ENHANCED PRODUCTION OF POLY(3-HYDROXYBUTYRATE-*co*-4-HYDROXYBUTYRATE) WITH HIGH 4HB UNIT COMPOSITION USING TRANSFORMANT *Cupriavidus* sp. USMAA1020

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by

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%	Percentage
3HO	3-hydroxyoctanoate
ω	Omega
ß	Beta
γ	Gamma
^{o}C	Degree celcius
µL	Microliter
µm	Micrometer
^{13}C	Carbon-13
$(NH_4)_2SO_4$	Ammonium sulphate
3HB	3-hydroxybutyrate
4HB	4-hydroxybutyrate
4HB-CoA	4-hydroxybutyrate-Coenzyme A
Acetyl-CoA	Acetyl-Coenzyme A
C	Carbon
$CoSO_4 \cdot 7H_2O$	Cobalt (II) sulphate heptahydrate
$CaCl_2 \cdot 2\ H_2O$	Calcium chloride dihydrate
$CuCl_2 \cdot 2\ H_2O$	Copper (II) chloride dihydrate
C/N	Carbon to nitrogen
CDW	Cell dry weight
CME	Caprylate methyl ester
Da	Dalton

DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DSC	Differential scanning calorimeter
EtBr	Ethidium bromide
FID	Flame ionization detector
g	Gram
GC	Gas chromatography
GPC	Gel permeation chromatography
h	Hour
HA	Hydroxyalkanoate
H_2O	Water
Hz	Hertz
J/g	Joule per gram
kb	Kilobyte
KA	Kanamycin sulphate
kPa	Kilo Pascal
kDa	Kilo Dalton
K_2HPO_4	Dipotassium hydrogen phosphate
KH_2PO_4	Potassium dihydrogen phosphate
L	Liter
L/min	Liter per minute
M	Molar
mm	Millimeter
min	Minute
mV	Milivolt

M_w	Weight –average molecular weight
M_n	Number –average molecular weight
mol%	Mol percentage
mcl-PHA	Medium chain length polyhydroxyalkanoate
$MgSO_4 \cdot 7H_2O$	Magnesium sulphate heptahydrate
mL	Milliliter
mg	Milligram
MSM	Mineral salt medium
mg/mL	Milligram to milliliter
$MnCl_2 \cdot 4H_2O$	Manganese (II) chloride tetrahydrate
NA	Nutrient agar
NADPH	Nicotinamide adenine dinucleotide phosphate
N	Newton
NR	Nutrient rich
NaCl	Sodium chloride
Na_2SO_4	Sodium sulphate
ND	Non-detectable
OD	Optical density
PGA	Polyglycolic acid
P(3HB)	Poly(3-hydroxybutyrate)
P(3HB-*co*-4HB)	Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate)
P(4HB)	Poly(4-hydroxybutyrate)
PDI	Polydispersity index
PS	Polystyrene

pH	Power of hydrogen
PHA	Polyhydroxyalkanoate
PLA	Polylactic acid
PE	Polyethylene
PBS	Polybutylene succinate
PTT	Polytrimethylene terephthalate
PHB	Polyhydroxybutyrate
Pa	Pascal
psi	Pounds per square inch
PTFE	Polytetrafluoroethylene
PhaA;*phaA*	ß-ketothiolase; gene encoding ß-ketothiolase
PhaB; *phaB*	NADPH-dependent acetoacetyl-CoA dehydrogenase; gene encoding NADPH-dependent acetoacetyl-CoA dehydrogenase
PhaC; *phaC*	PHA synthase; gene encoding PHA synthase
RCDW	Residual cell dry weight
rpm	Rotation per minute
scl	Short chain length
TCA	Tricarboxylic acid
TEM	Transmission electron microscope
T_m	Melting temperature
T_g	Glass transition temperature
T_c	Crystallization temperature
UV	Ultraviolet
vvm	Volume per volume per minute

v/v	Volume to volume
wt%	Weight percentage
$Y_{p/x}$	Yield of product per biomass
$Y_{p/s}$	Yield of product per substrate
$ZnSO_4 \cdot 7H_2O$	Zinc sulphate heptahydrate

PENINGKATAN PENGHASILAN POLI(3-HIDROKSIBUTIRAT-*ko*-4-HIDROKSIBUTIRAT) DENGAN KOMPOSISI UNIT 4HB YANG TINGGI MENGGUNAKAN TRANSFORMAN *Cupriavidus* sp. USMAA1020

ABSTRAK

Poli (3-hidroksibutirat-*ko*-4-hidroksibutirat) [P(3HB-*ko*-4HB)] kopolimer adalah salah satu biopolimer yang mempunyai ciri-ciri yang sesuai diaplikasikan di dalam bidang perubatan. P(3HB-*ko*-4HB) dengan unit 4HB yang tinggi adalah baik kerana ia meningkatkan ciri biokompabiliti dan ciri mekanikal kopolimer ini untuk dijadikan sebagai bahan implan dan bahan mampu serap. Walau bagaimanapun, biosintesis P(3HB-*ko*-4HB) melalui fermentasi satu peringkat telah menghasilkan komposisi unit 4HB dan produktiviti yang rendah. *Cupriavidus* sp. USMAA1020, bakteria yang diperolehi daripada persekitaran Malaysia, mempunyai kemampuan untuk menghasilkan kopolimer P(3HB-*ko*-4HB) dengan komposisi unit 4HB yang berbeza tetapi hanya sehingga 70% mol melalui fermentasi satu peringkat. Kajian ini mengkaji keupayaan transforman *Cupriavidus* sp. USMAA1020 yang mempunyai tambahan gen PHA synthase, *phaC* daripada jenis *Cupriavidus* sp. USMAA2-4 liar untuk menghasilkan kopolimer P(3HB-*ko*-4HB) dengan komposisi unit 4HB lebih daripada 80 mol%. Dengan adanya penambahan *phaC* secara heterologus, transforman ini didapati mampu menghasilkan kandungan PHA sebanyak 69 wt% dengan komposisi unit 4HB sebanyak 86 mol% daripada fermentasi dalam kelalang goncang menggunakan kombinasi karbon 1,6-hexandiol dan 1,4-butandiol. Fermentasi satu peringkat di dalam fermentasi 3 L menunjukkan bakteria

transforman ini mampu menghasilkan komposisi unit 4HB yang tinggi dengan penghasilan sebanyak 95 mol% dengan kandungan PHA sebanyak 75% wt% dan kepekatan PHA sebanyak 18.7 g/L. Bakteria transforman ini juga didapati mampu hidup dalam kepekatan karbon yang lebih tinggi (1.05wt% C) berbanding jenis bakteria liar (0.69 wt% C). Penghasilan kopolimer P(3HB-*ko*-4HB) dengan unit 4HB yang tinggi dipertingkatkan menggunakan kaedah fermentasi suapan sesekelompok melalui strategi suapan dua kali. Strategi suapan sumber karbon dan sumber nitrogen sebanyak dua kali ini menghasilkan kandungan PHA yang tinggi dengan kandungan dan kepekatan PHA masing-masing sebanyak 92 wt% dan 46.4 g/L yang mana 1.2 kali ganda dan 2.3 kali ganda lebih tinggi berbanding dengan fermentasi sesekelompok. Komposisi unit 4HB setinggi 97 mol% juga telah diperolehi melalui strategi ini. Penghasilan yang dipertingkatkan melalui fermentasi suapan sesekelompok mempunyai produktiviti keseluruhan dan hasil masing-masing sebanyak 0.35 g/L/h dan 11.6 g/g (produk/biomass) lebih tinggi daripada fermentasi sesekelompok dengan produktiviti dan hasil masing-masing sebanyak 0.19 g/L/ h dan 4.4 g/g (produk/biomass). Secara umumnya, kajian ini telah menghasilkan kopolimer P(3HB-*ko*-4HB) dengan komposisi unit 4HB di antara 85 mol% ke 97% mol. Sifat mekanikal dan haba kopolimer yang dihasilkan dari kedua-dua fermentasi sesekelompok dan suapan sesekelompok tidak mempunyai banyak perbezaan menunjukkan bahawa strategi suapan sumber karbon dan sumber nitrogen yang berbeza-beza tidak memberi banyak kesan kepada sifat mekanikal dan haba kecuali berat molekul. Berat molekul kopolimer yang dihasilkan daaripada fermentasi suapan seekelompok (175 kDa to 328 kDa) adalah lebih rendah berbanding dengan kopolimer yang dihasikan dari fermentasi sesekelompok (438 kDa).

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ABSTRACT

Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] copolymer is one of emerging biopolymer which possess suitable properties to be applied in medical applications. P(3HB-*co*-4HB) with high 4HB unit is favorable as this enhance the biocompatibility and mechanical properties of this copolymer as implantable and absorbable biomaterial. However, biosynthesis of P(3HB-*co*-4HB) in single-stage cultivation produces low 4HB unit composition with low productivity. *Cupriavidus* sp. USMAA1020, a bacterium isolated from Malaysian environment, was capable to synthesize P(3HB-*co*-4HB) with different 4HB unit compositions but only to a maximum of 70 mol% through single-stage cultivation. The present work studied the ability of transformant *Cupriavidus* sp. USMAA1020 which has additional PHA synthase gene, *phaC* of wild-type strain *Cupriavidus* sp. USMAA2-4 to produce P(3HB-*co*-4HB) with 4HB unit composition more than 80 mol%. With the presence of the extra PHA synthase gene, *phaC*, which was expressed heterologously, this strain was found to accumulate high PHA accumulation of 69 wt% with 86 mol% of 4HB unit composition in shake flask cultivation using mixed substrate of 1,6-hexanediol and 1,4-butanediol. Single-stage cultivation in 3 L fermentation confirmed the ability of this strain to produce high 4HB unit composition of 95 mol% with 75 wt% PHA content and high PHA concentration of 18.7 g/L. Interestingly, this strain was also found capable of surviving high carbon

concentration (1.05 wt% C) better than the wild-type strain (0.69 wt% C). The production of P(3HB-*co*-4HB) copolymer with high 4HB unit was enhanced using two times pulse feeding fed-batch fermentation and accumulated high PHA content as well as concentration of 92 wt% and 46.4 g/L, respectively; 1.2 fold and 2.3 fold higher compared to batch cultivation. Interestingly, 4HB unit composition as high as 97 mol% was also obtained through this feeding strategy. Enhanced production through fed-batch fermentation over batch operation highlighted overall productivity and yield of 0.35 g/L/h and 11.6 g/g (product/biomass), respectively better than the one obtained during batch cultivation with productivity and yield of 0.19 g/L/h and 4.4 g/g (product/biomass), respectively. Generally, the present study has produced P(3HB-*co*-4HB) with 4HB unit composition ranging from 85 mol% to 97 mol%. The mechanical and thermal properties of the copolymer produced from batch and fed-batch fermentation exhibited close range of values indicating that different feeding strategies employed in fed-batch fermentation did not affect their mechanical and thermal properties except for molecular weight. Lower molecular weight value of copolymer produced from fed-batch (175 kDa to 328 kDa) was observed compared to batch copolymer (438 kDa).

1.0 INTRODUCTION

Polyhydroxyalkanoates (PHA) is known as a 'green' or biopolymer due to its potential to substitute synthetic polymer as the film can be fully degraded in soil in less than 50 days (Sudesh & Iwata, 2008). From a well known biopolymer, PHA is currently being developed as absorbable biomaterial that suitable to be applied in medical and pharmaceutical applications. PHA based biomaterial possess a new variety of properties that broaden the design of existing biomaterial allowing the growth of new and improved products. PHA can be produced by a wide range of bacteria as their intracellular energy storage material when their environment was found insufficient of growth nutrients such as nitrogen, phosphorus or micro-elements whilst the carbon source was present in excess. PHA formed from this unfavorable environment can be seen in form of granules deposited in cell cytoplasm. PHA producing bacteria include *Ralstonia eutropha* (Insomphun *et al*., 2015), *Hydrogenophaga pseudoflava* (Choi *et al*., 1999; Amoli *et al*., 2013), *Comamonas acidovorans* (Saito *et al*., 1996; Lee *et al*., 2004) and *Alcaligenes latus* (Lefebvre *et al*., 1997; Wang *et al*., 2012).

Interestingly, even though PHAs are produced from microbial natural metabolism, their structures are comparable to synthetic polymer (Ojumu *et al*., 2004). Depending on the monomeric units, PHAs are categorized into short-chain-length (scl) and medium-chain-length (mcl) polymer. Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) P(3HB-*co*-4HB) is one of scl-PHAs which was produced from *Cupriavidus necator* (formerly known as *Ralstonia eutropha*) since 1988. *C. necator* has been studied comprehensively in PHA production due to its easy growth and ability to accumulate large amount of polymers from single carbon source (Kunioka

et al., 1989; Amirul *et al*., 2008; Md. Iqbal & Amirul, 2013). P(3HB) homopolymer is the most known PHA which is relatively stiff and rigid that has a tensile strength as good as polypropylene. On the other hand, the P(4HB) homopolymer is a highly ductile, flexible polymer with an extension at break of around 1000% compared with P(3HB), which has an extension at break of less than 10% (Saito *et al*., 1996). Combining these two different units to form a copolymer, as in P(3HB-*co*-4HB), produces materials with extensive range of useful mechanical properties that can be adapted to particular needs (Sudesh *et al*., 2000; Huong *et al*., 2013).

P(3HB-*co*-4HB) with high 4HB unit composition has been found to have desirable mechanical properties to be used in medical field, especially as implantable and absorbable biomaterial (Stock *et al*., 2000). In fact, P(4HB) is the first biopolymer to be approved for clinical applications as absorbable suture (TephaFLEX) by Food and Drug Administration (FDA) (Martin & Williams, 2003). Biocompatibility is the most important properties for the biopolymer if a medical application is being considered. Interestingly, P(4HB) is biocompatible and extremely well tolerated *in vivo* due to the fact that hydrolysis of P(4HB) produces 4HB acid, a common metabolite in the human body and it is naturally distributed in the mammalian body particularly in brain, kidney, heart, liver and muscle (Nelson *et al*., 1981; Martin & Williams, 2003). In addition, high 4HB unit in P(3HB-*co*-4HB) also improved surface wettability and support cell growth of P(3HB-*co*-4HB) nano-fiber construct to be used as biodegradable wound dressing (Vigneswari *et al*., 2016).

1.1 Problem statement

Due to the good potential of this biomaterial for medical applications, obtaining P(3HB-*co*-4HB) with high 4HB unit is highly favorable during microbial synthesis by using strain which has capability to accumulate biopolymer. Several attempts had been done by researchers to increase the 4HB unit composition in P(3HB-*co*-4HB) including cultivation using sole and mixture of carbon sources, addition of additives/precursor, use of recombinant strains and production by single and two-stage cultivation. In single-stage cultivation, Huong and co-workers had extensively studied the production of P(3HB-*co*-4HB) in both shake flask and bioreactor using wild-type *Cupriavidus* sp. USMAA1020 where only 7 mol% to 70 mol% of 4HB unit was achieved (Huong *et al*., 2015; Huong *et al*., 2013). By using *Wautersia eutropha* strain, Kimura and co-workers managed to produce 4HB unit in the range of 72 mol% to 86 mol% with 47.2 wt% of CDW cultured from mixed substrate and α-amino acids during cultivation (Kimura *et al*., 2008). Another study had attempted to increase 4HB unit composition using recombinant *E. coli* strain but yielded only up to 68.2 mol% 4HB using glucose as sole carbon source (Zhou *et al*., 2012). Yet, not more than 80 mol% of 4HB unit can be produced from any studies in single-stage cultivation.

The present study's aim was to increase the production of P(3HB-*co*-4HB) with high 4HB unit by using sole and mixed substrates at varying concentration using transformant *Cupriavidus* sp. USMAA1020 which was incorporated with extra *phaC* gene as being constructed by Syafiq *et al.* (2017). Transformant *Cupriavidus* sp. USMAA1020 was constructed by heterologous expression of cloned PHA synthase gene from *Cupriavidus* sp. USMAA2-4 ($phaC_{24}$) into *Cupriavidus* sp. USMAA1020 wild-type strains. This heterologous expression caused transformant *Cupriavidus* sp.

USMAA1020 to have an extra PhaC, an enzyme which is responsible for PHA production. The ability of this transformant strain to biosynthesize P(3HB-*co*-4HB) with high 4HB unit was investigated and compared to the wild-type strain. The polymers produced then were characterized.

Apart from that, many fermentation studies that had been carried out focusing on the normal batch fermentation. In batch fermentation of P(3HB-*co*-4HB), all necessary medium components are added at the beginning and not during the fermentation period. Therefore, their concentrations are not being controlled throughout the fermentation but vary as the cells use them up. The normal batch fermentation usually produces low yield of P(3HB-*co*-4HB) and low 4HB unit composition. Alternatively, fed-batch fermentation could be considered and further studied. A fed-batch culture is a semi batch operation in which the nutrients necessary for cell growth and product formation are fed either intermittently or continuously via one or more feed streams during the batch operation. Through the manipulation of one or more feed rates, the fed-batch operation can provide ways of regulating the concentration of substrates that limiting the production rates and therefore provide a real advantage over the batch operation. In this study, different feeding strategies were used to enhance the production of this copolymer while taking into consideration its simple operation.

1.2 Objectives

In this study, different means of fermentation strategy for the production of P(3HB-*co*-4HB) via batch and fed-batch fermentation was employed to increase PHA accumulation and 4HB unit and the polymer produced was characterized.

Objectives of this research were:

i. To produce P(3HB-*co*-4HB) with high PHA content and 4HB unit in shake flask and 3 L fermentation via batch fermentation.

ii. To increase P(3HB-*co*-4HB) production with high PHA content with high 4HB unit composition via fed-batch fermentation in 3 L fermentation by manipulating substrate feeding strategy.

iii. To characterize P(3HB-*co*-4HB) produced from batch and fed-batch fermentation.

2.0 LITERATURE REVIEW

2.1 Historical outline of polyhydroxyalkanoate (PHA) research

Polyhydroxyalkanoate (PHA) was first recognized by Lemoigne as polyhydroxybutyrate, P(3HB) which was found in *Bacillus megaterium* in the form of lipid-like inclusions before 1950. This finding became a well-known phenomenon when the presence of these inclusions was seen in most gram negative bacteria. The function of this inclusion known as P(3HB) polymer was also described by Dawes & Senior on 1973 (Sudesh *et al*., 2000). In the beginning, researchers thought that P(3HB) was the only form of hydroxyalkanoate (HA) that exists until other form of HA (3-hydroxyvalerate (3HV) and 3-hydroxyhexanoate (3HHx)) were discovered from extracted activated sludge (Wallen & Rohwedder, 1974). The finding of new type of PHA continued when Findlay & White (1983) in their report found 11 short-chain beta-hydroxy acids in polymer extracted from marine sediments in which 3HB and 3HV units were dominant. Four to eight carbons chain length polymer which extracted from *Bacillus megaterium* were also described in the same report which comprised of 3HB, 3-hydroxyheptanoate (3HHp) and eight carbons HA. In accordance to this report, Findlay mentioned that the polymer that previously referred as P(3HB) should be called PHA. Since then, the interest on the application of PHA has begun with the investigation on the properties of PHA.

The next stage of PHA development started when P(3HB) was studied for its function and properties. In a study done by Saito and co-workers it was reported that P(3HB) homopolymer is a brittle and stiff material with limited applications (Saito *et al*., 1996). In order to improve these properties, a study was done involving the incorporation of other unit, 3HV into 3HB which managed to produce a polymer

with good flexibility and less stiffness (Kim *et al*., 2009). This has marked an onset to PHA as a polymer with various characteristics that can be tailored into many kinds of applications.

2.2 PHA and its properties

Huge variety of polymers can be synthesized by various organisms where these polymers can be categorized based on their chemical structures in eight classes. They are polynucleotides, polyamides, polysaccharides, polyoxoesters, polythioesters, polyphosphate, polyisoprenoids and polyphenols (Steinbüchel, 2001). Hydroxyalkanoate (HA) as in PHA will be formed from the excess carbon and polymerize into PHA granules in cell cytoplasm. These granules can be easily seen under phase contrast light microscope due to its high refractivity (Loo & Sudesh, 2007). Other than PHA, polylactic acid (PLA), polybutylene succinate (PBS) and polytrimethylene terephthalate (PTT) are also examples of biopolymer with at least one of their units capable to be produced biologically. Among all, PHA has attracted huge attention over many years for its high biodegradability and its biocompatibility. PHA also exists as the only biopolymer that can be synthesized and polymerized completely from various bacteria (Chen & Wu, 2005). This includes both Gram positive and Gram negative bacteria, prokaryotic and eukaryotic bacteria from various environments such as soil, activated sludge, seas and even extreme environments.

PHA monomeric building block, HA is not usually stand alone but will mostly polymerize to form PHAs. Looking at its structure as in Figure 2.1, R can be replaced by alkyl or benzyl group while *n* can vary from 0 to 5 showing that this monomer has huge structural variations. This allows PHA to have a huge variety in terms of their structures and resulting in variety of PHA characteristics (Hazer &

Steinbüchel, 2007). So far, more than 150 constituents of PHA in terms of homopolymers or copolymers had been identified (Steinbüchel & Lütke-Eversloh, 2003). PHA can be categorized in three groups distinguished by its side chain length which are short-chain-length (scl-PHA) and medium-chain-length (mcl-PHA). Scl-PHA consists of not more than 5 carbons while mcl-PHA consists up to 14 carbons.

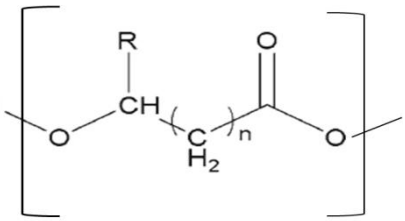

Figure 2.1 Polyhydroxyalkanoate (Loo & Sudesh, 2007)

2.2.1 Short-chain-length (scl-PHA)

Scl-PHAs are mostly produced by strain *R. eutropha* which possess PHA synthase, an enzyme responsible for PHA production which is active towards scl-HA monomer. P(3HB) homopolymer is the most common example of scl-PHA with high tensile strength comparable to polypropylene but has low elongation to break (Anderson & Dawes, 1990). The high melting point of 178ºC also makes P(3HB) difficult to process and limits its applications (Sudesh *et al*., 2000). Introduction of other monomers into P(3HB) such as in P(3HB-*co*-4HB), P(3HB-*co*-3HV) and P(3HB-*co*-4HV) produced scl-PHA with improved properties which is less stiff and tougher compared to P(3HB).

2.2.2 Medium-chain-length (mcl-PHA)

Pseudomonas strain has being recognized to be able to produce mcl-PHA since 1983 when polymer containing mainly 3-hydroxyoctanoic acid (3HO) was extracted from *Pseudomonas oleovorans* grown on *n*-octane (Smet *et al*., 1983). This strain possess PHA synthase that efficiently incorporated larger (*R*)-3HA monomer (6-12 carbons) compared to *Ralstonia eutropha* strain (Sudesh *et al*., 2000). Mcl-PHAs have low tensile strength but high elongation to break with elastomeric and semicrystalline properties. Examples of mcl-PHA are P(3HHx-*co*-3HO-*co*-3-hydroxydecanoate) and P(3HHx-*co*-3HO-*co*-3HD-*co*-3-hydroxydodecanoate).

2.3 PHA biosynthesis and its pathway

Various studies had been conducted to investigate in details the biosynthetic pathway and enzymes involved in the production of PHA. Most studies agreed that an enzyme called PHA synthase plays a key role in production of PHA which catalyzes various PHA biosynthesis pathways. With the finding of various type of PHA monomers since 1974, it was acknowledged that PHA synthase has extensive substrate specificity which results in polymerization of various PHA monomers (Sudesh *et al*., 2000). Growth conditions also play an important role in PHA accumulation where an important finding found by Wilkinson saying that PHA content was higher in medium with deficient nitrogen source while external carbon was in excess (Wilkinson, 1958). Generally, PHA production begins with the formation of (*R*)-hydroxyacyl-CoA from intermediate activated carbon source metabolism before polymerization by PHA synthase occurs which latter produce PHA granules (Steinbüchel, 2001). PHA will be obtained only when PHA structurally related substrates were provided during microbial synthesis which

supports the formation of hydroxyacyl-CoA thioesters from intermediate carbon metabolism. Not only that, type of carbon also determines the type of PHA produced where different carbon substrate creates different metabolic pathway which leads to formation of different PHA monomers (Sudesh *et al*., 2000).

Cupriavidus necator formerly known as *Ralstonia eutropha* has been studied broadly in PHA production for its easy growth and ability to accumulate large amount of polymers from single carbon source (Kunioka *et al*., 1989). *C. necator* possess class I PHA synthase which polymerize the scl HA monomers with 3 to 5 carbon atoms. This strain also able to accumulate large amount of P(3HB) from a simple carbon. *C. necator* in the presence of carbon sources produce two moles of acetyl-CoA where these acetyl-CoA will be condensed to acetoacetyl-CoA by an enzyme of ß-ketothiolase which is PhaA. This acetoacetyl-CoA will be reduced to (*R*)-3-hydroxybutyryl-CoA by NADPH-reductase (PhaB) before the PHA synthase, PhaC polymerizes it to become PHA inclusion in Pathway I (refer Figure 2.2).

The involvement of other enzymes apart from PhaA, PhaB and PhaC in production of PHA was also observed as being summarized in Figure 2.2. For instance, PhaJ and FabG enzyme are required in *Rhodospirillum rubrum* where these two enzymes help to convert (*S*)-isomer of 3-hydroxybutyryl-CoA into (*R*)-isomer of hydroxybutyryl-CoA before it was polymerized to form PHA (pathway II). This is different from *C. necator* which can directly produce (*R*)-3-hydroxybutyryl-CoA by an enzyme called PhaB.

On the other hand, several *Pseudomonas* strains such as *P. putida*, *P. aerugenosa* and *P. mendocina* which capable of producing mcl-PHA from unrelated carbon sources most likely undergo PHA biosynthesis pathway just like pathway III (Anderson & Dawes, 1990). The 3-hydroxyacyl monomer was obtained from the

fatty acid biosynthesis pathway where the intermediate is in form of (*R*)-3-hydroxyacyl-ACP. This (*R*)-3-hydroxyacyl-ACP needs to be converted into (*R*)-3-hydroxyacyl-CoA first, the only form of isomer that could be polymerized by PhaC to form PHA inclusion. Thus, a supplementary step is needed where an enzyme called PhaG channels this (*R*)-3-hydroxyacyl-ACP for PHA biosynthetic pathway (Rehm *et al*., 1998).

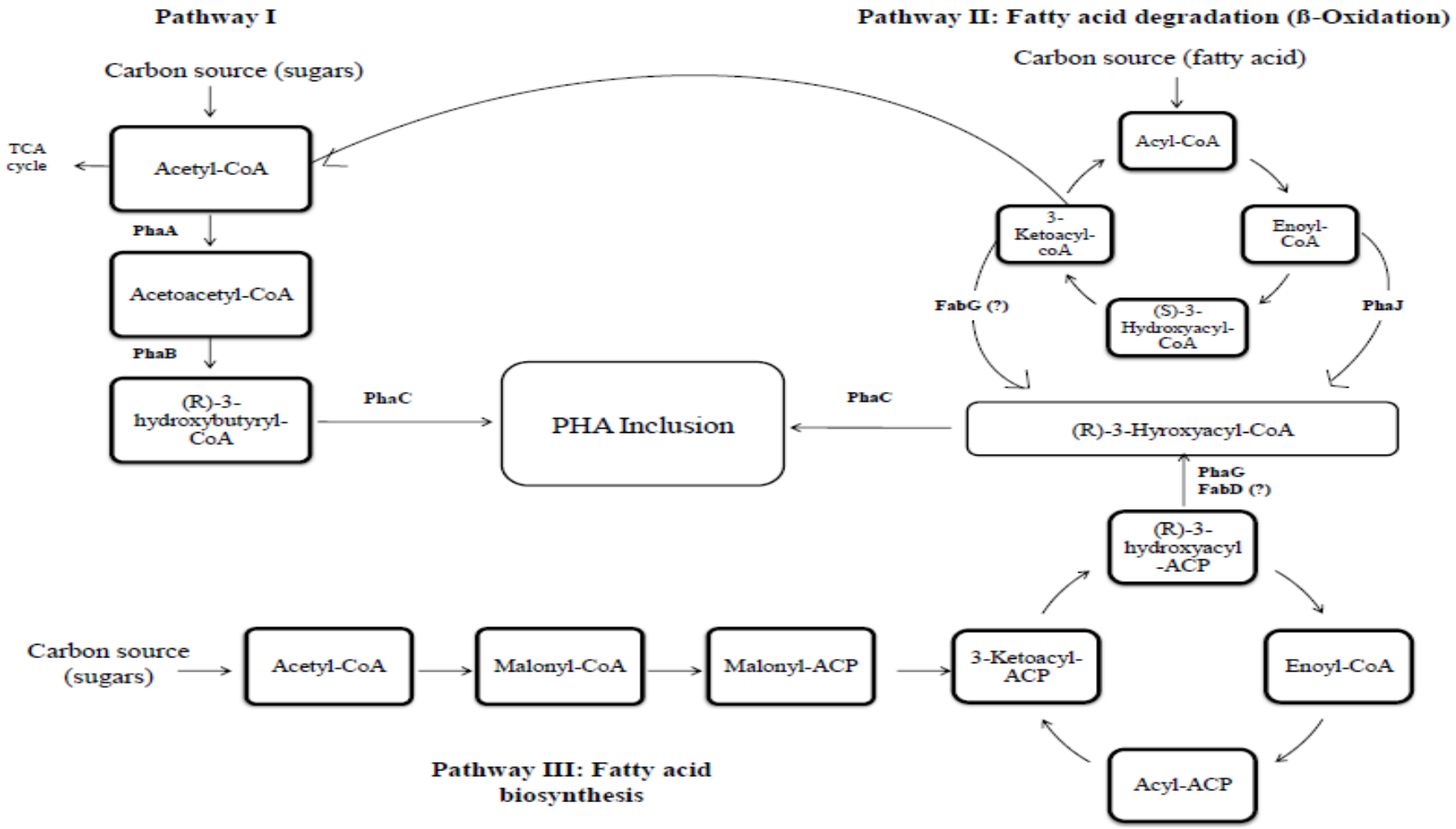

Figure 2.2 Metabolic pathway that are involved in the formation of HA monomers for PHA biosynthesis (Sudesh *et al*., 2000)

2.4 PHA production using genetically engineered bacteria

Even though PHA is recognized as an environmentally friendly polymer with huge range of characteristics which some are as good as synthetic polymer, the large scale production of this polymer was hampered by these following factors; high production cost, low yield and difficulty to control their structures and properties. The high production cost was mainly due to the usage of expensive substrates. Wild-type bacteria are able to utilize cheap substrate but produces low yield of PHA. Due to this, microbial metabolic engineering has been studied extensively to develop a recombinant bacteria which capable of utilizing cheap substrate and managed to produce higher yield.

For example, gene manipulation has been performed by Slater and co-workers when they constructed a recombinant *E. coli* by inserting P(3HB) biosynthetic genes from *C. necator* into both wild-type *E. coli* and *E. coli fadhR atoC* (Con) mutant to produce P(3HB-*co*-3HV) using medium containing glucose and propionate. As a result, P(3HB-*co*-3HV) was produced by *E. coli fadhR atoC* (Con) mutant with a composition of 10 to 40 mol% of 3HV total polymer while no 3HV was detected in polymer produced by wild-type *E. coli*. This mutant strain was genetically modified to eliminate the regulation of *E. coli* gene required for propionate metabolism and resulted in the uptake of propionate into P(3HB-*co*-3HV) synthesis. The increment in total PHA accumulation obtained also showed *E. coli fadhR atoC* (Con) mutant harboring *C. necator* P(3HB) biosynthetic genes capable to express the enzyme responsible for the utilization of short-chain fatty acids (Slater *et al*., 1992).

A transformant strain of *C. necator* was constructed by Lee and co-workers harboring the cloned genes related to P(3HB) biosynthesis which are *phbCAB*, *phbAB* and *phbC* to produce both P(3HB-*co*-3HV) and P(3HB-*co*-4HB). Interestingly, transformant strain of *C. necator* with cloned *phbC* gene managed to significantly increase both 3HV (1.5 fold higher) and 4HB (2.4 fold higher) unit in P(3HB-*co*-3HV) and P(3HB-*co*-4HB), respectively compared to parent strain. Other transformant strains with cloned *phbAB* and *phbCAB* showed slight increment and reduction of 3HV and 4HB unit composition, respectively compared to parent strain. This work has proved that P(3HB) synthase gene, *phbC* is the most critical gene which responsible for regulating unit composition of P(3HB-*co*-3HV) and P(3HB-*co*-4HB) (Lee *et al.*, 1997).

Amplification of some important genes for P(3HB) and P(3HB-*co*-3HV) biosynthesis was also done by Choi and co-workers where *phbC* gene encoding P(3HB) synthase and *zwf* gene encoding glucose-6 phosphate dehydrogenase generating NADPH from the metabolism of fructose were amplified separately and concurrently in *C. necator*. The biosynthesis of P(3HB) was enhanced significantly by the amplification of both genes separately and concurrently in the following order; *phbC* + *zwf* genes > *phbC* gene > *zwf* gene. This was due to the accelerated polymerization reaction of 3HB by the over-expressed P(3HB) synthase, along with sufficient supplementation of NADPH from the metabolism of fructose. On the other hand, 3HV unit in P(3HB-*co*-3HV) was also increased significantly after the co-amplification of both genes. The *zwf* gene alone was observed to not seriously affect the 3HV unit composition. This clearly proved that *phbC* gene has great influence in both P(3HB) and P(3HB-*co*-3HV) biosynthesis compared to the *zwf* gene generating NADPH (Choi *et al.*, 2003).

Recombinant bacteria were also constructed to achieve hyperproduction of PHA using cheap substrates to reduce production cost. As for instance, recombinant *E. coli* JM109 with succinate semialdehyde dehydrogenase gene (*sad* and *gabD*) deficient by co-expressing succinate degradation genes and P(3HB) synthase gene together with four PHA binding proteins (PhaP) which are PhaP1, PhaP2, PhaP3 and PhaP4 from *C. necator* were constructed to improve P(4HB) production from glucose. PhaPs are small amphipilic proteins which functioning in PHA synthesis and granule formation. The usage of glucose as substrate can lower the production cost of P(4HB) instead of using expensive 4HB precursor such as 4-hydroxybutyric acid, γ-butyrolactone and 1,4-butanediol. This study highlighted a significant improvement on the 4HB synthesis due to the inactivation of *sad* and *gabD* gene in recombinant *E. coli* compared to wild-type *E. coli* as succinate semialdehyde can be degraded to succinate, decreasing metabolic flux to 4HB production. The role of PhaP1 as dominant function for PHA granule formation was also proven when recombinant strain harboring PhaP1 binding protein was found useful to increase P(4HB) content among the other four PhaPs (Zhou *et al.*, 2012).

As a conclusion, the construction of recombinant bacteria with studied metabolic pathway successfully showed good potential to replace wild-type bacteria in PHA biosynthesis as they managed to increase PHA accumulation, able to produce PHA from cheap substrate and manage to synthesis new type of PHA thus allowing further exploitation of PHA for commercial applications.

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

As mentioned earlier, P(3HB) is a brittle and stiff material with high melting point which limits its application in material engineering. Due to this, researchers had studied in details the production of various copolymer to see their potential to improve the weak properties of P(3HB) such as P(3HB-*co*-3HV), P(3HB-*co*-3HH) and P(3HB-*co*-4HB). Among all studied copolymers, P(3HB-*co*-4HB) has been found to have interesting properties where the presence of 4HB unit produces a polymer with various mechanical properties ranging from highly crystalline to strong elastomeric rubber-like material which increases its applications (Saito *et al*., 1996). In addition, interesting finding by Williams & Martin (1996) highlighted P(3HB-*co*-4HB) is potentially applicable in medical and pharmaceutical applications.

2.5.1 P(3HB-*co*-4HB) production pathway

P(3HB-*co*-4HB) was first discovered from *C. necator* by Kunioka in 1989 when *C. necator* was grown in nitrogen free medium with 4-hydroxybutyric acid as carbon source (Doi *et al*., 1988). The incorporation of 4HB unit in P(3HB-*co*-4HB) is mainly due to the usage of 4HB related precursors substrates as carbon source such as 4-hydroxybutyric, γ-butyrolactone, 1,4-butanediol and other ω-alkanediols in many bacteria which possess PHA_{SCL} synthase for example *Cupriavidus* sp. As for instance, 4-hydroxybutyric will be converted into 4HB-CoA by a transferase or a thiokinase before being polymerized by PHA_{SCL} synthase and become 4-hydroxybutyrate (refer Figure 2.3). On the other hand, 3HB was resulted from the intermediates of catabolism of 4-hydroxybutyric acid where 3-hydroxybutyryl-CoA was synthesized leading to the formation of P(3HB-*co*-4HB) (refer Figure 2.4).

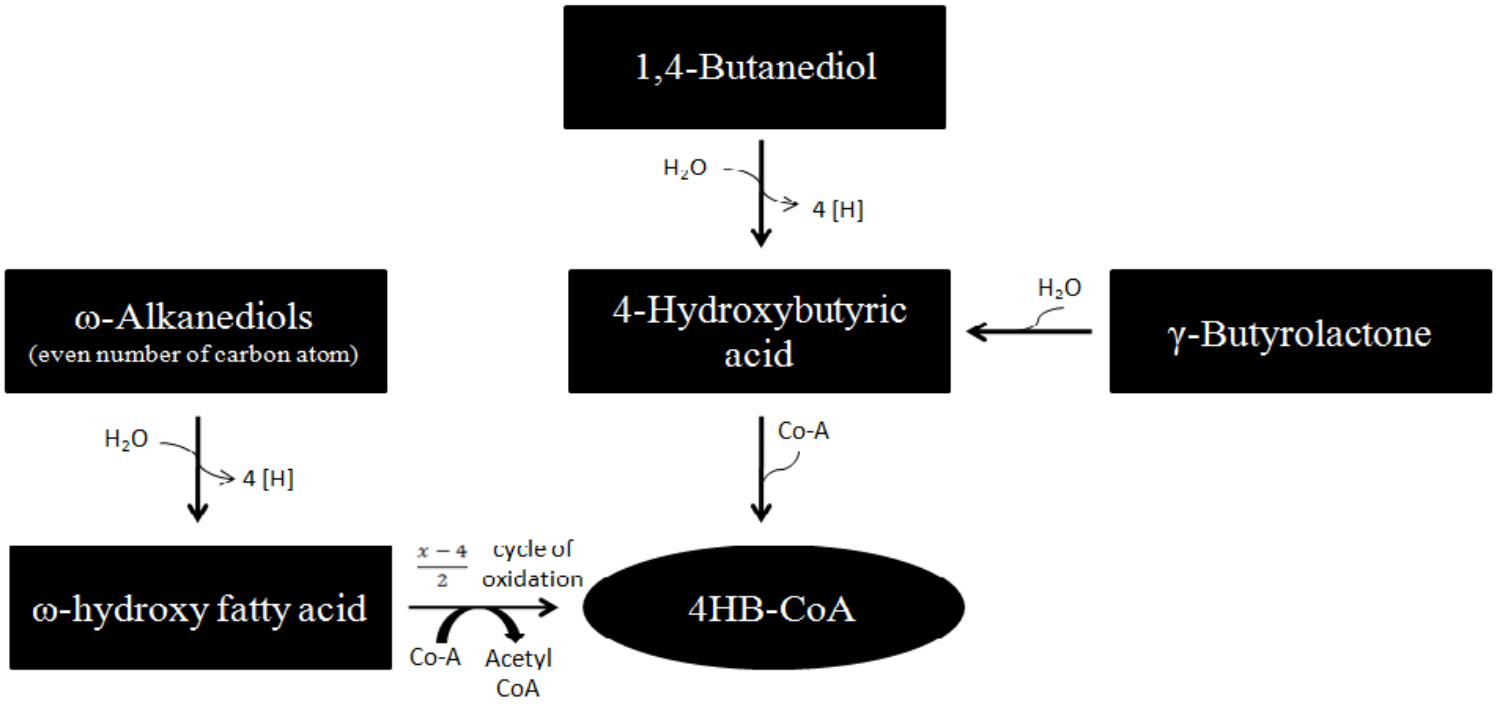

Figure 2.3: Sources of 4HB-CoA for biosynthesis of P(3HB-*co*-4HB) (Steinbüchel & Lütke-Eversloh, 2003)

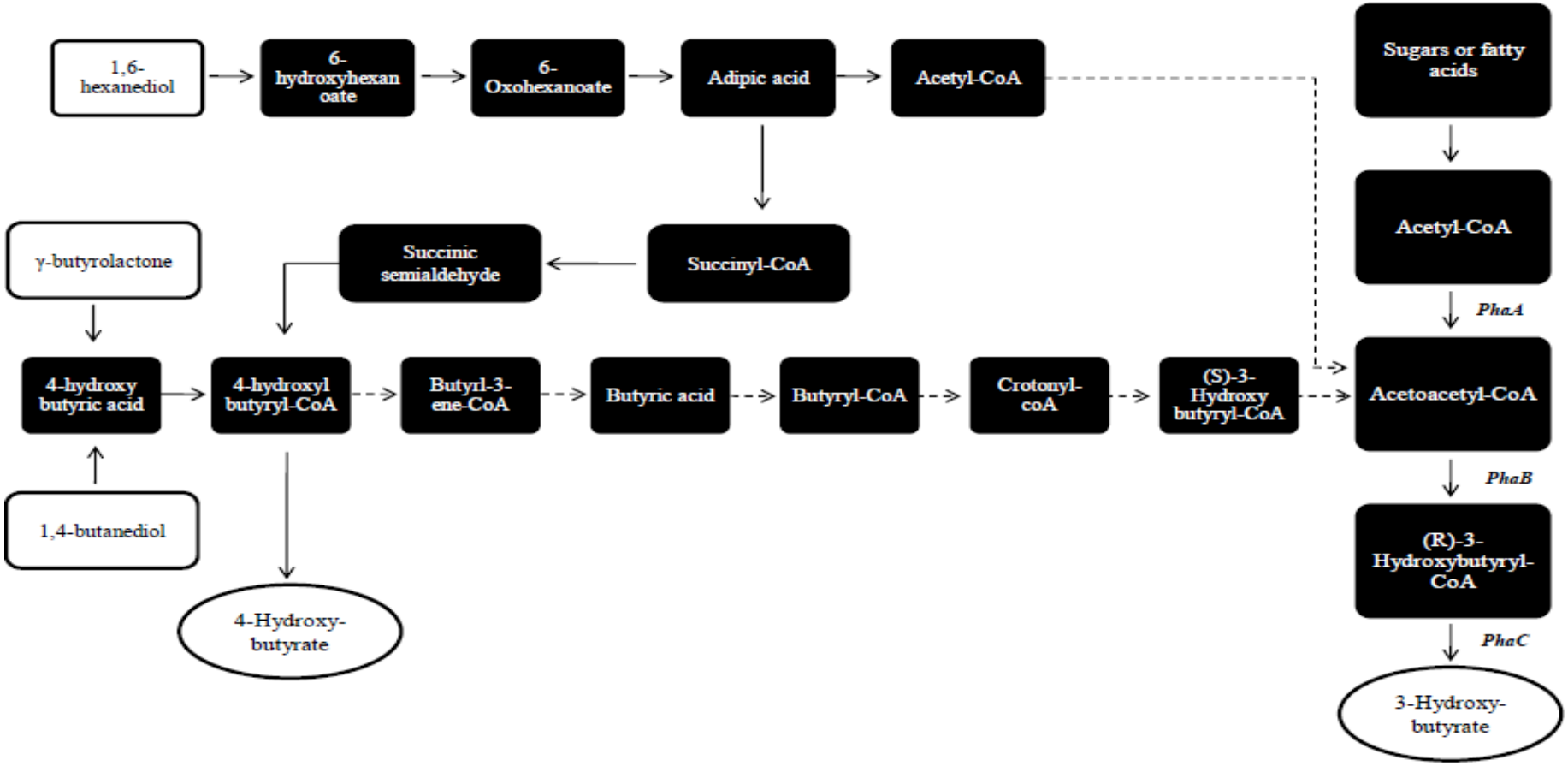

Figure 2.4: Proposed biosynthetic pathway of P(3HB-*co*-4HB) using related carbon sources (Steinbüchel & Lütke-Eversloh, 2003; Md. Iqbal & Amirul, 2013)

Another 4HB related substrates, γ-butyrolactone which hydrolytically cleaved to hydroxybutyric acid by esterase or lactonase also capable of producing 4HB-CoA which latter produces 4HB (refer Figure 2.3). Catabolized 4HB-CoA again can result in formation of 3HB (refer Figure 2.4). 4HB unit is also produce when 1,4-butanediol and other ω-alkanediols are used as substrate where this ω-alkanediol will be converted to 4HB-CoA through oxidation in two enzymatic reactions. Longer ω-alkanediol with even number of carbon atoms such as 1,6-hexanediol and 1,8-octanediol also ending up with the formation of 4HB-CoA in which they first oxidized to respective ω-hydroxyfatty acid and converted into a coenzyme A thioester before undergo ß-oxidation to form 4HB-CoA (refer Figure 2.3) (Steinbüchel & Lütke-Eversloh, 2003).

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

The assimilation of 4HB unit in P(3HB-*co*-4HB) results in polymer with huge range of properties as shown in Table 2.1. In terms of thermal properties, melting temperature, T_m, and glass transition temperature, T_g are important to be determined. Both are influenced by polymer chemical structure which related to polymer stiffness, presence of polar group and type as well as side group size. Unlike P(3HB) which exhibits high T_m, the incorporation of 4HB unit into P(3HB) makes P(3HB-*co*-4HB) having lower melting point which ease the melt-processing of the polymer for wider applications. When the 4HB unit increasing from 0 mol% to 38 mol%, the T_m, decreased from 178.2ºC to 152.2ºC while the T_g was also decreased from 2.4ºC to -9.9ºC. With the increment of 4HB unit from 0 to 27 mol%, Saito and co-workers highlighted that the crystallinity of copolymer decreased and crystallinity increased when 4HB composition increased from 64 mol% to 100 mol% (Saito *et al*., 1996).

The stress-strain behavior of a polymer was also examined to provide useful information regarding its tensile strength, elongation at break and Young's modulus. In terms of polymer tensile strength, Saito *et al.*, (1996) observed an increment in tensile strength when 4HB molar composition was increased from 64 mol% to 100 mol% which relates to the formation of crystalline region in the P(3HB-*co*-4HB). Chanprateep *et al*., (2010) demonstrated that Young's modulus of P(3HB-*co*-4HB) decreased from 1497 MPa to 655 MPa as the 4HB unit increased from 0 mol% to 38 mol% showing that the polymer exhibited more flexible and elastic properties compared to P(3HB) homopolymer. According to Young and Lovell (1991), Young's modulus of a polymer is influenced by the degree of cystallinity, shape, size and distribution of the crystals in the polymer. However, Saito *et al.*, (1996) found that the elongation at break value increased from 591% to 1000% as 4HB unit composition increased.

Not only that, the presence of 4HB unit in P(3HB-*co*-4HB) also speed up chain scission and enzyme hydrolysis process rate (Doi *et al*., 1990). Nishida *et al.* (1998) also mentioned highly crystalline material such as P(3HB) have lower biodegradability. Based on Table 2.1 the introduction of 4HB unit seem to lower the crystallinity in P(3HB-*co*-4HB) hence increase its biodegradability compared to P(3HB) homopolymer. In conclusion, the introduction of 4HB unit in P(3HB-*co*-4HB) showed an improved mechanical properties while the thermal properties can be regulated by varying the monomer compositions.

Table 2.1 Physical and thermal properties of P(3HB-*co*-4HB) with various 4HB unit composition (Saito *et al*., 1996; Vigneswari *et al*., 2009; Chanprateep *et al*., 2010)

Properties	4HB unit composition (mol%)										
	0	5	10	16	24	38	64	78	82	90	100
Mechanical											
a) Tensile strength (MPa)	43	1.36	24	26	48	2.98	17	42	58	65	104
b) Elongation at break (%)	5	11	242	444	2.98	48	591	1120	1320	1080	1000
Thermal											
a) Melting temperature (ºC)	178	169	-	130	161.1	48	50	49	52	50	53
b) Glass transition temperature (ºC)	4	-2	-	-7	-5	2.98	-35	-37	-39	-42	-48
c) Crystallinity (%)	60	-	45	45	-	-	15	17	18	28	34

Biocompatibility is one of the most important biological characters of a polymer to be considered for medical applications since it involves the *in vivo* biological response. As in P(3HB-*co*-4HB) which consists of both monomeric components of 3-hydroxybutyric acid and 4-hydroxybutyric acid makes P(3HB-*co*-4HB) to posses good biocompatibility properties. The 3-hydroxybutyric acid is a ketone body which can be found in human blood in range of 3 to 10 mg per 100 mL of blood in a healthy adult while in a report highlighted 4-hydroxybutyric acid to be widely distributed naturally in mammalian body which mainly present in heart, brain, kidney, liver, lung and muscle (Nelson *et al*., 1981).

2.5.3 Improvements in P(3HB-*co*-4HB) production strategies

Due to good properties of P(3HB-*co*-4HB) which results from the incorporation of 4HB unit, several fermentation strategies had been employed to increase the production of this and molar fraction of 4HB. This includes the usage of 4HB precursors during biosynthesis, employment of mixed substrates culture strategy and cultivation in fed-batch instead of normal batch operation. In biosynthesis of P(3HB-*co*-4HB) using wild-type strain of PHA producer, high 4HB molar fraction could not be achieved as the conversion of 4-hydroxybutyryl-CoA to 4HB is low due to conversion of 4-hydroxybutyryl-CoA to acetyl-CoA for 3HB formation through ketolysis reaction (Doi *et al*., 1990). Thus, to prevent the ketolysis reaction from occurring, small amount of stimulator such as propionate, acetate and α-amino acids are used. Lee and co-workers had studied the usage of propionate in the effort of increasing molar fraction of 4HB in P(3HB-*co*-4HB) with addition of small amount of propionate using γ-butyrolactone as substrate. As a result, 4HB molar fraction has increased significantly from 12.3 mol% to 51.8 mol%. The addition of propionate was observed to increase both P(3HB) synthase and acetyl-

CoA concentration. This is due to the metabolism of the propionate itself where it will condensed to acetyl-CoA for 3HB ketolysis and the other is to aceto-propionyl-CoA for 3HV formation. The abundant of acetyl-CoA seems to inhibit the ketolysis reaction hence 4HB fraction available for polymerization increased and consequently increasing 4HB fraction (Lee *et al*., 2000).

Another fermentation strategy that recently being used to increase both the PHA production and 4HB unit composition is mixed substrates cultivation strategy. Huong and co-workers in a study employed the mixture of two substrates in a single-stage fermentation and proved that this strategy worked well to overcome the limitation (low PHA production and low 4HB unit fraction) faced by single-stage fermentation of P(3HB-*co*-4HB). They highlighted an increment of PHA accumulation (as high as 73 wt%) in relative to the biomass formation when using combination of two 4HB related substrates (1,4-butanediol, 1,6-hexanediol and γ-butyrolactone) in three different combination ratios. This strategy also accumulated PHA with wider 4HB unit fractions in the range of 12 to 55 mol%. The mixed substrates strategy was observed to favor the channeling of carbon flow towards PHA biosynthetic pathway compared to the TCA cycle for cell growth hence increasing the PHA accumulation (Huong *et al*., 2015). Babel and co-workers also mentioned that the biotransformation of desired product can be enhanced when there is simultaneous utilization of the substrate mixture in a production culture (Babel *et al*., 1993).

Fed-batch fermentation is an efficient and reliable strategy to get high product concentration as a result of regulating the concentration of substrates that limits the production rates. Generally, fed-batch fermentation in PHA production depends on the characteristics of the PHA producer where they are divided into two groups. The

first group of bacteria is able to synthesis PHA in an environment with limitation of nutrients (nitrogen, phosphorus, magnesium or sulphur) from an excess of carbon sources. The second group of bacteria does not require nutrient limitation and can accumulate PHA during cell growth. To achieve high PHA accumulation using bacteria from the first group, a proper nutrient feeding strategy in fed-batch fermentation is needed in which the ratio of carbon source and nutrient mixture must be optimal for PHA accumulation. Usually the fermentation will be divided into two stages. The first stage involves the cell growth without nitrogen limitation which allows the cell to grow until a desired concentration of biomass is achieved. The second stage begins when the essential nutrients is kept in limiting concentration to allow maximum PHA accumulation. On the other hand, bacteria which belong to the second group only need a proper nutrient feeding strategy since the PHA accumulation does not depend on the nutrient limitation. However, cell growth and PHA accumulation need to be balanced to avoid incomplete accumulation of PHA or the premature termination of fermentation at low cell concentration (Khanna & Srivastava, 2005).

However, there is limited studies in fed-batch fermentation of P(3HB-*co*-4HB) production. Kim and co-workers had studied the fed-batch fermentation of P(3HB-*co*-4HB) using *C. necator* ATCC 17699 in order to increase both cell and PHA concentration. Two types of feeding; DO-stat and constant feeding were carried out using two substrates; fructose and γ-butyrolactone. In this study, they found the cell growth was inhibited by high concentration of fructose and γ-butyrolactone giving an idea that feeding rate of both substrates should be controlled in fed-batch fermentation. In the first stage, cell was grown to a desired concentration using DO-stat method where fructose was fed using dissolved oxygen as indicator. In DO-stat