BIOSYNTHESIS AND CHARACTERISATION OF POLYHYDROXYBUTYRATE [P(3HB)] PRODUCED BY *Cupriavidus necator* H16 FROM WASTE COOKING OIL

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
RM	Ringgit Malaysia
$	Dollar
±	Plus-minus
x g	Times gravity
β	Beta
˚C	Degree Celcius
˚C/min	Degree Celcius per minute
ΔH_m	Heat of fusion
µg	Microgram
µL	Microliter
µM	Micromolar
µm	Micrometer
µmol	Micromole
^{1}H	Proton
^{13}C	Carbon-13
3HB-CoA	3-hydroxybutyryl-CoA
ANOVA	Analysis of variance
A_{3HB}	Area of 3HB monomer peak
A_{CME}	Area of CME peak
ATP	Adenosine triphosphate
AV	Acid value
BOD	Biological oxygen demand

C	Carbon atom
COD	Chemical oxygen demand
C/N	Carbon to nitrogen
C12:0	Lauric acid
C16:0	Palmitic acid
C14:0	Myristic acid
C18:1	Oleic acid
C18:2	Linoleic acid
C18:3	Linolenic acid
$CaCl_2$	Calcium chloride
$CDCl_3$	Deuterated chloroform
$CHCl_3$	Chloroform
CH	Methine group
CH_2	Methylene group
CH_3	Methyl group
CH_3COOH	Acetic acid
cm	Centimeter
cm^{-1}	Reciprocal centimeter
CME	Caprylic methyl ester
CO_2	Carbon dioxide
CoA	Coenzyme-A
CoA-SH	Coenzyme-A with sulfhydryl functional group
$CoCl_2.6H_2O$	Cobalt (II) chloride hexahydrate
CPKO	Crude palm kernel oil
CPO	Crude palm oil

$CrCl_3.6H_2O$	Chromium chloride hexahydrate
$CuSO_4.5H_2O$	Copper sulfate pentahydrate
Da	Dalton
DAG	Diacylglycerol
DCW	Dry cell weight
dH_2O	Distilled water
DSC	Differential scanning calorimetry
DMSO	Dimethyl sulfoxide
EU	European Union
E_a	Activation energy
FAD	Flavin adenine dinucleotide
FADH	Reduced flavin adenine dinucleotide
FAO	Food and Agriculture Organization
FCO	Fresh cooking oil
$FeCl_3$	Iron (II) chloride
FFA	Free Fatty Acid
FT-IR	Fourier transform mid-infrared spectrometry
g	Gram
GC	Gas chromatography
GPC	Gel permeation chromatography
GPa	Gigapascal
h	Hour
ha	Hectare
HA	3-(*R*)-hydroxyalkanoic acids
HCl	Hydrochloric acid

HDL	High density lipoprotein
HA-CoA	Hydroxyacyl-CoenzymeA
H_2O	Water
H_2SO_4	Sulphuric acid
I	Iodine
IV	Iodine value
J	Joule
J/g	Joule per gram
K	Gas chromatography factor
Kg	Kilogram
K_{3HB}	3-hydroxybutyrate factor
KBr	Potassium bromide
KCl	Potassium chloride
kDa	Kilodalton
kg	Kilogram
KH_2PO_4	Potassium dihydrogen phosphate
KOH	Potassium hydroxide
kPa	Kilopascal
L	Liter
LDL	Low-density lipoprotein
Ltd.	Limited
M	Molar
MAG	Monoacylglycerols
Meq	Milliequivalents
MUFA	Monounsaturated fatty acid

Mg	Magnesium
M_n	Number-average molecular weight
M_w	Molecular weight
M_w/ M_n	Polydispersity index
mcl-	Medium-chain-length
mcl-PHA	Medium-chain-length PHA
MHz	Megahertz
min	Minute
mg	Milligram
mg/L	Milligram per liter
Mg	Magnesium atom
$MgSO_4$	Magnesium sulphate
$MgSO_4.7H_2O$	Magnesium sulphate heptahydrate
mL	Milliliter
ml/L	Milliliter per liter
mL/min	Milliliter per minute
mm	Millimeter
mm/min	Millimeter per minute
mm^3	Millimeter cube
mM	Millimolar
MM	Mineral medium
MPa	Megapascal
mol%	Mole percent
N	Normality
NAD^+	Nicotinamide adenine dinucleotide

NADH	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
ng	Nanogram
nm	Nanometer
NMR	Nuclear magnetic resonance
NR	Nutrient rich
OD	Optical density
OD_{600}	Optical density at wavelength of 600 nm
OD_{410}	Optical density at wavelength of 410 nm
P/S	Polyunsaturated and saturated ratio
PAV	*p*-Anisidine value
PBS	poly(butylene succcinate)
PCL	poly(Ɛ-caprolactone)
PDI	Polydispersity index
ppm	Parts per million
psi	Pounds per square inch
PV	Peroxide value
P(3HB)	Poly(3-hydroxybutyrate)
P(3HB-*co*-3HV)	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)
P(3HB-*co*-3HHx)	Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate)
P(3HB-*co*-3MP)	Poly(3-hydroxybutyrate-*co*-3-mercaptopropionic)
P(3HB-*co*-4HB)	Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate)

PE	Polyethylene
PET	Poly(ethylene terephtalate)
PUFA	Polyunsaturated fatty acid
PHA	Polyhydroxyaalkanoate
PhaA	*β* -ketothiolase
PhaB	NADPH-dependent acetoacetyl-CoA dehydrogenase
PhaC	PHA synthase
PhaG	3-hydroxyacyl-ACP-CoA transferase
PLA	Polylactide
*p*NPL	*p*-nitrophenyl laurate
PVA	Polyvinyl alcohol
PAO	Palm acid oil
PKO	Palm kernel oil
PO	Palm olein
PP	Polypropylene
PPDO	poly(p-dioxanone)
PS	Palm stearin
PTFE	Polytetrafluoroethylene
PV	Peroxide value
rpm	Revolutions per minute
(R)-	*Rectus*- isomer
(S)-	*Sinister*- isomer
scl-	Short-chain-length
scl-PHA	Short-chain-length PHA

SFA	Saturated fatty acid
sp.	Species
TAG	Triacylglycerols
TCA	Tricarboxylic acid
TEM	Transmission electron microscope
TGA	Thermogravimetric analysis
TLC	Thin layer chromatography
Tukey's HSD	Tukey's honestly significant difference
T_d	Decomposition temperature
T_g	Glass transition temperature
T_m	Melting temperature
U	One unit of enzyme activity
UV	Ultraviolet
UV-Vis	Ultraviolet-visible
V	Volt
v/v	Volume per volume
WCO	Waste cooking oil
wt%	Dry weight percent
w/v	Weight per volume
w/w	Weight per weight
yr	Year

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BIOSINTESIS DAN PENCIRIAN POLIHIDROKSIBUTIRAT [P(3HB)] DARIPADA SISA MINYAK MASAK MENGGUNAKAN *Cupriavidus necator* H16

ABSTRAK

Lambakan sisa plastik sintetik yang mencemarkan alam sekitar telah memberi tekanan untuk menggunakan plastik boleh reput sebagai material alternatif. Polihidroksialkanoat (PHA): Polihidroksibutirat [P(3HB)] mempunyai kelebihan untuk reput secara semulajadi dan tidak membahayakan alam sekitar. Penghasilan PHA daripada sumber karbon yang mahal atau bahan makanan (cth: minyak kelapa sawit atau gula) telah membebankan perkembangan industri ini. Oleh itu, penggunaan sisa minyak masak (WCO) sebagai sumber bahan mentah yang murah dan boleh diperbaharui bersesuaian untuk digunakan dalam penghasilan P(3HB). Sebelum analisis pencirian dan penghasilan P(3HB) daripada WCO, WCO hendaklah dikumpul daripada komuniti tempatan. WCO daripada beberapa tempat telah dianalisa secara proksimat dan fisikokimia sebelum proses biosintesis P(3HB) menggunakan *Cupriavidus necator* H16 dijalankan. Minyak masak segar (FCO) telah digunakan sebagai perbandingan. Analisa bagi komposisi pigmen, metil ester asid lemak (FAME), suhu pencairan dan suhu kristal, asid lemak bebas (FFA), dan nilai iodin (IV) memberikan perbezaan antara WCO dan FCO. Keputusan menunjukkan semua bahan sampingan dalam sampel-sampel WCO mempunyai kadar yang lebih tinggi berbanding FCO. Ini adalah berikutan reaksi yang telah berlaku dalam proses penggorengan. Analisa FAME menunjukkan komposisi tepu bagi asid lemak palmitik, C16:0 telah meningkat dalam lingkungan 39% hingga 45% dan komposisi tidak tepu bagi asid linoleik, C18:2 telah menurun dari 11% hingga

7%. Paten yang sama telah diperhatikan bagi IV, yang mana FCO memperoleh 56 g I_2/100g dan IV untuk WCO adalah lebih rendah iaitu 44-54 g I_2/100g. Ini menunjukkan WCO mempunyai lebih banyak ikatan tepu berbanding FCO. Setiap sampel WCO telah digunakan untuk penghasilan P(3HB). Urea dengan kepekatan 1 g/L dan 12.5 g/L minyak masak telah ditambah ke dalam medium mineral sebelum tempoh inkubasi selama 48 jam untuk pembiakan sel dan penghasilan P(3HB). *Cupriavidus necator* H16 telah menghasilkan 60-80 wt% P(3HB) dengan berat sel kering (DCW) 14-17 g/L. Dicadangkan bahawa peratusan asid lemak bebas yang telah sedia ada (0.3-0.6%) merangsang penghasilan P(3HB). Pemerhatian subjektif daripada lapisan nipis kromatografi menunjukkan bahawa asid lemak bebas telah tersedia ada ketika jam sifar dalam medium mineral yang telah dibekalkan WCO dan ia juga telah digunakan oleh *Cupriavidus necator* H16 secara efisien pada jam ke-36. Prestasi FCO sebagai bekalan karbon adalah tidak setanding WCO. Dalam FCO, asid lemak bebas hanya terbentuk pada jam ke-12 yang mana ia tidak terurai sepenuhnya sehingga tempoh inkubasi tamat. Efisiensi WCO sebagai sumber karbon bagi penghasilan P(3HB) juga telah disokong melalui analisis aktiviti lipase. Jumlah lipase yang banyak (24 U/mL) telah dapat dikesan pada jam ke-12 inkubasi, manakala aktiviti lipase bagi FCO mula meningkat kepada tahap yang lebih tinggi pada jam ke-36 iaitu sebanyak 14 U/mL. Suhu pencairan, suhu kristal, dan daya regangan bagi P(3HB) yang terhasil adalah hampir sama dengan hasil penyelidikan sebelum ini yang mana masing-masing memberikan keputusan 173 °C, 60 °C, dan 37 MPa. Berat purata bagi berat molekular adalah 1.8×10^6 g mol^{-1} dan penyebaran polimer (PDI) adalah 2.74 apabila WCO digunakan sebagai sumber karbon. Sebagai kesimpulan, WCO merupakan alternatif sesuai dan sumber karbon yang boleh diperbaharui untuk digunakan dalam biosintesis P(3HB).

BIOSYNTHESIS AND CHARACTERISATION OF POLYHYDROXYBUTYRATE [P(3HB)] PRODUCED BY *Cupriavidus necator* H16 FROM WASTE COOKING OIL

ABSTRACT

Accumulation of synthetic plastic waste that pollutes the environment has urged the need to use biodegradable polymers as substitute materials. Polyhydroxyalkanoates (PHA): poly(3-hydroxybutyrate) [P(3HB)] has the ability to bio-degrade and is environmentally friendly. The production of PHA from high price carbon source or food source (e.g. palm oils or sugars) has hampered the development of this industry. Waste cooking oil (WCO) is a low cost feedstock that comes from renewable sources and is suitable for the production of P(3HB). Prior to the characterisation and synthesis of P(3HB) from WCO, the WCO has to be collected from local community. WCO from several locations was characterised via proximate and physicochemical analysis prior to biosynthesis of P(3HB) using *Cupriavidus necator* H16. Fresh cooking oil (FCO) was used as the comparison. The analyses of pigments content, fatty acid methyl ester (FAME), melting and crystallisation temperatures, free fatty acid (FFA), and iodine value (IV) indicate difference between WCO and FCO. It was shown that the by-products in all WCO are higher as compared to those in FCO. This is due to the reactions that occur during the frying process. FAME shows that the saturated composition of palmitic acid in WCO, C16:0 was increased within range of 39% to 45% and unsaturated composition of linoleic acid, C18:2 were reduced from 11% to 7%.A similar trend was observed in IV, in which FCO obtained was 56 g I_2/100g while the IV of WCO was lower at 44-54 g I_2/100g. This means that WCO contained more saturated bonds compared to FCO. All WCO

samples were utilised for P(3HB) synthesis. A concentration of 1 g/L urea and 12.5 g/L of cooking oil were added to mineral medium prior to incubation period of 48 h for cell growth and P(3HB) production. *Cupriavidus necator* H16 accumulated 60-80 wt% P(3HB) with a dry cell weight (DCW) of 14-17 g/L. It was suggested that the high percentage and readily available FFA (0.3-0.6%) enhanced the production of P(3HB). Subjective observation from thin layer chromatography showed that FFA was readily available at 0 h in mineral medium that was fed with WCO, and was efficiently consumed by *Cupriavidus necator* H16 at 36 h. The performance of FCO as carbon feedstock to *Cupriavidus necator* H16 was not comparable to that of WCO. In FCO, the FFA was formed only at 12 h and was not totally degraded until the end of incubation period. The efficiency of WCO as the carbon source for P(3HB) production was also supported by the analysis of lipase activity. Lipase activity shows high amount of lipase (24 U/mL) was detected at 12 h of incubation, while the lipase activity in FCO accelerated at 36 h was 14 U/mL. The melting temperature, crystallisation temperature, and tensile strength of synthesised P(3HB) was comparable to the values reported in literature, which was 173 °C, 60 °C, 37 MPa, respectively. Weight average molecular weight of 1.8 x 10^6 g mol^{-1} and polydispersity (PDI) of 2.74 were obtained when WCO was used as the carbon source. In conclusion, WCO is an alternative and renewable carbon source for biosynthesis of P(3HB).

1.0 INTRODUCTION

Excessive disposal of petroleum-based polymers in environment has raised the awareness of researchers to figure out alternative materials which are biodegradable, environmentally friendly and have similar characteristics as the current synthetic polymers. The presence of these artificial plastic as a deleterious factor to aquatic population and a cause of environment disasters, have raised the attention of the communities that concern. However, the durability and stability of the plastic is undeniable, but its inability to degrade demands the substitution of this material to an environmental-friendly plastic such as polyhydroxyalkanoates (PHA) which, have the closest similarities to petroleum plastic (Loo and Sudesh, 2007; Park and Kim, 2011).

PHA is one of the many biopolymers that possess not only biodegradability and non-toxicity, but are also thermoplastics, moldable, and flexible for numerous applications ranging from stiff packaging to highly elastic materials for coatings (Doi, 1990; Sudesh *et al.*, 2000). The complete degradability of PHA and its renewable carbon sources that come from either sugars, plant oils or vegetables oil differentiate itself over the preference to synthetic petroleum-based polymers (Loo and Sudesh, 2007). PHA is produced by microorganisms in the form of granules when there is a depletion of essential nutrients such as nitrogen, phosphorus, and magnesium and excess amount of carbon source (Sudesh *et al.*, 2000; Rao *et al.*, 2010). PHA is known as a non-toxic polymer (Doi, 1990). However, interests to commercialize PHA have been hampered since few years ago due to the unusually high production cost (Lee *et al.*, 1999). The entire process of PHA production has been analysed, which includes substrate selections, type of strains, progress of fermentation strategies, and recovery processes (Akaraonye *et al.*, 2010; Park and

Kim, 2011). The main contribution to higher production cost of PHA as compared to that of petrochemical-synthetic polymers is carbon source, which is approximately half part of the capital (Song *et al.*, 2008). The cost of PHA was reported to be approximately $4-8/kg, while the cost of petrochemical-synthetic polymers was reported to be $0.6-0.9/kg (Liu *et al.*, 1998; Song *et al.*, 2008; Akaraonye *et al.*, 2010)

Vegetable oils such as palm olein, besides being a favorable and inexpensive choice in various bio-products, offer excellent conversion in the synthesis of PHA. It is due to higher number of carbon atoms per weight as compared to that of, for instance, sugar. It was reported that microorganism utilizing vegetable oils produced 1.0 g-PHA/g-vegetable oils while the microorganism utilizing sugars produced 0.32-0.48 g-PHA/g-sugar used (Yamane, 1992; Akiyama *et al.*, 2003; Kahar *et al.*, 2004).

Although the utilization of vegetable oils in PHA production show good prospect (such as high conversion, abundance and renewable) (Loo *et al.*, 2005), consumption of this food-grade oil may cause imbalance and depletion to the food supply worldwide. It will form a competition in human food-chain in order to convert the food-grade vegetable oils to bioplastics (Ng *et al.*, 2010). An alternative route to address this issue was then proposed by using the waste vegetable oils or commonly known as waste cooking oil (WCO) in PHA production (Song *et al.*, 2008). WCO can be easily collected from different household communities therefore it serves as a good choice of carbon source for PHA production (Wiltsee, 1998; Zhang *et al.*, 2003). Besides, improper disposal of WCO into drainage and river systems causes danger and extinction to aquatic life (Akaraonye *et al.*, 2010). This can be detected when there is an increase of chemical oxygen demand (COD) and biological oxygen demand (BOD) within aquatic environment.

The amount of WCO accumulation increases from year to year and it varies from country to country. The approximate amount of WCO accumulation in EU was 700,000 – 1,000,000 tonnes/yr. In terms of general health, the use of WCO in cooking activities leads to global health risks. The chemical compositions of WCO are comprised of peroxides, aldehydes, *trans*-fatty acids and other by-products that may stimulate various kinds of diseases in the long-term period (Kulkarni and Dalai, 2006).

Thus, in this study, *Cupriavidus necator* H16 was used to produce a common type of PHA, polyhydroxybutyrate [P(3HB)] by utilizing WCO which is palm olein-based as the carbon source. The aim is to evaluate the characteristics of fresh cooking oil (FCO) (palm olein-based) and different sources of WCO which appear to have been less studied concerning the detailed characteristic, and to evaluate the potential of WCO as the carbon source for PHA biosynthesis.

1.1 Objectives

The aims of this study are:

1. To evaluate the efficiency of polyhydroxybutyrate [P(3HB)] production from *C. necator* H16 by using waste cooking oil (WCO) as the carbon (C) source.
2. To maximize the production of P(3HB) with the selected parameters.
3. To characterize different types of WCO sources and to understand the effects of it towards the P(3HB) production.
4. To characterize the P(3HB) produced from WCO as the C source and to evaluate the characteristics of the P(3HB) produced.

2.0 LITERATURE REVIEW

2.1 Biodegradable polymers

Biodegradable polymers (or often called as biopolymers) are defined as polymers that can be fully-degraded in environment and do not cause any negative side effects to the surrounding flora and fauna. There are many examples of bio-based and natural polymers, such as polyhydroxyalkanaotes (PHA), polylactide (PLA), poly(*ε*-caprolactone) (PCL), poly(*p*-dioxanone) (PPDO), poly(butylene succcinate) (PBS), natural fibres, hydrogels, starch cellulose, chitin, chitosan, and lignin (Tsuge, 2002).

In general, biopolymers can be divided into three groups which consist of bio-chemosynthetic polymers, biosynthetic polymers, and modified natural polymers. Firstly, bio-chemosynthetic polymers are produced by chemical polymerisation of monomers derived from biological processes, for example PLA and PBS. These polymers are sold in the United States of America and Japan with the trade names of NatureWorks®, Hycail HM; Hycail LM, Lacea®, U'z, GS Pla and Bionolla. Secondly, biosynthetic polymers are produced from microorganisms through biosynthesis, such as PHA. PHA are sold under the trade names of Biomer®, Mirel™. Biogreen®, Biocycle®, and Biopol®. The last group, modified natural polymers, consists of starch and cellulose derivatives. These polymers are available under the trade names of Mater-Bi®, Solanyl®, BIOpar®, Cornpol®, and Cellgreen. They have better properties that might be suitable as bio-based polymers (Sudesh and Iwata, 2008).

It is well known that not all bio-based polymers are biodegradable. This includes PLA, polythioesters and cellulose ester derivatives. PLA can be hydrolysed

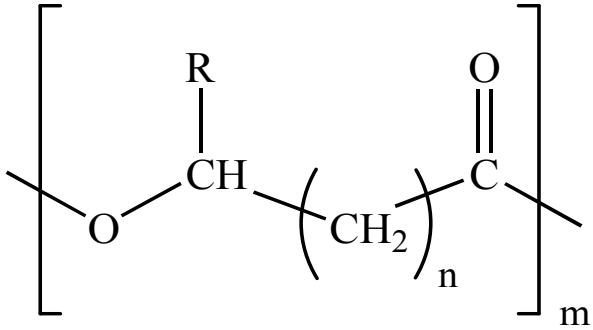
in water but it is not biodegradable, while the modified natural polymers can only be enzymatically degraded (Sudesh and Iwata, 2008). Contrary to PHA, it undergoes both hydrolysis and enzymatically reactions. Besides, Woolnough and coworkers reported that PHA has the shortest period of degradation (40-50 days) as compared to other bio-based polymers (Woolnough *et al.*, 2010). Having such unique properties, PHA are among the material of interests chosen to replace petrochemical synthetic polymers (Sudesh *et al.*, 2000; Sudesh and Doi, 2000; Tsuge, 2002).

2.2 PHA – overview

Existence of PHA in microorganisms was known since 1926 (Sudesh and Doi, 2000). It was identified as poly(3-hydroxybutyrate) [P(3HB)] in *Bacillus megaterium* by Maurice Lemoigne (Doi, 1990). These plastics are detrimental to environment if they were discarded without a proper procedure. PHA are produced inside the cytoplasm of microorganisms when there is a depletion of nutrients such as nitrogen, phosphorus, and magnesium and excess of carbon source (Anderson and Dawes, 1990; Doi, 1990; Sudesh *et al.*, 2000).

Following this discovery, other copolymers were subsequently reported. Some PHA such as P(3HB), poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)], poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)], and poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] have similar mechanical properties to that of artificial non-degradable plastics such as poly(propylene) (PP), poly(ethylene) (PE), and poly(styrene) (PS) (Sudesh and Iwata, 2008).

PHA consist of 3-(*R*)-hydroxyalkanoic acids (HA) linked by hydroxyl and carboxyl groups of an adjacent monomer. The chemical structure of PHA is shown in Figure 2.1. 'R' refers to the length of the side chain and 'n' refers to the size of the alkyl group. Both 'R' and 'n' determine the type of HA monomeric unit (Loo and Sudesh, 2007), the physical/chemical properties of chain length, type of functional group and degree of unsaturated bonds. Higher degree of unsaturation increases the flexibility of a polymer. Different functional groups change the physical and chemical properties of a polymer (Yunus *et al.*, 2008).



	R	**Monomer unit**
n = 1	hydrogen	3-hydroxypropionate
	methyl	3-hydroxybutyrate
	ethyl	3-hydroxyvalerate
	propyl	3-hydroxyhexanoate
	pentyl	3-hydroxyoctanoate
	nonyl	3-hydroxydodecanoate
n = 2	hydrogen	4-hydroxybutyrate
	methyl	4-hydroxyvalerate
n =3	hydrogen	5-hydroxyvalerate
	methyl	5-hydroxyhexanoate
n = 4	hexyl	6-hydroxydodecanoate

Figure 2.1: General structure of PHA (modified from Lee, 1996) (Lee, 1995)

According to the length of monomer, PHA can be divided into two categories, short chain length (SCL) and medium chain length (MCL) PHA (Doi, 1990; Sudesh and Doi, 2005). SCL-PHA consists of HA monomer unit equal to or less than five carbon atoms. This includes 3-hydroxybutyrate and 3-hydroxyvalerate.

MCL-PHA consists of at least six to fourteen carbon atoms, for example 3-hydroxyhexanoate and 3-hydroxytetradecanoate (Loo and Sudesh, 2007; Yunus *et al.*, 2008).

SCL-PHA are commonly produced in *Cupriavidus necator* H16. This is due to its specificity of PHA synthase from sugars, fatty acids, and other carbon sources as the substrate. Meanwhile, MCL-PHA are naturally produced by *Aeromonas caviae*, *Nocardia coralline* etc. (Sudesh and Abe, 2010). A more complete classification of PHA is shown in Table 2.1.

Table 2.1: Classification of PHA according to different criteria (modified from Loo and Sudesh, 2007) (Loo and Sudesh, 2007)

Classification according to	**Explanations**
Biosynthetic origin	1. Natural PHA: produced naturally by microorganisms from general substrates, i.e. P(3HB) 2. Semi-synthetic PHA: addition of unusual precursors (e.g. 3-mercaptopropionic acid) to promote the biosynthesis of poly(3-hydroxybutyrate-*co*-3-mercaptopropionic) [P(3HB-*co*-3MP)]
Monomer size	1. SCL-PHA (as explained in the text) 2. MCL-PHA
Number of different monomers in PHAs	1. Homopolymer: polymerisation begins with the linkage of a small monomer through ester bonds to the carboxylic group of the next monomer. A homopolymer is produced when single monomeric units are linked together, i.e. P(3HB) 2. Heteropolymer: two or more monomeric units linked together, i.e. P(3HB-*co*-4HB)
Chemical nature of the monomers	1. PHA containing aliphatic fatty acids, i.e. P(3HB) 2. PHA containing aromatic fatty acids 3. PHA containing aliphatic and aromatic fatty acids, i.e. P(3HB-*co*-3MP)

At present, more than 150 PHA monomers had been reported (Sudesh *et al.*, 2000; Sudesh *et al.*, 2011). The diversity of PHA monomers varies depending on substrate specificity of PHA synthase and carbon sources, which in turn influence the

metabolic pathways of microorganisms (Loo and Sudesh, 2007). PHA synthases are usually highly stereospecific, and they can only polymerise (*R*) enantiomer of HA monomer (Sudesh and Abe, 2010). During polymerisation, PHA synthase determines the sizes of monomers. In other words, it influences the final molecular weight of PHA (Loo and Sudesh, 2007).

Different carbon sources can be used to produce PHA, for example sugars, whey, molasses, starch, or triacylglycerol. Many studies agreed that plant oils gave 2 folds higher PHA conversion (0.6-0.8 g PHA/g plant oil) as compared to that of sugars (0.3-0.4 g PHA/g sugar) (Yamane, 1992; Akiyama *et al.*, 2003). These include non-edible oils (e.g. jatropha oil, crude palm kernel oil, and palm oil mill effluent), and vegetable oils (e.g. palm oil, soy bean oil, olive oil, and rapeseed oil) (Akaraonye *et al.*, 2010). Vegetable oils are renewable and can be easily obtained in large quantity. Besides, they can be converted efficiently by microorganisms. When microorganisms are fed with vegetable oils, the oils are converted into PHA through *de novo* fatty acid, ß-oxidation, and PHA cycle, in the condition that is excess of carbon (C), and limited of nitrogen (N) (Loo and Sudesh, 2007).

Based from the record of PHA production, up to 90 wt% of dry cell weight (DCW) can be obtained from shake-flask fermentation (Sudesh and Abe, 2010). Despite its high efficiency in shake-flask, careful considerations (e.g. effective feeding strategies, cheaper carbon sources and robust engineered microorganisms) are needed in large scale fermentation. The cost of carbon sources contributes approximately 28-50% of the total production cost (Sudesh and Abe, 2010). PHA has been commercialised by several companies such as Monsanto, Zaneca, Chimie Linz and others have been commercializing PHA for many years. However, the challenge of expensive production cost, which is within range of $9/kg have hampered the

marketing development (Sudesh *et al.*, 2000; Yaoping, 2007). Thus, it is important thorough research of utilizing type of strain which produce high yield production been used, good fermentation strategies, consumption of inexpensive C source should be done in order to reduce the selling price of PHA (Anderson and Dawes, 1990).

As mentioned, PHA are accumulated in the cytoplasm of microorganisms. They exist as amorphous polymers in the form of water-insoluble granules (Dawes, 1988; Anderson and Dawes, 1990; Doi, 1990). The granules can be observed using a phase-contrast microscope (equipped with oil immersion objective lens at magnification of 100×). Due to the difference in density between PHA and cytoplasm, PHA appear to be light refractive inclusions while the cytoplasm appears to be dark (Sudesh and Abe, 2010). It should be noted that certain biological features such as spores may appear to be actively refractive or sensitive towards lights. This can be differentiated by staining method. A common method to colourise lipid inclusion body (PHA granules) is by using an oxozine dye (such as Nile blue A and Sudan black) (Loo and Sudesh, 2007). Nile blue A has a greater affinity and higher specificity towards PHA as compared to Sudan black, and it does not stain glycogen and polyphosphate (Dawes, 1988). Under ultraviolet (UV) light microscope, PHA granules appear to be fluorescence bright orange (Sudesh and Abe, 2010). More detailed formation of PHA granules is given later in Section 2.3.1.

PHA undergoes total biodegradation and are non-toxic (Sudesh *et al.*, 2000; Amirul *et al.*, 2007; Yaoping, 2007). PHA can be degraded into carbon dioxide and water (in aerobic condition) and methane (in anaerobic condition) in diverse habitats such as soil, sea, stagnant water or sewage water (Lee, 1995; Sudesh *et al.*, 2000).

2.3 Biosynthesis of PHA

In normal practice, PHA are produced when carbon sources in the culture medium are in excess and other nutrients such as oxygen, nitrogen, phosphorous, sulphur or magnesium are depleted. Carbon sources are assimilated, converted into HA compounds and finally polymerised into high molecular weight PHA and stored as water-insoluble granules in the cell cytoplasm (Amirul *et al.*, 2007; Loo and Sudesh, 2007).

2.3.1 Initiation and formation of PHA granules

PHA granules are surrounded by phospholipids monolayer. The layer accommodates specific granule-associated proteins such as PHA synthase, depolymerases, structural proteins, regulator proteins and cytosolic proteins (Brockelbank *et al.*, 2006; Loo and Sudesh, 2007). PHA in cytoplasm do not disturb the osmotic pressure of the cell (Rehm and Steinbüchel, 2005; Loo and Sudesh, 2007). Depending on the species of bacteria, the numbers of PHA granules in each cell vary. In general approximation, each cell contains 8-12 granules with the measurement ranging from 0.24 to 0.50 μm (Anderson and Dawes, 1990).

There are two models for the formation of granules, micelle model (Figure 2.2) and budding model (Figure 2.3). The two models were proposed based from the location of PHA synthase. Micelle model is characterised by the presence of micelle-like structure formed during the aggregation of P(3HB)-linked synthase in cytosol. The synthase protein that resides on a micelle acquired the 3-hydroxybutyrate-CoA that scattered in cytosol and form the water insoluble PHA granules within the micelle. In budding model, the protein synthase is adhered to the inner phase of

plasma membrane. Once the PHA chain elongates and phasin proteins are produced, the PHA granules are formed from the plasma membrane in budding form into granules together with a phospholipid layer. Phasin is a specific protein that plays important role in the formation of PHA granules (Sudesh and Abe, 2010).

In an optimised (fermentation) condition, PHA granules can fill up to 85% of DCW in *Ralstonia eutropha*. Beeby and coworkers (2012) showed that the PhaP1 and other phasins in matured granules act as chaperon in order to prevent P(3HB) crystallisation (Beeby *et al.*, 2012). It was also reported that *Bacillus megaterium* had high amount of PHA granules, 98% P(3HB) and 2% protein (Griebel and Merrick, 1971).

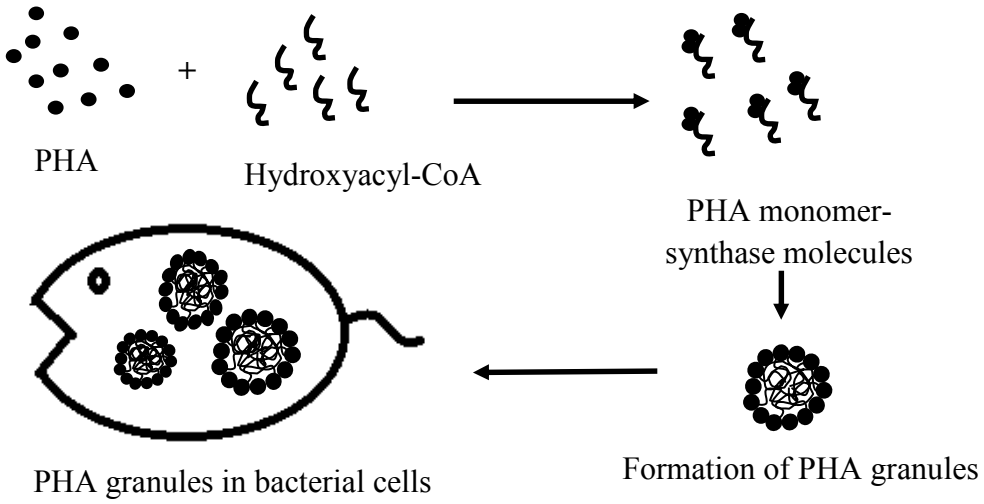


Figure 2.2: Micelle model of PHA granules formation

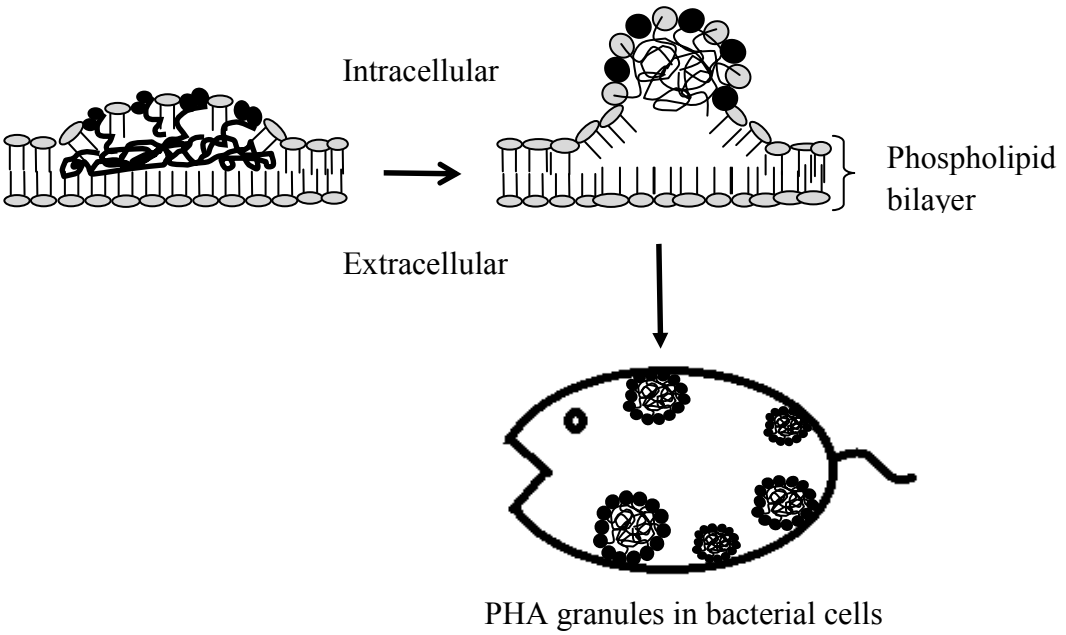


Figure 2.3: Budding model of PHA granules formation

2.3.2 PHA synthase

Presence of PHA synthase determines the type of polymerisation in PHA. PHA synthase reacts with hydroxyacyl-CoenzymeA (HA-CoA) that is formed *via* the synthesis pathway and produce PHA (Taguchi and Doi, 2004; Jendrossek, 2009). There are 4 major classes of PHA synthase (Table 2.2.). This classification of PHA synthase is based on the primary structure, substrate specificity, and the subunit composition (Rehm, 2003; Potter and Steinbüchel, 2005; Sudesh and Doi, 2005).

Table 2.2: Classes of PHA synthase in PHA pathway and the representative species

Class	Gene structure	Subunits	Preferred substrate	Representative species
I	*phaC*$_{Cn}$	~ 60-73 kDa	SCL-HA-CoA	*C. necator*
II	*phaC1*$_{Pa}$ – *phaC2*$_{Pa}$	~ 60-65 kDa	MCL-HA-CoA	*P. aeruginosa*
III	*phaC*$_{Cv}$ – *phaE*$_{Cv}$	PhaC~ 40 kDa PhaE~ 40 kDa	SCL-HA-CoA; MCL-HA-CoA	*Chromatium vinosum*
IV	*phaC*$_{Bm}$ – *phaR*$_{Bm}$	PhaC~ 40 kDa PhaR~ 22 kDa	SCL-HA-CoA	*B. megaterium*

Class I PHA synthase is consist of one subunit (PhaC) in which the size is in a range of 60-73 kDa. The Class I synthase is usually represented by *C. necator* for its ability to produce SCL-HA-CoA (3C-5C). In contrary to Class I, the Class II PHA synthase is consist of two similar subunits (PhaC1 and PhaC2) with the size range of 60-65 kDa. This synthase produces MCL-HA-CoA (6C-14C) and is represented by *P. aeruginosa*.

Another synthase, Class III, is different from the two former groups since Class III has two different subunits (PhaC and PhaE). Both Class I and Class II are consist of one subunit (PhaC) or two similar subunits (PhaC1 and PhaC2), respectively. PhaC and PhaE are PHA synthase expression proteins with the size of 40 kDa each and are responsible for the production of SCL-HA-CoA and MCL-HA-CoA. It was reported that the PHA synthase from Class III is more efficient to produce SCL PHA similar as the PHA synthase from Class I (Yuan *et al.*, 2001). For instance, *C. vinosum* which was previously known as *Allochromatium vinosum* represents the PHA synthase from the Class III (Liebergesell *et al.*, 1991; Sudesh *et al.*, 2000). Having similar subunit to Class III synthase, the Class IV synthase has two different subunits but the second subunit (which is PhaR) has shorter size at 22 kDa. This class preferred the substrate of SCL-HA-CoA. An example of bacteria possessing Class IV synthase is *B. megaterium* (McCool and Cannon, 1999).

2.3.3 Pathway for PHA synthesis

Figure 2.4 shows the pathway of PHA synthesis. It is well-known that the metabolic pathways are important determination to the type of PHA synthesised by microorganisms (Sudesh and Doi, 2005).

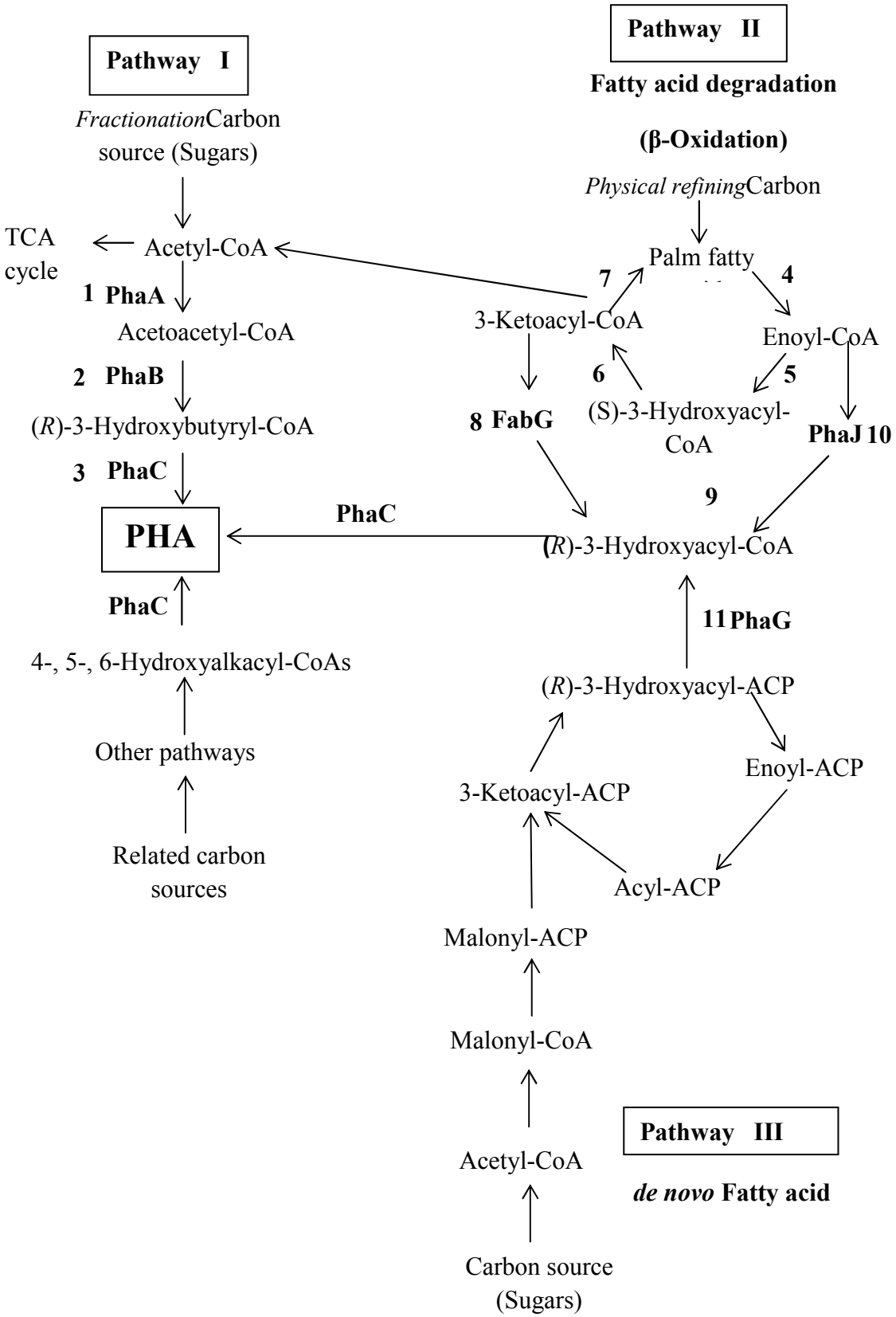


Figure 2.4: Overall pathways of PHA biosynthesis for PHA microorganisms. Enzymes: 1. β-ketothiolase; 2. NADPH-dependent acetoacetyl-CoA reductase; 3. PHA Synthase; 4. Acyl-CoA dehydrogenase; 5. Enoyl-CoA hydratase; 6. 3-hydroxyacyl-CoA dehydrogenase; 7. 3-ketoacyl-CoA thiolase; 8. 3-ketoacyl-CoA reductase; 9.Epimerase; 10.(*R*)-specific enoyl-CoA hydratase; 11.(*R*)-3-hydroxyacyl-

ACP-CoA transferase (Steinbüchel and Lütke-Eversloh, 2003; Sudesh and Doi, 2005; Riedel *et al.*, 2014).

PHA can be produced by utilising variety of carbon sources such as sugars (Lutke-Eversloh *et al.*, 2002; Brigham *et al.*, 2012), plant oils (Sudesh *et al.*, 2011; Riedel *et al.*, 2012), animal fats (Taniguchi *et al.*, 2003), fatty acids (Brigham *et al.*, 2010), and glycerol (Cavalheiro *et al.*, 2009; Cavalheiro *et al.*, 2012; Riedel *et al.*, 2014). When the carbon source is sugars, Pathway I is activated. Pathway I involves 3 main enzymes for the production of PHA, such as PhaA (β-ketothiolase), PhaB (NADPH Reductase), and PhaC (PHA Synthase) (Anderson and Dawes, 1990; Doi, 1990). This pathway will be discussed in detail in Section 2.4.

Pathway II is activated when long chain fatty acid or plant oils are fed to the microorganism as carbon source. Pathway II, which is also known as β-oxidation, channels the pathway intermediates into PHA biosynthesis (Sudesh and Doi, 2005). When the oil is fed to the culture, the lipases of microorganisms degrade the oil into glycerol and free fatty acids. This free fatty acid is then activated by thiokinase and CoA-transferase to produce acyl-CoA. The acyl-CoA is later used in catabolism process *via* β-oxidation through four enzymatic reactions. In four enzymatic reactions, the acyl-CoA is oxidised to enoyl-CoA by the assistance of acyl-CoA dehydrogenase enzyme. Then the intermediate is converted to (*S*)-3-hydroxyacyl-CoA by the reaction of enoyl-CoA hydratase. The (*S*)-3-hydroxyacyl-CoA that was oxidised by 3-hydroxyacyl-CoA dehydrogenase to form 3-ketoacyl-CoA. This compound is cleaved by 3-ketoacyl-CoA thiolase to a molecule of acetyl-CoA comprising two carbon atoms and a molecule of acyl-CoA, in which it lacks of 2 carbon atoms as compared to the initial acyl-CoA. The acetyl-CoA is transferred to

TCA cycle, PHA pathway, or other related pathways. Last, the acyl-CoA continues to be channeled into β-oxidation pathway in order to produce more acetyl-CoA.

MCL-PHA and co-polymer can be produced *via* β-oxidation pathway but not in a complete manner. The 3-ketoacyl-CoA and enoyl-CoA is converted to (*R*)-3-hydroxyacyl-CoA with the assistance of 3-ketoacyl-CoA reductase and (*R*)-specific enoyl-CoA hydratase, respectively. Then, the PhaC polymerises the compounds into PHA and form co-polymer. Production of co-polymer such as P(3HB-*co*-3HV) from propionic acid or valeric acid begin from the formation of 3-hydroxyvaleryl-CoA followed by polymerisation. Other MCL polymer such P(3HB-*co*-3HHx) can be obtained from the intermediates of β-oxidation pathway with the assistance of (*R*)-specific enoyl-CoA hydratase. However, MCL-PHA is also produced from Pathway III *via de novo* fatty acids. The β-oxidation pathway and the *de novo* fatty acids produce similar chemistries of the polymer but are regulated by different enzymes (Steinbüchel and Lütke-Eversloh, 2003; Riedel *et al.*, 2011).

Pathway III has the ability to produce variety of monomers from simple carbon sources such as gluconate, fructose, acetate, glycerol, and lactate. The starting material of *de novo* fatty acids are acetyl-CoA, however, the intermediates (*R*)-3-hydroxyacyl-ACP is not recognised by the PHA synthase. Thus, PhaG (also known as 3-hydroxyacyl-ACP-CoA transferase) converts the intermediates into (*R*)-3-hydroxyacyl-CoA before it is further polymerised by PHA synthase (Sudesh and Doi, 2005). As for several others metabolic pathways, it can be manipulated to produce substrate for PHA biosynthesis. The intermediates such as 4-hydroxybutyryl-CoA from TCA cycle can produce 4HB monomers. It can be done by providing external precursors, such as 4-hydroxybutyric acid, 1,4-butanediol, and

γ-butyrolactone, to certain microorganisms or the recombinants to produce the monomer (Steinbüchel and Lütke-Eversloh, 2003; Sudesh and Doi, 2005).

2.4 Poly(3-hydroxybutyrate)

P(3HB) is a homopolymer that has been extensively studied among other PHA and will be discussed further in this study. Formation of P(3HB) is based on the repeating units of (*R*)-3HB monomer. The biosynthesis of P(3HB) involves a three-step process which is also known as PHA pathway I and is regulated by β-ketothiolase (PhaA), NADPH-dependent acetoacetyl-CoA reductase (PhaB) and PHA synthase (PhaC) (Anderson and Dawes, 1990; Doi, 1990). Initially the β-ketothiolase condenses two molecules of acetyl-CoA which have two carbons each into acetoacetyl-CoA (four carbon atoms). Then, the acetoacetyl-CoA is reduced to (*R*)-hydroxybutyryl-CoA by the assistance of NADPH-dependent acetoacetyl-CoA reductase. The formation of (*R*)-hydroxybutyryl-CoA is signaled by PHA synthase and further the process of polymerisation prior to produce P(3HB) (c.f. Figure 2.5) (Steinbüchel and Lütke-Eversloh, 2003; Riedel *et al.*, 2014).

During imbalanced growth conditions (high carbon and low nitrogen), the citrate synthase are inhibited, and the levels of NADH and NADPH are increased. Therefore, it reduces the carbon-flux into the TCA cycle. The reduced concentration of free CoASH releases the inhibition of β-ketothiolase, and the overproduced acetyl-CoA is channeled into the P(3HB) biosynthesis pathway. In balanced growth conditions, the acetyl-CoA is channeled towards TCA cycle and increases the amount of free Coenzyme A. The activity of β-ketothiolase is inhibited by high amount of coenzyme A. Synthesis of P(3HB) is not active as β-ketothiolase is the key regulatory enzyme in the biosynthesis of P(3HB) (Doi, 1990; Loo and Sudesh,

2007). Acetyl-CoA is the precursor for P(3HB) in a bacteria (Doi, 1990; Yunus *et al.*, 2008).

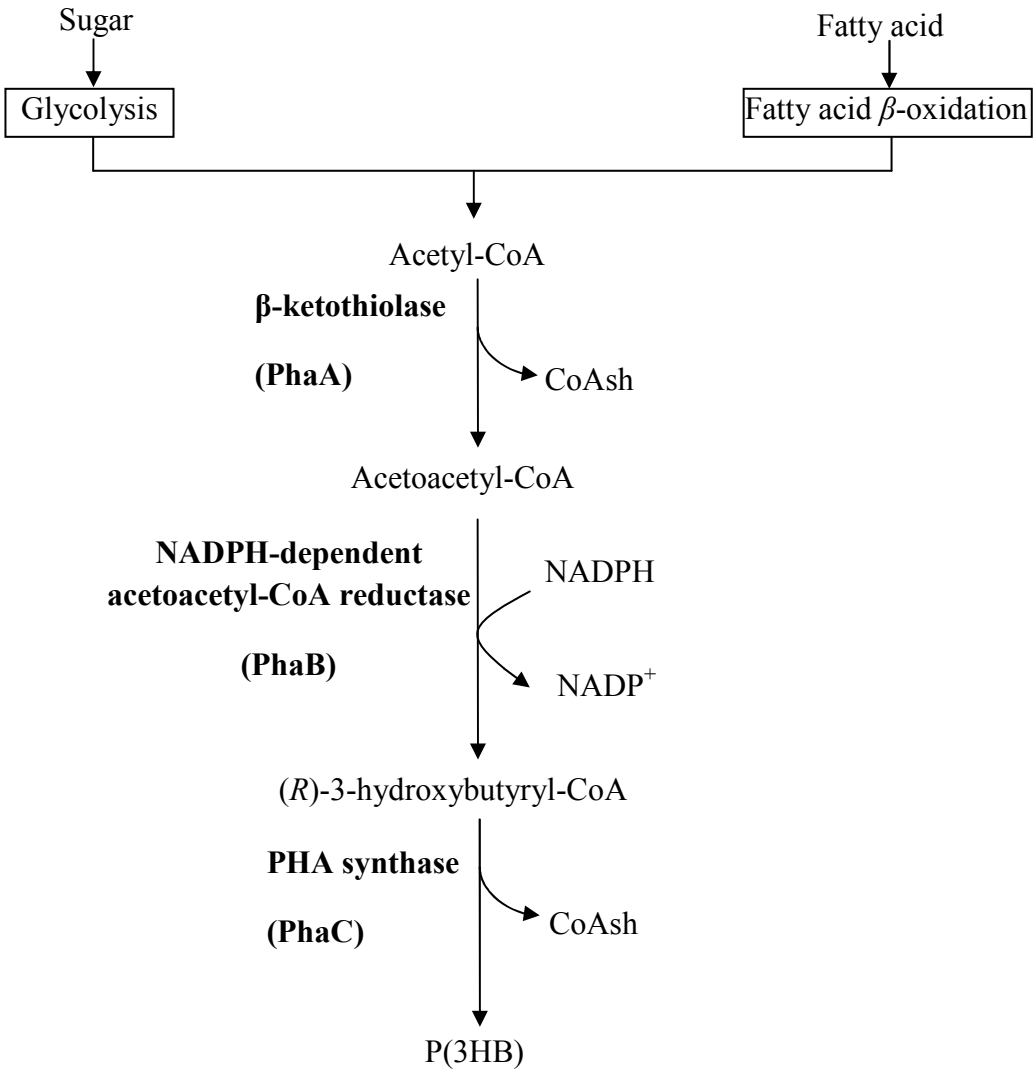


Figure 2.5: Pathways of P(3HB) biosynthesis (Anderson and Dawes, 1990; Doi, 1990)

The mechanical properties of P(3HB) can be altered by incorporation of second monomers such as 3-hydroxyvalerate. Table 2.3 shows some characteristics comparison between P(3HB), P(3HB-*co*-3HV) and synthetic PP (Sudesh *et al.*, 2000; Nikel *et al.*, 2006).

Table 2.3: The properties of P(3HB), (P3HB-*co*-3HV), and polypropylene (Tsuge, 2002)

Analysis	P(3HB)	P(3HB-*co*-3HV)*	PP
Melting temperature (°C)	177	145	176
Glass transition temperature (°C)	4	−1	−10
Crystallinity (%)	60	56	50-70
Tensile strength (MPa)	43	20	38
Elongation to break (%)	5	50	400

*20 mol% of 3HV

2.5 Physical properties of P(3HB)

P(3HB) is known to have similar degree of crystallinity as compared to PP, nylon 6, and poly(ethylene terephtalate) (PET), which is around 55-70% crystallinity (Barham *et al.*, 1984; Tsuge, 2002). In the bacteria cell, however, P(3HB) granules appeared in rubbery amorphous. The inhibition of nucleation is due to the hydrogen bond between PHA and water (Lauzier *et al.*, 1992; Jurasek *et al.*, 2001). Weight-average molecular weight (M_w) of P(3HB) produced by wild type bacteria is within range of 1×10^4 Da to 3×10^6 Da with polydispersity (PDI) (M_w/M_n) of around 2 (Tsuge, 2002).

2.5.1 Thermal properties

It is common to use differential scanning calorimeter (DSC) to characterise the thermal properties of a polymer. The melting temperature (T_m) of PHA is usually in the range of 50-180 °C depending on the length of side chain, composition of copolymers, and molecular weights. T_m decreases with increasing percentage of copolymer fractions. As for the solution-cast film, P(3HB) has a melting point of 177-180 °C. The glass transition (T_g) can also be characterised using DSC. T_g represents the amorphous fraction of a polymer. The T_g of P(3HB) is approximately

0-4 °C. The T_g also decreases as the fraction of copolymer increases (Sudesh and Abe, 2010).

At nearby T_m, there is a rapid reduction of M_w. This applies to many PHA such as P(3HB), P(3HB-*co*-3HV), P(3HB-*co*-3HHx) etc. The reduction of M_w is caused by chain scission mechanism, in which the polymer chains are cut short. The thermal decomposition of P(3HB), obtained from thermogravimetric (TGA) analysis at a heating rate of 10 °C/min, shows weight loss at approximately 275 °C in inert condition, and no residues were left at 305 °C (Sudesh and Abe, 2010).

As for copolymer, the TGA curve increases to higher temperature. Activation energy (E_a) for copolymer in TGA increased as the copolymer fraction increases. This is due to the production of volatile components such as monomers and oligomers (Sudesh and Abe, 2010).

2.5.2 Mechanical properties

P(3HB) have similar Young Modulus (3.5 GPa) and tensile strength (43 MPa) with isotactic PP (iPP). However, the elongation at break is far less than iPP, which is 5% and 400%, respectively. Thus, P(3HB) is considered as stiff and brittle polymer (Tsuge, 2002; Sudesh and Abe, 2010). Incorporation of second monomer such as 3-hydroxyvalerate, 4-hydroxybutyrate, and 3-hydroxyhexanoate increases the elongation at break.

2.6 Degradation of PHA

2.6.1 Intracellular degradation of P(3HB)

P(3HB) can be degraded intracellularly in cytosol or extracellularly in appropriate conditions by PHA-degrading bacteria. Before degradation takes place, bacteria produce PHA depolymerases. The depolymerases degrade the inclusions of P(3HB) granules in a condition whereby of limited carbon can be obtained internally or externally. As mentioned by Macrae and Wilkinson (1958), P(3HB) granules act as carbon source storage in the condition of limited growth nutrient (Macrae and Wilkinson, 1958). The intracellular P(3HB) content determines the survival of bacteria in the absence of exogenous carbon. The growth of P(3HB) rich cells is then inhibited slowly compared to the P(3HB)-poor cells. It was reported that *Cupriavidus necator* H16 can survive for at least 600 days in the absence of carbon source by utilising the accumulated P(3HB) (James *et al.*, 1999; Handrick *et al.*, 2000). Other than that, P(3HB) granules in cells will be degraded with the addition of nitrogen source, that it is suspected to be consumed for protein synthesis (Sudesh and Abe, 2010).

Intracellular PHA depolymerase can only degrade the native, amorphous PHA granules, while extracellular PHA depolymerase can degrade the crystalline or semi-crystalline granules (Merrick and Doudoroff, 1964). This is because the intracellular P(3HB) depolymerase is inactive with semi-crystalline or crystalline P(3HB) as it had no lipase, protease, or esterase activity when tested with *p*-nitrophenylacyl ester (Jendrossek and Handrick, 2002).

Cupriavidus necator H16 which is P(3HB)-rich bacteria have low-self hydrolysis rate of PHA granules. However, the hydrolysis process of PHA granules

can be accelerated during carbon starvation up to three-fold (Handrick *et al.*, 2000). The known P(3HB) depolymerases in *Cupriavidus necator* H16 are PhaZa1 to PhaZa5, PhaZd1 and PhaZd2. Both PhaZd1 and PhaZd2 have 2 amino-acid sequences that is similar to catalytic domain of extracellular P(3HB) depolymerase (Jendrossek *et al.*, 1995; Shinohe *et al.*, 1996; Abe *et al.*, 2005).

2.6.2 Extracellular degradation of P(3HB)

During extracellular degradation of P(3HB), the polymers with high M_w cannot be transported through the cell wall. The bacteria in soil, compost, aerobic and anaerobic sewage sludge, fresh and marine water, and estuarine sediment have to excrete extracellular PHA depolymerase to hydrolyse P(3HB) into water soluble oligomers or monomers and consume the degraded products as nutrients (Delafield *et al.*, 1965; Briese *et al.*, 1994; Sudesh *et al.*, 2000; Sudesh and Abe, 2010).

Most extracellular PHA-depolymerase microorganisms are specific for P(3HB) degradation or other similar MCL-PHA. However, *Xanthomonas*-like bacterium can degrade PHA with aromatic side chains and *Comamonas* sp. strain is able to degrade both of SCL-PHA and MCL-PHA (Doi *et al.*, 1992; Schirmer *et al.*, 1995; Jendrossek, 1998; Quinteros *et al.*, 1999). Extracellular PHA depolymerase is capable to hydrolyse *p*-nitrophenylester of fatty acid with six or more carbon atoms. This PHA depolymerase also hydrolyse the polymer into monomer, monomers and dimers, or mixture of oligomers. PHA depolymerase can be repressed when the carbon source is in excess (Bachmann and Seebach, 1999; Sudesh and Abe, 2010).

Degradation of P(3HB) in aquatic condition can be measured based on biochemical oxygen demand (BOD), weight loss (erosion) of polyester sample, and

dissolved organic carbon (DOC). It is influenced by the environmental conditions (e.g. temperature, moisture level, pH, and nutrient supply. These environmental factors also influence the growth of PHA-degrading microorganisms, the production and secretion of PHA depolymerase, and degradation activity of the enzymes (Sudesh and Abe, 2010).

PHA depolymerases consist of single polypeptide chain with molecular weights around 26000-63000 Da (Briese *et al.*, 1994; Jendrossek *et al.*, 1995; Klingbeil *et al.*, 1996; Kasuya *et al.*, 1997; Shinomiya *et al.*, 1997). These enzymes have good stability at wide range of pH, temperature, and ionic strength. Optimum results for the enzyme activity were obtained when the pH was 7.5-9.8 and the PHA depolymerases have affinity toward hydrophobic materials (Sudesh and Abe, 2010).

The structural genes of extracellular PHA depolymerase is consist of nitrogen-terminal catalytic domain, a carbon-terminal substrate binding domain, and a linker region connecting the two domains. The catalytic and substrate domain are also present in other depolymerising enzymes such as cellulase, xylanase and chitinase that hydrolysed water insoluble polysaccharide (Kellett *et al.*, 1990; Watanabe *et al.*, 1990; Gilkes *et al.*, 1991). The catalytic domain contains lipase box pentapeptides [Gly-X1-Ser-X2-Gly] which have the role as an active site for serine hydrolase. The position of lipase box determines the type of extracellular PHA depolymerase (c.f. Figure 2.6). Type A extracellular PHA-depolymerase has the lipase box located at the centre of the enzyme, whilst Type B extracellular PHA-depolymerase lipase box is located adjacent to nitrogen-terminal of the catalytic domain. The bacteria that represent the type of PHA depolymerase is shown in the Figure 2.6. The carbon-terminal domain acts as a substrate-binding domain water insoluble P(3HB) substrate (Brucato and Wong, 1991; Miyazaki *et al.*, 2000). In the