BIOSYNTHESIS OF HIGH MOLECULAR WEIGHT POLY(3-HYDROXYBUTYRATE-*co*-4-HYDROXYBUTYRATE) COPOLYMER BY *Cupriavidus* sp. USMAA1020 USING 1,8-OCTANEDIOL AS THE CARBON PRECURSOR

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
$(NH_4)_2SO_4$	Ammonium sulphate
°C	Degree celcius
µL	Microliter
µm	Micrometer
^{13}C	Carbon-13
3HB	3-hydroxybutyrate
4HB	4-hydroxybutyrate
4HB-CoA	4-hydroxybutyryl-CoenzymeA
acetyl-CoA	acetyl-CoenzymeA
C	carbon
C/N	Carbon to nitrogen
$CDCl_3$	Deuterated chloroform
CDW	Cell dry weight
cm	centimeter
CME	Caprylate methyl ester
CoA	CoenzymeA
DO	Dissolved oxygen
DSC	Differential scanning calorimeter
g	gram
g/V	gram per volume

GC	gas chromatography
GPC	gas permeation chromatography
h	hour
HA-CoA	hydroxyalkanoyl CoenzymeA
H_m	Enthalphy of fusion
Hz	hertz
J/g	Joule per gram
K_2HPO_4	Dipotassium hydrogen phosphate
kDa	kilo Dalton
KH_2PO_4	Potassium dihydrogen phosphate
kPa	kilo pascal
L	liter
L/min	liter per minute
M	molar
mcl-PHA	medium chain length polyhydroxyalkanoate
MDa	Mega Dalton
$MgSO_4 \cdot 7H_2O$	Magnesium sulphate heptahydrate
min	Minute
mL	mililiter
mm	milimeter
M_n	number-average molecular weight
mol%	mol percentage
MSM	Mineral salt medium
mV	miliVolt

M_w	molecular weight
M_w/M_n	Polydispersity index
NA	Nutrient agar
NADH	Nicotinamide adenine dinucleotide
nm	nanometer
NMR	Nuclear magnetic resonance
NR	Nutrient rich
OD	Optical density
P(3HB)	Poly(3-hydroxybutyrate)
P(3HB-*co*-4HB)	Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate)
P(4HB)	Poly(4-hydroxybutyrate)
PDI	Polydispersity index
PGA	Polyglycolic acid
PHA	Polyhydroxyalkanoate
phaA; *phaA*	3-ketothiolase; gene encoding *β*-ketothiolase
phaB; *phaB*	NADPH-dependent acetoacetyl-CoA dehydrogenase; gene encoding NADPH-dependent acetoacetyl-CoA dehydrogenase
phaC; *phaC*	PHA synthase; gene encoding PHA synthase
PHB	Polyhydroxybutyrate
PLLA	Poly-L-Lactide
psi	Pounds per square inch
PTFE	Polytetrafluoroethylene
RCDW	Residual cell dry weight

rpm	Rotation per minute
scl-PHA	Short chain length polyhydroxyalkanoate
ß	Beta
TCA	Tricarboxylic acid
T_g	Glass transition temperature
T_m	Melting temperature
UHMW	ultrahigh molecular weight
v/v	volume per volume
wt	Weight
wt%	Weight percentage

BIOSINTESIS KOPOLIMER POLI(3-HIDROKSIBUTIRAT-*ko*-4-HIDROKSIBUTIRAT) DENGAN BERAT MOLEKUL YANG TINGGI OLEH *Cupriavidus* sp. USMAA1020 MENGGUNAKAN 1,8-OKTANADIOL SEBAGAI PEMANGKIN KARBON

ABSTRAK

Polihidroksialkanoat (PHA) merupakan termoplastik bakteria yang terbiodegradasi secara semulajadi. Kopolimer poli(3-hidroksibutirat-*ko*-4-hidroksibutirat) P(3HB-*ko*-4HB) merupakan sejenis PHA yang dikenali dengan sifat kebioserasian yang dapat ditransformasikan sebagai produk biofarmaseutikal dan digunakan dalam bidang perubatan. Berat molekul yang tinggi (>1MDa) adalah sifat yang diidamkan kerana ia berpotensi untuk pelbagai aplikasi penggunaan memandangkan 1,8-oktanadiol dapat menghasilkan 4HB monomer dengan komposisi yang tinggi tetapi mempunyai kepekatan PHA yang rendah. Kajian ini dijalankan untuk mengkaji potensi 1,8-oktanadiol untuk menghasilkan P(3HB-*ko*-4HB) dengan berat molekul yang tinggi serta kepekatan PHA yang lebih baik menggunakan kultur substrat campuran. Proses pengkulturan satu peringkat telah dilakukan dengan menggabungkan karbon 1,8-oktanadiol dengan asid oleik, γ-butirolakton, 1,4-butanadiol atau natrium 4-hidroksibutirat. Kesan nisbah sumber karbon kepada nitrogen (C/N) oleh 1,8-oktanadiol telah dikaji dan menunjukkan C/N=10 memberikan pertumbuhan dan kepekatan PHA yang optimum. Kajian pengkulturan substrat campuran ke atas biosintesis kopolimer menggunakan kepekatan dan substrat campuran yang berbeza melalui kajian kelalang goncangan menghasilkan 4 hingga 52 mol% komposisi 4HB monomer oleh kultur sesekelompok dan kultur suapan sesekelompok. Dua kepekatan sumber karbon terpilih dari

gabungan substrat campuran telah dikaji menggunakan 3 L bioreaktor. Gabungan 1,8-oktanadiol dengan γ-butirolakton menghasilkan berat molekul dan pemanjangan sehingga putus yang tinggi sebanyak 1060 kDa dan 970% bagi P(3HB-*ko*-22%4HB) kopolimer manakala gabungan 1,8-oktanadiol dan natrium 4-hidroksibutirat menghasilkan 643 kDa dan 747% P(3HB-*ko*-28%4HB). Suapan sesekelompok sumber karbon dan nitrogen telah meningkatkan keseluruhan kepekatan PHA. Walau bagaimanapun, sifat-sifat mekanikal kopolimer menurun dengan peningkatan kadar suapan sesekelompok. Sifat-sifat mekanikal yang diperolehi melalui suapan sesekelompok 1,8-oktanadiol dan γ-butirolakton adalah sama dengan keputusan yang diperolehi daripada fermentasi substrat campuran dengan 1060 kDa kepada 1052 kDa dengan peningkatan berat keseluruhan sel kering dan kandungan PHA. Pemanjangan sehingga putus dan berat molekul untuk sekali suapan sesekelompok karbon adalah 944% P(3HB-*ko*-35%4HB) dengan 885 kDa dan 840% P(3HB-*ko*-30% 4HB) dengan 1052 kDa untuk dua kali suapan sesekelompok karbon. Kopolimer ini secara terbukti sebagai kopolimer selang-seli dan kopolimer rawak memandangkan nilai D yang diperolehi adalah berkisar di antara 0.23 hingga 1.0. Penemuan ini menunjukkan bahawa kombinasi 1,8-oktanadiol dan γ-butirolakton berupaya untuk menghasilkan P(3HB-*ko*-4HB) kopolimer dengan berat molekul yang tinggi serta berpotensi meluaskan penggunaan kopolimer berberat molekul yang tinggi dalam bidang bioperubatan.

BIOSYNTHESIS OF HIGH MOLECULAR WEIGHT POLY(3-HYDROXYBUTYRATE-*co*-4-HYDROXYBUTYRATE) BY *Cupriavidus* sp. USMAA1020 USING 1,8-OCTANEDIOL AS THE CARBON PRECUSOR

ABSTRACT

Polyhydroxyalkanoate (PHA) is a bacterial derived thermoplastic that is naturally biodegradable. Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] copolymer is a PHA known for its biocompatibility that can be transformed into biopharmaceutical products and be used in medical applications. High molecular weight polymer (>1 MDa) is highly desired for its many potential applications usage as 1,8-octanediol could produce high 4HB composition but low PHA concentration. This study investigates the potential of 1,8-octanediol to produce high molecular weight P(3HB-*co*-4HB) copolymer with improved PHA concentration using mixed substrate cultivation. One-stage cultivation process was performed using different carbon combination of 1,8-octanediol either with oleic acid, γ-butyrolactone, 1,4-butanediol or sodium 4-hydroxybutyrate. The effect of carbon to nitrogen (C/N) ratios of 1,8-octanediol was investigated and it was found that C/N=10 gave the optimum growth and PHA concentration. Biosynthesis of copolymer using different concentration of mixed-substrate by shake-flask cultivation resulted in 4 to 52 mol% 4HB monomer compositions by batch and fed-batch fermentation. Two selected mixed-substrate combination were studied in 3 L bioreactor. The combination of 1,8-octanediol with γ-butyrolactone was found to produce high molecular weight and elongation at break of 1060 kDa and 970%, respectively for P(3HB-*co*-22%4HB) copolymer while combination of 1,8-octanediol and sodium 4-hydroxybutyrate resulted in 643 kDa and 747%, respectively for P(3HB-*co*-28% 4HB). Feeding of carbon and nitrogen sources has enhanced the overall PHA concentration to 5.07

g/L. However, the molecular weight of copolymer decreased with increasing feeding rates. The molecular weight obtained through the feeding of 1,8-octanediol and γ-butyrolactone was similar to the results obtained from batch fermentation containing 1060 and 1052 kDa with improved overall residual biomass and PHA content. The elongation at break and molecular weight for single feeding of carbon was 944% for P(3HB-*co*-35%4HB) with 885 kDa and 840% for P(3HB-*co*-30%4HB) with 1052 kDa for twice the feeding of carbon sources. The copolymer was confirmed as alternating and random copolymers since the D values obtained were in the range of 0.2 to 1.0. This study showed that the combination of 1,8-octanediol and γ-butyrolactone can improve the production of P(3HB-*co*-4HB), and this discovery has the potential to enlarge the applications of high molecular weight copolymers in the biomedical fields.

CHAPTER 1

1.0 INTRODUCTION

The advancement of modern technology has led to many great inventions and innovations that shaped the world we live in today. There comes the discovery of plastics to improve the lifestyle and standard of living of human around the world. A biodegradable plastic can be degraded naturally in a certain period of time by a wide variety of microorganisms through their intracellular or extracellular biological process. However, a recent study showed that after food and organic waste, plastics are the second major contributor of municipal solid waste in Malaysia (Chikere and Hussain, 2014; Fauziah and Agamuthu, 2013; Johari *et al.*, 2014). This waste problem has caught the attention of scientists and has led to the development and production of biodegradable polymer.

The main component of a biodegradable polymer is polyhydroxyalkanoate (PHA). PHA is accumulated in the form of discrete water-insoluble granules in cytoplasm and the view of the granules can be visualised via phase-contrast optical microscopy (Sudesh *et al.*, 2000). Their inherent desirable properties such as non-toxic, biocompatible and biodegradable thermoplastic has caused an extensive attention that created a demand for them to be used in pharmaceutical and medical fields. Eventhough many PHAs are existed, not every types of PHA can be useful for medical purposes (Vert, 2005). The most promising types of PHA for medical fields are poly(4-hydroxybutyrate) P(4HB) and P(3HB-*co*-4HB).

PHAs have various medical applications. The well-oriented P(4HB) polymer chains showing promises as sutures and medical textile products making. Besides that, low molecular weight and high molecular weight P(4HB) shows it potential use in augmentation, soft tissue repair and bulking applications. Research shows that astonishing results were obtained when P(3HB-*co*-4HB) were used in heart valves development, cardiovascular patches, wound dressings, adhesion barriers and drug loading application (Chee *et al.*, 2008; Chen and Wu, 2005; Martin and Williams, 2003).

The biocompatibility and biodegradability effect of PHA through in vivo and in vitro study proved that it does not need any removal once it was inserted into the human or mammals. There are high levels of qualifications needed for plastic used in the human body. Therefore, the PHA extraction and purification methods are the critical things to be considered as the PHA must be free of bacterial endotoxins before it can be used in contact with blood in the human body (Sevastianov *et al.*, 2003).

1.1 Problem statements

PHAs can be produced by various Gram positive and Gram negative bacteria. The PHAs are produced under stress condition through fermentation when there is a limitation of nitrogen but in excess of carbon source (Doi, 1990). However, toxicity of carbon source to the bacteria will occur if excessive carbon source were present in the culture medium. This will stop the production of PHA and inhibit the growth of the bacteria (Stanbury and Whitaker, 1995). The composition of PHAs produced can be manipulated by supplying different types of carbon sources in cultivation

condition. There are numerous types of carbon precursors that have been reported that can promote and enhance the production of PHA.

In the biosynthesis process of P(3HB-*co*-4HB) copolymer, the commonly used carbon precursors are a mixture of oleic acid and γ-butyrolactone, sodium 4-hydroxybutyrate, 1,4-butanediol or 1,6-hexanediol. Besides that, 1,8-octanediol, 1,10-decanediol and 1,12-dodecanediol are the most unfavourable ω-alkanediols been studied. Among all carbon sources, 1,8-octanediol produced the highest 4HB content but low in PHA concentration (Chai *et al.*, 2009; Nurhezreen and Amirul, 2013; Nurhezreen, 2013). Hence, 1,8-octanediol was chosen to be the main focus of this study in order to improve the overall PHA concentration. There are also limited publications using the mixed-substrate cultivation strategy to synthesize P(3HB-*co*-4HB) copolymer even though it is not a new concept (Huong *et al.*, 2013).

Many P(3HB) producers have been found to a synthesize wide range of high molecular weight homopolymer P(3HB) (up to 20000 kDa) by manipulating their cultivation conditions (Kusaka *et al.*, 1997). To the best of our knowledge, high molecular weight (M_w) P(3HB-*co*-4HB) copolymer of 1110 kDa has only been reported by Volova *et al.* (2011). The P(3HB-*co*-4HB) copolymer was produced by optimizing the cultivation conditions of hydrogen-oxidizing bacteria, *Ralstonia eutropha* B5786. Moreover, detailed investigations on the the ability of bacteria and carbon sources to produce high M_w PHAs have been scarce despite it being the most important characteristic to determine applications offered by the polymer (Sudesh *et al.*, 2000). A characterization of various compositions of P(3HB-*co*-4HB) copolymer produced throughout this study is needed to add valuable information and to know the applications offered by this copolymer.

Therefore, in this study the ability of wild strain bacterium *Cupriavidus* sp. USMAA1020 to produce high molecular weight P(3HB-*co*-4HB) copolymer by combination of 1,8-octanediol with selected carbon sources by batch and fed-batch fermentation through one-stage cultivation method was evaluated. Finally, the mechanical and thermal properties of copolymer P(3HB-*co*-4HB) produced were characterized.

1.2 Objectives of this study

The objectives of this study are:

i. To screen different combinations of carbon sources with 1,8-octanediol for the production of P(3HB-*co*-4HB) copolymer in the shake-flask study.
ii. To enhance the selected production of high molecular weight P(3HB-*co*-4HB) copolymer by batch and fed-batch fermentation through 3 L bioreactor.
iii. To evaluate the characteristics of the P(3HB-*co*-4HB) copolymer.

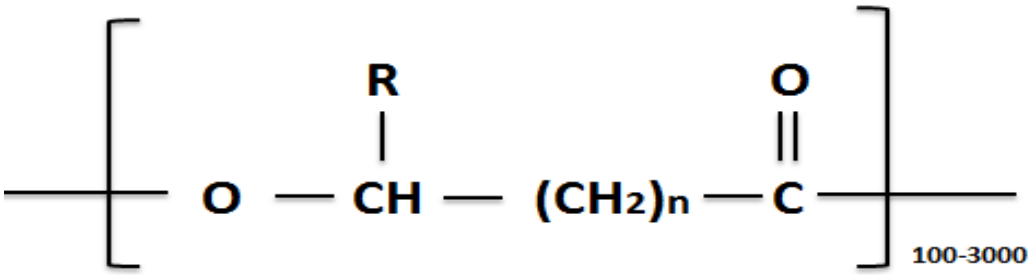
CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Polyhydroxyalkanoate (PHA)

PHAs are polyesters that are formed from several monomer units named hydroxyalkanoates (HAs). The evolution of PHAs started when Lemoigne discovered and reported that a polyester of β-hydroxybutyric acid, P(3HB), was a major component of *Bacillus megaterium* in 1926 (Lenz and Marchessault, 2005). Later, he confirmed that P(3HB) was a homopolymer of β-hydroxybutyrate. PHAs are accumulated by numerous Gram negative and Gram positive bacterial cells from areas such as activated sludge, sediment, soil and water. They are accumulated in the form of water-insoluble granules as carbon and energy storage materials for sporulating and non-sporulating bacteria.

PHAs are classified into two groups namely short chain length PHAs (scl-PHAs) and medium chain length PHAs (mcl-PHAs), based on the carbon numbers in their monomer structure. Scl-PHAs polymer consist of 3 to 5 carbon atoms containing monomers whereas mcl-PHAs polymer consist of 6 to 14 carbon atom containing monomers (Khanna & Srivastava, 2005). Incorporating of both scl-PHA and mcl-PHA monomers will result in scl-mcl PHAs copolymer having the properties of both polyesters. However, the properties will depend on the different ratio of scl and mcl monomers (Hema, 2014). Different types of PHA can be synthesized from a wide range of microorganism, which can produce the PHAs in the form of homopolymer or heteropolymer such as copolymer and terpolymer. The general chemical structure of PHA is shown in Figure 2.1.



Monomer

n=1	R=hydrogen	poly(3-hydroxypropionate)	P(3HP)
	R=methyl	poly(3-hydroxybutyrate)	P(3HB)
	R=ethyl	poly(3-hydroxyvalerate)	P(3HV)
	R=propyl	poly(3-hydroxycaproate)	P(3HC)
	R=butyl	poly(3-hydroxyheptanoate)	P(3HH)
	R=pentyl	poly(3-hydroxyoctanoate)	P(3HO)
	R=hexyl	poly(3-hydroxynonanoate)	P(3HN)
	R=heptyl	poly(3-hydroxydecanoate)	P(3HD)
	R=octyl	poly(3-hydroxyundecanoate)	P(3HUD)
	R=nonyl	poly(3-hydroxydodecanoate)	P(3HDD)
n=2	R=hydrogen	poly(4-hydroxybutyrate)	P(4HB)
n=3	R=hydrogen	poly(5-hydroxyvalerate)	P(5HV)

Figure 2.1: The general chemical structure of PHA (Wu *et al.*, 2003).

2.2 Biosynthetic pathway of PHA

To date, various kinds of wild type strains and recombinant bacterial strains were found to be able to produce PHA. Different strains require different substrates to produce the PHA. Different types of carbon substrates have been used such as chemical based, natural and unnatural based, sugar, alcohols, organic acids, domestic waste and plant oils. The commonly used carbon substrates includes oleic acid, γ-butyrolactone, 1,4-butanediol and 1,6-hexanediol (Huong *et al.*, 2013; Nurhezreen and Amirul, 2013; Vigneswari *et al.*, 2009b), while domestic waste includes glycerine pitch (Hema and Amirul, 2014), waste cooking oil (Kamilah *et al.*, 2013), glycerol, and plant oil. Furthermore, the composition of the PHA produced and their properties are strongly dependent on the bacterial and carbon substrates available in generating the monomers.

Generally, there are three well-known pathways in PHA biosynthesis. Pathway I involves the tricarboxylic acid (TCA) cycle while pathway II involves fatty acid degradation (β-oxidation) pathway. Pathway III involves carbon source (fatty acids) biosynthesis pathway using non-sugar molecules. Pathway I is the most commonly known among all the biosynthetic pathways (Philip *et al.*, 2007).

The production of P(3HB) homopolymer starts with acetylcoenzyme A (acetyl-CoA) which is an intermediate for biosynthesis and degradation of P(3HB). A pair of acetyl-CoA are coupled and condensed into acetoacetyl-CoA by 3-ketothiolase (PhaA) by releasing the CoA (Anderson and Dawes, 1990). Consequently, the acetoacetyl-CoA is reduced to (*R*)-3-hydoxybutyryl-CoA by stereospecific acetoacetyl-CoA reductase (PhaB) (Doi, 1990). PHA synthase (PhaC) then polymerizes 3-hydroxybutyryl-CoA to P(3HB) by associated release of CoA. The biosynthesis pathway of P(3HB) is shown in Figure 2.2.

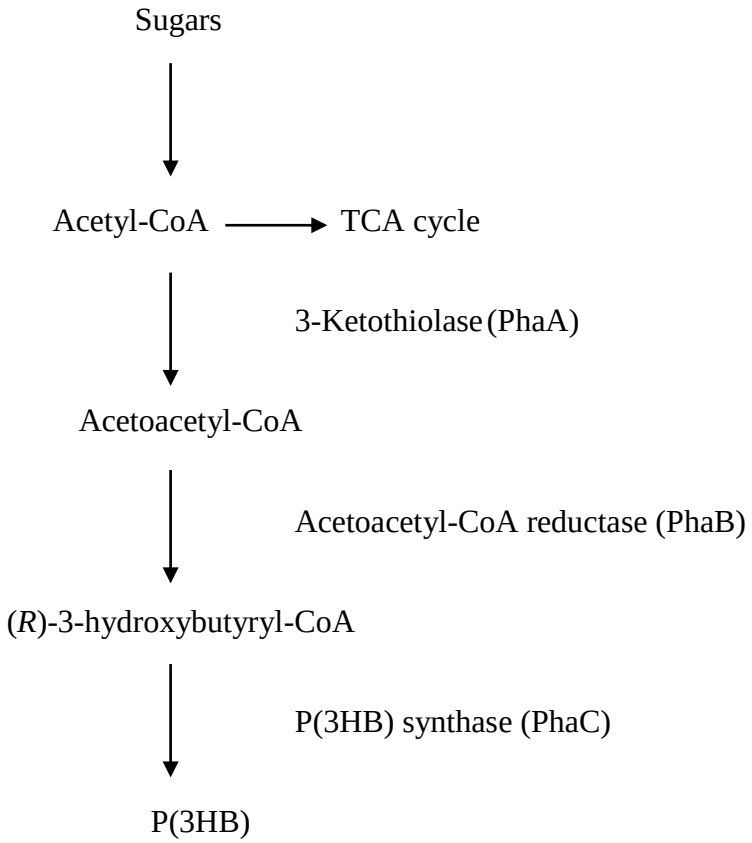


Figure 2.2: Metabolic pathway of P(3HB) (Verlinden *et al.*, 2007).

Acetoacetyl-CoA is generated in all living organism regardless of the type of carbon sources. This explains the extensive presence of P(3HB) in most environmental samples. There are three biosynthetic enzymes involved in the metabolic pathway for P(3HB) accumulation named PhaA, PhaB and PhaC. Among these three enzymes, PhaA is the key regulatory enzyme in P(3HB) synthesis that is inhibited by high concentrations of free coenzyme A.

Acetyl-CoA enters the tricarboxylic acid (TCA) cycle under balanced growth conditions for the formation of amino acids and energy generation. Thus, the synthesis of P(3HB) is inhibited due to high concentration of free coenzyme A. In contrast, under nutrient limitations with an excess of carbon sources, citrate synthase is inhibited by high concentration of NADH. Hence, acetyl-CoA levels increase the concentration of free coenzyme A. Eventually, the PhaA that is inhibited by free coenzyme A is activated and the P(3HB) synthesis is initiated (Doi, 1990; Verlinden *et al.,* 2007).

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

P(3HB) homopolymer is the most common type of PHA studied that can be synthesized from environment samples, transgenic plants and bio-renewable agricultural feedstock. It is also known as the commonest type of PHA that are produced and best characterized by most microorganisms. P(3HB) extracted from bacteria is an amorphous molecules that exists as water-insoluble granules and is kept within cell cytoplasm. In addition, P(3HB) has a fully isotactic structure that is only present in *R*-configuration (Sudesh *et al.*, 2000). This characteristic allows P(3HB) to possess high level of degradability.

P(3HB) is identified as partially crystalline, fairly brittle, stiff and it exhibits some mechanical properties closely related to a synthetic polymer named polypropylene. The properties of scl-PHAs like P(3HB) are contrary to mcl-PHAs which are more flexible and elastic. P(3HB) has a tensile strength of 40 MPa and Young's modulus of 3.5 GPa compared to polypropylene which exhibits values 34.5 MPa and 1.7 GPa, respectively (Akaraonye, Keshavarz, & Roy, 2010). However, P(3HB) has a recorded low elongation at break of 5% compared to polypropylene which is at 400%. Table 2.1 shows the comparison of thermal and mechanical properties between P(3HB) and polypropylene.

Table 2.1: Comparison of thermal and mechanical properties of P(3HB) and polypropylene (Akaraonye *et al.*, 2010)

Properties	**P(3HB)**	**Polypropylene**	**Polystyrene**
Melting temperature, T_m (°C)	180	176	240
Glass transition temperature, T_g (°C)	4	-10	100
Young's modulus (GPa)	3.5	1.7	3.1
Tensile strength (MPa)	40	34.5	50
Elongation at break (%)	5	400	0

2.2.2 Poly(4-hydroxybutyrate) [P(4HB)]

P(4HB) homopolymer is the most attractive PHA that has been studied due to its desirable properties. The 4-hydroxybutyric acid is a natural human metabolite that is present in the brain, lung, heart, kidney, liver and muscle (Nelson *et al.*,1981). The *In vivo* study this homopolymer showing its desirable properties of having good resorbability, biodegradability and biocompatibility proved its practical applications for pharmaceutical and medical fields such as in cardiovascular, wound management, drug delivery and tissue engineering.

This homopolymer has a promising and big opportunity extended far beyond those currently offered by their synthetic counterparts. For instance, P(4HB) has been tested to be less acidic than the lactic acid and glycolic released from poly-L-lactic acid (PLLA) and poly-glycolic acid (PGA) implants (Taylor *et al.,* 1994). Hence, the prospect of synthesizing PHA using mixed carbon substrates could widen the variety of the monomer units copolymer produced and widen the usefulness of this copolymer. Polymer with high molecular weight is preferable in most medical applications. Therefore, P(4HB) is preferably produced by fermentation rather than chemical synthesis, which can result in low molecular weight polymer.

A well known medical device company named Tepha Inc. (Cambridge, MA) is commercially producing P(4HB) by using recombinant *Escherichia coli* K12 via transgenic fermentation process and marketing it for medical applications (Martin and Williams, 2003). P(4HB) homopolymer is a strong and flexible thermoplastic material with a tensile strength of 104 MPa, which is closely comparable to that of polyethylene or other synthetic polymers (Philip *et al.,* 2007). It is also a highly ductile, malleable polymer with an elongation at break of around 1000% unlike

P(3HB), which has an elongation at break of less than 10% (Saito *et al.*, 1996; Williams and Martin, 1996).

The biosynthesis pathway of P(4HB) copolymer is shown in Figure 2.3. The P(4HB) biosynthesis pathway generally derives 4-hydroxybutyric acid from 4HB carbon precursors. The 4-hydroxybutyric acid is converted to 4-hydroxybutyryl-CoA either by transferase or thiokinase. Then, it is converted to 4-hydroxybutyrate monomer. However, when 4-hydroxybutyric acid, γ-butyrolactone or ω-alkanediol are used as carbon precursors, a part of it are converted into 4-hydroxybutyric acid and their intermediary are catabolized to 3-hydroxybutyryl-CoA. Firstly, ω-alkanediol is oxidized to ω-hydroxyfatty acids and converted to coenzyme A thioester before undergoing β-oxidation to form 4HB-CoA. On the other hand, γ-butyrolactone is metabolized to 4HB-CoA by lactonase or esterase. A portion of the 3HB and 4HB produced are then polymerized to form P(3HB-*co*-4HB) copolymer by the reaction of PHA synthase (Steinbüchel and Eversloh, 2003).

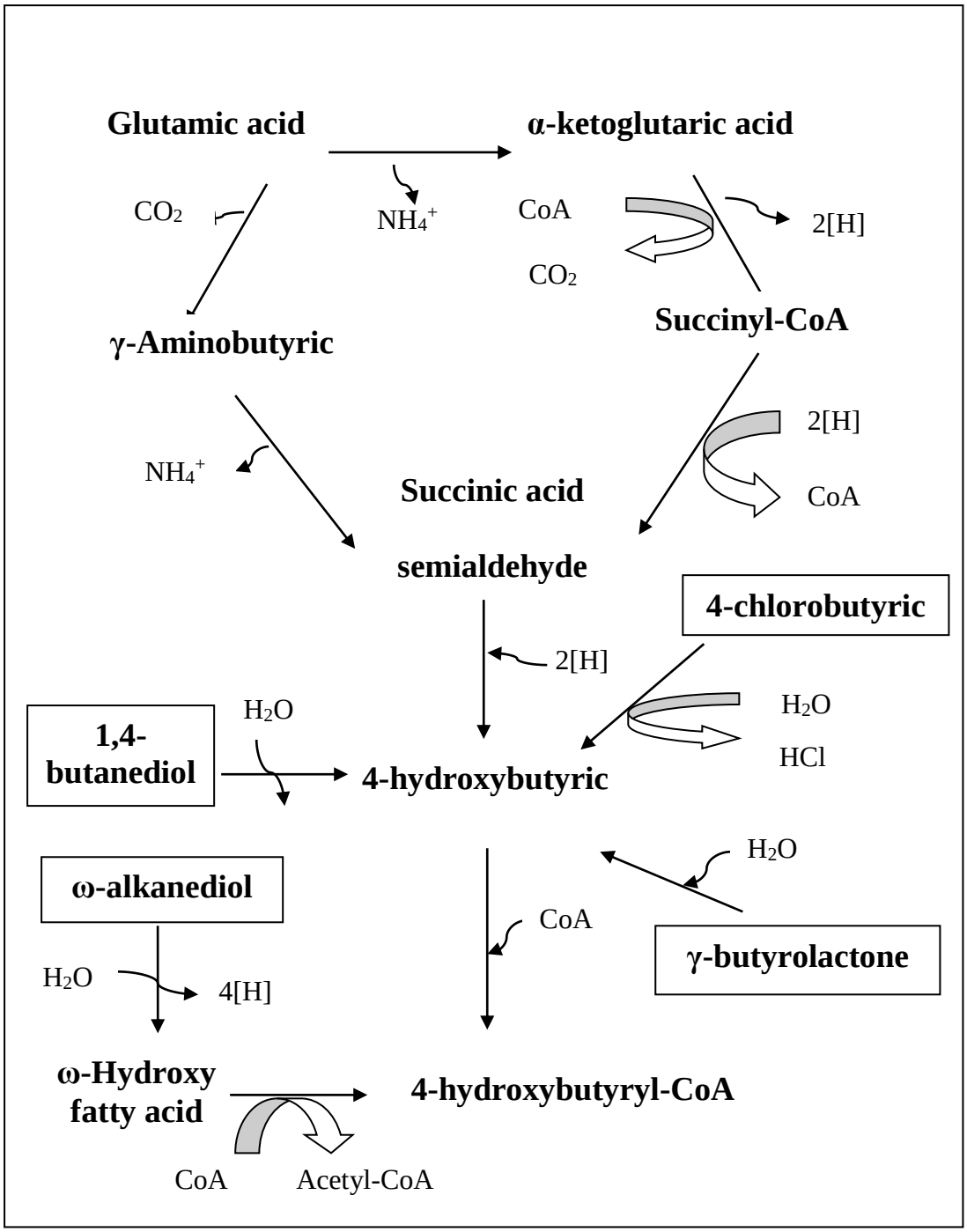


Figure 2.3: Production of 4-hydroxybutyryl-CoA (4(HB-CoA) by *Cupriavidus* sp. USMAA1020 (Steinbüchel and Eversloh, 2003).

2.2.3 Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) (P(3HB-*co*-4HB))

P(4HB) homopolymer alone is known to possess high biocompatibility properties while P(3HB) homopolymer alone has a high level of biodegradability. As a result, production of copolymer P(3HB-*co*-4HB) can integrate both properties of P(3HB) and P(4HB), while customizing their level of biocompatibility and biodegradability for applications in the medical and pharmaceutical fields. Besides that, study has shown that 3-hydroxybutanoic acid and 4-hydroxybutanoic acid which are natural metabolites present in the human body are produced in the degradation process of P(3HB-*co*-4HB) (Martin and Williams, 2003). This strong evidence proves the biocompatibility of P(3HB-*co*-4HB) copolymer.

Various bacterial strains are capable of synthesizing P(3HB-*co*-4HB) copolymer using different type of carbon precursors and bacterial strains. *Cupriavidus* sp. USMAA1020, *Cupriavidus* sp. USMAA2-4 and *Cupriavidus* sp. USMAHM13 are the three *Cupriavidus* strains isolated from Malaysian environment which are capable of producing P(3HB-*co*-4HB) copolymer (Amirul *et al.*, 2008; Hema and Amirul, 2012; Rahayu *et al.*, 2008). Formerly, there are many wild producers that successfully produced this copolymer such as *Alcaligenes latus*, *Comamonas testosteroneii, Hydrogenophaga pseudoflava*, *Comamonas acidovorans* and *Cupriavidus necator* (Choi *et al.*, 1999; Kang *et al.*, 1995; Kim, Lee and Kim, 2005).

The properties of P(3HB-*co*-4HB) copolymer can be tailored by manipulating the carbon substrate concentration. Besides that, the 4HB monomer composition will also varied as high composition of 4HB monomer composition was produced by *Cupriavidus necator* using 4-hydroxybutyric acid and presence additives as shown in Table 2.2 (Nakamura & Doi, 1992).

Table 2.2: Thermal properties of different compositions of P(3HB-*co*-4HB) copolymer (Nakamura and Doi, 1992).

Monomer Composition (mol%)		**Melting point (°C) (T_m)**	**Glass transition temperature (°C) (T_g)**	**ΔH_m (cal/g)**	**Crystallinity (%)**
3HB	**4HB**				
100	0	177	4	20.8	59 ± 5
94	6	162	-1	13.5	56 ± 5
90	10	159	-3	13.0	46 ± 5
72	28	0	-15	-	23 ± 5
15	85	48	-41	8.6	29 ± 5
10	90	50	-44	10.2	-
6	94	51	-46	11.0	42 ± 5
0	100	54	-50	11.0	-

2.3 Biodegradability and biocompatibility of P(3HB-*co*-4HB) copolymer

Biopolymers have been confirmed to exhibit biodegradability, biocompatibility and biomaterial properties in a number of *in vitro* studies (Chanprateep, 2010). PHAs can be formulated and processed for use in many applications including packaging, moulded goods, paper coatings, non-woven fabrics, adhesives, films and performance additives (Bugnicourt *et al.*, 2014). However, their applications are strongly dependent on the types of biopolymers produced. Besides that, the biodegradability of polymer is dependent on its molecular weight and degree of crystallinity (Tokiwa *et al.*, 2009). It has been found that PHAs with low molecular weight are more susceptible to biodegradation (Philip *et al.*, 2007).

Among the various types of biopolymers, P(3HB-*co*-4HB) has gained much attention as a biomaterial with high level of biocompatibility and inert *in-vivo* degradation products (Martin and Williams, 2003). In addition, P(3HB-*co*-4HB) copolymer has further advantage as it can be degraded by either PHA depolymerase or lipase (Saito *et al.*, 1996). Moreover, the overview of PHA properties, mostly the biocompatibility and biodegradability of P(3HB-*co*-4HB) presents a promising potential in drug delivery system (Nigmatullin *et al.*, 2015).

2.4 Biosynthesis of P(3HB-*co*-4HB) copolymer using common carbon sources

PHA biosynthesis is closely dependent on the carbon source due to the broad substrate specificity of PHA synthases and the metabolic pathways operating in bacterial cells (Potter and Steinbüchel, 2006). The composition of the polymer is greatly affected by the type of substrate used and its physiological conditions (Mamtesh *et al.*, 2015). In general, PHAs are produced in conditions that are unfavourable for growth; where there is a limit in certain nutrients, normally nitrogen, but in the presence of excess carbon source.

Different bacteria have their own capability to produce PHA depending on types of carbon sources utilized. Therefore, selection of carbon sources is one of the crucial aspects that can reduce the cost of PHA production and determine the type of PHA monomer. The commonly used carbon sources are sugar, molasses, plant oils, fatty acids, ω-alkanediols and simple carbohydrates. Ammonium sulphate is frequently used as nitrogen substrate (Shantini *et al.*, 2012; Nurhezreen and Amirul, 2013). However, Hema and Amirul (2014) revealed that increasing the amount of ammonium acetate concentration have increased the cell productivity of *Cupriavidus* sp. USMAHM13.

Biosynthesis of P(3HB-*co*-4HB) copolymer commonly used mixed-substrate cultivation concept because it is expected that the degree of substrate utilization and copolymer production to be different, subsequently this will contribute to the production of copolymer with a wider range of 4HB monomer compositions (Huong *et al.,* 2013). Table 2.3 summarizes different carbon sources used by bacterial strain for the production of P(3HB-*co*-4HB) copolymer using sole carbon and mixed substrates cultivation.

Table 2.3: List of bacterial strain capable of synthesizing P(3HB-*co*-4HB) copolymer and the carbon sources involved.

Bacterial strain	**Carbon sources combination**	**References**
Cupriavidus necator	γ-butyrolactone, 4-hydroxybutyric acid, ω-alkanediols	(Saito *et al.*, 1996)
	Fructose and ɤ-butyrolactone	(Kim *et al.*, 2005)
	γ-butyrolactone and propionate	(Lee *et al.*, 2000)
Cupriavidus sp. USMAA1020	γ-butyrolactone	(Amirul *et al.*, 2008)
	γ-butyrolactone, 4-hydroxybutyric acid, 1,4-butanediol	(Amirul *et al.*, 2009)
	γ-butyrolactone, 1,4-butanediol, 1,6-hexanediol	(Huong *et al.*, 2013)
Cupriavidus sp. USMAHM13	Glycerine pitch and 1,4-butanediol	(Hema and Amirul, 2013, 2014)
	γ-butyrolactone, glycerine pitch, simple sugars, glycerol derivatives, palm oils, fatty acids	(Hema and Amirul, 2012)
Cupriavidus sp. USMAA2-4	γ-butyrolactone, 4-hydroxybutyric acid, ω-alkanediols	(Chai et al., 2009)
	Oleic acid and 1,4-butanediol	(Rahayu *et al.*, 2008)
	γ-butyrolactone and 1,4-butanediol	(Vigneswari *et al.*, 2009b; Vigneswari *et al.*, 2009a)
Delftia acidovorans	1,4-butanediol and glucose	(Lee *et al.*, 2007)
	Glucose, saccharose, fructose, glutaric acid, n-butyric acid, 4-hydroxybutyric acid, 1,4-butanediol, γ-butyrolactone	(Hsieh *et al.*, 2009)
Hydrogenophaga pseudoflava	γ-butyrolactone and glucose	(Choi *et al.*, 1999)
Alcaligenes latus	3-hydroxybutyric acid and 4-hydroxybutyric acid	(Kang *et al.*, 1995)
Comamonas testosteronii	n-butyric acid and 1,4-butanediol	(Mitomo *et al.*, 2001)

2.5 Biosynthesis of P(3HB-co-4HB) copolymer using 1,8-octanediol

1,8-octanediol is a 4HB carbon precursor that is suitable for biosynthesis of PHA. According to Steinbüchel and Eversloh (2003), ω-alkanediols with greater carbon chain length, especially those with an even number of carbon atoms of more than 8, seem not to necessarily be oxidized to the ω-hydroxyfatty acid and to convert to 4HB-CoA through β-oxidation cycles. This ω-alkanediol has been tested in *Cupriavidus* sp. USMAA1020 by Amirul (2008) and *Cupriavidus* sp. USMAA2-4 (Rahayu *et al.*, 2008; Fahima *et al.*, 2011).

There are limited publications that studied the ability of ω-alkanediols with greater even number of carbon atoms to produce PHA. Production of P(3HB-*co*-4HB) copolymer from 1,8-octanediol by *Cupriavidus necator* (formerly known as *Wautersia eutropha, Ralstonia eutropha, Alcaligenes eutrophus and Hydrogenomonas eutropha*) has been studied (Saito *et al.*, 1996). Furthermore, ω-alkanediols with greater carbon chain length, but in an even number of carbon atoms are suitable precursor substrates for the biosynthesis of the P(3HB-*co*-4HB) copolymer with high 4HB fraction.

An early study by Amirul (2007) revealed the effect of different carbon sources towards the production of PHA at 0.56% of carbon concentration. It showed that ω-alkanediols with long carbon chain length like 1,8-octanediol, 1,10-decanediol and 1,12-dodecanediol did not enhance the accumulation of PHA even though 1,12-dodecanediol produced the highest PHA concentration of 3.93 g/L.

It is proposed that ω-alkanediols like 1,8-octanediol, 1,10-decanediol and 1,12-dodecanediol caused in a skewed PHA composition (Chai *et al.*, 2009). Among these three carbon sources, 1,8-octanediol produced the highest 4HB composition but lowest cell dry weight (CDW) and PHA content for P(3HB-*co*-4HB) copolymer

produced from *Cupriavidus* sp. USMAA2-4 by two-stage cultivation (Chai *et al.*, 2009). Therefore, 1,8-octanediol was further studied to improve the overall PHA concentration.

There were few studies done to elucidate on the capability of *Cupriavidus* sp. USMAA2-4 and *Cupriavidus* sp. USMAA1020 to produce P(3HB-*co*-4HB) copolymer using 1,8-octanediol as a carbon precursor. A recent study proposed that biosynthesis of the P(3HB-*co*-4HB) copolymer by *Cupriavidus* sp. USMAA2-4 resulted in high 4HB portion but lowest in PHA concentration and residual cell dry weight (RCDW) when 1,8-octanediol was used as 4HB precursors (Nurhezreen, 2013). In another study by Nurhezreen and Amirul (2013), 1,8-octanediol produced the lowest CDW and PHA content at C/N ratio 20 using one-stage cultivation, suggesting that the C/N ratio used had inhibited the bacterial growth.

Feeding of sole 1,8-octanediol by *Cupriavidus* sp. USMAA1020 resulted in the lowest CDW and PHA content of P(3HB-*co*-4HB) copolymer (Vigneswari *et al.*, 2009a). It also resulted in the accumulation of copolymer with low compositions of 4HB units. Thus, it can be assumed that 1,8-octanediol is capable of producing P(3HB-*co*-4HB) copolymer but in low biomass and overall PHA production. It is suggested that the production of P(3HB-*co*-4HB) copolymer using 1,8-octanediol should be further studied in an optimized condition.

2.6 Biosynthesis of P(3HB-*co*-4HB) copolymer through fed-batch fermentation

Batch fermentation is considered to be a closed system, where there is no addition or removal of media, nutrients and cells into the culture medium. In exclusion, only oxygen, antifoam agent and acid or base was added to control the foaming and pH. In contrast, fed-batch fermentation was carried out by the addition of critical elements such as fresh media and substrates intermittently depending on the feeding strategy (Chen, *et al.*, 2001). Meanwhile, continuous fermentation is a process where the critical elements were added continually with the removing of the culture medium until the end of fermentation process.

For production of PHAs, there are many advantages of fed-batch fermentation over batch fermentation. Fed-batch fermentation offers higher yields and productivity of PHA which demonstrated by Lenczak *et al.* (2013) trough manipulation of feeding strategy. The production of highest possible amount of PHA is then followed by balanced cell growth and PHA accumulation thus reducing the production cost (Lee and Choi, 1998). Batch fermentation is not a preferred method for PHA production because some organism cannot grow in nitrogen restricting condition. In fed-batch fermentation, the growth limiting substrates such as carbon and nitrogen sources can be controlled. Thus, this is an ideal method for PHA production as it only occurs under nutrient restriction but surplus of carbon source (Doi, 1990). In addition, the production of by products can be controlled.

Production of P(3HB-*co*-4HB) have been employed through fed-batch fermentation using several feeding methods (Hsieh *et al.*, 2009; Kim *et al.*, 2005; Park and Kim, 2011). Different feeding strategies were tested using different types of

carbon sources and bacterial strain. The most common methods used are pO_2 cascade control and DO-stat strategy.

Park and Kim (2011) reported the production of 6 to 10mol% of 4HB composition by *Ralstonia Eutropha* in addition of 20 g/L of soy bean oil and 10 g/L of γ-butyrolactone. However, the cell biomass and PHA yield decreased when supplemented with high γ-butyrolactone. In other experiment conducted by Kim *et al.*(2005), production of P(3HB-*co*-4HB) were tested using two feeding strategy (DO-stat and constant feeding) via *Cupriavidus necator*. At first, DO-stat feeding of sucrose as carbon source was used to grow cells to in the range of 38 to 48 g/L. During nitrogen limitation condition, the fructose feed was changed to combination of fructose and γ-butyrolactone to induce PHA accumulation. The agitation speed was varied while the dissolved oxygen was maintained above 20%. Increase in the ratio of γ-butyrolactone resulted in increase of 4HB composition from 1.64 to 25.2 mol%. Meanwhile, the cell biomass, PHA content and productivity decreased from 49.1 g/L to 33.6 g/L, 24.4 to 14.0 g/L and 0.55 to 0.35 g/L/h, respectively.

2.7 Molecular weight of PHAs

Molecular weight (M_w) determines many physical properties of the polymer produced (Tokiwa *et al.*, 2009). A study by Saito *et al.* (1996) showed that high molecular weight P(3HB-*co*-4HB) copolymer from 0 to 100% of 4HB monomer composition can be easily produced hence the degradation rate can also be controlled. A year later, an ultra-high molecular weight (UHMW) P(3HB) of 20 MDa was reported as the largest M_w value for biologically synthesized PHA (Kusaka *et al.*, 1997). UHMW polymer is an extremely long chains polymer with molecular

weight higher than 10 million Dalton (MDa). The longer the polymer chains, the higher the polymer monomer units.

Molecular weight is the most important characteristic of a polymer. Therefore, the molecular mass of the pure copolymer is determined by gel permeation chromatography (GPC). GPC can be used to determine the molecular weight (M_w), the number average molecular weight (M_n) and polydispersity index (M_w/M_n) of a polymeric material. The polydispersity index (M_w/M_n) is an estimation of the width of the molecular weight distributions in the polymer (Rogošić *et al.*, 1996).

UHMW and high molecular weight value is a promise for the polymer to be useful. High molecular weight polymer ranges from 1 MDa and above while medium molecular weight polymer consists of 500 kilo Dalton (kDa) to 999 kDa molecular weight of the polymer. Meanwhile, a polymer with lower than 500 kDa molecular weight is considered as low molecular weight polymer. Normally, most of the polymer produced are low molecular weight polymer.

For instance, high molecular weight polymers are suitable candidates for implants and tablet makings (Martin and Williams, 2003). Besides that, P(3HB) with molecular weight more than 3 MDa can be processed into strong fibers and films (Hiroe *et al.*, 2012). Moreover, a recent study showed that P(3HB) with high molecular weight of 1.14 MDa can be applied as scaffolds for nerve cells (Peña *et al.*, 2014). On the other hand, high molecular weight polymer have lower degradation rates compared to low molecular weight polymer.