FABRICATION AND CHARACTERIZATION OF POLY(3-HYDROXYBUTYRATE-*co*-4-HYDROXYBUTYRATE/COLLAGEN) BLENDED MICROSPHERES FOR TISSUE ENGINEERING APPLICATION

NOOR AIDDA BINTI OMAR

UNIVERSITI SAINS MALAYSIA

2023

FABRICATION AND CHARACTERIZATION OF POLY(3-HYDROXYBUTYRATE-*co*-4-HYDROXYBUTYRATE/COLLAGEN) BLENDED MICROSPHERES FOR TISSUE ENGINEERING APPLICATION

by

NOOR AIDDA BINTI OMAR

Thesis submitted in fulfillment of the requirements
for the Degree of
Master of Science

June 2023

ACKNOWLEDGEMENT

First and foremost, I offer my gratitude to Allah S.W.T for the guidance and good health that had been given to complete this project. I would like to express my appreciation to my supervisor, Prof .Dr. Amirul Al-Ashraf Abdullah for his guidance and support throughout this project. His advice, comments and scientific views are very much helpful and cherished.I am most grateful to my family for their encouragement and moral support. I also would like to take this opportunity to give my appreciation to all my lab members especially Aiman and Jeremy for their continuous help and support along this project. Special thanks to Julia, Priya , Umie, Aliaa, Dr Kai Hee, Azuraini ,my labmates and friends for their helped and support .My grateful thanks also go to some technical staff in School Biological Science, School of Chemical Sciences, Centre for Chemical and Biology USM and Pusat Pengajian Arkeologi Global (PPAG) for allowing me to use their facilities.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS AND ABBREVIATIONS

°C	Degree celcius
g	Gravity force
mol%	Mol percentage
mL	Mililiter
mg	Miligram
µg	Microgram
v/v	Volume per volume
rpm	Revolution per minute
wt%	Weight percentage
psi	Pound per Square inch
CME	Caprylic methyl ester
CoA	Coenzyme A
ECM	Extracellular matrix
GA	Gluteraldehyde
GC	Gas chromatography
kDA	Kilo Dalton
LCL	Long chain length
MCL	Medium chain length
MSM	Mineral salt medium
NR	Nutrient rich
PBS	Phosphate buffer saline

PCL	Polycaprolactone
PHA	Polyhydroxyalkanoate
PLA	Poly(lactic acid)
PLGA	Poly(lactic-glycolic-acid)
PVA	Polyvinyl alcohol
P(3HB)	Poly(3-hydroxybutyrate)
P(3HB-*co*-4HB)	Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate)
P(3HHX)	Poly(3-hydroxyhexanoate)
P(3HO)	Poly(3-hydroxyoctanoate)
P(3HD)	Poly(3-hydroxydecanoate)
SCL	Short chain length
SEM	Scanning electron microscope
T*g*	Glass transition temperature
T*m*	Melting temperature
UV	Ultraviolet

FABRIKASI DAN PENCIRIAN MIKROSFERA CAMPURAN POLI(3-HIDROKSIBUTIRAT-*ko*-4-HIDROSIBUTIRAT)/KOLAGEN UNTUK KEGUNAAN DALAM KEJURUTERAAN TISU

ABSTRAK

Perancah adalah salah satu komponen utama dalam kejuruteraan tisu yang memberikan sokongan struktur kepada penapakan sel dan perkembangan tisu. Tetapi, oleh kerana kelemahan dalam pengangkutan nutrisi dan bahan buangan dalam perancah menyebabkan sel nekrosis berlaku terutamanya di bahagian tengah perancah yang bersaiz besar.Tambahan pula pemindahan perancah sering melibatkan pembedahan invasif yang mengambil masa yang panjang untuk pulih dan menyukarkan peasakit. Lalu dengan menggunakan perancah kecil yang kebolehsuntikan dan mampu memberikan ruang untuk pergerakan oksigen dan bahan buangan seperti mikrosfera boleh mengurangkan nekrosis sel.Tambahan pula, perancah yang kebolehsuntikan juga dapat memudahkan prosedur pembedahan lalu mempercepatkan masa penyembuhan dan memberikan keselesaan kepada pesakit.

Dalam kajian ini, mikrosfera dihasilkan daripada P(3HB-*ko*-4HB) kopolimer dengan dua komposisi 4HB monomer berbeza iaitu 21 mol % dan 91mol % dengan menggunakan kaedah penyejatan pelarut-pengemulsian. Memandangkan P(3HB-*ko*-4HB) kopolimer adalah hidrofobik, kolagen dicampurkan dengan P(3HB-*ko*-4HB) kopolimer untuk menambah hidrofilik dan menyediakan permukaan untuk sel melekat. Maka dua jenis, mikrosfera campuran kolagen dihasilkan dengan menggunakan kepekatan kolagen dan polimer yang berbeza. Mikrosfera dengan perangkapan kolagen tertinggi kemudian dicirikan untuk menentukan saiz, morpologi permukaan, kapasiti penyerapan air dan pengekalan kolagen dalam mikrosfera lalu

dibandingkan dengan mikrosfera kosong.Diantara mikrosfera campuran kolagen, P(3HB-*ko*-4HB) pada kepekatan 4% menunjukkan perangkapan kolagen tertinggi. Saiz mikrosfera didapati meningkat daripada 89 µm ke 161 µm dengan meningkatnya kepekatan kolagen untuk P(3HB-*ko*-21mol%4HB) manakala untuk P(3HB-*ko*-91mol%4HB), saiznya meningkat daripada 49 µm ke 140 µm. Imej mikroskop pengimbas elekron (SEM) pula menunjukkan penambahan kolagen menjadikan permukaan mikrosfera campuran kolagen untuk P(3HB-*ko*-21 mol%4HB) lebih berongga berbanding P(3HB-*ko*-91 mol%4HB) yang hanya ada beberapa lubang. Sementara itu, mikrosfera kosong mempunyai permukaan yang licin tanpa rongga. Peningkatan kepekatan kolagen juga meningkatkan kapasiti penyerapan air oleh mikrosfera dalam 90 % ke 450 % untuk P(3HB-*ko*-21 mol%4HB) dan antara 115% ke 140 % untuk P(3HB-*ko*-91 mol%4HB). P(3HB-*ko*-21 mol%4HB) mikrosfera yang kehilangan banyak kolagen daripada 80 % ke 30% semasa ujian pengekalan kolagen di silang kait meggunakan gluteraldehid dan didapati silang kait dengan gluteraldehid mampu mengekalkan lebih banyak kolagen antara 70 % ke 85%. Mikrosfera dengan campuran 4% P(3HB-*ko*-21 mol%4HB)/ 1% kolagen yang menunjukkan perangkapan kolagen dan pengekalan kolagen yang tinggi dinilaikan dengan ujian sitotoksik menggunakan L929 fibroblast. Sel didapati hidup dalam semua sampel diuji dan kadar pertumbuhan sel lebih ketara pada sampel mikrosfera campuran kolagen dengan 5.9 x 10^5 ke 6.18 x10^5 berbanding dengan mikrosfera kosong dengan jumlah sel daripada 4.3 x10^5 ke 4.4 x10^5. Peningkatan pertumbuhan sel dalam mikrosfera campuran kolagen yang di silang kait dengan gluteraldehid menandakan bahawa mikrosfera tidak toksik kepada sel dan mempunyai potensi untuk kegunaan dalam kejuruteraan tisu.

FABRICATION AND CHARACTERIZATION OF POLY(3-HYDROXYBUTYRATE-*co*-4-HYDROXYBUTYRATE) /COLLAGEN BLENDED MICROSPHERES FOR TISSUE ENGINEERING APPLICATION

ABSTRACT

The scaffold is one of the main components in tissue engineering that provides structural support for cell attachment and tissue development. However, due to poor transportation of nutrients and waste products in the scaffold, cell necrosis often occurs in the center of large sized scaffolds. Additionally, transplanting scaffolds usually involves invasive surgery which can incur a prolonged healing period and is inconvenient to the patient. Hence, by using small injectable scaffold which can provide the space for movement of oxygen and waste product such as microspheres can reduce the cell necrosis. Furthermore, the injectablity scaffold also can facilitate the surgical procedure thus speed up the time recovery and convenient to the patient. In this study, microspheres were fabricated from the P(3HB-*co*-4HB) copolymer with two different 4HB monomer compositions of 21 mol % and 91 mol% using the emulsification of solvent evaporation method. Since P(3HB-*co*-4HB) is hydrophobic, the collagen was blended with the P(3HB-*co*-4HB) copolymer to add hydrophilicity and provide the surface for cell attachment.Thus, two types of collagen-blended microspheres were fabricated by varying the concentrations of polymer and collagens used .The collagen-blend microspheres with the highest collagen entrapment efficiency were then characterized to determine their size, surface morphology, water absorption capacity, and retention of collagen and their properties were compared with blank microspheres. Among the collagen-blended microspheres, P(3HB-*co*-4HB) at

4% concentration showed the highest entrapment efficiency. The size of the microspheres was found to increase from 89 µm to 161 µm with the increase of collagen concentration for P(3HB-*co*-21mol%4HB) while for P(3HB-*co*-91mol%4HB) the size increase from 49 µm to 140 µm. Scanning Electron Microscopy images showed that the addition of collagen created a more porous surface morphology of microspheres for P(3HB-*co*-21mol%4HB) compared to P(3HB-*co*-91 mol%4HB). While the blank microspheres have a smooth surface without pores. Increasing collagen concentration also increased water absorption capacity for microspheres around 91% to 450 % for P(3HB-*co*-21mol%4HB) and from 115% to 140 % for P(3HB-*co*-91mol%4HB). P(3HB-*co*-21mol%4HB), which was found to have lost a lot of collagen from 80 % to 33% during the collagen retention test, was cross linked with gluteraldehyde and it was found that crosslinking the collagen-blend microspheres with gluteraldehyde retained more collagen from 70 % to 85%. The blend of 4 % P(3HB-*co*-21 mol%4HB)/1 % collagen microspheres, that showed the highest entrapment efficiency of collagen and retention of collagen, were evaluated with a cytotoxicity test using L929 fibroblast cells. The cells were found to be viable in all sample and the increase of proliferation rate was prominent in the collagen blend microspheres sample from 5.9 x 10^5 to 6.18 x10^5 compared to blank microspheres which is from 4.3 x10^5 to 4.4 x10^5. The increasing number of cell in collagen blend cross-linked with gluteraldehyde microspheres indicating that the microspheres are not toxic to the cell and have potential in tissue engineering applications.

CHAPTER 1 INTRODUCTION

Advances in modern technology have contributed to improvements in the medical sector to provide treatments that are safe, fast, efficient, and easy to administer. That being said, tissue engineering, which aims to repair and restore the function of lost or damaged tissue, is continually being developed to meet the increasing demand for human tissue and organ substitutes (Rodriguez-Vazquez et al., 2015). Using modern technology and techniques, the usage and properties of scaffolds in tissue engineering have improved drastically. However, despite best efforts, it has been reported that cell necrosis frequently occurs due to the insufficient supply of nutrients or oxygen, as well as the build-up of metabolic waste, especially in the center of larger scaffolds (Choi et al., 2010). Besides that, the implementation of a large preformed scaffold often involves an invasive surgical procedure (Wei et al., 2018). To overcome this problem, it is necessary to come up with an improvised scaffold that is easier to administer and decreases the occurrence of cell necrosis, and one such candidate is the microsphere.

Microspheres are defined as spherical particles with a size range from 1 to 1000 µm (Singh et al., 2011). In tissue engineering, they can provide appropriate spaces for attachment, migration, proliferation, and differentiation of cells to restore damaged tissues (Wei et al., 2018). In addition to that, their size and surface can also be customized to be porous, hollow, or blended with other materials to suit their intended function. Their spherical shape is also particularly useful as it allows the microspheres to be easily injected into the body without obstructions compared to other geometrical shapes (Saralized et al., 2010). This also allows them to be site–targeted delivery vessels which can provide minimally invasive surgical implantation thus decreasing operation time, improving the patient's comfort and healing process (Kang et al.,

2009). To create microspheres which can support cell growth, they have to be designed appropriately using a material with suitable properties, and this is where polyhydroxyalkanoates come into the picture.

Polyhydroxyalkanoates (PHAs) are biopolymers that are naturally synthesized intracellularly by microorganisms when certain nutrients become limited but with the carbon source is in excess (Liu et al., 2016). These biopolymers possess a large range of chemical and physical properties that make them biodegradable and biocompatible with living systems (Ali & Jamil, 2016). Over the years, PHAs have been developed as useful materials in the medical field for different applications due to their exceptional properties. PHAs' bioactivity and their lower acidity have presented them with minimal risk of cell necrosis compared to other biopolymers such as poly-glycolic acid (PGA) and poly-lactic acid (PLA) (Martin & William, 2003). The versatility of PHAs in terms of their ideal physical and chemical properties, non-toxic degradation products, biocompatibility, desired surface area alterations, and cellular growth support has encouraged their usage in tissue engineering (Elmowafy et al., 2019). Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate), P (3HB-*co*-4HB), especially is known to possess a high rate of *in vivo* degradation and very good biocompability compared to other types of PHA (Saito et al., 1996). Moreover, the biodegradation rate and physical characteristics of P(3HB-*co*-4HB) can be manipulated based on its 4HB composition which provides the polymer with brittle and elastomeric properties (Vigneswari et al., 2011). Despite having many redeeming qualities, P(3HB-*co*-4HB), like other PHAs, lacks cell adhesion sites due to its innate hydrophobicity (Winnacker & Rieger, 2016). Hence, blending it with other hydrophilic polymers such as collagen could overcome this problem.

Collagens are a type of protein that can be found abundantly in the body. Collagen is biodegradable and biocompatible and also possesses low antigenicity and negligible cytotoxicity. This makes them an ideal blending material for P(3HB-*co*-4HB) (Rezaei & Mohd Ishak, 2014). Furthermore, blending P(3HB-*co*-4HB) with collagen not only can enhance the hydrophilicity of the produced microspheres but also its natural ability to interact with cells (Vigneswari et al., 2015).

Previous studies have proved that microspheres have been successfully produced using PHA (Wei et al., 2018). However, no studies have been reported for the preparation of microspheres from P(3HB-*co*-4HB) blended with collagen. Therefore, in this study, the fabrication and characterization of P(3HB-*co*-4HB) microspheres blended with collagen will be conducted, and these fabricated P(3HB-*co*-4HB) microspheres may potentially serve as a valuable scaffold in tissue engineering applications.

The objectives of this study are:

1) To optimize the fabrication of P(3HB-*co*-4HB) and P(3HB-*co*-4HB)/collagen microspheres.
2) To characterize the fabricated P(3HB-*co*-4HB) and P(3HB-*co*-4HB)/collagen microspheres.
3) To evaluate the cytotoxicity of fabricated microspheres using L929 fibroblast cells.

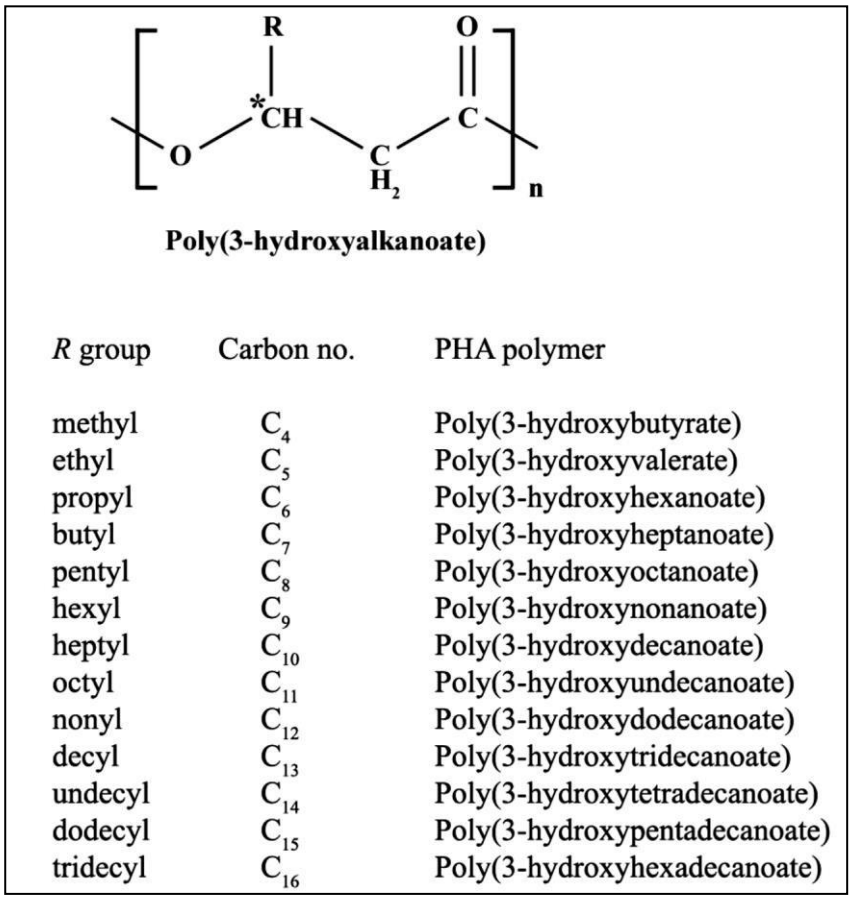
CHAPTER 2 LITERATURE REVIEW

2.1 Polyhydroxyalkanoates (PHAs)

Polyhydroxyalkanoates (PHAs) are biopolymers synthesised by microbes under the stress condition when they were forced to grow in restricted conditions of some essential nutrients such as nitrogen, magnesium, oxygen, phosphorus, or sulfur but with abundant resources of carbon (Liu et al., 2015; Nigmatullin et al., 2015). PHA production is one of the bacteria strategies to cope with environmental stress such as starvation. Under the poor nutrient condition, the accumulated PHA was eventually broken down to serve as a carbon and energy source which promotes the long- term of bacterial survival (Kadouri et al., 2005; Tan et al., 2014). In bacteria, PHA can be found in the cytoplasm of the cell in the form of inclusion bodies (lipid granules) with a diameter of 0.2 microns to 0.5 microns (Nigmatullin et al., 2015). These granules have high refractivity and can be viewed using the phase contrast and electron microscope (Sudesh et al., 2000).

At present, PHA-producing microorganisms have been discovered in nature or engineered ecosystems such as in soil, activated sludge processes, wastewater from the full-scale treatment plant, and salt marsh (Liu et al., 2015). To date, there are more than 90 genera of PHA-producing microorganisms have been discovered worldwide from the eubacteria and archaebacteria domains (Zinn et al., 2001). This includes Gram-positive and Gram-negative bacteria such as *Bacillus* sp., *Corynebacterium* sp., *Nocardia* sp., *Rhodococcus* sp., *Azobacter* sp., *Burkholderia* sp., *Cupriavidus* sp., *Methylobacterium* sp., *Pseudomonas* sp., and recombinant *Escherichia coli* (Tan et al., 2014; Liu et al., 2016).

PHAs are biopolymers made up of a repeated chain of hydroxyalkanoates (HAs) monomers where the ester bond was formed between the carboxylic group of a monomer and the hydroxyl group of a neighboring one (Philip et al., 2007; Mozejko-Ciesialska & Kiewiz, 2016) Figure 2.1 show the general structure of PHA, where *R* is an alkyl group and n is a number of polymerization. The PHA compound was named based on the number of monomers in the functional alkyl *R* group (Tan et al., 2014). The alkyl group is a monomer that includes saturated or unsaturated aliphatic, branched, aromatic, halogenated, or epoxidized monomers (Mozejko-Ciesielska & Kiewiz, 2016). There are more than 150 different PHA monomers that have been found that make PHAs the largest group of natural polyesters (Kim et al., 2007; Li et al., 2016). Homopolymer, copolymer, heteropolymer, random polymer, and even block polymer is the variety of PHAs form which can be produced influenced by the type of PHA synthase owned by the bacteria, substrate fed, and even modes of fermentation (Nigmatullin et al., 2015; Sharma et al., 2017).



R group	Carbon no.	PHA polymer
methyl	C_4	Poly(3-hydroxybutyrate)
ethyl	C_5	Poly(3-hydroxyvalerate)
propyl	C_6	Poly(3-hydroxyhexanoate)
butyl	C_7	Poly(3-hydroxyheptanoate)
pentyl	C_8	Poly(3-hydroxyoctanoate)
hexyl	C_9	Poly(3-hydroxynonanoate)
heptyl	C_{10}	Poly(3-hydroxydecanoate)
octyl	C_{11}	Poly(3-hydroxyundecanoate)
nonyl	C_{12}	Poly(3-hydroxydodecanoate)
decyl	C_{13}	Poly(3-hydroxytridecanoate)
undecyl	C_{14}	Poly(3-hydroxytetradecanoate)
dodecyl	C_{15}	Poly(3-hydroxypentadecanoate)
tridecyl	C_{16}	Poly(3-hydroxyhexadecanoate)

Figure 2.1:General structure of PHA and monomer. The asterisk denotes the chiral center of the PHA-building block (Tan et al., 2014).

Besides that, PHAs display plastic-like feature that is similar to thermal and mechanical properties to those of petroleum-based plastics such as polypropylene (Raza et al., 2018). They are resistant to water, impermeable to gases and act as piezoelectric materials (Bugnicourt et al., 2014). Unlike conventional plastic, PHAs are recognized for their biodegradable and biocompatibility properties. In a natural environment, PHAs are degraded by various microorganisms into harmless by-products producing water and carbon dioxide in aerobic conditions while water and methane was released in anaerobic conditions (Volova et al., 2010). While *in vitro* and *in vivo* studies, many have shown that PHAs capable to support the growth of various mammalian cells efficiently, (Sodian et al., 2000; Wei et al., 2009; Liu et al., 2010; Ribeiro-Samy et al., 2013) and are well accepted by the organism after implantation

(Wu et al., 2007; Li et al., 2015) made PHAs are excellent candidate material for tissue engineering.

2.2 Properties and Classification of Polyhydroxyalkanoates (PHAs)

After the discovery of the very first PHA, 3-hydroxybutyrate, (3HB) unit in 1926, more than 150 hydroxyalkanoates (HA) units have been found (Figure 2.1)(Tan et al., 2014; Li et al., 2016). Identification of more HA units has increased the research and commercial interest in this polymer. This is because the diversity of the monomer structure and composition of PHAs provides the polymer with a wide range of thermal and mechanical properties. Thermal properties such as melting temperature, T*m*; glass transition temperature, T*g;* and degradation temperature, T*d,* is important as they provide information regarding the condition of temperature at which the polymer can be processed and applied (Tan et al., 2014).

Meanwhile, mechanical properties such as elongation at break, tensile strength, and Young's modulus indicate the elasticity of the polymer. Elongation at break measures the degree of material to stretch before it breaks and is expressed as a percentage of the material's original length whereas tensile strength measures the amount of force needed to pull a material until it breaks, expressed in MPa unit and Young modulus measure the stiffness of material expressed in MPa unit or sometimes in GPa unit (Tan et al., 2014). Nevertheless, the properties of PHAs rely on the bacterial host and the fermentation strategy used for their production (Mozejko-Ciesielska & Kiewiz, 2016).

PHAs can be divided into three groups consisting of short-chain-length-PHA (SCL-PHA), medium-chain-length-PHA (MCL-PHA), and long-chain-length-PHA (LCL-PHA) based on the number of carbon atom in the monomeric constituents.

However, the information about long-chain-length-PHA is still limited (Wang et al., 2016).

2.2.1 Short-chain-length-PHA (SCL-PHA)

SCL-PHAs consist of three to five number of carbon atoms in the monomer. SCL-PHAs are generally stiff, brittle, and highly crystalline materials that have methyl or ethyl groups as a side chain (Verlinden et al., 2007). Homopolymer Poly(3-hyroxybutyrate), P(3HB), is one of the SCL-PHA examples. It was the first PHA monomer discovered by a French scientist, Lemoigne in 1926 (Raza et al., 2018). Biologically, P(3HB) has a perfect isotactic structure composed only of *(R)*-3-hydroxybutyrate units (Sudesh et al., 2000). The isotactic structure has caused the formation of large spherulites during the crystallization process (Rigouin et al., 2019). This leads to a highly crystalline polymer (about 55 – 80 %) (Ciesielski et al., 2015). The mechanical properties of P (3HB) material such as tensile strength (43 MPa) and Young‘s modulus (3.5 GPa) is near to those of polypropylene. However, the elongation at break (5%) is much lower compared to polypropylene (400%). Thus, makes P(3HB) more brittle and stiffer materials compared to polypropylene (Sudesh et al.,2000; Mozejko-Ciesielska & Kiewiz, 2016).

Besides that, P(3HB) possesses a glass transition temperature (T_g) at 4 °C and a high melting temperature (T_m) from 173 °C to 180 °C which is close to its decomposition temperature (T_d) (185 °C). This narrow range of temperature between T_m *and* T_d makes P(3HB) experience thermal instability during processing and vulnerable to thermal degradation thus limiting its uses in the industrial field (Surendran et al., 2020). However, incorporating other monomers such as 3-hydroxyvalerate (3HV) or 4-hydroxybutyrate (4HB) to form copolymer can decrease

the crystallinity and melting temperature, T_m, of the polymer, hence producing a more elastic polymer and easier to utilize (Choi et al., 2020).

.

2.2.2 Medium-chain-length-PHA (MCL-PHA)

MCL-PHA consist of six to 14 number of carbon atom in the monomer. It was first found produced by *Pseudomonas putida* (formerly *Pseudomonas oleovorans*) (Rigouin et al., 2019). MCL-PHAs are structurally more diverse compared to SCL-PHAs. This is because the '*R*' group of MCL-PHAs can hold different types of side chains. Based on the side chain of MCL-PHAs, it can be classed into five groups consisting of 1) saturated aliphatic such as poly(3-hydroxyoctanoate), P(3HO), 2) unsaturated aliphatic containing carbon with a double or triple bond, 3) halogenated group such as bromine, 4) aromatic side chains such as nitrophenyl group and 5) branched monomer (Rai et al., 2011).

Generally, MCL-PHAs are semi-crystalline polymers having low crystallinity (about 25 %), low tensile strength (5 to 16.3 MPa), Young's modulus (1 to 103.1 MPa), and high elongation to break (88 to 350 %) (Liu et al., 2011; Ouyang et al., 2007; Ma et al., 2009). MCL-PHAs can exhibit flexible or even sticky properties based on the type of monomer and composition they possess. MCL-PHAs and its copolymer have low crystallinity due to the existence of large and irregular side chain groups that inhibit the polymer chain to form a regular structure and prevent crystallization (Van der Walle et al., 2001).

MCL-PHAs have a low melting temperature range from 38 °C to 80 °C and glass transition temperature below room temperature ranging from − 52 °C to − 25 °C (Liu et al., 2011; Rai et al., 2011). The low crystallinity and low glass transition temperature make MCL-PHA exhibit elastomeric properties. Nevertheless, MCL-

PHAs only behave as elastomers at a narrow range of temperatures corresponding to their low melting temperature, and at above or close to melting temperature the polymer becomes fully amorphous and too sticky hence making them difficult to utilize (Hazer & Steinbüchel, 2007). Typical examples of MCL-PHAs are poly(3-hydroxyhexanoate), P(3HHx), poly(3-hydroxyoctanoate), P(3HO), poly(3-hydroxydecanoate), P(3HD), poly(3-hydroxydodecanoate), P(3HDD) and their copolymers (Nigmatullin et al., 2015).

2.3 Biosynthesis of Polyhydroxyalkanoates (PHAs)

The biosynthesis of PHA is conducted by several enzymes such as PHA synthases (PhaC), PHA depolymerase (PhaZ), phasins (PhaP), and regulators (PhaR). Among the enzymes, the key enzyme for PHA synthesis is PhaC which catalyzes the polymerization of β-hydroxyalkanolyl-CoA monomers into poly-β-hydroxyalkanoate (Kadouri et al., 2005). PHA synthases can be divided into four classes that have known as Class I, Class II, Class III, and Class IV. These classes were differentiated based on the composition of subunit (either only PhaC or PhaC/PhaE or PhaC/PhaR) and substrate specificity (synthesize SCL-PHAs or MCL-PHAs) (Surendran et al., 2020). Class I, III, and IV PHA synthases polymerize SCL-PHAs using substrates containing three to five carbon monomers while Class II PHA synthase polymerizes MCL-PHAs with monomers consisting of six to 14 carbon as substrates. Class I and Class II are composed of one subunit, PhaC, meanwhile, Class III and IV are composed of two subunits, PhaC with PhaE, and PhaC with PhaR. Class I, II, III, and IV PHA synthases can be found in bacteria such as *Cupriavidus necator*, *Pseudomonas putida*, *Allochromatin vinosum* and *Bacillus megaterium* respectively (Jendrossek,2009; Mozejko-Ciesielska & Kiewiz,2016).

Microorganisms can synthesize PHA from different carbon sources such as organic acid, alcohol, animal and plant oils, carbohydrate as well as simple sugar such as sucrose (Jiang et al., 2016). This is because promiscuity of PHA synthase allows the wide range utilization of substrates for the synthesis of various polymers. Another characteristic of PHA synthase is its stereospecificity that only receives monomers with *(R)* configuration to form the PHA polymer (Choi et al., 2020). Therefore the substrates must be transformed into the *(R)* configuration form before synthesizing into PHA polymer.

Three types of PHA biosynthetic pathways can occur in the microorganism which are Pathway I, II and III (Sudesh et al., 2000). Pathway I usually used to indicate the synthesis of the most common PHA which is P(3HB) monomer, the SCL-PHA produced by *Cupriavidus necator* (previously known as *Ralstonia eutropha*) (Sudesh et al., 2000). In this pathway, two molecules of acetyl-CoA are condensed into acetoacetyl-CoA by the enzyme β-ketothiolase (PhaA). Then, the acetoacetyl-CoA is reduced to *(R)* - 3-hydroxybutyryl-CoA by the NADPH-dependent reductase enzyme (PhaB). Finally *(R)*- 3-hydroxybutyryl-CoA is esterified to the P(3HB) polymer by PHA synthase (PhaC) (Sudesh et al., 2000; Zinn et al., 2001; Sagong et al., 2018).

Meanwhile, pathways II and III both are involved in the biosynthesis of MCL-PHA. Pathway II catalyzes the intermediate products coming from fatty acid *β*-oxidation that is produced by related carbon sources such as alkanes, alkanals, or alkanoates (Philip et al., 2007). In this pathway, the intermediates from *β*-oxidation (enoyl-CoA and 3-Ketoacyl-CoA) which produces *(S)*-3-hydroxyacyl-CoA were converted into *(R)*-3-hydroxybutyryl-CoA by enzymes (R)-specific enoyl CoA hydratase (PhaJ) (Fukui et al., 1999) and 3-ketoacyl-CoA reductase (FabG)

respectively (Taguchi et al., 1999). Only then, the polymerization of MCL-PHA takes place by PHA synthase (PhaC).

On the other hand, Pathway III involves the production of MCL-PHA from unrelated carbon sources such as fructose, glucose and sucrose through fatty acid biosynthesis. The intermediates from fatty acid biosynthesis are in the form of *(R)*-3-hydroxyacyl-ACP which is eventually converted into *(R)*-3-hydroxyacyl-CoA form by an enzyme known as acyl-ACP-CoA transacylase (PhaG) which can be utilized by PHA synthase (PhaC) (Philip et al.,2007). Figure 2.2 shows the pathway for biosynthesis of PHA.

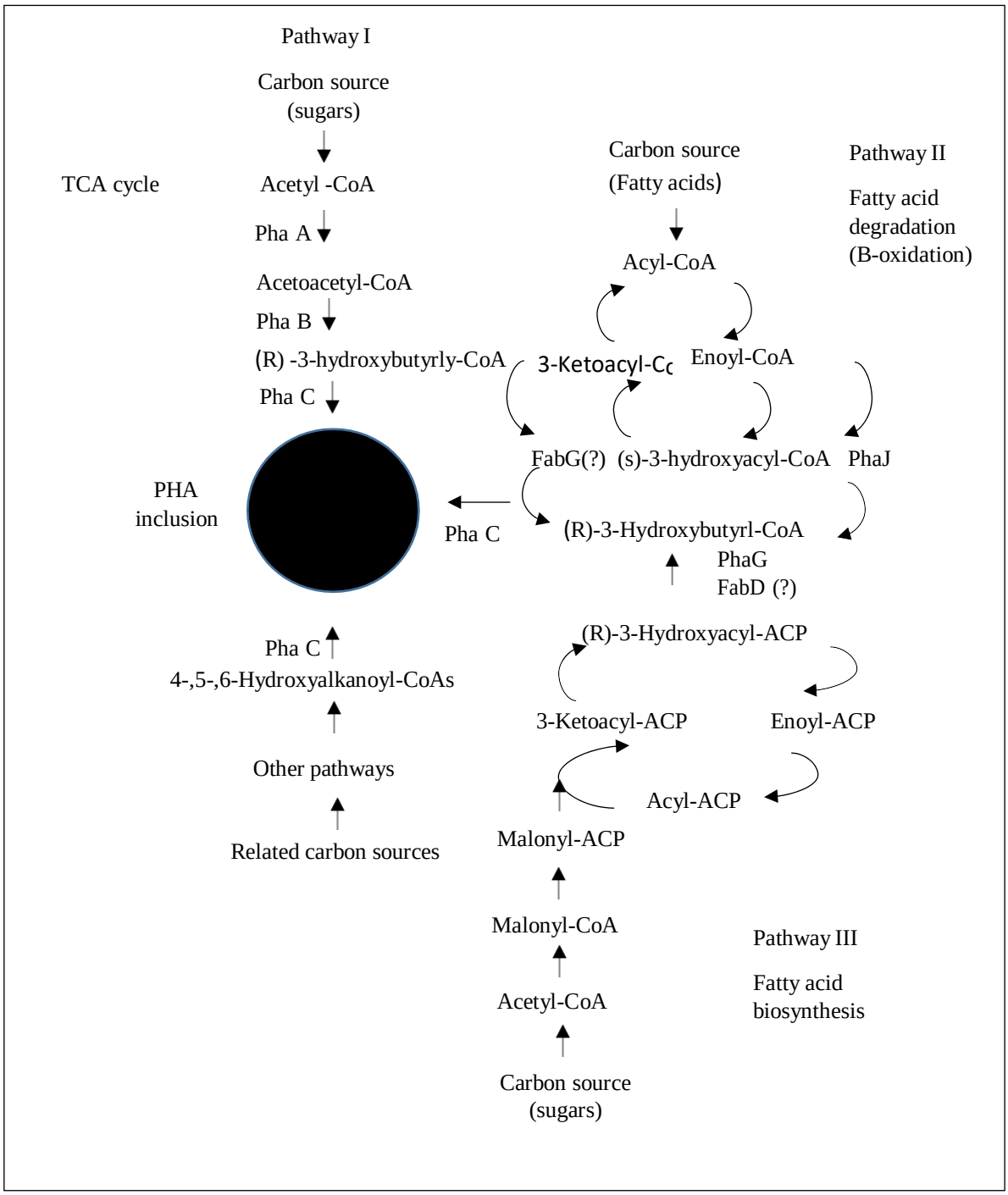


Figure 2.2: Pathway for biosynthesis of PHA. PhaA, B-ketothiolase; PhaB, NADPH-dependent acetoacetyl-CoA reductase; PhaC, PHA synthase; PhaG, 3-hydroxyacyl-ACP-CoA transferase; PhaJ, *(R)*-enoyl-CoA hydratase; FabD, malonyl-CoA-ACP transacylase; FabG, 3-ketoacyl-CoA reductase (Sudesh et al.,2000)

2.4 Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate), P(3HB-*co*-4HB)

Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) copolymer is one of the SCL-PHAs that is formed from the combination of two different monomers which are 3-hydroxybutyrate (3HB) and 4-hydroxybutyrate (4HB). Figure 2.3 shows the structure of the P(3HB-*co*-4HB) copolymer. The structure of 4HB is different from the 3HB monomer where the 4HB monomer does not possess the methyl side chain that attaches to the respective polymer backbone (Philip et al., 2007).

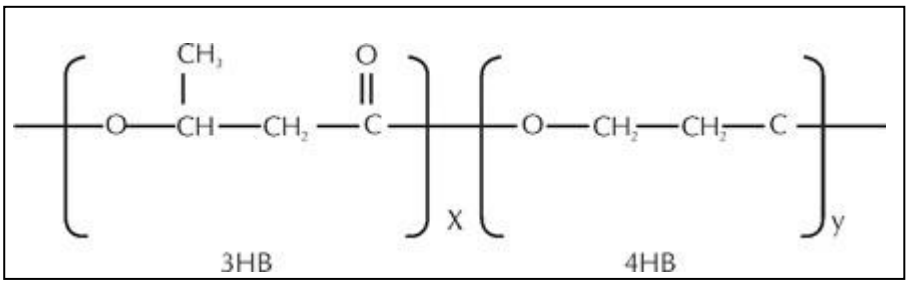


Figure 2.3: Structure of Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) P(3HB-*co*-4HB) (Vigneswari et al., 2014).

2.4.1 P(3HB-*co*-4HB) producers

Various microorganisms have been investigated to be able to produce P(3HB-*co*-4HB) such as *Alcaligenes latus* (Kang et al., 1995), *Hydrogenophaga pseudoflava* (Choi et al.,1999), *Comamonas acidovoras* (Mitomo et al., 2001), *Cupriavidus malaysiensis* USMAA1020 (Amirul et al., 2008), *Cupriavidus necator* (Chanprateep et al., 2010) *C. malaysiensis* USMAA2-4 (Iqbal & Amirul, 2014) and *C. malaysiensis* USMAHM13 (Iszatty et al., 2017). P(3HB-*co*-4HB) can be synthesized using the substrates such as 4-hydroxybutyric acid, γ-butyrolactone or from alkanediols such as 1,4-butanediol; 1,6-hexanediol; 1,8-octanediol; 1,10-decanediol and 1,12-dodecanediol (Chai et al., 2009; Huong et al., 2015). These substrates can be used as sole carbon sources or co-substrate with 3HB carbon precursors. Several factors such as bacteria strain, substrate (type, concentration, mixed strategy), and culture condition

(incubation period, aeration, pH, single or two-stage cultivation) influence the P(3HB-*co*-4HB) production, 4HB composition, and even its properties (Iqbal & Amirul, 2014; Huong et al., 2015; Huong et al., 2017).

2.4.1(a) *Cupriavidus malaysiensis* USMAA1020

Cupriavidus malaysiensis USMAA1020 (previously known as *Cupriavidus* sp. USMAA1020) is one of the bacteria that is used in this study to produce P(3HB-*co*-4HB). This bacteria is Gram-negative and was isolated from a sludge sample obtained from Lake Kulim, Kedah, Malaysia. Amirul et al (2008), performed the production of the copolymer by using γ-butyrolactone as a sole carbon source. In this experiment, the production was done via single and two cultivation stages. It was found that two-stage cultivation produces more 4HB composition compared to single-stage cultivation. The composition of 4HB in single-stage cultivation range from 6 mol % to 14 mol % while in two-stage cultivation, 25 mol % to 60 mol % was attained. Vigneswari et al. (2009) have conducted a study using the same bacteria to enhance the 4HB composition in copolymer by manipulating the condition of fermentation such as the size of the inoculum, the ratio of phosphate, and aeration. The production was done using two-stage cultivation. From this study, generally, all the variables caused an impact on 4HB composition, and a high 4HB monomer composition range from 23 mol % to 75 mol % was produced.

2.4.1(b) *Cupriavidus malaysiensis* USMAA1020 Transformant

Cupriavidus malaysiensis USMAA1020 transformant is the genetically modified wild-type *C. malaysiensis* USMAA1020. These bacteria have been modified to be able to produce a much higher 4HB composition. The strain was modified by

inserting the plasmid containing extra gene phaC (PHA synthase) into the bacteria through the transconjugation process from competent *Escherichia coli* S17-1 (Syafiq et al., 2017). After that, Syafiq & coworkers (2017) worked on the production of copolymer using 1, 6-hexanediol, and γ –butyrolactone as a carbon source. An increase in 4HB monomer composition was obtained at 93 mol % using the shake-flask fermentation. Meanwhile, in a large-scale production system using 5 L and 30 L bioreactors, the 4HB monomer composition at a range of 83–89 mol % was acquired. Norhafini et al (2017) also have done the study using the same bacteria with 1,4-butanediol and 1,6-hexanediol as a carbon source. The transformant strain was able to survive in a high concentration of carbon compared to the wild-type strain where the growth of the wild-type strain was constrained at the concentration of total carbon at 0.69 wt % (Huong et al., 2015). This is because the existence of an extra gene in bacteria can act as a backup for functional enzymes and pathways during stress conditions (Zhu et al., 2012).This study shows the highest 4HB composition production (up to 95 mol %) via single-stage cultivation. Based on the result from both experiments, the transformant was able to produce high 4HB monomer composition 2.1- fold higher compared to the original strain.

2.4.2 Biosynthesis of P(3HB-*co*-4HB)

Biosynthesis of P(3HB-*co*-4HB) was first discovered when *Cupriavidus necator (formerly known as Ralstonia eutropha and Alcaligenes eutrophus)* was cultivated using 4-hydroxybutyric acid or *γ*-butyrolactone as a carbon source (Doi et al., 1990). Later many other bacteria also can synthesize P(3HB-*co*-4HB) as mentioned in section 2.5.1. The incorporation of 4HB monomer to form P(3HB-*co*-4HB) highly depends on the use of particular carbon sources as a precursor such as 4-

hydroxybutyric acid, *γ*-butyrolactone, 1,4-butanediol, and other ω-alkanediols and 4-chlorobutyric acid (Steinbüchel & Lütke-Eversloh,2003; Huong et al., 2014).

All the carbon precursors must first be converted into 4-hydroxybutyric acid and then enzymes transferase or thiokinase will catalyze the conversion of 4-hydroxybutyric acid into 4- hydroxybutyryl-CoA. Finally, 4- hydroxybutyryl-CoA will be polymerized by PhaC into a 4HB monomer. Different types of metabolic pathways were involved in the biosynthesis of P(3HB-*co*-4HB) based on the type of carbon precursor used as shown in Figure 2.4.

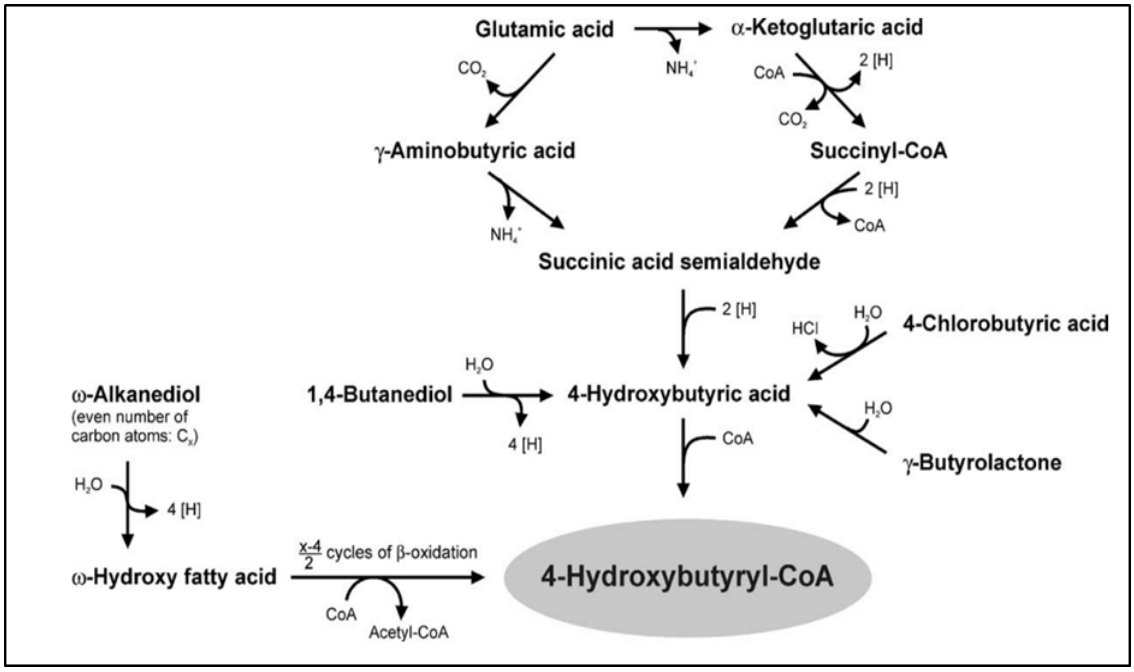


Figure 2.4: Sources of 4-hydroxybutyryl-CoA for biosynthesis of PHAs containing 4HB as constituent (Steinbüchel & Lütke-Eversloh, 2003)

Substrates such as *γ*-butyrolactone and 4-chlorobutyric acid were converted into 4-hydroxybutyric acid via a hydrolytic mechanism. *γ*-butyrolactone is cleaved into 4-hydroxybutyric acid by the reaction of enzyme esterases or lactonases while 4-chlorobutyric acid was converted into 4-hydroxybutyric acid by the haloalkane dehalogenase enzyme (Steinbüchel & Lütke-Eversloh, 2003). As for 1,4-butanediol

and other ω-alkanediols (even number of carbon), they must be oxidized via enzymatic reactions including *β*-oxidation to form 4-hydroxybutyric acid before being converted into 4-hydroxybutyryl-CoA (Saito et al., 1996). Then 3-hydroxybutyrate (3HB) monomer was formed when 4-hydroxybutyric acid was catabolized into intermediates leading to the formation of 3-hydroxybutyryl-CoA catalyzed by 4HB dehydrogenase (4HB-DH) and succinic acid semialdehyde dehydrogenase enzymes through succinic acid semialdehyde pathway (Valenti et al., 1995; Lütke-Eversloh & Steinbüchel, 1999).

On the other hand, there is also an interesting attempt to produce P(3HB-*co*-4HB) from unrelated carbon such as glucose through the citric acid cycle intermediate succinyl-CoA. The pathway in Figure 2.4 shows that succinyl-CoA is reduced to succinic acid semialdehyde and can be converted to 4HB-CoA where eventually polymerize by PHA synthase.

In 1997, a recombinant strain *Escherichia coli* (*E.coli*) which expressed succinic acid semialdehyde dehydrogenase, 4HB-DH, and 4-hydroxybutyric acid-CoA:CoA transferase gene from *Clostridium kluyveri* (*C. kluyveri*) that encoded for the enzyme for succinyl-CoA conversion to succinic acid semialdehyde and PHA synthase from *Ralstonia eutropha* (*R. eutropha)* is able produced P(3HB-*co*-4HB) up to 2 mol % 4HB using glucose as a carbon source (Valentin & Dennis, 1997). It was suggested that the content of 4HB in copolymer could be increased if the metabolic flux from succinyl-CoA to 4HB-CoA could be increased.

Therefore in 2010, Li & co-worker has construct metabolically engineered *E.coli* that express genes from *C.kluyveri* that involve in succinate degradation and P(3HB) accumulation from *R. eutropha* to synthesis P(3HB-*co*-4HB) using glucose as carbon source. To increase the carbon flux to 4HB biosynthesis, *E. coli* native

succinate semialdehyde dehydrogenase genes *sad* and *gabD* were both deleted to eliminate succinate formation from succinate semialdehyde, thus increasing the content of 4HB-CoA converted from succinate semialdehyde. Through shake flask and 6 liter bioreactor fermentation 11.1 mol% 4HB and 12.5 mol% 4HB were successfully obtained (Li et al., 2010). Even though the content of 4HB mol% obtained from the synthesis of P(3HB-*co*-4HB) from an unrelated carbon source is lower compared to the usage of a related carbon source, these studies provide evidence of enzymes versatility which are expressed in genetically modified organism able to produce P(3HB-*co*-4HB) using unrelated carbon sources.

2.4.3 Properties of P(3HB-*co*-4HB)

P(4HB) possesses high strength and exhibits flexible thermoplastic with Young's modulus of 149 MPa, a tensile strength of 104 MPa, and an elongation at break of 1000% (Saito & Doi 1994; Saito et al.,1996). Therefore copolymerizing P(4HB) monomer with P(3HB) can improve the flexibility of the polymer. The incorporation of a 4HB monomer into P(3HB) at various molar fractions has provided the polymer with a variety of physical and mechanical properties.

Since homopolymer P(3HB) possesses high crystallinity that leads to stiff and brittle properties, the addition of a 4HB monomer has decreased the crystallinity of the polymer making it less brittle and more flexible. This is because the presence of a 4HB monomer has disturbed the regularity structure of P(3HB) hence reducing the crystallization process (Zhang et al., 2012). Saito et al (1996), reported that when the molar fraction of 4HB monomer in P(3HB-*co*-4HB) increased from 0 to 27 mol %, the crystallinity of the polymer decreased from 60 to 40 %. Meanwhile, when the molar

fraction of 4HB in the polymer increase from 64 to 100 mol %, the crystallinity of the polymer increase from 15 to 34 %.

Besides that, the tensile strength of P(3HB-*co*-4HB) at 0 to 16 mol % 4HB decreases from 43 to 26 MPa (Saito et al., 1996). Likewise, Young's modulus also declined from 66 MPa to 13 MPa as the 4HB monomers increased from 10 to 65 mol % (Iqbal & Amirul, 2014). However, from 64 to 100 mol % 4HB, the tensile strength increase from 17 to 104 MPa (Saito et al., 1996). Furthermore, it was found that at high 4HB monomer composition, the polymer displays elastomeric properties. This is because as 4HB composition increase from 0 to 100 mol % the elongation at break of P(3HB-*co*-4HB) also increase from 5 to 1320 % (Saito & Doi,1994). Therefore, high tensile strength and elongation at break make P(3HB-*co*-4HB) a strong elastic polymer that can return to its original position after being stretched out (Surendran et al., 2020).

On the other hand, the presence of 4HB in P(3HB-*co*-4HB) reduces the melting temperature (T_m) of the polymer. It was reported as the composition of 4HB increased from 0 to 100 mol % the T_m also decrease from 178 ºC to 50 ºC. The glass transition temperature (T_g) of P(3HB-*co*-4HB) ranges from 4 ºC to 48 ºC (Saito et al.,1996). In a conclusion, it shows that depending on the 4HB monomer composition in the copolymer, the properties of the copolymer can be tailored to be hard crystalline to the elastomeric properties which can suit the application.

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

Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate), P(3HB-*co*-4HB), possess remarkable characteristics as biodegradable materials due to their ability to degrade in different environments such as sludge, soil, and seawater (Doi et al., 1990; Doi et al., 1992; Saito et al., 1996). Besides PHB depolymerase, P(3HB-*co*-4HB), also can be

degraded by other enzymes such as lipase. Based on the study conducted by Nakamura & Doi (1992) and Saito & Doi (1994), the erosion rate of the copolymer by PHB depolymerase increase when the 4HB monomer composition increase from 0 mol % to 28 mol %. This is because the crystallinity of the copolymer has been reduced due to the existence of the 4HB monomer. However further increase of 4HB monomer composition from 64 mol % to 100 mol %, caused the erosion rate to decrease. A similar trend also was found in a study performed by Mitomo et al. (2001). This is due to the depolymerase enzyme being nearly insusceptible to 4HB sequence thus decreasing the degradation rate of polymer with higher 4HB monomer composition. Meanwhile, the degradation rate by lipase enzyme shows the contradict trend where the increase of 4HB monomer composition shows the increase of lipase activity. This shows that the crystallinity of the material hardly influences the erosion rate by lipase enzyme as 3HB monomers are not eroded by this enzyme. Furthermore, the different trend of these biodegradation rates is because of differences in functional structure and biodegradation mechanism of both enzymes (Saito and Doi 1994; Saito et al., 1996; Mitomo et al., 2001; Vigneswari et al., 2009).

2.4.5 Biocompability of P(3HB-*co*-4HB)

Degradation of P(3HB-*co*-4HB) releases its monomer product, 3HB, and 4HB units which are the natural metabolite that can be found in the human body. The 3HB monomer can be a trace in the bloodstream while 4HB exists in the kidney, liver, lung, heart, and brain (Martin &Williams, 2003). Besides that, P(3HB-*co*-4HB) possesses a higher degradation rate *in vivo* compared to other PHA and its degradation rate also could be controlled by manipulating the 4HB composition in the copolymer (Mitomo et al., 2001). Various studies *in vitro* and *in vivo* also have revealed surface properties

of P(3HB-*co*-4HB) copolymer to be favorable for attachment and proliferation of cells.

In a study performed by Chee et al. (2008), a mouse fibroblast cell line (L929) was grown on P(3HB-*co*-4HB) film at various 4HB compositions from 11 mol % to 54 mol %. The result demonstrated the poor growth of cells on P(3HB) copolymer while the number of cell viability increases as the 4HB content in the copolymer increase. This is because the smoother and more regular surface morphology of the film produced by the 4HB monomer presence in the copolymer provides a more favorable place for the cell to attach and grow compared to P(3HB). Furthermore, the same trend was also displayed by Chanprateep et al. (2010), where the cell number increase proportionally as the 4HB content in the copolymer increased from 5 mol % to 64 mol %.

Besides that, the biocompatibility of copolymer was tested using the P(3HB-*co*-4HB) film treated with an oxidizing agent, hydrogen peroxide, H_2O_2. In a study conducted by Rao et al. (2010a), four different types of P(3HB-*co*-4HB) film consist of untreated P(3HB-*co*-4HB), treated P(3HB-*co*-4HB), P(3HB-*co*-4HB) /vitamin E blend and P(3HB-*co*-4HB) /collagen blend were fabricated. The studies show the films are greatly different in terms of their ability to stimulate inflammatory responses as well as encourage angiogenesis. The untreated film shows an acute inflammatory response however with treatment and blending process implemented were capable of reducing the inflammation significantly. The result also shows the ability of all films to induce angiogenesis with P(3HB-*co*-4HB)/collagen blend presents the better proangiogenic effect indicated by the highest Metalloproteinase activity. This is because of the RGD sequence of collagen which improves endothelial attachment and migration with corresponding increased MMP 2 activity. The *in vitro* cytotoxicity

evaluation study done by Vigneswari et al. (2011) also present the ability of the cell to grow and proliferate on the different surface morphology of the P(3HB-*co*-4HB) scaffold. The scaffolds were fabricated with different techniques consisting of the film (solvent-casting), salt-leached, enzyme degradation, electrospun and a combination of salt-leached/ enzyme degradation as well as different 4HB compositions from 16 mol % to 91 mol %. The result shows the scaffold fabricated with the combination of salt-leached / degradation technique is preferable compared to other scaffolds. Meanwhile, the number of viable cell increase with an increase in the composition of 4HB, with 91 mol % 4HB having the highest cell viability number. This finding also proved that this polymer does not toxic to the cell.

2.4.6 Application of PHA

The outstanding biocompatibility and biodegradability, as well as non-toxicity properties of PHA have made them promising biomaterials to be applied in medical areas. For example, monofilament sutures made from P(3HB), and a copolymer of P(3HB-*co*-3HV), implanted intramuscularly in Wistar rats up for one year period. The tissue reactions were investigated and the result shows that the P(3HB) and P(3HB-*co*-3HV), sutures did not trigger any acute vascular reaction and were able to provide enough support in terms of mechanical strength and wound healing. (Shishatskaya et al., 2004).

The polyester films of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate-*co*-3-hydroxyhexanoate) (P(3HB-*co*-4HB-*co*-3HHx), poly(L-lactic acid) (PLA) and poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) P(3HB-*co*-3HHx) were tested for biocompatibility using Human bone marrow mesenchymal stem cells (MSCs). Based on the study it was found that more MSCs were attached on (P(3HB-*co*-4HB-*co*-

3HHx) film than those on P(3HB-*co*-3HHx) and PLA films. Cell proliferation on (P(3HB-*co*-4HB-*co*-3HHx) film was the highest compared to PHBHHx film, and PLA film after three-day culture. These data proved that (P(3HB-*co*-4HB-*co*-3HHx) could significantly promote more cell adhesion and proliferation compared with PLA, and P(3HB-*co*-3HHx). It was also found that MSCs could differentiate into osteoblasts on (P(3HB-*co*-4HB-*co*-3HHx) films .The (P(3HB-*co*-4HB-*co*-3HHx) polymer was suggested to be useful for bone tissue engineering (Wei et al., 2009).

In a study conducted by Xu el at (2010), the nanofibre scaffold and film was fabricated from three different types of PHA consist of poly(3-hydroxybutyrate), P(3HB); poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate),P(3HB-*co*-4HB); poly(3-hydroxyvalerate) P(3HV), poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate), P(3HB-*co*-3HHx) and other polymer, poly(lactic acid), PLA. The study was done to evaluate the response of cells toward the film and nanofibre of the polymer. The neural stem cell from Sprague – Dawley rats was seeded on the film and nanofibre scaffold. After 10 days of incubation, it was found that a nanofibre scaffold made from PHA polymer, P(3HB-*co*-3HHx) show a higher cell viability rate compared to other polymers. This is because of comparing to film, nanofibre consists of a continuous fibrous network that can provide an interconnected porous structure that enables the cell to penetrate the interior of the scaffold and establish the connection between the cell as well as nutrient and gas exchange and waste disposal. Together with strong mechanical properties and biodegradability, PHA polymer, P(3HB-*co*-3HHx) can be applied for repairing injuries in the central nervous system.

P(3HB-*co*-4HB) has been well document not only for its excellent biocompatibility and non-toxicity properties but as well as its biodegradability which also can be degraded by other enzymes such as lipase and esterase (Martin & Williams,