EVALUATION OF *Burkholderia* sp. USM (JCM 15050) FOR THE PRODUCTION OF COPOLYMERIC POLYHYDROXYALKANOATE CONTAINING 3-HYDROXY-4-METHYLVALERATE MONOMER

LAU NYOK SEAN

UNIVERSITI SAINS MALAYSIA

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by

LAU NYOK SEAN

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LIST OF SYMBOLS AND ABBREVIATIONS

SYMBOLS AND ABBREVIATIONS	FULL NAME
%	Percentage
× *g*	Times gravity
β	Beta
°C	Degree Celcius
ΔH_m	Enthalpy of fusion
µg	Microgram
µL	Microliter
µM	Micromolar
µmol	Micromole
^{1}H	Proton
^{13}C	Carbon-13
3HHx	3-hydroxyhexanoate
3HV	3-hydroxyvalerate
3H4MV	3-hydroxy-4-methylvalerate
4HB	4-hydroxybutyrate
3HB-CoA	3-hydroxybutyryl-CoA
BSA	Bovine serum albumin
C	Carbon atom
C/N	Carbon to nitrogen
$CDCl_3$	Deuterated chloroform
$CaCl_2$	Calcium chloride

CH	Methine group
CH_2	Methylene group
CH_3	Methyl group
cm	Centimetre
CME	Caprylic methyl ester
CO_2	Carbon dioxide
CoA	Coenzyme-A
CoASH	Free coenzyme-A
$CoCl_2 \cdot 6H_2O$	Cobalt (II) chloride hexahydrate
CPKO	Crude palm kernel oil
$CrCl_3 \cdot 6H_2O$	Chromium chloride hexahydrate
$CuSO_4 \cdot 5H_2O$	Copper sulphate pentahydrate
dH_2O	Distilled water
ddH_2O	Sterile distilled water
DNA	Deoxyribonucleic acid
DTNB	5,5’-dithio-bis(2-nitrobenzoic acid)
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
$FeCl_3$	Iron (III) chloride
FT-IR	Fourier transform infrared spectrometry
g	Gram
GC	Gas chromatography
GPC	Gel permeation chromatography
h	Hour
HCl	Hydrochloric acid

H_2O	Water
H_2SO_4	Sulphuric acid
ICI	Imperial Chemical Industries
IPTG	isopropyl-β-D-thiogalactopyranoside
J	Joule
kb	Kilo base pairs
kDa	Kilodalton
kg	Kilogram
KH_2PO_4	Potassium dihydrogen phosphate
KOH	Potassium hydroxide
L	Liter
LB	Luria Bertani
M	Molar
M_n	Number average molecular weight
M_w	Weight average molecular weight
M_w/M_n	Polydispersity index
mcl-	Medium chain length
min	Minute
mg	Miligram
$MgSO_4 \cdot 7H_2O$	Magnesium sulphate heptahydrate
mL	Mililiter
mm	Milimeter
mM	Milimolar

MM	Mineral salts medium
mol%	Mol percent
N	Normality
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide phopshate
NADPH	Reduced nicotinamide adenine dinucleotide phopshate
NAOH	Sodium hydroxide
NH_4Cl	Ammonium chloride
NH_4OH	Ammonium hydroxide
$NiCl_2 \cdot 6H_2O$	Nickel chloride hexahydrate
nm	Nanometer
NMR	Nuclear magnetic resonance
NR	Nutrient rich
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
ppm	Parts per million
psi	Pounds per square inch

P(3HB)	Poly(3-hydroxybutyrate)
P(3HB-*co*-3HHx)	Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate)
P(3HB-*co*-3HV)	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)
P(3HB-*co*-4HB)	Poly(3-hydorxybutyrate-*co*-4-hydroxybutyrate)
P(3HB-*co*-3H4MV)	Poly(3-hydroxybutyrate-*co*-3-hydroxy-4-methylvalerate)
P(3HB-*co*-3H4MV-*co*-3HHx)	Poly(3-hydroxybutyrate-*co*-3-hydroxy-4-methylvalerate-*co*-3-hydroxyhexanoate)
P(3HB-*co*-3HV-*co*-3H4MV)	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-3-hydroxy-4-methylvalerate)
PHA	Polyhydroxyalkanoate
PhaA; *phaA*	PHA synthase; gene encoding PHA synthase
PhaB; *phaB*	β-ketothiolase; gene encoding β-ketothiolase
PhaJ; *phaJ*	(*R*)-specific enoyl-CoA hydratase; gene encoding (*R*)-specific enoyl-CoA hydratase
PhaP; *phaP*	Phasin; gene encoding phasin
PhaR; *phaR*	PHA-synthesis regulator; gene encoding PHA-synthesis regulator
PhaZ; *phaZ*	PHA depolymerase; gene encoding PHA depolymerase

rpm	Revolutions per minute
s	Second
scl-	Short chain length
TAE	Tris-acetate-EDTA
TCA	Tricarboxylic acid
T_g	Glass transition temperature
T_m	Melting temperature
U	One unit of enzyme activity
UV	Ultraviolet
V	Volt
v/v	Volume per volume
wt%	Dry weight percent
w/v	Weight per volume
w/v	Weight per weight
X-Gal	5-bromo-4-chloro-3-indolyl-β-^{D}-galactopyranoside

PENILAIAN *Burkholderia* sp. USM (JCM 15050) UNTUK PENGHASILAN POLIHIDROKSIALKANOAT KOPOLIMER YANG MENGANDUNGI 3-HIDROKSI-4-METILVALERAT

ABSTRAK

Kepelbagaian penggunaan nutrient bagi strain *Burkholderia* sp. USM (JCM 15050) telah mencetuskan permulaan kajian mengenai penggunaan bakteria ini untuk penghasilan polihidroksialkanoat. Penyelidikan ini telah dijalankan untuk menilai kemampuan *Burkholderia* sp. USM (JCM 15050) dan transformannya yang mengandungi gen PHA sintase daripada *Aeromonas caviae* untuk menghasilkan satu jenis monomer baru iaitu 3-hidroksi-4-metilvalerat (3H4MV). Monomer yang mempunyai rantai sisi metil ini menjadi tarikan memandangkan ia dapat mengelakkan kemerosotan poli(3-hidroksibutirat-*co*-3-hidroksi-4-metilvalerat), P(3HB-*co*-3H4MV) semasa proses penuaan. Bagi menggalakkan akumulasi PHA yang mengandungi monomer 3H4MV, asid 4-metilvalerik telah dibekalkan sebagai sumber ko-karbon. Polimerisasi monomer 3H4MV oleh *Burkholderia* sp. USM (JCM 15050) telah mengesahkan kebolehan sintase *Burkholderia* sp. USM (JCM 15050) untuk menerima tioester CoA 3H4MV yang mengandungi monomer enam atom karbon sebagai substrat. Pelbagai parameter kultur seperti kepekatan medium NR, fruktosa dan asid 4-metilvalerik serta masa penuaian telah dimanipulasikan untuk menghasilkan P(3HB-*co*-3H4MV) dengan komposisi 3H4MV yang berbeza. Ciri-ciri struktur PHA yang mengandungi monomer 3H4MV telah dikaji dengan menggunakan nuklear magnetik resonansi (NMR) dan fourier transformansi inframerah spektroskopi (FTIR). Intensiti relatif band pada 1183 dan 1228 cm^{-1} dalam spektrum FTIR membezakan P(3HB-*co*-3H4MV) daripada jenis PHA yang

lain. Selain itu, kehadiran unit 3H4MV dalam kopolimer didapati dapat merendahkan suhu lebur (T_m) dan entalpi pelakuran (ΔH_m) berbanding dengan poli(3-hidroksibutirat), P(3HB). Bagi meneruskan pencirian pada peringkat molekul, gen biosintesis PHA *Burkholderia* sp. USM (JCM 15050) telah berjaya diklon. Gen biosintesis PHA bakteria ini terdiri daripada sintase PHA (*phaC*), β-ketotiolase (*phaA*), reduktase asetoasetil-CoA (*phaB*) dan pengawalatur sintesis PHA (*phaR*). Produk translasi gen menunjukkan persamaan identiti kepada protein yang berkenaan, dengan *Burkholderia vietnamiensis* (99−100%) dan *Cupriavidus necator* H16 (63−89%). Keupayaan PHA-negatif *C. necator* PHB^{-}4 mensintesis PHA dengan ekspresi $phaC_{Bs}$ telah mengesahkan bahawa $phaC_{Bs}$ mengekod protein aktif yang berfungsi. Transforman *C. necator* PHB^{-}4 yang mengandungi gen PHA sintase *Burkholderia* sp. USM (JCM 15050) telah menghasilkan poli(3-hidroksibutirat-*co*-4-hidroksibutirat) dengan monomer 4-hidroksibutirat telah memperoleh sebanyak 87 mol% daripada natrium 4-hidroksibutirat. Penyelidikan ini telah membuktikan buat pertama kalinya bahawa PHA sintase *Burkholderia* sp. USM (JCM 15050) mempunyai kekhususan substrat yang lebih luas daripada dianggap sebelum ini dan ia mampu mempolimerasikan monomer 3H4MV dan 4HB.

EVALUATION OF *Burkholderia* sp. USM (JCM 15050) FOR THE PRODUCTION OF COPOLYMERIC POLYHYDROXYALKANOATE CONTAINING 3-HYDROXY-4-METHYLVALERATE MONOMER

ABSTRACT

The nutrition-versatility of *Burkholderia* sp. strain USM (JCM 15050) has initiated the studies on the use of this bacterium for polyhydroxyalkanoate (PHA) production. This study was carried out to evaluate the ability of *Burkholderia* sp. USM (JCM 15050) and its transformant harboring the PHA synthase gene of *Aeromonas caviae* to incorporate a new type of PHA monomer, 3-hydroxy-4-methylvalerate (3H4MV). This monomer with methyl side chain is of interest since the monomer can prevent the deterioration of poly(3-hydroxybutyrate-*co*-3-hydroxy-4-methylvalerate), P(3HB-*co*-3H4MV) during aging. To promote the accumulation of PHA containing 3H4MV monomer, 4-methylvaleric acid was provided as co-carbon source. The polymerization of 3H4MV monomer by *Burkholderia* sp. USM (JCM 15050) confirmed the ability of *Burkholderia* sp. USM (JCM 15050) synthase to accept CoA thioester of 3H4MV, a six-carbon atom monomer, as substrate. Various culture parameters such as concentrations of NR medium, fructose and 4-methylvaleric acid as well as harvesting time were manipulated to produce P(3HB-*co*-3H4MV) with different 3H4MV compositions. The structural properties of PHA containing 3H4MV monomer were investigated by using nuclear magnetic resonance (NMR) and fourier transform infrared spectroscopy (FTIR). The relative intensities of the bands at 1183 and 1228 cm^{-1} in the FTIR spectra enabled the rapid detection and differentiation of P(3HB-*co*-3H4MV) from other types of PHA. In addition, the presence of 3H4MV units in the copolymer was found to considerably lower the

melting temperature (T_m) and enthalpy of fusion (ΔH_m) compared to those of poly(3-hydroxybutyrate), P(3HB). In order to further characterize at the molecular level, the PHA biosynthetic genes of *Burkholderia* sp. USM (JCM 15050) were successfully cloned. The PHA biosynthetic cluster of this strain consisted of a PHA synthase (*phaC*), β-ketothiolase (*phaA*), acetoacetyl-CoA reductase (*phaB*) and PHA synthesis regulator (*phaR*). The translated products of these genes revealed identities to corresponding proteins of *Burkholderia vietnamiensis* (99−100%) and *Cupriavidus necator* H16 (63−89%). Heterologous expression of $phaC_{Bs}$ conferred PHA synthesis to the PHA-negative *Cupriavidus necator* PHB^{-}4, confirming that $phaC_{Bs}$ encoded functionally active protein. Interestingly, the transformant *C. necator* PHB^{-}4 harbouring *Burkholderia* sp. USM (JCM 15050) PHA synthase gene accumulated poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) with 4-hydroxybutyrate (4HB) monomer as high as up to 87 mol% from sodium 4-hydroxybutyrate. This study has revealed for the first time that the PHA synthase of *Burkholderia* sp. USM (JCM 15050) has a wider range of substrate specificity than it was previously thought and the synthase was capable of polymerizing 3H4MV and 4HB monomer.

Chapter 1.0: Introduction

Since the introduction of phenoformaldehyde plastic in 1909 by Leo Hendrik Baekeland, petrochemical plastics have developed into a major industry and an indispensable commodity for modern life (Meikle, 1995). One of the reasons that cause the popularity of plastics is their structures can be chemically manipulated to have a wide range of strengths and shapes, including fibers and thin films to suit different usages. The widespread applications of synthetic plastics are not only due to their favourable mechanical and thermal properties but also their stability and durability. However, these very desirable properties of synthetic plastics have now become the greatest problem for mankind. It was estimated that around 205 million tons of plastics are produced and used globally in 2006 and the number is projected to increase in the coming years (Snell & Peoples, 2009). Plastics being xenobiotic, are resistant to microbial degradation and persist in the environment for a long time (Flechter, 1993). Large molecular size seems to be the factor that is responsible for the resistance of these chemicals to enzymatic attack (Atlas, 1993). The synthetic polyethylene, polyvinyl chloride and polystyrene, which are widely used in the manufacture of plastics, have molecular weights ranging from $5x10^4$ to $1x10^6$ Da (Madison & Huisman, 1999). Most of these plastics end up after their useful life as discarded waste and some are disposed into the aquatic environment and pose threats to the aquatic wildlife. In recent years, the widespread and increasing use of petrochemical plastics has raised concerns about the adverse impact of these recalcitrant plastics on the environment. The current solutions to plastic waste management include source reduction, incineration and recycling. However, most of these solutions are not sustainable and have problems associated with them. Incinerating plastics has been the traditional way in disposing non-degradable

plastics, but the process releases chemicals like hydrogen chloride and hydrogen cyanide that are hazardous to health. On the other hand, post-consumer recycling is impractical as it is difficult to sort the different types of plastics and the further application range of recycled plastics is also limited (Braunegg et al., 1998; Khanna & Srivastava, 2005). In such a scenario, the use and development of biobased and biodegradable plastics offer a better solution to the environmental problems posed by petrochemical plastics.

The term “biobased polymers” encompass not only polymeric materials that are found naturally but also natural substances that have been polymerized into high molecular weight materials by chemical and/or biological systems. Biobased polymers can be classified into three major groups based on their production methods: (i) bio-chemosynthetic polymers, e.g. poly(lactic acid) (ii) biosynthetic polymers, e.g. polyhydroxyalkanoate (PHA) and (ii) modified natural polymers, e.g. starch polymers (Sudesh & Iwata, 2008). The examples of biobased polymers include polyhydroxyalkanoate (PHA), polylactides, aliphatic polyesters and polysaccharides. Among all the biobased polymer, PHA has gained major interest since it is has been shown to be completely biodegradable. In addition, the synthesis of PHA from renewable resources such as agricultural and industrial wastes makes them more sustainable (Braunegg et al., 1998). In contrast, synthetic plastics are derived from non-renewable fossil fuels, which are anticipated to deplete in the near future. The production of PHA from renewable carbon sources also uses less fossil fuels and at the same time releases less greenhouse gas than the production of synthetic plastics (Sudesh & Iwata, 2008; Chen, 2009).

Despite the numerous advantages of using biodegradable plastics, the commercialization of PHA has been ongoing since 1980s with limited success. The

high production cost of PHA has been a major drawback to their replacement of petrochemical plastics. The price of most commodity plastics derived from petroleum, such as polyethylene and polypropylene are below US$1 kg^{-1}, whereas most biodegradable plastics cost around US$2.20-6.60 kg^{-1} (Steinbüchel & Füchtenbusch, 1998). Considering the cost differences between PHA and petrochemical plastics, PHA cannot currently compete with the bulk production of petrochemical plastics. To achieve the commercial application and wide use of PHA, efforts are directed on effectively lowering the production cost of PHA. Much research has been focused on reducing the production cost by strain development, developing more efficient fermentation and recovery processes and using inexpensive carbon sources. At the same time, more PHA applications are being developed, including the high value applications such as medical and pharmaceutical field at which cost of production is not the main concern (Chen, 2009).

Poly(3-hydroxybutyrate) [P(3HB)], the most common type of PHA, has mechanical properties such as Young's modulus and tensile strength similar to polypropylene. Nevertheless, P(3HB) is a stiffer and more brittle plastic material compared to polypropylene (Tsuge, 2002). Many other types of PHA with improved mechanical properties are synthesized by incorporating co-monomers such as 3-hydroxyvalerate, 3-hydroxyhexanoate and 4-hydroxybutyrate. Although approximately 150 different hydroxyalkanoic acids have been identified as constituents of PHA (Rehm & Steinbüchel 2002), only few of them can be produced from simple carbon sources. Exceptions are some Gram-positive bacteria such as *Rhodococcus ruber*, which are able to synthesize poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), P(3HB-*co*-3HV) from simple carbon sources such as glucose (Haywood et al., 1991). Many of the unusual monomers are only produced when

structurally related precursor substrates are supplied as carbon sources to the microorganisms (Sudesh & Doi, 2005). *Cupriavidus necator* strain PHB^{-}4 expressing *Aeromonas caviae* and *Chromobacterium* sp. PHA synthase have been shown to synthesize PHA containing new 3-hydroxy-4-methylvalerate (3H4MV) monomer when 4-methylvaleric acid was provided as precursor substrates (Tanadchangsaeng et al., 2009; Chia et al., 2010). The characteristics of these precursor substrates are the structural similarity of their carbon skeletons to those of the hydroxyalkanoic acid to be incorporated into PHA. However, these precursor substrates are usually more expensive than simple carbon sources and many of them are toxic to the microorganism (Steinbüchel & Lütke-Eversloh, 2003).

1.1 Rationale and objectives

Following the discovery of poly(3-hydroxybutyrate), P(3HB) as a storage compound in *Bacillus megaterium*, more than 150 different hydroxyalkanoic acids are known to occur as constituents of PHA (Lemoigne, 1926; Rehm & Steinbüchel, 2002). Many of the unusual monomers are produced when structurally related precursor substrates are supplied as carbon sources to the microorganisms (Sudesh & Doi, 2005). This study was carried out to evaluate the ability of *Burkholderia* sp. USM (JCM15050) and its transformant harboring the PHA synthase gene of *A. caviae* (*phaC*$_{Ac}$) to biosynthesize PHA containing 3H4MV monomer from structurally related carbon source, 4-methylvaleric acid. The synthesis of PHA terpolymers containing 3H4MV and other monomers e.g. 3-hydroxyvalerate (3HV) and 3-hydroxyhexanoate (3HHX) was explored by co-feeding of other carbon sources together with 4-methylvaleric acid.

The properties of PHA are important aspects that should be taken into consideration for the industrial applications of PHA. The types of monomer incorporated and the monomer compositions affect the structural and physical properties of the PHA synthesized. PHA containing 3H4MV monomer was extracted from wild type and transformant *Burkholderia* sp. USM (JCM 15050) and subjected to characterization by nuclear magnetic resonance spectroscopy (NMR), fourier transform infrared spectroscopy (FT-IR), gel permeation chromatography (GPC), differential scanning calorimetry (DSC), thermogravimetric (TGA) analysis and tensile testing. A rapid method for the detection and differentiation of P(3HB-*co*-3H4MV) from other types of PHA was suggested based on the relative intensities of specific bands in FTIR spectra.

The PHA biosynthetic cluster of bacteria harbouring type I PHA synthase e.g. *C. necator* and *Azohydromonas lata* consisted of a PHA synthase (*phaC*), β-ketothiolase (*phaA*) and acetoacetyl-CoA reductase (*phaB*) that are often found clustered in the bacterial genomes. In this study, the PHA biosynthetic genes from locally isolated *Burkholderia* sp. strain USM (JCM 15050) were successfully cloned and characterized. *In vivo* functional expression of the cloned PHA biosynthetic genes was carried out with PHA-negative *C. necator* PHB^{-}4.

The specific objectives of this project can be summarized as follow:

1. To investigate the ability of *Burkholderia* sp. USM (JCM15050) and its transformant harboring the PHA synthase gene of *A. caviae* to biosynthesize PHA containing 3H4MV monomer

2. To synthesize and characterize the physical and structural properties of PHA containing 3H4MV monomer by NMR, FT-IR, GPC, DSC, TGA and tensile testing

3. To clone the PHA biosynthetic cluster of *Burkholderia* sp. USM (JCM 15050) consisting of *phaC*, *phaA*, *phaB* and *phaR* and functional study of *phaC*

Chapter 2.0: LITERATURE REVIEW

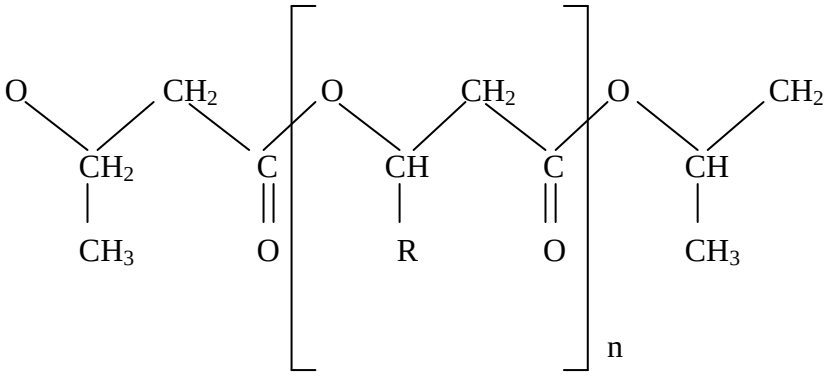
2.1 Polyhydroxyalkanoate (PHA)

Living organisms are able to synthesize a large variety of biopolymers which can be classified into eight major classes based on their chemical structure: (i) polynucleotides, (ii) polyamides, (iii) polysaccharides, (iv) polyoxoesters, (v) polythioesters, (vi) polyanhydrides, (vii) polyisoprenoids and (viii) polyphenols (Steinbüchel, 2001). PHA represents a class of natural polyesters that is synthesized by numerous bacteria under unbalanced growth conditions. Some bacteria have been reported capable of producing PHA as much as 90% (w/v) of dry cells during depletion of an essential nutrient such as nitrogen, phosphorus or magnesium (Madison & Huisman, 1999). Besides functioning as storage compounds of carbon and energy, PHA also serves as sink for reducing equivalents for some microorganisms (Schubert et al., 1988). PHA is an ideal storage compound due to its insolubility, which exerts negligible increase in osmotic pressure inside bacterial cytoplasm (Anderson & Dawes, 1990). The existences of PHA are not limited to intracellular granules only. Studies have revealed that poly(3-hydroxybutyrate) P(3HB) with lower molecular weight (cPHB), have been detected in the cytoplasmic membrane of enterobacteria like *Escherichia coli* (Reddy et al., 2003). Besides eubacteria, cPHB is also found in eukaryotic cells, ranging from yeast, peanut, spinach, sheep (intestine) and cat muscles (Reusch, 1995). This low molecular weight P(3HB), with molecular mass less than 15000 Da, contributes only 0.1% or less of the dry cell weight (Pötter & Steinbüchel, 2006). The putative functions of cPHB include roles in voltage-dependent calcium channel or DNA transport (Anderson & Dawes, 1990).

2.1.1 Structures of PHA

To date, approximately 150 different hydroxyalkanoic acids are known to occur as constituents of PHA (Rehm & Steinbüchel, 2002). The monomer compositions of PHA produced depend on the availability of carbon sources, flexibility of the organism's intermediary metabolism and the substrate specificity of the PHA synthase (Anderson & Dawes, 1990). PHA can be classified into two major groups based on the length of the carbon chain of their monomer. The short chain length PHA (scl-PHA) is composed of 3 to 5 carbon atoms. In contrast, the medium chain length PHA (mcl-PHA) has monomers ranging from 6 to 14 carbon atoms (Steinbüchel & Lütke-Eversloh, 2003). Scl-PHA was first described by Lemoigne whom discovered that *Bacillus megaterium* produced poly(3-hydroxybutyrate), P(3HB) as a storage compound (Lemoigne, 1926). Several decades later, the production of mcl-PHA in laboratory was first reported for *Pseudomonas putida* GPo1 (De Smet et al., 1983). Recent advances in genetic engineering have enabled the biosynthesis of a new type of PHA containing both scl- and mcl-monomer units (Tsuge, 2002).

The monomer units are all in the (*R*)-configuration due to the stereospecificity of the biosynthetic enzymes (Reddy et al., 2003). Most of the PHA are aliphatic polyesters consist of carbon, oxygen and hydrogen. Their general formula is shown in Fig. 2.1. The composition of the side chain or atom R determines the identity of a monomer unit. To date, monomers with saturated, unsaturated, aromatic, halogenated, epoxidized or branched alkyl side chain have been reported (Zinn et al., 2001).



n varies from 100 to 30000

n = 1	R = hydrogen	Poly(3-hydroxypropionate)
	R = methyl	Poly(3-hydroxybutyrate)
	R = ethyl	Poly(3-hydroxyvalerate)
	R = propyl	Poly(3-hydroxyhexanoate)
	R = pentyl	Poly(3-hydroxyoctanoate)
	R = nonyl	Poly(3-hydroxydodecanoate)
n = 2	R = hydrogen	Poly(4-hydroxybutyrate)
	R = methyl	Poly(4-hydroxyvalerate)
n = 3	R = hydrogen	Poly(5-hydroxyvalerate)
	R = methyl	Poly(5-hydroxyhexanoate)
n = 4	R = hexyl	Poly(6-hydroxydodecanoate)

Figure 2.1 Structure of PHA (Lee, 1996; Sudesh et al., 2000).

2.1.2 Properties of PHA

PHA granules exist as amorphous and water-insoluble inclusions in the cytoplasm of bacterial cells (Sudesh et al., 2000). Studies with numerous organisms have shown that PHA exists as light refractive granules inside the cell with diameters of 0.2 to 0.5 mm and the number of granules per cell seems to be strain specific (Byrom, 1994). The microorganisms accumulating PHA can be identified easily by staining with Sudan Black or light fluorescent stain such as Nile Blue or Nile Red (Spiekermann et al., 1999). The PHA granules appear as electron-dense bodies under transmission electron microscope observation. The molecular mass of PHA is in the range of 2×10^5 to 2×10^6 Da, depending on the microorganism and growth condition (Braunegg et al., 1998). PHA is also enantiomeric, biocompatible, non-toxic, piezoelectric and exhibits a high degree of polymerization (Hocking & Marchessault, 1994).

The study on PHA granules isolated from *B. megaterium* had demonstrated that the surface of the granules was surrounded by lipid monolayer (Lundgren et al., 1964). Subsequent structural analyses in recent years revealed that different types of granule-associated proteins are located on the surface of PHA granules. The proteins include PHA synthases, involve in the polymerization of PHA; intracellular PHA depolymerases, responsible for degradation of accumulated PHA; phasins, prevent the coalescing of PHA granules and PHA-specific regulator proteins (Pötter & Steinbüchel, 2006; Rehm, 2006; Jendrossek, 2009).

2.1.3 Types of PHA

P(3HB) is the most extensively studied and well characterized PHA. The mechanical properties of P(3HB) such as Young's modulus and tensile strength are similar to polypropylene. Nevertheless, the extension to break of P(3HB) is significantly lower than that of polypropylene. Hence, P(3HB) is a stiffer and more brittle plastic material compared to polypropylene (Tsuge, 2002). The melting temperature of P(3HB) is 179 °C (Table 2.1), which is close to the thermal decomposition temperature of this polymer and this property makes its processing difficult (Khanna & Srivastava, 2005). Therefore efforts are directed towards improving the mechanical and thermal properties of P(3HB) by incorporating co-monomer such as 3-hydroxyvalerate (3HV). The industrial process for the synthesis of poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate), P(3HB-*co*-3HV) from a mixture of glucose and propionic acid by fed-batch culture of *C. necator* was developed by Imperial Chemical Industries (Holmes, 1988). P(3HB-*co*-3HV) has lower melting points compared to P(3HB), and is less crystalline, more ductile, easier to mold and tougher than P(3HB) homopolymer (Luzier, 1992). The properties of the copolymer are determined by the mol fraction of 3HV. As the fraction of 3HV monomer increases, the copolymers become more flexible (decrease in Young's modulus) and the melting temperature decreases as can be seen from Table 2.1.

The propyl group in P(3HB-*co*-3HHx) is more effective than the ethyl group in P(3HB-*co*-3HV) in improving the polymer's elongation at break. A random copolymer of poly (3-hydroxybutyrate-*co*-3-hydroxyhaxanoate), P(3HB-*co*-3HHx) is produced by *A. caviae* when alkanoic acids of even carbon numbers or plant oils are fed. Studies have demonstrated that P(3HB-*co*-3HHx) is a flexible material and is characterized by a low elastic modulus, high elongation at break, and relatively low

Table 2.1 Comparison of polymer properties (Kunioka et al., 1989; Doi et al., 1990; Byrom, 1994; Saito & Doi, 1994; Tsuge, 2002).

Polymer	**Melting point (°C)**	**Young's modulus (GPa)**	**Tensile strength (MPa)**	**Elongation to break (%)**	**Glass transition temperature (°C)**
P(3HB)	179	3.5	40	5	4
P(3HB-*co*-3HV)					
3 mol% 3HV	170	2.9	38	–	–
9 mol% 3HV	162	1.9	37	–	–
14 mol% 3HV	150	1.5	35	–	–
20 mol% 3HV	145	1.2	32	50	-1
25 mol% 3HV	137	0.7	30	–	–
P(3HB-*co*-4HB)					
3 mol% 4HB	166	–	28	45	–
10 mol% 4HB	159	–	24	242	–
16 mol% 4HB	150	–	26	444	-7
64 mol% 4HB	50	30	17	591	–
90 mol% 4HB	50	100	65	1080	–
P(4HB)	53	149	104	1000	–
P(3HHx-*co*-3HO)	61	–	10	300	–
P(3HB-*co*-6 mol% 3HA)	133	0.2	17	680	-8
P(3HB-*co*-67 mol% HP)	44	–	–	–	-19
P(3HB-*co*-10 mol% 3HHx)	127	–	21	400	-1
Polypropylene	170	1.7	34.5	400	45
Polyethylene–terepthalate	262	2.2	56	7300	3400
Polystyrene	110	3.1	50	–	21
LDPE	130	0.2	10	620	-3

tensile strength (Doi et al., 1995).

Another efficient method to improve the physical properties of P(3HB) is by the incorporation of different 3-hydroxyalkanoates (3HA) units into 3HB-based polymers. *Pseudomonas* sp. 61-3 has been demonstrated to produce a polyester blend of P(3HB) homopolymer and a random copolymer containing 3HB and 3HA units of C6 to C12 from glucose (Kato et al., 1996). It is interesting to note that P(3HB-*co*-6 mol%3HA) copolymer has properties including melting temperature, Young's modulus, tensile strength, elongation to break and glass transition temperature similar to low-density polyethylene (LDPE).

2.2 Metabolic pathways involved in PHA synthesis

The biosynthetic pathway of P(3HB) has been studied well in *C. necator*. In *C. necator*, P(3HB) synthesis occurs in a three-step reaction and starts with the condensation of two acetyl-CoA that are derived from tricarboxylic acid cycle (TCA) to acetoacetyl-CoA as shown in pathway I (Figure 2.2). This step is catalyzed by β-ketothiolase. The second step is the reduction of acetoacetyl-CoA to (*R*)-3-hydroxybutyryl-CoA by the NADPH-dependent acetoacetyl-CoA reductase. This reaction occurs stereospecifically and only the (*R*)-isomers are produced. The last step is the incorporation of (*R*)-3-hydroxybutyryl-CoA into the polymer chain by PHA synthase (Tsuge, 2002). The synthesis of P(3HB) is regulated by the intracellular concentration of acetyl-CoA. Under growth without nutrient limitation, there is high level of free coenzyme A. However, when growth is restricted by the limitation of nutrient such as ammonium or phosphate, coenzyme A level is reduced and this condition favors the synthesis of P(3HB) (Zinn et al., 2001). The genes

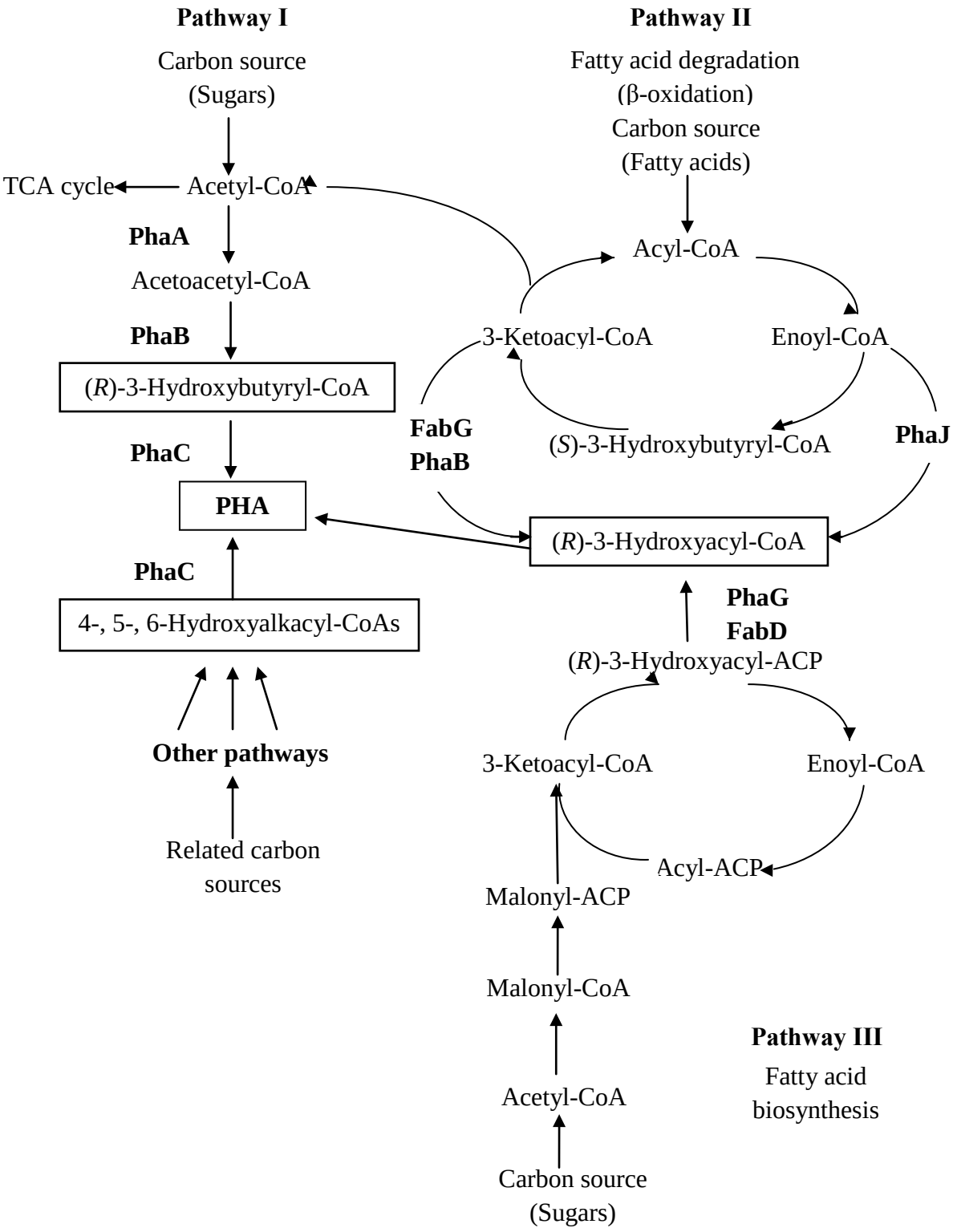


Figure 2.2 Metabolic pathways that supply various hydroxyalkanoate (HA) monomers for PHA biosynthesis. PhaA, β-Ketothiolase; PhaB, NADPH-dependent acetoacetyl-CoA reductase; PhaC, PHA synthase; PhaG, 3-hydroxyacyl-ACP-CoA transferase; PhaJ, (*R*)-specific enoyl-CoA hydratase; FabG, 3-ketoacyl-ACP reductase (Tsuge, 2002; Sudesh & Doi, 2005).

involved in the biosynthesis of PHA have been designated *phaA*, *phaB* and *phaC* for β-ketothiolase, acetoacetyl-CoA reductase and PHA synthase respectively (Tsuge, 2002).

The synthesis of mcl-PHA relies mainly on β-oxidation pathway (pathway II in Figure 2.2) when fatty acids are used as carbon source, or on fatty acid *de novo* synthesis (pathway III) when simple carbon sources, such as gluconate, fructose, glucose, acetate, glycerol and lactate are employed. Pseudomonads that belong to the rRNA-homology group I can synthesize mcl-PHA when grown on alkanes, alkanols, and alkanoates. Most of the pseudomonads belonging to this group, except *Pseudomonas oleovorans*, can also produce PHA when grown on unrelated carbon sources such as carbohydrates (Steinbüchel & Lütke-Eversloh, 2003). When fatty acids are supplied as exogenous substrate, the fatty acids are first converted to the corresponding acyl-CoA thioesters and then undergo oxidization by fatty acid β-oxidation via enoyl-CoA and (*S*)-3-hydroxyacyl-CoA to 3-ketoacyl-CoA. After that, 3-ketoacyl-CoA is cleaved by a β-ketothiolase to acetyl-CoA and acyl-CoA. These intermediates of the β-oxidation cycle are withdrawn and enzymatically converted to (*R*)-3-hydroxyacyl-CoA, which serves as substrate for PHA synthase (Steinbüchel & Lütke-Eversloh, 2003). If the fatty acids are not completely degraded to acetyl-CoA, the intermediates are not accepted as substrate by the mcl-PHA synthases. Therefore, a metabolic pathway is required to convert the intermediates into (*R*)-3-hydroxyacyl-CoA which is accepted by PHA synthase. In *A. caviae,* the β-oxidation cycle intermediate, trans-2-enoyl-CoA is converted to (*R*)-3-hydroxyacyl-CoA by (*R*)-specific enoyl-CoA hydratase encoded by *phaJ* (Fukui & Doi, 1997*)*. In recombinant *E. coli*, it was reported that 3-ketoacyl-CoA is converted to (*R*)-3-hydroxyacyl-CoA

by a NADPH-dependent 3-ketoacyl-acyl carrier protein (ACP) reductase that is encoded by *fabG*. Furthermore, previous study shows that the acetoacetyl-CoA reductase (PhaB) of *C. necator* can also carry out the conversion of 3-ketoacyl-CoA to the corresponding (*R*)-3-hydroxyacyl-CoA in recombinant *E. coli* (Ren et al., 2000).

Precursor for fatty acid *de novo* synthesis is derived from the acetyl-CoA pool. The first step involves the conversion of acetyl-CoA to malonyl-CoA, which is used to elongate the fatty acid carbon chain. In *P. putida and Pseudomonas aeruginosa*, an acyltransferase transfers the hydroxyacyl moiety from (*R*)-3-hydroxydecanoyl-acyl carrier protein to coenzymeA, forming (*R*)-3-hydroxydecanoyl-CoA, which is a substrate for the mcl-PHA synthases. This key enzyme is encoded by the *phaG* gene and it links fatty acid *de novo* synthesis to medium chain length PHA biosynthesis (Rehm et al., 1998).

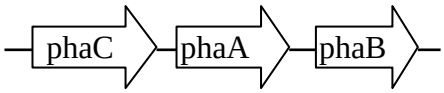
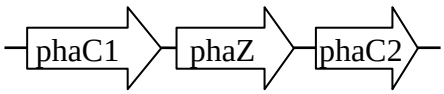
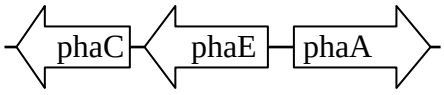
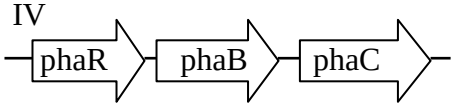
2.3 PHA synthase

PHA synthases are the most important enzymes in PHA biosynthesis. They use coenzyme A (CoA) thioesters of hydroxyalkanoic acids (HA) as substrates and catalyze the polymerization of HA into PHA with the release of CoA. More than 88 PHA synthase genes have been cloned from 68 species of bacteria and can be grouped into four classes based on three criteria: their *in vivo* substrate specificities, primary amino acid sequences and subunit composition (Table 2.2) (Rehm, 2007). Class I synthases, which are represented by the PHA synthase from *C. necator*, are active towards short-chain length (*R*)-hydroxyacyl-CoA consist of three to five carbon atoms (Pötter & Steinbüchel, 2006). Class II synthases are active towards medium-chain length (*R*)-3-hydroxyacyl-CoA that contain six to fourteen carbon

atoms and are represented by *P. aeruginosa*. Class I and II PHA synthases comprise enzymes consisting of only one type of subunit (PhaC) with molecular masses (M_w) between 61 and 68 kDa. Class III PHA synthases, represented by the *Allochromatium vinosum* PHA synthases, comprise two subunits: PhaC and PhaE. The PhaC subunit of Class III PHA synthases, with M_w about 40 kDa, exhibits 21 to 28% sequence similarity to type I and II PHA synthases. Class III PHA synthases prefer short-chain length (*R*)-hydroxyacyl-CoA. Class IV PHA synthases, represented by the enzyme of *B. megaterium*, consist of two different types of subunits (PhaC and PhaR). Class IV PHA synthases exhibit a similar substrate range as class III PHA synthases, which are also active towards short-chain length (R)-hydroxyacyl-CoA (Rehm, 2003).

A few exceptions to the above classification with respect to substrate specificity are the PHA synthases from *Thiocapsa pfennigii* and *A. caviae*. The PHA synthase of *T. pfennigii* exerts a broad substrate specificity and is capable of utilizing CoA thioesters of short-chain-length and medium-chain-length 3-hydroxyalkanoic acids. On the other hand, the PHA synthase of *A. caviae* is characterized by its ability to polymerize copolymer containing 3HB and 3HHx monomer (Rehm & Steinbüchel, 1999).

Table 2.2 The four class of polyester synthase (adapted from Pötter & Steinbüchel, 2006).

Class/ general structures of PHA operon	Subunits	Substrates	Representative species
I 	60-73 kDa	$3HA_{SCL}$-CoA $4HA_{SCL}$-CoA $5HA_{SCL}$-CoA $3MA_{SCL}$-CoA - C3-C5	*Cupriavidus necator* *Sinorhizobium melioti* *Burkholderia sp.* *Aeromonas caviae* many other bacteria
II 	60-65 kDa	$3HA_{MCL}$-CoA - C5-C14	*Pseudomonas aeruginosa* *Pseudomonas putida* all pseudomonas belonging to rRNA homologous group I
III 	PhaC - 40 kDa PhaE - 40 kDa	$3HA_{SCL}$-CoA - C3-C5 $3HA_{MCL}$-CoA - C5-C8 $4/5HA_{SCL}$-CoA	*Allochromatium vinosum* *Thiocapsa pfennigii* *Synechocystis* sp. PCC6803 purple sulfur bacteria all cyanobacteria
IV 	PhaC - 40kDa PhaR – 22kDa	$3HA_{SCL}$-CoA - C3-C5	*Bacillus megaterium* *Bacillus* sp. INT005 other strains are unknown

2.4 PHA granule-associated proteins

2.4.1 Phasin (PhaP)

Investigation of *Rhodospirillum rubrum* cells by transmission electron microscopy revealed that the PHA granules were covered by surface layer with thickness of approximately 4 nm (Mayer & Hoppert, 1997). Freeze-fracture electron microscopy of *P. putida* cells had shown that the boundary layer of PHA granules was too thin to be bilayer membrane. These observations are the basis for the assumption that PHA granules are surrounded by lipid monolayer. Subsequent structural analyses indicated that the phospholipid monolayers are embedded with a set of granule-associated proteins consisted of phasin, PHA synthesis regulator and PHA depolymerase (Figure 2.3). To date, *phaP*1 from *C. necator* is one of the most studied phasin. The availability of *C. necator* genome information has allowed the identification of other phasin homologous (*phaP*2, *phaP*3, *phaP*4 and *phaP*5). Although the function of phasin remains unknown, extensive studies have been performed to elucidate the role of phasin in PHA metabolism. It was reported that the disruption of *phaP*1 by Tn5 insertion mutagenesis had resulted in PHA-leaky mutants that accumulate PHA at lower rate compared to the wild type and the synthesis of one large single granule in the cells was observed (Wieczorek et al., 1995). On the other hand, overexpression of *phaP*1 had led to the formation of smaller PHA granules accompanied by an increase in the number of granules (Pötter et al., 2002). Therefore, it was suggested that phasin might involve in the determination of surface-to-volume ratio of PHA granules. Phasin prevents the coalescence of PHA granules by stabilizing the dispersion of hydrophobic PHA in the hydrophilic cytoplasm (Jendrossek, 2009).

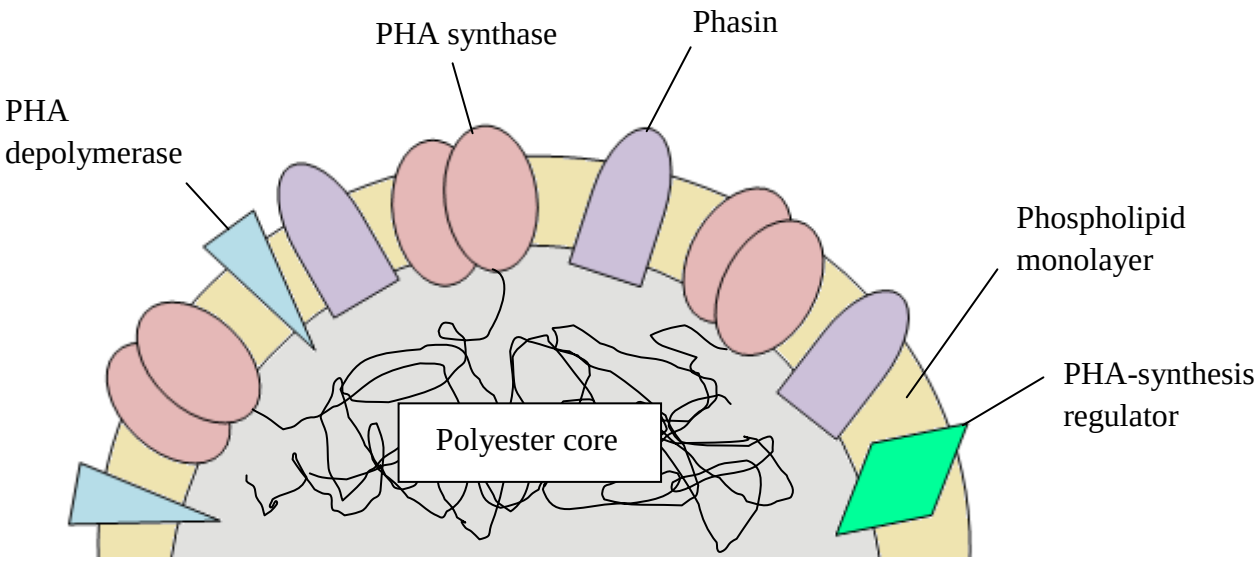


Figure 2.3 Schematic presentation of a PHA granule (adapted from Rehm, 2003).

2.4.2 PHA-synthesis regulator (PhaR)

Studies on regulation of phasin expression had led to the discovery of a PHA-synthesis regulator, PhaR. Proteins homologous to PhaR of *C. necator* were identified in other PHA-producing bacteria e.g. *A. vinosum, Paracoccus denitrificans, Sinorhizobium meliloti* and *Burkholderia cepacia* (Pötter & Steinbüchel, 2006). Insight into the role of PhaR in PHA metabolism was provided by the construction of several deletion mutants of *C. necator*. It was shown that the deletion of *phaR* resulted in the constitutive expression of PhaP at high levels. In the case of *phaC* deletion mutant, no PhaP was synthesized (Wieczorek et al., 1995; York et al., 2001). Genetic studies also demonstrated that the deletion of both *phaR* and *phaC* resulted in high level of PhaP expression even though PHA accumulation was not observed. Western immunoblotting analysis using antibodies raised against PhaR provided evidence that PhaR binds to PHA granules. Further support was provided by *in vitro* studies which reveal that PhaR was able to cause a gel shift of a sequence of DNA to which PhaR is proposed to bind to and this gel shift was reversible in the presence of PHA (Maehara et al., 2002). A model for the regulation of phasin expression was deduced based on all these results. PhaR is proposed to be a regulatory protein which binds to the promoter regions of PhaP, PhaR and surface of PHA granules. When conditions are not permissive for PHA accumulation or in PHA-negative mutants, the transcription of *phaP* is repressed by the binding of PhaR to the promoter region of *phaP*. When cultivation conditions are permissive for PHA accumulation, the PHA granules formed are suggested to be responsible for the de-repression of PhaR (Stubbe & Tian, 2003; Pötter et al., 2005; Pötter & Steinbüchel, 2006).

2.4.3 PHA depolymerase (PhaZ)

The most important physiological function of PHA to its producing microorganisms is to provide carbon and energy sources in conditions unfavorable for growth. The key enzymes that are capable of hydrolyzing previously accumulated PHA are intracellular PHA depolymerases (i-PHA depolymerases) (Jendrossek & Handrick, 2002). These enzymes show no similarity to the protein sequence of extracellular PHA depolymerases and differ from extracellular PHA depolymerases in term of substrates hydrolyzed. It is remarkable to note that the accumulated PHA had enabled the survival of *Legionella pneumophila* cells in tap water for up to 600 days (James et al., 1999). The first intracellular depolymerase that had being characterized genetically and assayed *in vitro* was PhaZ1 (Saegusa et al., 2001). To date, seven putative i-PHA depolymerases and two 3HB oligomer hydrolases have been identified in *C. necator*. The nomenclature of these enzymes is based on the similarities of the primary amino acid sequences of the enzymes to each other. The i-PHB depolymerases are named PhaZ1 to PhaZ5. On the other hand, the 3HB oligomer hydrolases are assigned as PhaY1 and PhaY2 to prevent confusion with the naming of PHA depolymerases (Pohlmann et al., 2006). In a recent report on the function of PhaY2, this enzyme was found to degrade not only trimers of HB, but also amorphous P(3HB) under both *in vivo* and *in vitro* experimental conditions (Kobayashi et al., 2003). Besides that, this oligomer hydrolase show completely different protein size and sequences from previously identified PhaZ1, PhaZ2 and PhaZ3. The availability of genomic sequences in the databases due to extensive genomic sequencing activities has lead to a significant increase in the number of putative i-PHA depolymerases annotated or identified. However, only a few of these i-PHA depolymerases had been biochemically or *in vivo* characterized, e.g. PhaZ1 of

C. necator, *P. denitrificans* and *Rhodobacter sphaeroides* (Handrick et al., 2000; Gao et al., 2001; Kobayashi et al., 2004). Among the i-PHA depolymerases characterized, PhaZ1 is the most-studied enzyme and it is bound to native PHA granules *in vivo*. In a *phaZ*1 deletion mutant, a significant decrease in the release of 3HB from P(3HB)-accumulated cells was demonstrated compared to the wild type phenotype. On the contrary, the expression of *phaZ*1 in recombinant PHA-accumulating *E. coli* showed an increase in the release of 3HB. In a similar experiment, various *C. necator* putative i-PHA depolymerases (ΔphaZ1phaZ2phaZ3, ΔphaZ1phaZ2, ΔphaZ1phaZ3, ΔphaZ2phaZ3) and each single deletion mutants were constructed to examine their function *in vivo*. PhaZ2 was shown to exhibit intracellular depolymerizing activity but no phenotype was observed for PhaZ3 despite its similar sequence identity with PhaZ1 (45% identity) (Stubbe & Tian, 2003).

2.5 PHA granule formation

Despite the increase in the understanding of PHA synthase and PHA granule associated proteins e.g. phasin, PHA synthesis regulator, PHA depolymerase and etc. due to extensive studies in recent years, the formation of PHA granules *in vivo* is still not very well understood. Currently, two models are being proposed in the literature to describe the formation of PHA granules. They are the micelle model and budding model (Stubbe & Tian, 2003) (Figure 2.4). The former model was supported by experimental data on *in vitro* formation of PHA granules in the absence of membranes. In the micelle model, PHA biosynthesis proceeds with the reactions of

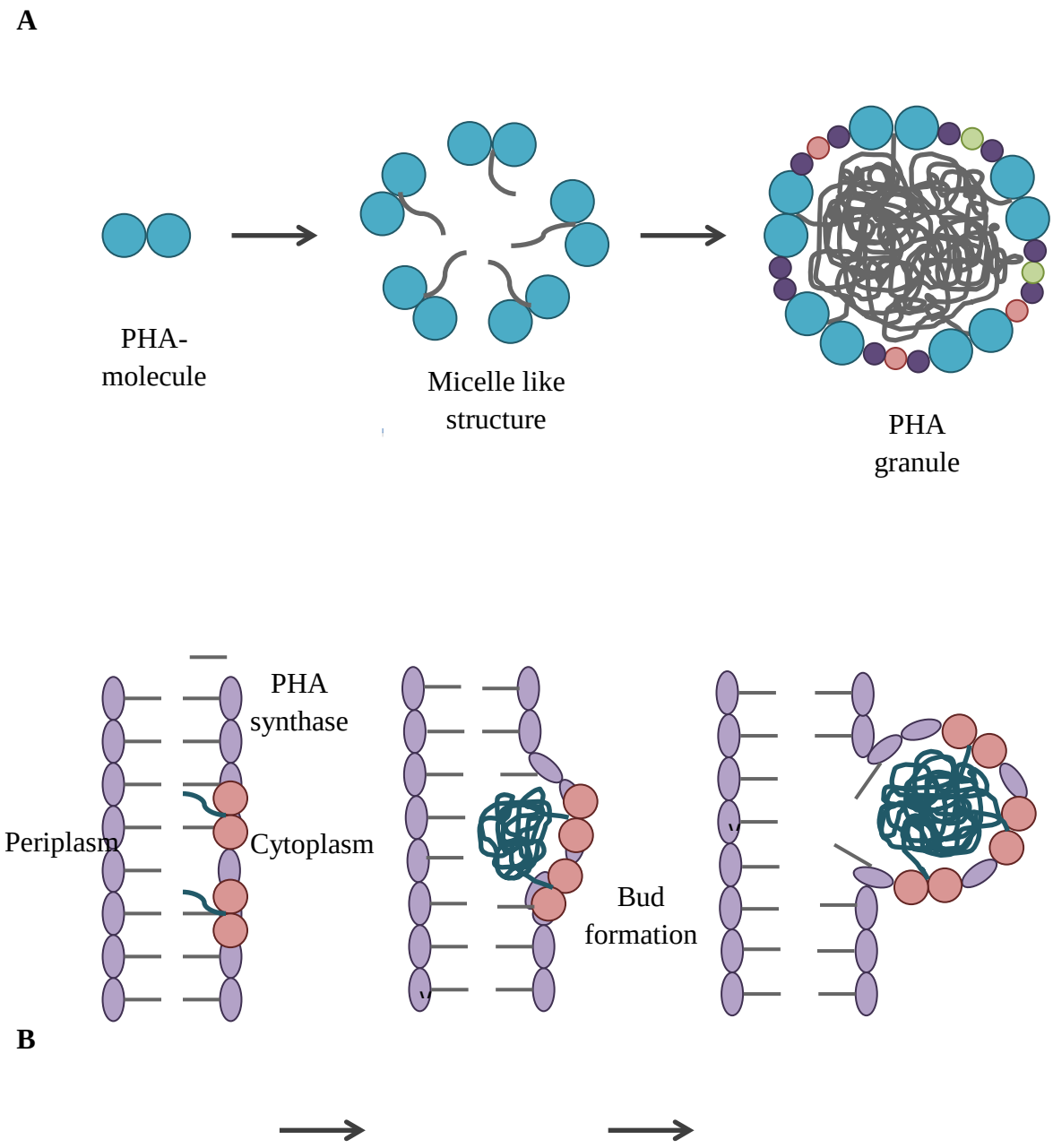


Figure 2.4 Models for the formation of PHA granules in bacteria. A. micelle model; B. budding model (adapted from Pötter & Steinbüchel, 2006; Rehm, 2007).