USJ) and USB, Universidade Catolica Portuguesa, Porto professors and students for providing me with a healthy and productive research environment during my PhD research.

In the ISE (USJ), I am thankful to all my friends and teammates/ lab mates for their valuable friendship, sharing of ideas, and support that kept me motivated through tough times. Everyone has helped me in one way or the other. Here I enlist them in no particular order; Pedro Quelhas, Ricardo Borges, Alexandre Lebel, Chan Weng Sam, Yolanda Lau, Susana Wong, Lúcia Gonzalez, Andreia Ramos, Guilherme Martins, Pedro Vieira, Vânia Barradas,

Vivian, Rafael Ayala Lara, Varon Lou, Lukas Breitzler, Jason Lam and more recently, Sofia and Steve. I will always cherish every moment we have shared.

In UCP, I want to thank Ana Couto, Miguel, and Ana Oliveira, for their friendship, cheerful mood, and valuable discussions on topics related to my thesis.

At last, I thank my mother, Hajiya Halima Muhammad, for all the love and support and for always being prayerful. Special thanks to my wife, Zainab Ho, for her love, patience, encouragement, and always being there for me over the years. And my son, Muhammad Ali, for helping me type and messy my writing. I love you all.

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Glossary of acronyms and abbreviations

ANOVA: Analysis of variance

ATR: Attenuated Total Reflectance

BHET: Bis(2-hydroxyethyl) terephthalate

CFU/ml: Colony Forming Unit per millilitre

CO_2: Carbon dioxide

DCW: Dry Cell Weight

EG: Ethylene Glycol

EPS: Exopolysaccharides

FT-IR: Fourier-Transform Infrared Spectroscopy

HDPE: High Density Polyethylene

HPLC: High-Performance Liquid Chromatography

IR: Infrared

LCL: Long Chain Length

LDPE: Low-Density Polyethylene

MCL: Medium Chain Length,

MEG: Monoethylene Glycol

mg/L: Miligram per liter

MHET: Mono (ethylene terephthalate)

MM: Minimal Media

NA: Not Applicable

NAFTA: North American Free Trade Agreement

NaOCl: Sodium Hypochlorite

OD: Optical Density

PET: Polyethylene Terephthalate

PHAs: Polyhydroxyalkanoates, 24

PHB: Poly-3-hydroxybutyrate

PHBV: Poly (3 hydroxybutyrate-co-3-hydroxyvalerate)

PHH: Polyhydroxyhexanoate

PHV: Polyhydroxyvalerate

PS: Polystyrene

PUR: Polyurethane

PVC: Polyvinyl chloride

SCL: Short Chain Length

SDS: Sodium Dodecyl Sulphate

TEM: Transmission Electron Microscopy

TOC: Total Organic Carbon

TPA: Terephthalic acid

RNA: Ribonucleic acid

SAR: Special Administrative Region

SD: Standard Deviation

SEM: Scanning Electron Microscope

UV: Ultraviolet

WWTP: Wastewater Treatment Plant

1. Chapter One - General introduction and thesis scope

1.1. Research background

Plastics are synthetic materials that use polymers as the main constituents. Plastic polymers are chemical arrangements based on the repetition of many monomeric units forming chemically bonded long-chain structures (Crawford & Quinn, 2016; McKeen, 2019). Conventional plastics are mostly petroleum-based. Examples within this group are polypropylene (PP), polyethylene (PE) polyvinyl chloride (PVC), polyethylene terephthalate (PET), polystyrene (PS), and polyurethane (PUR). Because of their outstanding properties, which include toughness, durability, tensile strength, and relatively low cost, among others, plastics provided humanity with immeasurable benefits and have become indispensable to society. These properties make plastic materials highly versatile and suitable for various applications. Since the beginning of their industrial implementation in 1950, the world has seen a steady increase in plastic production, reaching 390.7 million metric tons in 2021 (PlasticEurope, 2022) and estimated to reach 590 million metric tons in 2050 (Statista, 2023). Plastic types differ in their monomeric units and polymer types. Polyethylene terephthalate (PET) is one of the world's most widely produced and used plastics (Chen et al., 2020; Castro et al., 2017) and plays a central role in the global packaging industries, especially for beverages, fresh food, and water. PET is synthesised by the esterification reaction of terephthalic acid (TPA) and ethylene glycol (EG), which is the focus of this study.

However, despite the many advantages and benefits of synthetic plastic materials, the world has also witnessed the daunting challenges of plastic waste in the environment leading to air, land, and marine pollution. Water bodies are the main receptacles for plastics waste, as 4–12 million tonnes of plastics go into the seas and oceans yearly (Picó & Barceló, 2019),

causing severe damage to aquatic life (Allen et al., 2012; Krueger et al., 2015). The combined effects of plastic litter threaten coastal ecosystems (Menicagli et al., 2020), with significant environmental and potential human health impacts. Plastics are difficult to degrade and can remain in the environment for hundreds of years.

Many efforts have been undertaken to provide solutions to the plastic waste problem. One approach is to recycle plastic waste, but the overall global recycling rate is low, at 16% in 2018, with 66% of plastics estimated to be landfilled or leaked into the environment (Nicholson et al., 2021). Microorganisms have evolved to interact with or grow on plastic (Didier et al., 2017; Dussud et al., 2018; Oberbeckmann et al., 2018; Oberbeckmann et al., 2016); break it down (Farzi et al., 2019; Liu et al., 2018; Urbanek et al., 2018; Veethahavya et al., 2016), or even use it as an energy source (Yoshida et al., 2016). It is essential to deepen the knowledge involved in plastic biodegradation to increase the development of techniques for mitigating this growing global problem, which is one of the main aims of this study.

In addition to biodegradation, another approach to mitigate plastic waste problems in the environment is the development of biodegradable plastics or biopolymers. Biodegradable plastics can reduce the persistence of plastic wastes in the environment, as they are more easily degraded by the soil microorganisms (compared to conventional plastics). Production of biodegradable polymers is an innovative approach to solving plastic disposal and environmental pollution problems, delivering better environmental outcomes, and minimizing the consumption of natural resources, depending on which materials are used for their production. However, the effectiveness of biodegradation can vary depending on the type of plastic. Bioplastics or biopolymers are currently produced from plant biomass and microorganisms. This study explores the production of biopolymers, such as polyhydroxyalkanoates (PHAs), by bacterial isolates. PHAs are polyoxoesters produced by

several microorganisms as reserve food material (Trivedi et al., 2016; Hawas et al., 2016), a mechanism of carbon storage during unbalanced growing conditions (i.e. carbon excess and limited nutrients availability such as nitrogen). PHAs are biodegradable and biocompatible and have a wide range of applications (Muhammadi et al., 2015).

This study aims to further explore natural environments as a source of microorganisms that may be able to use synthetic polymers and their monomers as carbon and energy sources, converting them into biopolymers (PHA), which could be a sustainable approach to address the challenges of the conventional plastic pollution. Significantly less research attention has been given to this integrated solution for the degradation of synthetic polymers and the production of biopolymers. Within the possible natural ecosystems hosting microorganisms with these characteristics, this work focused on mangrove forests. Mangroves are a unique type of coastal ecosystem found in tropical and subtropical regions around the world. Mangroves play essential roles in ecosystem functioning, including serving as nursery grounds for fish and crustaceans, habitat to an abundant variety of birds, protecting coastlines from erosion and storms, and capturing and sequestering carbon (Bayen, 2012). In addition to their ecological importance, these ecosystems are characterised by salt-tolerant trees and shrubs and harbour diverse microorganisms that are capable of breaking down organic and some inorganic matter, including plastics (Pramanik et al., 2018; Thatoi et al., 2020; Helen et al., 2017). These characteristics are partly due to the abundance of waste that accumulates in mangroves and the high levels of moisture, nutrients, and sunlight in these ecosystems. As a result, mangroves may provide a rich source of bacteria capable of degrading plastic waste (do Carmo et al., 2011).

Given the potential of mangroves as a source of plastic-degrading bacteria, the plastic degradation capabilities of bacteria isolated from mangroves in Macao is central to this thesis. This research could provide insights into the potential of the mangrove environment as a source of microorganisms that are able to degrade conventional plastic and, at the same time, of biodegradable plastic-producing bacteria.

1.2. Research questions

The thesis consists of a combination of fieldwork and laboratory experiments on the biodegradation of PET and its monomers and the production of PHAs.

The main questions addressed in this thesis are listed below, along with the steps followed to answer them.

A. Can bacterial consortia in liquid matrix degrade PET plastic into its monomers, terephthatlic acid (TPA) and monoethylene glycol (MEG) and intermediate, bis(2-hydroxyethyl terephthalate (BHET)?

Steps to answer the question included:

1) collection of water and sediment in different sampling points in Macao;
2) isolation of degrading bacterial consortia;

PET degradation experiments and analysis.

B. Can mangrove sediments be considered sources of bacterial consortia that can degrade PET plastic and its monomers (TPA, MEG) and intermediate (BHET)?

Steps to answer the question include:

1) experiment with buried PET films in sediment with and without mangrove plants for an extended period;

2) isolation of bacterial consortia from the sediment with and without mangrove plants;
3) evaluation of the degradation of TPA, MEG, and BHET by the bacterial consortia;

C. Can mangrove sediments be considered sources of bioplastic (PHAs) producing bacteria?

Steps to answer the question include:

1) collection of sediment samples and bacterial isolation;
2) screening for PHA-producing isolates.

D. Can bacterial isolates from mangrove sediment degrade and convert PET monomers to produce PHA (bioplastic)?

Steps to answer the question include:

1) experiments to evaluate the degradation of PET monomers (TPA, MEG) by the PHA-producing isolates;
2) identification of the selected PHA-producing isolate;
3) fermentation cultures and extraction of PHA using MEG;
4) analysis of the extracted PHA.

1.3. Thesis outline

This thesis addresses the questions mentioned above and is summarised into the following six chapters:

Chapter 1 explains the research background, questions, and objectives of the thesis.

Chapter 2 presents the state-of-the-art on the biodegradation of conventional plastics and biopolymer production within the past ten years. It also addresses the possibility of integrating both approaches and highlights future trends.

Chapter 3 presents the methodologies and results of isolating bacterial consortia from various environmental samples, including an old landfill, coastal water, mangrove sediment, sediment scrapings adhered to plastic waste, and sludge samples, and assessing the potential to degrade PET granules.

Chapter 4 presents the methodologies and results of PET film biodegradation in soil with and without mangrove plants and with and without bioaugmentation. This chapter also assesses the degradation of PET monomers, terephthalic acid (TPA) and monoethylene glycol (MEG), and intermediate, Bis(2-hydroxyethyl) terephthalate (BHET) with bacterial consortia from mangroves soil.

Chapter 5 presents the methodologies and results of screening for bacterial isolates to produce polyhydroxyalkanoates (PHAs). This chapter also evaluates the degradation of terephthalic acid (TPA) and monoethylene glycol (MEG) and further integrates the conversion of monoethylene glycol (MEG) into the production of polyhydroxyalkanoates (PHAs).

Chapter 6 summarises the key findings of this research and discusses their implications for answering the research questions. Further research opportunities identified based on this research are also presented.

2. Chapter Two - A review on microbial degradation of conventional plastics and production of bioplastics

2.1. Introduction

Due to demands and increased population, plastic use has become indispensable in the twenty-first century. In most applications, conventional plastics have displaced many traditional materials, including leather, paper, bone, metal, glass, ceramic, wood, stone, horn and others. Plastics are crucial and essential materials for society in many sectors like packaging, building and construction, automotive, electrical and electronics, agriculture, household, sports, and leisure. The world saw increased plastic production and distribution for many decades, but a slight decrease occurred in 2020. Figure 2.1 shows the distribution of plastic materials' global production (not included: polyethylene terephthalate (PET), Polyacrylic, and polyacryl-fibres). China is indicated as the largest producer of plastic materials (32%), followed by NAFTA (18%) and the rest of Asia (17%) (PlasticEurope, 2022). As for (PET), the global annual production capacity stood at 30.5 million metric tons in 2019. In four years, the figure is expected to increase to approximately 35.3 million metric tons (Statista, 2023). Coal, natural gas, and oil are still the sources of most conventional plastic materials. The world's largest demands are thermoplastics (polypropylene, polyethylene, polyvinyl chloride, polyolefin, etc.) and thermosetting plastics (polyurethanes, etc.) (Lithner, 2011; PlasticEurope, 2016; Schwarz et al., 2019; PlasticsEurope, 2021).

Although plastics have enabled advances in the quality of life via advantages in processing, booming industries, ease in packaging and overall handling convenience, relatively low cost, versatility, and imperviousness to water, they are considered major environmental pollutants.

Due to inappropriate and uncontrolled waste dumping, a significant amount of plastic waste has found its way into the environment, contaminating terrestrial and marine environments (Geyer et al., 2017). Recently, a report has been published on the combined effects of plastic litter threatening coastal ecosystems (Menicagli et al., 2020). Plastic pieces carried by river currents accumulate in the sea, eventually harming marine ecosystems. Plastic waste materials may get lost at all life cycle stages and enter the environment where large amounts of plastic waste end up in the open oceans, either being washed or blown from the land into the sea or dumped there directly (Krueger et al., 2015). Most plastics are lightweight and durable, the qualities that make them buoyant in the aquatic environment and may eventually become waterborne, circulating in one of the five major ocean gyres (Sigler, 2014). In fact, the convergence zones of each of the five subtropical gyres have been a critical point where the worldwide accumulation of plastics on the ocean surface occurs (Cózar et al., 2014). Hundreds of thousands of plastic debris per square kilometre have been found in the Greenland and Barents seas (Cózar et al., 2017). Microplastics (<5 mm) and mesoplastics (< 5 cm) have been found in deep-sea sediments and on the surface of the Antarctic marine system (Waller et al., 2017). The amount of plastic detected on the ocean's surface is far less than that entering the marine system (Eriksen et al., 2014). Plastics will likely stay longer than hundreds to thousands of years in deep-sea and non-surface polar environments (Barnes et al., 2009).

Many ecologically damaging effects on the marine environment are caused by plastic pollution (Webb, et al., 2013). Plastic wastes affect marine biodiversity as many sea animals are entangled in fishnets, and plastic foil restricts their mobility, in extreme cases leading to drowning (Allen et al., 2012; Krueger et al., 2015). Plastics debris can also lead to coastal pollution, as reported by several studies around the world (Chitaka and Blottnitz, 2019;

Esiukova, 2017; Fauziah and Nurul, 2015; Vidyasakar et al., 2020; Lee et al., 2013; Reinold et al., 2020), and may present hazardous effects to terrestrial animals, especially after breaking down into microplastics (Zhang et al., 2016; Zhang et al., 2018; Zhang et al., 2018; Zhang et al., 2018).

Multiple processes, such as the synergistic actions of abiotic (light, seawater, mechanical) and possibly biotic weathering, cause the disintegration of large plastic materials into smaller pieces, eventually giving rise to microplastics (<5mm). An increasing number of reports have shown that tiny pieces of plastics are ingested by organisms such as fish (Naidoo et al., 2016; López-López et al., 2018; Forrest and Hindell, 2018; Saturno et al., 2020; Vries et al., 2020), crustaceans (Frydkjær et al., 2017), detritivores (Hodgson et al., 2018), birds (Franco et al., 2019; Provencher et al., 2018; Ballejo et al., 2021), turtles (Jung et al., 2018; Rizzi et al., 2019), and may also remain in their digestive tract, reducing the quantity of food that can be taken up and thereby lowering animal fitness (Derraik, 2002).

Most conventional plastics are non-biodegradable or poorly biodegradable (Gonzaga Diego, 2017; Sharon & Sharon, 2012). However, research has investigated the possible breaking down of plastic materials into smaller units by the actions of microorganisms. *Bacillus weihenstephanensis* has the potential to degrade Low-Density Polyethylene (LDPE) (Mukherjee and Chatterjee, 2014) and High-Density Polyethylene (HDPE) (Ingavale and Raut, 2018). Research has also shown that *Ideonella sakaiensis* could degrade and assimilate PET plastics (Yoshida et al., 2016).

Conversely, bioplastics or biopolymers with biodegradable properties are produced from plant biomass and microorganisms. Polyhydroxyalkanoates (PHAs) are biopolymers that belong to the group of polyoxoesters produced by several microorganisms as reserve food material (Trivedi et al., 2016; Hawas et al., 2016).

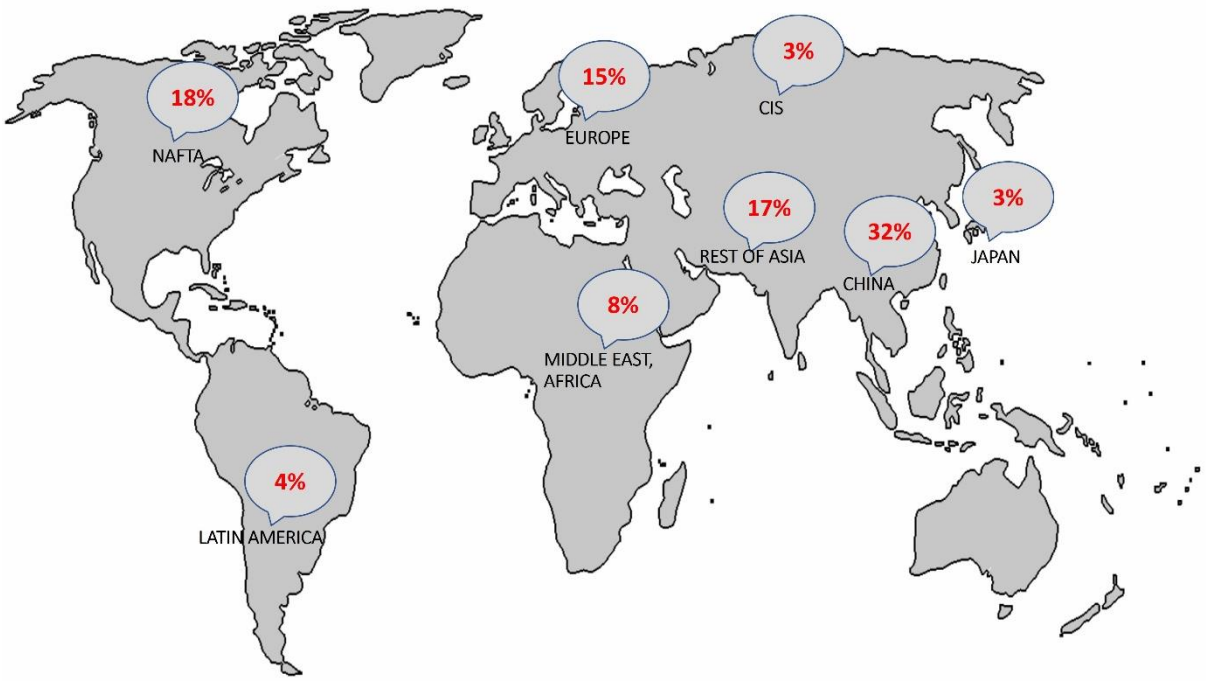


Figure 2.1: Global distribution of plastic materials production (2022: 390.7 Mt). From (PlasticsEurope, 2022).

Biopolymers are biodegradable and biocompatible and have a wide range of applications. The main constraints for the industrial scale-up of bioplastics are technological complexities and high production and recovery costs (Rodriguez-Perez et al., 2018; McAdam et al., 2020). However, biosynthesis in several recombinant organisms (bacteria, yeasts, or transgenic plants) was made possible through metabolic and genetic engineering (Zheng et al., 2020) and mutation (Bashir et al., 2014), thereby increasing the yields of production and decreasing overall costs (Takahashi et al., 2017).

Studies on integrating the biodegradation of conventional plastics and biopolymer production are scarce. To this aim, the microorganisms use the plastics degradation products or metabolites as the carbon source for the production of polyhydroxyalkanoates, such as poly-3-hydroxybutyrate (PHB), polyhydroxyvalerate (PHV), polyhydroxyhexanoate (PHH),

and the copolymer poly (3 hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) (Guzik et al., 2014).

2.2. Biodegradation of conventional plastics

Plastics are polymers, molecules consisting of a specific arrangement of monomers with repeated linear chains or branches. The result is a large molecule composed of 2000 or more monomers (GLAD, 2000). For 30 years, scientists have been developing ways to degrade long-chain natural and synthetic polymers into their respective monomers or intermediates using microorganisms. Plastics contain inert biochemical structures that minimise and resist noticeable microbial degradation and persist in the environment for many years (Krueger et al., 2015; (Kavitha et al. 2014). Limited bioavailability of the densely cross-linked polymers to microorganisms or their enzymes restricts their accessibility to the outer layer (Krueger et al., 2015), posing a challenge for the microbial breakdown of conventional plastics. Moreover, the solid nature of plastics poses difficulties to the detection/quantification of microbial degradation, which has to rely on the determination of mass loss, surface modifications, the appearance of metabolic products, or the observation of microbial growth at the expense of the polymer (Krueger et al., 2015).

Although the plastic surface is a poor substrate for the growth of microorganisms (Biffinger et al., 2014), recent research on plastisphere has indicated that microorganisms and diatoms can colonise the surface of plastic debris (Hansen et al., 2021; Roager and Sonnenschein, 2019; Wright et al., 2020; Zettler et al., 2013). Plastic debris acts as a novel ecological habitat for microorganisms in marine waters and as new floating vehicles for microbial transportation and colonization (Dussud and Ghiglione, 2016). Microorganisms may form a niche on the surface of particles offering support for growth and a protected area with limited

predation (Zettler et al., 2013). Most commonly made thermoplastics and thermosets have been reported to be susceptible to microbial attack (Table 2.1).

Table 2.1: Summary of studies on the biodegradation of different polymers.

Polymers	**Microorganisms/ enzymes**	**Weight loss/ % degraded**	**References**
Polyethylene (PE)	*Zalerion maritimum*, 28 days at 25 °C	43 %	(Paço et al., 2017)
	M. hydrolyticus IRE-31, 30 days at 37 °C	Not reported	(Li et al., 2020)
	Aspergillus sp. & *Fusarium* sp., 60 days at 33.3 °C	Ave. 7.25 %	(Das and Kumar, 2014)
	Consortium, 6 weeks at 28-37 °C	20.28 – 36.4 %	(Muhonja et al., 2018)
	Bacillus sp. & *Pseudomonas* sp. 3 months at 30 °C	1.54 - 40.39 %	(Sangeetha Devi et al., 2019)

	Consortium, *5-7 days at* 30 °C	Not reported	(Ameen et al., 2015)
Polyethylene terephthalate (PET)	*Ideonella sakaiensis* 201-F6, 6 weeks at 30 °C	75 %	(Yoshida et al., 2016)
	Streptomyces sp., 18 days at 28 °C	49.2 - 68.8 %	(Farzi et al., 2019)
	Delftia sp., *2 months at* 30 °C	Not reported	(Liu et al., 2018)
	*PETase (*from *ideonella sakaiensis),*	Not reported	(Austin et al., 2018)
	Consortia, 270 days at 30 °C	0.6 & 0.1 %	(Taghavi et al., 2021)
	Bacillus sp., 40 days at 29 °C	3.0 %	(Auta et al., 2017)
Polypropylene (PP)	*Pseudomonas* sp. & *Rhodococcus* sp., 40 days at 10 °C	7.3 - 17.3 %	(Habib et al., 2020)

	Aspergillus niger & pencillium funculosum, 45 days at 58 °C	24 %	(Santhoskumar & Palanivelu, 2012)
Polystyrene (PS)	*Pseudomonas. aeruginosa* strain DSM 50071, 15 days at 25 °C	2.6 %	(H. R. Kim et al., 2020)
	Consortia, 12 months at oligotrophic conditions	4.7 %	(Syranidou et al., 2017)
	Serratia sp., 20 days at 24 °C,	Not determined	(Woo et al., 2020)
	Pseudomonas sp., 30 days at 30 °C,	10 %	(Mohan et al., 2016)
	Bacillus sp., 30 days at 30 °C	23.7 %	(Mohan et al., 2016)
Polyvinylchloride (PVC)	White-rot fungi, 30 days at 30 °C	11.1-53.7 %	(Kirbaş et al., 1999)
	Pseudomonas fluorescens CBCC 1237, 14 days at 30 °C,	11 %	(Sharpe & Woodrow, 1971)
		17 %	(Giacomucci et al., 2020)

	Consortia, 7 months at 20 °C		
Polyurethane (PU)	*Bacillus safensis, 10 days at* 37 °C, *Aspergillus flavus G10*, 15 weeks at 25 °C Fungi consortium, 14 days at 30 °C	Not reported 1.9 % per week 87 %	(Nakkabi et al., 2015) (Schaefer et al., 2020) (Álvarez-Barragán et al., 2016)

Biodegradation can be influenced by both the physical and chemical properties of plastics. The main factors that affect biodegradation are exposure conditions and polymer

characteristics (Rajendran et al., 2015), categorised further, adapted from Kijchavengkul T and Auras R. (2008) (Figure 2.2).

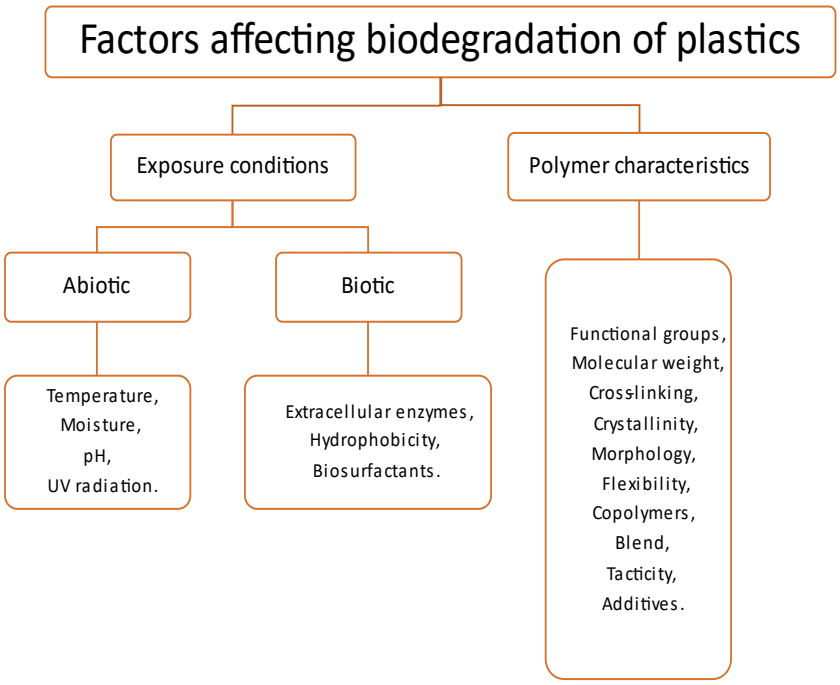


Figure 2.2: Factors affecting biodegradation of plastics. (Adapted from Kijchavengkul T and Auras R. (2008).

2.2.1. Plastic degrading microorganisms

Microorganisms are at the forefront of preventing the accumulation of various inorganic and organic compounds in the environment, including plastic polymers (Amobonye et al., 2021). They are involved in degrading both natural and synthetic plastics (Pathak and Navneet, 2017; Shah and Alshehrei, 2017) and, more importantly, evolved strategies to degrade plastics (Zeenat et al., 2021). Many reports demonstrate microbial degradation of synthetic plastics in natural environments, which depends on the ability of the microorganism to

produce extracellular enzymes (Mohanan et al., 2020). Biofouling and biodegradation have been suggested as processes of plastic elimination from surface water bodies (Krueger et al., 2015; Cózar et al., 2014). Species of the genera *Pseudomonas, Streptomyces, Corynebacterium, Arthrobacter, Micrococcus*, and *Rhodococcus* have been used in biodegradation assays (Pathak & Navneet, 2017). Bacterial isolates such as *Polaromonas, Micrococcus, Subtercola, Agreia, Leifsonia, Cryobacterium,* and *Flavobacterium* were isolated from the cryoconite of three glaciers located in northwest Spitsbergen; 12 strains from those isolates were able to produce lipase, an enzyme that hydrolyses ester bonds in lipids and some polyesters (Singh et al., 2014). Kavitha et al. (2014) reported more than 3% mass loss in polyethylene due to biodegradation by microbes from contaminated soil. Ambika et al. (2014) also reported a significant weight loss of PE due to biodegradation by bacteria from marine water.

2.2.2. Mechanisms of plastic biodegradation

Biodegradation is the breakdown of organic compounds or contaminants into some intermediates or monomers by microorganisms. Most polymers are too large to pass through cellular membranes, so extracellular enzymes are secreted to first depolymerise them into smaller units (Mohanan et al., 2020) before they can be assimilated and biodegraded within microbial cells (Shah et al., 2008) and ultimately be mineralised as CO_2 (Yoshida et al., 2016) or be used for the biosynthesis of valuable products through various metabolic pathways (Sangale, 2012). Microorganisms have evolved to produce specific enzymes to act on different plastic types (Bhardwaj et al., 2013).

Plastic biodegradation can occur in aerobic or anaerobic conditions. Anaerobic biodegradation occurs in the absence of oxygen, usually producing methane. Certain compounds, such as nitrate, sulphate, iron, manganese, etc., are essential as electron

acceptors. In aerobic biodegradation, microorganisms use oxygen as an electron acceptor, often producing CO_2 and water as the final products. Polymers undergo chemical reactions, and the chains are broken down into monomers or other intermediates, which can enter cellular metabolic processes (such as the Krebs cycle), generating energy, water, carbon dioxide, biomass, and other essential products (Pramila et al., 2012). The products formed after complete degradation are non-toxic to the environment and living organisms.

Biodeterioration of plastics entails the action of decomposers and other microbial communities responsible for physical or/and chemical alterations that result in a superficial degradation that modifies the plastic's mechanical, physical, and chemical properties (Allsopp et al., 2010; Ashter, 2016). Biodeterioration has been reported to occur on some polymers like PE (Allsopp et al., 2010; Anwar et al., 2013; Das and Kumar, 2014), PP (Habib et al., 2020), and PVC (Anwar et al., 2013). Other mechanisms of plastic biodegradation include biofragmentation (secretion of extracellular enzymes or free radicals by microorganisms for catalytic actions that cleave polymeric plastics into oligomers, dimers, or monomers), assimilation (integration of molecules transported in the microbial cytoplasm), and mineralization (complete degradation of molecules) (Lucas et al., 2008; Dussud & Ghiglione, 2016; Ahmed et al., 2018) (Figure 2.3).

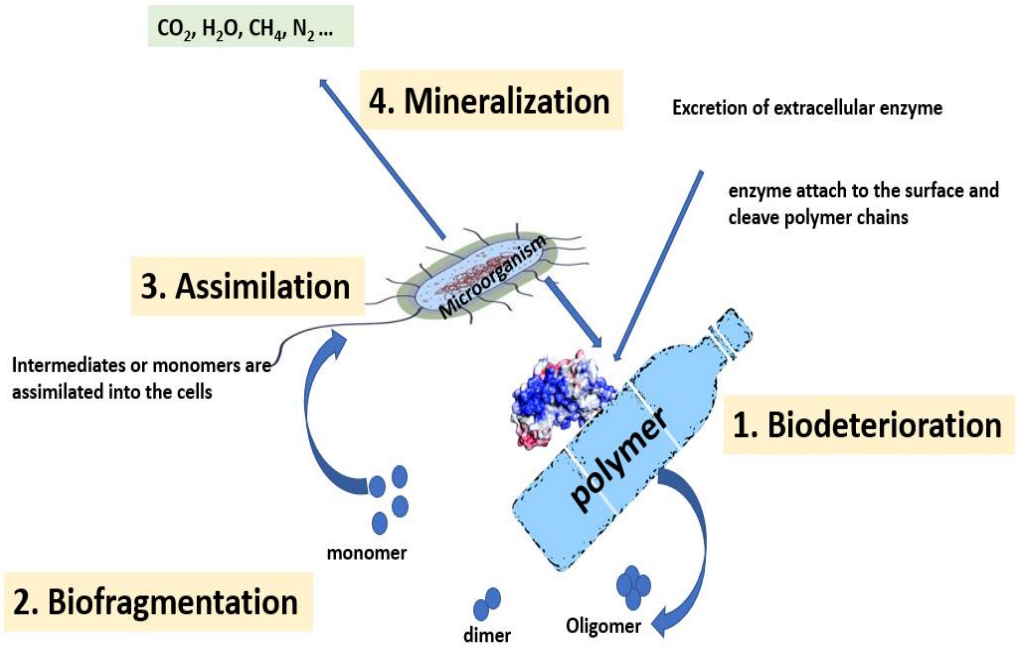


Figure 2.3: Mechanisms of plastic biodegradation by microorganisms (Dussud & Ghiglione, 2016).

2.3. Bioplastics and biopolymers

The emergence of bioplastics as alternatives to conventional plastics has increased over the last decade. These biopolymers are large macromolecules composed of single, repeating monomer units. They are of exceptionally high molecular weight, and their material characteristics vary according to the nature of their monomer composition (Basnett and Roy, 2010). Large-scale production of these biobased polymers and their extensive use is critical to ensure alternative sources of plastics and the severe environmental threats posed by conventional plastics (Zinn et al., 2001). Bioplastics are not just one single substance; they comprise a whole family of materials with differing properties and applications. According to the European Bioplastics organization, a plastic material is bioplastic if it is either biobased, biodegradable or features both properties (EuropeanBioplastics, 2014). Biobased plastics constitute a class of plastics that are produced from renewable biomass sources. The

biomass includes vegetable fats and oils (Elvistia Firdaus, 2019), vegetable wastes (Perotto et al., 2018), cornstarch (Abotbina et al., 2021; Hayati and Lazulva, 2018), sugarcane (Dalsasso et al., 2019), potato starch (Muneer, 2014; Bezirhan and Bilgen, 2019), cellulose (Isroi et al., 2017), straws (Bilo et al., 2018; Inayati et al., 2021), cotton (Rumi et al., 2021) or microorganisms (Bashir et al., 2014; Getachew and Woldesenbet, 2016).

2.3.1. Biopolymers from microorganisms

Microorganisms have been explored as sources of natural polymers, formed within microbial cells by complex metabolic processes. More complex polymers produced by bacteria and fungi are increasingly gaining attention, particularly polysaccharides such as xanthan, curdlan, pullulan, chitin, chitosan, hyaluronic acid, and polyhydroxyalkanoates (PHAs) (GOPAL, 2015; Balaji et al., 2013). Biodegradable polymers have been synthesised or formed in nature during the growth cycles of some microorganisms (Bender et al., 2019). Intracellularly produced polyhydroxyalkanoates (PHAs) and extracellularly produced exopolysaccharides (EPS) attract attention over the other biopolymers. These naturally produced polymers can replace plant-based or petroleum-derived polymers. Substantial reports have demonstrated the production of PHA and EPS by several microorganisms, such as bacteria, fungi, actinomycetes, and algae (Angelina & Vijayendra, 2015; Basnett & Roy, 2010).

2.3.2. Polyhydroxyalkanoates (PHAs)

Polyhydroxyalkanoates (PHAs) are polyesters of 3-, 4-, 5- and 6-hydroxyalkanoic acids produced by various bacterial species under nutrient-limiting conditions with excess carbon (Raza et al., 2018; G. Q. Chen, 2009). PHAs are intracellular carbon storage (Figure 2.4) mechanisms for many species of microorganisms (Brigham and Sinskey, 2012) and potential

candidates for an environmentally friendly replacement of petroleum-based plastics in numerous applications. Under different growth conditions, both aerobic and anaerobic, more than 90 genera of Gram-negative and Gram-positive bacteria have been identified for PHAs production, and more than 150 monomers have been reported (Ng and Sudesh, 2016; Agnew and Pfleger, 2013; Kim DY et al., 2007; Raza et al., 2018). Microorganisms store intracellular inclusions as an organic substance, such as PHAs, or an inorganic substance, such as magnetosomes. For PHAs, the core of the polyester is surrounded by either phospholipids or proteins. The PHA storage in bacterial cytoplasm exists in granular forms ranging from 0.2 to 0.5 μm (Poli et al., 2011; Rehm, 2007).

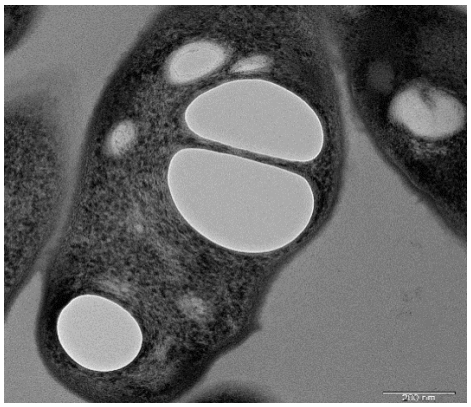
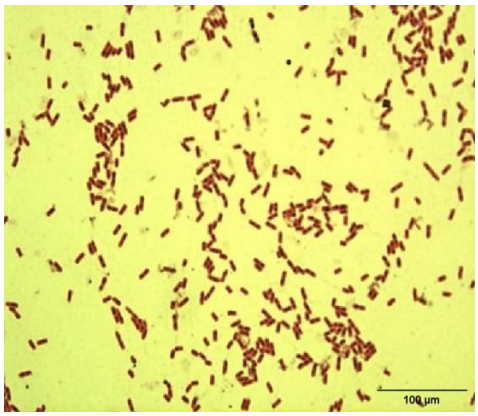
A  B 

Figure 2.4: Morphology of PHA granules in the bacterial cells observed under (a) Transmission Electron Microscope and (b) bright-field microscope stained with Sudan Black B solution.

There are many PHAs, characterised distinctly by chain length, type of functional group, and degree of unsaturated bonds. As for the carbon (C) chains, they are short (SCL-PHAs; C_3–C_5), medium (MCL-PHAs; C_6–C_{14}), and long (LCL-PHAs; C_{17}–C_{18}) (Singh et al., 2015). The most explored and most typical among the polyhydroxyalkanoates are poly(3-

hydroxybutyrate) (PHB), poly(3-hydroxyvalerate) (PHV), and their copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) (Figure 2.5). The molecular weight varies from 50000 to over a million, depending on the microorganism, fermentation conditions, and extraction method (Bugnicourt et al., 2014).

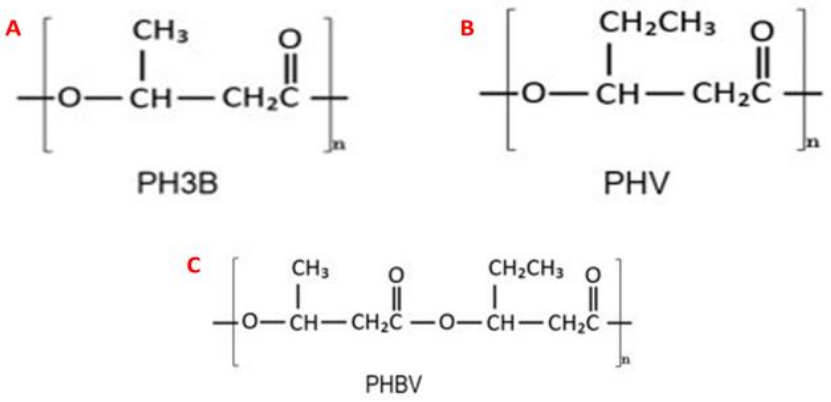


Figure 2.5: The most common polyhydroxyalkanoates (A) poly(3-hydroxybutyrate), (B) poly(3-hydroxyvalerate), and (C) poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (M. Singh et al., 2015).

2.3.2.1. Recovery of PHAs

Microbial PHA is stored as insoluble intracellular inclusions. Typically, the methods to extract PHA would involve lysis of cell wall/membrane, solubilization and purification of PHA component, and precipitation of PHA polymer. The production of PHAs needs to align with eco-friendly downstream operations (Koller et al., 2013). Technological constraints are the main concerns regarding the microbial production of PHAs on a large scale. The recovery efficiency and purity mainly depend on the extraction methods employed to recover the polymer from bacterial cells (Aramvash et al., 2018). Successful large-scale production of PHAs depends on three crucial factors: substrate cost, downstream processing cost, and

process development (Kaur and Roy, 2015). The most common methods for PHA recovery from microbial biomass are solvent extraction and chemical and enzyme-based digestions methods (Tan et al., 2014), and a later approach using mealworms to selectively digest bacterial cell (Ong et al., 2018).

2.3.2.2. Solvent extraction

Solvent extraction remains the most used method of PHA recovery due to its high recovery yield and purity (Abate et al., 2022). Certain solvents that can change the permeability of the cell membrane and selectively dissolve the polymer stored inside are used. These can be chlorinated or non-chlorinated solvents. Pretreatment may be employed to increase the accessibility of the solvent to the polymer. After dissolving the polymer, it is then precipitated at low temperatures with precipitating agents such as methanol or ethanol (Pérez-Rivero et al., 2019). Halogenated solvents, especially chloroform, have been explored to recover PHAs (Desouky et al., 2014; Ratnaningrum et al., 2020; Moorkoth and Nampoothiri, 2016) because they provide good recovery yields, high purity of the extracted polymer, and allow low endotoxin content of the extracted polymer (Rebois et al., 2017). However, halogenated solvents can affect the morphology of PHA granules and are suspected of inducing cancer (Barham, 1990). Non-halogenated solvents are generally considered less harmful than chlorinated solvents. Advantages of halogen-free extraction include solvent reutilization and reduced extraction time, from 12 h to 20 minutes (Koller et al., 2013), safe and environmentally friendly (Mongili et al., 2021), high PHA yield (Filippi et al., 2021), and improves molecular weight characteristic (Bartels et al., 2020).

2.3.2.3. Chemical digestion method

The digestion method is a well-studied alternative to solvent extraction for PHA recovery. In chemical digestion, sodium hypochlorite is used to dissolve non-PHA biomass such as proteins, lipids, carbohydrates, and nucleic acids, thus effectively separating PHA content, which can be recovered by centrifugation. Sodium hypochlorite (NaOCl) digestion has been demonstrated as an efficient and effective method for the disruption of *Burkholderia* sp. B37 (Ratnaningrum et al., 2020), *Bacillus* sp. (Raj et al., 2014; Chang et al., 2021), *Alcaligenes* sp. and *Pseudomonas* sp. (Sayyed et al., 2021) cell walls. However, the hypochlorite digestion method can result in lower molecular masses of the extracted polymer due to severe polymer degradation (Berger et al., 1989).

Surfactants digestion methods are other chemical methods for disrupting bacterial cell walls to recover intracellular PHA. Surfactants enter the bacterial lipid membrane, increasing the cell envelope volume until it bursts. A good example is anionic sodium dodecyl sulphate (SDS), which is the most widely used surfactant for PHA recovery. SDS can recover PHB from *Ralstonia eutropha* high cell density without pretreatment steps and achieve purity of over 95% (Kim et al., 2003).

2.3.2.4. Enzymatic digestion method

An enzymatic process requires milder operating conditions than a chemical process. Enzymes such as proteases, nucleases, phospholipases, lysozymes, and other enzymes can be used to lyse and digest non-PHA biomass and release polymers into the solution (Samrot et al., 2021). The active site of lysozyme and cellulase can hydrolyse the structural bonds of peptidoglycan (a structural polymer that makes up the cell wall of most bacteria) and specifically break the β-1,4-glycosidic bonds between the N-acetylglucosamine and N-acetylmuramic acid of peptidoglycan (Lee et al., 2019). The mechanism could be employed to release the intracellular inclusions of the PHA-producing bacteria. The enzymatic method

can achieve negligible product degradation, high specificity, and low energy requirements (Gonzalez et al., 2021). High recovery (93.2%) of 3-hydroxybutyrate-co-3-hydroxyvalerate (a type of PHA) with 94% purity was recovered from *Cupriavidus necator* using the enzymatic digestion method (Neves and Müller, 2012). After enzymatic hydrolysis, the P(3HB-co-3HV) polymer was recovered by centrifugation. Enzyme-based PHA recovery methods are safer, pose lower health risks, and have a lower environmental footprint as compared to chemical recovery (Tan et al., 2014). However, the enzymes' high costs may increase the extraction cost.

2.3.2.5. General properties and applications of PHAs

Among PHAs, polyhydroxybutyrates (PHB) are comparable to polypropylene (PP), presenting good moisture resistance and excellent gas and aroma barrier properties. PHB is a fragile polymer material due to re-crystallization with ageing at room temperature. Thus, mechanical properties change with time. The considerable interest in commercially exploring these polyesters is because these water-insoluble storage polymers are biodegradable and behave as piezoelectric materials (Bugnicourt et al., 2014). In addition, they are also biocompatible and have the potential for biomedical applications (Brigham and Sinskey, 2012). PHAs have good resistance to hydrolytic attack, UV resistance, and sinks in water, facilitating anaerobic biodegradation in sediments. PHAs also have been shown to possess chiral characteristic molecules that are not superimposable (Boyandin et al., 2013); therefore, their degradation is affected by the composition of the polymer, type of microorganisms, and environmental conditions. It has been reported that different microorganisms produce different PHA-depolymerases to degrade PHAs (Masood et al., 2014). PHAs can dissolve in chloroform and other chlorinated solvents. They have glass

transition temperature, which varies from −50 to 4 °C, and melting temperature from 40 to 180 °C (Padermshoke et al., 2005).

Research has been conducted to incorporate other monomeric units into HB polymer chains leading to copolymers with improved properties (Haddouche et al., 2011; Sheu et al., 2012). The PHBV copolymer has better physical properties such as impact resistance, toughness, flexibility, and other properties involved in the manufacturing process when compared to the PHB homopolymer. In addition, the performance of PHBV can vary greatly when this polymer contains different proportions of the HV monomer, and increasing the HV content in PHBV from 0 to 50% can significantly lower the melting point of the resulting polymer (Wang et al., 2013).

2.3.2.6. Biosynthetic pathways to PHB

PHBs were the first PHA to be discovered, considerably gaining interest, and most studied among other PHAs. PHB was first isolated and characterised by Lemoigne as lipid-like intracellular storage granules from *Bacillus megaterium* (Lemoigne, 1926). PHB is produced in high amounts by microorganisms under optimum conditions, such as physical (pH, temperature, and incubation times), nutritional (carbon, nitrogen sources, and C/N ratio), and biochemical (Hawas et al., 2016). Concerning the metabolic pathways, bacteria produce acetyl-coenzyme-A (acetyl-CoA), which is converted into PHB by three biosynthetic enzymes (Figure 2.6). Under unfavourable conditions of nutrient deficiency and higher carbon sources, microorganisms produce polymers using acetyl-CoA as the initial material. First, the enzyme 3-ketothiolase (phaA) condenses two acetyl-CoAs to acetoacetyl-CoA, acetoacetyl-CoA is reduced by acetoacetyl reductase (phaB) to (R)-3- hydroxybutyryl-CoA, and PHB polymerase (phaC) polymerises (R)-3-hydroxybutyryl-CoA to PHB. All the

enzymes of the metabolic pathway of PHB synthesis are encoded by phaCAB operon (Verlinden et al., 2007).

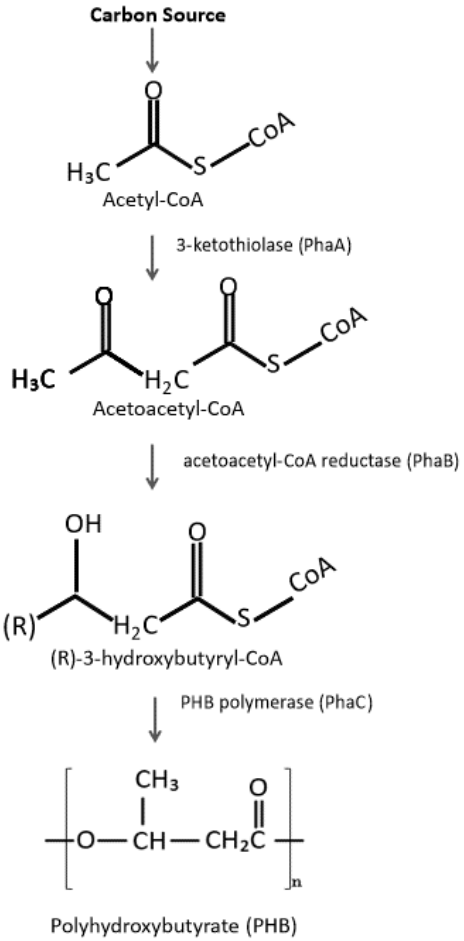


Figure 2.6: Biosynthetic pathways to Polyhydroxybutyrate (Verlinden et al., 2007).

2.4. Integrating conventional plastics degradation and bioplastics production

Many studies have been conducted across the spectrum of plastic degradation and bioplastic production, identifying a wide range of potential bacteria. Many bacteria have produced biopolymers using various carbon sources ranging from sugars and fatty acids (Vastano et al., 2019; Chin et al., 2022; Mohammed & Ray, 2022) to industrial (Bhuwal et al., 2013; Zhu et al., 2013; Clifton-García et al., 2020) and agricultural wastes (Sayyed et al., 2021; Danial et al., 2021). The product price of pure PHAs is influenced by the cost of the carbon

source used, which is a critical challenge in the microbial fermentation process (Kaur & Roy, 2015). Plastic waste is cheap and abundant but detrimental to the environment; thus, plastic wastes have the potential to be used as carbon sources for bioconversion into PHAs. Integrating plastic waste degradation and the production of biopolymers may contribute to mitigating the environmental problem of plastic waste disposal (Figure 2.7). This involves using organic waste to produce high-quality bioenergy and biopolymers. Few studies have concomitantly investigated the integration of plastic degradation and biopolymer production. An approach to achieve this is pyrolysis, in which bacteria use the generated pyrolysis products to accumulate biopolymers. Studies reported the production of 9.8 ± 0.2 % (w/w) PHA from pyrolysed polyethylene (PE) (Guzik et al., 2014) and 0.25 g/L of PHA from pyrolysed polyethylene terephthalate (PET) (Kenny et al., 2008). Another approach was explored with pro-degraded polypropylene (PP), in which oxidative treatment was conducted on PP before being used as an additional carbon source by *C. necator* H16 for PHA production (Johnston et al., 2019). Recent studies have examined the use of microbial enzymes for mixed plastics biodegradation and upcycling, which can provide functional materials that are durable, lightweight, and low-cost (Ballerstedt et al., 2021). To reduce the plastic pretreatment costs, Fujiwara et al. (2021) investigated the direct conversion of PET into PHA by *Ideonella sakaiensis* and reported 0.75 ± 0.09 g/L PHA production in addition to PET weight loss of 7.6 ± 0.6 g/L.

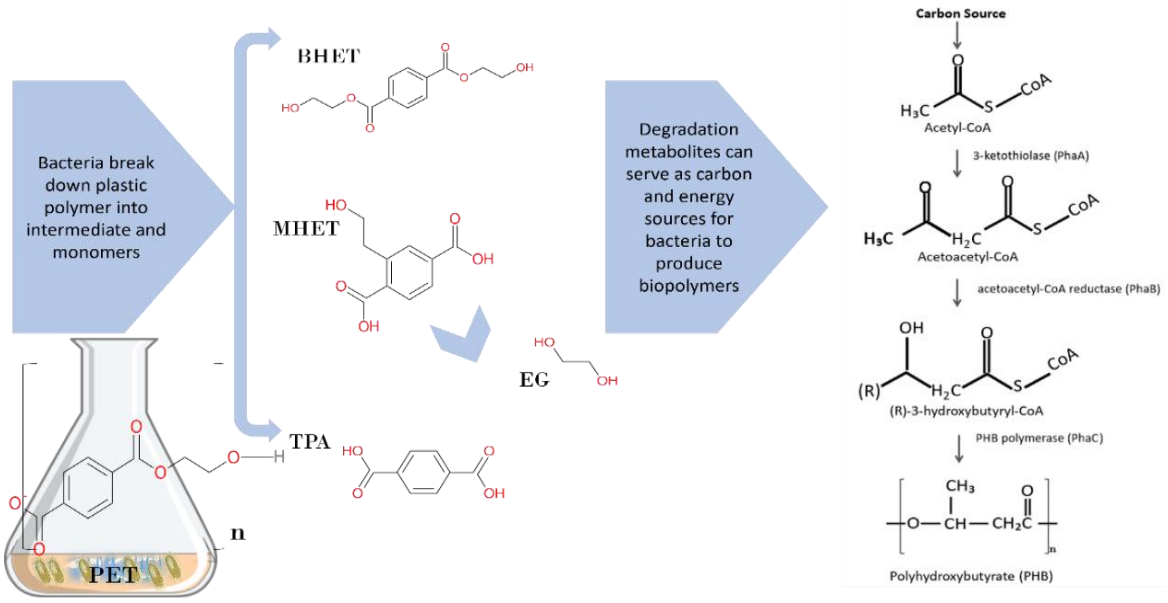


Figure 2.7: Bioconversion of PET into PHB by bacteria. PET is first depolymerised into the intermediates (MHET and BHET) and then into the monomers (TPA and EG). PET depolymerization is based on the model proposed by Austin et al. (2018) and PHB synthesis adapted from (Verlinden et al., 2007).

Other benefits of integrating plastic waste degradation and biopolymer production include; biodegradable polymers are sustainable, renewable, and have good oxygen barrier properties (Samir et al., 2022); the adoption of biodegradable plastics can help with sustainability in materials production and waste management (Moshood et al., 2022), chemical recycling can produce value-added materials and has the potential for a higher yield than mechanical recycling (Lamberti et al., 2020), innovations in bioplastics can provide social benefits while addressing environmental concerns (Nanda et al., 2022).

2.5. Conclusion and future outlooks

Plastics will continue to provide a lot of convenience to people's lives. However, it is also conspicuous that plastics cause detrimental effects and pose ecological threats to the environment. Although many reports demonstrate microbial plastic degradation potentials, none have proven large scale applications. Biodegradation is a slow process and cannot be the solution for the enormous amount of plastic waste dispersed through the ecosystems. Bioplastics must become a real solution, but major research advances are still needed. Reduction in the cost of production, especially in the polymer extraction and blending, resulting in competitive prices of biodegradable polymers, will broaden their range of applications.

3. Chapter Three - Biodegradation of polyethylene terephthalate (PET) by environmentally isolated bacterial consortia

3.1. Introduction

Extensive production and use of plastic materials mismanaged plastic wastes, and inadequate recycling technologies have led to severe environmental plastic pollution. Plastic waste particles have gone beyond just polluting the environment; they directly or indirectly interact with living cells in the form of microplastics (Dehaut et al., 2016; Forrest and Hindell, 2018; Karami et al., 2017; Lehner et al., 2019; Teng et al., 2019; Walkinshaw et al., 2020; Cole and Galloway, 2015; Lusher et al., 2017; Avio et al., 2015; Catarino et al., 2017). In 2020 alone, 367 metric tons (Mt) of plastics have been estimated to be produced (PlasticsEurope, 2021). The following year, global plastic production increased by 6% (PlasticEurope, 2022), and estimates for 2050 are to reach 590 million metric tons (Statista, 2023). Polyethylene terephthalate (PET) is a synthetic aromatic polyester (Nexant, 2009; Yan et al., 2021; Roberts et al., 2020) and one of the most widely produced and used plastics in the world (Chen et al., 2020; Castro et al., 2017). It is synthesised by the esterification reaction of terephthalic acid (TPA) and ethylene glycol (EG). PET can exist in an amorphous and semi-crystalline form (Webb *et al*., 2013). In addition to polyolefins and polystyrene, PET plays a central role in the global packaging industries, especially for beverages, fresh food, and water (Ellen MacArthur Foundation., 2017). Laville and Taylor have reported that more than 480 bn plastic drinking bottles were sold in 2016 across the world, up from about 300bn a decade ago (Taylor & Laville, 2018). Tiseo also reported that in 2019 the global production capacity of PET reached up to 30.5 million metric tons annually and was expected to increase to approximately 35.3 million metric tons by 2024 (Tiseo, 2021). PET contributes to more than 70 % global production of synthetic fibres used mainly in the textile industry (Geyer et al., 2017). These synthetic fibres may end up polluting the natural environment as

microplastics (Zhang et al., 2019; Deng et al., 2020). Although PET is made from highly recyclable materials, only a small proportion is actually recycled because of its high cost and perhaps, loss of properties after multiple recycling cycles (Awoyera & Adesina, 2020).

PET contains a high proportion of aromatic components, which are chemically inert, resulting in resistance to microbial degradation (Webb et al., 2013; Austin et al., 2018; Castro et al., 2019). However, since the microbial colonization of plastic bottles was first reported in the 1970s (Carpenter and Smith, 1972), studies for identifying plastic-degrading microorganisms have not stopped. Several strains from the genera *Aspergillus, Pseudomonas, Penicillium, Clostridium, Ideonella, Streptomyces*, *Delftia*, *Microbacterium, Thermobifida*, and *Bacillus* were reported in recent years to have the capabilities of degrading PET (Yan et al., 2021; Yoshida et al., 2016; Taghavi et al., 2021; Wilkes and Aristilde, 2017; Yan et al., 2021). Several reports on PET biodegradation use enzymes isolated from microorganisms (Teotia et al., 2017). Fungal and bacterial polyester hydrolases have exhibited hydrolytic activity against PET films and fibres (Chen et al., 2013; Wei et al., 2014; Wei and Zimmermann, 2017). For instance, Barth et al. (2015) used polyester hydrolase from *Thermobifida fusca* to hydrolyse PET films.

Identifying bacterial consortia that possess the enzymatic capability to degrade PET and its monomers can facilitate the development of recycling and remediation methods for plastic wastes. The present study aimed to isolate bacterial consortia from different environmental samples and to assess their biodegradation capacity of highly crystalline PET in a liquid matrix.

3.2. Materials and methods

3.2.1. Chemicals

PET granules (nominal granule size: 3-5mm and >50% crystallinity) were obtained from Goodfellow (Cambridge, England), and terephthalic acid (TPA) (185361-500G, purity >98%, Saint Louis, USA), Sodium acetate trihydrate (193643), Sodium dodecyl sulphate (8170341000), Darmstadt, Germany, Orthophthalic acid (402915- ST. Louis United USA) were purchased from Sigma.

3.2.2. Pretreatment of PET granules

PET granules were exposed to ultraviolet (UV) radiation for 24 hours with continuous stirring in distilled water to obtain uniform exposure, followed by sterilization by autoclave. The weight of PET granules in each experimental replicate was determined by placing eight granule pieces on a weighing scale and recorded.

3.2.3. Sediment and water sample collection

Samples were collected from different sites in Macao SAR, China (table 3.1); sediment samples were collected at City green space municipal park (22°12'58.7"N 113°32'37.7"E) (GPB12), water samples at Cheoc Van (22°06'47.2"N 113°33'41.0"E) (CW1), mangrove sediment at Ka'Ho (22°08'07.1"N 113°34'50.9"E) (KH27), mangrove sediment at ecological zone 2 (22°08'30.8"N 113°33'05.8"E) (EC2-30), sediment scrapings adhered to a plastic waste surface at wetland Avenida da Praia (22°09'12.2"N 113°33'36.0"E) (LZG16), and sludge samples from a wastewater treatment plant (22°12'47.2"N 113°32'05.6"E) (WTP31B-5) (Figure 3.1). Sediment samples were collected from a 3-4 cm depth using a stainless spatula, wrapped in aluminium foil, and stored in a refrigerator until the commencement of the experiments.

Table 3.1: Sampling sites in Macao for environmental consortia and their designated codes.

S/N	Sample sources	Codes
1	Mangrove sediment ecological zone 2	EC2-30
2	Mangrove sediment Ka'Ho	KH27
3	Sediment scraping from a plastic surface on a wetland	LZG16
4	Sediment from Cheoc Van	CW1
5	City green space municipal park	GPB12
6	Wastewater treatment plant	WTP31B-5

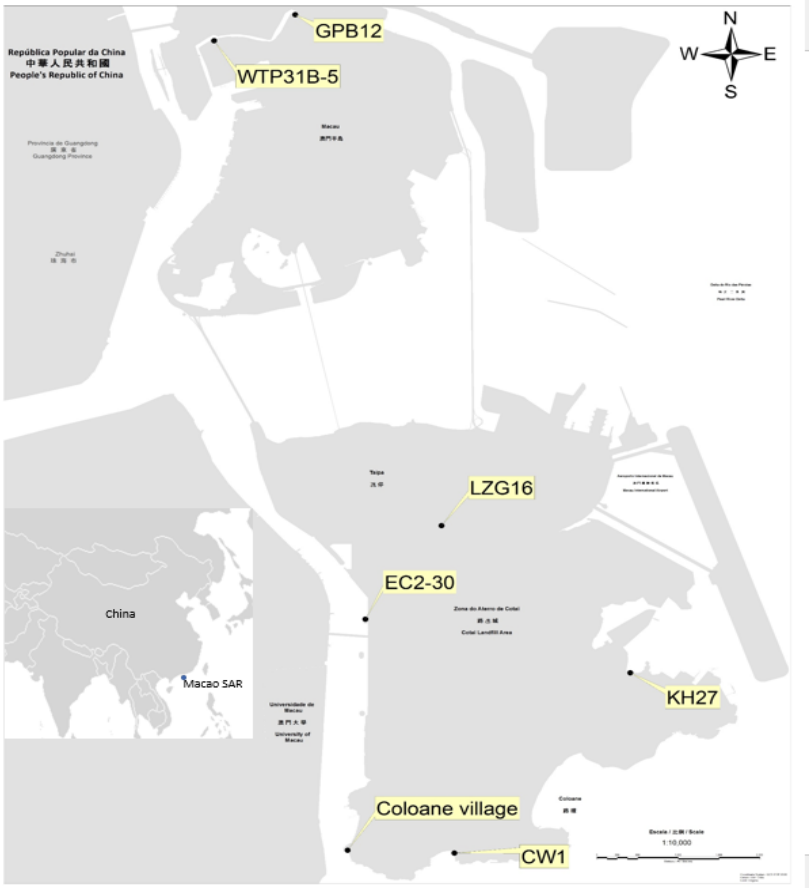


Figure 3.1: Locations of sampling sites for environmental consortia around Macao SAR.

3.2.4. Isolation of bacterial consortia

For the isolation of bacteria, 1g of all samples were suspended separately in 9ml sterile saline solution and serially diluted. For water samples, 1 ml was mixed in 9 ml sterile saline solution and serially diluted. Dilutions 10^{-3} to 10^{-5} were plated on nutrient agar (agar 15 g/L and nutrient broth 25 g/L) and incubated at 30 ºC for 24-48 hrs.

3.2.5. Screening of PET-degrading bacteria

The obtained bacterial consortia were screened for their ability to degrade PET on sterile minimal salt media (MM) (Moreira et al., 2013) on agar plates. Plate cultures were incubated

at 30ºC for 10-15 days. The bacteria that grew around the edges of the PET granules were selected, subcultured in nutrient broth, and stored at -80ºC for further experiments.

3.2.6. Microbial degradation of PET granules by consortia

The inoculum culture of the isolated consortia CW1, KH27, LZG16, and EC2-30 were pre-grown for 24 hours in a 100ml Erlenmeyer flask containing 10 ml minimal media and 5.9 mM sodium acetate as carbon source. After 24 hours, each consortium was inoculated at a concentration of 8.0 x 10^7 cells/ml into a 250 ml Erlenmeyer flask containing 75 ml of sterile carbon-free minimal media at a pH of 7.0 (Vague et al., 2019). Approximately 0.15 g of the pretreated PET granules were supplied to the flasks. Flasks were incubated at 30 ºC with an agitation of 130 rpm for 55 days. All the experiments were performed in triplicate. The bacterial growth was monitored by measuring optical density (OD) at 600 nm. An aliquot of the culture media was periodically collected, centrifuged at 13500 x g for 10 minutes, and stored for further quantitative analysis. Triplicate control flasks without inoculation were also performed. Sodium acetate solution (5.9 mM) was added to the cultures on days 3, 14, 24, and 31 to boost bacterial growth.

3.2.7. Degradation of the PET granules

After the incubation period of 55 days, the PET granules were washed with 0.2 % aqueous sodium dodecyl sulphate (SDS) solution for 4 hrs; then rinsed with distilled water and 75 % alcohol (Ambika et al., 2014). The washed PET granules were placed on a glass petri dish and dried overnight at 60 ºC before weighing. After drying, the PET granules' weight loss expressed as a percentage (%) was the difference in weight of the granules before and after the experiment. The following formula was used to determine the percentage degradation:

$$Percentage(\%)\ degradation = \frac{Initial\ Weight - Final\ Weight}{Initial\ Weight} X\ 100$$

3.2.8. FTIR analysis

The infrared absorption (IR) spectrum of the PET granules was recorded with a Perkin Elmer Spectrum 100, USA. The washed and dried PET granules were scanned between 450 and 4000 cm^{-1}.

3.2.9. High-performance liquid chromatography (HPLC) analysis

High-Performance Liquid Chromatography was performed to evaluate the presence of the PET degradation by-product, terephthalic acid (TPA). A reverse-phase SymmetryShield RP8 5 µm 3.9x150 mm column (Lot No. 0153300741, Part No. WAT200655) was used, and a detection at wavelength 242 nm. Orthophthalic acid was used as the internal standard, and terephthalic acid had retention times of 7.2 min and 8.4 min, respectively. The injection volume was 20 µl, and the flow rate was 0.8 ml/min. The mobile phase was prepared by mixing an equal volume of methanol with sodium acetate trihydrate buffer solution. The pH was maintained at 3.6.

The terephthalic acid stock solution was prepared by dissolving terephthalic acid in methanol (500 mg/L). To obtain the calibration graphs, the terephthalic acid's peak area was divided by the orthophthalic acid’s peak area.

3.2.10. Statistical analysis

One-way ANOVA was computed using AI-therapy statistics (https://www.ai-therapy.com), to compare the PET weight loss difference among the consortia. A significance level of $p < 0.05$ was used.

3.3. Results

3.3.1. Bacterial growth

Bacterial growth was not observed in the first few days of the experiment. At the third day of the experiment, sodium acetate (5.9 mM) was added, to promote bacterial growth (Figure 3.2). The bacteria remained viable at day 55 in the consortia CW1, KH27, LZG16, and EC2-30 with a density of 1.62 x 10^8, 1.3 x 10^8, 1.33 x 10^8, 1.67 x 10^8 cells/ml, respectively. The bacterial viability may be caused by the presence of acetate in the medium.

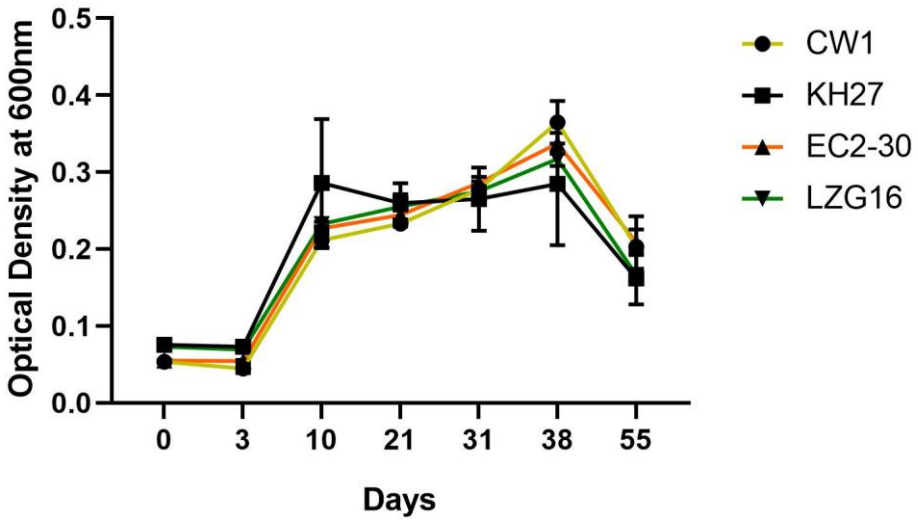


Figure 3.2: The graph shows the growth pattern of the four bacterial consortia assayed in minimal media with crystalline PET granules for 55 days. Sodium acetate was added to boost the growth of the consortia.

3.3.2. Weight variation of PET granules

The PET weight loss observed after 55 days was negligible (Table 3.2). Statistical analysis revealed no differences in the weight loss among the consortia and in relation to the controls without bacteria, which was not statistically significant ($F_{(4,10)} = 0.424$, $p = 0.788$).

Table 3.2: PET weight differences observed after 55 days of biodegradation experiment.

S/N	Consortia	Initial weight (g)	Final weight (g)	Weight difference (g)	Weight loss (%)
1	EC2-30	0.1502 ± 0.00	0.1488 ± 0.00	0.0014 ± 0.00	0.9097 ± 0.54
2	KH27	0.1501 ± 0.00	0.1482 ± 0.00	0.0017 ± 0.00	1.1545 ± 0.95
3	LZG16	0.1492 ± 0.00	0.1477 ± 0.00	0.0015 ± 0.00	1.0004 ± 1.67
4	CW1	0.1501 ± 0.00	0.1488 ± 0.00	0.0014 ± 0.00	0.9127 ± 1.47
5	Control	0.1504 ± 0.00	0.1503 ± 0.00	0.0001 ± 0.00	0.0887 ± 0.08

3.3.3. Chemical and structural changes of the PET granules

FTIR analysis of the PET granules revealed spectra stretch and shifts. Peak spectra were compared with UV-pretreated PET granules and the control to detect the main spectral changes. The consortium KH27 induced major stretching, indicating C-H vibrations at the peak region of 2800 to 3000 cm^{-1}, less pronounced with consortia LZG16 and EC2 (Figure 3.3). The consortia induced a shift in several bands, as shown in Table 3.3.

Table 3.3: Summary of the spectral shifts, stretches, and intensities obtained when the crystalline PET was subjected to FTIR analysis after 55 days of biodegradation experiment.

Consortia	Observed Peak characteristics		
	Shifts (cm^{-1})	**Stretches (cm^{-1})**	**Higher intensities (cm^{-1})**

CW1	1096 to 1092		
	1505 to 1506		
	845 to 848		
LZG16	1243 to 1241		3419
KH27	1505 to 1506	2800 to 3000	1963
	845 to 846		3422
EC2-30	1505 to 1504		3443
	1712 to 1711		
	845 to 849		

Disappearance of peaks were observed in the experimental PET granules exposed to the consortia. For instance, the peaks observed in the UV-treated PET granules at 1505, 1471, and 845cm^{-1} disappeared in the assay with the consortium LZG16; peaks at 1043 and 791cm^{-1} disappeared with the consortium EC2-30; and peak 2969 cm^{-1} disappeared in the assay with the consortium KH27. The band 1712cm^{-1} was induced by UV pretreatment.

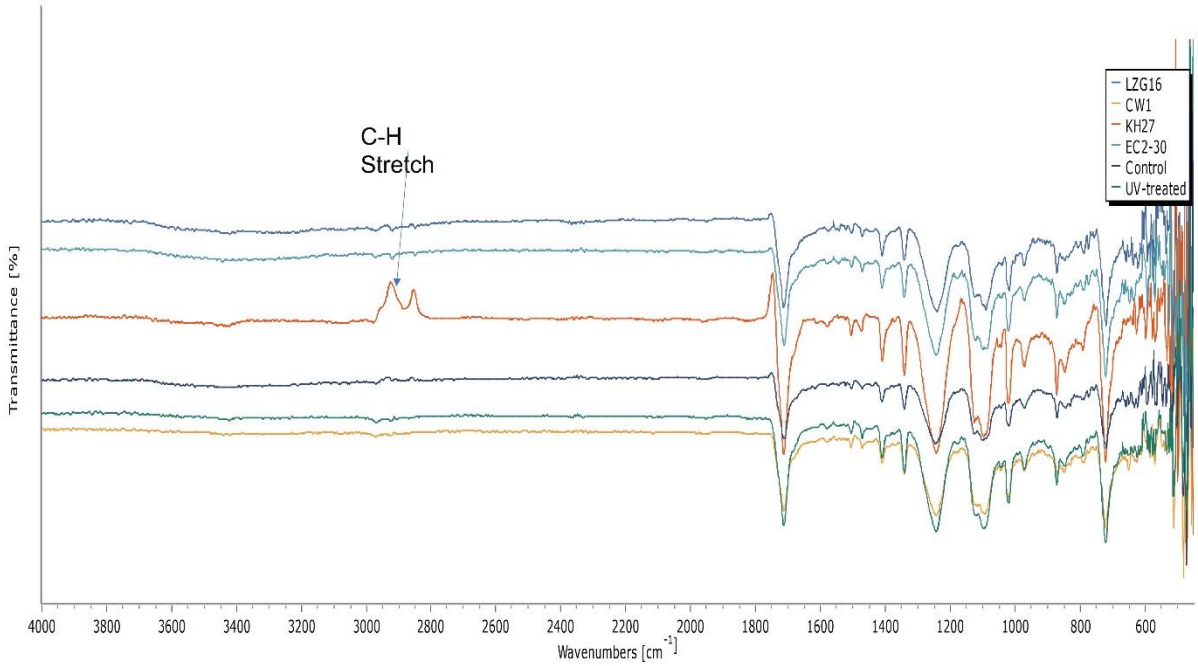


Figure 3.3: FTIR spectra of UV-treated PET granules before and after incubation in minimal media with consortia CW1, KH27, LZG16, EC2-30 for 55 days, and control without consortium.

3.3.4. Quantification of TPA

HPLC analysis was performed targeting the detection of TPA as one of the possible PET degradation products. TPA was not detected in any of the samples (Annex).

3.4. Discussion

This study assessed the potential of biodegradation of PET by bacterial consortia obtained from different environments. The highest PET weight loss observed was 1.07 % in 55 days by consortium KH27 (table 3.2), obtained from a mangrove environmental sample. However, PET weight differences among the different consortia and the control without bacteria was not statistically significant. The very low degradation observed might be due to the high

crystallinity of the PET samples or to the low metabolic activity of the consortia toward PET, as crystalline PET has limited accessibility of the ester linkages to catalytic depolymerisation. Anyway, the observed PET weight loss for the consortium KH27 was higher than the PET degradation (0.6%) reported by Taghavi et al. (2021), where it was demonstrated the biodegradation of PET and other plastics materials using naturally occurring microbial strains examined in mixed and individual systems for 270 days. A study by Auta et al. (2017) demonstrated a 3.0% PET weight loss after 40 days under the influence of *Bacillus* strains. Moreover, the PET degradation product TPA was not detectable in any of the samples after the degradation experiment. However, the presence of other PET degradation products, such as bis(2-hydroxyethyl terephthalate (BHET) and mono(ethylene terephthalate) (MHET), which were not evaluated in this study, or the transient formation of these molecules, would still be possible. Other researchers have reported the release of MHET and TPA during the enzymatic degradation of PET film/powder (Pellis et al., 2016). Yoshida et al. (2016) also reported the release of BHET, MHET, and TPA during the degradation of PET film by *Ideonella sakaiensis*.

The FTIR spectroscopic analysis conducted in the range 400 to 4000 cm^{-1}indicated some signs of bacterial degradation of the PET film. Prior to the biodegradation experiment, PET granules were subjected to pretreatment by UV radiation to enhance plastic degradation (Falkenstein et al., 2020; Esmaeili et al., 2013; Hadad et al., 2005). Photodegradation-based pretreatment has proven beneficial (Restrepo-Flórez et al., 2014) and is thus considered a potential to mitigate the challenges of biodegradation caused by polymer microstructures (Gewert et al., 2015). Based on the FTIR results information (Figure 3.3), it appears that there were changes in the chemical structure of the crystalline PET granules. The evolution of new infrared bands at 1963 cm^{-1}, assigned to C=C=C stretching, and 3422 cm^{-1}, assigned to N-H stretching (primary aliphatic amine) (consortium KH27), 3419 cm^{-1} (consortium

LZG16), and 3443 cm^{-1} (consortium EZ2-30) were observed. The appearance of these new bands suggests the occurrence of changes in the chemical structure of the crystalline PET granules. Additionally, the C-H vibrations at the peak region of 2800 to 3000 cm^{-1} observed in consortium KH27 also indicate changes in the chemical structure of the crystalline PET granules. The appearance of new peaks can be used to identify oxidation products formed at different frequencies (Torena et al., 2021). The disappearance or addition of functional groups is considered essential to the mechanism of biodegradation and related to microbial activities (Helen et al., 2017; Naz et al., 2013; Skariyachan et al., 2017). These changes were due to the adherence of the microorganisms, which altered the PET through oxidation reactions (Wilkes and Aristilde, 2017). Therefore, in the present study, the FTIR results indicated an interaction between the consortia and the PET samples, supporting the assumption of the ability of the consortia for PET biodegradation.

Thermophilic microorganisms are quite often employed for studies of polymer biodegradation, including bacteria of the genus *Thermobifida*, *Actinomycetes*, *Brevibacillus*, and *Geobacillus* (Wei et al., 2014; Yan et al., 2021; Muhonja et al., 2018; Pramila et al., 2012). Our research examined the biodegradation of PET plastics using mesophilic bacteria isolated from an anthropogenically stressed environment, including mangroves coastal areas and old waste landfill sites (Vague et al., 2019; Esmaeili et al., 2013; Then et al., 2015, 2016). We also explored the diverse microorganisms from sludge samples with the potential to degrade plastic polymers. Microbial communities from sludge samples have been shown to have biodegradation capabilities when exposed to PET (Yoshida et al., 2016; Torena et al., 2021).

Microbial attachment and subsequent degradation of polymers are most likely to occur at the surface as opposed to the bulk material. Surface availability could be explained as a crucial factor. The role of microbial attachment to plastic surfaces and its correlation to biodegradation was well studied by several researchers (Das & Kumar, 2015; Ghosh et al., 2019; Tribedi & Sil, 2013).

Limited reports on the bacterial degradation of highly crystalline PET with significant weight loss are available. For instance, *Vibrio* sp. has been reported to degrade 35% of PET from bottle samples for a period of six weeks (Sarkhel et al., 2020). The result outcomes of our study may be attributed to polymer characteristics, the activity of microorganisms, and degradation conditions. Crystallinity is one of the vital polymer characteristics that can affect microbial attacks on PET polymers (Wei and Zimmermann, 2017; Kawai et al., 2019). PET biodegradation rate depends on polymer crystallinity, purity, and orientation (Mohanan et al., 2020). The high degree of crystallinity is representative of the dense structure of the PET, which is a significant reason why PET is not readily biodegradable (Gong et al., 2018; Maurya et al., 2020). Hence, biodegradation may be hampered.

Taken together, the results suggest that some of the tested consortia had the ability to modify the crystalline PET granules as indicated by the changes observed in the chemical structure, as revealed by the FTIR data. The high crystallinity of the PET granules used in this study might underly the absence of more significant biodegradation.

4. Chapter Four - The role of mangroves-associated bacteria in the biodegradation of PET

4.1. Introduction

The mangrove ecosystem is a unique coastal wetland which acts as a vital carbon reservoir with high productivity (Alongi, 2014). Mangroves also play a crucial role in coastal protection and ecological purification of water (Barbier, 2016). In addition, the well-developed root system enables mangrove plants to thrive against dynamic environmental stress and act as traps for marine litter (Martin et al., 2019; Srikanth et al., 2015). Phytoremediation is a sustainable remediation process that uses plants and rhizosphere microorganisms to degrade, metabolise, transform, assimilate, or remove harmful contaminants from various environments (Jha, 2020). A study on microplastic degradation pointed out mangrove rhizospheres as potential candidates for plastic degradation (Xie et al., 2021).

Polyethylene terephthalate (PET) monomers, monoethylene glycol (MEG) and terephthalic acid (TPA), and intermediate Bis(2-hydroxyethyl) terephthalate (BHET) are essential ingredients for PET synthesis. The latter is a commercial product with a similarity to the core structure of PET and has been widely used as a model for studying PET biodegradation (Joo et al., 2018). BHET, mono-(2-hydroxyethyl) terephthalate (MHET), and TPA are reported as the major degradation products of PET during the enzymatic hydrolysis and chemical recycling process (Vertommen et al., 2005; Salvador et al., 2019). BHET has also been studied as a model molecule for PET recycling (Ion et al., 2021). Currently, there are no reports of toxicity and pollution from BHET (Qiu et al., 2020), while TPA has been reported to cause bladder stones and bladder cancer, as well as impairment of liver and

testicular functions (Zhang et al., 2010; Ordaz-Cortés et al., 2014). Prolonged MEG exposures can cause varying toxicity levels depending on the exposure concentration (Iqbal et al., 2021). Only a few reports are available showing a bacterial strain or consortia to degrade PET monomers and intermediate.

Identifying bacterial consortia that possess the enzymatic capability to degrade PET and its monomers can facilitate the development of recycling and remediation methods for plastic wastes. The present study aimed to assess crystalline PET films biodegradation in mangrove soil with and without mangrove plants and bioaugmentation with bacterial consortium. Bacterial consortia were retrieved from the soil of all treatments and further used to evaluate the biodegradation of PET monomers, terephthalic acid, monoethylene glycol, and bis (2-hydroxyethyl) terephthalate.

4.2. Materials and methods

4.2.1. Chemicals

Monothylene glycol (MEG) (1075477-500 ml, International Laboratory, USA), Bis(2-hydroxyethyl) terephthalate (BHET) (465151-100G, Saint Louis, USA), and Terephthalic acid (TPA) (185361-500G, purity >98%, Saint Louis, USA) were purchased from Sigma. PET films (ES30-FM-000200, thickness 0.1mm, crystalline) were obtained from Goodfellow (Cambridge, England). PET films were cut into $1cm^2$ squares, and their initial weights were determined and recorded.

4.2.2. Experiment 1 - Biodegradation of PET in soil

4.2.2.1. Plant material and soil

Mangrove plants (*Kandelia* spp.) were collected from the plants nursing centre, Helen Garden Coloane, Macao SAR, and 12 saplings were selected for the experiment. Biometric parameters height and weight of the mangrove plants were recorded. Non-sterile garden soil (decomposed leaves humus) was purchased from, DAISO Japan.

4.2.2.2. Microorganisms

Bacterial consortia for bioaugmentation consisted of isolates Bacillus sp. GPB12 (isolated from city Green park samples - an old landfill, Macao SAR, China) and Enterococcus sp. WTP31B-5 (wastewater sludge samples, Macao SAR, China) were grown in Nutrient broth for 24 hours and an equal proportion of 2.3 x 109 CFU/ml cell density was inoculated in the soil for the bioaugmented treatment.

4.2.2.3. Experimental design

Each experimental glass and round bottom pot (24 x 24 x20 cm) is half-filled with soil (3.5 kg) and contains six pieces of pre-weight PET films, each buried at 4 cm depth and 4 cm apart, following the method by Janczak et al. (2018) with some modifications by adjusting the size and number of plastic films, experimental treatments, replicates, bacterial consortium, and plants. Three treatments were conducted: treatment A pots contained only soil and PET films (control); treatment B pots contained soil, PET films, and one mangrove plant; treatment C pots contained soil, PET films, one mangrove plant, and the bacterial consortium described above (Figure 4.1). The consortium was inoculated at a cell density of 2.3×10^9 CFU/ml, following the protocol of Dąbrowska with some modification (Dabrowska

et al., 2014) by augmenting with more than one bacterial sp. The experiment was performed in quadruplicate. The experimental pots were evenly arranged in the open university garden on the levelled ground under a roof to prevent waterlogging from raining. The experiment was set under environmental conditions for 270 days. After the experimental period of 270 days, the mangrove plants were uprooted, and the final lengths and weights were determined. The pH of the soil was also measured at the end of the experiments.

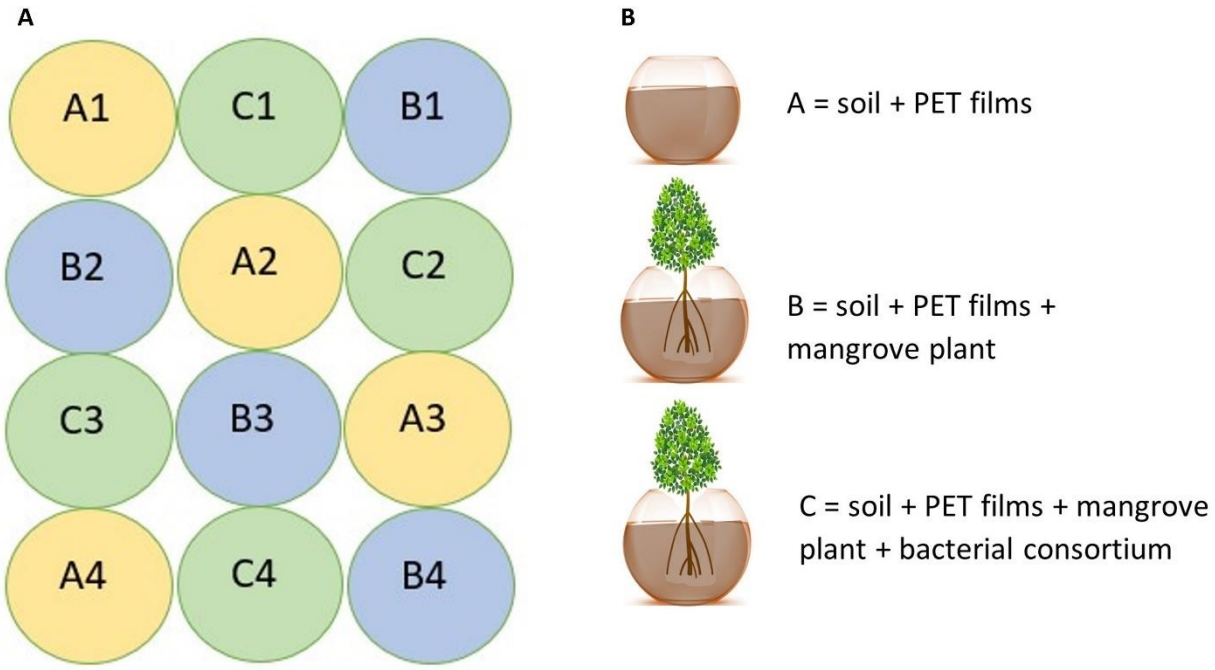


Figure 4.1: (A) Experimental pots arrangement for biodegradation of PET films and (B) experimental design.

4.2.2.4. Molecular characterisation of soil bacterial community

4.2.2.4.1. Sample collection

Soil samples were collected from all the treatments at the experiment's beginning and end. The soil samples were taken at 4 cm depth. Approximately 1g of the soil samples was collected in a cleaned plastic bag and stored at -20 ^{o}C for further analysis.

4.2.2.4.2. DNA extraction

To isolate metagenomic DNA, soil samples were subjected to DNA isolation using Power soil DNA isolation kits (Qiagen, USA) in accordance with the manufacturer's protocol. The purity and integrity of isolated DNA were determined using Nanodrop (Qiagen, USA). DNA samples were then sent for V3 and V4 16s rRNA sequencing.

4.2.2.5. Determination of the dry weight of the residual PET films

After the incubation period of 270 days, the PET films were manually retrieved from the soil and washed with 1% aqueous sodium dodecyl sulphate (SDS) solution for 4 hrs; then rinsed with distilled water and 75% alcohol (Ambika et al., 2014). The washed PET films were placed on a glass petri dish and dried overnight at 60ºC before weighing. After drying, the final weight of the PET films was determined. The weight loss expressed as a percentage (%) was the difference in weight of the PET films before and after incubation, using the formula below.

$$Percentage(\%)\ degradation = \frac{Initial\ Weight - Final\ Weight}{Initial\ Weight} X\ 100$$

4.2.2.6. ATR-FTIR Measurements

ATR-FTIR spectra were recorded with Nicolet IS50 (Thermo Scientific, USA) spectrometer, coupled with an Attenuated Total Reflectance (ATR) sampling accessory using a wavenumber range of 500–4000 cm^{-1}. The carbonyl index was calculated as the ratio of the intensity at the band absorbance of the carbonyl group (C=O, 1715 cm^{-1}) and the methylene group (CH_2, 1465 cm^{-1}) (Katarzyna et al., 2018)

4.2.2.7. Scanning electron microscopy (SEM)

The recovered PET films were washed with 1 % aqueous SDS solution for 4hrs, rinsed with distilled water and 75% alcohol, and then dried at 60^{o}C. The PET films were sprayed with gold particles and subjected to SEM observation.

4.2.3. Experiment 2 - Biodegradation of bis(2-hydroxyethyl) terephthalate (BHET), monoethylene glycol (MEG), and terephthalic acid (TPA) with consortia from mangroves soil.

4.2.3.1. Screening for TPA, MEG, and BHET degrading bacteria.

Bacterial consortia were isolated from soil samples after the PET film biodegradation experiment (section 4.2.4). A soil sample (1 g) was collected from each replicate and screened for BHET, MEG, and TPA degrading bacteria.

The soil samples were inoculated into a 250 ml flask containing 90 ml of sterilised minimal media (Moreira et al., 2013) at pH 7. Three set of flasks were used. Each set of flasks was supplied with one of the compounds - 1000 mg/L of BHET, terephthalic acid TPA, and 1113 mg/L of monoethylene glycol MEG.

Flasks were incubated at 30°C and 200 rpm for five days. After this period, the cultures were plated on agar media (15 g/L) containing the respective compounds (TPA, MEG, and BHET) and further incubated for five days. After incubation, colonies were aseptically retrieved and stored in 20 % glycerol stock at -80°C for further assays.

4.2.3.2. Biodegradation assays of TPA, MEG, and BHET

The isolated bacterial consortia were subcultured and used for biodegradation assay of BHET, MEG, and TPA. Three assays were conducted and each biodegradation assay contains one of the compounds at a concentration of 1000 mg/L for TPA and BHET and 1113 mg/L for MEG as the carbon sources in minimal media (Suwanawat et al., 2019; Qiu et al., 2020). The bacterial consortia were inoculated at an initial density of 8.0 x 10^7 cells/mL. The assay was conducted in quadruplicate. After inoculation, culture flasks were incubated at 30°C and shaken at 200 rpm for ten days. Three control sets were performed: minimal media and the target compounds (TPA, BHET, MEG) without bacterial inoculation; minimal media and bacterial inoculation without the compounds; minimal media, the target compounds, and heat-inactivated consortia to evaluate adsorption.

Bacterial growth was monitored by spectrophotometry at $OD600_{nm}$, (Biowave II, UK). Aliquot samples were aseptically collected daily and centrifuged at 13500 x g for 10 min. Supernatants were stored at -20 °C for further analysis.

4.2.3.3. HPLC and GC analysis

The residual concentration of BHET and TPA was analysed by high-performance liquid chromatography (HPLC).- The HPLC analyses were performed on a System Gold 126 (Beckman Coulter, Fullerton, USA) using a reversed phase 250–4 HPLC Cartridge LiChrospher 100 RP-18 column (Merck, Darmstadt, Germany), operated in isocratic mode at room temperature, with a flow rate of 0.8 ml/min and an injection volume of 20 µL. Acetonitrile/water (60:40, V/V) acidified to pH 2 with TFA was used as the mobile phase.

MEG was analysed by gas chromatography (GC) using a gas chromatograph Varian CP-3800 (Agilent Technologies, California, USA) and a CP-Wasc 52 CP capillary column (Chrompack International B.V., Middelburg, The Netherlands), using a temperature

program starting at 80 ºC for 2 min, increasing to 180 ºC at a rate of 10 ºC min^{-1} with 5 min hold. Injector and detector temperatures were 250 ºC. Degradation rate constants were calculated assuming first-order kinetics. With this model, the residual concentration changes with time (t) were determined according to the following relationship: $C = C_{0e}$-kt, where C_0 is the initial concentration and k is the degradation rate constant. The half-life of biodegradation ($t_{1/2}$) is estimated from k using $t_{1/2} = \ln 2/k$.

4.2.3.4. Total organic carbon (TOC)

TOC was evaluated with a Vario TOC cube (Elementar Analysensysteme GmbH, Langenselbold, Germany). The combustion tube with the sheath tube, ash crucible, quartz chips (15 mm), Pt-Kat (25 mm), quartz chips (85 mm), and quartz wool (5 mm) was set up from top to bottom. A standard working solution (500 mg/L) of KHP and Na_2CO_3 in Milli-Q water was prepared and further diluted with Milli-Q for the standard curve measurements. Synthetic air (around 1000 mbar, purity 99.995%) was used as the operating gas with a gas flow of 200 mL/min and the combustion tube temperature set to 680 ºC. The instrument to measure TC (total carbon), TIC (total inorganic carbon), and TOC (total organic carbon) and samples were analysed (time zero and final) by recording the levels of TC and TIC. TOC was calculated by subtracting TIC from TC. TOC was also assessed in the control experiment with heat-inactivated consortia to check for adsorption.

4.2.4. Statistical analysis

A paired t-test (two-tailed) was conducted to compare the overall initial and final weight of PET films. A general linear model with the repeated measured test was performed using

SPSS (IBM SPSS Statistics 28.0.0.0) to compare the degradation of TPA, MEG, and BHET within the time points and between the treatments.

4.3. Results

4.3.1. Biodegradation of PET in soil

4.3.1.1. Mangrove plants and soil parameters

After the experimental period of 270 days, the mangrove plants were uprooted, and the final lengths and weights were determined (table 4.1). The pH of the soil was also measured at the end of the experiments.

Table 4.1:Parameters of soil and mangrove plants before and after the experimental period of 270 days. Abbreviations: Li – initial length, Lf – final length, Wi – initial weight, Wf – final weight, pHi – initial pH, pHf – final pH.

Treatments	Mangrove L^i (cm)	Mangrove L^f (cm)	Mangrove W^i (g)	Mangrove W^f (g)	Soil pH^i	Soil pH^f
A	NA	NA	NA	NA	7.57 ±0.01	7.22 ±0.09
B	56.50 ± 16.23	43.27 ±15.98	56.45 ±34.08	50.67±27.94	7.40 ±0.02	7.36 ±0.09
C	49.07 ± 1.67	45.50 ±13.50	40.64 ±24.26	39.08±25.94	7.36 ±0.02	7.53 ±0.03

4.3.1.2. Residual weights of PET films

A low PET weight loss in all treatments was observed (table 4.2). There was a slightly higher weight loss of PET films in the presence of mangrove plants (treatments B and C). The overall PET weight difference between the initial and final in all treatments was statistically significant ($p<0.001$). However, multiple weight difference comparisons among the treatments were not statistically significant ($F_{(2,9)} = 0.273$, $p = 0.767$).

Table 4.2: Initial and final average weight of PET films after the experimental period of 270 days. Abbreviations: Wi – initial weight, Wf – final weight.

Treatments	N	PET films W^i (g)	PET films W^f (g)	Weight loss (%)
A	4	0.0840 ± 0.00	0.0839 ± 0.00	0.0899 ± 0.10
B	4	0.0854 ± 0.00	0.0853 ± 0.00	0.1162 ± 0.10
C	4	0.0847 ± 0.00	0.0846 ± 0.00	0.1182 ± 0.00

4.3.1.3. Chemical and structural changes on the pet films

The chemical structures of the biodegraded PET films were analysed through ATR-FTIR spectra. The analysis was performed to examine the surface changes in the chemical structures of the PET films after 270 days. Figure 4.2 shows peak shifts observed in all the treatments compared to the control PET film (not buried in the soil). Peaks at 746 cm^{-1} – assigned to C-H bending, shifted to 744 cm^{-1} - (treatment A), 745 cm^{-1} (treatments B and C); peaks at 1429 cm^{-1} – assigned to O-H bending (carbonyl group), shifted to 1421 cm^{-1} (all treatments); 1491 cm^{-1} shifted to 1490 cm^{-1} (treatment A), 1480 cm^{-1} (treatments B and C); 1519 cm^{-1} shifted to 1511 cm^{-1} – assigned to N-O stretching (treatments B and C). New peak intensities appeared at 3992 cm^{-1} – assigned to O-H stretching (alcohol group) (treatment A) and 808 cm^{-1} – assigned to C-H stretching (aromatic ring) in treatment C. The carbonyl index is up to 1.17. All the surface chemical and structural changes detected by FTIR analysis confirm that PET films were degraded. These changes were more significant in samples from treatments with mangrove plants (B and C).

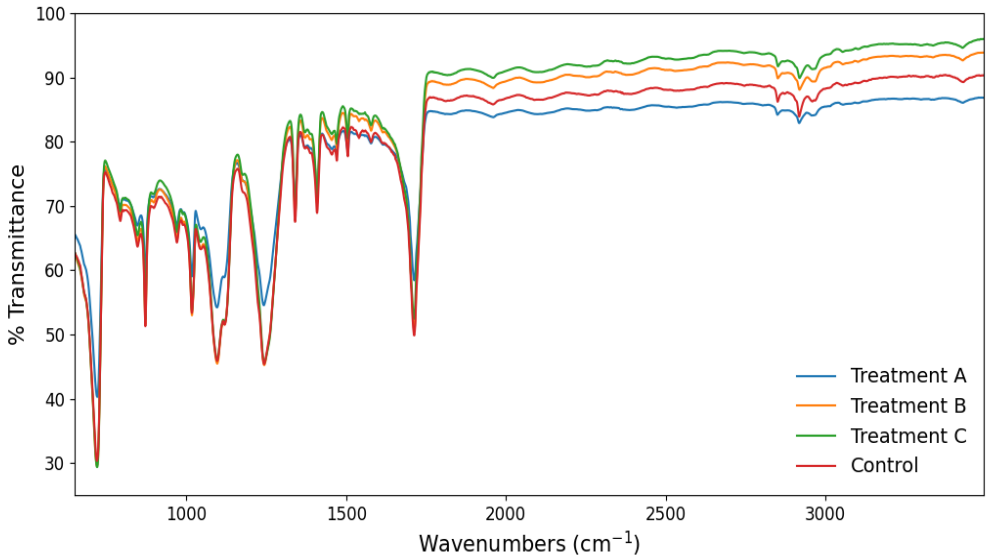


Figure 4.2: FTIR spectra of PET films for the experimental treatments A, B, C, and control after 270 days biodegradation period in soil with and without mangrove plants.

4.3.1.4. SEM analysis of PET films

The morphologies of the PET films were assessed after the experimental treatments. The surface of PET films in treatments with mangroves (B and C) showed roughness and abnormal drape, indicating disruption of PET films because of degradation. In comparison, no significant damages were observed on the PET in treatment (A) and untreated PET film (D), as shown in Figure 4.3.

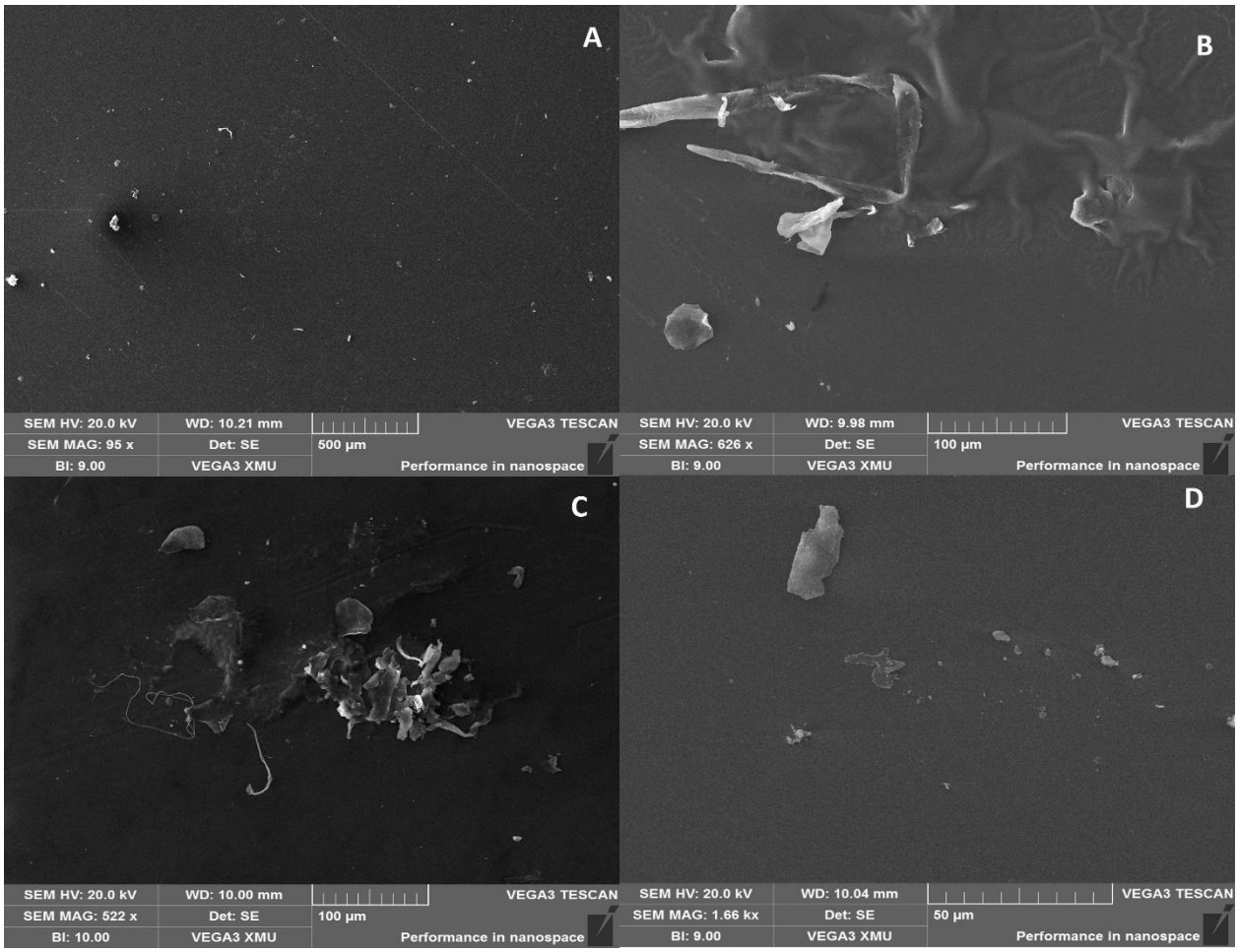


Figure 4.3: SEM of PET films after 270 days of biodegradation experiment. (A) treatment A, (B) treatment B, (C) treatment C, and (D) untreated PET. Treatment A had soil + PET, Treatment B had soil + PET + mangroves, and Treatment C had soil + PET + mangroves + bioaugmented consortium.

4.3.2. Biodegradation of BHET, MEG, and TPA by bacteria consortia from mangroves soil

Table 4.3 shows the final bacterial cell densities for all the compounds after the ten- days biodegradation experiments. Figure 4.4 shows that the bacterial consortia isolated from soil samples from all treatments grew in the presence of the compounds over the period of ten

days, which indicated that they could use the PET monomers and intermediate as carbon sources.

Table 4.3:Summary of the final bacterial cell densities in media containing TPA, MEG, and BHET inoculated with consortia from all the experimental treatments after ten days.

Treatments	**Cell densities (cells/mL) after ten days**		
	MEG	**TPA**	**BHET**
A	1.59×10^9	1.63×10^9	4.39×10^8
B	1.90×10^9	1.63×10^9	5.74×10^8
C	1.64×10^9	1.16×10^9	4.33×10^8

Bacterial consortia showed higher growth in TPA (Figure 4.4A) and MEG (Figure 4.4B) than BHET (Figure 4.4C). Bacterial consortia from all the treatments maintained steady growth in MEG for the ten days. As for TPA and BHET, bacterial growth was only significant in the first two days of the experiment and showed a prolonged stationary growth phase. Bacterial growth was not observed in the control assays without the compounds.

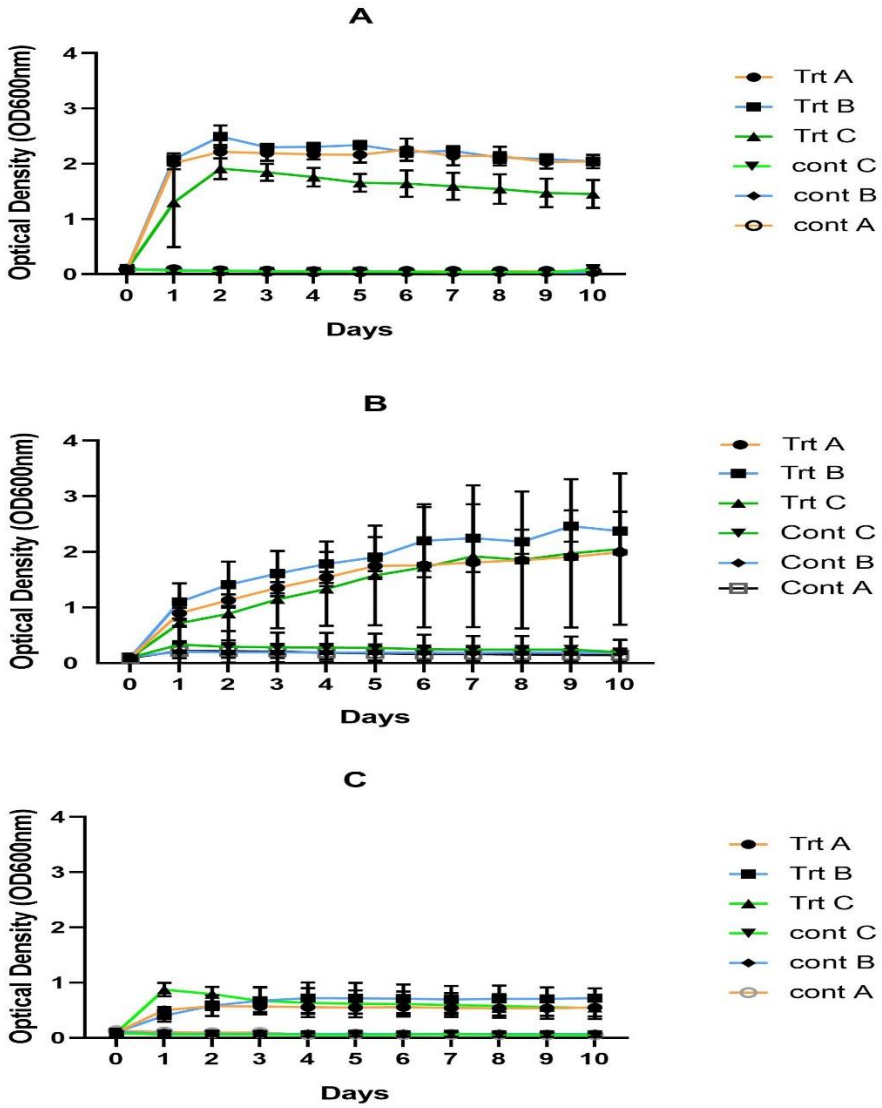


Figure 4.4: Bacterial growth of consortia from treatments A, B, and C in TPA, MEG, and BHET. (a) Consortia growth in TPA, (b) consortia growth in MEG, and (c) consortia growth in BHET. Treatment A had soil + PET, treatment B had soil + PET + mangroves, and treatment C had soil + PET + mangroves + consortia. The controls A, B, and C did not contain any compounds. Error bars represent the standard error among the independent biological replicates. Abbreviations: Trt - treatment, cont-control.

Biodegradation was evaluated by the residual concentration of the PET monomers and intermediates throughout the experiment. TPA, MEG, and BHET degradations were

observed for the three consortia, as shown in Figure 4.5. Complete TPA degradation (Figure 4.5A) was observed on day 3, irrespective of the consortia employed. The repeated measured test for TPA within-subject effects was statistically significant ($F_{(10,80)}$ = 498.11, P<0.001). However, the repeated measured test for TPA between-subject effects of the treatment was not statistically significant ($F_{(2,8)}$ = 0.60, P = 0.570), which was also confirmed by post hoc tests.

In relation to MEG, complete degradations were not observed for any of the consortia in the time course of the experiment (Figure 4.5B). Treatment A showed 75.88% MEG degradation, and treatments B and C, which had mangrove plants, showed 75.73 and 83.65% degradation, respectively. In comparison, the consortium from treatment C showed higher degradation than treatments A and B. The MEG degradation rate constants of these experiments were well fitted to the first-order kinetics (the value of R2 = 0.92–0.98). The rate constant k values followed the trend C>A>B, while the corresponding half-life t1/2 values decreased in that order, Table 4.4. The repeated measured test analysis for MEG degradation within the time points was statistically significant ($F_{(10,90)}$ = 31.99, P<0.001), while the repeated measured test comparing MEG degradation between the treatment was not statistically significant ($F_{(2,9)}$ = 3.99, P = 0.057), as confirmed by post hoc test.

As for BHET degradation (Figure 4.5C), a steady degradation was observed in all the treatments. Degradation was higher in treatment B, with a maximum BHET degradation of 96.09%, compared to treatments A and C, with a maximum degradation of 47.23% and 47.53%, respectively. The BHET degradation rate of consortium B assay followed first-order kinetics (the value of R2 = 0.96–0.99). The repeated measured test degradation analysis within the time points was statistically significant ($F_{(10,80)}$ = 53.325, P<0.001), and the repeated measured test comparing BHET degradation between the treatments was also statistically significant ($F_{(2,8)}$ = 11.629, P = 0.004).

Table 4.4: First-order rate constant (k) and half-life (t1/2) for TPA, MEG, and BHET degradation by bacterial consortia from sediment samples.

	TPA			MEG			BHET		
Consortium	**Degradation (%)**	**k**	**$t_{1/2}$**	**Degradation (%)**	**K**	**$t_{1/2}$**	**Degradation (%)**	**k**	**$t_{1/2}$**
A	100	NA	NA	75.9	0.148±0.033	2.60	47.2	NA	NA
B	100	NA	NA	75.7	0.099±0.008	3.00	98.9	0.466±0.030	1.46
C	100	NA	NA	83.7	0.194±0.029	2.33	47.5	NA	NA

In relation to the control experiments, no significant decrease was observed in TPA, MEG and BHET, which suggests that negligible or no abiotic degradation or adsorption has occurred (Figure 4.6). Overall, the three bacterial consortia were able to degrade the target compounds (TPA, MEG, and BHET).

Total organic carbon (TOC) also showed a significant decrease in all the assays (Figure 4.6). The TOC analysis results validate that the bacterial consortia used the compounds as the only carbon sources. The results suggest complete mineralisation of the compounds. The reduction in TOC for TPA from the consortia A, B, and C are 93.8 %, 91.9 %, and 89.1 %, respectively. The decrease in TOC for MEG from the consortia A, B, and C are 40.8 %, 62.3 %, and 57.2 %, respectively. Regarding the TOC of BHET, consortium B was observed to have the maximum reduction of 95.3 %, compared to consortia A and C, with the maximum TOC reduction of 55.5 % and 53.2 %, respectively.

As for the control experiments of the heat-inactivated consortia, no significant decrease was observed in the assayed compounds (Figure 4.6).

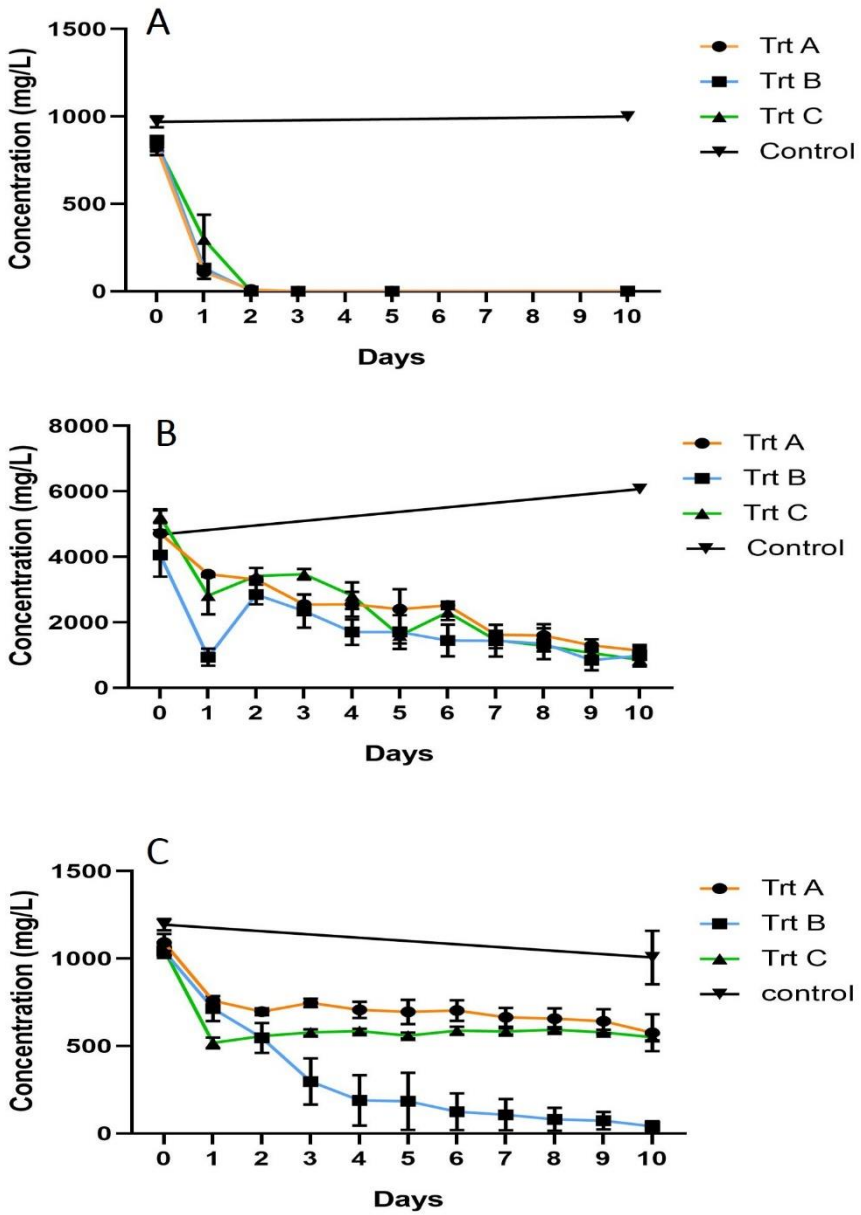


Figure 4.5: Degradation of (a) TPA, (b) MEG, and (c) BHET. During the degradation experiment, treatment A contained soil + PET, treatment B contained soil + PET + mangroves, and treatment C contained soil + PET + mangroves + consortia. The controls had only the target compounds without bacterial inoculations. Error bars represent the standard error among the independent biological replicates. Abbreviations: Trt – treatment.

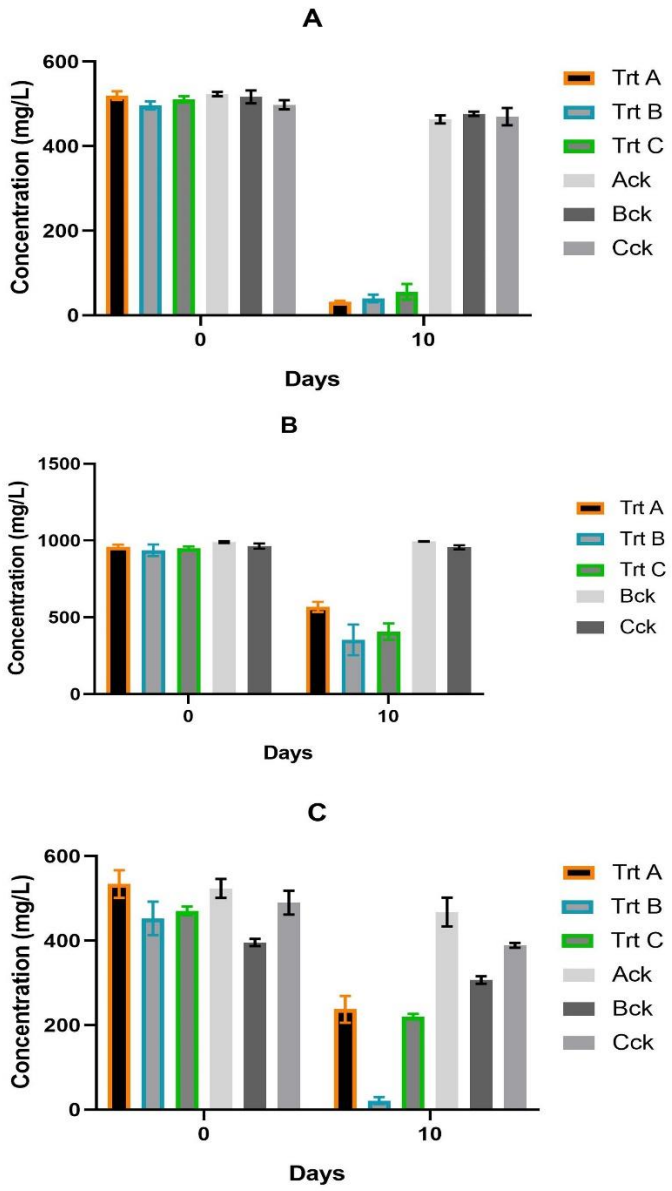


Figure 4.6: Total Organic Carbon analysis shows declines in the organic carbon concentration of the compounds. However, sharp decreases were not observed in heat-inactivated experiments (a) TPA, (b) MEG, and (c) BHET. During the degradation experiment, treatment A contained soil + PET, treatment B contained soil + PET + mangroves, and treatment C contained soil + PET + mangroves + consortia. The controls had only the target compounds without bacterial inoculations. Error bars represent the

standard error among the independent biological replicates. Abbreviations: Trt – treatment, ck – heat-inactivated.

4.4. Abundance of microbial community between the treatments

Figure 4.7 shows the relative abundance of soil bacterial taxa classified to phylum level: top 20 phyla (A), family level: top 20 families (B). Heatmap illustrating the relative abundance of the 20 most abundant bacterial phyla (C) at the beginning and end of the experiment. Relative abundance is indicated by a colour gradient from blue to red, with blue representing low abundance and red representing high abundance. The results showed that microorganism taxa in the soil samples were relatively abundant, with the relative abundance of different bacterial families in the initial and final soil samples. Actinobacteria dominated in all the treatments from the initial soil samples. The results also showed that Micrococcaceae dominated in all the treatments from the initial soil samples. However, Proteobacteria dominated in all the treatments from the final soil samples. Bacillaceae was observed to show abundance in all the treaments of the soil samples at different time points. Others include Longimicrobiaceae and Hyphomicrobiaceae.

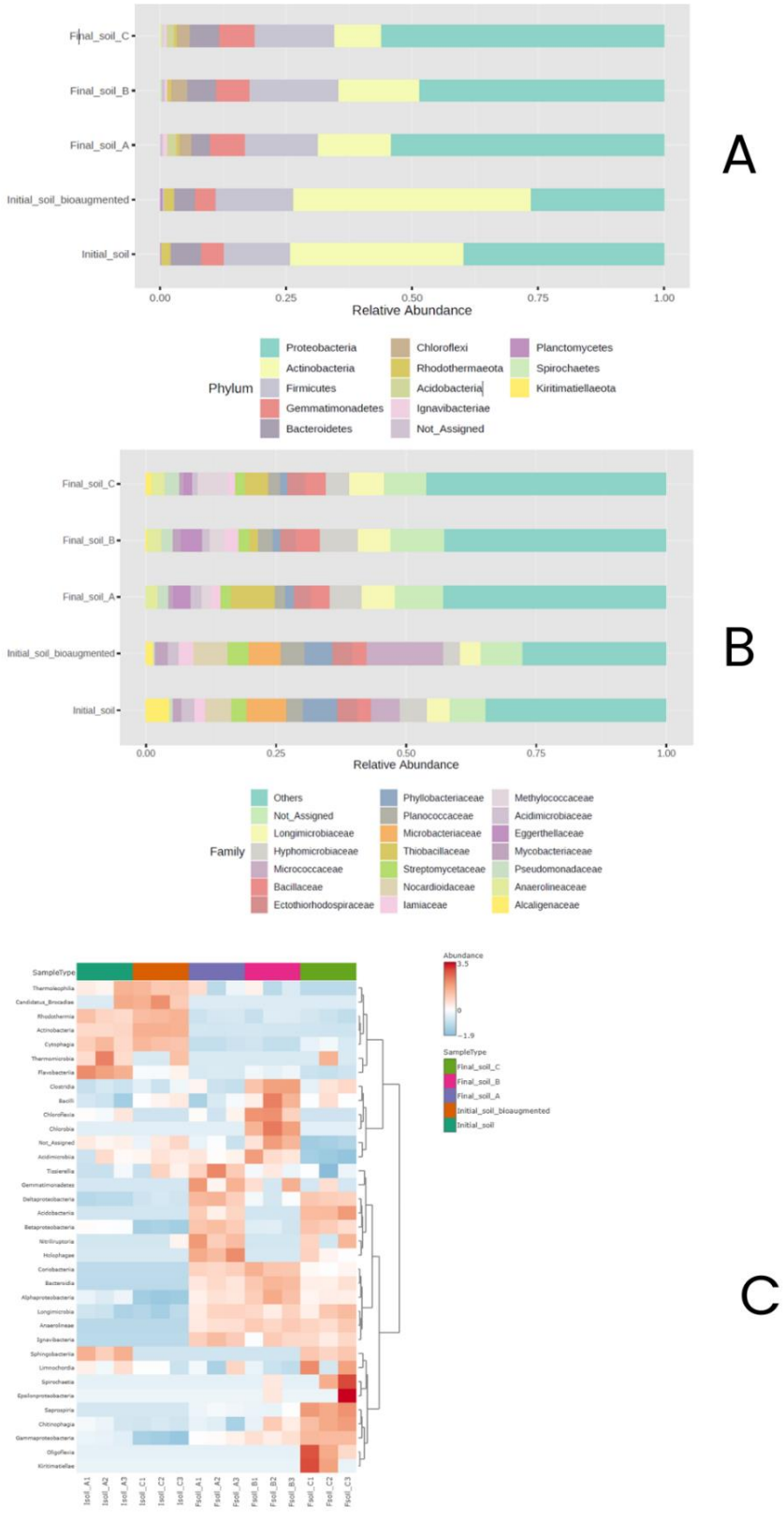


Figure 4.7: Relative abundance of soil bacterial taxa classified to phylum level: top 20 phyla (A), family level: top 20 families (B). Heatmap illustrating relative abundance of the 20 most abundant bacterial phyla (C) at the beginning and end of the experiment. Relative

abundance is indicated by a colour gradient from blue to red with blue representing low abundance and red representing high abundance.

4.5. Discussion

Mangroves are known to harbour a high diversity of microbes capable of breaking down organic and some inorganic matter. Our choice of mangrove species, *Kandelia,* was based on its resistance to harsh environmental conditions and growth characteristics. In this report, we presented results for crystalline PET film degradation by soil microorganisms, which include PET weight loss (Table 4.2), ATR-FTIR for evaluating chemical structure changes (Figure 4.2), and surface modification determined by SEM (Figure 4.3). The highest PET weight loss of 0.116% and 0.118% (Table 4.2) were observed in treatments B and C, respectively. The observed weight loss results were similar to that reported by Taghavi et al. (2021), which achieved a weight loss of 0.6 and 0.1% using naturally occurring microbial strains in mixed and individual systems for 270 days. However, the weight loss difference between the treatments was not statistically significant, which means that the presence of mangroves and bioaugmentation did not contribute to the possible PET degradation.

It is important to notice that our study employed crystalline PET samples for the biodegradation experiment. Crystallinity is one of the vital polymer characteristics that can affect microbial attacks on PET polymers (Wei and Zimmermann, 2017; Kawai et al., 2019). PET biodegradation rate depends on the polymer's crystallinity, purity, and orientation (Mohanan et al., 2020). The high degree of crystallinity means a dense structure of the PET, which is a significant reason why PET is not readily biodegradable (Gong et al., 2018; Maurya et al., 2020).

Mangrove forests possess significant diversity of microorganisms (Thatoi et al., 2012), which play essential roles in numerous environmental processes and applications. The development of microbial populations in the mangrove environment is favoured by high temperature, salinity, pH, and organic matter content and low aeration and moisture levels,

which also improve the substrate conditions (Ghizelini et al., 2012). In a study to assess the impact of plastics on mangroves, Van Bijsterveldt et al. (2021) pointed out that plastic was abundant, covering up to 50% of the mangrove forest floor, with 27 plastic items per m^2 on average. Microorganisms in the mangrove environment could evolve and survive heavy plastic pollution. In our study, the presence of mangrove plants and their rhizosphere caused structural and chemical changes on the PET surface, not observed in soil without the plants. Moreover, the weight loss observed was also higher in the treatment with mangroves, although not statistically significant.

At the end of the PET degradation experiments, a slight pH change (table 4.1) was observed in treatment C, which may have been caused by the activities of the mangrove rhizosphere microorganisms and the augmented consortia (Miloševí et al., 2017). The consortia were composed of two bacterial species, *Enterococcus* sp. WTP31B-5 and *Bacillus* sp. GPB12 isolated from the activated sludge of a wastewater treatment plant and an old landfill sample, respectively. *Bacillus* sp. has demonstrated PET degradation potential (Dąbrowska et al., 2021; Roberts et al., 2020; Helen et al., 2017). (Dąbrowska et al., 2021) observed intense PET film degradation in the presence of bacteria and plants and further concluded that the *Bacillus* strain combined with miscanthus plantings may be a promising method for accelerating PET degradation in compost soil (Dąbrowska et al., 2021).

Leaf falls were observed during the experiment following a storm in the first sixth months, which may have led to a decrease in the mangroves' lengths and weights in all treatments with mangrove plants at the end of the experiment. The reduction in biomass raises some topics for further research: it would be interesting to understand the direct effect of plastic levels on the overall life of mangrove plants, and long-term studies are needed to investigate other factors, such as the potential leakage of chemicals from degrading plastics (Gao & Wen, 2016). Other studies have shown that plastic pollution causes stress on mangrove

plants and that prolonged suffocation by plastic causes rapid pneumatophore growth and potential leaf loss (Van Bijsterveldt et al., 2021).

The FTIR spectroscopic analysis was conducted in the range 400 to 4000 cm^{-1}. The appearance of new infrared bands at 3992 cm^{-1} (alcohol group) (treatment A, Figure 4.2) and 808 cm^{-1} (aromatic ring) (treatment C, Figure 4.2) were observed. The new peaks can be associated with oxidation products formed at different frequencies (Torena et al., 2021). The disappearance or addition of functional groups is a strong indication of biodegradation mechanism and related to microbial activities (Helen et al., 2017; Naz et al., 2013; Skariyachan et al., 2017). The formation of oxidation products, such as carbonyls, hydroxyls, esters, aromatics, and alcohols, and the observed peaks shift in the PET films reflect changes in the chemical structure of the polyethylene terephthalates (Helen et al., 2017). These changes were due to the adherence of the microorganisms, which altered the PET plastics through oxidation reactions (Wilkes and Aristilde, 2017). Therefore, the FTIR results indicated an interaction between the microorganisms and PET film samples.

The SEM analysis of PET films showed modification of the PET morphology in the treatment with mangrove plants (Figure 4.3). Changes in the surface structure were observed in the form of bumps, dulling, or abnormal drapes. In comparison, the most significant roughness was observed in the film from the treatment with mangrove and inoculated with the bacterial consortium. Similar changes on PET surfaces have been reported by Torena et al. (2021) in a study on PET biodegradation for 168 days. PET surface roughness and cracks are considered evidence of biodegradation (Sarkhel et al., 2020; Yan et al., 2021)

There are various methods for degrading PET and its monomers and intermediates, among which biodegradation is more environmentally friendly and technologically suitable. A very

low amount of PET was degraded based on the gravimetry analysis, which could be caused by various factors. A possible reason could be a low affinity and concentration of the microbial enzyme towards the highly crystalline PET films. In addition to the crystallinity, the hydrophobicity of the PET films could be a crucial factor in the binding of the microbial enzymes onto the PET films. Other constituents present in the soil may also affect the activities of the microorganisms. Therefore, this study used TPA, MEG, and BHET as model substrates to assay biodegradation by the bacterial consortia isolated from the soil after the PET degradation experiments. The results describe the ability of the bacterial consortia to grow and utilise different monomers and intermediates of PET as sole carbon sources. The bacterial consortia grew when cultured in media containing either of the compounds, TPA, MEG, and BHET.

The results highlight that the secreted enzymes by the consortia can efficiently convert BHET to TPA and provide insights into the biodegradation and bioconversion of PET and the role of bacterial consortia in this process. In this study, we demonstrated a complete degradation of TPA by the consortia. The result is higher than what was reported by Yang's team in their research on the biodegradation of TPA conducted in batch experiments for 162 days, which reported 92.1-98.5 % degradation when TPA (400 mg/L) was fed as the sole substrate (Yang et al., 2014). In other studies, *Rhodococcus* sp. SSM1 was used to achieve 100% (Kumar et al., 2020) and 99.6% (Suwanawat et al., 2019) TPA degradation from an initial concentration of 4994.29 mg/l and 1000 mg/L, respectively. Another study has demonstrated the degradation of 100 mg/L of TPA by a few bacterial strains and reported a total removal within 24 hours (Aksu et al., 2021).

The degradation percentage of MEG increased with respect to the incubation period of up to 10 days by all bacterial consortia (Figure 4.5B). Treatment C showed higher degradation

(83.65 %) than treatments A and B. These results may be attributed to the presence of mangrove plants and the consortium in this treatment. A similar degradation extent of MEG at varying concentrations (0.25-1.0 % (v/v)) has been reported by (Ghogare and Gupta, 2012) when working with bacterial isolates and a consortium. The bacterial consortium showed 75.49 % MEG degradation at 2226 mg/L and approximately 5 % more MEG degradation by the best bacterial degrader, *Oliptrichum macrosporum*, when tested individually for seven days. MEG degradation by bacterial oxidation using *Pseudomonas putida* strains KT2440 and JM37 has also been reported. The bacterial strains converted MEG into a more valuable product (Mückschel et al., 2012). In another study, the engineered *Pseudomonas putida* KT2440 has demonstrated complete degradation and, in addition, enables the conversion of MEG to medium-chain-length polyhydroxyalkanoates (Franden et al., 2018). MEG has also been used as a sole carbon and energy source by *Acenetobacterium woodii*. This organism metabolised and disproportionately converted MEG to ethanol and acetyl coenzyme A (acetyl-CoA), which further converted to acetate (Trifunović et al., 2016).

A steady degradation of BHET was observed in all the consortia. Bacterial consortia from treatments B and C (which have mangrove plants) showed significant degradation compared to consortium from treatment A without mangroves (Figure 4.5). Even though treatment C was bioaugmented, consortium from treatment B showed higher BHET degradation, up to 96 % (Figure 4.5C). The complete degradation of BHET was not observed in the experimental period of ten days. However, the consortium from treatment B may show a complete degradation if allowed for additional few days. Our result on BHET degradation by bacterial consortia was higher than what has been reported by individual bacterial strains. For instance, Qiu et al. have investigated the degradation of BHET at an initial concentration of 1000 mg/L and reported 80.8 % removal by *Enterobacter* sp. (Qiu et al., 2020). Other microorganisms reported in previous studies as degrading BHET include *I. sakaiensis*

(Yoshida et al., 2016), *Humicola insolens* (Castro et al., 2017), and *B.subtilis* (Ribitsch et al., 2011). However, those studies did not report the actual degradation rate of BHET as BHET removal only took place in the process of PET degradation. In the present study, the bacterial consortia retrieved from the soil with minute evidence of PET degradation have further demonstrated the ability to biodegrade TPA, MEG, and BHET.

As for the abundance of microorganisms, a relative abundance of different bacterial families in the initial and final soil samples was observed. Gammaproteobacteria dominated in all the treatments due to the abundance of Longimicrobiaceae, Hyphomicrobiaceae, Micrococcaceae, Bacillaceae, and others in the soil samples at different time points. Our finding is consistent with the previous study (Wright et al., 2021) while working on microbial community succession within the PET plastisphere. Acidobacteria and Chloroflexi, as well as Verrumicrobia and Gemmatimonadetes are present in our samples and are considered oligotrophic microbes (Hegyi et al., 2021). Bacilli were observed in our samples and have been previously found to colonise plastics in the marine (Carson et al., 2013; Syranidou et al., 2019, 2017) and terrestrial (Nakkabi et al., 2015) environments. An increase in the relative abundance of Proteobacteria was also observed in the soil which can be attributed to different factors.

5. Chapter Five - Closing the loop: bacterial conversion of PET monomers into polyhydroxyalkanoates (PHAs)

5.1. Introduction

The deteriorating impact of conventional plastic wastes on the environment and the potential adverse effects on human health have led researchers to explore other alternative sources of plastic materials for various applications. Polyhydroxyalkanoates (PHAs) are biopolymer polyesters produced by many Gram-negative and Gram-positive bacterial species under nutrient-limiting conditions with excess carbon (Raza et al., 2018; G. Q. Chen, 2009). PHAs are intracellular carbon storage mechanisms for many species of microorganisms (Brigham and Sinskey, 2012). There are many PHAs, characterised distinctly by chain length, type of functional group, and degree of unsaturated bonds. As for the carbon (C) chains, they are short (SCL-PHAs; C_3–C_5), medium (MCL-PHAs; C_6–C_{14}), and long (LCL-PHAs; C_{17}–C_{18}) (Singh et al., 2015). The most explored and typical among the PHAs are poly(3-hydroxybutyrate) (PHB), poly(3-hydroxyvalerate) (PHV), and their copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV). The molecular weight varies from 50000 to over a million, depending on the microorganism, fermentation conditions, and extraction method (Bugnicourt et al., 2014).

PHAs exhibit extraordinary similarities to well-known conventional polymers, such as low-density polyethylene and polypropylene. PHA disposal as bio-waste and biocompatibility made them increasingly attractive in pursuing sustainable development of biodegradable plastics. PHAs produced from standard carbon sources, such as glucose, have influenced the high cost of production. Carbon source plays a vital role in the PHA production process as it impacts not only the yield and composition of the recovered biopolymers (Raza et al., 2018)

but also constitutes about half of the overall production costs (Schmidt et al., 2016). Plastic waste utilization to produce a highly enriched biodegradable organic compounds is a plausible way to reduce production costs. Waste polypropylene has been used in laboratory and pilot-scale experiments to produce PHAs (Johnston et al., 2019). Polyethylene terephthalate (PET) is one of the world's most widely produced and used plastic (Chen et al., 2020; Castro et al., 2017). PET plays a central role in global packaging (Taylor and Laville, 2018; Ellen MacArthur Foundation., 2017) and textile industries (Geyer et al., 2017). The monomers of PET (monoethylene glycol and terephthalic acid) could serve as carbon and energy sources for bacteria and subsequent bioconversion to PHA. Research on the production of PHA using PET monomers is scarcely explored. The cost of PHA production through fermentation is inextricably linked to the cost of the starting substrate. Much effort has been committed to reducing the production cost of PHA by using low-cost carbon sources (Getachew & Woldesenbet, 2016b). Here, we investigate using PET monomers as bacterial feedstocks to produce PHA. The first part of this study focuses on screening and isolating bacteria with the potential to produce PHA. The study also explores the potential of the promising isolate to use PET monomers as carbon sources and further perform separate fermentation experiments to integrate PET monomers biodegradation assay and production of PHA.

5.2. Materials and method

5.2.1. Chemicals

Monothylene glycol (MEG) (1075477-500ml, International Laboratory, USA), glucose-dextrosol, (Darmstadt, Germany), terephthalic acid (TPA) 185361-500G, purity >98%, (Sigma, Saint Louis, USA), Sudan Black B-199664, (Sigma, Saint Louis, USA), Safranin

O-84120 (Sigma, Darmstadt, Germany) and polyhydroxybutyrate (PHB) granules (LS565256, size: 5 mm, GoodFellow, England) were commercially obtained.

5.2.2. Sample collection

Sediment samples were collected from different mangroves sites (22°08'30.8"N 113°33'05.8"E), and sludge samples were collected from the wastewater treatment plant (22°12'47.2"N 113°32'05.6"E) in Macao, SAR China. Sediment samples were collected from a 3-4 cm depth using a cleaned stainless spatula, wrapped in aluminium foil. Activated sludge samples were collected in sterile containers. All the samples were stored in a refrigerator until the commencement of the experiments.

5.2.3. Culture and isolation of bacteria

In 9 ml distilled water, 1g of the sediment samples were thoroughly mixed, and serial dilution was performed, followed by plating on nutrient agar medium for 24-48 hours. In the case of the sludge sample, 1 ml was used for the serial dilutions. After the incubation period, each plate was carefully observed, and a series of streaking was performed to obtain pure isolate cultures. Each pure culture was then inoculated in a 50 ml Erlenmeyer flask containing 10 ml Nutrient Broth. The flasks were incubated at 30 ^{o}C with agitation at 200 rpm for 24-48 hours. Isolates were stored in glycerol stock at -80 ^{o}C for further analysis.

5.2.4. Screening for PHA producing bacteria

5.2.4.1. Sudan Black B staining on bacterial colonies

Colonies were screened for PHA-producing bacteria following the protocol by Bhuwal et al. (2013), with some modifications. Briefly, overnight bacterial isolate culture was spread onto nutrient agar plates containing 1 % glucose. The plates were incubated at 30^{o}C for 24 hours. After incubation, bacterial colonies on the plates were stained by flooding with Sudan Black B solution (3 g/L, in 75 % ethyl alcohol) for 30 minutes. Plates were washed with ethanol 98% to remove the excess stain. The bluish-black colouration of colonies indicates a positive PHA stain. All tests were conducted in triplicate. A total of 35 isolates were screened for PHA inclusions. Based on colour intensity, isolate EC2-30-3 was considered the most promising and was selected for further studies.

5.2.4.2. Bacterial fix smear staining

A loopful of the isolate broth culture was spread on clean, grease-free slides, covering an area of about 10 mm^2. The smears were allowed to air dry and fixed by passing horizontally through spirit flame 2–3 times. The slides were flooded with Sudan black solution and allowed for 10 minutes. Excess Sudan Black stain was drained off, and the slides were immersed in xylene until completely decolourised (about 10 seconds) and allowed to dry. The slides were further counterstained with safranine 0.5 % (w/v) solution for 10-15 seconds, followed by gentle rinsing with running distilled water and allowed to air dry. After drying completely, a drop of immersion oil was added directly and examined with an oil immersion lens (100X). Fluorescent microscopy in bright field observations showed lipid inclusion granules as stained blue-black, while the bacterial cytoplasm was stained light pink by safranine counterstain.

5.2.5. Transmission electron microscopy (TEM)

Transmission Electron Microscopy (Philips CM100, USA) was performed to confirm the intracellular inclusions.

5.2.6. Molecular identification of the PHA-accumulating bacteria

The identification was performed based on 16S rRNA gene sequence analysis at Macrogen Inc. laboratory. PCR amplification was performed using universal primers: 27F 5' (AGAGTTTGATCMTGGCTCAG) 3' and 1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'. The sequences obtained were then aligned with known 16S rRNA gene sequences in GenBank database using the basic local alignment search tool (BLAST) of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST). The phylogenetic tree was constructed using MEGA11 (Tamura K & Stecher G, 2021) and sequence alignment with MUSCLE and 1000 replications of the bootstrap method. The 16S rRNA gene sequence of strain EC2-30-3 was submitted to the GenBank database under the accession number NR157734.1.

5.2.7. Biodegradation of MEG and TPA by isolate EC2-30-3

The bacterial isolate EC2-30-3 was subcultured and used for MEG and TPA biodegradation assay. The biodegradation assay was conducted following the protocols by (Suwanawat et al., 2019; Qiu et al., 2020) with some modifications, at a concentration of 1000 mg/L of TPA and 1113 mg/L of MEG as the carbon sources in minimal media and pH 7. The isolate was inoculated at an initial 8.0 x 10^7 cells/mL density. The assay was conducted in triplicate. Culture flasks were incubated at 30 °C and shaken at 200 rpm for eight days. Control flasks contained minimal media and the target compounds (TPA and MEG), without bacterial inoculation.

Bacterial growth was monitored by spectrophotometry (Biowave II, UK) at $OD600_{nm}$. Aliquot samples were aseptically collected daily and centrifuged at 13500 x g for 10 min. Supernatants were stored at -20 °C for further analysis.

5.2.7.1. Production of PHA with glucose and MEG as carbon sources

The inoculum was prepared by growing the bacterial isolate (EC2-30-3) in 250 ml flasks containing 100 ml Nutrient broth. The flasks were incubated at 30 ^{o}C with agitation 200 rpm for 24 h. After 24 h, the culture was centrifuged to remove all the nutrient broth and resuspended in PHA production media. PHA production media was composed of minimal media (Moreira et al., 2013) with 0.25 % yeast extracts and 0.4 % peptone. The addition of 0.2 % glucose and 0.2 % MEG as carbon sources were conducted in separate flasks. Control flasks without additional carbon sources were also prepared. The flasks were inoculated with bacterial suspension at an initial concentration of about 6.43 x 10^{8} CFU/ml. All experiments were conducted in triplicate, and flasks were incubated at 30 ^{o}C with an agitation of 200 rpm. Samples were collected at time zero, 48 h, and 96 h. Supplementary addition of carbon sources (MEG, glucose, yeast extract, and peptone) was provided to the respective flasks after 48h of incubation.

5.2.7.2. Extraction and recovery of PHA

At the end of the experiments, the bacteria cells were harvested according to the protocol by Ratnaningrum et al. (2020) with some modifications. Briefly, bacteria cells were separated by centrifugation (Eppendorf 5810R, Germany) at 3061 g for 15 min. The supernatant was removed, and the pellet was washed using distilled water. The collected pellet was then

lyophilised. About 1 g of the dried pellet was mixed with a solution containing 50 mL chloroform and 50 mL NaOCl (12.5 % v/v) for digestion. The digestion was conducted in a shaker incubator (180 rpm for 24 h). The mixtures were centrifuged at 3061 x g for 15 min, resulting in three layers. The bottom layer (chloroform containing PHA) was collected, and a mixture of cold methanol and water (7:3, v/v) was added to the PHA in chloroform to precipitate the PHA. The precipitated PHA was dried at 40 °C. Subsequently, the dried PHA was rinsed with acetone to remove impurities. The purified PHA was then dried in a controlled oven (40 °C) and used for further characterization.

5.2.7.3. Quantitative analysis of PHA

The lyophilised cell pellet was used to estimate the dry cell weight (DCW) in units of g/L. The percentage of intracellular PHA accumulation was estimated as the percentage composition of PHA present in the dry cell weight (Bhuwal, et al., 2013) using the following formula.

$$\text{PHA accumulation (\%)} = \frac{\text{Dry weight of extracted PHA } \left(\frac{\text{g}}{\text{L}}\right)}{\text{DCW } \left(\frac{\text{g}}{\text{L}}\right)} X\ 100$$

5.2.7.4. FTIR analysis

The extracted PHA was analysed through FTIR (VERTEX 70 IR spectrometer, Bruker, Germany) by following the protocol described by Porras et al. (2015) with some modifications under ambient conditions. Briefly, the specimens were prepared by grinding and mixing 0.9 mg of extracted PHA with 80 mg of spectroscopic grade KBr, followed by mechanical pressing (Specac, England) of the mixtures into pellets. Transmission mode was used. Each spectrum measurement was collected over 64 scans in the range of 4000–400

cm^{-1} at a resolution of 4 cm^{-1}. The vacuum environment was measured and used as the background.

5.2.8. Statistical analysis

All the experiments were performed in triplicate, and the average values were statistically analysed by a general linear model (GLM) with univariate analysis of variance using SPSS (IBM SPSS Statistics 28.0.0.0) followed by a Tukey test. Using AI-Therapy Statistics, Spearman's rho correlation coefficient was computed to assess the relationship between bacterial growth density, dry cell weight (DCW), and PHA accumulations. A significance level of $p < 0.05$ was used.

5.3. Results

5.3.1. Screening and isolation of PHA-producing bacteria.

A total of 35 isolates were screened by Sudan Black B staining on both colonies and the smeared glass slide for PHA inclusions, and 22 isolates showed a bluish-black colouration indicative of positive PHA-accumulation (Table 5.1 and Figure 5.1A-B). TEM images of the intracellular PHA inclusions were also captured Figure 5.1C-D. Based on colour intensity; isolate EC2-30-3 was selected as the most promising for further analysis.

Table 5.1: Bacterial isolates screened for PHA inclusions using Sudan Black B stain. Abbreviations: + (positive) and - (negative).

Isolate ID	SAMPLE DESCRIPTION	PHA screening
31B-5	Activated Sludge WWTP (liquid)	+++
31B-3	Activated Sludge WWTP (liquid)	+++

31A-3A	Sludge WWTP (solid)	+++
A27-1	Sediment Mangrove Ka'Ho	
31A-7B	Sludge WWTP (solid)	+++
31A-7C	Sludge WWTP (solid)	+++
A19	Mangroves from Coloane power station	+++
EC2-A25	Sediment Mangrove Ecological zone2 site D	+++
EC2-B25	Sediment Mangrove Ecological zone2 site D	+
B28-1	Sediment Mangrove Taipa Cycling Site B	-
B28-2	Sediment Mangrove Taipa Cycling Site B	-
31A-1A	Activated Sludge WWTP (liquid)	+
31A-1B	Activated Sludge WWTP (liquid)	+
31A-7A	Activated Sludge WWTP (liquid)	+
31A-2A	Activated Sludge WWTP (liquid)	-
31B-1	Activated Sludge WWTP (liquid)	-
31B-2	Activated Sludge WWTP (liquid)	-
31A-3B	Activated Sludge WWTP (liquid)	+
31B-4B	Activated Sludge WWTP (liquid)	-
31B-4A	Activated Sludge WWTP (liquid)	-
31A-5	Activated Sludge WWTP (liquid)	-
31A-2B	Activated Sludge WWTP (liquid)	+
31B-6A	Activated Sludge WWTP (liquid)	++
31A-6B	Activated Sludge WWTP (liquid)	+
31A-6A	Activated Sludge WWTP (liquid)	+
31A-4	Activated Sludge WWTP (liquid)	-
EC2-30—1	Mangrove sediment Ecological zone2 Site E	++
EC2-30—2	Mangrove sediment Ecological zone2 Site E	+++
EC2-30—3	Mangrove sediment Ecological zone2 Site E	+++

EC2-30—4	Mangrove sediment Ecological zone2 Site E	+
EC2-30—5	Mangrove sediment Ecological zone2 Site E	-
EC2-30—6	Mangrove sediment Ecological zone2 Site E	-
EC2-30—7	Mangrove sediment Ecological zone2 Site E	-
EC2-30—8	Mangrove sediment Ecological zone2 Site E	++
EC2-30—9	Mangrove sediment Ecological zone2 Site E	++

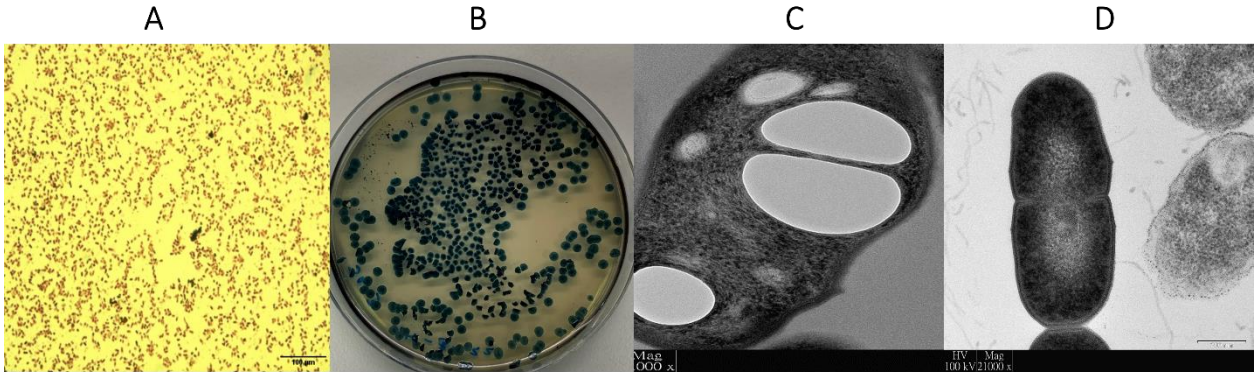


Figure 5.1: Bluish-black stain of the PHA-producing isolate. (A) Sudan black stain of bacteria showing intracellular PHA inclusion under bright field microscope. (B) Sudan black stain of the bacterial colonies on Petri dish. (C) TEM image of isolate 31A-7C showing intracellular PHA inclusions. (D) TEM image of isolate B28-1 without intracellular PHA inclusions.

5.3.2. Identification of the isolate EC2-30-3 based on 16s rRNA gene sequence.

The alignment results of the 16S rRNA sequences showed that strain EC2-30-3 is a member of the genus *Bacillus* and formed a consistent cluster with *Bacillus paramycoides* strain MCCC 1A04098 (Accession number: NR157734.1; similarity 99%; Figure 5.2).

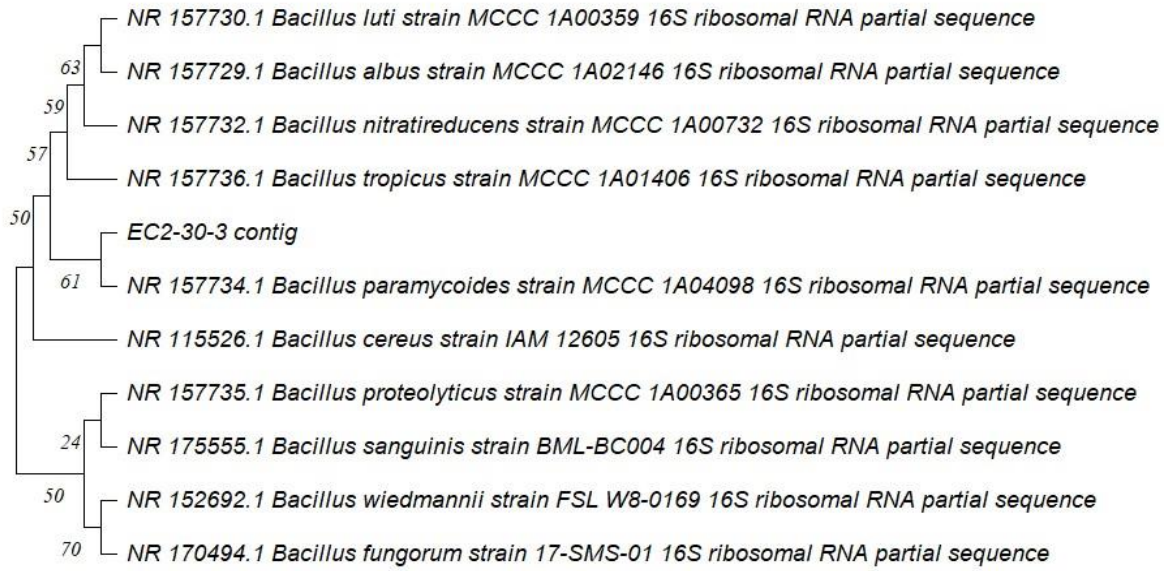


Figure 5.2: Neighbour-joining phylogenetic tree showing the nearest neighbours of strain EC2-30-3. The numbers indicate the evolutionary distance, whereas the labels at the end of the arms represent the Genbank accession number.

5.3.3. Biodegradation of TPA and MEG by *Bacillus* sp. EC2-30-3

It was observed that *Bacillus* sp. EC3-30-3 showed better growth in the media containing MEG than TPA. The growth entered the stationary phase after 24 hours. However, rapid exponential growth was not observed in the media containing TPA (Figure 5.3A). Concerning the degradation, *Bacillus* sp. EC3-30-3 was able to use MEG as a carbon source and complete degradation was observed within 48 h. However, *Bacillus* sp. EC3-30-3 does not seem to utilise TPA as a carbon, and degradation was not observed (Figure 5.3B).

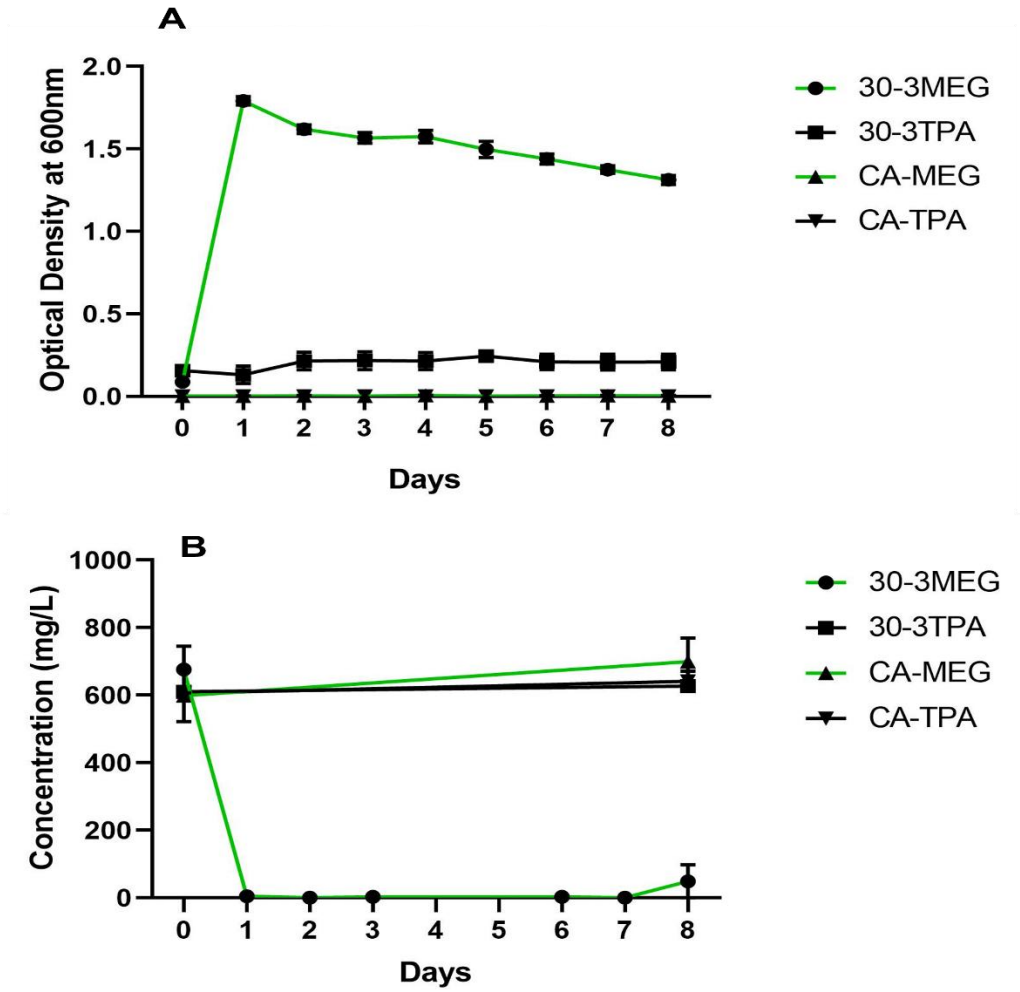


Figure 5.3: (A) Growth of Bacillus sp. EC2-30-3 in media supplied with MEG and TPA as the sole carbon sources. (B) degradation of MEG and TPA by Bacillus sp. EC2-30-3. Error bars represent the standard error among the replicates. Abbreviations: MEG (monoethylene glycol), TPA (terephthalic acid), CA (control without bacterial inoculum).

5.3.4. Growth of *Bacillus* sp. EC2-30-3, quantification, and analysis of the PHA produced.

The growth of *Bacillus* sp. EC2-30-3 in media supplied with different carbon sources during PHA production is depicted in Figure 5.4. The isolate showed exponential growth within the

first 24 hours but slightly dropped. The second exponential growth was observed 48 hours after the addition of MEG and glucose.

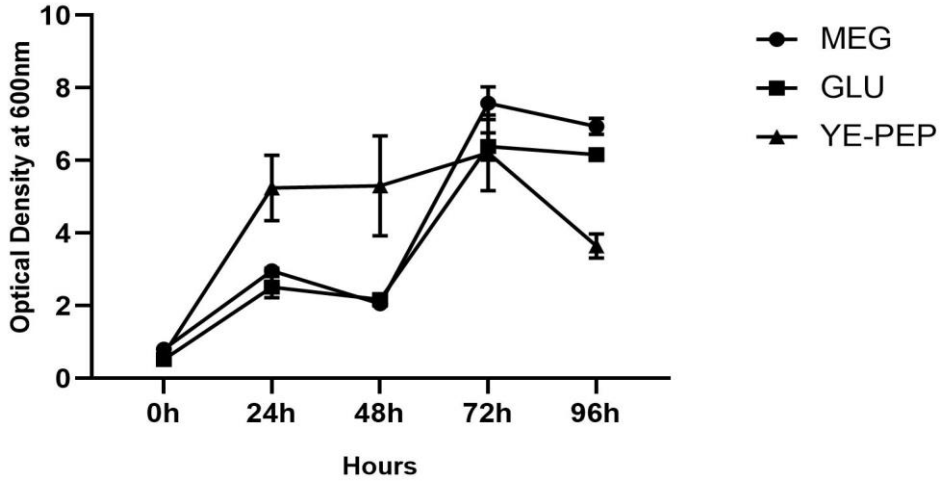


Figure 5.4: Growth of Bacillus sp. EC2-30-3 in PHA fermentation media. Error bars represent the standard error among the replicates. Abbreviations: MEG (monoethylene glycol), GLU (glucose), and YE-PEP (yeast extract and peptone).

After PHA production, pellet samples were lyophilised at different time points during fermentation culture, and biomass concentration was evaluated based on gravimetry analysis (Figure 5.5). The biomass difference among the carbon sources was statistically assessed at time zero ($F_{(2,6)} = 4.882$, $P = 0.055$), 48h ($F_{(2,6)} = 3.292$, $P = 0.108$), and 96h ($F_{(2,6)} = 4.838$, $P = 0.056$).

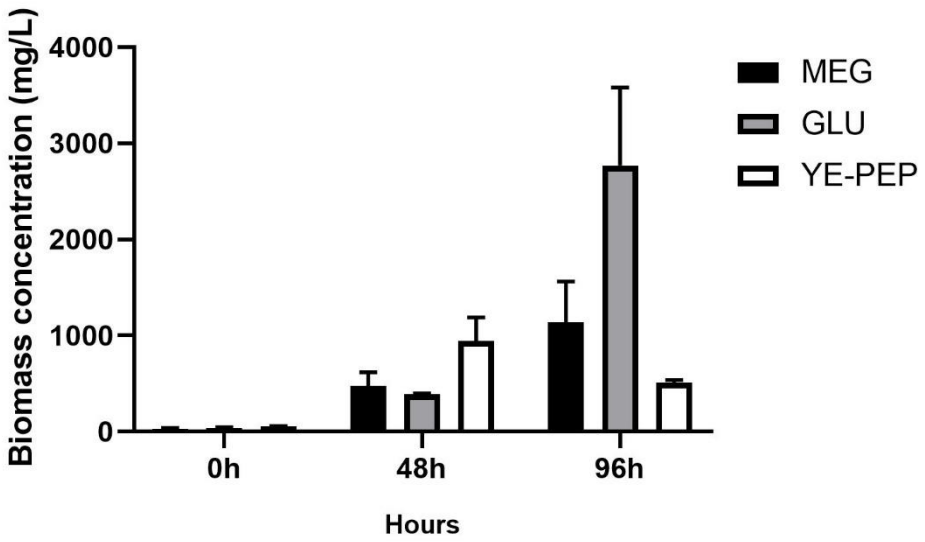
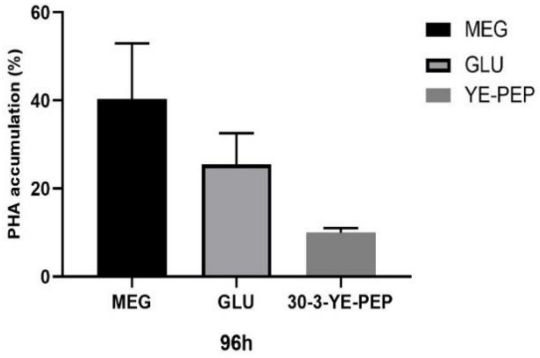


Figure 5.5: Biomass accumulation of Bacillus sp. EC2-30-3 collected at different time points (time zero, 48 h, and 96 h) during the fermentation culture in media supplied with varying carbon sources. Error bars represent the standard error among the replicates. Abbreviations: MEG (monoethylene glycol), GLU (glucose), and YE-PEP (yeast extract and peptone).

The amount of PHA was evaluated in relation to the biomass (Figure 5.6A). *Bacillus* sp. EC2-30-3 showed higher PHA accumulation in media supplied with MEG (40.31 %) than with glucose (25.53 %) and then with yeast extract plus peptone (9.99 %). The univariate test for PHA accumulation difference was not statistically significant ($F_{(2,6)} = 3.269$, $P = 0.110$), which was also confirmed by post-hoc tests between groups. A correlation between the PHA accumulation and bacterial growth density was observed ($r_s = 0.845$, $N = 9$, $p = 0.004$). However, no strong correlation was observed between the dry cell weight and PHA accumulations ($r_s = 0.067$, $N = 9$, $p = 0.865$).

A.



B.

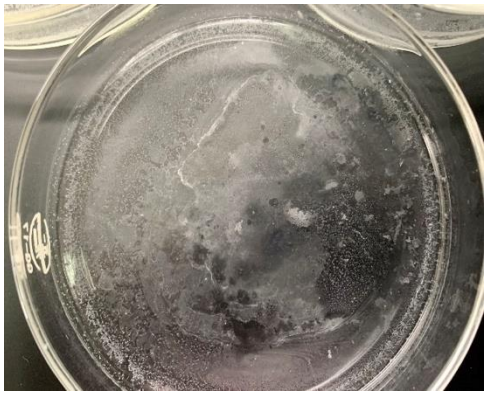


Figure 5.6: (A) PHA accumulation of Bacillus sp. EC2-30-3 collected at 96 h after fermentation with added MEG or glucose and without either of the main carbon sources. Error bars represent the standard error among the replicates. (B) PHA granules on Petri Dish after extraction. Abbreviations: MEG (monoethylene glycol), GLU (glucose), and YE-PEP (yeast extract and peptone).

The FTIR profile for PHA recovered from *Bacillus* sp. EC2-30-3 is shown in Figure 5.7, alongside standard PHB polymer. The extracted PHA fed with MEG and glucose showed that the band at 1278 cm^{-1} corresponds to CH, showing asymmetrical stretching and bending vibration in the CH_3 group. The extracted PHA also showed an intense absorption peak with

a C=O bond, which is common in the carbonyl group at 1720.45 cm^{-1} for both PHA from MEG and glucose (Figure 5.7) that closely matches with 1722.31 cm^{-1} for the standard PHB. However, the 1600 cm^{-1} and 3500 cm^{-1} absorptions showed C=O and O-H stretching in the extracted PHA fed with glucose and MEG, respectively. The observed stretchings may be caused by different carbon sources or technical issues while using the equipment. The overall FTIR spectra revealed that the band at 3436 cm^{-1} corresponds to the hydroxyl (O-H) group of alcohol, whereas the bands at 2977 and 2933 cm^{-1} are because of the methylene (C-H) stretch of alkanes. The functional group of PHB is confirmed as C=O groups, and these absorption bands obtained are closely related to the structure of PHB.

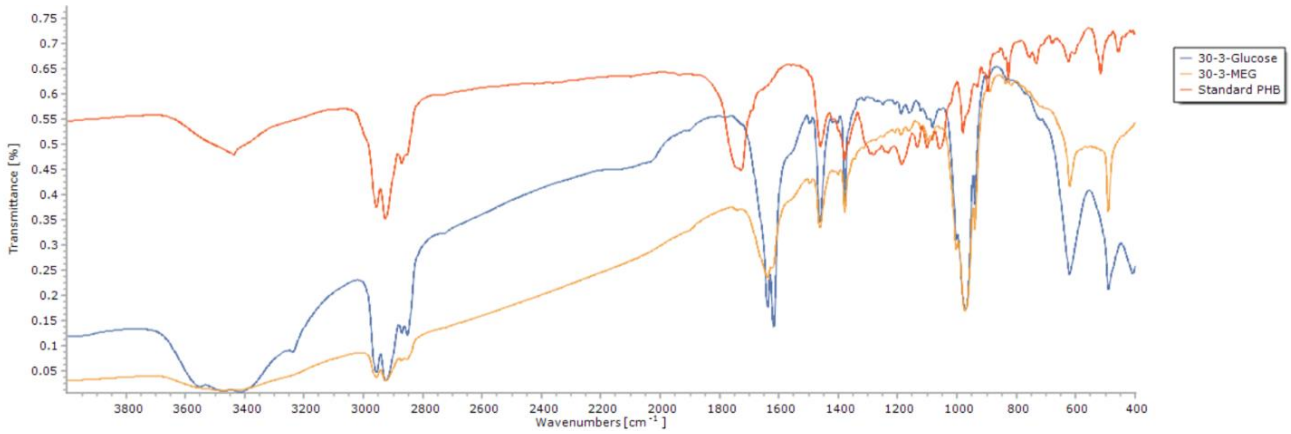


Figure 5.7: Fourier-transformed infrared spectrum of polymer extracted from Bacillus sp. EC2-30-3 and standard PHB. Abbreviations: MEG (monoethylene glycol).

5.4. Discussion

The present study demonstrates for the first time the capacity of the isolate EC2-30-3, with 99 % similarity to *Bacillus paramycoides* to metabolise a PET monomer to synthesise PHAs. To achieve this, we started by testing 35 isolates from different environmental sources, with 22 showing the ability to produce PHA inclusions. Isolates 31A-7C and EC2-30-3 revealed

a considerable amount of PHA inclusions when viewed under a bright field microscope and appeared circular in the central region, occupying the maximum space within the cell. Moreover, PHA inclusions in typical PHA producers can also be observed as hollow circles within the cytoplasm when observed under a phase contrast microscope (Arshad et al., 2017). From these isolates, *Bacillus* sp. EC2-30-3 was selected for further analysis because it showed more promising PHA accumulation—the potential of *Bacillus* sp. EC2-30-3 to use MEG and TPA to produce PHA was first investigated. The experiment was conducted in aerobic conditions as aerobic degradation of MEG was reported to be more efficient than anaerobic (Huang et al., 2005). *Bacillus* sp. EC2-30-3 exhibited better growth performance towards MEG compared to TPA. The growth result of the bacterial isolate in TPA may be due to the toxic effect of TPA at the studied concentration level (1000 mg/L) or its low metabolic ability towards TPA as a substrate. Although other bacteria, such as engineered *E. coli* (Pandit et al., 2021), *Acetobacterium woodii* (Trifunović et al., 2016), and *Pseudomonas putida* (Mückschel et al., 2012), have been reported to metabolise MEG, to the best of our knowledge, this is the first study describing the potential of *Bacillus* sp. EC2-30-3 to metabolise MEG and produce PHA. However, *P. umsongensis GO16 KS3* was reported to slowly grow and metabolise MEG after undergoing some adaptive laboratory evolution approach. In their bio-upcycling study (Tiso et al., 2021) performed 15 sequential batch cultivations for about 45 days and later reported that the evolved (after evolving for approximately 100 generations) strain *P. umsongensis* GO16 KS3 was able to grow on EG at a rate of 0.4 1/h. The present study demonstrated the ability of *Bacillus* sp. EC2-30-3 to completely metabolise MEG within 48 h without adapting any metabolic improvement approach. The strain *P. umsongensis* GO16 was further reported to covert MEG combined with TPA and produced PHA (Tiso et al., 2021). Even though the present study showed low

metabolic activity of *Bacillus* sp. EC2-30-3 towards TPA but demonstrated a better MEG metabolic performance and conversion to PHA.

Nevertheless, the phylogenetic tree indicated that isolate EC2-30-3 and *Bacillus paramycoides* shared a cluster (Figure 5.2), and *B. paramycoides* has recently been demonstrated to produce PHB from agricultural wastes (Djerrab et al., 2022; Chekroud et al., 2022; El-kadi et al., 2021).

Based on the growth of *Bacillus sp.* EC2-30-3, MEG was selected as a substrate model for studies on PHA production. We also employed 0.2 % glucose for comparison. Glucose is a traditional and readily used feedstock for most bacterial fermentation experiments.

Regarding the dynamics of PHA production by the isolate, the DCW results revealed an increase along time, reaching a maximum up to 1136.3 mg/L and 2768.9 mg/L at 96 h for MEG and glucose, respectively. Maximum PHA content obtained (40.31 w/w % of the DCW) was for the cultures supplemented with MEG, which is higher than that recently obtained from *I. sakaiensis* when grown in MEG (4.9 ± 0.1 %) and closer when *I. sakaiensis* was grown on PET granules (48 ± 5 %) (Fujiwara et al., 2021). The result confirmed that *Bacillus sp.* EC2-30-3 could use the feedstocks (MEG) as a carbon and energy source. PHA content may vary according to the carbon source used. Glucose is a readily usable carbon source that encourages bacterial production of PHA. Even with the relatively low amount of glucose (0.2 %) used, 25.52 % (w/w) PHA accumulation was observed. This result is higher than 14.2 % (w/w) obtained from *Burkholderia* sp B37 when 2 % of glucose was used as a carbon source. The result is also consistent with the reported maximum PHB (about 36 %) production from *Bacillus wiedmannii* AS-02 OK576278 when glucose (2%) was used as a carbon source (Danial et al., 2021). However, peptone and yeast extract did not show high

DCW (509.2 mg/L), or PHA accumulation (9.99 %), indicating that these substrates contribute to cell growth but not to PHA accumulation.

Scaling up the PHA production levels to an industrial scale remains challenging. Sato et al., (2013) propose a potentially useful approach in which the metabolic and fermentation engineering strategies previously applied for *C. necator* H16 strain, enable the production of PHA copolymer (> 100 g/L) by high-density fed-batch cultivation under nitrogen limitation.

The polymer extracted from *Bacillus* sp. EC2-30-3 in MEG and glucose media were used for recording IR spectra in the range 4000–600 cm^{-1}. The FTIR spectra peaks (Figure 5.7) give information about the structure of PHA by showing bands at about 1720.45 cm^{-1} and 1278 cm^{-1} corresponding to the ester carbonyl group and the –CH group, respectively. Other studies have reported similar bands as characteristic of PHAs (Bhuwal et al., 2013; López et al., 2012). The spectrum pattern obtained was similar to the PHB standard.

The results suggest that MEG degradation by *Bacillus* sp. EC2-30-3 provide a promising method to improve PET degradation while producing PHA.

6. Chapter Six - Conclusions, limitations, and future directions

6.1. Conclusions

This thesis demonstrates that it should be possible to use a single bacterial strain to biodegrade PET plastic and simultaneously produce bioplastics. Although the results obtained suggest direct microbial degradation of highly crystalline PET, present in many plastic products, would be difficult, degradation of the PET monomers and intermediates was shown to be possible, suggesting that closing the loop on the bacterial degradation of conventional plastics to bioplastics production is feasible.

To answer the research questions initially posed, field and laboratory work on isolating bacterial consortia from different environmental samples were conducted. The bacteria consortia were employed for the biodegradation of crystalline PET samples in laboratory-controlled conditions and soil microcosmos in open areas. In Chapter Three, we assessed the PET degradation potential of four bacterial consortia isolated from different environmental samples. In Chapter Four, we evaluated the potential of the mangrove rhizosphere (using *Kandelia* sp. as the model plant) for the degradation of crystalline PET films. Based on gravimetry analysis, the biodegradation results of the crystalline PET in liquid media and mangrove soil were negligible. The consortia KH27, isolated from mangrove environmental samples, showed the maximum PET weight loss (1.1%) of the four consortia employed. The very low PET degradation in the present study may be due to the limited accessibility of the ester linkage in the crystalline PET for bacterial attack, or the low metabolic activity of the consortia towards the crystalline PET. TPA, one of the PET degradations metabolites, was not detected in the media by HPLC analysis, indicating that the PET granules were not thoroughly degraded to their monomer units in concentrations above the limited of detection

or it was consumed by the consortia. Keeping in mind our initial research question, "Could bacterial consortia in liquid matrix degrade PET plastic into its monomers (TPA, MEG) and intermediate (BHET)?" we could say our findings answered the question halfway, considering other analyses such as the FTIR showing indications of crystalline PET degradation. To further investigate the degradation of crystalline PET, we hypothesised that the mangrove ecosystem might harbour potential microorganisms that can degrade plastic. The overall PET film weight difference between the initial and final in all treatments was statistically significant. However, the PET weight difference between the treatments was not statistically significant.

The FTIR and SEM analyses showed changes in the chemical and morphological structure of the crystalline PET granules and films. The formation, shift, and disappearance of spectral peaks belonging to esters, aldehydes, carboxylic acids, aromatic, alcohols, ethers, and alkene groups at different frequencies suggest the initiation of degradations of PET plastics by the consortia and mangrove rhizospheres from the experimental treatments. SEM analysis of the PET films showed the formation of bumps, dulling, and roughness on the surfaces, demonstrating the ability of the rhizosphere to adhere, colonise and modify the surfaces of the crystalline PET films.

However, although our results indicated the initiation of crystalline PET film degradations, we could not observe a significant weight loss of the PET plastic samples in any of the experimental treatments. Therefore, the study further investigated whether bacteria could degrade PET monomers (TPA, MEG) and intermediate (BHET). Our findings demonstrated complete degradation of TPA and significant degradation of BHET and MEG by bacteria consortia isolated from soil. Even though BHET and MEG did not completely degrade, the consortium from experimental treatment B (with mangrove plants) showed significant

BHET degradation and the consortium from treatment C (with mangrove plants and bioaugmentation) showed significant MEG degradation. The overall results showed the ability of the isolated bacterial consortia to utilise TPA, MEG, and BHET as sole carbon sources, irrespective of the experimental treatments. The key findings answered our initial research question "Could mangrove sediments be considered sources of bacterial consortia to degrade PET plastic and its monomers (TPA, MEG) and intermediate (BHET)"?

Chapter Five investigated the ability of bacterial isolates from various environmental samples to produce biopolymer polyhydroxyalkanoates (PHAs). Of the bacterial isolates screened for PHA inclusions, 62.9% showed positive for PHA inclusions. Isolate EC2-30-3, identified as *Bacillus sp*, was shown to be able to produce PHA in high amounts. These findings also answered the initial research question, "Could mangrove sediments be considered sources of bioplastic (polyhydroxyalkanoates) producing bacteria"?

To address our last question, "Could bacterial isolates from mangrove sediment degrade and convert PET monomers to produce PHA (bioplastic)"?, it was evaluated the degradation of TPA and MEG by the PHA-producing isolate, *Bacillus sp.* EC2-30-3. The results showed that the bacterium could use MEG as a carbon source over TPA. MEG was selected as a model substrate for PHA production. *Bacillus sp.* EC2-30-3 was further grown in MEG media and showed PHA accumulation of up to 40.31 w/w % of the DCW. Chloroform and 12.5 % v/v NaOCl approach were employed for cell wall disruptions and recovery of PHA. Peptone and yeast extracts in the fermentation culture media may contribute to cell growth but not PHA accumulation. The FTIR analysis provided information about the structure of the extracted PHA by showing bands corresponding to the ester carbonyl and the –CH groups indicating a spectrum pattern similar to the PHB standard. The results showed that

Bacillus sp. EC2-30-3 did not only metabolise MEG but also produced PHA, demonstrating the integration of PET monomer degradation and the production of biopolymers.

However, it is essential to point out that the degradation of crystalline PET to its monomer units is challenging. To achieve the up-cycling of PET plastic wastes, an additional approach is required to enhance the complete depolymerization of PET to its monomers so the monomers can be used as feedstock for biopolymer production. This approach can contribute to the development of new PHA production pathways can help reduce plastic pollution and increase the production of inexpensive, biodegradable plastics while increasing PET recycling.

6.2. Limitations and future directions

During this study, it would be relevant to highlight some of the circumstances and identify some future research opportunities:

I. The environmental samples contain a diverse community of microorganisms, some culturable while others may be difficult to culture. Moreover, some organisms can be cultured in the laboratory within a short time, while others tend to have a more extended incubation period. A metagenomic study of the environmental samples would offer vital information about the relevant microorganisms that are not culturable in the laboratory condictions and have the potential to degrade PET plastic material.

II. This study showed that bacterial consortia could degrade PET plastic samples minimally (Chapters Three and Four). The study also demonstrated the biodegradation of PET monomers (MEG and TPA) and intermediate (BHET) by

bacterial consortia, as seen in Chapter Four. More information would be relevant to understanding which bacterial strains and their metabolic pathways contributed to PET degradation.

III. We also observed changes in the biomass of mangrove plants at the end of the experiments in Chapter Four. It would be interesting to investigate the direct effect of plastic levels on the overall physiological and genetic aspects of mangrove plants. Moreover, given that different mangrove species have peculiar characteristics, it would be interesting to explore the rhizospheres of other species of mangrove plants and other factors, such as the potential leakage of chemicals from degrading plastics.

IV. In Chapter Five, we demonstrated the screening of biopolymer-producing bacteria and the production of PHA. It would be relevant to understand the pathways and gene expression of the different enzymes at all stages of the PHA synthesis. The information would help to enhance and improve PHA production. It would also be interesting to optimise PHA production by varying various parameters such as temperature, pH, carbon sources, C/N ratios, inoculum concentrations, etc.

V. This study aims to integrate the degradation of PET plastics with the production of PHAs. Further studies are needed to improve the effectiveness of PET depolymerization into its monomer units and functionally link the two metabolic pathways. Research on the enzymatic depolymerization of PET plastics would offer a helpful approach.

VI. We employed chlorinated solvent approaches for PHA recovery. More experiments would be needed to utilise environmentally friendly and sustainable approaches for

PHA recovery from intact bacterial cells. Scaling up the production and recovery of PHA using inexpensive strategies would help to mitigate the impact of the plastic waste problems and recycling challenges.

VII. During the PHA fermentation culture, it was not easy to control some parameters, such as the pH, because the fermentation was not performed in a bioreactor. It would be relevant to conduct the fermentation in a bioreactor to maintain the optimum growth and performance of the bacterial isolate.

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8. Annex

Table S1: HPLC data displaying average peak areas of samples taken at day 55 of degradation experiments. Abbreviations: CA- control.

Consortia	**Peak areas and Retention times at Day 55**							
	1.3	2.9	3.5	3.8	4.7	5.3	6	6.5
CW1	185146.5	114098	73069	83820.5	19992.5	11835	6233	5917
LZG16	291287	97255	54238	51035	12692.67	9051.667	20930	10327
KH27	357482.3	94403	83393.67	64519	10256	8566.667	9097.667	10275.67
EC2-30	325631.3	116336.3	58722.33	96072.67	19456.67	14777.67	22959.5	9595
CA	14502	29129						

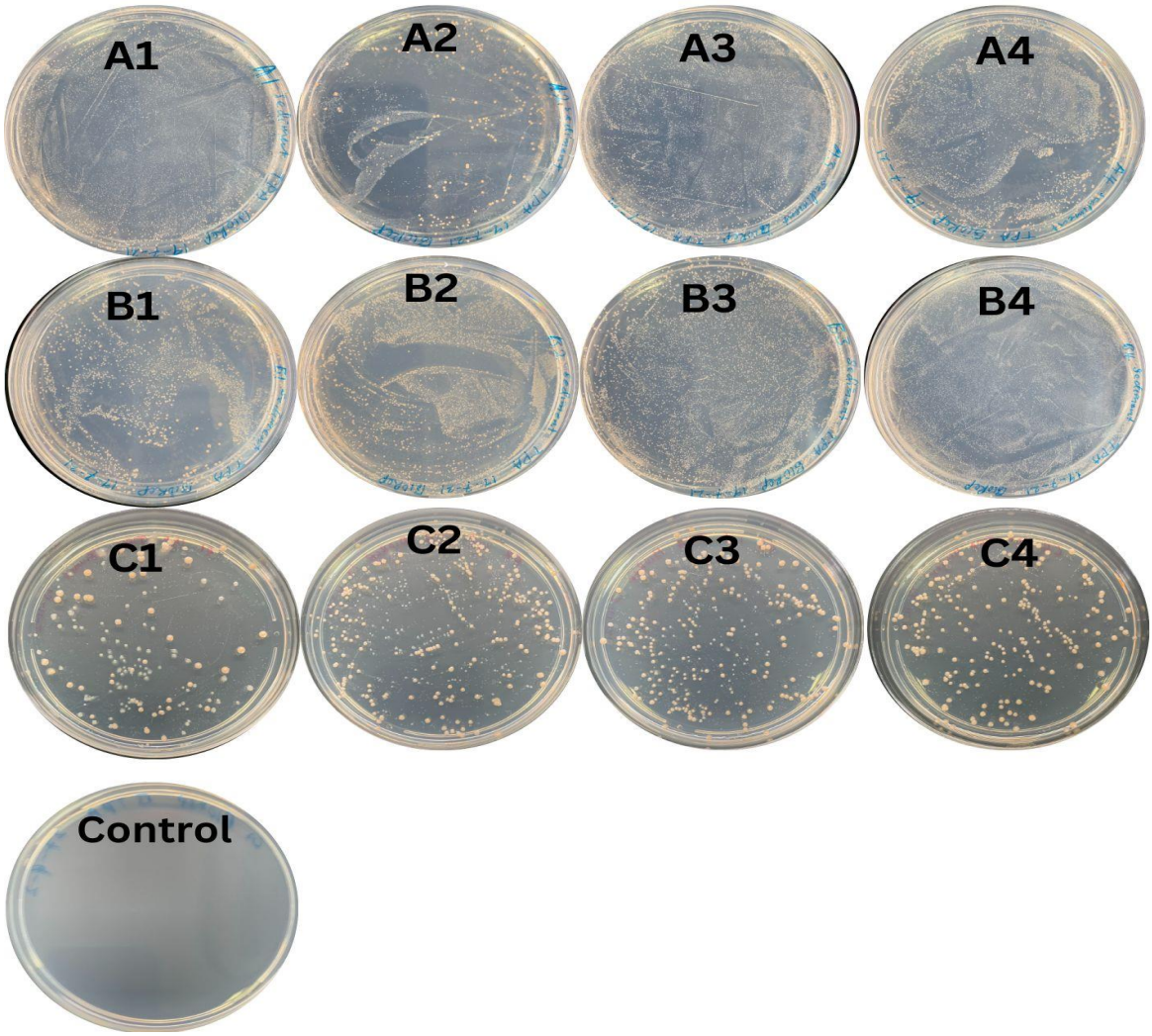

Figure S2: Bacterial colonies on plates enriched with TPA after the 10 days biodegradation experiment. Colonies were observed on the replicate plates independent of all treatment variants (A, B, and C).

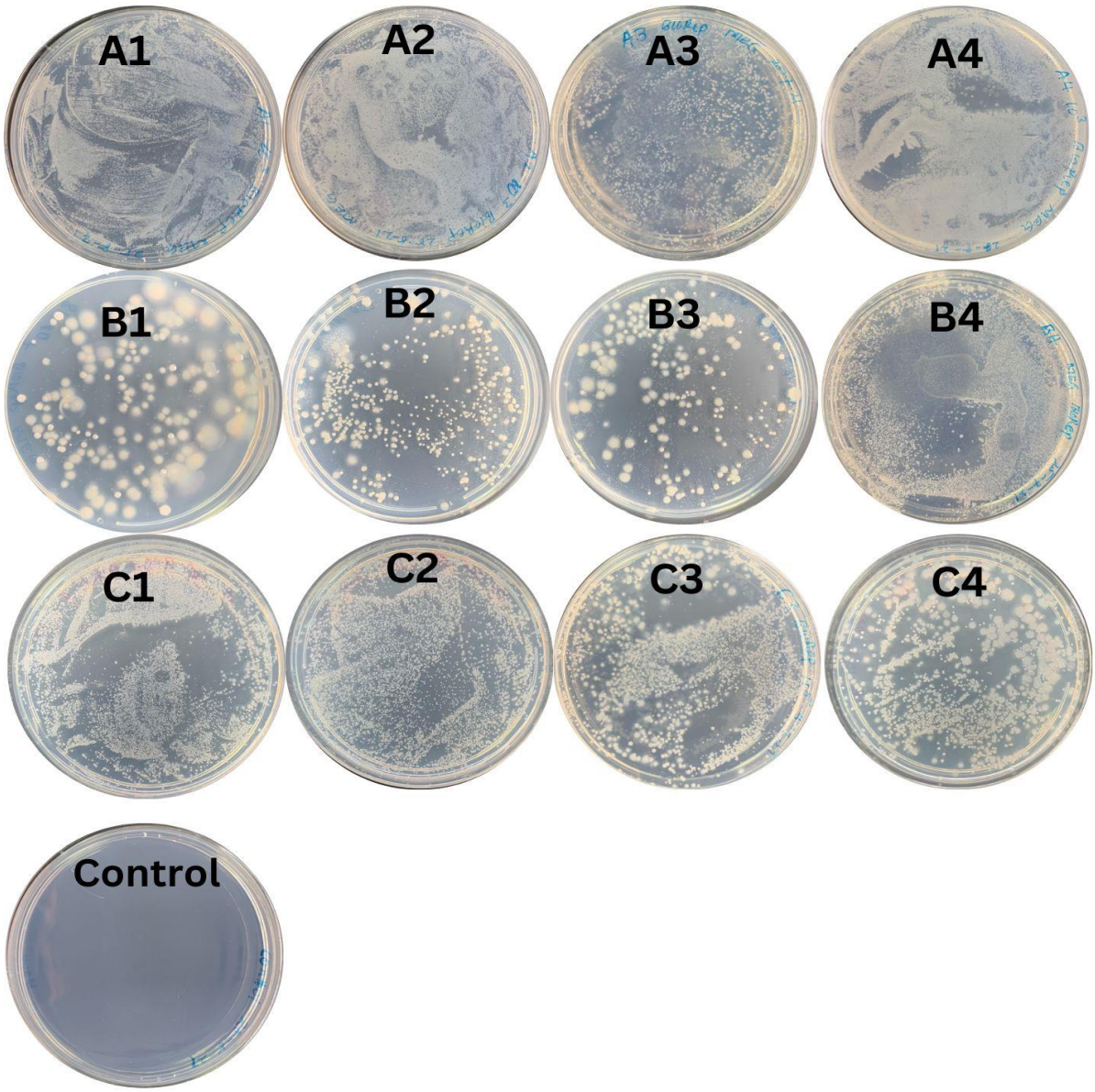

Figure S3: Bacterial colonies on plates enriched with MEG after the 10 days biodegradation experiment. Colonies were observed on the replicate plates independent of all treatment variants (A,B, and C)

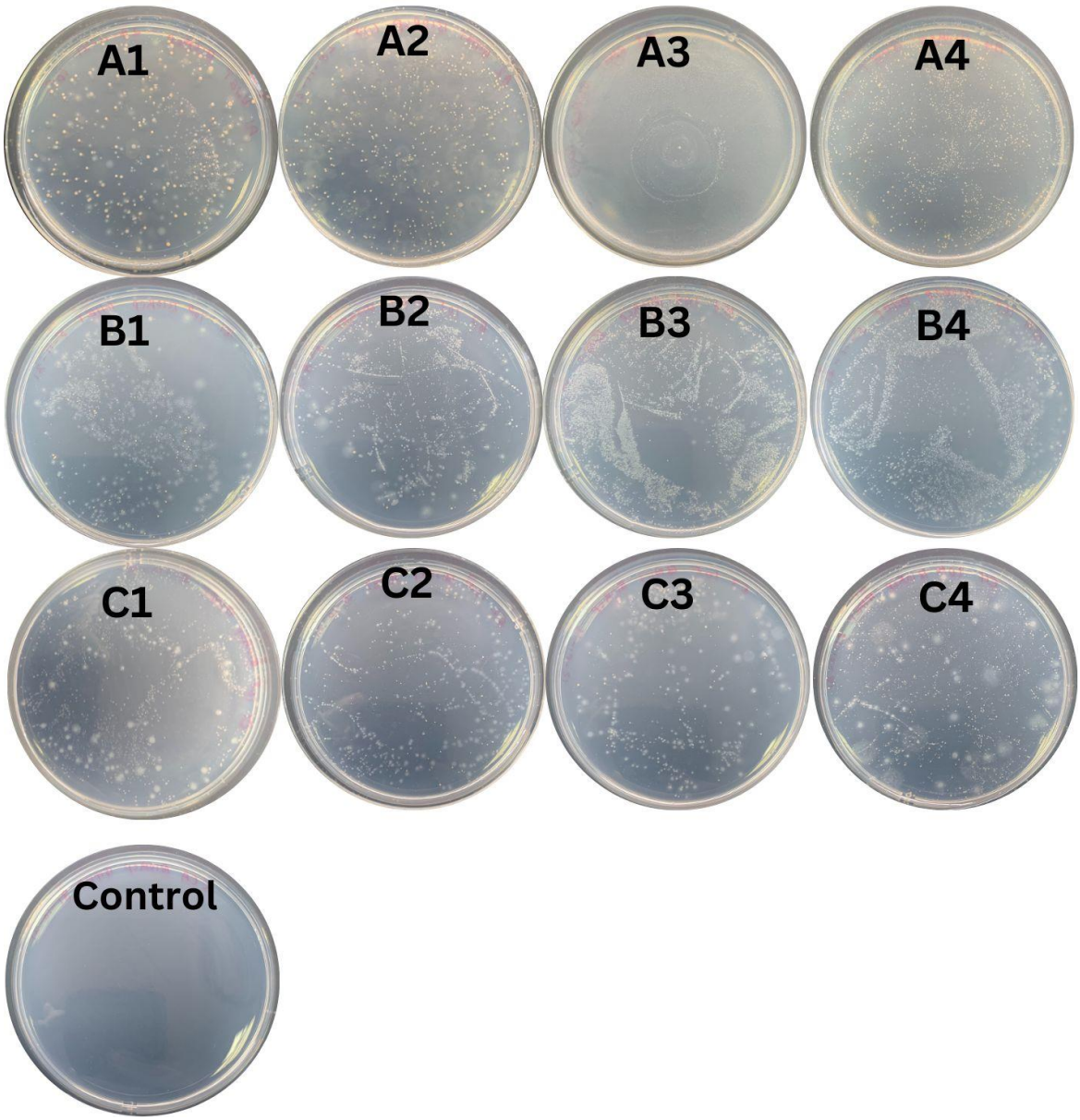

Figure S4: Bacterial colonies on plates enriched with BHET after the 10 days biodegradation experiment. Colonies were observed on the replicate plates independent of all treatment variants (A,B, and C)