POLYHYDROXYALKANOATES PRODUCTION BY Cupriavidus necator TRANSFORMANTS HARBORING POLYHYDROXYALKANOATE SYNTHASE OF Chromobacterium sp. USM2 ($PhaC_{Cs}$) AND ITS POTENTIAL APPLICATION FOR LIPASE ASSAY KIT

TANG HUI JIA

UNIVERSITI SAINS MALAYSIA

2024

POLYHYDROXYALKANOATES PRODUCTION BY *Cupriavidus necator* TRANSFORMANTS HARBORING POLYHYDROXYALKANOATE SYNTHASE OF *Chromobacterium* sp. USM2 ($PhaC_{Cs}$) AND ITS POTENTIAL APPLICATION FOR LIPASE ASSAY KIT

by

TANG HUI JIA

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

May 2024

ACKNOWLEDGEMENT

First and foremost, I would like to express my heartfelt gratitude and appreciation to my supervisor, Professor Dr. K. Sudesh Kumar A/L C. Kanapathi Pillai for his guidance, support, and mentorship throughout my master's study. Thanks to his insightful feedback and helpful suggestions, I have acquired valuable knowledge and experience in this field of study.

Next, I am also deeply grateful to the labmates of the Ecobiomaterial Research Laboratory, for establishing a productive and encouraging research atmosphere. I wish to extend my gratitude to all the laboratory members, especially Mr. Neoh Soon Zher, for their invaluable assistance and constructive comments that enriched the quality of my research.

Besides that, I would like to acknowledge the Electron Microscopy Unit at the School of Biological Sciences, USM for their assistance in using the facilities.

Last but not least, I would like to thank my family for their continuous support and encouragement, which has served as a constant source of motivation for me to complete my master's study. Their emotional support provides me with the strength to endure and overcome obstacles.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS AND ABBREVIATIONS

α	Alpha
~	Approximately
β	Beta
Da	Dalton
℃	Degree Celsius
Δ	Delta
wt%	Dry weight percent
γ	Gamma
GPa	Gigapascal
g	Gram
g/L	Gram per liter
Hz	Hertz
h	Hour
kDa	Kilodalton
kPa	Kilopascal
L	Liter
MPa	Megapascal
µg	Microgram
µL	Microliter
µm	Micrometer
mg	Milligram
mL	Milliliter
mM	Millimolar
min	Minute
−	Minus
M	Molar
mol%	Mole percent
ng	Nanogram
nm	Nanometer
%	Percentage
±	Plus-minus

psi	Pounds per square inch
(*R*)	*Rectus*-isomer
rpm	Revolutions per minute
s	Second
(*S*)	Sinister isomer
×	Times
× *g*	Times gravity
V	Volt
v/v	Volume per volume
w/v	Weight per volume
3H4MV	3-hydroxy-4-methylvalerate
3HB	3-hydroxybutyrate
3HB-CoA	3-hydroxybutyryl-CoA
3HD	3-hydroxydodecanoate
3HHx	3-hydroxyhexanoate
3HHx-CoA	3-hydroxyhexanoyl-CoA
3HO	3-hydroxyoctanoate
3HV	3-hydroxyvalerate
4HB	4-hydroxybutyrate
4HB-CoA	4-hydroxybutyryl-CoA
5HV	5-hydroxyvalerate
ACP	Acyl-carrier-protein
ANOVA	One-way analysis of variance
CDW	Cell dry weight
CME	Caprylic methyl ester
C/N ratio	Carbon-to-nitrogen ratio
CoA	Coenzyme-A
CPKO	Crude palm oil kernel
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
EDTA	Ethylenediaminetetraacetic acid
GC	Gas chromatography
gDNA	Genomic DNA
HA	Hydroxyacyl

HA-CoA	Hydroxyacyl-CoA
LB	Lysogeny broth
MCL	Medium-chain-length
MM	Mineral medium
NADPH	Nicotinamide adenine dinucleotide phosphate
NR	Nutrient rich
OD	Optical density
OD_{600nm}	Optical density at wavelength 600 nm
P(3HB)	Poly(3-hydroxybutyrate)
P(3HB-*co*-3H4MV)	Poly(3-hydroxybutyrate-*co*-3-hydroxy-4-methylvalerate)
P(3HB-*co*-3HHx)	Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate)
P(3HB-*co*-3HV)	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)
P(3HB-*co*-4HB)	Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate)
P(3HB-*co*-5HV)	Poly(3-hydroxybutyrate-*co*-5-hydroxyvalerate)
PCR	Polymerase chain reaction
PP	Polypropylene
pH	Potential of hydrogen
ProC	Pyrroline-5-carboxylate reductase
P-value	Probability value
PHA	Polyhydroxyalkanoate
PhaA	Beta-ketothiolase
PhaB	NADPH-dependent acetoacetyl-CoA reductase
PhaC	PHA synthase
PhaJ	(*R*)-specific enoyl-CoA hydratase
PhaR	PHA synthase subunit R
FadA	3-ketoacyl-CoA thiolase
FadB	enoyl-CoA hydratase
FadD	acyl-CoA synthetase
FadE	acyl-CoA dehydrogenase
FabG	3-ketoacyl-CoA reductase
sucD	succinic semialdehyde dehydrogenase
4hbD	4-hydroxybutyrate dehydrogenase
OrfZ	succinyl-CoA: CoA transferase
RBS	Ribosomal binding sites

SCL	Short-chain-length
SD	Standard deviation
sp.	Species
TAE	Tris-acetate-EDTA
TCA	Tricarboxylic acid
T_g	Glass transition temperature
T_m	Melting temperature
T_c	Crystalline temperature
TSB	Tryptic soy broth

LIST OF APPENDICES

PENGHASILAN POLIHIDROKSIALKANOAT OLEH TRANSFORMAN *Cupriavidus necator* YANG MENGANDUNGI POLIHIDROKSIALKANOAT SINTASE *Chromobacterium* sp. USM2 ($PhaC_{Cs}$) DAN POTENSI APLIKASINYA UNTUK KIT ASAI LIPASE

ABSTRAK

Polihidroksialkanoat (PHA) adalah biopoliester yang disintesis oleh mikroorganisma sebagai simpanan karbon di bawah keadaan sumber karbon yang berlebihan tetapi sumber nutrient penting yang terhad. Disebabkan oleh kesifatan biodegradasi, poli(3-hidroksibutirat-*ko*-4-hidroksibutirat) [P(3HB-*ko*-4HB)] telah digunakan dalam aplikasi industri termasuk pembungkusan, plastik yang boleh terbiodegradasi, peranti perubatan, dan aplikasi bioperubatan. *Chromobacterium* sp. USM2 telah dipencilkan dari Langkawi, Malaysia dan didapati berupaya menghasilkan PHA. PHA sintase *Chromobacterium* sp. USM2 berupaya mempolimerisasikan pelbagai monomer seperti 3-hidroksibutirat (3HB), 3-hidroksivalerat (3HV) dan 3-hidroksiheksanoat (3HHx) daripada sumber karbon atau prekursor. Sehingga kini, tiada laporan berkaitan potensi $PhaC_{Cs}$ untuk menghasilkan P(3HB-*ko*-4HB). Dalam kajian ini, ujian siri pengoptimuman dan biosintesis PHA telah dijalankan untuk menilai keupayaan ekspresi heterolog $PhaC_{Cs}$ dalam transforman *C. necator* menghasilkan P(3HB-*ko*-4HB) dengan menggunakan prekursor 4HB yang berkaitan secara struktural seperti 1,6-heksanediol, 1,4-butanediol, γ-butirolakton dan natrium 4-hidroksibutirat. Polimer yang diperolehi kemudiannya dinilai ciri-cirinya untuk menentukan komposisi monomer, analisa termal dan aktiviti depolimerasi lipase. *C. necator* Re2058 yang mengandungi *phaC*$_{Cs}$ didapati berupaya menghasilkan 5.9 g/L berat kering sel dan kandungan PHA

sebanyak 53.4 wt% dengan komposisi monomer 4HB yang tinggi (25 mol% 4HB) dengan menggunakan campuran 10 g/L fruktosa dan 5 g/L natrium 4-hidroksibutirat sebagai sumber karbon. Oleh itu, keupayaan transforman ini telah dinilai ciri-cirinya dengan menggunakan pelbagai kepekatan prekursor 4HB yang berkaitan secara struktur, kepekatan fruktosa dan analisis selang masa. *C. necator* Re2058 yang mengandungi *phaC*$_{Cs}$ dilaporkan berupaya menghasilkan P(3HB-*ko*-4HB) dengan maksimum komposisi monomer 4HB sebanyak 52 mol% dengan menggunakan campuran 10 g/L fruktosa dan 20 g/L natrium 4-hidroksibutirat pada nisbah C/N 10 selama 48 jam. Analisa termal pada filem campuran P(3HB-*ko*-4HB)/P(3HB-*ko*-8 mol% 3HHx) yang mengandungi pelbagai komposisi monomer 4HB telah diuji untuk menentukan kestabilan termal. Kaedah pencampuran ini dapat meningkatkan kestabilan polimer dan jangka hayatnya untuk digunakan sebagai bahan biologi. Aktiviti depolimerasi lipase komersial pada filem polimer PHA menunjukkan penghasilan tompok hidrolisis yang legap. Campuran polimer PHA didapati boleh digunakan dalam kit asai lipase sebagai substrat untuk menentukan aktiviti lipase dan ia menyediakan pilihan yang mesra alam untuk komersialisasi kit asai lipase.

POLYHYDROXYALKANOATES PRODUCTION BY *Cupriavidus necator* TRANSFORMANTS HARBORING POLYHYDROXYALKANOATE SYNTHASE OF *Chromobacterium* sp. USM2 (PhaC$_{Cs}$) AND ITS POTENTIAL APPLICATION FOR LIPASE ASSAY KIT

ABSTRACT

Polyhydroxyalkanoates (PHAs) are biopolyesters produced by microorganisms as carbon storage in the presence of abundant carbon sources and the limitation of growth nutrients. Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] has been used for industrial applications, including packaging, biodegradable plastics, medical devices, and biomedical applications, owing to its biodegradability properties. *Chromobacterium* sp. USM2 was isolated in Langkawi, Malaysia, and was able to accumulate PHA. PHA synthase of *Chromobacterium* sp. USM2 (PhaC$_{Cs}$) was able to polymerize various monomers such as 3-hydroxybutyrate (3HB), 3-hydroxyvalerate (3HV), and 3-hydroxyhexanoate (3HHx) from carbon sources or precursors due to its broad substrate specificity. To date, there is no report on the potential of PhaC$_{Cs}$ to produce P(3HB-*co*-4HB) copolymer. In this study, a series of optimization tests and PHA biosynthesis were performed to investigate the potential of the heterologous expression of PhaC$_{Cs}$ in *C. necator* transformants to produce P(3HB-*co*-4HB) using 4HB-structurally related precursors such as 1,6-hexanediol, 1,4-butanediol, γ-butyrolactone, and sodium 4-hydroxybutyrate. The polymers obtained were then further characterized to determine their monomer compositions, thermal properties, and lipase depolymerizing activity. It was found that *C. necator* Re2058 harboring *phaC*$_{Cs}$ was able to produce 5.9 g/L of cell dry weight (CDW) and 53.4 wt% of PHA content with high 4HB monomer composition (25 mol%

4HB) from a mixture of 10 g/L of fructose and 5 g/L of sodium 4-hydroxybutyrate. Therefore, the ability of this transformant was further characterized using various concentrations of 4HB-structurally related precursors, concentrations of fructose, and time course analysis. It was reported that *C. necator* Re2058 harboring *phaC*$_{Cs}$ could produce P(3HB-*co*-4HB) with maximum 4HB monomer composition (52 mol%) using a mixture of 10 g/L of fructose and 20 g/L of sodium 4-hydroxybutyrate at C/N ratio of 10 for 48 hours. The thermal properties of the thin cast film of P(3HB-*co*-4HB)/P(3HB-*co*-8 mol% 3HHx) blend with various 4HB monomer compositions were investigated to determine their thermal stability. This blending method could improve polymer stability and increase its shelf life to be used as biomaterials. The depolymerizing activity of different commercial lipases on the thin cast film of polymer blends showed the formation of opaque hydrolysis spots. It was found that PHA polymer blends can be used in lipase assay kits as substrates to detect lipase activity, which provides an environmentally friendly alternative for the commercialization of lipase assay kits.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Lipases are enzymes that break down triglycerides into free fatty acids and glycerol (Singh & Mukhopadhyay, 2012). They are produced by microorganisms, plants, and animals (Haki & Rakshit, 2003). Lipases are soluble in water and can react with substrates that do not dissolve in water, leading to the formation of an enzyme-substrate complex at the interface of an emulsion. This proves advantageous when reacting with substrates that are insoluble in water but can dissolve in other organic that do not mix with water (Rodríguez-Contreras *et al*., 2012). They are widely used in various industries, including food processing, baking, detergent, pharmaceuticals, leather, and biodiesel industry (Houde *et al*., 2004; Aravindan *et al*., 2007; Singh & Mukhopadhyay, 2012). Measurement of lipase activity is important for quality assurance, research, and advancement in these fields. One such approach for evaluating lipase activity is the use of lipase assay kits, which provide convenience and rapid detection of lipase activity (Ch'ng & Sudesh, 2013). The lipase assay kit can be developed by employing polyhydroxyalkanoate (PHA) as a substrate (Ch'ng & Sudesh, 2013; Mok *et al*., 2016). PHA, a biopolymer, has attracted attention owing to its biodegradable nature and its adaptability as a substrate for lipase assay. The applications of lipase assay using PHA as a substrate can be used as a diagnostic tool to detect conditions related to lipid metabolism and digestion such as pancreatic lipase levels (Singh & Mukhopadhyay, 2012).

PHAs are produced and accumulated by most bacteria and archaea under excessive carbon sources and limitation of essential nutrients (Anderson & Dawes, 1990; Sudesh *et al*., 2000). PHAs can be classified into three groups based on the

number of carbons: short-chain-length PHAs (SCL-PHAs), medium-chain-length PHAs (MCL-PHAs), and a combination of SCL- and MCL-PHA (Madison & Huisman, 1999; Sudesh, 2012). The types of monomers in the PHA polymer chain can affect the thermal and mechanical properties of the PHA polymer produced (Tan *et al*., 2020). Poly(3-hydroxybutyrate) [P(3HB)] is the most common PHA homopolymer accumulated by many PHA producers. It is brittle, highly crystalline, and has a high melting temperature thus limiting its applications (Reinecke & Steinbüchel, 2009). One method of improving the properties of the P(3HB) homopolymer is by copolymerization with other PHA monomers such as 3-hydroxyhexanoate (3HHx), 4-hydroxybutyrate (4HB) and many more monomer units. Copolymerization with other monomers can improve the physicochemical properties of the PHA making them better than that of P(3HB) homopolymer. The copolymer is more flexible and applicable in industries such as poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] and poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] (Noda *et al*., 2005).

P(3HB-*co*-4HB) is SCL-PHAs, composed of two different PHA monomers, 3HB and 4HB monomer units. The copolymerization of 4HB monomer with 3HB monomer increases tensile strength and elastomeric properties, making it more flexible than P(3HB) homopolymer (Loo & Sudesh, 2007). Depending on the 4HB monomer composition, the properties of P(3HB-*co*-4HB) vary from crystalline to elastic. The most outstanding properties of P(3HB-*co*-4HB) are its ability to be degraded by lipase, biocompatible, and bioabsorbable (Martin & Williams, 2003). P(3HB-*co*-4HB) can be degraded by both lipase and PHA depolymerase giving them an extra advantage compared to most other PHAs. The biodegradability rate of P(3HB-*co*-4HB) relies on factors such as 4HB monomer composition, concentration, and the type of lipase, as

well as environmental conditions (Ch'ng *et al*., 2012). Since P(3HB-*co*-4HB) can be degraded by lipase and hence, is commonly used for screening of lipase depolymerizing activity and in medical applications such as tissue engineering, drug delivery, sutures, scaffolds, and wound care (Williams *et al*., 2013).

PHA synthase (PhaC) is the key enzyme in PHA biosynthesis and catalyzes the conversion of (*R*)-HA-CoA to PHA with a concomitant release of CoA (Rehm, 2003; Sudesh, 2012). Some PhaCs prefer to polymerize SCL-PHA monomers whereas some prefer to polymerize MCL-PHA monomers. There are also a few PhaCs that can polymerize both SCL- and MCL-PHA. In this study, one such PhaC from *Chromobacterium* sp. USM2 ($PhaC_{Cs}$) which is used in this study (Bhubalan *et al*., 2010). There are some efforts in the engineering of PhaC have been made to broaden their substrate specificity and improve PHA accumulation (Rehm, 2003; Harada *et al*., 2021).

Chromobacterium sp. USM2 is a Gram-negative bacterium, that was isolated from a waterfall in Langkawi, Malaysia. The $PhaC_{Cs}$ is a highly active PhaC with the highest polymerizing ability so far (Bhubalan *et al*., 2011). Several studies have been done to broaden the substrate specificity of $PhaC_{Cs}$. Chuah and co-workers reported that performing saturation point mutagenesis at position A479 in $PhaC_{Cs}$ could result in an improved substrate specificity towards 3HHx, leading to a notable increase in 3HHx content of up to 6.6 mol% when compared to the wild-type $PhaC_{Cs}$. In a previous study, it had been reported to incorporate 3HB, 3HHx, 3-hydroxyvalerate (3HV), and 3-hydroxy-4-methylvalerate (3H4MV) when supplied with appropriate carbon sources or precursors (Yong, 2009; Bhubalan *et al*., 2010; Ling *et al*., 2011). In another study by Bhubalan *et al*. (2010), $PhaC_{Cs}$ was studied for the production of copolymer or

terpolymer with better thermal and mechanical properties from suitable carbon sources and precursors.

On the other hand, *C. necator* is the best candidate for PHA production as it shows robust cell growth and PHA accumulation from various carbon sources (Reinecke & Steinbüchel, 2009; Morlino *et al*., 2023). *C. necator* is also the most well-studied organism in PHA production and accumulation. The heterologous expression of PhaC$_{Cs}$ in PHA-negative mutant *C. necator* PHB^{-}4 transformants could produce poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] with 3HV monomer composition as high as 60 mol% when fed with a mixture of fructose and sodium valerate. Furthermore, *C. necator* PHB^{-}4 transformant harboring *phaC*$_{Cs}$ could also produce poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] with 4 mol% of 3HHx from crude palm kernel oil (CPKO) (Bhubalan *et al.*, 2010). So far, there is no report that the *C. necator* transformants harboring *phaC*$_{Cs}$ could produce any P(3HB-*co*-4HB) or P(3HB-*co*-5HV) with structurally related precursors. Therefore, in this study, PhaC$_{Cs}$ was characterized for its substrate specificity using *C. necator* PHB^{-}4 as the host. The ability of PhaC$_{Cs}$ to accumulate P(3HB-*co*-4HB) was further characterized using *C. necator* Re2058 and Re2160 as hosts. The 4HB-structurally related precursors such as 1,6-hexanediol, 1,4-butanediol, γ-butyrolactone, and sodium 4-hydroxybutyrate were fed to the transformants. Besides that, the ability of PhaC$_{Cs}$ to accumulate P(3HB-*co*-4HB) was further characterized using various concentrations of precursor, concentrations of fructose, and time course analysis. The P(3HB-*co*-4HB) copolymers with various 4HB monomer compositions obtained were blended physically with P(3HB-*co*-8 mol% 3HHx) copolymers and the blend films were prepared using a solvent-casting method to characterize their degradability by various lipases and thermal properties.

1.2 Problem statement

The ability of the PHA synthase of *Chromobacterium* sp. USM2 to produce P(3HB-*co*-4HB) has not been determined. P(3HB-*co*-4HB) copolymer is a well-known PHA to be degraded by lipases. However, the biodegradability of P(3HB-*co*-4HB) and P(3HB-*co*-8 mol% 3HHx) polymer blends by lipases are unknown. Hence, there is a need to study its potential to be used as substrate in rapid microassay which provides a more environmentally friendly option for lipase activity screening. It is also unclear whether blended PHA can offer improved polymer stability and shelf-life over the individual PHA.

1.3 Objectives

a) To investigate the ability of PhaC$_{Cs}$ to polymerize various PHA monomers from structurally related precursors and carbon sources.

b) To evaluate the potential of the heterologous expression of PhaC$_{Cs}$ in *C. necator* transformants for P(3HB-*co*-4HB) production using structurally related 4HB substrates.

c) To produce and characterize P(3HB-*co*-4HB) and P(3HB-*co*-8 mol% 3HHx) polymer blends.

d) To evaluate the lipase depolymerizing activity on the thin cast film of P(3HB-*co*-4HB)/P(3HB-*co*-8 mol% 3HHx) blends for the development of rapid lipase assay.

CHAPTER 2

LITERATURE REVIEW

2.1 Polyhydroxyalkanoates (PHAs)

Polyhydroxyalkanoates (PHAs) are environmentally friendly and sustainable biopolymers that are synthesized by microorganisms and accumulated in the cytoplasm as carbon and energy storage. PHA accumulation occurs when the essential nutrients are limited, such as nitrogen, magnesium, or oxygen, and the carbon sources are excessive (Anderson & Dawes, 1990; Doi, 1990). PHA granules can accumulate discretely within cells, reaching levels of up to 90% of the cell dry weight (Madison & Huisman, 1999). The general chemical structure of PHA is shown in Figure 2.1.

The PHA was first discovered by a French scientist, Maurice Lemoigne in 1926 (Lemoigne, 1926; Palmeiro-Sánchez *et al*., 2022). P(3HB) homopolymer was first found in *Bacillus megaterium*, which is the most typical type and well-characterized PHA homopolymer (Lemoigne, 1926). In 1974, Wallen and Rohwedder identified 3HV and 3HHx from the PHA biosynthesis using activated sewage sludge (Wallen & Rohwedder, 1974). In 1983, Witholt and coworkers discovered 3-hydroxyoctanoate (3HO) and minor constituents of 3HHx units in *Pseudomonas oleovorans* when the cells were cultured on *n*-octane (de Smet *et al*., 1983; Lageveen *et al*., 1988). To date, many monomer units can be incorporated into the PHA polymer chain which provides different physicomechanical properties of PHA polymers and it is believed more to come soon (Rehm, 2003).

PHAs can be categorized into 3 main groups, which are determined by the number of carbon atoms present in the monomer unit. They are SCL-PHAs, containing PHA monomers of 3 – 5 carbon atoms, MCL-PHAs, consisting of 6 – 14 carbon atoms and a combination of SCL- and MCL-PHA, consisting of 3 – 14 (Madison & Huisman,

1999; Sudesh, 2012). The examples of the monomer units in SCL-PHAs are 3HB, 3HV, and 4HB while the monomer units in MCL-PHAs are 3HHx, 3HO, and 3-hydroxydodecanoate (3HD) (Sudesh, 2012). The mixture of SCL- and MCL-PHA is well represented in P(3HB-*co*-3HHx) (Anderson & Dawes, 1990; Steinbüchel, 1991). PHA can be produced as a homopolymer or heteropolymer. A homopolymer consists of the repetition of the same monomer whereas a heteropolymer consists of two or more different monomers. Different types of PHA have different thermal and mechanical properties based on their respective monomer compositions (Sudesh *et al*., 2000). SCL-PHAs are brittle, rigid, high melting temperature, high crystallinity, and high glass transition temperature. In contrast, MCL-PHAs are elastic, flexible, have lower crystallinity, and have lower glass transition temperature (Yu, 2007; Reddy *et al*., 2022). Nevertheless, it was found that the properties of the SCL-MCL-PHA copolymers were better than the homopolymers of SCL-PHA and MCL-PHA. These copolymers are more ductile, moldable, and durable than PHA homopolymers, which are preferred for wider applications (Tsuge, 2002).

Generally, PHAs possess superior properties including biodegradability, biocompatibility, thermoplasticity, good ultraviolet resistance, and non-toxicity. PHAs can be degraded, which makes them eco-friendly and reduces plastic pollution. Besides that, the biocompatibility and bioresorbability of PHAs make them applicable in the medical field such as scaffolds and sutures as they are not harmful to the human body. PHA can also be melted and reshaped into desired shapes for bottles, packaging, and agriculture sheets (Poltronieri & Kumar, 2017). Furthermore, they are hydrophobic polymers that cannot be soluble in water, making them resistant to hydrolytic degradation (Sudesh *et al*., 2000; Chen, 2010).

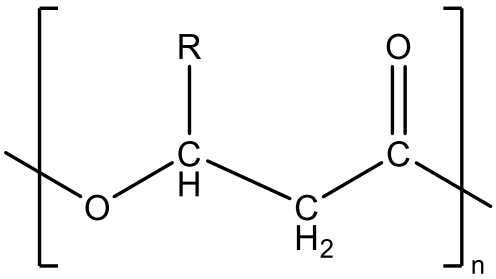
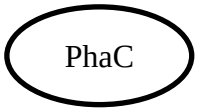
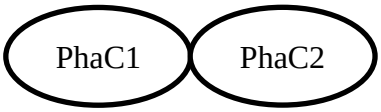
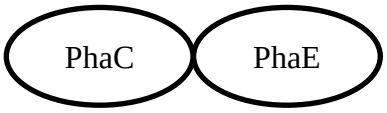
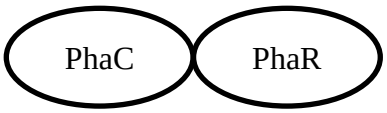


Figure 2.1: General chemical structure of PHAs. Functional alkyl R group determines the number of carbon atoms and PHA designations. The n symbol refers to the number of repeating units.

2.2 PHA synthase (PhaC)

The PHA synthases (PhaCs) have a significant role in PHA biosynthesis. They catalyze the polymerization of 3-hydroxyacyl-CoA (3HA-CoA) into PHA. PhaCs can be categorized into four major groups depending on their primary structure, subunit composition, and substrate specificities as shown in Table 2.1 (Sudesh *et al*., 2000; Rehm, 2003). Class I, III, and IV PhaCs prefer the SCL-PHA monomers while class II prefers MCL-PHA monomers. Although the substrate specificities of class I and class II PhaCs are different, they have one similarity, whereby both comprise one type of subunit (Sudesh *et al*., 2000; Sudesh, 2012). Examples of class I PhaCs are PhaCs from *Cupriavidus necator* ($PhaC_{Cn}$) while class II can be found in *Pseudomonas aeruginosa* ($PhaC_{Pa}$). On the other hand, Class III PhaC comprises two subunits, forming a heterodimer. The heterodimer is formed by PhaC and PhaE subunits, which are identified in *Allochromatium vinosum* while class IV PhaCs consist of PhaC and PhaR subunits, which can be found in *Bacillus megaterium* (McCool & Cannon, 2001). However, there are also a few PhaCs which can polymerize both SCL- and MCL-PHA such as PhaC from *Chromobacterium* sp. USM2 ($PhaC_{Cs}$) and PhaC from mangrove soil metagenome ($PhaC_{BP\text{-}M\text{-}CPF4}$) (Bhubalan *et al*., 2010; Foong *et al*., 2018).

Table 2.1: Classes of PhaCs

Class	Subunits	Substrate	Representative species
I	 ~60 – 73 kDa	SCL-HA-CoA	*Cupriavidus necator*
II	 ~60 – 65 kDa	MCL-HA-CoA	*Pseudomonas aeruginosa*
III	 ~ 40 kDa ~ 40 kDa	SCL-HA-CoA; MCL-HA-CoA	*Allochromatium vinosum*
IV	 ~ 40 kDa ~ 22 kDa	SCL-HA-CoA	*Bacillus megaterium*

2.2.1 PhaC from *Chromobacterium* sp. USM2 (PhaC$_{Cs}$)

Chromobacterium sp. USM2 is a PHA producer discovered in Langkawi, Malaysia. It was reported to produce P(3HB) homopolymer from glucose, fructose, hexanoic acid, or CPKO. A previous study reported that the PhaC$_{Cs}$ has high preference towards 3HV monomers when cultured with sodium valerate, sodium propionate, or valeric acid as carbon sources (Steinbüchel *et al*., 1993; Kolibachuk *et al*., 1999; Bhubalan *et al*., 2010). It can accumulate up to 98 mol% of 3HV from sodium valerate. Nevertheless, when valeric acid is used as the sole carbon source, it affects cell growth when used in a high concentration. Higher concentrations of 3HV precursors will inhibit cell growth due to toxicity, resulting in low PHA content and cell dry weight (Park *et al*., 2001). Besides that, PhaC$_{Cs}$ was also reported to incorporate 3HB, 3HV, and 3HHx when heterologous expressed in *C. necator* PHB^{-}4. The heterologous expression of PhaC$_{Cs}$ in *C. necator* PHB^{-}4 could produce P(3HB-*co*-3HV) with up to 60 mol% of 3HV monomer composition in the presence of fructose and sodium valerate. Besides that, *C. necator* PHB^{-}4 harboring *phaC*$_{Cs}$ could produce P(3HB-*co*-3HHx) with 4 mol% of 3HHx from CPKO (Bhubalan *et al*., 2010). Furthermore, *C. necator* PHB^{-}4 harboring *phaC*$_{Cs}$ could produce P(3HB-*co*-3HV-*co*-3HHx) terpolymer from the combination of CPKO and sodium valerate or sodium propionate (Bhubalan *et al*., 2010). These findings showed the potential of PhaC$_{Cs}$ to incorporate both SCL- and MCL-PHA. Bhubalan *et al*. (2011) also reported that the polymerization activity of purified PhaC$_{Cs}$ towards 3HB-CoA was 8-fold higher than that of PhaC from *C. necator* (PhaC$_{Cn}$) and also has the highest enzymatic activity among all reported purified PhaCs with superior polymerizing ability (Bhubalan *et al*., 2011).

2.2.2 PhaC from *Aquitalea* sp. USM4

Aquitalea sp. USM4 was first isolated from a waterfall in Perak, Malaysia. It is a Gram-negative bacterium that possesses a high sequence identity which is nearly 99% to *Aquitalea magnusonii*. Later, the whole genome sequence of *Aquitalea* sp. USM4 was further analyzed and renamed as *Aquitalea pelogenes* USM4 (Wan *et al*., 2023). *Aquitalea* sp. USM4 was able to produce PHA using sugars such as glucose, fructose, and sucrose, but it was unable to accumulate PHA when CPKO was utilized as a carbon source (Ng & Sudesh, 2016). However, the heterologous expression of the PhaC of *Aquitalea* sp. USM4 ($PhaC_{As}$) in *C. necator* $PHB^{-}4$ could produce PHA from CPKO. It was also reported that *C. necator* $PHB^{-}4$ harboring $phaC_{As}$ was able to accumulate PHA at 2-fold higher than *Aquitalea* sp. USM4 from sugar. It was reported that the synthase activity of the *C. necator* transformant harboring $phaC_{As}$ was higher than that of wild-type *Aquitalea* sp. USM4 which was suggested due to higher enzyme expression level. The synthase activity of the heterologous expression of the $PhaC1_{As}$ in *C. necator* $PHB^{-}4$ (1402 U/g) was comparable to the PhaC from *A. caviae* ($PhaC_{Ac}$) (1600 U/g) (Kichise *et al*., 1999; Ng & Sudesh, 2016). Both the wild-type *Aquitalea* sp. USM4 and *C. necator* $PHB^{-}4$ harboring $phaC_{As}$ could incorporate 3HB, 3HV, 4HB, and 3H4MV when appropriate precursors were supplemented (Ng & Sudesh, 2016; Lim *et al*., 2021). Overall, it showed that $PhaC_{As}$ has high activity and wide substrate specificity (Ng & Sudesh, 2016; Teh *et al*., 2018).

2.2.3 PhaC from mangrove metagenome ($PhaC_{BP\text{-}M\text{-}CPF4}$)

$PhaC_{BP\text{-}M\text{-}CPF4}$ was isolated from the metagenome of the mangrove soil from Balik Pulau, Penang, Malaysia. This PhaC was reported to have a wide substrate specificity enabling it to accumulate SCL-PHA and MCL-PHA from suitable precursors, including P(3HB), P(3HB-*co*-3HV), P(3HB-*co*-4HB), P(3HB-*co*-3H4MV), P(3HB-*co*-5HV), and P(3HB-*co*-3HHx). This PhaC has the potential to be used for the production of PHA for medical applications due to its ability to incorporate 4HB and 5HV monomer units which are susceptible to lipase degradation (Foong *et al*., 2018). $PhaC_{BP\text{-}M\text{-}CPF4}$ could incorporate also up to 23 mol% of 5HV when supplemented with fructose and sodium 5-hydroxyvalerate. When fructose and sodium 4-hydroxybutyrate were used as substrates, it also accumulated up to 14 mol% of 4HB. Foong *et al*. (2018) also reported that *C. necator* $PHB^{-}4$ harboring $PhaC_{BP\text{-}M\text{-}CPF4}$ was able to produce 44 wt% of P(3HB-*co*-3HHx) with 18 mol% of 3HHx when supplemented with fructose and sodium hexanoate whereas when supplied with CPKO as sole carbon source, 62 wt% of P(3HB-*co*-3HHx) with 7 mol% was accumulated. Furthermore, Tan *et al*. (2020) reported that the co-expression of PhaJ from *P. aeruginosa* and $PhaC_{BP\text{-}M\text{-}CPF4}$ in *C. necator* transformants could increase the 3HHx monomer composition in PHA copolymer.

2.3 PHA biosynthesis pathways

Different carbon sources and precursors uptake by microorganisms will enter different pathways and eventually form HA-CoA which are PHA monomers. These HA-CoA will then be polymerized into PHA polymer by PhaCs (Sudesh *et al*., 2000). Various pathways can be used by the bacteria to supply the PHA monomers for PHA biosynthesis. For instance, tricarboxylic acid (TCA) cycle, fatty acid β-oxidation, and fatty acid *de novo* synthesis (Madison & Huisman, 1999).

The simplest pathway is P(3HB) biosynthesis pathway. This pathway involved three PHA main genes which are *phaA*, *phaB*, and *phaC* that encode β-ketothiolase (PhaA), nicotinamide adenine dinucleotide phosphate (NADPH) reductase (PhaB), and PhaC, respectively. When sugar or oil or any carbon source is supplied, the most common intermediate formed will be acetyl-CoA. The conversion of acetyl-CoA into PHA involved three enzymatic steps (Anderson & Dawes, 1990; Sudesh *et al*., 2000). First, PhaA catalyzes the condensation of two molecules of acetyl-CoA into acetoacetyl-CoA followed by the reduction of acetoacetyl-CoA into (*R*)-3-hydroxybutyryl-CoA [(*R*)-3HB-CoA] through a reduction reaction by PhaB. Lastly, PhaC will catalyze the polymerization of (*R*)-3HB-CoA into P(3HB) (Figure 2.2, pink colour pathway).

In the fatty acid β-oxidation pathway, triglycerides will first be broken down into glycerol and fatty acids. Acyl-CoA synthetase (FadD) catalyzes the conversion of fatty acids to acyl-CoA. Acyl-CoA dehydrogenase (FadE) catalyzes the oxidation of acyl-CoA into *trans*-2-enoyl-CoA. Then, enoyl-CoA hydratase (FadB) catalyzes the hydration of *trans*-2-enoyl-CoA into (*S*)-3-HA-CoA. Next, 3-HA-CoA dehydrogenase (Had) catalyzes the oxidation of (*S*)-3-HA-CoA into 3-ketoacyl-CoA. Then, 3-ketoacyl-CoA thiolase (FadA) will catalyze the conversion of 3-ketoacyl-CoA back

into acyl-CoA by releasing one acetyl-CoA. The acetyl-CoA will be converted into P(3HB) homopolymer using the *phaA, phaB,* and *phaC* genes. (*R*)-specific enoyl-CoA hydratase (PhaJ) is an enzyme that catalyzes the hydration of six carbons *trans*-2-enoyl-CoA into (*R*)-3-HA-CoA (Fukui *et al*., 1998; Tang *et al*., 2022). PhaC will then polymerize the (*R*)-3-HA-CoA into PHA (Figure 2.2, purple colour pathway).

In fatty acids *de novo* synthesis pathway, acetyl-CoA will convert into malonyl-CoA followed by conversion into malonyl-ACP *via* catalytic reaction by malonyl-CoA-ACP transacylase (FabD). β-ketoacyl-ACP synthase (FabB) catalyzes the conversion of malonyl-ACP to 3-ketoacyl-ACP followed by conversion into (*R*)-3-HA-CoA, catalyzed by 3-ketoacyl-ACP reductase (FabG). PhaC polymerizes (*R*)-3-HA-CoA into PHA (Figure 2.2, purple colour pathway) (Rehm *et al*., 2001; Tsuge, 2002).

When sodium propionate or sodium valerate is supplemented, it leads to the formation of propionyl-CoA (Steinbüchel & Lütke-Eversloh, 2003). Propionyl-CoA and acetyl-CoA will be condensed to form 3-ketovaleryl-CoA, catalyzed by 3-ketothiolase (BktB). Ketovaleryl-CoA will be reduced to form (*R*)-3-hydroxyvaleryl-CoA by PhaB, then polymerized by PhaC (Figure 2.2, orange colour pathway).

When 4HB precursors are used as carbon sources, microorganisms that can express succinic semialdehyde dehydrogenase (*sucD*), 4-hydroxybutyrate dehydrogenase (*4hbD*), and succinyl-CoA: CoA transferase (*orfZ*) can produce P(3HB-*co*-4HB) (Valentin *et al*., 1995). First, succinyl-CoA from the tricarboxylic acid (TCA) cycle is converted to succinic semialdehyde, catalyzed by *sucD.* Then, succinic semialdehyde is converted to 4-hydroxybutyrate *via* the catalytic reaction of *4hbD* followed by conversion to 4HB-CoA *via* a catalytic reaction of *orfZ* (Figure 2.2, dark blue colour pathway). With the supplies of γ-butyrolactone, the lactone is cleaved into 4-hydroxybutyric acid, catalyzed by esterases or lactonases, and then converted into

4HB-CoA by transferase or thiokinase (Figure 2.2, green colour pathway). When ω-alkanediols such as 1,4-butanediol and 1,6-hexanediol are used as 4HB precursors, they will be oxidized to 4-hydroxybutyric acid to form 4-hydroxybutyryl-CoA and then converted to 4-hydroxybutyrate (Figure 2.2, green and dark red colour pathway) (Kunioka *et al*., 1989). With the incorporation of 3HB monomers, P(3HB-*co*-4HB) copolymer is formed. The PHA biosynthesis pathways are shown in Figure 2.2.

With the supplies of the precursors or structurally related substrates, the copolymers can be produced from biosynthesis. For example, sodium propionate or sodium valerate are the precursors that are used to produce P(3HB-*co*-3HV). Sodium 4-hydroxybutyrate, 1,4-butanediol or γ-butyrolactone are used to synthesis P(3HB-*co*-4HB) whereas isocaproic acid is used to produce P(3HB-*co*-3H4MV) (Kunioka *et al*., 1989; Lütke-Eversloh *et al*., 2001; Bhubalan *et al*., 2008; Ling *et al*., 2011).

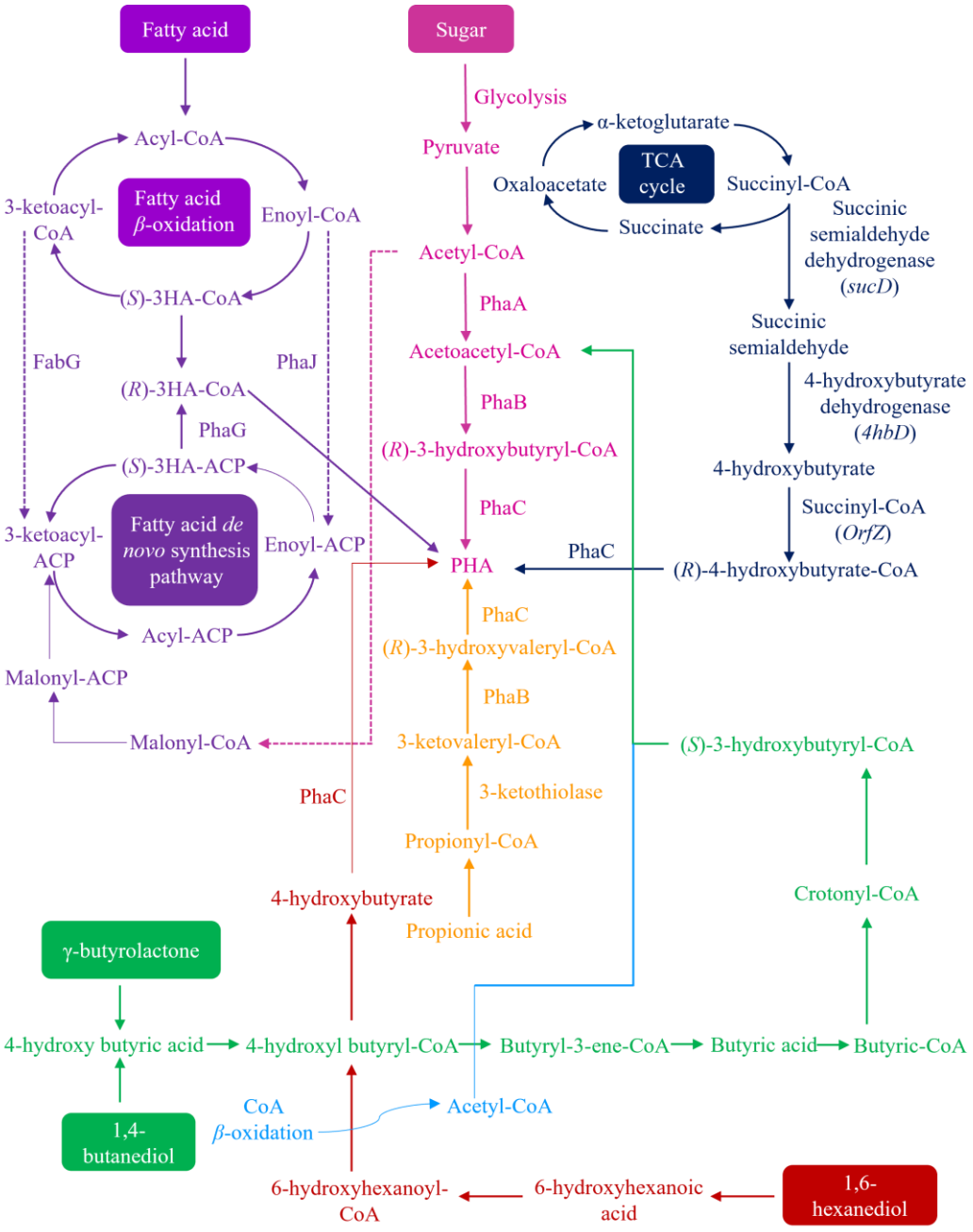


Figure 2.2: Metabolic pathway of PHA biosynthesis. Pink, purple, and orange colour represent the pathway of P(3HB), P(3HB-*co*-3HHx), and P(3HB-*co*-3HV), respectively. Dark blue, green, and dark red colour represent the pathway of P(3HB-*co*-4HB). Abbreviation: PHA, polyhydroxyalkanoate; PhaA, β-ketothiolase; 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,

(*R*)-3-hydroxybutyryl-CoA; PhaJ, (*R*)-specific enoyl-CoA hydratase; *sucD*, succinic semialdehyde dehydrogenase; *4hbD*, 4-hydroxybutyrate dehydrogenase; *OrfZ*, succinyl-CoA: CoA transferase. Adapted from Sudesh *et al*. (2000) and Tsuge (2002)

2.4 Types of PHA

2.4.1 P(3HB)

P(3HB) homopolymer was first discovered by Maurice Lemoigne in the year 1925 (Lemoigne, 1926). It is the most common, widely studied, and well-characterized PHAs (Madison & Huisman, 1999). The chemical structure of P(3HB) homopolymer is shown in Figure 2.3. In the 1980s, it was produced by Imperial Chemical Industries through an industrial-scale fermentation process. It appears in an amorphous state in its granule (Holmes, 1988). The melting temperature of the polymer is approximately 180 ℃ and its glass transition temperature is around 4 ℃. The mechanical properties of P(3HB) are close to polypropylene (PP). The Young's modulus of P(3HB) is 3.5 GPa whereas the tensile strength is 43 MPa. However, the elongation at break of P(3HB) is lower than that of PP (5% versus 400%) (Sudesh *et al*., 2000). Hence, P(3HB) is stiffer and more brittle than PP. Since P(3HB) is brittle, has high melting temperature, and hard to be heat processed, it has limited application. Therefore, extensive work on improving the properties of PHA has been done. For instance, the incorporation of other PHA monomers such as 3HV, 3HHx, and 4HB into P(3HB) homopolymer had caused changes in the mechanical and physical properties, attracting more attention from academic and industrial (Anderson & Dawes, 1990; Madison & Huisman, 1999; Loo & Sudesh, 2007). Besides that, the recombinant bacteria had been constructed to produce P(3HB) with better physical and mechanical properties. A previous study showed that a recombinant *E. coli* harboring PHA biosynthesis gene from *C. necator* was capable of producing P(3HB) homopolymer with ultra-high molecular weight (Kusaka *et al*., 1998).

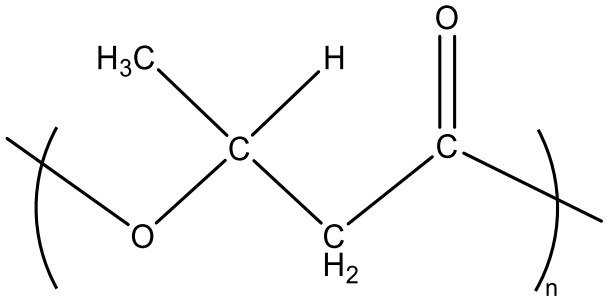


Figure 2.3: Chemical structure of P(3HB). 'n' represents number of repeating units.

2.4.2 P(3HB-*co*-3HV)

P(3HB-*co*-3HV) is made up of 3HB and 3HV monomer units. The chemical structure of this copolymer is shown in Figure 2.4. In the 1980s, Imperial Chemical Industries (ICI) introduced it to the market as Biopol^{TM}, marking its initial commercialization. The substrates supplemented for the production of P(3HB-*co*-3HV) random copolymers were propionic acid and glucose (Holmes, 1988). The 3HV monomer compositions obtained ranged from 0 – 30 mol%. P(3HB-*co*-3HV) copolymer shows isodimorphism, causing both 3HV and 3HB monomer units to co-crystallize in either of the polymer crystal lattices. Therefore, the degree of crystallinity of P(3HB-*co*-3HV) remains almost similar to P(3HB), which means this property of this copolymer did not change as compared to P(3HB) (Bluhm *et al.*, 1986; Sudesh *et al.*, 2000). However, varying 3HV monomer shows a significant effect on P(3HB-*co*-3HV) copolymer. The tensile strength and Young's Modulus of P(3HB-*co*-3HV) films decreased as the 3HV monomer composition increased from 0 to 25 mol%, which also means that the flexibility of (3HB-*co*-3HV) films increased too. Furthermore, P(3HB-*co*-3HV) with 28 mol% of 3HV has increased elongation at break as high as 700 %. When 3HV monomer composition increased from 0 to 25 mol%, the melting temperature (T_m) decreased without affecting the thermal degradation temperature (Sudesh & Abe, 2010).

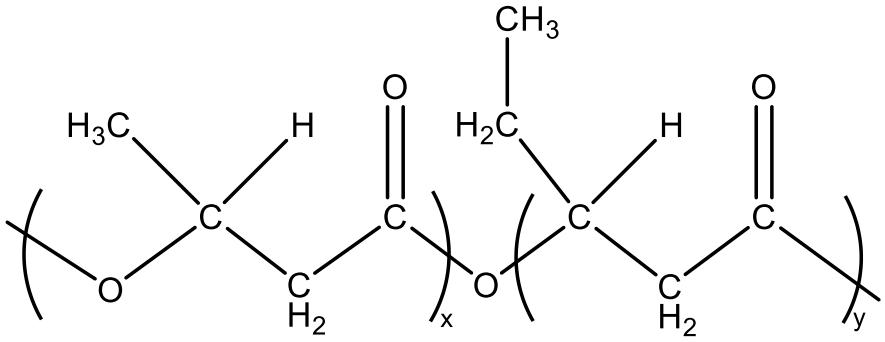


Figure 2.4: Chemical structure of P(3HB-*co*-3HV). 'x' and 'y' represent repeating unit of each monomer.

2.4.3 P(3HB-*co*-3HHx)

P(3HB-*co*-3HHx) copolymer is comprised of 3HB and 3HHx monomers. The chemical structure of this copolymer is shown in Figure 2.5. Due to its longer alkyl side chain, 3HHx prevents isodimorphism between 3HB and 3HHx monomer units. Both monomer units are not able to fit into the crystalline lattices of each other (Doi *et al*., 1995). When 3HHx increased from 0 to 25 mol%, the crystallinity of this copolymer decreased from 60 to 18 %. As 3HHx molar compositions increased from 0 to 17 mol%, the elongation at break of this copolymer increased from 6 to 850 % while its tensile strength decreased from 43 to 20 MPa (Sudesh & Abe, 2010). The copolymer becomes softer and more flexible as 3HHx increases (Asrar *et al*., 2002). The melting temperature of P(3HB-*co*-3HHx) copolymer with 5 mol% of 3HHx is less than 155 ℃ and it can be as low as 52 ℃ when 3HHx molar composition further increased to 25 mol% (Doi *et al*., 1995; Loo *et al*., 2005). P(3HB-*co*-3HHx) is a potential copolymer to be used in blending to obtain a better-quality polymer (Freier, 2006).

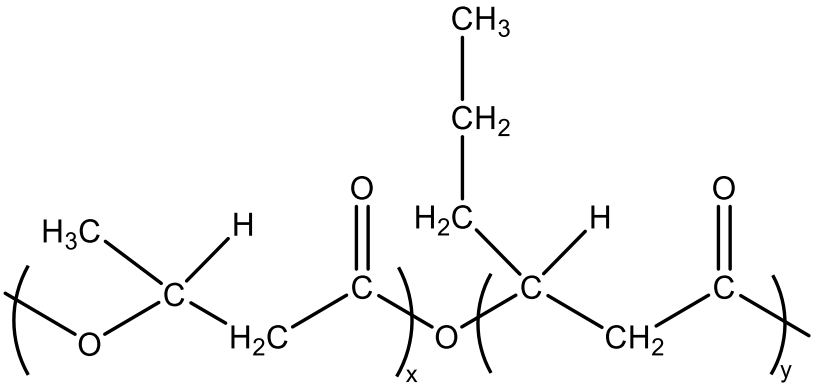


Figure 2.5: Chemical structure of P(3HB-*co*-3HHx). 'x' and 'y' represent repeating units of each monomer.

2.4.4 P(3HB-*co*-4HB)

P(3HB-*co*-4HB) copolymer is made up of 3HB and 4HB monomer units. The number of carbon atoms of 4HB monomer units is the same as 3HB except that it does not have an alkyl side group. The structure of 4HB is linear thus, avoiding the co-crystallization between 4HB and 3HB monomer units in the 3HB crystal lattice. 4HB has altered the 3HB crystal lattice thoroughly. There is a deformation process in the 4HB lattice for them to fit into the 3HB monomer lattice (Mitomo *et al*., 2001). The chemical structure of this copolymer is shown in Figure 2.6. It was reported that the increment of 4HB molar fractions from 0 to 64 mol% showed a declining trend in the crystallinity of P(3HB-*co*-4HB) from 60 to 15 %. However, when 4HB molar fractions increased from 78 to 100 mol%, the crystallinity of P(3HB-*co*-4HB) increased from 15 to 34 %. The T_m of P(3HB-*co*-4HB) copolymer is lower than P(3HB) homopolymer. As 4HB monomer compositions increase from 0 to 100 mol%, T_m decreases from 178 to 50 °C. Meanwhile, the glass transition temperature (T_g) also showed a declining trend. The incorporation of 4HB monomer composition into P(3HB) homopolymer makes the copolymer more elastomeric and flexible. The elongation at break values of

P(3HB-*co*-82 mol% 4HB) could be as high as 1320 % showing that this copolymer was very flexible. However, as 4HB monomer composition further increases from 82 to 100 mol%, it shows a declining trend in the elongation at break from 1320 to 1000 %. When 4HB monomer compositions in the P(3HB-*co*-4HB) copolymer are more than 64 mol%, the tensile strength increases up to 104 MPa (Saito *et al*., 1995). P(3HB-*co*-4HB) copolymers are biocompatible and bioabsorbable, making them suitable for various medical applications. Examples of medical applications are surgical sutures, implantation, and gauzes (Jaeger *et al*., 1995; Martin & Williams, 2003).

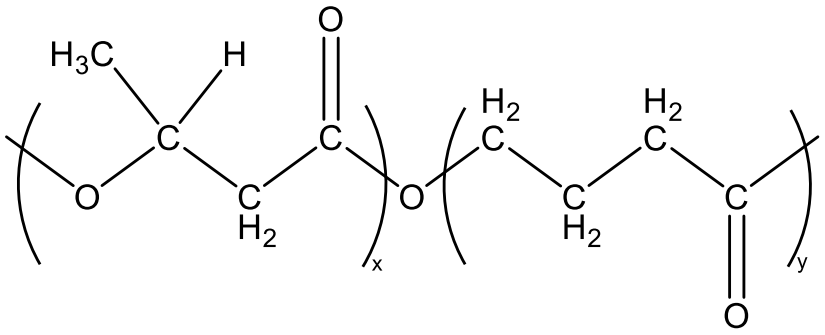


Figure 2.6: Chemical structure of P(3HB-*co*-4HB). ‘x’ and ‘y’ represent repeating units of each monomer.

2.5 Application of PHA

PHA has a wide range of interesting properties and can be applied in various fields such as packaging, single-use items, tissue engineering, the skin care industry, and agriculture. It has been commercialized in the United States (Danimer Scientific), Japan (KANEKA), Germany (Biomer), China (Bluepha), and Italy (Bio-On) (Tan *et al*., 2021; Koller & Mukherjee, 2022). Table 2.2 shows the applications of PHA and its producing companies.

In single-use applications, P(3HB-*co*-3HHx) was used as shopping bags, bottles, food containers, drinking straws, and coffee capsules by KANEKA Corporation, Danimer Scientific, and Metabolix due to its close resemblance to PP and low-density polyethylene (Reddy *et al*., 2022; Tang *et al*., 2022). In addition, P(3HB-*co*-3HHx) has been evaluated for potential utilization in the cosmetics and skin care industry as a facial oil-absorbing film (Sudesh *et al*., 2007). It was also reported that PHA films can absorb sebum from the skin even in the absence of any supplementary lipophilic additives. The transparency on P(3HB-*co*-3HHx) films indicated that oil was absorbed into the films. Besides facial oil-blotting film, P(3HB) is also applied as micro powder-based sun creams, developed by Unilever to increase water resistance and minimize their effects to the environment (Reddy *et al*., 2022).

In the agricultural field, PHAs are used as eco-friendly mulch because of their potential to improve the soil structure, retain moisture, and reduce environmental pollution (Samrot *et al*., 2021). Biodegradable mulch made of P(3HB-*co*-3HHx) copolymer had been patented by Danimer Scientific (Chen, 2009). PHAs are also used to make agricultural nets to improve crop yield and protect them from harmful factors such as insects and birds. The PHA-based agricultural nets are compostable and can be disposed of directly into the soil (Guerrini *et al*., 2017). Furthermore, growing bags

made of PHA do not release hazardous substances into the soil once they are degraded. The degraded PHA monomers can also serve as a carbon source which can improve microbial growth and increase the fertility of the soil (Samrot *et al*., 2021). PHAs can also be applied as a coating for controlled-release fertilizers, pesticides, or herbicides. Due to the biodegradability of PHA, as PHA degrades, it releases the compounds slowly and efficiently to the target site, minimizing the negative impact on the plants and environment (Grillo *et al*., 2011).

Due to the biocompatibility and bioresorbability of PHA such as P(4HB) and P(3HB-*co*-4HB), it has the potential to be applied in tissue engineering scaffolds, osteosynthetic materials, surgical sutures, and cardiovascular patches (Martin & Williams, 2003). In the medical field, the scaffold and sutures made from P(3HB-*co*-4HB) encourage cell attachment, proliferation, and differentiation. They can be implanted into the human body without triggering any severe immune response or side effects. For example, the tissue response towards P(3HB-*co*-4HB) copolymers electrospun implant in a rat model showed improvement with increasing 4HB monomer composition (Tang *et al*., 2008). Besides that, TephaFLEX^{TM} absorbable suture made from P(4HB) is one excellent example of a PHA-based biomaterial that has received United States Food and Drug Administration approval (Tang *et al*., 2010).