THE USE OF POLYHYDROXYALKANOATE SYNTHASE ($PHAC_{BP\text{-}M\text{-}CPF4}$) AND NEWLY IDENTIFIED ENOYL-COA HYDRATASE ($PHAJ_{Ss}$) FOR THE PRODUCTION OF POLY[(*R*)-3-HYDROXYBUTYRATE-*CO*-(*R*)-3-HYDROXYHEXANOATE]

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UNIVERSITI SAINS MALAYSIA

2020

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by

TAN HUA TIANG

Thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy

December 2020

ACKNOWLEDGEMENT

The completion of my doctoral degree in Universiti Sains Malaysia would not have been possible without the support and guidance from many wonderful individuals. I am grateful for their supports throughout the whole journey of my doctoral study.

I would like to take this opportunity to express my sincere gratitude and deepest appreciation to my research supervisor Professor Dr. K. Sudesh Kumar for accepting me as a doctoral student and for introducing me to polyhydroxyalkanoates (PHA). His valuable advice, constructive suggestions and encouragement have enabled me to complete my research project successfully.

I would like to express my heartfelt appreciation to members of Ecobiomaterial Research Laboratory, for creating such a collegial work environment, especially to Dr. Foong Choon Pin who had helped me get started with PHA research. I would also like to convey my thanks to members of the laboratory for their help and I would forever cherish the wonderful moments we've spent together.

I would also like to express my deepest appreciation to Professor Dr. Toshio Hakoshima for allowing me to conduct part of my study in his laboratory at Nara Institute of Science and Technology (NAIST). His guidance and scientific views are very helpful and valuable. My utmost gratitude goes to Dr. Chek Min Fey, Dr. Kim Sun Yong and Dr. Tomoyuki Mori for their time, patience and guidance. Their willingness in teaching me skills and techniques which are indispensable to the field of my study has enabled the efficient execution of my project. I would also like to convey my thanks to the members of Structural Biology Laboratory, for their kindness and warm hospitality which has made my stay in Japan a pleasant and enjoyable one.

I am grateful to the Ministry of Higher Education, Malaysia for awarding me with MyBrainSc scholarship, which has provided me financial support throughout my research period. I would also like to acknowledge Universiti Sains Malaysia and NAIST for the funding throughout my 14-month research period in Japan.

Last but not least, I would like to express my deepest gratitude to my family for their boundless support and love which has kept me motivated to complete this research project.

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LIST OF UNITS AND SYMBOLS

α	Alpha
~	Approximately
β	Beta
Da	Dalton
°C	Degree Celsius
Δ	Delta
wt%	Dry weight percent
γ	Gamma
GPa	Gigapascal
g	Gram
Hz	Hertz
h	Hour
∞	Infinity
kDa	Kilodalton
kPa	Kilopascal
L	Liter
MPa	Megapascal
µg	Microgram

µL	Microliter
µM	Micromolar
mg	Milligram
mL	Milliliter
mM	Millimolar
min	Minute
mol%	Mole percent
ng	Nanogram
nm	Nanometer
nM	Nanomolar
%	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

LIST OF ABBREVIATIONS

3H4MV	3-hydroxy-4-methylvalerate
3HB	3-hydroxybutyrate
3HB-CoA	3-hydroxybutyryl-CoA
3HHp	3-hydroxyheptanoate
3HHx	3-hydroxyhexanoate
3HHx-CoA	3-hydroxyhexanoyl-CoA
3HV	3-hydroxyvalerate
4HB	4-hydroxybutyrate
4HB-CoA	4-hydroxybutyryl-CoA
5HV	5-hydroxyvalerate
ANOVA	One-way analysis of variance
β-ME	β-mercaptoethanol
CME	Caprylic methyl ester
CoA	coenzyme A
CPKO	Crude palm kernel oil
C-terminus	Carboxy-terminus
D	Aspartic acid
DCW	Dry cell weight

DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
DTNB	5,5'-dithio-bis-(2-nitrobenzoic acid)
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
GC	Gas chromatography
gDNA	Genomic DNA
GPC	Gel permeation chromatography
H	Histidine
HA	Hydroxyacyl
HA-CoA	Hydroxyacyl-CoA
HPLC	High-performance liquid chromatography
IPTG	Isopropyl-β-D-thiogalactopyranoside
LB	lysogeny broth
LDPE	Low-density polyethylene
MALDI-TOF MS	Matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry
MEGA	Molecular Evolutionary Genetic Analysis
MM	Mineral salts medium

M_w	Weight-average molecular weight
M_n	Number-average molecular weight
M_w/M_n	Polydispersity index
NADPH	Reduced form nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
Ni-NTA	Nickel-nitrilotriacetic acid
NR	Nutrient rich
OD_{412nm}	Optical density at wavelength 412 nm
OD_{600nm}	Optical density at wavelength 600 nm
P(3HB)	Poly(3-hydroxybutyrate)
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)
PA	polyamide
PCR	Polymerase chain reaction
PE	polyethylene
PET	polyethylene terephthalate
pH	Potential of hydrogen
PHA	Polyhydroxyalkanoate

PhaA	Beta-ketothiolase
PhaB	NADPH-dependent acetoacetyl-CoA reductase
PhaC	PHA synthase
PhaE	PHA synthase subunit E
PhaG	3-hydroxyacyl-ACP-CoA transferase
PhaJ	*(R)*-specific enoyl-CoA hydratase
PhaP	Phasin
PhaR	PHA synthase subunit R
PhaZ	PHA depolymerase
PO	Palm olein
PP	Polypropylene
ProC	Pyrroline-5-carboxylate reductase
PS	Polystyrene
P-value	Probability value
PVC	Polyvinyl chloride
RBS	Ribosomal binding sites
SCL	Short-chain-length
SD	Standard deviation
SDS	Sodium dodecyl sulfate

SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOB	Super Optimal Broth
TAE	Tris-acetate-EDTA
TCA	Tricarboxylic acid
T_g	Glass transition temperature
T_m	Melting temperature
TSB	Tryptic soy broth
UHMW	Ultrahigh-molecular-weight
UV	Ultraviolet

LIST OF APPENDICES

PENGGUNAAN SINTASE POLIHIDROKSIALKANOAT ($PHAC_{BP\text{-}M\text{-}CPF4}$) DAN ENOIL-KOA HIDRATASE ($PHAJ_{Ss}$) YANG BARU DITEMUI UNTUK PENGHASILAN POLI[(*R*)-3-HIDROKSIBUTIRAT-*KO*-(*R*)-3-HIDROKSIHEKSANOAT]

ABSTRAK

Kebanyakan plastik industri dihasilkan daripada sumber yang tidak boleh diperbaharui seperti petroleum. Oleh itu, proses penghasilan polimer berasaskan sumber yang boleh diperbaharui perlu dibangunkan untuk mengurangkan jejak karbon yang ditinggalkan oleh aktiviti manusia. Polihidroksialkanoat (PHA) adalah biopoliester intrasel yang disintesis oleh pelbagai jenis mikroorganisma sebagai simpanan karbon di bawah keadaan kultur yang kekangan sumber nutrien penting tetapi berlebihan sumber karbon. Selain itu, PHA boleh dibiodegradasikan. Antara pelbagai jenis PHA, poli[(*R*)-3-hidroksibutirat-*ko*-(*R*)-3-hidroksiheksanoat] [P(3HB-*ko*-3HHx)] mempunyai potensi yang tinggi untuk berfungsi sebagai bioplastik komersial kerana ia mempunyai sifat yang paling serupa dengan plastik berasaskan petroleum. PHA sintase *Chromobacterium* sp. USM2 ($PhaC_{Cs}$) dan dipencilkan dari metagenom paya bakau ($PhaC_{BP\text{-}M\text{-}CPF4}$) telah dilaporkan mampu menghasilkan P(3HB-*ko*-3HHx). Dalam kajian ini, $PhaC_{Cs}$ dan $PhaC^{BP\text{-}M\text{-}CPF4}$ serta enoil-KoA hidratase *Streptomyces* sp. strain CFMR 7 ($PhaJ_{Ss}$) telah dicirikan. Oleh itu, lima transforman *Cupriavidus necator* yang mengandungi $phaC_{BP\text{-}M\text{-}CPF4}$ dengan genotip yang berbeza dan tiga transforman *C. necator* yang mengandungi gen $phaC_{Cs}$ dengan genotip yang berbeza telah dihasilkan untuk menilai kecekapan inkorporasi monomer 3-hidroksiheksanoat (3HHx). Jumlah monomer 3HHx yang diinkorporasikan dalam PHA yang disintesis oleh transforman *C. necator* tersebut telah diperiksa

menggunakan minyak kelapa sawit sebagai sumber karbon tunggal. PhaC$_{BP\text{-}M\text{-}CPF4}$ membolehkan inkorporasi monomer 3HHx yang lebih tinggi daripada PhaC$_{Cs}$ (sehingga 18 mol% 3HHx). Selain itu, berat molekul (M_w) P(3HB-*ko*-3HHx) yang dihasilkan oleh transforman yang mengandungi *phaC*$_{BP\text{-}M\text{-}CPF4}$ dapat mencapai sehingga 1.8×10^6 Da, enam kali ganda lebih tinggi daripada P(3HB-*ko*-3HHx) yang dihasilkan oleh transforman yang mengandungi *phaC*$_{Cs}$. Enoil-KoA hidratase sangat penting untuk pengumpulan 3HHx semasa penghasilan P(3HB-*ko*-3HHx). Enzim ini menyalurkan laluan untuk membekalkan unit monomer (*R*)-3-hidroksiasil-KoA, terutamanya (*R*)-3-hidroksiheksanoil-KoA dari pengoksidaan-β asid lemak. Dalam kajian ini, *phaJ*$_{Ss}$ telah dikenal pasti dari bakteria Gram-positif pendegradasi getah yang tidak mampu menghasilkan PHA, *Streptomyces* sp. strain CFMR 7. Pengekspresan bersama gen hidratase enoil-KoA tersebut dengan sintase PHA, *phaC*$_{BP\text{-}M\text{-}CPF4}$, dalam *C. necator* PHB^{-}4, telah meningkatkan komposisi 3HHx dengan ketara tanpa pengurangan kandungan PHA. Transforman ini dapat menghasilkan P(3HB-*ko*-3HHx) dengan 18 mol% 3HHx dan M_w menghampiri satu juta Da lantas mendedahkan bahawa kedua-dua PhaC$_{BP\text{-}M\text{-}CPF4}$ dan PhaJ$_{Ss}$ berpotensi digunakan untuk aplikasi industri.

THE USE OF POLYHYDROXYALKANOATE SYNTHASE ($PhaC_{BP\text{-}M\text{-}CPF4}$) AND NEWLY IDENTIFIED ENOYL-COA HYDRATASE ($PHAJ_{Ss}$) FOR THE PRODUCTION OF POLY[(*R*)-3-HYDROXYBUTYRATE-*CO*-(*R*)-3-HYDROXYHEXANOATE]

ABSTRACT

Most industrial plastics are produced using non-renewable resources such as petroleum. Hence, polymer production processes based on renewable resources must be developed to reduce the carbon footprints left by human activities. Polyhydroxyalkanoates (PHAs) are intracellular biopolyesters synthesized by numerous microorganisms as carbon storage under culture conditions of limiting essential nutrients but with excess carbon source. Besides, PHA is biodegradable. Among the various types of PHA, poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyhexanoate] [P(3HB-*co*-3HHx)] has a high potential to serve as a commercial bioplastic due to it having the most identical properties to petroleum-based plastics. PHA synthase of *Chromobacterium* sp. USM2 ($PhaC_{Cs}$) and PHA synthase isolated from mangrove metagenome ($PhaC_{BP\text{-}M\text{-}CPF4}$) have been reported to be able to produce P(3HB-*co*-3HHx). In this study, $PhaC_{Cs}$ and $PhaC_{BP\text{-}M\text{-}CPF4}$ as well as enoyl-CoA hydratase of *Streptomyces* sp. strain CFMR 7 ($PhaJ_{Ss}$) were characterized. Thus, five different genotypes of *Cupriavidus necator* transformants harboring $phaC_{BP\text{-}M\text{-}CPF4}$ gene and three different genotypes of *C. necator* transformants harboring $phaC_{Cs}$ gene were developed to evaluate the incorporation efficiency of 3-hydroxyhexanoate (3HHx) monomers. The amount of 3HHx monomer incorporated in the PHA synthesized by these *C. necator* transformants were examined using palm oil as the sole carbon source. $PhaC_{BP\text{-}M\text{-}CPF4}$ enabled the incorporation of higher 3HHx monomer

than PhaC$_{Cs}$ (up to 18 mol% 3HHx). Besides, the molecular weight (M_w) of P(3HB-*co*-3HHx) produced by transformants harboring *phaC*$_{BP\text{-}M\text{-}CPF4}$ could reach up to 1.8 × 10^6 Da, which was six times higher than the P(3HB-*co*-3HHx) produced by transformants harboring *phaC*$_{Cs}$. Enoyl-CoA hydratase is crucial for 3HHx accumulation during the production of P(3HB-*co*-3HHx). This enzyme channels the pathway for supplying (*R*)-3-hydroxyacyl-CoA monomer units, especially (*R*)-3-hydroxyhexanoyl-CoA from fatty acid β-oxidation. In this study, *phaJ*$_{Ss}$ was identified from the rubber degrading Gram-positive non-PHA producing bacterium, *Streptomyces* sp. strain CFMR 7. Co-expression of this enoyl-CoA hydratase gene with the chosen PhaC above, *phaC*$_{BP\text{-}M\text{-}CPF4}$, in *C. necator* PHB^{-}4, significantly increased 3HHx composition without decreasing the PHA content. This transformant could produce P(3HB-*co*-3HHx) with 18 mol% of 3HHx and has a M_w of nearly one million Da revealing that both PhaC$_{BP\text{-}M\text{-}CPF4}$ and PhaJ$_{Ss}$ could potentially be used for industrial applications.

CHAPTER 1

INTRODUCTION

1.1 Overview

Most industrial plastics are produced using non-renewable resources such as petroleum. Hence, polymer production processes based on renewable resources must be developed to reduce the dependence on the fossil-based plastic (Sudesh *et al.*, 2000). Solid wastes from non-biodegradable plastics also cause major impact on both terrestrial and marine environment as they affect wildlife via entanglement and ingestion (Derraik, 2002; Hahladakis, 2020) Polyhydroxyalkanoates (PHAs) are bio-based polyesters synthesized by numerous microorganisms as carbon storage, using renewable carbon resources, for example, plant oils, sugars, plant biomass and even carbon dioxide (Anderson and Dawes, 1990; Fukui and Doi, 1998; Albuquerque *et al.*, 2007; Miyasaka *et al.*, 2013; Heng *et al.*, 2017). The unique attributes of PHA such as excellent biodegradability, good thermostability and processability make it a worthwhile alternative to replace conventional plastics (Sudesh *et al.*, 2000).

In general, there are three types of PHAs, namely the short-chain-length PHA (SCL-PHA) consisting of three to five carbons in the monomer units, medium-chain-length PHA (MCL-PHA) consisting of six to 14 carbons in the monomer units, as well as the mixture of both SCL- and MCL-PHA (Sudesh, 2012). The types of monomer present in the polymer chain are the determining factor for the physicochemical properties of the resulting PHAs. For instance, poly[*(R)*-3-hydroxybutyrate)] [P(3HB)], is a type of SCL-PHA homopolymer consisting of four carbons in its monomer unit and is produced by most PHA producers. P(3HB) polymer is highly crystalline and holds high melting temperature (Reinecke and Steinbüchel, 2009). However, the brittle and stiff nature of P(3HB) makes it difficult to be processed and used for industrial

applications. In contrast, copolymerization of MCL monomers with 3HB leads to dramatic changes in the properties of the polymer, making it softer and more flexible compared to P(3HB) homopolymer (Noda *et al.*, 2005).

Among the various types of PHAs, poly[*(R)*-3-hydroxybutyrate-*co*-*(R)*-3-hydroxyhexanoate] [P(3HB-*co*-3HHx)] has been identified as a commercially useful PHA copolymer since it has a close resemblance to commodity plastics such as polypropylene (PP) and low-density polyethylene (LDPE) (Doi, 1990). Depending on the 3-hydroxyhexanoate (3HHx) compositions, the polymer P(3HB-*co*-3HHx) vary substantially in its properties ranging from the strong but less flexible to the more flexible and strong variety (Asrar *et al.*, 2002). It has been shown that high 3HHx levels (17 mol%) in this copolymer results in properties which are similar to LDPE (Doi *et al.*, 1995). Besides, molecular weight is one of the most important criteria in selecting polymeric materials as it can significantly influence the physical and mechanical strength of the bioplastic produced (Hahn *et al.*, 1994). PHAs with higher molecular weight are known to possess better mechanical properties and are preferred for practical applications (Aoyagi *et al.*, 2003). In particular, it is highly desired to develop a microbial strain that is capable of producing high molecular weight P(3HB-*co*-3HHx) copolymer.

PHA synthase (PhaC) is the key enzyme for PHA production. It determines the composition of monomer that is incorporated into the PHA polymer chain. Since the PHA polymer properties are greatly affected by the composition of the monomer units, PhaC is one of the determining factors that affect the properties of the polymers synthesized. PhaCs are categorized into four classes based on their primary sequences, subunit compositions and substrate specificities (preferences for SCL [C3 to C5] or MCL [C6 to C14] monomers) (Rehm, 2003). However, some PhaCs are difficult to be

classified based on these criteria as they are able to polymerize both SCL- and MCL-monomers, for example, PhaCs of *Aeromonas caviae*, *Aeromonas hydrophila* 4AK4, *Rhodococcus aetherivorans* I24, *Chromobacterium* sp. strain USM2 and the recently reported $PhaC_{BP\text{-}M\text{-}CPF4}$ that was isolated from mangrove soil metagenome (Doi *et al.*, 1995; Han *et al.*, 2004; Chia *et al.*, 2010; Budde *et al.*, 2011; Foong *et al.*, 2018). Among these synthases, $PhaC_{BP\text{-}M\text{-}CPF4}$ has attracted much interests due to its ability to incorporate up to six different types of PHA monomers, i.e. 3-hydroxybutyrate (3HB), 3HHx, 3-hydroxyvalerate (3HV), 3-hydroxy-4-methylvalerate (3H4MV), 4-hydroxybutyrate (4HB) and 5-hydroxyvalerate (5HV), with appropriate precursors. The ability of this PhaC for the production of P(3HB-*co*-3HHx) copolymer, has intrigued scientists to study these enzymes in detail. Prior to the discovery of $PhaC_{BP\text{-}M\text{-}CPF4}$, various efforts such as enzyme evolution and strain engineering have been made to enable the production of P(3HB-*co*-3HHx) copolymer with a regulated monomer composition and ultrahigh-molecular-weight (UHMW) using plant oils as the sole carbon source (Tsuge *et al.*, 2007; Mifune *et al.*, 2008; Budde *et al.*, 2011; Chuah *et al.*, 2013).

Cupriavidus necator showed better growth and PHA accumulation when using plant oils as carbon sources, as compared to sugars (Fukui and Doi, 1998). The benefit of using plant oil is that it contains higher carbon content per weight than sugars. It is proposed that the yield of PHA by microorganism from plant oils could be at least two-fold higher than those that was produced from sugar (Akiyama *et al.*, 2003). Besides, the metabolism of plant oil can influence the monomer composition of the resulting PHA. For example, 3HHx monomers could be supplied as metabolites without using expensive precursor compounds such as sodium hexanoate, sodium octanoate and sodium dodecanoate (Fukui and Doi, 1997; Akiyama *et al.*, 2003; Sun *et al.*, 2010).

Plant oil is a preferable carbon source as it is cheaper and does not require expensive precursor for production of P(3HB-*co*-3HHx). Hence, it is necessary to develop an efficient recombinant strain that can produce P(3HB-*co*-3HHx) copolymer with variable molar fractions of 3HHx, preferably from 10 – 20 mol% 3HHx, by using plant oil as the sole carbon source. Different applications require P(3HB-*co*-3HHx) copolymer with different composition of 3HHx. In order to have good mechanical properties, it is necessary to produce P(3HB-*co*-3HHx) copolymer with sufficiently high M_w of more than 500,000 Da.

In this study, five *C. necator* transformants with $PhaC_{BP\text{-}M\text{-}CPF4}$ in two different plasmids have been developed to evaluate the composition of 3HHx monomer and the molecular weight of P(3HB-*co*-3HHx) copolymer produced by this PhaC when using palm oil as the sole carbon source. In addition, the capability of $PhaC_{BP\text{-}M\text{-}CPF4}$ to incorporate 3HHx was compared with corresponding *C. necator* transformants which harbors $phaC_{Cs}$ (*Chromobacterium* sp. strain USM2). The factors affecting the 3HHx composition were investigated based on the different host strains, PhaCs, plasmids and carbon sources. The *C. necator* mutant hosts used in this study were H16ΔC, $PHB^{-}4$, Re2058 and Re2160. Previous studies have shown that these mutant hosts affect the incorporation of 3HHx monomers in P(3HB-*co*-3HHx) copolymer owing to the activity differences in PhaA and PhaB (Mifune *et al.*, 2008; Budde *et al.*, 2011). Another enzyme that is crucial for 3HHx accumulation during production of P(3HB-*co*-3HHx) is enoyl-coenzyme A (enoyl-CoA) hydratase (PhaJ) (Fukui and Doi, 1997). This enzyme creates a pathway for supplying (*R*)-3-hydroxyacyl-CoA [(*R*)-3HA-CoA] monomer units, especially 3HHx-CoA from fatty acid β-oxidation. In this study, putative enoyl-CoA hydratase genes from the rubber degrading Gram-positive, non-PHA producing bacterium, *Streptomyces* sp. strain CFMR 7 were analyzed through

bioinformatics. The enoyl-CoA hydratase gene that could possibly be functional was cloned into *C. necator* PHB^{-}4 and co-expressed with *phaC*$_{\text{BP-M-CPF4}}$ to investigate the uncharacterized PhaJ.

1.2 Objectives of this study

a) To determine the efficiency of PhaC$_{\text{BP-M-CPF4}}$ and PhaC$_{Cs}$ for the production of P(3HB-*co*-3HHx) from plant oil.

b) To determine the relationship of enzymatic activity of PhaC with M_w of the PHA produced.

c) To determine the ability of uncharacterized enoyl-CoA hydratase genes from *Streptomyces* sp. strain CFMR 7 on the production of P(3HB-*co*-3HHx) from plant oil.

CHAPTER 2

LITERATURE REVIEW

2.1 Plastics

Plastics is a group of polymers with industrially desirable properties such as chemical and light-resistance, inexpensive, strong, durable, high thermostability and electrical insulation. The large varieties of polymers and the versatility of their properties accelerate the production of plastic products which continue to benefit human society with expanding applications and create a better quality of life. Since 2018, world plastics production has increased nearly 180-fold from 1950 (2 million metric tons), reaching up to 359 million metric tons (Garside, 2019).

Although plastics are very important in today's society, production of plastics from non-renewable resources is one of the major concerns of the society. Moreover, its non-biodegradability has caused major impact on environment, such as waste accumulation, marine pollution, greenhouse gases production, and human-health risk (Rodrigues *et al.*, 2019; Shen *et al.*, 2020).

The continued increase in disposable, short-life consumer products with ultra-durable property limits its recyclability, further highlighting that plastics are unsustainable (Halden, 2010). More than 33 % of the production volume of plastics are for disposable items. The useful life span for many plastic items such as plastic bags, disposable cups, and plastic utensils are generally very short. These products are known to persist and pollute for extremely long period of time. Release of these products to the environment generates many plastics-exposed biotas. Weathering of plastic debris causes fragmentation of the plastic wastes into smaller particles that can be ingested by small marine animals (Laist, 1997). There are over 260 species of fish, seagulls, invertebrates, turtles, condor, and mammals which have been documented as

to having ingested or being entangled by plastic debris. Although incineration is the most effective way for removing plastic wastes, this method generates chemicals which will cause severe health issues to human such as carcinogenic polychlorinated dibenzo-*p*-dioxins/furans (PCDD/Fs) and organohalogens (Takasuga *et al.*, 2007).

2.2 Bio-based polymer

Bio-based polymers are polymers that have similar properties with petrochemical-based plastics, which are made from biological renewable resources, and polymerized by either chemical or biological methods (Harmsen *et al.*, 2014). Hence, they are one of the best alternative materials to replace petrochemical-based plastics such as polyamide (PA), polyethylene (PE), polyethylene terephthalate (PET), PP, polystyrene (PS), and polyvinyl chloride (PVC).

Bio-based polymers can be classified into three groups: modified natural polymers, biosynthetic polymers, and bio-chemosynthetic polymers (Table 2.1) (Sudesh and Iwata, 2008). Biomass such as cellulose and starch need to undergo chemical modifications to produce modified natural polymers (Lu *et al.*, 2005). However, the use of modified natural polymers has been restricted to only a few applications such as packaging, textiles and construction. Biosynthetic polymers, on the other hand, are totally synthesized through biological process, whereby the entire process of the polymer production occurs in bacterial cells, right from the beginning (production of monomers) up to the end (polymerization of the monomers) (Sudesh *et al.*, 2000). One of the well-known biosynthetic polymers is PHA. For bio-chemosynthetic polymers, the monomers are first synthesized biologically in bacterial cells and then polymerized chemically (Jem *et al.*, 2010). Poly(lactic acid), PLA, for example, is produced in a way that lactic acid monomers are initially produced by

microbial fermentation and the monomers produced are then chemically polymerized into PLA by the aid of a metal catalyst.

Table 2.1: Examples of bio-based polymers.

Adapted from Sudesh (2012).

Type	Processes involved	Example
Modified natural polymers	Chemical modification of natural polymer	Starch polymer, cellulose derivatives, proteins
Biosynthetic polymers	Biosynthesis of polymer by microorganisms	Poly(3-hydroxybutyrate)
Bio-chemosynthetic polymers	Biosynthesis of monomers and polymerized chemically	Poly(lactic acid), polyvinyl alcohol

2.3 PHA

PHA was first discover by Maurice Lemoigne in *Bacillus megaterium* in 1926 (Lemoigne, 1926). PHAs, which are biopolymeric chains composed of various hydroxyalkanoates, are accumulated as intracellular carbon storage compound (Anderson and Dawes, 1990). Since the first discovery of PHA back in 1926, the number of different genera of both Gram-positive and Gram-negative PHA producing bacteria which have been documented had increased to more than 75 (Reddy *et al.*, 2003). Up to now, there are more than 150 different monomeric blocks of PHA identified (Rehm, 2003). The general chemical structure of PHA is made up of one hydroxyl group and one carboxyl group as shown in Figure 2.1. PHAs are naturally produced by many bacteria and archaea under condition with excess supply of carbon source, but with the limitation of other nutritional elements, such as nitrogen, phosphorus, sulfur, magnesium or oxygen (Anderson and Dawes, 1990; Doi, 1990).

In contrast with deoxyribonucleic acid, ribonucleic acid, and polypeptide, which synthesis are directed by information encoded in other biopolymers, PHAs are synthesized in a non-template-dependent manner (Stubbe *et al.*, 2005). PHAs are stored as carbon and energy reserves intracellularly (cytoplasm) in the form of water insoluble inclusions or granules. Studies had shown that almost 90 % of the bacterial dry cell weight (DCW) could be comprised of PHA, without causing any significant effect to the osmotic pressure in the cell (Madison and Huisman, 1999). These water insoluble PHAs will later be degraded by PHA depolymerase (PhaZ) when required for survival during carbon starvation (Anderson and Dawes, 1990).

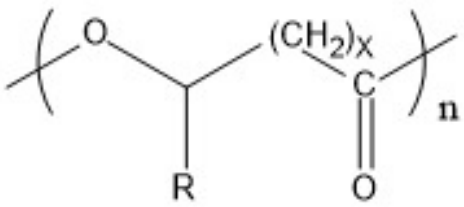


Figure 2.1: General chemical structure of PHA. ‘x’ represents the carbon chain in the linear polyester structure while ‘R’ represents the variable hydrocarbon side chains. ‘n’ represents number of repeating units.

Generally, PHA can be categorized into three main groups based on the carbon numbers in the monomer building blocks: SCL-PHA, MCL-PHA, and combination of SCL-PHA and MCL-PHA (SCL-MCL-PHA) (Sudesh, 2012). SCL-PHA consists of monomers with 3 to 5 carbon atoms, MCL-PHA consists of monomers with 6 to 14 carbon atoms and SCL-MCL-PHA consists of monomers with 3 to 14 carbon atoms. The physical and thermal properties of PHAs are dependent on the monomer type, monomer composition and molecular weight of the polymer (Sudesh *et al.*, 2000). SCL-PHAs have thermoplastic properties such as high crystallinity, stiff, brittle, high melting temperature, high tensile modulus and low elongation at break while MCL-PHAs have elastomeric properties such as low crystallinity, low melting temperature and high elongation at break (Yu, 2007). However, the SCL-MCL-PHA copolymers which are composed of both SCL-PHA and MCL-PHA monomers generally possess better thermal and mechanical properties compared to homopolymers like SCL-PHA and MCL-PHA (Kellerhals *et al.*, 2000). Depending on the composition of MCL-PHA, the SCL-MCL-PHA copolymers vary substantially in their properties ranging from the strong but less flexible to the more flexible and strong variety (Asrar *et al.*, 2002).

Besides, the molecular weight of PHA substantially affect its physical and mechanical strength (Hahn *et al.*, 1994). PHAs with higher molecular weight are known to possess better mechanical properties and are preferred for practical applications (Aoyagi *et al.*, 2003). One of the unique properties of PHA is that it is completely biodegradable (Sudesh *et al.*, 2000). It can be biodegraded into carbon dioxide and water under aerobic condition or into methane and carbon dioxide under anaerobic condition by microorganisms. Hence, these polymers are gaining global attention and are being studied extensively for developing PHAs for commercial use.

2.4 Metabolic pathways of PHA biosynthesis

Cupriavidus necator H16 is the paradigm for studying PHA biosynthesis. Wild type *C. necator* H16 can only produce P3HB homopolymer. This PHA biosynthesis pathway comprises of three main enzymatic steps which will convert acetyl-CoA intermediate into P3HB (Figure 2.2) (Anderson and Dawes, 1990; Sudesh *et al.*, 2000). The genes for these three important steps were discovered as an operon (*phaC1-phaA-phaB1*) in *C. necator* H16. They are *phaA, phaB* and *phaC*, which encode *β*-ketothiolase (PhaA), NADPH-dependent acetoacetyl-CoA reductase (PhaB) and PhaC, respectively. First, two molecules of acetyl-CoA undergo condensation which is catalyzed by *β*-ketothiolase (PhaA) to form a molecule of acetoacetyl-CoA. Then, the acetoacetyl-CoA is reduced by PhaB to form *(R)*-3-hydroxybutyryl-CoA (3HB-CoA). Lastly, 3HB-CoAs are polymerized by PhaC to form P3HB (Figure 2.2, black box).

In addition to P3HB, there are many types of copolymers such as poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)], P(3HB-*co*-3HHx) and poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] which can be produced by PHA producing microorganisms (Doi *et al.*, 1988a; Doi *et al.*, 1988b; Doi *et al.*, 1995). The substrates or monomers for the biosynthesis of these copolymers were supplied from various metabolic pathways, such as citrate acid cycle, fatty acid β-oxidation and fatty acid *de novo* biosynthesis (Figure 2.2).

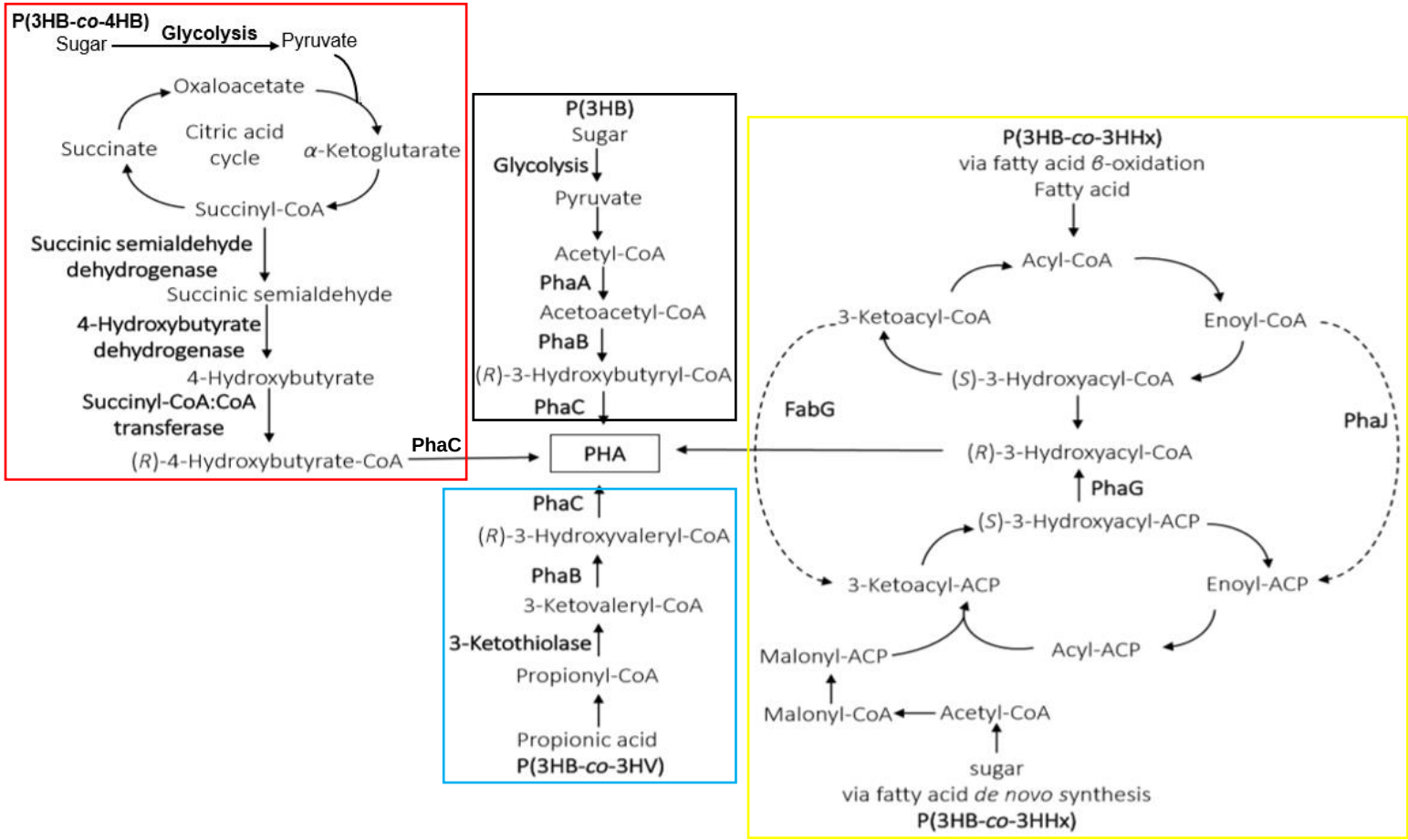


Figure 2.2: Metabolic pathway of PHA biosynthesis. Black box, blue box, red box and yellow box represent the pathway of P(3HB), P(3HB-*co*-3HV), P(3HB-*co*-4HB) and P(3HB-*co*-3HHx) production, respectively. Adapted from Sudesh *et al.* (2000) and Valentin *et al.* (1995).

P(3HB-*co*-3HV) copolymer can be produced when odd chain organic acids are used as the carbon source (Steinbüchel and Lütke-Eversloh, 2003). Metabolism of these acids leads to the formation of propionyl-CoA. Propionyl-CoA and acetyl-CoA then undergo condensation which is catalyzed by *β*-ketothiolase (BktB) to form ketovaleryl-CoA (Figure 2.2, blue box). Reduction of the ketovaleryl-CoA by PhaB produces (*R*)-3-hydroxyvaleryl-CoA, the substrate intermediate that is ready to be incorporated into the polymer chain.

Meanwhile, P(3HB-*co*-4HB) can be produced if the PHA-producing microorganism can express succinic semialdehyde dehydrogenase, 4-hydroxybutyrate dehydrogenase, and succinyl-CoA:CoA transferase (Valentin *et al.*, 1995; Lütke-Eversloh and Steinbüchel, 1999). The 4-hydroxybutyrate monomer is synthesized by conversion of succinyl-CoA from tricarboxylic acid (TCA) or citric acid cycle to 4-hydroxybutyryl-CoA (4HB-CoA) through a series of enzymatic reaction with catalysis by the enzymes previously mentioned (Figure 2.2, red box). Firstly, succinyl-CoA (the intermediate of TCA or citric acid cycle) is converted to succinic semialdehyde and then 4-hydroxybutyrate by the catalytic reaction of succinic semialdehyde dehydrogenase and 4-hydroxybutyrate dehydrogenase, respectively. Lastly, the 4HB intermediate is converted to 4HB-CoA by reacting with acetyl-CoA via the catalytic reaction of succinyl-CoA:CoA transferase.

For the production of MCL-PHA monomers such as 3HHx (Figure 2.2, yellow box) and 3-hydroxyheptanoate (3HHp), oil or fatty acids are generally used as the carbon sources (Doi *et al.*, 1995; Fukui *et al.*, 1997). Trans-2-enoyl-CoA intermediates synthesized from fatty acid β-oxidation pathway can be converted to [(*R*)-3HA-CoA] via the catalysis reaction of *(R)*-specific enoyl-CoA hydratase (PhaJ) (Fukui *et al.*, 1998). Besides, (*S*)-3HA-CoA can be converted to 3-ketoacyl-CoA intermediates and

then *(R)*-3HA-CoA in the same pathway via the catalytic reaction of epimerase and 3-ketoacyl-CoA reductase (FabG), respectively (Tsuge *et al.*, 2003). Moreover, MCL-PHA monomers could also be channelled from the fatty acid *de novo* biosynthesis pathway, whereby *(R)*-3-hydroxyacyl-ACP intermediates can be converted to *(R)*-3HA-CoA via the catalysis reaction of 3-hydroxyacyl-ACP-CoA transferase (PhaG).

The substrates or monomers of the copolymers can also be supplied through the addition of precursors or structurally-related substrates as exogenous carbon sources to the microorganisms during biosynthesis. There are also some unusual copolymers which can only be produced through the addition of precursors or structurally-related substrates because their substrates or monomers cannot be supplied through the metabolic pathway. For example,

(i) Sodium propionate or sodium valerate are the precursors for the synthesis of P(3HB-*co*-3HV) (Bhubalan *et al.*, 2008)

(ii) Sodium 4-hydroxybutyrate, γ-butyrolactone or 1,4-butanediol are the precursors for the synthesis of P(3HB-*co*-4HB) (Doi *et al.*, 1989; Kunioka *et al.*, 1989)

(iii) 3-mercaptopropionic acid is the precursors for the synthesis of poly(3hydroxybutyrate-*co*-3-mercaptopropionate) (Lütke-Eversloh *et al.*, 2001)

(iv) Isocaproic acid is the precursor for the synthesis of poly(3-hydroxybutyrate-*co*-3-hydroxy-4-methylvalerate) (Ling *et al.*, 2011)

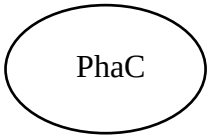
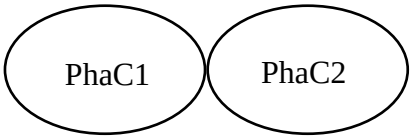
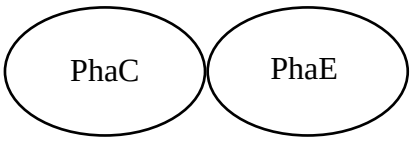
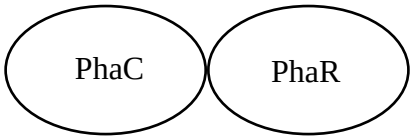
However, this also depends on the substrate specificity of the PhaC.

2.5 The key enzyme in PHA biosynthesis: PhaC

PhaC is the key enzyme involved in the process of PHA biosynthesis. PhaC's function is to catalyze the polymerization of the (*R*)-enantiomer form of hydroxyacyl (HA) moiety in (*R*)-HA-CoA to PHA with a concomitant release of CoA (Rehm, 2003; Sudesh, 2012). PhaCs can be grouped into four classes. The categorization is based on their primary sequences, substrate specificity, and subunit composition (Table 2.2). Class I and class II PhaCs contain only one type of subunit (PhaC) (Sudesh, 2012). However, class I PhaC comprises of only a single PhaC subunit while class II PhaC comprise of two PhaC subunits. In contrast, class III and class IV PhaCs contain two different types of subunits, forming heterodimers, comprising of PhaC-PhaE and PhaC-PhaR, respectively (Sudesh, 2012). Interestingly, PhaE and PhaR subunits have no similarity to PhaC subunit. Meanwhile, all the PhaC subunit consists of a conserved lipase-box-like motif "G-X-[S/C]-X-G" (whereby X represents any amino acid residues, and serine is substituted with cysteine in PhaC) (Rehm, 2003). Many studies have shown that PhaCs possess catalytic triads with a nucleophile (cysteine), an acid (aspartic acid), and a base (histidine) that are required for catalytic activity.

Generally, class I, III and IV PhaCs can only produce SCL-PHA while class II PhaCs can only produce MCL-PHA (Sudesh, 2012). However, there are always exceptions. For examples, PhaCs from *A. caviae*, *Chromobacterium* sp. strain USM2, *R. aethirovorans* I24 and mangrove metagenome ($PhaC_{BP\text{-}M\text{-}CPF4}$) have strong similarity to class I synthases, but they are able to catalyze the copolymerization of P(3HB-*co*-3HHx) (Doi *et al.*, 1995; Bhubalan *et al.*, 2010; Budde *et al.*, 2011; Foong *et al.*, 2018). Besides, PhaC1 and PhaC2 from *Pseudomonas* sp. 61-3 also have strong similarity to other class II synthases but are able to catalyze the mixed copolymerization of SCL 3HB with other MCL monomers (Matsusaki *et al.*, 1998).

Table 2.2: Classes of PhaCs based on substrate specificity and subunit composition.

Class	Subunits	Substrate[a]
I	 ~ 60 – 73 kDa	SCL-monomer: 3HA-CoA (C3 – C5), 4HA-CoA, 5HA-CoA
II	 ~ 60 – 65 kDa	MCL-monomer: 3HA-CoA (≥ C5)
III	 ~ 40 kDa ~ 40 kDa	SCL-monomer: 3HA-CoA, 4HA-CoA, 5HA-CoA MCL-monomer: 3HA-CoA (C6 – C8)
IV	 ~ 40 kDa ~ 22 kDa	SCL-monomer: 3HA-CoA

[a]Abbreviations: SCL, short-chain-length; MCL, medium-chain-length; 3HA, 3-hydroxyalkanoate; 4HA, 4-hydroxyalkanoate, 5HA, 5-hydroxyalkanoate. C3, monomer with 3 carbons such as 3-hydroxypropionate; C5, monomer with 5 carbons such as 3-hydroxyvalerate; C6, monomer with 6 carbons such as 3-hydroxyhexanoate; C8, monomer with 8 carbons such as 3-octanoate. Adapted from Rehm (2003).

2.6 Types of PHA

2.6.1 P(3HB)

P(3HB) is the best characterized biopolymer as extensive researches had been conducted revolving around it, in contrast to the other PHAs. P(3HB) is a polyester with an ester group composed of four carbon atoms with a methyl group as the side chain group at the *β*-position. The chemical structure of this polymer is illustrated in Figure 2.3. The crystallinity of this polymer was reported to be in the range of 55 to 80 % (Holmes, 1988). The melting temperature of this polymer is close to 180 °C and it's glass transition temperature is close to 4 °C. The tensile strength, Young's Modulus, and elongation at break of P(3HB) homopolymer were found to be 43 MPa, 3.5 GPa, and 5 %, respectively (Sudesh *et al.*, 2000). The Young's Modulus and tensile strength of P(3HB) are similar to PP but PP has 80 times higher value of elongation at break (400 %) than P(3HB). Hence, P(3HB) is more brittle and harder than PP. The formation of large crystalline domains in the form of spherulites causes the P(3HB) films to be brittle. It was reported that P(3HB) can be produced with weight average molecular weight (M_w) up to 1.1×10^7 Da by recombinant *Escherichia coli* harboring *C. necator*'s *phaC1* (Kusaka *et al.*, 1998). This ultra-high-molecular-weight (UHMW) ($3 \times 10^6 – 1.1 \times 10^7$) P(3HB) has better mechanical properties compared to P(3HB) with low M_w. The tensile strength, Young's Modulus, and elongation at break of the UHMW P(3HB) were 62 MPa, 1.1 GPa, and 58 %, respectively.

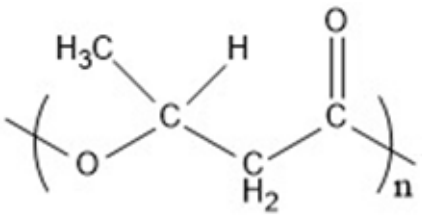


Figure 2.3: Chemical structure of P(3HB). 'n' represents number of repeating units.

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

P(3HB-*co*-3HV) is a copolymer composed of two types of monomers, which are 3HB and 3HV (an ester group composed of five carbon atoms with an ethyl group as the side chain group at the *β*-position). The chemical structure of this copolymer is illustrated in Figure 2.4. Imperial Chemical Industries (ICI) has commercialized P(3HB-*co*-3HV) under the trade name Biopol™ since 1980s. Glucose and propionic acid could be used for the production of P(3HB-*co*-3HV) random copolymers (Holmes, 1988). Co-crystallization of 3HV and 3HB monomer units in either of the polymer crystal lattices due to the isodimorphism of both these units causes the degree of crystallinity of P(3HB-*co*-3HV) to remain almost similar to P(3HB) (50 to 70 %) (Doi and Steinbüchel, 2001). Monomer composition of 3HV strongly affects the mechanical properties of P(3HB-*co*-3HV) copolymer. When 3HV content was increased from 0 to 25 mol% in solution cast films of P(3HB-*co*-3HV), the value of tensile strength and Young's Modulus decreased. These results indicate that increase in 3HV monomer composition will cause an increase in the flexibility of P(3HB-*co*-3HV) films. For P(3HB-*co*-3HV) with 28 mol% of 3HV monomer composition, the elongation at break reached 700 % and the toughness of the film was significantly increased. Furthermore, increase in 3HV content from 0 to 25 mol% lowers the melting temperature (T_m) but does not affect the thermal degradation temperature. This provides more room for thermal processing (Sudesh and Abe, 2010).

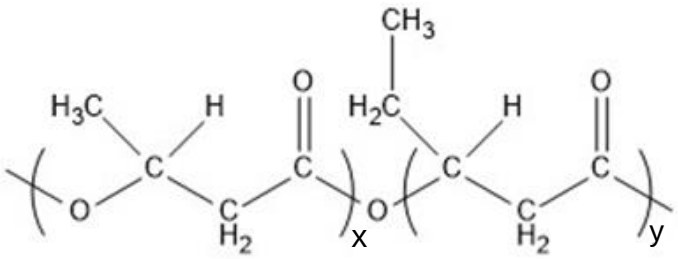


Figure 2.4: Chemical structure of P(3HB-*co*-3HV). 'x' and 'y' represent repeating unit of each monomer.

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

P(3HB-*co*-3HHx) is a copolymer composed of two types of monomers, which are 3HB and 3HHx (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 this copolymer is illustrated in Figure 2.5. 3HHx monomer has a longer alkyl side chain, so 3HB and 3HHx monomer units are not able to fit into the crystalline lattices of each other (Doi *et al.*, 1995). This avoids isodimorphism between 3HB and 3HHx monomer units. The crystallinity of P(3HB-*co*-3HHx) decreased (60 to 18 %) as the 3HHx monomer composition was increased (0 to 25 mol%). Besides, increase in the 3HHx content of the solution-cast films of P(3HB-*co*-3HHx) from 0 to 17 mol% will increase the elongation at break of the copolymer from 6 to 850 % while decreasing the tensile strength of the copolymer from 43 to 20 MPa. Thus, increasing 3HHx content in P(3HB-*co*-3HHx) copolymer causes the copolymer to become softer and more flexible (Asrar *et al.*, 2002). P(3HB-*co*-3HHx) that is composed of small amounts of 3HHx monomer fraction (5 mol%) has a T_m less than 155 °C and the T_m can be further reduced to 52 °C when the 3HHx monomer fraction is increased to 25 mol% (Doi *et al.*, 1995; Loo *et al.*, 2005). Hence, P(3HB-*co*-3HHx) which possesses better mechanical property and processability is a suitable candidate for blending in order to enhance the ductility of brittle and hard polyesters (Freier, 2006).

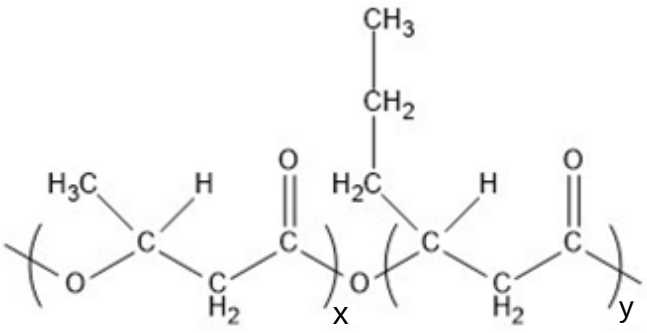


Figure 2.5: Chemical structure of P(3HB-*co*-3HHx). ‘x’ and ‘y’ represent repeating unit of each monomer.

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

P(3HB-*co*-4HB) is a copolymer composed of two types of monomers, which are 3HB and 4HB (a linear ester group composed of four carbon atoms). The chemical structure of this copolymer is illustrated in Figure 2.6. The crystallinities of P(3HB-*co*-4HB) were reported to be in the range of 15 to 60 % (Saito and Doi, 1994). When 4HB was incorporated into P(3HB), the resulting copolymer exhibits elastomeric property. The elongation at break values of P(3HB-*co*-4HB) copolymers showed an increasing trend (from five to 1320 %) from 0 to 82 mol% of 4HB monomer composition, but a declining trend (from 1320 to 1000 %) when the 4HB monomer composition was further increased from 82 to 100 mol%. However, the tensile strength of these copolymers showed a declining trend (from 43 to 17 MPa) from 0 to 64 mol% of 4HB monomer composition but increasing trend (from 17 to 104 MPa) when the 4HB monomer composition was further increased from 64 to 100 mol%. The T_m of P(3HB-*co*-4HB) copolymers is lower compared to P(3HB) homopolymer. The T_m showed a declining trend from 178 to 50 °C when the 4HB monomer composition was increased from zero to 100 mol%. Besides, the glass transition temperature (T_g) also exhibited a similar declining trend from 4 to – 48 °C when the 4HB monomer composition was increased. P(3HB-*co*-4HB) is lipase-degradable and hence, it is biocompatible and bioabsorbable (Jaeger *et al.*, 1995; Martin and Williams, 2003). These unique features made it applicable for various medical applications.

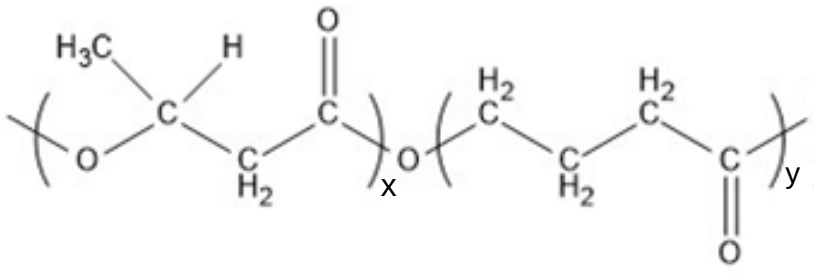


Figure 2.6: Chemical structure of P(3HB-*co*-4HB). 'x' and 'y' represent repeating unit of each monomer.

2.6.5 PHA with unusual monomers

There are numerous types of PHA with different functional groups in the side chain such as carboxyl, hydroxyl, phenoxy, epoxy, halogens, nitrophenoxy, thiophenoxy, cyanophenoxy, and methylester groups. These types of PHA were synthesized by bacteria possessing different metabolic pathways (Kessler *et al.*, 2001). For example, *Pseudomonas oleovorans* was reported to be able to synthesize various types of PHA with unusual monomers (Kim *et al.*, 1991). *P. oleovorans* is able to produce PHA polymer containing 3-hydroxy-5-phenylvalerate when supplemented with mixtures of 5-phenylvaleric acid with either n-octanoic acid or n-nonanoic acid as the carbon source. Besides, *P. oleovorans* is able to produce PHA polymer containing 9-cyano-3-hydroxynonanoate and 7-cyano-3-hydroxyheptanoate when supplemented with mixtures of 11-cyanoundecanoic acid and n-nonanoic acid as the carbon feedstock (Lenz *et al.*, 1992). Furthermore, *P. oleovorans* was also reported to be able to produce PHA polymer containing halogenated functional group, 3-hydroxy-8-chlorooctanoate (Doi and Abe, 1990). This polymer was synthesized by *P. oleovorans* when octane and 1-chlorooctane were supplemented as the carbon feedstock.

2.7 Application of PHA

Since PHA has similar properties with petrochemical-based synthetic plastic and is biodegradable, biocompatible and sustainable, it has been studied as a potential candidate to replace conventional plastics (Anderson and Dawes, 1990). Since 1982, PHA has been commercialized by many companies in several countries such as Georgia (Danimer Scientific), United States (Yield10 Bioscience), Germany (BioMatera), and China (TianAn Biologic Materials, Tianjin GreenBio Materials)

(Maida, 2017). Due to the unique features of PHAs, they can be used for many applications such as disposable items, coating and packaging materials, bio-implants, drug carriers and encapsulated fertilizer, pesticides or herbicides (Martin and Williams, 2003; Voinova *et al.*, 2009; Grillo *et al.*, 2011). Packaging and disposable items such as utensils, bottles, cups, razors, mulch films, diapers and feminine hygiene products are the most common applications of PHA. Besides, study by Sudesh and his colleague (2007) has shown that PHA has the potential to be used as facial oil-blotting film in cosmetic industry. Moreover, the biocompatibility and biodegradability of PHA make it suitable for osteosynthetic materials, bone plates, cardiovascular patches, wound dressings, surgical sutures and tissue engineering scaffolds (Martin and Williams, 2003). For example, P(3HB-*co*-4HB) is suitable to be used for suture and implants because of its lipase-degradable properties (Jaeger *et al.*, 1995). Hence, this biodegradable plastic can be implanted and does not need to be removed. PHA could also be used as drug carrier by adjusting the ratio of drug and PHA polymer to control the release formulations (Shrivastav *et al.*, 2013). In the agricultural field, PHA can be used to encapsulate fertilizers, pesticides or herbicides for controlled release (Voinova *et al.*, 2009; Grillo *et al.*, 2011). This controlled release system provides sufficient pesticides and herbicides that in turn reduces the negative impact on plants since these chemicals can be harmful to the plants in high dosage.

2.8 PhaC for SCL-MCL-PHA production

2.8.1 PhaC from *Aeromonas caviae*

P(3HB-*co*-3HHx) was first found to be synthesized in *A. caviae* (Doi *et al.*, 1995). *A. caviae* accumulated P(3HB-*co*-3HHx) when grown on plant oils or fatty acids. Studies on *A. caviae* have revealed that the PhaC of *A. caviae* ($PhaC_{Ac}$) has substrate specificity

toward 3HB-CoA and (*R*)-3-hydroxyhexanoyl-CoA (3HHx-CoA). Advances in genetic engineering have allowed the construction of recombinant bacteria capable of synthesizing P(3HB-*co*-3HHx) by expressing this heterologous gene (Fukui and Doi, 1997). This study has shown that *C. necator* harboring *phaC*$_{Ac}$ could accumulate more P(3HB-*co*-3HHx). PhaC$_{Ac}$ NSDG mutant [PhaC$_{Ac}$ with double mutation at the 149th and 171st amino acid, which is a substitution of asparagine 149 by serine (N149S) and aspartic acid 171 by glycine (D171G)] is able to accumulate 82 wt% of P(3HB-*co*-3HHx) DCW with up to 12.2 mol% 3HHx content when supplemented with octanoate as the carbon source (Tsuge *et al.*, 2007). The M_w of this copolymer almost reached the UHMW, which is 2.55×10^6 Da. This strain was also able to accumulate 82 wt% of P(3HB-*co*-3HHx) from soybean oil. However, the 3HHx content was only up to 3.5 mol% and the M_w was only 4.7×10^5 Da.

2.8.2 PhaC from *Chromobacterium* sp. strain USM2

Chromobacterium sp. strain USM2 was isolated in an island named Langkawi in Kedah,Malaysia and was found to be able to accumulate PHA (Yong, 2009). The PhaC of *Chromobacterium* sp. strain USM2 (PhaC$_{Cs}$) was found to favour 3HV substrate monomer. Constructed transformant, PHB^{-}4 harboring *phaC*$_{Cs}$ was reported to be able to synthesize P(3HB-*co*-3HHx) from plant oil (Bhubalan *et al.*, 2010). The transformant was able to accumulate 63 wt% of P(3HB-*co*-3HHx) DCW with 4 mol% 3HHx content from CPKO. Furthermore, Strep2-tagged PhaC$_{Cs}$ showed an enzymatic activity of 238 U/mg toward (*R*)-3HB-CoA, which is almost five-fold higher than the activity of PhaC from *C. necator* (PhaC$_{Cn}$) (Bhubalan *et al.*, 2011). This *in vitro* activity result indicates that this highly active PhaC possesses superior polymerizing ability. To date, PhaC$_{Cs}$ is the most active PhaC among the purified PhaC ever reported.

2.8.3 PhaC from *Rhodococcus aetherivorans* I24

R. aetherivorans I24 contained two PhaC genes, *phaC1*$_{Ra}$ and *phaC2*$_{Ra}$ (Budde *et al.*, 2011). The study has shown that these genes were functional and were able to polymerize PHA. The constructed recombinant strains, Re2000 and Re2001 (harboring *phaC1*$_{Ra}$ and *phaC2*$_{Ra}$, respectively), were reported to be able to synthesize P(3HB-*co*-3HHx) from both hexanoate and octanoate. Many recombinant strains and transformants were constructed to study the ability of *phaC1*$_{Ra}$ and *phaC2*$_{Ra}$. Studies have shown that PhaC2$_{Ra}$ can produce P(3HB-*co*-3HHx) with a broader range of 3HHx monomer composition (1 – 70 mol%) from plant oil compared to PhaC1$_{Ra}$. However, the molecular weight of the copolymer produced by PhaC2$_{Ra}$ was lower than 4×10^5 Da. High cell density fermentation of Re2058/pCB113, a strain harboring *phaC2*$_{Ra}$, achieved 102.9 g/L P(3HB-*co*-3HHx) with 19 mol% 3HHx monomer composition from plant oil (Riedel *et al.*, 2012).

2.8.4 BP-M-CPF4 PhaC

PhaC$_{BP\text{-}M\text{-}CPF4}$ is a PhaC isolated from the mangrove metagenome in a mangrove forest at Balik Pulau, Penang, Malaysia (Foong *et al.*, 2018). PhaC$_{BP\text{-}M\text{-}CPF4}$ showed extremely wide substrate specificity. It was able to incorporate six types of PHA monomers namely, 3HB, 3HHx, 3HV, 3H4MV, 4HB and 5HV, with appropriate precursors. The study also shown that constructed transformant, PHB^{-}4 harboring *phaC*$_{BP\text{-}M\text{-}CPF4}$ was able to accumulate 62 wt% of P(3HB-*co*-3HHx) DCW with 7 mol% 3HHx content from CPKO. When fructose was supplemented together with sodium hexanoate, the strain can synthesize P(3HB-*co*-3HHx) with 3HHx content up to 18 mol% but yield only 44 wt% of DCW (Foong *et al.*, 2018).

2.8.5 PhaC from *Pseudomonas* sp. 61-3

Study by Matsusaki *et al.* (1998) has confirmed that *Pseudomonas* sp. 61-3 contained two PhaC genes, $phaC1_{Ps}$ and $phaC2_{Ps}$. The PhaCs of *Pseudomonas* sp. 61-3 belong to class II PhaCs, which differ from class I PhaCs as they prefer MCL-PHA monomers. Constructed transformants, PHB^{-}4 harboring $phaC1_{Ps}$ and $phaC2_{Ps}$, respectively, have been reported to be able to synthesize P(3HB) homopolymer and [P(3HB-*co*-3HA)] random copolymer consisting of 3HA units of C4 to C12 when supplemented with sugars and alkanoic acids (Matsusaki *et al.*, 1998). This has shown that $PhaC1_{Ps}$ and $PhaC2_{Ps}$ exhibited similar substrate specificities and are thus, able to incorporate a wide range of 3HA units into PHA.

2.9 Metabolic engineering of PHA producing strains

SCL-MCL-PHA shows better thermal and physical properties than SCL-PHA and MCL-PHA (Asrar *et al.*, 2002). A lot of efforts have been made to produce SCL-MCL-PHA with desired properties such as through metabolic engineering by genetic manipulation of PHA-production-related genes to optimize the bacterial production system (Fukui *et al.*, 1998; Tsuge, 2002; Budde *et al.*, 2010; Budde *et al.*, 2011; Kawashima *et al.*, 2015). Through metabolic engineering, metabolic process of the production strains can be modified in order to improve the PHA yield as well as to alter the PHA monomer composition for production of tailor-made PHA. Since the amount of the substrates (e.g. 3HV-CoA, 3HHx-CoA and 4HB-CoA) that can be synthesized from common intermediates of the central metabolism are limited or even not synthesizable in naturally occurring PHA-producing microorganisms, modification or introduction of new metabolic pathways into the PHA-producing microorganisms can help in supplying or enhancing these monomeric substrates.