ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF POLY(3-HYDROXYBUTYRATE-*co*-3-HYDROXYHEXANOATE) [PHBHx] DEGRADING BACTERIA FROM SOIL, PLASTISPHERE, AND BRACKISH WATER SAMPLES

AHMAD SYAUQI BIN TAUFIQ

UNIVERSITI SAINS MALAYSIA

2025

ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF POLY(3-HYDROXYBUTYRATE-*co*-3-HYDROXYHEXANOATE) [PHBHx] DEGRADING BACTERIA FROM SOIL, PLASTISPHERE, AND BRACKISH WATER SAMPLES

by

AHMAD SYAUQI BIN TAUFIQ

Thesis submitted in fulfilment of the requirements
for the degree of
Master of Science

July 2025

ACKNOWLEDGEMENT

In the name of Allah, the Most Compassionate and the Most Merciful. All praise is to Allah for His blessings throughout my journey in completing this thesis.

I am deeply grateful to my supervisor, Prof. Dr. K. Sudesh Kumar, for his unwavering patience, invaluable guidance, and continuous support. His profound knowledge and insightful feedback have greatly shaped my research and academic development. My heartfelt appreciation extends to all members of the Ecobiomaterial Lab, SATREPS-OPTRL, and PTC Lab, especially Dr. Chee Jiun Yee, Dr. Manoj Lakshmanan, Dr. Tan Hua Tiang, Dr. Zainab, Dr. Ann Basik, Dr. Chanaporn (Sine), Dr. Azura Ahmad, Dr. Norhaida, Dr. Faisal, Shaik Ling, Billy Neoh Soon Zher, Wan Jia Hui, Tang Hui Jia, Nur Shahira Sani, Nur Amirah Fazilla, and Hanis Alisa for their friendship, support, and constructive discussions. I also thank Chin Guan Ming, Lee Joyyi, Iffa Farahin, Ong Su Yean, Chai Hong Xuan, and Tan Kar Ling for their assistance throughout this journey.

I am also thankful to the academic, technical, and administrative staff of the School of Biological Sciences, including the Electron Microscopy Unit and Centre for Global Archaeological Research, for their kind support in facilitating my research. Most importantly, I would like to express my deepest appreciation to my family for their endless love, support, and prayers. Their encouragement has been my foundation through all the highs and lows of this journey.

This research was supported by the Ministry of Education Malaysia (203/PBIOLOGI/67811001), SATREPS (JPMJSA1801) under JST/JICA, and USM GRA-Assist. I sincerely acknowledge this financial support.

To everyone who contributed directly or indirectly— Thank you.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF UNITS AND SYMBOLS

α	Alpha
~	Approximately
β	Beta
Tc	Crystallization temperature
℃	Degree Celsius
D	Dextrorotatory form
wt%	Dry weight percent
Tg	Glass transition temperature
GPa	Gigapascal
g	gram
h	Hour
∞	Infinity
kDa	Kilodalton
kPa	Kilopascal
L	Levorotatory form
L	Litre
Dv (50)	Mass median diameter
MPa	Megapascal
T_m	Melting temperature
Mt	Metric tons
K_m	Michaelis constant
µg	Microgram
µL	Microliter
µm	Micrometer
µM	Micromolar

mg	Milligram
mL	Milliliter
min	Minute
mol%	Mole percent
ng	nanogram
nm	Nanometer
M_n	Number-average molecular weight
%	Percentage
±	Plus-minus
$Đ$	Polydispersity index
psi	Pounds per square inch
(*R*)	Rectus isomer
M_r	Relative Molecular Mass
rpm	Revolutions per minute
s	Second
(*S*)	Sinister isomer
T_d	Thermal degradation temperature
×	Times
× *g*	Times gravity
V	Volt
v/v	Volume per volume
W	Watt
M_w	Weight-average Molecular Weight
w/v	Weight per volume

LIST OF ABBREVIATIONS

3HA	3-hydroxyalkanoic acids
3HB	3-hydroxybutyrate
3HB-CoA	3-hydroxybutyrl-CoA
3HD	3-hydroxydecanoate
3HHx	3-hydroxyhexanoate
3HHx-CoA	3-hydroxyhexanoyl-CoA
3HO	3-hydroxyoctanoate
3HV	3-hydroxyvalerate
4HB	4-hydroxybutyrate
aa	Amino acid
AACS	Acetoacetyl-CoA synthethase
AMP	Adenosine 5'-monophosphate
ASTM	American Society for Testing and Materials
ATP	Adenosine 5'-triphosphate
ATR	Attenuated total reflectance
bdhA	D-(-)-3-hydroxybutyrate dehydrogenase
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BP	Bootstrap percentage
C1	Catalytic domain type I
C2	Catalytic domain type II
ca.	Circa (about)
Cad	Cadherin-like domain
Ccr	Crotonyl-CoA reductase
CDW	Cell dry weight
CFU	Colony forming unit
CME	Caprylic methyl ester
CO_2	Carbon dioxide
CoA	Coenzyme-A
COOH	Carboxyl group
CPKO	Crude palm kernel oil

Crt2	Crotonase
CSI	Conserved signature indel
DDT	Dichlorodiphenyltrichloroethane
DEAE	Diethylaminoethyl cellulose
DFP	Diisopropylfluorophosphate
dH_2O	Distilled water
DIY	Do it yourself
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dPHA	Denatured PHA
DSC	Differential scanning calorimetry
EB	Elution buffer
EDTA	Ethylenediaminetetraacetic acid
FadA	3-ketoacyl-CoA thiolase
FadB	Enoyl-CoA hydratase
FadD	Acyl-CoA synthetase
FadE	Acyl-CoA dehydrogenase
FabG	3-ketoacyl-CoA reductase
FEG	Field emission gun
Fn3	Fibronectin type III domain
FTIR	Fourier-transform infrared spectroscopy
GC	Gas chromatography
gDNA	Genomic DNA
Gly	Glycine
GPC	Gel permeation chromatography
Had	3-hydoxyl-CoA dehydrogenase
HMDS	Hexamethyldisilazane
HPLC	High-performance liquid chromatography
ISO	International Organization for Standardization
mcl	Medium-chain-length
MEGA	Molecular Evolutionary Genetic Analysis
MET	Metribuzin
MM	Mineral medium
MMD	Mass median diameter

MNR	Marine nutrient-rich medium
mPHA	Marine PHA medium
NA	Not available
NCBI	National center for Biotechnology Information
n.d.	Not determined
nPHA	native PHA granules
NR	Nutrient-rich
OD	Optical density
OH	Hydroxyl group
OPTRL	Oil palm trunk research laboratory
P&G	Procter & Gamble
P(3HB)	Poly(3-hydroxybutyrate)
P(3HB/4HB)	Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate)
P(3HB/3Hx/4Hx)	Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate-*co*-4-hydroxyhexanoate)
P(3HD/3HO)	Poly(3-hydroxydecanoate-*co*-3-hydroxyoctanoate)
P(3HO)	Poly(3-hydroxyoctanoate)
P(3HO/3Hx)	Poly(3-hydroxyoctanoate-*co*-3-hydroxyhexanoate)
P(3HP)	Poly(3-hydroxypropionate)
P(4HB)	Poly(4-hydroxybutyrate)
PA	Polyamide
PAHs	Polycyclic aromatic hydrocarbons
PBAT	Polybutylene adipate terephthalate
PBS	Polybutylene succinate
PCA	Poly(crotonic acid)
PCBs	Polychlorinated biphenyls
PCL	Poly(caprolactone)
PCR	Polymerase chain reaction
PDLA	Poly(D-lactic acid)
PE	Polyethylene
PES	Poly(ethylene succinate)
PET	Polyethylene terephthalate
pH	Potential of hydrogen
PHA	Polyhydroxyalkanoate
PhaA	β-ketothiolase

PhaB	NADPH-dependent acetoacetyl-CoA reductase
PhaC	PHA synthase
PHACOS	Poly(OH-Alk-*co*-OH-6ATH-OH4ATB)
PhaJ	(*R*)-specific enoyl-CoA hydratase
PhaZ	PHA depolymerase
PHBA	Poly(3-hydroxybutyratee-*co*-3-hydroxyalkanoate)
PHBHx	Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate)
PHBV	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)
PHPV	Poly(β-hydroxy-5-phenylvalerate)
PLA	Poly(lactic acid)
PLLA	Poly(L-lactic acid)
PO	Palm olein
POPs	Persistent organic pollutants
PP	Polypropylene
PPE	Personal protective equipment
PPi	Diphosphate
PS	Polystyrene
PSA	Particle Size Analysis
PTFE	Polytetrafluoroethylene
PTT	Polytrimethylene terephthalate
PVC	Polyvinyl chloride
rRNA	Ribosomal nucleic acid
RNA	Ribonucleic acid
SATREPS	Science and technology research partnership for sustainable development
SARS-Cov-2	Novel severe acute respiratory syndrome coronavirus
SBD1	Substrate-binding domain type 1
SBD2	Substrate-binding domain type 2
scl	Short-chain-length
SCP	Single-cell protein
SDS	Sodium dodecyl sulfate
SEC	Size exclusion chromatography
SEM	Scanning Electron Microscopy
Ser	Serine
SFCA	Surfactant free cellulose acetate

SUPs	Single-use plastics
TAE	Tris-acetate-EDTA
TCA	Tricarboxylic acid
TE	Tris-Cl EDTA buffer
Thr	Threonine-rich region
UMB	Ultramicrobacteria
USM	Univeristi Sains Malaysia
UV	Ultraviolet
WAXD	Wide angle X-ray diffraction
XRD	X-ray diffraction

LIST OF APPENDICES

PEMENCILAN, IDENTIFIKASI DAN PENCIRIAN BAKTERIA PENGURAI POLI(3-HIDROKSIBUTIRAT-*ko*-3-HIDROKSIHEKSANOAT) [PHBHx] DARIPADA TANAH, PLASTISFERA DAN SAMPEL AIR PAYAU

ABSTRAK

Polihidroksialkanoat (PHA) adalah bioplastik yang menjadi alternatif kepada plastik konvensional yang berasaskan petroleum dan berpotensi untuk mengurangkan kesan pencemaran plastik terhadap alam sekitar. Pengkomersilan produk PHA menjadikan sistem pengurusan sisa yang cekap suatu keperluan utama bagi menangani peningkatan pengeluaran dan penggunaan bioplastik ini. Penubuhan sistem sebegini memerlukan bakteria pengurai PHA yang versatil dan mampu menguraikan pelbagai jenis PHA dalam pelbagai keadaan persekitaran. Kajian ini bertujuan untuk memencilkan bakteria pengurai PHBHx daripada tanah, plastisfera dan air payau. Sebanyak 22 bakteria pengurai PHA telah disaring bagi menguji keupayaan mereka menguraikan pelbagai jenis PHA, khususnya PHBHx dengan komposisi monomer 3HHx yang berbeza, menggunakan kaedah zon jelas. Keputusan menunjukkan bahawa apabila kandungan monomer 3HHx meningkat, bilangan bakteria yang mampu menguraikan kopolimer tersebut semakin berkurangan. Empat belas pencilan menunjukkan keupayaan untuk menguraikan PHBHx (27 mol% 3HHx), namun hanya tujuh daripadanya membentuk zon jernih yang ketara. Antara pencilan tersebut, strain USM5 telah dipilih kerana kemampuannya menguraikan kedua-dua P(3HB) dan PHBHx (sehingga 27 mol% 3HHx) pada agar PHA laut. Analisis jujukan 16S rRNA dan filogenetik menunjukkan bahawa strain tersebut tergolong dalam genus *Priestia*. Strain ini telah dipencilkan dari ekosistem paya bakau di Balik Pulau, Pulau Pinang. Dalam kajian penguraian secara *in vitro* menggunakan filem PHBHx sebagai sumber

karbon tunggal dalam kaldu MM laut, strain USM5 berjaya menguraikan filem PHBHx (8 mol% 3HHx) sepenuhnya, manakala filem PHBHx (11 mol% 3HHx) dan (27 mol% 3HHx) masing-masing mencapai kadar penguraian sebanyak 97.4% dan 42% dalam tempoh 14 hari. Strain ini tidak menunjukkan pertumbuhan pada suhu melebihi 50 °C, manakala suhu optimum bagi pertumbuhannya ialah 37 °C. Namun begitu, julat suhu bagi penguraian PHBHx (8 mol% 3HHx) adalah antara 25 °C hingga 37 °C. Strain ini dapat tumbuh dalam kepekatan NaCl antara 0% hingga 15%, namun hanya mampu menguraikan PHBHx (8 mol% 3HHx) sebagai sumber karbon tunggal dalam julat kepekatan NaCl 0% hingga 5%. Kemampuan *Priestia* sp. USM5 untuk menguraikan PHBHx dalam pelbagai keadaan saliniti menyerlahkan peranannya dalam kitaran PHA di ekosistem paya bakau. Penemuan ini bukan sahaja menunjukkan potensi bakteria ini dalam menyumbang kepada pengurusan sisa bioplastik yang mampan, tetapi juga merupakan kajian pertama yang meneroka bakteria pengurai PHA yang dipencilkan dari persekitaran paya bakau.

ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF POLY(3-HYDROXYBUTYRATE-*co*-3-HYDROXYHEXANOATE) [PHBHX] DEGRADING BACTERIA FROM SOIL, PLASTISPHERE, AND BRACKISH WATER SAMPLES

ABSTRACT

Polyhydroxyalkanoates (PHAs) are a promising bioplastic alternative to conventional petroleum-based plastics, offering the potential to mitigate the environmental impact of plastic pollution. Among PHAs, poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [PHBHx] stands out due to its superior thermal and mechanical properties compared to poly(3-hydroxybutyrate) [P(3HB)], making it suitable for a wider range of applications. The commercialization of PHA products necessitates efficient waste management systems to handle their increasing production and consumption. Establishing such a system requires versatile PHA-degrading bacteria capable of effectively breaking down various PHA types under diverse environmental conditions. This study aimed at isolating potential PHBHx-degrading bacteria from soil, plastisphere and brackish water samples. In this study, a total of 22 PHA-degrading bacteria were screened for their ability to degrade different types of PHA, particularly PHBHx with different 3HHx monomeric compositions, using clear zone assays. Results showed that as the 3HHx monomer composition increased, the number of bacteria capable of degrading the copolymer decreased. Fourteen isolates were able to degrade PHBHx (27 mol% 3HHx), with only seven forming distinct clear zones. Among these, strain USM5 was selected due to its ability to degrade both P(3HB) and PHBHx (up to 27 mol% 3HHx) on marine PHA agar. The 16S rRNA sequencing and phylogenetic analysis revealed that the strain belonged to the genus *Priestia*. This

strain was previously isolated from the mangrove ecosystem of Balik Pulau, Pulau Pinang. In *in vitro* biodegradation of PHBHx films as the sole carbon source in marine MM broth, strain USM5 completely degraded PHBHx films (8 mol% 3HHx), while films with 11 mol% and 27 mol% 3HHx showed 97.4% and 42% degradation, respectively, within 14 days. The strain did not grow above 50 °C, and the optimum temperature for growth was 37 °C. However, the temperature range for PHBHx (8 mol% 3HHx) degradation was 25 °C - 37 °C. The strain grew in NaCl concentrations from 0% - 15%, but only degraded PHBHx (8 mol% 3HHx) as the sole carbon source within a 0% - 5% NaCl range. The ability of *Priestia* sp. USM5 to degrade PHBHx across varying salinity conditions underscores its ecological role in the PHA cycle within mangrove ecosystems. This finding not only highlights the bacterium's potential for contributing to sustainable bioplastic waste management but also represents the first study to explore PHA-degrading bacteria isolated from a mangrove environment.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Petroleum-based plastics have become deeply embedded in modern society, with widespread applications in packaging, healthcare, agriculture, and household goods due to their durability, versatility, and low production costs (Geyer et al., 2017). However, these plastics are derived from non-renewable resources and are highly resistant to degradation. Their persistence in the environment, combined with inadequate disposal and recycling systems, has resulted in the accumulation of plastic waste in landfills and aquatic ecosystems. This persistent pollution poses serious threats to both terrestrial and marine environments, affecting biodiversity and disrupting ecological functions (Derraik, 2002; Gregory, 2009). Furthermore, microplastics originating from fragmented plastic waste have been detected in human tissues, including the blood, lungs, and placenta. These findings have raised growing concerns about the potential health risks associated with plastic pollution (Ragusa et al., 2021; Wu et al., 2022).

In response to the growing environmental crisis, there is a strong push toward the development and adoption of sustainable alternatives. Biodegradable plastics, particularly polyhydroxyalkanoates (PHAs), have emerged as a promising solution. PHAs are a family of bio-based, biodegradable polyesters synthesized by a wide range of microorganisms as intracellular carbon and energy storage compounds (Anderson & Dawes, 1990; Fukui & Doi, 1998; Ishizaki & Tanaka, 1991). These polymers exhibit mechanical properties comparable to conventional plastics such as polypropylene (PP) and polyethylene (PE), but with the crucial advantage of being biodegradable under

natural environmental conditions (Sudesh et al., 2000). As such, PHAs offer a viable route toward reducing the environmental burden of conventional plastic waste.

Among the various types of PHAs, poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (PHBHx) has attracted considerable attention due to its improved flexibility, reduced brittleness, and enhanced processability compared to the homopolymer poly(3-hydroxybutyrate) [P(3HB)] (Doi et al., 1995; Tang et al., 2022). These advantageous properties result from the incorporation of 3-hydroxyhexanoate (3HHx) monomers into the polymer backbone, and the tunable composition of these monomers enables the customization of polymer characteristics for diverse industrial applications (Chen, 2009). However, as PHBHx continues to gain traction in commercial and industrial sectors, there is a growing need to understand its environmental fate, particularly its biodegradation.

Although numerous studies have investigated the microbial degradation of polyhydroxyalkanoates (PHAs), particularly poly(3-hydroxybutyrate) [P(3HB)], research on the biodegradation of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (PHBHx) by PHA-degrading bacteria remains relatively limited. Most existing studies have focused on PHBHx with low 3-hydroxyhexanoate (3HHx) content, while the degradation behavior and microbial interactions with PHBHx containing higher 3HHx monomeric compositions especially those exceeding 20 mol%, are still poorly understood. This knowledge gap restricts our ability to fully evaluate the environmental performance and biodegradability of commercially relevant PHBHx formulations.

While several studies have examined PHA degradation in tropical environments, including soil and marine ecosystems, research involving the isolation

and identification of PHBHx-degrading bacteria from these environments remains limited. Moreover, no reports to date have documented the isolation of such bacteria from mangrove brackish water environments. This highlights the importance of exploring underrepresented ecosystems to discover novel microbial strains with the potential to degrade PHBHx, particularly those with high 3HHx content.

To address this gap, the present study aims to isolate, screen, and characterize PHA-degrading bacteria capable of degrading PHBHx, with a focus on polymers containing high 3HHx monomeric compositions. Gaining insights into how these bacterial strains interact with and degrade PHBHx can inform the development of targeted bioremediation strategies and sustainable waste management practices. Ultimately, this study aims to contribute to broader efforts to mitigate plastic pollution and promote environmental sustainability.

1.2 Objectives

The objectives of this study were:

1. To isolate, screen, and identify PHA-degrading bacteria using agar media prepared with different types of polyhydroxyalkanoates (PHAs).
2. To investigate the ability of isolated bacteria to degrade different types of PHAs using solid-based media culture.
3. To characterize the selected bacterium and evaluate its PHBHx film degrading ability in liquid media.

CHAPTER 2

LITERATURE REVIEW

2.1 An overview of plastics

Plastics are a class of polymers that possess valuable industrial qualities, including light and chemical resistance, low production cost, strength and durability, high thermostability, electrical insulation and resistance to biological decay. Although their large-scale production and usage only began during the 1950s, their rapid increase in plastics manufacturing surpasses any man-made materials. It has become one of the most important manufactured materials in the 21st century, reaching a production of 390 million tons in 2021 (Plastics Europe, 2022).

The exponential increase in polymer production, driven by the versatility of its properties, is particularly notable in the packaging sector, which is the largest market for plastics. Although plastics are vital in modern society and are almost impossible to completely replace, they were made from fossil hydrocarbons which is a non-renewable resource. Plastics such as polypropylene (PP), polyethylene (PE), polystyrene (PS) and polyvinyl chloride (PVC) were derived from fossil hydrocarbons and as such, are non-biodegradable. These plastics do not degrade nor decompose, resulting in plastics accumulation in landfills or worse, in the environment causing plastic pollution (Barnes et al., 2009).

2.1.1 Plastic waste and pollution

It has been proposed that plastics are a key geological indicator of the Anthropocene epoch, attributed to the ubiquitous presence of plastic waste in the environment (Zalasiewicz et al., 2016). It is estimated that over 400 million metric tons

(Mt) of plastic are manufactured each year, of which 50% are reportedly thrown away after just one use (i.e. single-use plastics) (Geyer et al., 2017). Additionally, it is believed that between 60 and 80 percent of waste produced worldwide consists of plastic debris (Derraik, 2002). Furthermore, research has shown that by 2015, approximately 6.3 billion metric tons of plastic polymers had been produced globally. A significant portion of this production has ended up as waste, with only 9% being recycled and 12% being incinerated. The remaining 79% accumulated in the natural environments or landfills, with roughly 10% of that entering the marine and coastal environments (Geyer et al., 2017; Mendenhall, 2018). Recent predictions indicated that the quantity of mismanaged plastic waste entering the oceans due to human activities on land could range between 100 and 250 million metric tons by 2025 (Jambeck et al., 2015). This prediction might be a reality particularly exacerbated by the sudden plastic waste boom during the COVID-19 pandemic.

Single-use plastics (SUPs) have a very short lifespan and are discarded shortly after usage. SUPs consist of a wide range of plastic products including plastic bags, cutleries, polystyrene cups and food containers, straws, and even microbeads. The COVID-19 pandemic (which was caused by the outbreak of novel severe acute respiratory syndrome coronavirus (SARS-Cov-2) has also exacerbated the world plastic pollution problem caused by the SUPs (Shams et al., 2021). This has caused an increase in the manufacture and waste of SUP personal protective equipment (PPE) such as face masks, surgical masks, face shields and gloves used both by frontline health workers and normal citizens to stop the spread of the virus. These PPEs were mostly made of polymers such as PE, PP, PVC and PS which are rarely recycled (Kahlert & Bening, 2020). The World Bank even warned of the potential reversal of the years-long trend towards reducing the use of SUPs caused by this pandemic. This increase in the usage

of single-use plastic personal protective equipment and packaging-related items is particularly evident during this time. It is a result of the increasing trend of online shopping and takeout, spurred by the pandemic lockdown and a new hyper-sanitary lifestyle (Parashar & Hait, 2021).

Currently, the only viable method to permanently eradicate plastic waste is by destructive thermal processes such as burning or pyrolysis. Consequently, the overflow of plastic waste, which poses a risk of permanent contamination of the natural environment, has become a major global concern. Plastic waste is ubiquitous, contaminating the terrestrial and freshwater environments before entering and persists in the marine environment via wind, rivers, sewage and drainage systems (Rillig, 2012; Wagner et al., 2014; Zubris & Richards, 2005). Plastic waste has been reported in diverse locations, ranging from Mount Everest, remote coastlines, and mangroves to all major ocean basins, including the five gyres of the ocean (North and South Pacific, the North and South Atlantic, and the Indian Ocean) and even the Mariana trench (world's deepest oceanic trench) (Barnes et al., 2009; Chiba et al., 2018; Martin et al., 2020; Napper et al., 2020; Van Sebille et al., 2020).

Plastic debris can be classified into two major categories: macroplastic (size: > 5 mm) and microplastic (size: 0.1 μm to < 5mm) (Thompson et al., 2004). Microplastic itself consist of two sub-categories which are primary microplastics and secondary microplastics. Primary microplastics such as microbeads were purposely created to be used as exfoliants in cosmetics and personal hygiene products, in cleaning products, printer toners, medical applications and industrial products such as abrasive media (Gregory, 1996; Pettipas et al., 2016). Secondary microplastics are fragmented plastics derived from deteriorated macroplastic (Pettipas et al., 2016). This fragmentation is a result of the plastic debris undergoing embrittlement processes due to prolonged

exposure to UV light (photo-degradation) and physical damage (Colton et al., 1974; Gregory, 1978; Thompson et al., 2004). Further ongoing embrittlement also caused the microplastics to be nanofragmented into even smaller particles dubbed nanoplastic (1 nm to 1 μm) (Bouwmeester et al., 2015; Cózar et al., 2014; Gigault et al., 2018). Hence, microplastic and nanoplastic have become a major concern as they are almost impossible to remove from the environment. In addition, it is reported that approximately 8 trillion microbeads are released daily into the wastewater, making them the most abundant form of microplastics in the ocean (Cole et al., 2011; Rochman et al., 2015a; Rochman et al., 2015b).

2.1.2 The impact of plastic pollution

The pervasive impact of plastic pollution on the natural environment is an urgent and undeniable concern. Ecotoxicity, coexisting biological effects on keystone or endangered species, altered habitat in soils, sediments, and aquatic ecosystems, and changes in carbon and nutrient cycles are all potentially devastating impacts of plastic pollution (Aloy et al., 2011; Carson et al., 2011; Goldstein et al., 2012; MacLeod et al., 2021). In the marine environment, plastic debris has been reported to heavily impact marine life through entanglement and ghost fishing, ingestion and suffocation (Ayaz et al., 2006; Donohue et al., 2001; Good et al., 2010; Gregory, 2009). The fatalities of marine life due to plastic debris have ranged from marine mammals (54% of all species), fishes (0.68% of all species), sea turtles (100% of all known species) and seabirds (56% of all species) (Gall & Thompson, 2015; Laist, 1987). In addition, the surface of plastic debris could also serve as a substrate for the transport of alien invasive species that

might change or even destroy the local habitat (Barnes & Fraser, 2003; Barnes, 2002; Gregory, 2009; Winston et al., 1997).

The detrimental effects of plastic pollution on human health also cannot be overstated. Recent reports have revealed alarming findings, demonstrating that microplastics have the ability to penetrate biological membranes and have been detected even in human placenta (Ragusa et al., 2021). Moreover, plastic debris has been found to release and adsorb harmful chemicals (Napper et al., 2015; Teuten et al., 2007, 2009; Wang et al., 2016). These chemicals include additives such as phthalates, polybrominated diphenyl ethers, and bisphenol A, which leach from plastics and can directly impact the human body, leading to endocrine disruption and affecting mobility, reproduction, development, and even carcinogenesis (Lithner et al., 2009, 2011; Talsness et al., 2009). Furthermore, bisphenol A has been associated with alterations in hormone levels in the blood, as well as an increased risk of diabetes and heart disease (Galloway et al., 2010; Lang et al., 2008). Additionally, plastics can adsorb and desorb pollutants such as aqueous metals (e.g. Al, Fe, Mn) and persistent organic pollutants (POPs) [e.g. polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), Dichlorodiphenyltrichloroethane (DDT)] (Ashton et al., 2010; Betts, 2008; Rios et al., 2007). Human exposure to POPs has been linked to an increased risk of cancers, congenital malformations, and various respiratory diseases (Qing Li et al., 2006).

Overall, there is an urgent need to address plastic pollution and it demands innovative and sustainable solutions. Hence, bioplastics offer a promising alternative, presenting an opportunity to mitigate the detrimental effects of plastic pollution on both the environment and human health, paving the way towards a more sustainable future.

2.2 Bioplastics

While the term "bioplastic" has no set definition, it is generally accepted that a bioplastic is any polymer that is either biobased, biodegradable, or both (Nandakumar et al., 2021). Therefore, this broad definition can be divided into three categories; (i) bio-based and non-biodegradable; (ii) bio-based and biodegradable and (iii) fossil-based and biodegradable. This implies that not all bioplastics are biodegradable, and some non-biobased plastics are biodegradable. A schematic overview of the bioplastic definition is presented in Figure 2.1.

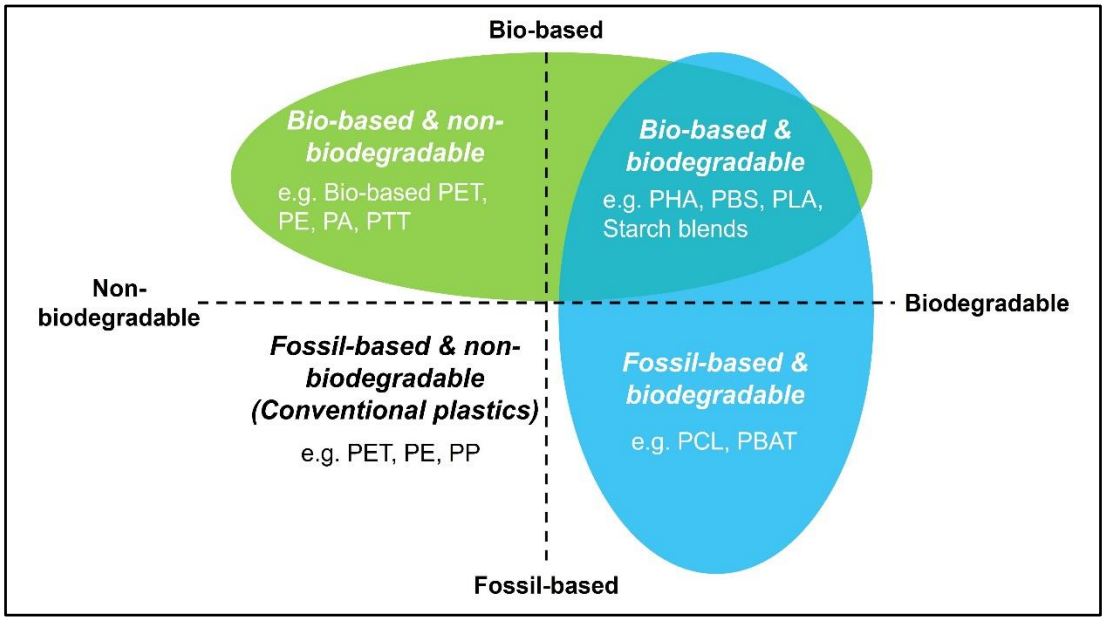

Figure 2.1 Schematic overview of bioplastic definition. PET: polyethylene terephthalate; PE: polyethylene; PA: polyamide; PTT: Polytrimethylene terephthalate; PHA: Polyhydroxyalkanoates; PBS: Polybutylene succinate; PLA: Polylactic acid; PCL: Polycaprolactone; PBAT: Polybutylene adipate terephthalate.

Although the broad definition of bioplastic, this review only focuses on bioplastics that are bio-based and biodegradable, which are relevant to the present study. Bio-based polymers are polymers that are derived from renewable biological resources and polymerized through chemical or biological processes. These polymers possess similar qualities as fossil-based plastics (Harmsen et al., 2014).

The bio-based polymers were categorized based on the type of polymers and the processes involved. Bio-based plastics were divided into three main categories: (i) biosynthetic polymers, (ii) bio-chemosynthetic polymers and (iii) modified natural polymers (Sudesh & Iwata, 2008). Biosynthetic polymers are polymers that are synthesized entirely through biological processes. Such polymer biosynthesis process occurs inside microorganisms from the monomer production until polymerization of monomers. Polyhydroxyalkanoates (PHAs) are one of the most well-known biosynthetic polymers. Bio-chemosynthetic polymers undergo a two-step process, (i) biological processes to derive monomers and (ii) chemical polymerization of monomers. An example of a bio-chemosynthetic polymer is poly(lactic acid) (PLA), where its lactic acid monomers were derived from microbial fermentation and agricultural waste (Gross & Kalra, 2002; Jem et al., 2010). Natural polymers from biomass such as starch and cellulose undergo chemical modifications including the cross-linking of two hydroxyl groups from neighbouring starch molecules, resulting in a modified natural polymer. Nevertheless, to decrease the hydrophilicity and increase processability, modified natural polymers like starch are combined with synthetic or bio-based polymers for most applications (Bastioli et al., 2020).

Bioplastics, particularly bio-based and biodegradable plastics present a solution to the issue of mismanaged waste leaking into the environment, where human intervention proved ineffective (Manfra et al., 2021). This is because products made

from bioplastics are capable of degrading naturally in the environment through the action of microorganisms such as bacteria, fungi and algae (Lambert & Wagner, 2017). Currently, bioplastics have been widely used throughout various industries including packaging, consumer electronics, toys, textiles, agriculture, consumer electronics, and automotive. In 2023, bioplastic production capacities reached 2.18 million metric tons, of which 52.1% were biodegradable. In addition, bioplastics for the packaging market account for the largest share at 43% of total production (European bioplastics, 2023). Lists of commercially important biobased and biodegradable polymers along with their producers are shown in Table 2.1.

Table 2.1 List of commercially important bio-based and biodegradable polymers, and their producers (Sudesh & Iwata, 2008).

Category	Polymer	Producer	Trade name
Biosynthetic polymers	Polyhydroxyalkanoate (PHA)	Kaneka, Japan	GreenPlanet™
		Metabolix, USA	Biopol®
		Biomer, Germany	Biomer®
		Mitsubishi Gas, Japan	Biogreen®
		PHB Industrial S/A, Brazil	Biocycle®
		Telles, USA	Mirel™
Bio-chemosynthetic polymers	Poly(butylene succinate) (PBS)	Showa High Polymer, Japan	Bionolla
		Mitsubishi Chemicals, Japan	GS Pla
	Poly(lactic acid) (PLA)	Toyota, Japan	U'z
		Mitsui Chemicals, Japan	Lacea®
		Hycail, Netherlands	Hycail HM; Hycail LM
		NatureWorks, USA	NatureWorks®
Modified natural polymers	Derivatives of cellulose	Daicel Chemical Industries, Japan	Cellgreen
	Starch polymers	BIOP, Germany	BIOpar®
		Novamont, Italy	Mater-Bi®
		Japan Corn Starch, Japan	Cornpol®
		Rodenburg, Netherlands	Solanyl®

2.3 Polyhydroxyalkanoate (PHA)

Among all the biosynthetic polymers, polyhydroxyalkanoate (PHA) is revered as the cornerstone for bioplastic innovation. PHA, a biopolymer chain composed of various hydroxyalkanoates, is synthesized, accumulated, and stored intracellularly by microorganisms as a source of carbon and energy (Anderson & Dawes, 1990). Maurice Lemoigne first discovered PHA in 1926, identifying lipid-like inclusions in *Priestia megaterium* (formerly known as *Bacillus megaterium*) (Lemoigne, 1926, 1927). To date, over 300 species across at least 75 genera of both Gram-positive and Gram-negative bacteria, as well as archaea, have been identified as capable of synthesizing PHA (Reddy et al., 2003; Rehm, 2003). Furthermore, more than 160 distinct monomeric blocks of PHA have been identified (Miyahara et al., 2021; Rehm, 2003).

PHA accumulates within bacterial cells under various stress conditions, such as nitrogen, phosphorus, oxygen, or magnesium depletion, coupled with an excess supply of carbon (Anderson & Dawes, 1990; Kato et al., 1996; Madison & Huisman, 1999). Under unfavourable growth conditions, the excess carbon will be assimilated within the bacterial cells and biochemically converted into 3-hydroxyalkanoic acids (3HA) monomer units, which are then polymerized into high molecular weight, water-insoluble PHA molecules. These lipid-like molecules will be retained as inclusions or granules (diameter: 0.2 – 0.5 μm) in the cytoplasm of microbial cells, which can be easily observed under a phase contrast microscope due to its high refractivity (Dawes & Senior, 1973; Sudesh, 2013). Under laboratory settings, these granules can be accumulated up to a maximum of 90 wt% of the dry cell weight by some bacteria (Madison & Huisman, 1999). Generally, PHA consists of carbon, oxygen and hydrogen,

with the polymer structure containing at least one hydroxyl group (–OH) and a carboxyl group (–COOH) as depicted in Figure 2.2.

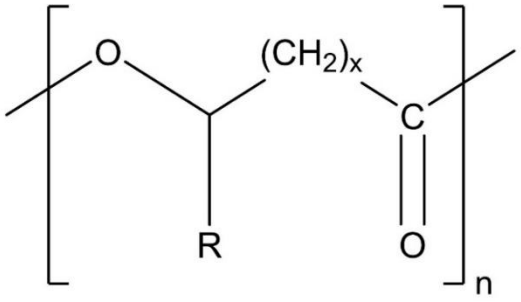

Figure 2.2 The general chemical structure of polyhydroxyalkanoate (PHA). R refers to the side chain that attached to the molecule. x refers to the carbon chain in the linear polyester structure. 'n' refers to the number of repeating units.

PHA are classified into three main types based on the number of carbon atoms present in their monomeric units: (i) short-chain-length (scl), (ii) medium-chain-length (mcl) and (iii) a combination of both (scl-mcl). Monomers of scl-PHAs consists of 3 – 5 carbon atoms whereas monomers of mcl-PHAs have 6 – 14 carbon atoms. Additionally, scl-mcl-PHAs monomers can range from 3 – 14 carbon atoms (Li et al., 2007; Sudesh, 2013). In nature, most PHA-producing microbes synthesize scl-PHAs, primarily contains 3HB units, or mcl-PHAs, primarily contain 3-hydroxyoctanoate (3HO) and 3-hydroxydecanoate (3HD) (Steinbüchel & Füchtenbusch, 1998).

The PHA synthesized by bacteria exhibits industrially desirable polymer properties, including high molecular weights comparable to petrochemical plastics such as PE and PP (Madison & Huisman, 1999; Qin et al., 2007). Additionally, PHA has been reported to be non-toxic, biocompatible, optically active, with a high degree of polymerization, and most importantly, capable of complete biodegradation in the natural environment (Steinbüchel & Füchtenbusch, 1998; Sudesh et al., 2000).

PHA properties such as thermal and mechanical properties are dependent on the type of monomers, composition and molecular weight (Sudesh et al., 2000). Generally,

scl-PHAs possess thermoplastic qualities such as high crystallinity, melting temperature and tensile strength. However, they are also stiff, brittle and have low elongation at break. Conversely, mcl-PHAs display more elastomeric qualities, such as low crystallinity and melting temperature, with a high elongation at break (Yu, 2007). Compared to the homopolymers of scl-PHAs and mcl-PHAs, scl-mcl-PHAs copolymers exhibit improved qualities such as enhanced thermal and mechanical properties (Kellerhals et al., 2000). However, the scl-mcl-PHAs copolymers properties varies significantly depending on the monomeric composition of mcl-PHA. The properties vary from a polymer that is strong with less flexibility to a polymer that is strong and more flexible (Asrar et al., 2002).

Given their remarkable properties, PHAs are increasingly attracting global attention and are the subject of extensive research aimed at developing them for commercial applications. These biopolymers not only offer a sustainable alternative to traditional plastics but also demonstrate versatility across various industrial uses due to their tuneable characteristics.

2.3.1 Poly(3-hydroxybutyrate) [P(3HB)]

Poly(3-hydroxybutyrate), or P(3HB), was the first of the 160 distinct monomeric blocks of PHA to be identified. Since then, P(3HB) has become the most thoroughly characterized and extensively studied among all PHAs. P(3HB) is a polyester comprising an ester group with four carbon atoms, along with a methyl group attached as the side chain at the β-position. The chemical structure of P(3HB) is shown in Figure 2.3. This polyester exhibit 55–80% crystallinity and a perfect isotactic structure with solely a (*R*)-configuration (Holmes, 1988).

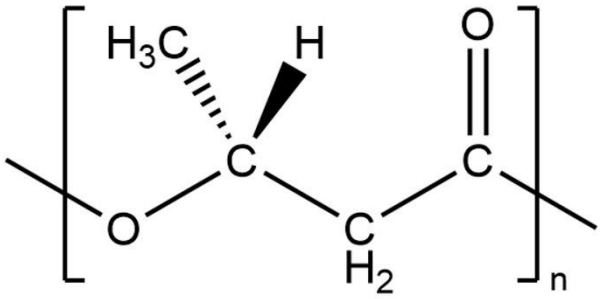

Figure 2.3 Chemical structure of P(3HB). 'n' refers to the number of repeating units.

Typically, wild-type bacteria will be able to produce P(3HB) with the molecular weight (M_w) ranging from 1×10^4 to 3×10^6 g/mol with a polydispersity index of approximately 2.0. P(3HB) exhibits mechanical properties akin to that of PP, with a Young's modulus of 3.5 GPa and a tensile strength of 43 MPa. However, P(3HB) exhibits a significantly lower elongation at break, measured at 5%, compared to PP at 400%. This disparity is attributed to the inherently brittle and stiff nature of P(3HB) when contrasted with the more conventional PP. One of the factors contributing to the polymer inherent embrittlement is the presence of cracks in the P(3HB) spherulites.

Inter-spherulitic cracks are visible in large spherulites due to the low nucleation density of the P(3HB) homopolymer (Barham et al., 1992; Barham & Keller, 1986; Martinez-Salazar et al., 1989). Additionally, after their initial crystallization, P(3HB) materials also experience embrittlement during storage (de Koning & Lemstra, 1993).

Moreover, P(3HB) polyester has a high melting temperature (T_m) at approximately 180 °C, with a glass transition temperature (T_g) at approximately 4 °C. However, its T_m is relatively close to its thermal degradation temperature (T_d), which is approximately 200 °C. This results in a narrow processing window of this homopolymer, due to its inherent thermal instability (Khanna & Srivastava, 2005; Wang et al., 2008, 2010).

While P(3HB) shares qualities with conventional plastics like PP, its inherent stiffness, brittleness and thermal instability pose significant limitations on its applicability. Hence, an effective strategy to overcome the inherent mechanical defect of P(3HB), copolymerization with other monomeric constituents will help to enhance the overall polymer properties (Miyahara et al., 2021).

2.3.2 Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (PHBHx)

Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate), or PHBHx, is classified as a scl-mcl PHA. This random copolymer is composed of two different types of monomer unit: (*R*)-3HB (scl-PHA) and (*R*)-3HHx (mcl-PHA). The 3HHx monomer consists of an ester group composed of six carbon atoms with a propyl group as the side chain group at the β-position. The chemical structure of the PHBHx copolymer is shown in Figure 2.4. The 3HHx monomer unit has a long alkyl side chain, preventing both 3HHx and 3HB monomer units from fitting into crystalline lattices of each other. This lack of mutual fit prevents isodimorphism between both monomer units (Doi et al., 1995).

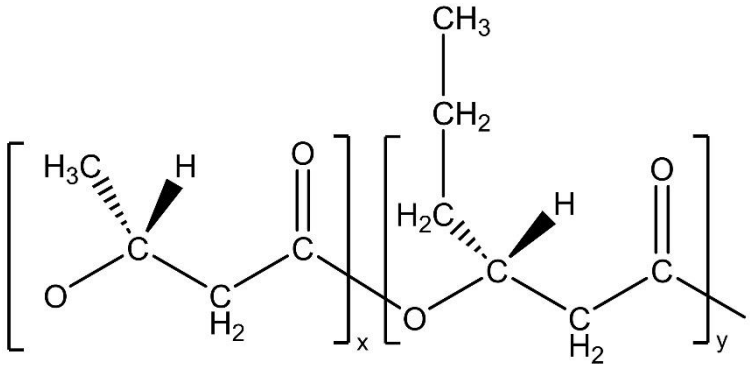

Figure 2.4 Chemical structure of PHBHx. Both 'x' and 'y' refers to the repeating unit of each monomer.

This copolymer was first discovered to be biosynthesized by *Aeromonas caviae* FA440 (Shimamura et al., 1994). This strain has been reported to synthesize PHBHx by utilizing olive oil and sodium salts of alkanoic acids with chain lengths ranging from 11 to 18 (C_{11} – C_{18}). It was able to accumulate up to 27 wt% of its dry cell weight (DCW) with 10 – 25 mol % 3HHx fractions (Doi et al., 1995). Since then, several recombinant bacteria have also been reported to be able to produce PHBHx including *Pseudomonas putida* GPp104 and *Ralstonia eutropha* (previously known as

Alcaligenes eutrophus) (PHB⁻4/pJRDEE32d13) (Fukui & Doi, 1997, 1998; Kichise et al., 1999).

The polymer properties of PHBHx copolymer are influenced by the molar fraction of 3HHx. With an increase in the 3HHx molar fraction from 0 to 25 mol%, the crystallinity of the PHBHx solvent-cast films decreased from 60 to 18%. This increase also impacted the mechanical properties of the copolymers, leading to a rise in the elongation at break from 5 to 850%, while the tensile strength decreased from 43 to 20 MPa with an increase in the 3HHx content from 0 to 17 mol%. Consequently, the PHBHx copolymer became softer and more flexible. Furthermore, changes in the 3HHx molar fraction influenced the thermal properties, resulting in a decrease in both the T_m from 177 to 52 °C and the T_g from 4 to –4 °C as the 3HHx molar fraction increased from 0 to 25% (Doi et al., 1995; Sudesh, 2013). The presence of 3HHx widens the processing window for PHBHx copolymer due to the lower T_m, enabling processing such as moulding at temperatures below the T_d (Miyahara et al., 2021).

The combination of scl- and mcl-PHA in this copolymer yields superior mechanical and thermal properties compared to the P(3HB) homopolymer. The inclusion of the 3HHx monomer renders the polymer softer, more flexible and enhances processability (Tang et al., 2022). Furthermore, the adjustment of the 3HHx molar fraction allows for the customization of polymer properties to suit specific applications. This versatility positions the copolymer as highly desirable for various industrial applications.

2.4 PHA biosynthesis

2.4.1 PHA-producing bacteria

The diverse array of bacteria capable of producing PHA spans multiple taxonomic groups, encompassing phototrophic bacteria, archaea, Gram-positive and Gram-negative bacteria, as well as both aerobic and anaerobic species (Anderson & Dawes, 1990; Brandl et al., 1990). Most PHA-producing bacterial species are capable of synthesizing scl-PHAs such as *Bacillus*, *Burkholderia*, *Aquitalea* and *Jeongeupia* (Chee et al., 2010; Ng & Sudesh, 2016; Tsuge et al., 2015; Wan et al., 2023; Zain et al., 2020). However, some bacterial species such as from the genus *Pseudomonas* are capable of synthesizing mcl-PHAs (de Smet et al., 1983; Khare et al., 2014; Lageveen et al., 1988; Sudesh et al., 2000). Additionally, there are also some wild-type species that are capable of synthesizing the scl-mcl-PHA copolymer (Doi et al., 1995; Eraslan et al., 2022; Gong et al., 2008; Shimamura et al., 1994; Steinbüchel & Valentin, 1995).

Cupriavidus necator H16 (formerly known as *Ralstonia eutropha*, *Wautersia eutropha*, or *Alcaligenes eutrophus*) serves as the model bacterium in PHA biosynthesis studies. While the wild-type strain can only produce P(3HB), it can be genetically engineered to produce other copolymers such as PHBHx, poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) and even poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB/4HB)] (Doi et al., 1990; Jawed et al., 2022; Kimura et al., 2008; Tan et al., 2020). Notably, *C. necator* H16 exhibits versatility in utilizing various carbon sources for PHA biosynthesis, including sugars (e.g., glucose, fructose, sucrose and xylose), fatty acids (e.g., coffee waste oil, free fatty acids and waste glycerol), industrial wastes (e.g., wheat straw, kenaf biomass, corn stover) and even carbon dioxide (Bhatia et al.,

2018; Diankristanti et al., 2024; Ishizaki & Tanaka, 1991; Saratale et al., 2019; Sharma et al., 2016; Sichwart et al., 2011; Soto et al., 2019; Weng et al., 2023). However, the type of carbon source supplied affects the composition of the produced PHAs.

2.4.2 Metabolic pathway of PHA production

As outlined in section 2.4.1, wild-type *C. necator* H16 produces P(3HB) homopolymer through a three-step enzymatic pathway converting acetyl-CoA into P3HB. This process involves the genes *phaA*, *phaB*, and *phaC*, organized as an operon (*phaC1-phaA-phaB1*) (Anderson & Dawes, 1990). Initially, pyruvate obtained from the glycolysis of sugars will be converted to acetyl-CoA by pyruvate dehydrogenase. Next, β-ketothiolase (PhaA) catalyzes the condensation of two acetyl-CoA molecules into acetoacetyl-CoA. Then, acetoacetyl-CoA is reduced by PhaB to form (*R*)-3-hydroxybutyryl-CoA [(*R*)-3HB-CoA]. Finally, PhaC polymerizes 3HB-CoA to produce P(3HB) (Sudesh et al., 2000). This process is labelled as pathway I in Figure 2.5.

Besides P(3HB), a recombinant strain of *C. necator* is also capable of synthesizing PHBHx. This copolymer is synthesized using substrates or monomers supplied from two metabolic pathways: fatty acid β-oxidation (pathway II) or fatty acid *de novo* biosynthesis (pathway III). Typically, PHBHx production utilizes oil or fatty acids, and it will undergo the fatty acid β-oxidation pathway (pathway II) (Doi et al., 1995; Fukui & Doi, 1997). Here, acyl-CoA synthetase (FadD) converts fatty acids into acyl-CoA, which is then oxidized to *trans*-enoyl-CoA by acyl-CoA dehydrogenase (FadE). In the subsequent step, *trans*-enoyl-CoA will undergo hydration by enoyl-CoA hydratase (FadB) into (*S*)-3-hydroxyacyl-CoA, followed by oxidation to 3-ketoacyl-CoA by 3-hydroxyacyl-CoA dehydrogenase (Had). Finally, 3-ketoacyl-CoA will be

converted into acyl-CoA by 3-ketoacyl-CoA thiolase (FadA). This process reduces acyl-CoA by two carbon atoms, yielding acetyl-CoA. This acetyl-CoA can be converted to P(3HB) homopolymer utilizing the previous pathway (pathway I). Subsequently, (*R*)-specific enoyl-CoA hydratase (PhaJ) converts the six carbons of (*S*)-3-hydroxyacyl-CoA or (*S*)-3-hydroxyhexanoyl-CoA [(*S*)-3HHx-CoA] into (*R*)-3-hydroxyhexanoyl-CoA [(*R*)-3HHx-CoA]. PhaC then incorporates (*R*)-3HHx-CoA into the PHBHx granule (Tang et al., 2022; Tsuge, 2002).

When sugar is used as the sole carbon source for PHBHx production, it follows an alternative engineered pathway which is the fatty acids *de novo* synthesis pathway (pathway III). The start of this pathway is similar to pathway I when sugars undergo glycolysis to form pyruvate, followed by decarboxylation by pyruvate dehydrogenase into acetyl-CoA. PhaA then converts acetyl-CoA into acetoacetyl-CoA. However, instead of reduction by PhaB to form (*R*)-3HB-CoA, acetoacetyl-CoA will undergo reduction by Had into (*S*)-3-hydroxybutyryl-CoA [(*S*)-3HB-CoA]. Then, (*S*)-3HB-CoA is further converted by crotonase (Crt2) into crotonyl-CoA. Following this, crotonyl-CoA is converted into (*R*)-3HB-CoA by either PhaJ or butyryl-CoA and subsequently incorporated into PHBHx as the 3HB monomers. Crotonyl-CoA reductase (Ccr) then reduces crotonyl-CoA to butyrl-CoA, which is further converted to 3-oxohexanoyl-CoA and reduced to (*S*)-3HHx-CoA by Had. This is followed by the conversion to 2-hexenoyl-CoA by Crt2 before being hydrated by PhaJ to (*R*)-3HHx-CoA. Finally, PhaC incorporates the (*R*)-3HHx-CoA into PHBHx as the 3HHx monomers (Tang et al., 2022; Zhang et al., 2019). The overview of the PHA metabolic pathway involving P(3HB) and PHBHx is shown in Figure 2.5.

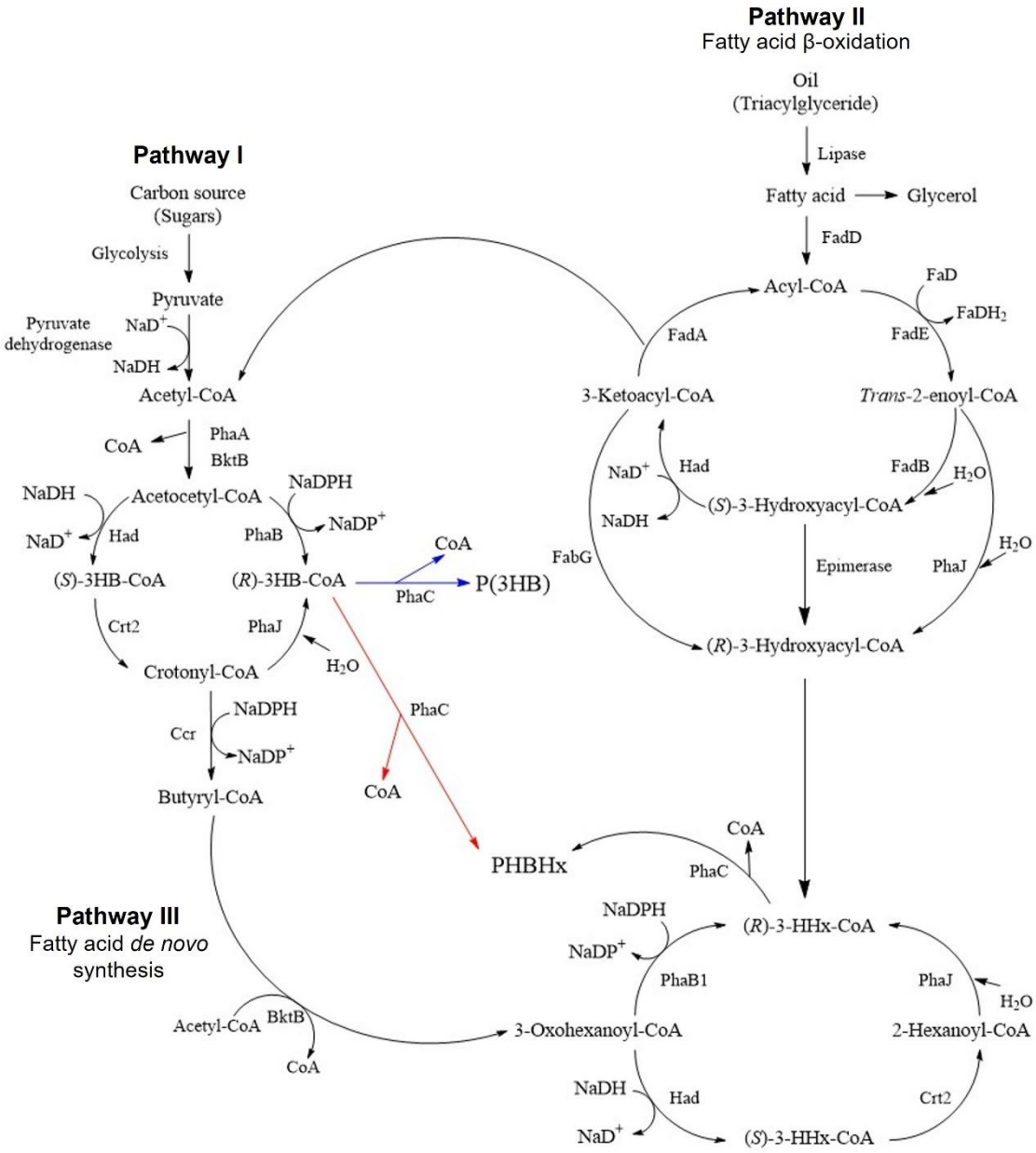

Figure 2.5 Metabolic pathway of P(3HB) and PHBHx production (Sudesh *et al*., 2000; Tang *et al*., 2022, Tsuge, 2002; Zhang *et al*., 2019). Abbreviation: PHA, polyhydroxyalkanoate; PhaA, β-ketothiolase; PhaB, NADPH-dependent acetoacetyl-CoA reductase; PhaC, PHA synthase; FadA, 3-ketoacyl-CoA thiolase; FadB, enoyl-CoA hydratase; FadD, acyl-CoA synthetase; FadE, acyl-CoA dehydrogenase; FabG, 3-ketoacyl-CoA reductase; 3HB-CoA, 3-hydroxybutyryl-CoA; 3HHx-CoA, 3-hydroxyhexanoyl-CoA; PhaJ, (*R*)-specific enoyl-CoA hydratase; Had, 3-hydroxyacyl-CoA dehydrogenase; Crt2, crotonase; Ccr, crotonyl-CoA reductase. Blue arrow: P(3HB) production; red arrow: PHBHx production.

2.5 PHA recovery and purification

It has been nearly a century since the discovery of PHA in 1926, yet its practical application as an alternative to conventional petrochemical plastics is still in progress. The primary challenge hindering PHA commercialization is its high production cost, with two main contributing factors: the type of carbon sources used for PHA biosynthesis and the polymer recovery process (Chen, 2009; Choi & Lee, 1997; Khatami et al., 2021). PHA downstream processing, constituting 30% of total production costs, is crucial as it determines polymer quality, subsequently influencing their final application and market value (Khatami et al., 2021; Sun et al., 2007). The recovery of PHA has been the focus of numerous studies and technological developments, ranging from laboratory to industrial scales. Various methods have been explored, including cell wall disruption using sodium hypochlorite and surfactants, enzymatic cell disruptions, bacteriophage-mediated lysis, induced cell autolysis, bead milling, high-pressure homogenization, ultrasonication, and solvent extraction (Hajnal et al., 2016; Hand et al., 2016; Kunasundari & Sudesh, 2011).

The solvent extraction method is commonly employed for polymer recovery and purification at the laboratory scale due to its rapid process and simplicity. Patented by Noel in 1692, this method utilizes chlorinated hydrocarbons such as methylene chloride, 1,2-dichloroethane, or chloroform to solvate the PHA granules inside cells by altering cell permeability. Following this purification technique, insoluble biomass is removed using methanol or ethanol precipitation (Noda, 1998; Ramsay et al., 1994). Advantages of this method include effective removal of bacterial endotoxins synthesized by gram-negative bacteria, obtaining pure polymer with high molecular weight, and minimizing PHA degradation (Lee et al., 1999). However, its industrial-scale application is limited

due to increased production costs and environmental concerns associated with the large quantities of chloroform used, which poses risks to operators and the environment as a potential carcinogen (Meek et al., 2002).

One recent advancement in the pursuit of lowering the cost of PHA downstream processing is a novel biological recovery method via animal models. This research area began by incorporating lyophilized *C. necator* H16 cells containing P(3HB) into the diet of Sprague-Dawley rats. Periodic observations suggested that the protein source present in the bacterial cells could positively contribute to the rats' growth. Moreover, this method allows for the purification of PHA granules, as non-polymeric materials are digested by the animals and the polymeric materials are excreted in pellet form (Kunasundari et al., 2013). The excreted whitish faecal pellet contains 82 – 97 wt% PHA and the impurities will be easily washed with water and/or a low concentration of detergent to obtain PHA granules with high purity (Kunasundari et al., 2017). Since then, similar studies have been conducted using different animal models including cockroaches, crickets, superworms and mealworms (Ong et al., 2018a).

The biological recovery method has further evolved with the use of yellow mealworms (*Tenebrio molitor*) as the animal model. Mealworms, the larvae of the darkling beetle, were chosen for their ease of maintenance and efficient polymer recovery. They can be grown in high densities, require less water and space and breed prolifically. Most importantly, they can consume up to 10 wt% feed of their body weight per day (Murugan et al., 2016). Mealworms were fed the lyophilized *C. necator* cells and excreted whitish faeces containing the PHA granules. Further purification using water and low concentrations of detergent resulted in more than 90% pure PHA granules with no molecular weight reduction while retaining their native spherical morphology (Murugan et al., 2016; Ong et al., 2018). The integration of yellow mealworms in PHA