BIOSYNTHESIS OF POLY(3-HYDROXYBUTYRATE-CO-3-HYDROXY-4-METHYLVALERATE) COPOLYMER BY Chromobacterium sp. USM2

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by

LING SIEW CHEN

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

SYMBOLS AND ABBREVIATIONS	FULL NAME
α	Alpha
β	Beta
°C	Degree Celsius
°C/min	Degree Celsius per minute
%	Percentage
±	Plus-minus
× *g*	Times gravity
ΔH_m	Enthalpy of fusion
μm	Micrometer
μL	Microliter
^{1}H	Proton
^{13}C	Carbon-13
3H4MV	3-hydroxy-4-methylvalerate
3H4MV-CoA	3-hydroxy-4-methylvalerate-CoA
3HB	3-hydroxybutyrate
3HB-CoA	3-hydroxybutyrate-CoA
3HD	3-hydroxydecanoate
3HHx	3-hydroxyhexanoate
3HO	3-hydroxyoctanoate
3HV	3-hydroxyvalerate
4HB	4-hydroxybutyrate

A_{3H4MV}	Area of 3H4MV monomer peak
A_{3HB}	Area of 3HB monomer peak
A_{CME}	Area of CME peak
ACP	Acyl carrier protein
ATP	Adenosine triphosphate
B	Dry cell weight used during methanolysis
BOD	Biochemical Oxygen Demand
$CaCl_2$	Calcium chloride
$Ca(NO_3)_2 \cdot 4H_2O$	Calcium nitrate tetrahydrate
$CDCl_3$	Deuterated chloroform
CH	Methine group
CH_2	Methylene group
CH_3	Methyl group
cm^{-1}	Reciprocal centimeter
CME	Caprylic acid methyl ester
CO_2	Carbon dioxide
CoA	Coenzyme A
CoA-SH	Coenzyme A with sulfhydryl functional group
$CoCl_2 \cdot 6H_2O$	Cobalt (II) chloride hexahydrate
COSY	Correlated spectroscopy
CPKO	Crude palm kernel oil
$CrCl_3 \cdot 6H_2O$	Chromium chloride hexahydrate
$CuSO_4 \cdot 5H_2O$	Copper (II) sulphate pentahydrate
Da	Dalton
DCW	Dry cell weight

DO	Dissolve oxygen
DSC	Differential scanning calorimetry
EDTA	Ethylenediaminetetraacetic acid
FabD	Malonyl-CoA-ACP transacylase
FabG	3-ketoacyl-CoA reductase
FabH	3-ketoacyl-ACP synthase III
FADH	Reduced flavin adenine dinucleotide
$FeCl_3$	Iron (III) chloride
FT-IR	Fourier transform infrared spectrometry
g	Gram
g/L	Gram per liter
GC	Gas chromatography
GPa	Gigapascal
GPC	Gel permeation chromatography
h	Hour
HCl	Hydrochloric acid
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Correlation
J/g	Joule per gram
K	Gas chromatography factor
K_{3HB}	3-hydroxybutyrate factor
K_{3H4MV}	3-hydroxy-4-methylvalerate factor
KBr	Potassium bromide
KCl	Potassium chloride
kDa	Kilodalton

KH_2PO_4	Potassium dihydrogen phosphate
KOH	Potassium hydroxide
kPa	Kilopascal
L	Liter
LB	Luria Bertani
LDPE	Low-density polyethylene
M_n	Number-average molecular weight
M_w	Molecular weight
M	Molar
mcl-	Medium-chain-length
mcl-PHA	Medium-chain-length polyhydroxyalkanoate
mcl-HA-CoA	Medium-chain-length hydroxyacyl-CoA
mg	Milligram
mg/L	Milligram per liter
$MgSO_4 \cdot 7H_2O$	Magnesium sulphate heptahydrate
MHz	Megahertz
min	Minute
mL	Milliliter
mL/L	Milliliter per liter
mL/min	Milliliter per minute
MM	Mineral medium
mM	Millimolar
mm	Millimeter
mm/min	Millimeter per minute
mm^3	Millimeter cube

mol%	Mole percent
MPa	Megapascal
N	Normality
Na_2HPO_4	Disodium hydrogen phosphate
Na_2SO4	Sodium sulphate
NaCl	Sodium chloride
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NH_4Cl	Ammonium chloride
$NiCl_2 \cdot 6H_2O$	Nickel chloride hexahydrate
nm	Nanometer
NMR	Nuclear magnetic resonance
OD	Optical density
OsO_4	Osmium tetroxide
P(3HB)	Poly(3-hydroxybutyrate)
P(3HB-*co*-3H4MV)	Poly(3-hydroxybutyrate-*co*-3-hydroxy-4-methylvalerate)
P(3HB-*co*-3H4MV-*co*-3HV)	Poly(3-hydroxybutyrate-*co*-3-hydroxy-4-methylvalerate-*co*-3-hydroxyvalerate)
P(3HB-*co*-3HHx)	Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate)
P(3HB-*co*-3HV)	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)
P(3HB-*co*-3HV-*co*-3H4MV-*co*-3HHx)	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-3-hydroxy-4-methylvalerate -*co*-3-hydroxyhexanoate)
P(3HB-*co*-3HV-*co*-3HHx)	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-3-hydroxyhexanoate)

P(3HB-*co*-4HB)	Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate)
P(3HV)	Poly(3-hydroxyvalerate)
P(4HB)	Poly(4-hydroxybutyrate)
PDI	Polydispersity index
PHA	Polyhydroxyalkanoate
PhaA	*β*-ketothiolase
phaA	Gene encoding *β*-ketothiolase
PhaB	NADPH-dependent acetoacetyl-CoA reductase
PhaB	Gene encoding NADPH-dependent acetoacetyl-CoA reductase
PhaC	PHA synthase
phaC	Gene encoding PHA synthase
PhaE	Subunit of PHA synthase
phaE	Gene encoding subunit of PHA synthase
PhaG	(*R*)-3-hydroxyacyl-ACP-CoA transferase
PhaJ	(*R*)-specific enoyl-CoA hydratase
PhaP	Phasin
phaP	Gene encoding phasin
PhaR	Regulator protein of the phasin expression
phaR	Gene encoding regulator protein of the phasin expression
PhaZ	PHA depolymerases
phaZ	Gene encoding PHA depolymerases
PLA	Polylactic acid
PP	Polypropylene
ppm	Parts per million

psi	Pounds per square inch
PTFE	Polytetrafluoroethylene
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
scl-	Short-chain-length
scl-PHA	Short-chain-length polyhydroxyalkanoate
scl-HA-CoA	Short-chain-length hydroxyacyl-CoA
SDS	Sodium dodecyl sulfate
sp.	Species
T_d	Decomposition temperature
T_g	Glass-transition temperature
T_m	Melting temperature
TCA	Tricarboxylic acid
TEM	Transmission electron microscope
TGA	Thermogravimetric analysis
TMS	Tetramethylsilane
v/v	Volume per volume
wt%	Weight percentage
w/v	Weight per volume

Biosintesis poli(3-hidroksibutirat-*ko*-3-hidroksi-4-metilvalerat) kopolimer oleh *Chromobacterium* sp. USM2

ABSTRAK

Polihidroksialkanoat (PHA) yang telah dipertingkatkan ciri-ciri fisikal-kimia merupakan polimer yang ideal untuk diaplikasikan dalam pelbagai sektor. Penyatuan monomer baru, 3-hidroksi-4-metilvalerat (3H4MV) pada rangkaian monomer PHA dapat memperbaiki ciri-ciri polimer secara keseluruhan. Namun begitu, mikroorganisma yang sesuai dan PHA sintase yang mampu mempolimerisasikan monomer ini dengan efisien masih belum dapat diketahui. Dalam kajian ini, keupayaan *Chromobacterium* sp. USM2 untuk mensintesis poli(3-hidroksibutirat-*ko*-3-hidroksi-4-metilvalerat) [P(3HB-*ko*-3H4MV)] dalam keadaan pengkulturan yang berbeza telah dianalisis dan dinilai. *Chromobacterium* sp. USM2 didapati berupaya menghasilkan P(3HB-*ko*-3H4MV) apabila glukosa dan asid isokaproik dibekalkan sebagai sumber karbon. Namun, komposisi monomer 3H4MV yang tertinggi iaitu sebanyak 22% telah dicatatkan apabila asid isokaproik digunakan sebagai sumber karbon tunggal. Selain itu, manipulasi kepekatan oksigen terlarut dalam media kultur telah dikenalpasti sebagai satu faktor penting yang boleh meningkatkan akumulasi monomer 3H4MV. Konfigurasi struktur P(3HB-*ko*-3H4MV) telah dikenalpasti dengan menggunakan analisa FT-IR, 1D dan 2D NMR. Di samping itu, kopolimer P(3HB-*ko*-3H4MV) yang dihasilkan telah dinilai ciri-cirinya melalui analisa termal dan analisa tegangan. Kopolimer P(3HB-*ko*-3H4MV) dengan berat molekul dalam julat antara 5.4×10^5–9.3×10^5 Da telah dihasilkan. Analisa TGA dan ujian tegangan menunjukkan penyatuan monomer 3H4MV dalam

rantai polimer 3HB menghasilkan polimer yang lebih fleksibel dan mempunyai kestabilan termal yang lebih baik berbanding homopolimer P(3HB).

Biosynthesis of poly(3-hydroxybutyrate-*co*-3-hydroxy-4-methylvalerate) copolymer by *Chromobacterium* sp. USM2

ABSTRACT

Polyhydroxyalkanoate (PHA) with enhanced physicochemical properties will be ideal for a wide range of practical applications. The incorporation of 3-hydroxy-4-methylvalerate (3H4MV), a branched C_6-monomeric unit into the polymer backbone is known to improve the overall properties of the resulting polymer. However, the most suitable microorganism and PHA synthase that can synthesize and polymerize this monomer efficiently still remain unknown at present. In this study, the ability of *Chromobacterium* sp. USM2 to synthesize poly(3-hydroxybutyrate-*co*-3-hydroxy-4-methylvalerate) [P(3HB-*co*-3H4MV)] was evaluated under different culture conditions. It was found that *Chromobacterium* sp. USM2 can synthesize P(3HB-*co*-3H4MV) when glucose and isocaproic acid were fed as carbon sources. However, the highest molar fraction of 3H4MV, 22 mol% was detected in *Chromobacterium* sp. USM2 when isocaproic acid was provided as the sole carbon source. In addition, dissolved oxygen concentration was identified as a crucial factor in initiating the accumulation of high 3H4MV molar fractions. The structural configuration of the resulting P(3HB-*co*-3H4MV) was confirmed by FT-IR, 1D and 2D NMR spectroscopy analysis. Subsequently, the thermal and tensile properties of P(3HB-*co*-3H4MV) copolymer was characterized. P(3HB-*co*-3H4MV) copolymer with M_w in the range of 5.4 x 10^5–9.3 x 10^5 Da were produced. TGA analysis and tensile test further revealed that the incorporation of small amounts of 3H4MV into 3HB polymer chain resulted in greater flexibility and thermal stability compared to P(3HB) homopolymer.

1.0 INTRODUCTION

Petroleum-based synthetic plastics have become an indispensable part in our daily life. They are being utilized in almost every sector from household applications to manufacturing industries. These plastics are very much advantageous in terms of their diverse properties such as being highly chemical resistant, durable and can be chemically manipulated and molded into desired strengths and shapes (Reddy et al., 2003). Due to their excellent properties and widespread applications, worldwide demand for plastics has dramatically increased from 1.5 million tons in 1950 to 245 million tons in 2008 (Chanprateep, 2010). Nevertheless, these materials are not biodegradable and they are often discarded indiscriminately causing severe problems both in terrestrial and marine environments. Petroleum-based plastics often have single-use applications, especially in food packaging and medical materials. It is very difficult to reduce the consumption of plastics due to their versatile properties and affordable price. Therefore, plastics have become an integral part of our daily living.

However, due to the accumulation of plastic waste and being non-biodegradable, there has been increasing public concerns over the harmful impacts of these recalcitrant materials to the environment. Although plastic waste can be disposed off by incineration and recycling, incineration of plastics is practically dangerous and recycling is a time-consuming and labour intensive process. Besides, these two processes become worse as incineration of plastics releases hazardous chemicals such as furans and dioxins from the combustion of polyvinylchloride, while plastics in the presence of pigments, coatings and other additives cannot be recycled (Ojumu et al., 2004; Jayasekara et al., 2005). Owing to environmental concerns, much effort has been dedicated towards the development of biobased and

biodegradable polymers in order to help us overcome the problem of plastic waste disposal.

Over the years, polyhydroxyalkanoate (PHA) has been studied extensively and is recognized as biobased and completely biodegradable plastics (Sudesh and Iwata, 2008). Therefore, PHA serves as a potential material for the replacement of some commodity plastics (Sudesh and Iwata, 2008). PHA appears to be one of the most fascinating polymers as it possess similar thermoplastic properties to some synthetic polymers and is completely biodegradable after being discarded in the environment (Khanna and Srivastava, 2005; Chanprateep, 2010). About 150 different types of PHA monomers classified as short-chain-length (scl) PHA and medium-chain-length (mcl) PHA have been reported (Steinbüchel and Lütke-Eversloh, 2003). These polymers are accumulated as intracellular inclusions, serving as carbon and energy reserve (Anderson and Dawes, 1990; Fuller, 1999).

Although many Gram-positive and Gram-negative bacteria have been identified to synthesize PHA, isolation and identification of new promising candidates with better or comparable ability to produce novel PHA are being continuously investigated. A free living, violacein producing, Gram-negative bacterium, *Chromobacterium* sp. USM2, which was isolated from a waterfall in Langkawi, Malaysia, was used throughout this study (Bhubalan et al., 2010a). A similar strain *Chromobacterium violaceum,* which can be found in a wide range of tropical and subtropical biodiversity (Carepo et al., 2004), is known to accumulate PHA (Steinbüchel et al., 1993) in both aerobic and anaerobic conditions. The ability of *C. violaceum* to produce high levels of poly(3-hydroxyvalerate) [P(3HV)] homopolymer and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] when fed with valeric acid have been reported (Steinbüchel et al., 1993). Besides

that, both *Chromobacterium* sp. USM2 and *C. violaceum* can produce P(3HB-*co*-3HV) when grown in sodium valerate and sodium propionate, as well as trace amounts of 3-hydroxyhexanoate (3HHx) (Kolibachuk et al., 1999; Bhubalan et al., 2010a). Additionally, *Cupriavidus necator* PHB^{-}4 transformant harboring the PHA synthase gene (*phaC*) of *Chromobacterium* sp. USM2 was discovered to produce copolymers and terpolymers containing 3HHx (Bhubalan et al., 2010b; Chia et al., 2010).

In this study, the ability of locally isolated *Chromobacterium* sp. USM2 to biosynthesize PHA containing 3-hydroxy-4-methylvalerate (3H4MV) monomer was evaluated. Using this newly isolated bacterial strain, the production of PHA containing various molar fractions of 3H4MV has been carried out by supplementing the culture medium with special chemical precursors under various culture conditions. Finally, the chemical and tensile properties of the resulting copolymers were also analyzed.

1.1 Objectives of this study

1) To study the ability of locally isolated *Chromobacterium* sp. USM2 to synthesize P(3HB-*co*-3H4MV)
2) To determine crucial culture parameters for improvement of 3H4MV monomer composition production by *Chromobacterium* sp. USM2
3) To analyze the properties of P(3HB-*co*-3H4MV) with various monomer compositions

2.0 LITERATURE REVIEW

2.1 Polyhydroxyalkanoate (PHA)

The occurrence of PHA was first observed by a French microbiologist Maurice Lemoigne in 1920s in the form of cytoplasmic inclusions in Gram-positive *Bacillus megaterium* (Lemoigne, 1926). Ever since then, numerous prokaryotic bacterial strains have been identified to synthesize various types of PHA under unfavorable growth conditions. PHA is deposited as water-insoluble cytoplasmic inclusions within the cells in the presence of excess carbon source and depletion of essential nutrients such as nitrogen, phosphorous, sulphur or dissolved oxygen (Senior and Dawes, 1971; Anderson and Dawes, 1990; Steinbüchel and Füchtenbusch, 1998). PHA is accumulated as intracellular granules in the bacterial cells, serves as carbon and energy reserve compound (Sudesh and Doi, 2000). Additionally, PHA also serves as a sink of reducing equivalents within the cells (Senior and Dawes, 1971).

Accumulation of intracellular PHA granules can be identified by a number of methods. A method known as rapid staining technique using lipophilic dyes such as Sudan black B, Nile blue and Nile red are commonly applied for detection and staining of intracellular PHA granules in bacterial cell (Burdon, 1946; Ostle and Holt, 1982; Kitamura and Doi, 1994; Gorenflo et al., 1999; Spiekermann et al., 1999). Besides detecting PHA granules using staining techniques, they also can be observed using transmission electron microscope (TEM). PHA granules appear as discrete and spherical particles with clear boundaries within the cell cytoplasm. The size and number of granules per cell varies depending on PHA-producer microorganisms. Although the presence of PHA can be identified using staining methods, chemical analyses are often required to determine intracellular PHA

content and its monomeric composition. Gas chromatography (GC) and nuclear magnetic resonance (NMR) spectroscopy analysis are widely used for this purpose.

Approximately 150 different constituents of PHA has been identified (Steinbüchel and Lütke-Eversloh, 2003). The chemical and physical properties of PHA depend strongly on the type and sequence distribution of comonomer in the polymer side chains, as well as the average molecular weight and molecular weight distribution (Noda et al., 2005; Lu et al., 2009a). The fact that these water insoluble PHA exhibit physical and mechanical properties comparable to petroleum-based synthetic plastics in addition to biodegradability and biocompatibility, has resulted in various applications in packaging industry, medical and pharmaceutical field, biofuel or as biocontrol agent in aquaculture systems (Holmes, 1985; Chen and Wu, 2005; Wang et al., 2008; Bian et al., 2009; Defoirdt et al., 2009; Zhang et al., 2009).

2.2 Diversity of PHA

PHA is mainly composed of (*R*)-3-hydroxyalkanoic acid monomers unit and the general structure of PHA is shown in Figure 2.1 (Madison and Huisman, 1999). In addition to saturated side groups, unsaturated and straight- or branched chain containing halogenated or aromatic side groups have been reported as well (Lageveen et al., 1988; Abe et al., 1990; Doi and Abe, 1990; Fritzsche et al., 1990a; Fritzsche et al., 1990b; Kim et al., 1991; Kim et al., 1992; Choi and Yoon, 1994; Hazer et al., 1994; Curley et al., 1996; Song and Yoon, 1996).

PHA can be classified into three groups based on the number of carbon atoms present in the monomer units: i) as short-chain-length PHA (scl-PHA), ii) medium-chain-length PHA (mcl-PHA) and iii) PHA comprising of both scl- and mcl- monomer units (scl-mcl-PHA). Historically, poly(3-hydroxybutyrate) [P(3HB)]

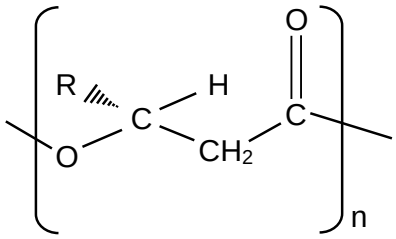


Figure 2.1 General structure of PHA. R refers to the composition of the side chain whereas 'n' refers to the number of repeating units. Both R and n determine the type of hydroxyalkanoic acid monomer unit.

was the most extensively studied PHA polymer. Apart from that, other PHA monomers such as 3-hydroxyvalerate (3HV), 4-hydroxybutyrate (4HB), poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] and poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] are among the commonly investigated polymers. Scl-PHAs are mostly accumulated by *Cupriavidus necator*, *Alcaligenes latus* and *Escherichia coli* transformant (Lee, 1996b; Lee, 1996a). Scl-PHA refers to hydroxyalkanoate monomers with 3 to 5 carbon atoms while mcl-PHA refers to monomers of 6 to 14 carbon atoms. *Pseudomonas oleovorans* and *P. putida* are well known to synthesize mcl-PHA such as 3-hydroxyoctanoate (3HO) and 3-hydroxydecanoate (3HD) from fatty acids (Sun et al., 2007; Chardron et al., 2010). To date, only a few microorganisms such as *Pseudomonas* sp. 61–3, *P. stuzteri*, *Aeromonas caviae*, *A. hydrophila*, and *Nocardia corallina* showed unique substrate specificity to produce scl-mcl-PHA (Abe et al., 1994; Fukui and Doi, 1997; Hall et al., 1998; Lee et al., 2000; Chen et al., 2004).

The possible formation of diverse types of PHA is strongly dependent on the extraordinary substrate specificity of PHA synthases as well as the availability of precursor substrate, which is particularly influenced by the metabolic background of the microorganism (Rehm and Steinbüchel, 1999). Thus, desirable PHA properties can be tailor-made by several means of protein engineering of the PHA biosynthetic

enzymes, manipulation of metabolic pathways and external substrates as well as recombinant gene expression and genome manipulation (Aldor and Keasling, 2003)

2.3 PHA granule formation

PHA granules are enveloped by a layer of phospholipids monolayer with four different types of granule-associated proteins consisting of PHA synthases, PHA depolymerases (PhaZ), phasin (PhaP), and regulator protein of the phasin expression (PhaR) (Luengo et al., 2003; Pötter and Steinbüchel, 2006). During the last two decades, investigation of PHA metabolism has led to a better understanding on the individual function of these four major granule-associated proteins (Stubbe and Tian, 2003; Rehm, 2006; Jendrossek, 2007). However, the initiation site of PHA synthesis and PHA granule formation remains unknown.

Two models of *in vivo* PHA granule formation have been proposed, namely, the micelle model (Gerngross et al., 1994) and the budding model (Stubbe and Tian, 2003). Both models consider the defined location of the PHA synthase (Figure 2.2). The micelle formation model (Figure 2.2 A) assumes that PHA synthase are randomly distributed in the cytoplasm and aggregates to micelle-like structures once the first polymerization reactions have occurred (Stubbe and Tian, 2003; Pötter and Steinbüchel, 2006; Jendrossek, 2009). In this model, the PHA synthase is converted into an amphipathic molecule upon polyester chain synthesis and remains covalently attached to the micelle. Thus, increase formation of the PHA chains will simultaneously lead to the increasing volume of the self-assembly micelles and formation of larger PHA granules (Stubbe and Tian, 2003; Pötter and Steinbüchel, 2006; Jendrossek, 2009). PHA granule formation *in vitro* and in the absence of

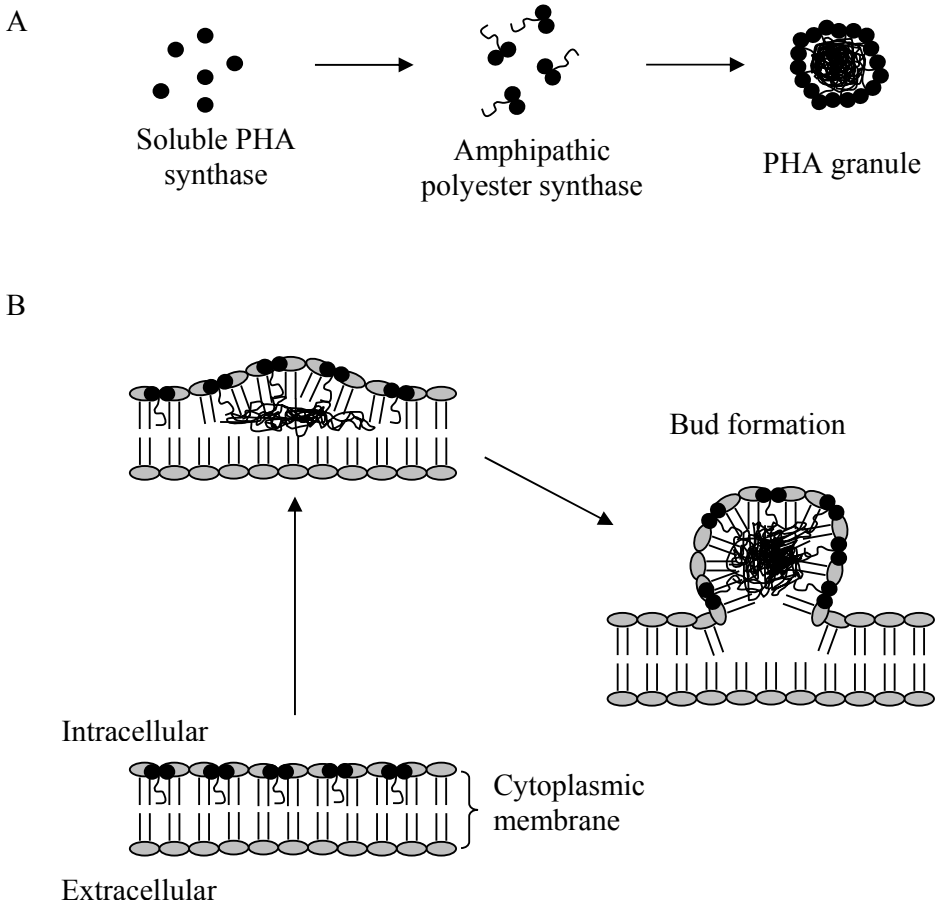


Figure 2.2 Working models for the formation of PHA granule in bacteria. (A) The micelle model. (B) The budding model. The irregular lines represent the elongation of PHA chains. ● , soluble polyester synthase; ● , PHA synthase with polymer chain; , phospholipid of cytoplasmic membrane (Rehm, 2006).

membranes provides experimental support for the micelle granule formation model (Gerngross and Martin, 1995; Qi et al., 2000).

On the other hand, the budding model (Figure 2.2 B) assumes that PHA synthase is attached to the cytoplasmic membrane, resulting in the formation of PHA granule between the two layers of the phospholipid bilayer. Eventually, the growing PHA granule will be released into the cytoplasm at the later stages of budding and PHA-specific proteins are attached to the surface of the granules (Jendrossek, 2005; Pötter and Steinbüchel, 2006; Jendrossek, 2009). Investigations on the early stages of PHA granules formation reported that early stage granules were found to be localized near to the cell wall and to the cell poles (Jendrossek, 2005; Peters and Rehm, 2005). These results suggest that PHA granule is synthesized in particular regions in the cell and supported the budding model.

Aside from these models, Tian et al. (2005) proposed a third model for granule formation. Tian and coworkers found granules formation close to unknown mediation elements located in the center cells when wild type *C. necator* are grown in PHA production medium. The granules were found to increase in size and stay close to these mediation elements for the first 24 h of incubation. It was proposed that these mediation elements played an important role in the nucleation of granule formation.

Phasin (PhaP), a structural protein that is attached non-covalently to the PHA granule surface layer, plays an important role in PHA biosynthesis (Pötter and Steinbüchel, 2005). The PhaP of *C. necator* is the best studied phasin although the structures of phasin proteins have not yet been reported (Stubbe and Tian, 2003). The exact function of PhaP was not understood but they have been shown to affect PHA biosynthesis and PHA granule size (Stuart et al., 1998). The lack of phasin in

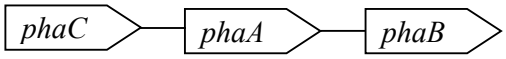
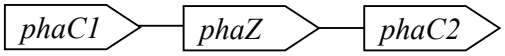
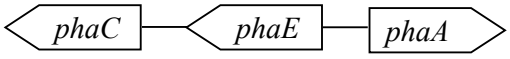
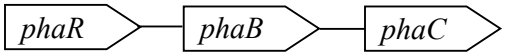
mutants had led to significant decreased in PHA content (York et al., 2001), while over expression of PhaP resulted in formation of many small PHA granules (Pötter et al., 2002). Alternatively, PhaR serves as auto-regulated repressor to control the expression of PhaP (Maehara et al., 2002).

2.4 PHA synthases – The key enzyme of PHA biosynthesis

PHA synthases, also referred to as PHA polymerases, has been identified as the key enzyme that catalyze the polymerization of hydroxyacyl-coenzyme A (CoA), provided by precursor pathways, into water insoluble PHA with the concomitant release of CoA (Rehm, 2003; Taguchi and Doi, 2004; Jendrossek, 2009). More than 59 different PHA synthases from 45 different bacteria have been cloned and sequenced (Rehm and Steinbüchel, 2001; Rehm, 2003). Four major classes of PHA synthase have been categorized based on their primary structure, substrate specificities and subunit composition (Table 2.1) (Rehm, 2003).

Class I PHA synthases, which can be represented by the well-characterized PhaC of *C. necator*, preferentially accept the CoA thioesters of short chain length hydroxyalkanoic acids (scl-HA-CoA, 3–5 carbon atoms); whereas Class II PHA synthases from most Pseudomonads preferentially polymerize the CoA thioesters of medium chain length hydroxyalkanoic acids (mcl-HA-CoA), comprising 6–14 carbon atoms. Class I PHA synthases occurs in most scl-PHA accumulating microorganisms. This type of PHA synthases comprises of only one type of subunit with molecular weights ranging from 60–73 kDa (Rehm, 2003). In contrast, class II PHA synthases comprises of two similar subunits, PhaC1 and PhaC2 with size ranging from 60–65 kDa (Sudesh et al., 2000). On the other hand, class III and class IV PHA synthases are composed of two heterosubunits that exhibit similar substrate

Table 2.1 Classification of PHA synthase (*phaC*) based on their primary structures, substrate specificities and the representative species (Pötter and Steinbüchel, 2006).

Class	General structure of PHA operon and representative species	Subunits	Substrate specificity
I	*C. necator* 	~60–73 kDa	scl-HA-CoA (C3–C5)
II	*P. oleovorans* 	~60–65 kDa	mcl-HA-CoA (C5–C14)
III	*Allochromatium vinosum* 	PhaC ~ 40 kDa PhaE ~ 40 kDa	scl-HA-CoA (C3–C5); mcl-HA-CoA (C5–C8)
IV	*Bacillus megaterium* 	PhaC ~ 40kDa PhaR ~22 kDa	scl-HA-CoA (C3–C5)

phaA, *β*-ketothiolase; *phaB*, NADPH-dependent acetoacetyl-CoA reductase; *phaC*, *phaC1*, and *phaC2*, PHA synthases; *phaE*, subunit of PHA synthases, *phaR*, regulator protein of the phasin expression; *phaZ*, PHA depolymerase

preference as class I PHA synthases (Rehm and Steinbüchel, 2001; Taguchi and Doi, 2004). Class III PHA synthases comprises of two different types of subunits; PhaC (~40 kDa) and PhaE (~40 kDa) and accept the scl-HA-CoA as substrates, while some of them accept the mcl-HA- CoA substrates. Steinbüchel and coworkers (1992) had reported that these two subunits are necessary for the expression of functional activity for Class III synthases. This type of PHA synthases has so far been identified in PHA-producing strains such as *C. vinosum*, *Thiocapsa violacea* and *Synechocystis* sp. PCC 6803 (Sudesh et al., 2000). Class IV synthases are represented by *B. megaterium*, exhibit similar subunit as those of class III synthases,

but PhaE is replaced by PhaR subunit with molecular weight of approximately 20 kDa (McCool and Cannon, 2001).

A phylogenetic tree based on multiple alignments of various PHA synthases supports the classification of PHA synthases (Figure 2.3). However, there are some exceptions to this classification such as the synthases from *Thiocapsa pfennigii* (two different subunits with strong similarity to class III PHA synathases), *A. caviae* (one type of subunit with strong similarity to class I PHA synathases) and *Pseudomonas* sp. 61–3 (PhaC1 and PhaC2 with strong similarity to class II PHA synathases), which showed an unusual substrate specificity of PHA synthases. Liebergesell and coworkers (1993) had reported the synthase of *T. pfennigii* exhibits a broad substrate specificity, which accepts CoA thioesters of scl- (3–5 carbon atoms) as well as mcl- (6–14 carbon atoms) 3-hydroxy fatty acids. Apart from that, the synthases of *A. caviae* and *Pseudomonas* sp. 61–3 also showed the ability to catalyze the polymerization of copolymer consisting of both scl- and mcl-PHA (Fukui and Doi, 1998; Matsusaki et al., 1998).

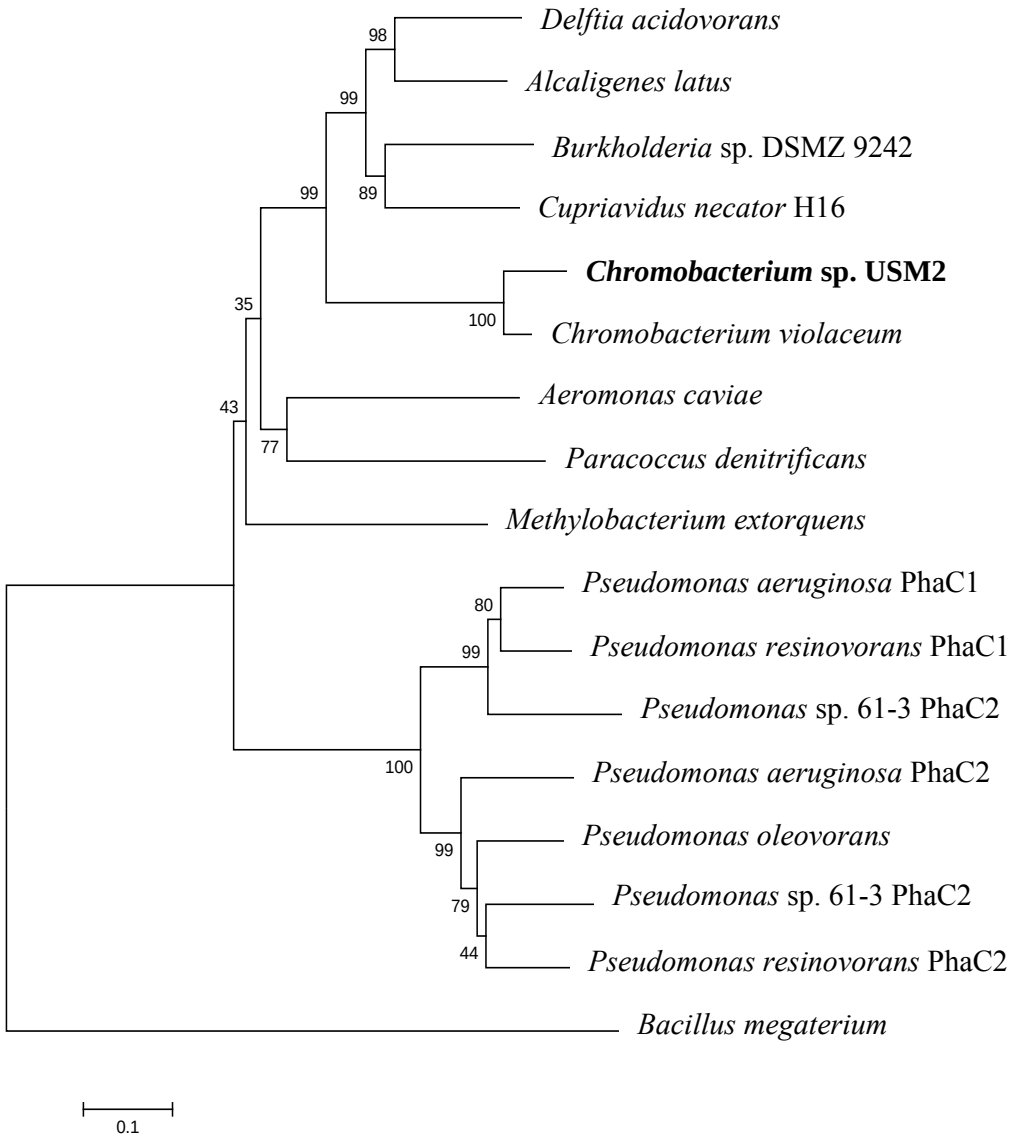


Figure 2.3 Phylogenetic relationship based on the analysis of 17 PHA synthases using neigbour-joining analysis. The number to the left at each node indicates the bootstrap values. The horizontal bar indicates a 0.1 evolutionary distance.

2.5 Metabolic pathways involved in PHA biosynthesis

Besides the specificity of the PHA synthase, it has become evident that the availability of the carbon sources and metabolic pathways in a particular microorganism are also important factors in PHA biosynthesis (Sudesh et al., 2000; Sudesh and Doi, 2000; Taguchi et al., 2001). In bacterial cell, different pathways are involved in the uptake and conversion of various carbon substrates ranging from inexpensive, complex waste effluents, to plant oils, and to alkanes as well as simple carbohydrates into hydroxyacyl-CoA and subsequently polymerized into PHA (Sudesh et al., 2000). Based on the incorporation of monomer into PHA, three well known metabolic pathways are involved in PHA biosynthesis are presented in Figure 2.4. These pathways are; i) three steps P(3HB) biosynthetic pathway, ii) fatty acid β-oxidation dependent mcl-PHA biosynthetic pathway, iii) *de novo* fatty acid biosynthesis dependent mcl-PHA biosynthetic pathway (Sudesh and Doi, 2000; Taguchi et al., 2001; Aldor and Keasling, 2003).

2.5.1 Short-chain-length PHA biosynthetic pathway

The biosynthesis pathway of P(3HB) is considered to be the simplest, which involves three distinct enzymatic reactions. The first reaction comprises of the condensation of two acetyl-CoA molecules derived from the metabolism of carbohydrates to acetoacetyl-CoA by *β*-ketothiolase (PhaA). This is followed by reduction step catalyzed by NADPH-dependent acetoacetyl-CoA reductase (PhaB) which produces (*R*)-3-hydroxybutyryl-CoA. Finally, P(3HB) is synthesized through the polymerization of (*R*)-3-hydroxybutyryl-CoA by PHA synthase (PhaC) (Anderson and Dawes, 1990; Braunegg et al., 1998). On the other hand, in some bacteria such as *Rhodospirillium rubrum*, an additional step is involved in P(3HB)

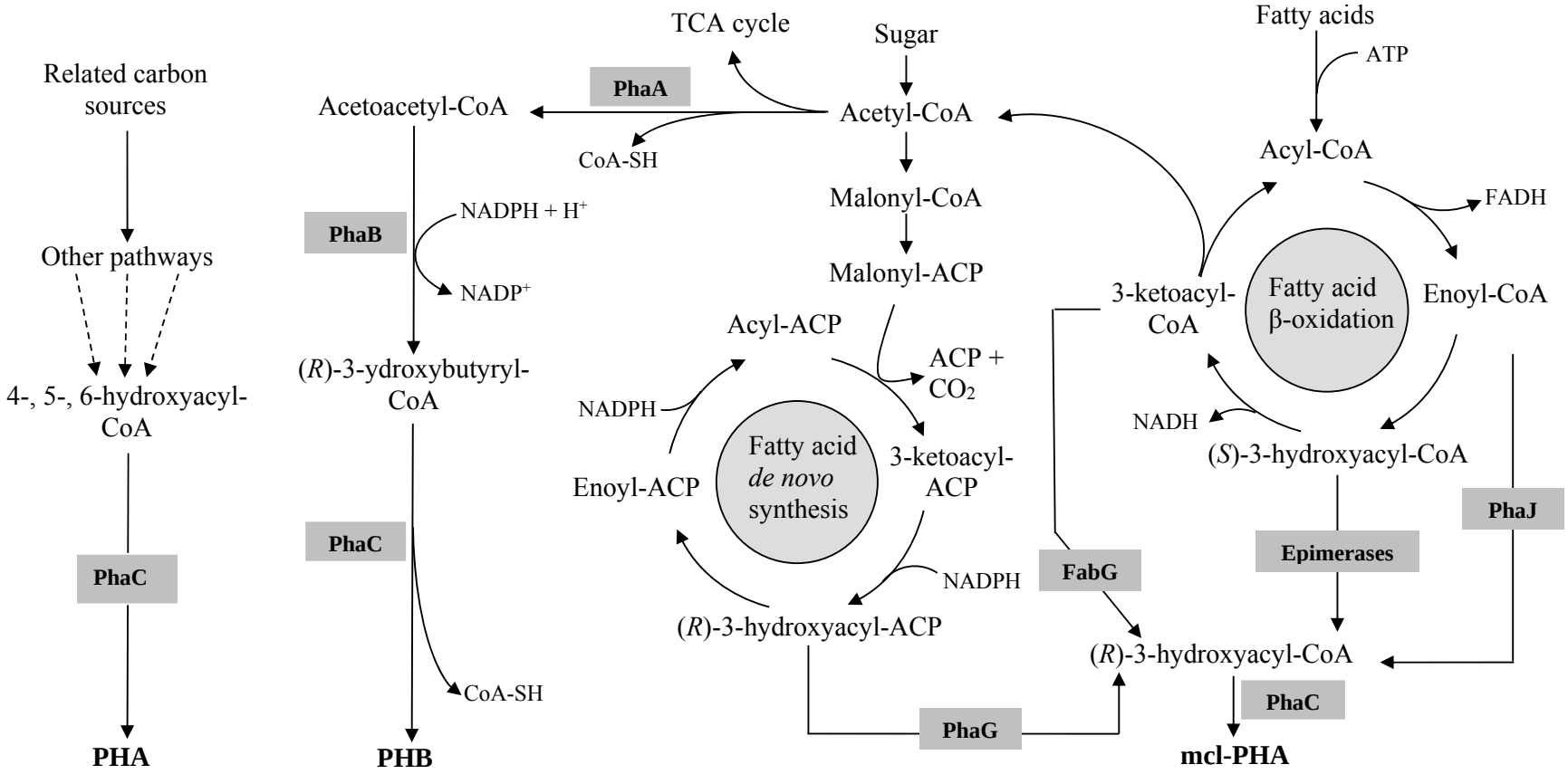


Figure 2.4 Metabolic pathway that supply hydroxyalkanoate monomers for PHA biosynthesis. PhaA, *β*-ketothiolase; PhaB, NADPH-dependent acetoacetyl-CoA reductase; PhaC, PHA synthase; PhaG, (*R*)-3-hydroxyacyl-ACP-CoA transferase; PhaJ, (*R*)-specific enoyl-CoA hydratase; FabG, 3-ketoacyl-CoA reductase; TCA cycle, tricarboxylic acid cycle (Sudesh et al., 2000; Rehm, 2006; Suriyamongkol et al., 2007).

biosynthesis because the reduction of acetoacetyl-CoA by PhaB produces S-(+)-hydroxybutyryl-CoA. Two enoyl-CoA hydratases will then catalyze the conversion of the (*S*)-isomer to the (*R*)-isomer of hydroxybutyryl-CoA and finally polymerized into P(3HB) (Moskowitz and Merrick, 1969).

Apart from P(3HB), bacteria also synthesizes various scl-PHA depending on the type and relative quantity of carbon sources in the growth media. For example, it had been reported that the addition of propionic acid or valeric acid in the growth media leads to the production of copolymer consists of 3HB and 3HV. If propionic acid is fed, the pathway involved is essentially identical with the P(3HB) biosynthesis pathway. In this pathway, propionic acid is initially converted to propionyl-CoA. Then, a distinct 3-ketothiolase, (BktB) will mediate the condensation of acetyl-CoA with propionyl-CoA to form 3-ketovaleryl-CoA. Reduction of 3-ketovaleryl-CoA to (*R*)-3-hydroxyvaleryl-CoA and subsequent polymerization to form 3HV monomer units are catalyzed by PhaB and PhaC (Steinbüchel and Lütke-Eversloh, 2003; Suriyamongkol et al., 2007). If valeric acid is fed, propionyl-CoA is derived from the fatty acid β-oxidation pathway and condensed with acetyl-CoA in order to generate 3HV monomer (Steinbüchel and Lütke-Eversloh, 2003; Thakor et al., 2006). Production of P(3HB-*co*-3HV) with high 3HV molar fractions has been reported using various microorganism such as *C. necator*, *C. violaceum*, *Delftia acidovorans* and *Caldimonas taiwanensis* (Doi et al., 1988; Steinbüchel et al., 1993; Loo and Sudesh, 2007; Sheu et al., 2010).

2.5.2 Medium-chain-length PHA biosynthetic pathway

Another type of PHA biosynthesis pathway is exhibited by the Pseudomonads of the rRNA homology group I. Bacteria in this group can synthesize

mcl-PHA from the intermediates of fatty acid β-oxidation pathway from various alkane, alkene, or fatty acid carbon sources (Brandl et al., 1988; Lageveen et al., 1988; Sudesh et al., 2000). Analysis on the co-monomer composition of mcl-PHA showed the direct relationship of the PHA produced with the structure of the growth substrate (Lageveen et al., 1988). The co-monomers in the resulting mcl-PHA were identical to the carbon substrate or shortened by one or more C_2 carbon units. Hence, it was proposed that the mcl-PHA biosynthesis pathway is closely related to the fatty acid β-oxidation pathway (Brandl et al., 1988; Lageveen et al., 1988; Huisman et al., 1989).

In β-oxidation pathway, fatty acids are initially activated by acyl-CoA synthetase into respective acyl-CoA thioesters before they enter the β-oxidation pathway. In the β-oxidation pathway, acyl-CoA is oxidized into enoyl-CoA by acyl-CoA dehydrogenase which is then converted into (*S*)-3-hydroxyacyl-CoA by enoyl-CoA hydratase. Oxidation of (*S*)-3-hydroxyacyl-CoA by 3-hydroxyacyl-CoA dehydrogenase results in the formation of 3-ketoacyl-CoA, which is cleaved by a *β*-ketothiolase to acetyl-CoA and acyl-CoA. However, this newly generated acyl-CoA is shortened by two carbons as compared to the acyl-CoA that were present during the first cycle (Steinbüchel and Lütke-Eversloh, 2003; Pötter and Steinbüchel, 2006). The β-oxidation pathway intermediates including enoyl-CoA, (*S*)-3-hydroxyacyl-CoA and 3-ketoacyl-CoA, can serve as precursors for the mcl-PHA synthesis. However, none of these intermediates are present in the form accepted as substrate by the PHA synthases. Therefore, an additional step is required to convert these intermediates into (*R*)-3-hydroxyacyl-CoA, which is polymerized by PHA synthase into corresponding monomers (Steinbüchel and Lütke-Eversloh, 2003; Suriyamongkol et al., 2007). Three different enzyme: (*R*)-specific enoyl-CoA

hydratase (PhaJ), 3-hydroxyacyl-CoA epimerase and 3-ketoacyl-CoA reductase (FabG) have been postulated to link the β-oxidation pathway with the mcl-PHA biosynthesis (Pötter and Steinbüchel, 2006; Rehm, 2007).

A second route of mcl-PHA synthesis in microorganisms is through the use of intermediates of *de novo* fatty acid biosynthesis pathway. In contrast to *P. oleovorans* and *P. fragii*, most of the pseudomonads such as *P. aeruginosa* and *P. putida* can synthesize mcl-PHA from structurally unrelated and simple carbon sources such as carbohydrates or sugars (Haywood et al., 1990; Timm and Steinbüchel, 1990; Huijberts et al., 1992). In this case, (*R*)-3-hydroxyacyl-CoA are derived from intermediates of *de novo* fatty acid biosynthesis (Huijberts et al., 1992). Since the metabolic pathway involved in fatty acid biosynthesis generates intermediate in the form of (*R*)-3-hydroxyacyl-ACP, an additional enzymatic activity is required to convert it into the (*R*)-3-hydroxyacyl-CoA. The linking enzyme, PhaG which encodes a (*R*)-3-hydroxyacyl-ACP-CoA transferase, has been shown to be the key enzyme linking *de novo* fatty acid biosynthesis pathway with PHA biosynthesis. This specific enzyme catalyzes the conversion of (*R*)-3-hydroxyacyl-ACP to its corresponding CoA derivative (Rehm et al., 1998; Hoffmann et al., 2002). However, this enzyme is only required when microorganisms are grown in carbon sources that are metabolized to acetyl-CoA such as acetate, gluconate or ethanol (Rehm et al., 1998). On the other hand, Taguchi and coworkers (1999) showed that transacylating enzymes, malonyl-CoA-ACP transacylase (FabD) and 3-ketoacyl-ACP synthase III (FabH) also played a role in biosynthesis of PHA via *de novo* fatty acid biosynthesis pathway.

2.6 Biosynthesis of PHA by microorganisms from various substrates

A large number of natural and transformant bacterial cultures have been evaluated for their ability to synthesize PHA. To date, many bacteria has been well documented to accumulate PHA under restricted growth conditions, with a few exceptions such as *A latus*, *Azotobacter beijerinkii* and *Bacillus* sp. (Bormann et al., 1998; Grothe and Christi, 2000; Tay et al., 2010). The production of PHA by various microorganisms is largely dependent on the type of substrate available in the medium, which can be referred to as structurally related carbon sources and unrelated carbon sources (Taguchi et al., 2001).

Sugars such as glucose, fructose and sucrose are the common substrates supplemented to the culture media as the main carbon sources (Tsuge, 2002). Continuous production of P(3HB) by *C. necator* in a two-stage cultivation on glucose giving the maximal accumulation of 47.6 g/L P(3HB) (Du et al., 2001a). Apart from *C. necator*, *A. latus* also can produce PHA using glucose and sucrose as carbon sources with good yields. Within five hours of cultivation, this strain was able to achieve PHA accumulation of 80% of cell biomass when grown on sucrose as the sole carbon source. Furthermore, it was also shown that P(3HB) productivity in *A. latus* can be further enhanced under nitrogen limited conditions (Wang and Lee, 1997). In addition, *Pseudomonas* sp. 61-3 has been found able to synthesize scl-mcl-PHA copolymer when sugars was fed as sole carbon sources (Kato et al., 1996). Huijberts and coworkers (1992) also revealed that *P. putida* growing on glucose was able to synthesize mcl-PHA composed of seven different monomers. For the production of P(3HB-*co*-3HV), precursor such as propionic acid and valeric acid are usually added to the growth media for the generation of 3HV monomer units. The feeding of propionic acid or valeric acid into growth media containing

glucose for the production of P(3HB-*co*-3HV) from *C. necator*, *A. latus*, *B. cereus*, *P. cepacia*, *P. pseudoflava*, *A. vinelandii* and *E. coli* transformant has been reported (Ramsay et al., 1990; Page et al., 1992; Madden and Anderson, 1998; Choi and Lee, 1999b; Liu et al., 2009). However, some Gram-positive bacteria belonging to the genera *Nocardia* and *Rhodococcus* synthesize P(3HB-*co*-3HV) from glucose with 3HV as the major constituent (Haywood et al., 1991; Alvarez et al., 1997). Recently, a locally isolated *Chromobacterium* sp. USM2 was reported to be able to synthesize P(3HB) from glucose and fructose (Bhubalan et al., 2010a).

In addition to sugars, various plant oil and their derived fatty acids were also shown to be feasible and suitable carbon sources for PHA biosynthesis (Solaiman et al., 2006). Doi and coworkers demonstrated the ability of *A. caviae* to synthesis P(3HB-*co*-3HHx) from olive oil (Doi et al., 1995). Also, Fukui and Doi found that both wild type and *C. necator* transformant in synthesizing high content of P(3HB) and P(3HB-*co*-3HHx) using olive oil, corn oil or palm oil (Fukui and Doi, 1998). Also, *P. stutzeri* 1317 was shown to accumulate variety of PHA when grown in fatty acids (Chen et al., 2001). Subsequently, Kahar and coworkers demonstrated the use of soybean oil to produce approximately 95.8 g/L P(3HB) and 102 g/L P(3HB-*co*-3HHx) from *C. necator* and its transformant strain (Kahar et al., 2004). Production of P(3HB) by *C. necator* and *Burkholderia cepacia* from various palm oil product also has been reported (Alias and Tan, 2005; Kek et al., 2008; Lee et al., 2008). Recently, Ng and coworkers showed that relatively high P(3HB) accumulation of approximately 11.4 g/L P(3HB) was obtained from *C. necator* fed with jatropha oil (Ng et al., 2010). In another instance, biosynthesis of P(3HB-*co*-3HV-*co*-3HHx) terpolymer from palm oil with co-feeding of sodium valerate from *C. necator* PHB^{-}4 transformant harboring the PHA synthase from *Chromobacterium* sp. USM2

has been evaluated (Bhubalan et al., 2010b). This strain also has been proven to be able to synthesize novel scl-mcl-PHA containing 3H4MV monomer from palm oil and isocaproic acid (Chia et al., 2010). Only a few studies had demonstrated the production of PHA containing 3H4MV monomer from isocaproic acid (Tanadchangsaeng et al., 2009; Lau et al., 2010).

Agricultural and industrial waste materials or by-products also being explored as inexpensive substrate for the synthesis of PHA (Akaraonye et al., 2010). Cheese whey, molassess, xylose and starchy wastewater are among the inexpensive substrates for PHA production (Lee, 1998; Quillaguamán et al., 2005; Albuquerque et al., 2007; Haas et al., 2008; Koller et al., 2008; Nath et al., 2008; Santimano et al., 2009). Besides that, the productions of P(3HB-*co*-3HV) from activated sludge have also been studied (Bengtsson et al., 2008) The feasibility of using waste oily substrates such as used frying oil or by-products from the vegetable oil refineries also has been studied (Taniguchi et al., 2003; Fernández et al., 2005; Hwan et al., 2008). The production of various PHA by natural and transformant bacterial strains from different substrates is summarized in Table 2.2.

Table 2.2 The production of various PHA by natural and transformant bacterial strains from different carbon substrates.

Bacterial strain	Type of PHA	Substrates	References
C. necator	P(3HB)	Glucose	(Du et al., 2001a)
		Jatropha oil	(Ng et al., 2010)
		Soybean oil	(Kahar et al., 2004)
	P(3HB-*co*-3HV)	Plant oils + sodium valerate/propionate	(Lee et al., 2008)
C. necator PHB^{-}4 transformant	P(3HB)	Fructose	(Bhubalan et al., 2010a)
	P(3HB-*co*-3HV)	Fructose + sodium valerate	(Bhubalan et al., 2010a)
	P(3HB-*co*-3H4MV)	Fructose + isocaproic acid	(Tanadchangsaeng et al., 2009)
	P(3HB-*co*-3HHx)	CPKO	(Bhubalan et al., 2010b)
		Soybean oil	(Kahar et al., 2004)
	P(3HB-*co*-3HV-*co*-3H4MV-*co*-3HHx)	CPKO + isocaproic acid	(Chia et al., 2010)
A. latus	P(3HB)	Sucrose	(Wang and Lee, 1997)
	P(3HB-*co*-3HV)	Glucose + propionic acid	(Ramsay et al., 1990)
A. caviae	P(3HB-*co*-3HHx)	Olive oil	(Doi et al., 1995)
	P(3HB-*co*-3HV)	Fatty acids (C11, C13, C15, C17)	(Doi et al., 1995)
Pseudomonas sp. 61-3	scl-mcl-PHA	Glucose/fructose	(Kato et al., 1996)
P. aeruginosa	scl-mcl-PHA	Sodium gluconate/sodium octanoate	(Timm and Steinbüchel, 1990)
P. putida	mcl-PHA	Glucose	(Huijberts et al., 1992)

Table 2.2 (continued)

Bacterial strain	Type of PHA	Substrates	References
P. oleovorans	mcl-PHA	Octanoic acid	(Chardron et al., 2010)
C. violaceum	P(3HV)	Valeric acid	(Steinbüchel et al., 1993)
	P(3HB-*co*-3HV)	Glucose/fructose/fatty acids	(Kolibachuk et al., 1999)
Chromobacterium sp. USM2	P(3HB)	Glucose/fructose/CPKO	(Bhubalan et al., 2010a)
	P(3HB-*co*-3HV)	Sodium valerate/sodium propionate/valeric acid	(Bhubalan et al., 2010a)
Burkholderia sp.	P(3HB-*co*-3H4MV)	Glucose + isocaproic acid	(Lau et al., 2010)
	P(3HB-*co*-3H4MV-*co*-3HV)	Sodium valerate/sodium propionate + isocaproic acid	(Lau et al., 2010)
Bacillus sp.	P(3HB)	Glucose/fructose	(Tay et al., 2010)
E. coli transformant	P(3HB)	Xylose	(Lee, 1998)
	P(3HB-*co*-3HV)	Glucose + propionic acid	(Liu et al., 2009)
	P(3HB-*co*-3HHx)	Dodecanoic acid	(Park et al., 2001)
	P(3HB-*co*-4HB)	Glucose + α-ketoglutarate	(Li et al., 2010)

CPKO, Crude palm kernel oil; 3HB, 3-hydroxybutyrate; 3HHx, 3-hydroxyhexanoate; 3H4MV, 3-hydroxy-4-methylvalerate; 3HV, 3-hydroxyvalerate; 4HB, 4-hydroxybutyrate

2.7 Extraction and purification of PHA

It is known that the method applicable for the effective recovery/separation of intracellular PHA from other cellular biomass components can be complex and costly (Chee et al., 2010). A number of separation methods have been developed for the recovery of PHA. The most common method for the extraction of PHA from biomass involves the use of chlorinated solvent such as chloroform and halogenated hydrocarbon solvents such as dichloromethane, dichloroethane and chloropropane (Stageman, 1985; Vanlautem and Gilain, 1987; Ramsay et al., 1994; Hahn and Chang, 1995). Chloroform extraction is a very simple and effective method, which is able to extract PHA with a high purity and without the degradation of PHA molecules (Ramsay et al., 1994; Hahn and Chang, 1995). The extracted PHA can be recovered from the solvent either by evaporation or by precipitation with the addition of methanol. However, this method is not suitable for large scale processing as large amount of hazardous solvent is needed (Ramsay et al., 1994).

Besides solvent extraction, another recovery method is by using sodium hypochlorite (Berger et al., 1989). Although this method is simple and effective, it causes severe degradation of PHA and reduction in the molecular weight (M_w). To take advantage of both differential digestions by hypochlorite and solvent extraction, another separation process was developed by using sodium hypochlorite together with chloroform (Hahn et al., 1993; Hahn et al., 1994). Purity of PHA recovered in this manner was approximately 97% (Hahn et al., 1993). It was suggested that the hydrophobic PHA will immediately dissolve into the chloroform phase, and thus protects the polymer from further degradation due to the strong oxidation effects of sodium hypochlorite (Hahn et al., 1993). Apart from this, chemicals such as NaOH

and KOH were found to exhibit high level of efficiency in recovering PHA with high purity (Choi and Lee, 1999a).

Enzymatic digestion method was developed as an alternative to solvent extraction. This recovery process involved a short period of thermal treatment on biomass, followed by enzymatic digestion and washing with anionic surfactant (Holmes and Lim, 1990; de Koning and Witholt, 1997; Yasotha et al., 2006). Various proteolytic enzymes (pepsin, trypsin, papain and mixtures thereof) have high activities on dissolution of proteins and with little product damage (Braunegg et al., 1998; Jacquel et al., 2008). Anionic surfactant such as proteinase K, sodium dodecyl sulfate (SDS) or ethylenediaminetetraacetic acid (EDTA) are generally employed to increase the efficiency of enzymatic treatment (Holmes and Lim, 1990; Yasotha et al., 2006). However, it is also possible to recover PHA with high purity without using any anionic surfactants (Kapritchkoff et al., 2006). It was reported that the purity of PHA recovered in this manner exceeded 90% (de Koning and Witholt, 1997; Kapritchkoff et al., 2006; Yasotha et al., 2006). Apart from these, other alternative recovery/separation methods such as mechanical disruption, dissolved-air flotation, air classification were also reported (Noda, 1998; Tamer et al., 1998; van Hee et al., 2006; Jacquel et al., 2008).

2.8 Properties of PHA

2.8.1 Thermal and mechanical properties

PHA exists as highly amorphous and water insoluble inclusions within the bacterial cells. Upon disruption of cells, rapid crystallization occurs when the PHA is extracted from the cells (Sudesh et al., 2000; Khanna and Srivastava, 2005). Owing to the diversity of PHA family, they exhibit a wide variety of material