METABOLIC ENGINEERING OF *Cupriavidus necator* FOR THE PRODUCTION OF POLY(3-HYDROXYBUTYRATE-*co*-3-HYDROXYHEXANOATE)

by

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A	Absorbance
mm^3	A cubic of millilitre
Amp	Ampicillin
bp	Basepair
β	Beta
CDSs	Coding DNA Sequences
Cm	Chloramphenicol
CME	Caprylate methyl ester
CoA	Coenzyme A
CPKO	Crude palm kernel oil
Da	Dalton
Δ	Delta
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate
DCW	Dry cell weight
EDTA	Ethylenediamine tetraacetic acid
GC	Gas chromatography
Gm	Gentamycin
GTE	Glucose Tris-EDTA
HCL	Hydrochloric acid
IPTG	Isopropyl-β-D-thiogalactoside
IS	Insertion sequence
Kan	Kanamycin
kb	Kilobase pairs

kDa	Kilo Dalton
kPa	Kilopascal
λ	Lambda
LA	Luria-Bertani agar
LB	Luria-Bertani broth
LCL	Long chain length
LDPE	Low density polyethylene
M	Molar
MCL	Medium chain length
MCS	Multiple cloning sites
µg/mL	Microgram per millilitre
µL	Microlitre
µM	Micromolar
mM	Millimolar
MS	Mineral salt
N	Normal
NA	Nutrient agar
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide (reduced form)
NaOH	Sodium hydroxide
Na_2SO_4	Sodium sulphate
ng/mL	Nanogram per mililitre
nm	Nanometer
NNG	1-nitroso-3-nitro-1-metylguanidine
NO_2^-	Nitrate
NO_3^-	Nitrite
OD	Optical density

ORF	Open reading frame
P(3HB)	Poly(3-hydroxybutyrate)
P(4HB)	Poly(4-hydroxybutyrate)
P(3HB-*co*-67 mol % HP)	Poly(3-hydroxybutyrate-*co*-hydroxypentanoate) containing 67 mol % of HP
P(3HB-*co*-4HB)	Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate)
P(3HB-*co*-3HHx)	Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate)
P(3HB-*co*-3HV)	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)
P(3HB-*co*-6 mol % 3HA)	Poly(3-hydroxybutyrate-*co*-3-hydroxyalkanoate) containing 6 mol % of 3HA
P(3HHx-*co*-3HO)	Poly(3-hydroxyhexanoate-*co*-3-hydroxyoctanoate)
PCR	Polymerase chain reaction
PHA	Polyhydroxyalkanoate
PhaC	Polyhydroxyalkanoate synthase
PHB	Polyhydroxybutyrate
psi	Pound per square inch
(*R*)-3HB-CoA	(*R*)-3-hydroxybutyrate-Coenzyme A
RBS	Ribosome binding site
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SCL	Short chain length
SDS	Sodium dodecyl sulfate
× *g*	Times gravity
TAE	Tris-Acetic Acid-EDTA
TCA	Tricarboxylic acid
TE	Tris-EDTA
TEM	Transmission electron microscopy

tRNA	Transfer ribonucleic acid
TSS	Transformation and storage solution
U/mg	Unit per milligram
U/mL	Unit per millilitre
V	Volt
v/v	Volume per volume
wt %	Weight percent
w/v	Weight per volume

KEJURUTERAAN METABOLIK *Cupriavidus necator* UNTUK PENGHASILAN POLI(3-HIDROKSIBUTIRAT-*ko*-3-HIDROKSIHEKSANOAT)

ABSTRAK

Polihidroksialkanoat (PHA) adalah keluarga poliester yang dihasilkan oleh bakteria yang mempunyai ciri-ciri fizikal untuk kegunaan pelbagai industri. Dalam kajian ini, gen PHA sintase telah dipencilkan daripada *Chromobacterium* sp. USM2 (*phaC*$_{Cs\ USM2}$) telah dikaji potensinya untuk mensintesiskan polimer dalam *Cupriavidus necator*. Plasmid berjulat luas pBBR1MCS-2 telah diubahsuai untuk membina plasmid pengekspresan pYEB-100, yang telah direka untuk memudahkan pengklonan PHA sintase dalam langkah untuk menilai prestasi pelbagai PhaC dan komposisi PHA terkumpul dalam keadaan piawai. Bagi membuktikan konsep ini, *phaC*$_{Cs\ USM2}$ telah diekspreskan dalam *C. necator* PHB^{-}4 yang menghasilkan 68% jisim kopolimer P(3HB-*ko*-3HHk) dengan menggunakan minyak isirong sawit mentah sebagai sumber karbon tunggal. Kesan terhadap enzim utama lain; β-ketotiolase (PhaA) dan NADPH-bersandar asetoasetil-KoA reduktase (PhaB1) terhadap pertumbuhan dan penghasilan PHA dalam *C. necator* turut dikaji. Penghapusan *phaA* dan *phaB1* dalam *C. necator* (kedua-dua strain H16 dan PHB^{-}4) telah dihasilkan daripada rekombinasi berhomolog menggunakan plasmid swa-hapus pDM4. Mutan delesi diperhatikan telah menghasilkan jisim kering sel yang lebih rendah tetapi bahagian 3HHk yang lebih tinggi dalam kopolimer P(3HB-*ko*-3HHk) yang terhasil. Gen (*R*)-spesifik enoil-KoA hidratase (*phaJ*) daripada *Aeromonas* sp. AE6, yang telah diekpres bersama dengan *phaC*$_{Cs\ USM2}$ telah meningkatkan kopolimer P(3HB-*ko*-3HHk) tetapi bahagian 3HHk masih kekal pada 2% mol.

Analisis mikroskop elektron transmisi (TEM), telah mendedahkan bahawa granul PHA P/$C_{Cs}J_{As}$ adalah lebih besar dan panjang berbanding granul P/C_{Cs}. Dalam mutan delesi, granul HΔAB1/$C_{Cs}J_{As}$ adalah bersaiz lebih kecil, tetapi mempunyai bilangan yang lebih banyak berbanding granul HΔAB1.

METABOLIC ENGINEERING OF *Cupriavidus necator* FOR THE PRODUCTION OF POLY(3-HYDROXYBUTYRATE-*co*-3-HYDROXYHEXANOATE)

ABSTRACT

Polyhydroxyalkanoate (PHA) is a family of polyesters produced by bacteria that possesses physical properties for various industrial applications. In this study, PHA synthase (PhaC) gene isolated from *Chromobacterium* sp. USM2 (*phaC*$_{Cs\ USM2}$) was explored for its potential to synthesize polymer in *Cupriavidus necator*. A broad-host range plasmid pBBR1MCS-2 was modified to construct an expression plasmid pYEB-100, which was designed to facilitate cloning of PHA synthases in order to assess the performance of various PhaCs and the composition of PHA accumulated in a standard environment. To prove this concept, *phaC*$_{Cs\ USM2}$ was expressed in *C. necator* PHB^{-}4, which produced 68 wt% of P(3HB-*co*-3HHx) copolymer using crude palm kernel oil as the sole carbon source. The effect of other key enzymes; β-ketothiolase (PhaA) and NADPH-dependent acetoacetyl-CoA reductase (PhaBI) on growth and PHA production in *C. necator* were also investigated. Deletion of *phaA* and *phaB1* in *C. necator* (both H16 and PHB^{-}4 strains) were generated by homologous recombination using the suicidal plasmid pDM4. The deletion mutants were observed to produce lower cell dry weights but higher 3HHx fraction in the P(3HB-*co*-3HHx) copolymer produced. The (*R*)-specific enoyl-CoA hydratase gene (*phaJ*) from *Aeromonas* sp. AE6, which was co-expressed with *phaC*$_{Cs\ USM2}$ increased P(3HB-*co*-3HHx) copolymer but the 3HHx fraction remained constant at 2 mol%. Transmission electron microscopy analysis revealed that PHA granules of P/C$_{Cs}$J$_{As}$ were larger and elongated compared to P/C$_{Cs}$ granules.

In deletion mutants, HΔAB1/$C_{Cs}J_{As}$ granules were smaller in size, but greater in number compared to HΔAB1.

CHAPTER 1

INTRODUCTION

The over production of petro-based polymers (commonly known as plastics) by dynamic growing human populations has considerably increased the number of landfills across the world. In most urban areas, plastic-based materials would be a major constitution of the landfills. The main problem of these waste materials is its poor biodegradable nature. They are not easily degraded and will be remained in the environment for many years.

Biological polymers such as polyhydroxyalkanoates (PHA) and their derivatives could be alternatives to plastics that are being used currently. These biological polymers receive great attention globally because they are viewed as environmentally friendly, non-toxic, and possess potential applications in various fields such as packaging, medical, pharmaceuticals, foods and agriculture (Khanna and Srivastava, 2005).

PHA is naturally synthesized by various microorganisms as storage materials under imbalanced growth conditions (Anderson and Dawes, 1990). These polymers are unique because they are synthesized from renewable resources and can be degraded into water and carbon dioxide by a wide range of microorganisms (Steinbuchel and Fuchtenbusch, 1998; Jendrossek, 2001).

The main obstacle in replacing plastics by PHA is the high cost of PHA production. Thus, many efforts have been expended in reducing the cost of PHA production by using cheap raw material resources, isolating better producer strains, improving fermentation and downstream processes as well as using metabolic engineering approaches to develop low cost production processes.

Generally, PHA can be divided into two major groups which are short-chain-length PHA (scl-PHA) and medium-chain-length PHA (mcl-PHA). Scl-PHA is composed of monomers with three to five carbon atoms, while mcl-PHA is composed of monomers with six to fourteen carbon atoms (Anderson and Dawes, 1990). Poly(3-hydroxybutyrate) [P(3HB)], which is a homopolymer of 3HB monomer has a high degree of crystallinity is very stiff and brittle (Doi, 1995). These properties will limit its commercial potential. Mcl-PHA more rubber or latex-like, has lower crystallinity and but higher elasticity compared to scl-PHA (Gross *et al*., 1989; Doi, 1990).

However, a blend polymer consisting of scl-PHA and mcl-PHA has more favourable properties for industrial applications (Steinbuchel and Valentin, 1995; Sudesh *et al*., 2000; Steinbuchel and Lutke-Eversloh, 2003). One such example of copolymer is poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)]. P(3HB-*co*-3HHx) copolymer is tougher, more ductile and flexible as compared to P(3HB) homopolymer (Shimamura *et al*., 1994). The incorporation of a small amount of 3HHx monomer fraction into the P(3HB) backbone polymer improved the properties of the copolymer similar to low-density polyethylene(LDPE) (Matsusaki *et al*., 2000).

The goal of this study was to synthesize P(3HB-*co*-3HHx) copolymer with enhanced 3HHx monomer fraction in the PHA model organism, *C. necator*. The strategy of the study was to reduce the synthesis of 3-hydroxybutyrate-CoA (3HB-CoA) from the PHA metabolic pathway by the deletion of β-ketothiolase (*phaA*) and NADPH-dependent acetoacetyl-CoA reductase (*phaB1*) genes. The incorporation of 3HHx monomer into the PHA production might be improved due to the reduction of PhaA and PhaB1 activities in the cells.

Hence, the aim of the study was carried out in three parts. The first part was to construct an expression plasmid based on a broad-host range vector suitable for PHA synthase (PhaC) functional study. This new expression plasmid would be useful to facilitate the cloning and evaluating the performance of PhaC from different bacteria. The second part was to observe the effect of β-ketothiolase (*phaA*) and NADPH-dependent acetoacetyl-CoA reductase (*phaB1*) genes deletion on PHA production in *C. necator*. Deletion mutants were generated by homologous recombination approach with the help of suicidal plasmid pDM4. The expression of PHA synthase gene (*phaC*) from *Chromobacterium* sp. USM2 using the newly constructed expression plasmid in deletion mutants and the wild-type was observed. The final part of the study was to increase 3HHx fraction in the copolymer production by the co-expression of (*R*)-specific enoyl coenzyme-A hydratase (*phaJ*) from *Aeromonas* sp. AE6 with $phaC_{Cs\ USM2}$.

CHAPTER 2

LITERATURE REVIEW

2.1 Background of polyhydroxyalkanoates (PHA)

Polyhydroxyalkanoates (PHA) are homo- or hetero-polymers synthesized from various carbon substrates by microorganisms under imbalanced growth conditions (Doi, 1990; Steinbüchel, 1991; Braunegg *et al*., 1998). Microorganisms accumulate PHA when there is excess carbon source and under the limitation of growth factors such as nitrogen (Schlegel *et al*., 1961), potassium or sulphur (Wilkinson and Munro, 1967), or oxygen (Senior *et al*., 1972). PHA are accumulated in the cytoplasm of the microorganisms as water insoluble inclusion bodies (referred to as PHA granules) and function as storage material for energy and carbon sources (Steinbüchel, 1991). PHA can be degraded completely into carbon dioxide and water in the environment by a wide variety of bacteria and fungi (Mergaert *et al*., 1995; Braunegg *et al*., 1998).

PHA becomes attractive due to its biodegradable, biocompatible and thermo-plasticity characteristics which are highly valued in terms of medical, agricultural and industrial applications (Reddy *et al*., 2003). Interestingly, PHA can be synthesized from renewable resources in contrast to synthetic petro-based polymers (Eggersdorfer *et al*., 1992). However, the cost of PHA production is still high and becomes the main obstacle to completely replace the petro-based plastics currently preferably used over the biodegradable plastics. Thus, a lot of studies are extensively done to reduce the cost of PHA production by developing an efficient fermentation and recovery processing techniques, as well as generating numerous

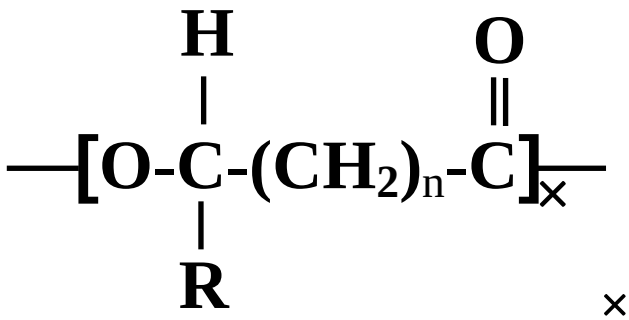
better strains and producing PHA in a large scale by transgenic plants in order to make it a success.

2.2 Classification of PHA

PHA can be divided into three groups based on the number of carbon atoms present in the monomer unit; short-chain-length PHA (scl-PHA) consists of monomer with carbon number in the range of 3 to 5, while medium-chain-length PHA (mcl-PHA) consists of 6 to 14 carbon atoms (Anderson and Dawes, 1990). However, there is another sub-group which comprises of both scl-PHA and mcl-PHA with the chain length ranging from 3 to 14 carbon atoms (Matsusaki *et al*., 1998).

Cupriavidus necator (formerly known as *Ralstonia eutropha*) represents bacterium producing scl-PHA, while mcl-PHA is produced by *Pseudomonas oleovorans* (Anderson and Dawes, 1990). The other example is *Aeromonas caviae*, which produces a combination of scl-mcl-PHA from oleic acids and olive oil as carbon sources (Doi *et al*., 1995). Each group is different depending on the PHA produced, and this is due to the substrate specificity of PHA synthases to incorporate 3-hydroxyalkanoates (3HAs) of a certain range of carbon length (Anderson and Dawes, 1990).

PHA is polymer consisting of *R*(-)-3-hydroxyalkanoic acid monomers with variety of saturated or unsaturated and straight or branched side chain of aliphatic or aromatic group (Kawaguchi and Doi, 1992; Steinbüchel and Valentin, 1995). The chemical structure of PHA is shown in Figure 2.1. PHA has an *R* absolute configuration in the chiral center of 3-hydroxybutyric acid. The component of the side (R) and the number of carbon atom determined the identity of monomer unit.



×= 100-30 000 Da

n = 1	R = hydrogen	poly(3-hydroxypropionate)
	R = methyl	poly(3-hydroxybutyrate)
	R = ethyl	poly(3-hyrdroxyvalerate)
	R = propyl	poly(3-hyrdroxyhexanoate)
	R = pentyl	poly(3-hyrdroxyoctanoate)
	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 The chemical structure of polyhydroxyalkanoates

*R refers to side group and n refers to the number of repeating units

(Source: Ojumu *et al*., 2004)

2.3 Biosynthesis of PHA

Generally, there are three major types of metabolic pathways which are responsible for the synthesis of PHAs. The three most well known metabolic pathways involved are; P(3HB) biosynthesis pathway, fatty acid β-oxidation pathway and fatty acid biosynthesis pathway. These metabolic pathways can be divided based on the monomer compositions of PHA produced by bacteria. Figure 2.2 summarized the metabolic pathways that supply hydroxyalkanoate monomers for PHA biosynthesis.

2.3.1 Biosynthesis of scl-PHA

Biosynthesis of scl-PHA or P(3HB) has been studied extensively and well established in *C. necator* (Steinbüchel and Schlegel, 1991; Steinbüchel, 1991). In Pathway I, biosynthesis of scl-PHA begins with the conversion of carbon substrate to acetyl-coenzyme A (acetyl-CoA) which is derived from tricarboxylic acid (TCA) cycle. Subsequently, two molecules of acetyl-CoA are condensed to acetoacetyl-CoA by β-ketothiolase (PhaA). Acetoacetyl-CoA is later reduced to (*R*)-isomer of 3-hyrdoxybutyryl-CoA by NADPH-dependent acetoacetyl-CoA reductase (PhaB). Finally, PHA synthase (PhaC) will polymerize the (*R*)-3-hydroxybutyryl-CoA to produce scl-PHA or P(3HB) (Madison and Huisman, 1999; Sudesh *et al*., 2000; Rehm, 2007).

2.3.2 Biosynthesis of mcl-PHA

Biosynthesis of mcl-PHA also has been studied in detail in pseudomonads such as *P. oleovorance*, *P. putida* and *P. aeruginosa* (de Smet *et al*., 1983; Haywood *et al*., 1990; Timm and Steinbüchel, 1990). Fluorescent pseudomonads which belong

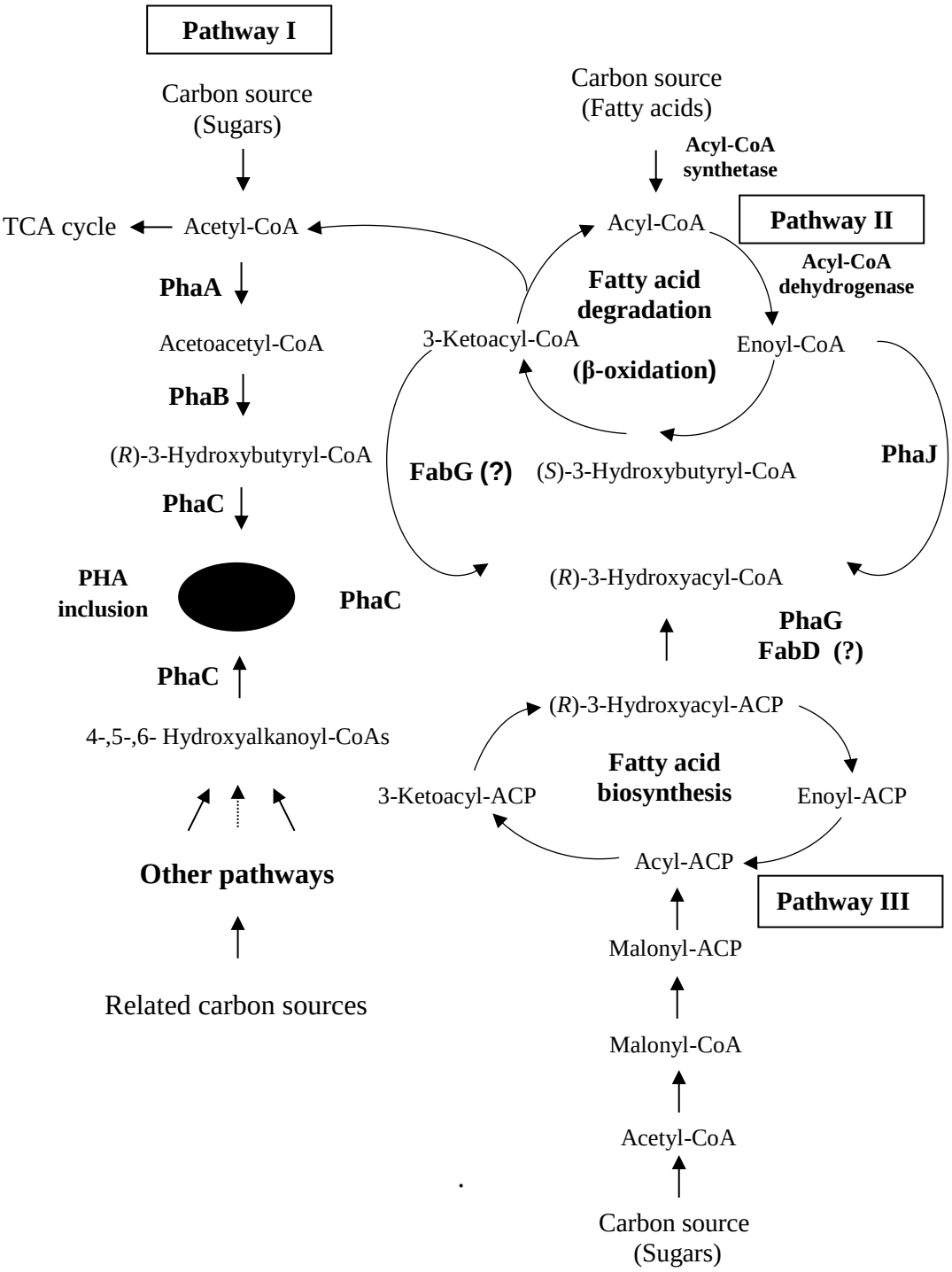


Figure 2.2 Metabolic pathways that supply hydroxyalkanoate monomers for PHA biosynthesis

Legends: PhaA, β-ketothiolase; PhaB, NADPH-dependent acetoacetyl-CoA reductase; PhaC, PHA synthase; PhaG, 3-hydroxyacyl-ACP-CoA transferase; PhaJ, (*R*)-specific enoyl-CoA hydratase; FabD, malonyl-CoA-ACP transacylase; FabG, 3-ketoacyl-CoA-reductase.

(Source: Sudesh *et al*., 2000)

to the rRNA-homology-group I are able to utilize various alkanes for the production of mcl-PHA (Lageveen *et al*., 1988). However, pseudomonads have a wider range of monomers incorporated as they can utilize fatty acids, glucose and unrelated carbon sources (Haywood *et al*., 1990) in order to produce mcl-PHAs.

There are three pathways found to be involved in the biosynthesis of mcl-PHAs; fatty acid degradation by β-oxidation, fatty acid biosynthesis and chain elongation. Fatty acid degradation pathway is also known as fatty acid β-oxidation (Pathway II) and it is represented by *P. oleovorans*. Initially, fatty acid is catalysed by acyl-CoA synthetase to form acyl-CoA. Acyl-CoA is then converted to 2-trans-enoyl-CoA by acyl-CoA dehydrogenase. Subsequently, (*R*)-specific enoyl-CoA hydratase (PhaJ) catalyses 2-trans-enoyl-CoA into (*R*)-3-hydroxyacyl-CoA and used as precursors to form the PHA polymerase substrates (Zinn *et al*., 2001; Kim *et al*., 2007). The other two precursors (*S*)-3-hydroxyacyl-CoA and 3-ketoacyl-CoA are produced from β-oxidation cycle.

Fatty acid biosynthesis pathway (Pathway III) is represented by *Pseudomonas aeruginosa, Pseudomonas mendocina* and *Pseudomonas putida*. A 3-hydroxyacyl-ACP-CoA transferase, PhaG is capable to channel the intermediates of the fatty acids biosynthesis to form mcl-PHA (Kim *et al*., 2007; Rehm *et al*., 1998). However, (*R*)-3-hydroxyacyl-ACP will be firstly converted to (*R*)-3-hydroxyacyl-CoA form before channelling to PHA biosynthesis cycle.

The third pathway is a chain elongation reaction. In this reaction, acyl-CoA is extended with acetyl-CoA to form ketoacyl-CoA. Subsequently, ketoacyl-CoA is converted to (*R*)-3-OH-acyl-CoA by ketoacyl-CoA reductase. This precursor will be incorporated into mcl-PHA under optimize condition.

2.3.3 Biosynthesis of scl-mcl-PHA; P(3HB-*co*-3HHx) copolymer

Biosynthesis of scl-mcl-PHA [P(3HB-*co*-3HHx)] copolymer has been well studied in *Aeromonas caviae* (Doi *et al*., 1995; Kobayashi *et al*., 1994) and *Aeromonas hydrophila* (Chen *et al.*, 2001). P(3HB-*co*-3HHx) is a unique copolymer which consists of both scl- and mcl-PHA. The physical properties and thermal process-ability of the resultant copolymer improved with the incorporation of at least 5 mol% of 3HHx monomer into P(3HB) polymer chain (Doi *et al*., 1995; Matsusaki *et al*., 2000; Loo *et al*., 2005).

Metabolic pathway of P(3HB-*co*-3HHx) copolymer in *Aeromonas caviae* has been proposed by Fukui and Doi (1997). Acyl-CoA, which is derived from alkanoics acids or oils is degraded through β-oxidation cycle to form enoyl-CoA intermediates with different chain lengths. Subsequently, the intermediates are converted to (*R*)-3HA-CoA by the (*R*)-specific enoyl-CoA hydratase which is encoded by *phaJ*. Then, the resultant (*R*)-3HA-CoA of four to six carbon atoms were incorporated into PHA copolymer(s) by PHA synthase. However, the small amount of 3HB unit which is found in the copolymers may be supplied from acetyl-CoA molecules through four-step reactions are catalyzed by β-ketothiolase, NADPH-dependent acetoacetyl-CoA reductase, crotonase [(*S*)-specific enoyl-CoA hydratase] and (*R*)-specific enoyl-CoA hydratase. The proposed metabolic pathway of P(3HB-*co*-3HHx) in *A. caviae* is illustrated in Figure 2.3.

2.4 PHA granules

PHA granules exist as spherical inclusion bodies with average diameters approximately 200-500 nm (Anderson and Dawes, 1990). These granules are surrounded by a phospholipid membrane (Griebel *et al*., 1968) with embedded or

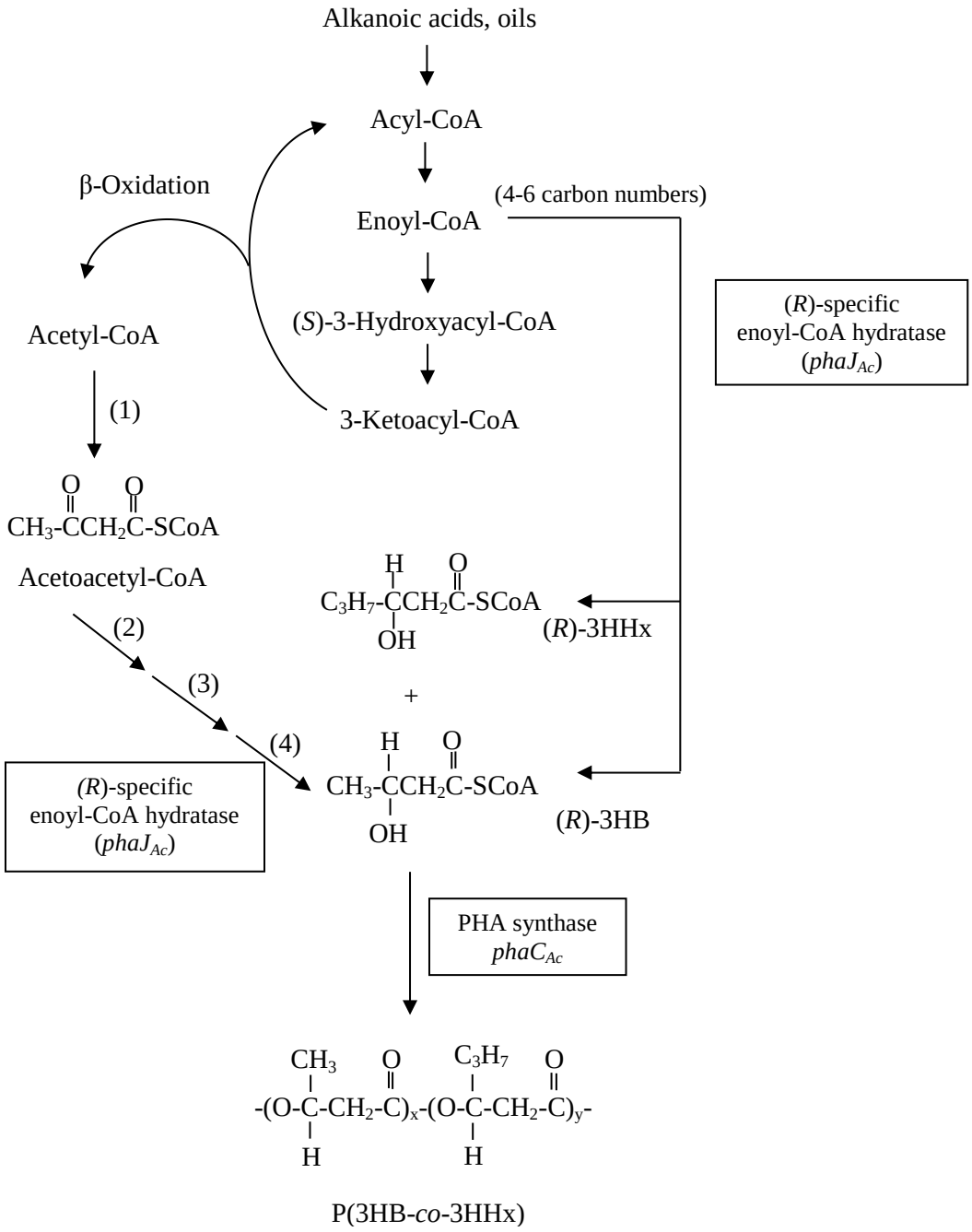


Figure 2.3 Pathway of P(3HB-*co*-3HHx) biosynthesis by *Aeromonas caviae* from alkanoic acids or oils 1) β-ketothiolase; 2) NADPH-dependent acetoacetyl-CoA reductase; 3) crotonase [(*S*)-specific enoyl-CoA hydratase]; 4) (*R*)-specific enoyl-CoA hydratase

(Source: Fukui *et al*., 1997)

attached proteins (Stuart *et al*., 1998). The attached proteins are composed of PHA synthase (Gerngross *et al*., 1993; Liebergesell *et al*., 1994), intracellular PHA depolymerase (Handrick *et al*. 2000; Saegusa *et al*., 2001; York *et al*., 2003), amphipathic phasin proteins (Wieczorek *et al*., 1996; York *et al*., 2001; Jurasek and Marchessault, 2002), PHA-specific regulator proteins (Maehara *et al*., 2002; York *et al*., 2002; Pötter *et al*., 2005) and other uncharacterized proteins (Klinke *et al*., 2000).

To date, there are two models of PHA granule formation have been proposed, namely, the micelle model and the budding model. These two models are considered at the defined location of the PHA synthase and the attached proteins at the surface of the granule. Both models are illustrated in Figure 2.4(A) and Figure 2.4(B), respectively. The micelle model was supported by PHA granules synthesis *in vitro* which was assayed without the presence of phospholipids membrane. In the micelle model, (*R*)-3-hydrocyacyl-CoA substrates required to attach to the free soluble PHA synthase, in order to form an amphiphathic PHA synthase which functional to initiate PHA granules formation. This formation of artificial granules was firstly demonstrated by Gerngross and Martin (1995).

However, the formation of PHA granule *in vivo* by the micelle model proposed that PHA synthase molecules and monomer substrates are randomly distributed in the cytoplasm (Gerngross *et al*., 1994). During the initial stage of 3-hydroxyacly-CoA polymerization, PHA synthase molecules aggregate into micelle-like structure. The elongation cycle of the polymer chain will continuously repeat the process to form small PHA granules by hydrophobic interactions. While, PHA synthase still attached to the surface of the PHA granules and the polymer chain will continue to grow. Finally, PHA granules become larger as they might be coalesced with their neighbouring granules. The other PHA surface proteins that attach to the

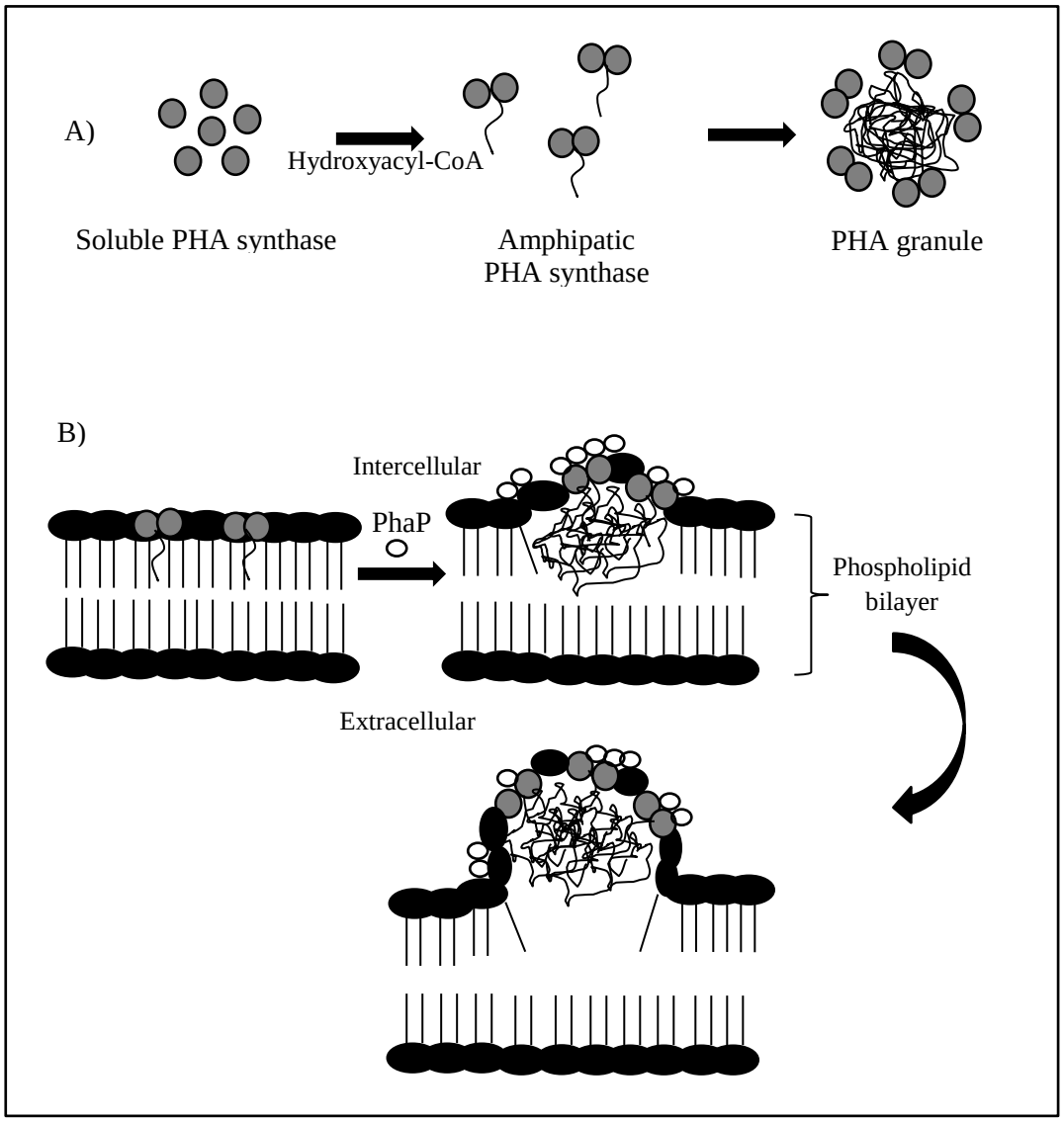


Figure 2.4 Model of PHA granule formation A) Micelle model representing *in vitro* formation in the absence of phospholipids. B) Budding model representing PHA granule formation at the cytoplasmic membrane. (Source: Tian *et al*., 2005; Rehm, 2007)

PHA growing granules are phasins (PhaP), PHA depolymerase (PhaZ) and PHA-spesific regulator proteins (PhaR).

In the budding model, it has been proposed that PHA synthases bind to the cytoplasmic membrane by hydrophobic interaction, then PHA granules accumulated and incorporated into the periplasm, a space in between of the phospholipid bilayer (Tian *et al*., 2005). Subsequently, the membrane-bounded PHA granules bud towards cytoplasm, and released inside it. Based on the first microscopy studies, it was showed that membrane-like material surrounding PHA granules either in intact cells or isolated granules supported the evidence of budding model (Boatman, 1964; Wang and Lundgren, 1969; Jensen and Sicko, 1971; Dunlop and Robards, 1973).

2.5 Properties of PHA

Naturally, PHA exist as highly amorphous and water-soluble inclusion within bacterial cell (Barnard and Sanders, 1989). A rapid crystallization occurs, when the disruption of cell to extract polymer (Hocking *et al*., 1996). The physical properties of PHA mainly depend on the composition of monomer units incorporated into the polymer chain and the molecular weight of the PHAs. Table 2.1 shows the comparison of various polymer properties.

The most well studied polymer is P(3HB) homopolymer. This is because most of the producing PHA bacteria accumulate P(3HB) in their cells, thus it makes an accessibility to study the polymer in great detail compared to mcl-PHA polymer or copolymer. P(3HB) homopolymer is stiffer, brittle and has high crystallinity properties compared to mcl-PHA. However, this homopolymer is highly viscous and and moldable at temperatures close to or above melting point. P(3HB) also has a high crystallinity properties compared to mcl-PHA. However, this homopolymer is highly

Table 2.1 Comparison of polymer properties

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	nd	nd
9 mol% 3HV	162	1.9	37	nd	nd
14 mol% 3HV	150	1.5	35	nd	nd
20 mol% 3HV	145	1.2	32	nd	nd
25 mol% 3HV	137	0.7	30	nd	nd
P(3HB-*co*-4HB)					
3 mol% 4HB	166	-	28	45	nd
10 mol% 4HB	159	-	24	242	nd
16 mol% 4HB	-	-	26	444	nd
64 mol% 4HB	50	30	17	591	nd
90 mol% 4HB	50	100	65	1080	nd
P(4HB)	53	149	104	1000	nd
P(3HHx-*co*-3HO)	61	-	10	300	nd
P(3HB-*co*-6 mol% 3HA)	133	0.2	17	680	-8
P(3HB-*co*-67 mol% HP)	44	nd	nd	nd	-19
P(3HB-*co*-3HHx)	52	nd	20	850	-4
Polypropelene	170	1.7	34.5	400	45
Polyethylene-terephalate	262	2.2	56	7300	3400
Polystyrene	110	3.1	50	-	21
LDPE	130	0.2	10	620	-30

*P(3HB) is poly(3-hydroxybutyrate), P(3HB-*co*-3HV) is poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), P(3HB-*co*-4HB) is poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate), P(4HB) is poly(4-hydroxybutyrate), P(3HHx-*co*-3HO) is poly(3-hydroxyhexanoate-*co*-3-hydroxyoctanoate), P(3HB-*co*-6 mol% 3HA) is poly(3-hydroxybutyrate-*co*-3-hydroxyalkanoate) containing 6 mol% of 3HA, P(3HB-*co*-67 mol% HP) is poly(3-hydroxybutyrate-*co*-hydroxypentanoate) containing 67 mol% of HP, P(3HB-*co*-3HHx) is poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) and LDPE is low density polyethylene.(Kunioka *et al.*, 1989; Doi *et al.*, 1990; Byrom, 1994; Saito & Doi, 1994) nd is not determined.

(Source: Khanna and Srivastava, 2005)

viscous and moldable at temperatures close to or above melting point. P(3HB) also has a high melting temperature about 177 °C similar to conventional plastic (polypropylene) (Doi *et al*., 1990). Mechanical properties of P(3HB) like Young's modulus and tensile strength are close to polypropylene, but extension to break is very low.

Mcl-PHA has much lower crystallinity and higher elasticity compared to P(3HB). It also has low melting point, low tensile strength and high elongation to be broken (Preusting *et al*., 1990). The physical properties of PHA can be improved by increasing the molecular weight or by incorporation of various hydroxyl-acids units to form PHA copolymer which is greatly needed for the industrial applications.

In P(3HB-*co*-3HV) copolymer, it is found to be less stiffer and tougher compared to P(3HB). It has higher elongation to be broken as well as its melting point decreased from 160 °C to 100 °C. The properties of this copolymer are greatly improved due to the presence of 3HV monomer units. The 3HV fraction needs to exceed at least 5 mol% in the copolymer to become an effective material (Mitsky *et al*., 1999). Glass transition temperature is reduced in order to allow them to be used at low temperature without to becoming brittle or glassy (Hartmann, 1998).

P(3HB-*co*-3HHx) copolymer exhibits lower crystallinity as compared to P(3HB) homopolymer. Incorporation of 3HHx monomer fraction into P(3HB) polymer reduces the crystallinity of the copolymer. According to Doi and co-workers, the crystallinity of P(3HB-*co*-3HHx) copolymer reduced from 60 to 18%, with the incorporation of 3HHx mol fraction increased from 0 to 25 mol% (Doi *et al*., 1995). Furthermore, P(3HB-*co*-3HHx) is a flexible material and shows a high degree of elongation to break, up to 850% with the incorporation of 17 mol% of

3HHx monomer (Doi *et al*., 1995). A good advantage of a longer chain length PHA is, that it is more ductile and easily molded (Zhao and Chen, 2007).

2.6 Applications of PHA

PHA is unique because of their biocompatibility, biodegradability and thermoplastic features. PHA has been used in a wide range of applications such as industrial, medical, pharmaceuticals, agricultural and food industries as well. In industrial applications, PHA has been used in packaging films such as bags, bottles, cosmetic containers, golf tees, paper coatings and pens (Holmes *et al*., 1981; Holmes, 1988). The usage of PHA also includes disposable items such as plastic bags, razors, drinking cups, plates, cutlery, fast-food containers and straws (Siracusa *et al*., 2008).

In agricultural applications, PHA is useful as biodegradable carriers for long term dosage of herbicides, fungicides, insecticides or fertilizers (Reddy *et al*., 2003). In medical applications, PHA can be used as osteosynthetic materials in stimulation of bone growth due to their piezoelectric properties, in bone plates, surgical sutures and blood vessel replacements . Other than that, P(3HB) also shows a high biocompatibility to various cell lines such as osteoblastic, epithelial cell and ovine chondrocytes (Knowles, 1993; Kunze *et al*., 2002; Wang *et al*., 2005; Cheng *et al*.; 2006).

2.7 Biodegradation of PHA

The most unique characteristic of PHA is its biodegradability property. PHA can be degraded intracellularly and extracellularly into water and carbon dioxide by various microorganisms in the environment. PHA biodegradation is influenced by their own properties such as crystallinity, stereoregularity, composition

and by the environmental conditions includes pH, temperature, moisture level as well as nutrient supply (Sudesh *et al*., 2000).

In aerobic condition, monomers will be used up and metabolized by the cells to yield carbon dioxide and water. In anaerobic condition, monomers will be metabolized by the cells to release methane, carbon dioxide and water into the environment.

Intracellular degradation is an active degradation, as it occurs in the bacterium which produces the polymer itself. This bacterium will hydrolyse the polymer by depolymerase enzyme in the cells and utilize it as carbon and energy sources. For the extracellular degradation, microorganisms from various environments will excrete depolymerase enzyme to hydrolyze PHA or polymers into monomers or dimers into the environment.

Some of microorganisms that are able to degrade PHA has been isolated from various ecosystems such as *Aspergillus fumigatus* (Mergaert *et al*., 1993) and *Comamonas testeroni* from soil (Kasuya *et al*., 1994), *Alcaligenes faecalis* (Kita *et al*., 1995) and *Pseudomonas fluorescens* from activated sludge, from soil, *Pseudomonas stutzeri* from lake water (Mukai *et al*., 1994) and *IIyobacter delafieldii* from anaerobic sludge (Janssen & Harfoot, 1990).

2.8 Classification of PHA synthases

PHA synthase is known to be the key enzyme of the PHA biosynthesis pathway. There are numerous of PHA synthase genes from various bacteria have been studied in detail at a molecular level (Steinbüchel & Hein, 2001). PHA synthase operon of *C. necator* was firstly cloned and characterized in three different laboratories approximately 25 years ago. PHA synthases can be classified into four

classes depending on their primary structures, subunit composition and substrate specificities as presented in Table 2.2.

Class I PHA synthase consists of one type of subunit with the molecular weight ranging from 60 to 73 kDa (Qi and Rehm, 2001). This class is represented by *Cupriavidus necator* that produces scl-PHA containing three to five carbon atoms. PHA synthase from this class favourably utilizes CoA thioesters from various (*R*)-3-hydroxyfatty acids comprise three to five carbon atoms.

Class II PHA synthase also consists of one type of subunit with the molecular weight ranging from 60 to 73 kDa. However, this class of enzyme is represented by two PHA synthases such as PhaC1and PhaC2. PHA synthases from this class is presented by *Pseudomonas oleovorans* which produces mcl-PHA containing 6 to 14 carbon atoms. This type of PHA synthases is favoured towards CoA thioesters of various (*R*)-3-hydroxyfatty acids comprise 6 to 14 carbon atoms (Rehm, 2003).

Unlike two other classes described earlier, class III PHA synthase consists of two different types of subunits namely PhaC-subunit and PhaE-subunit. Both of the subunits are approximately 40 kDa in molecular weight size and exist in large molecular mass aggregates (Müh *et al*., 1999). PhaC-subunit is found to have about 21 to 28% amino acid identity to the PHA synthases from class I and II. However, there is no similarity showed between PhaE-subunit with other classes of PHA synthases. This class III PHA synthase is represented by *Allochromatium vinosum* which produces scl-PHA containing three to five carbon atoms. This type of PHA synthase prefers CoA thioester of (*R*)-3-hydroxyfatty acids which comprise three to

Table 2.2 Classification of PHA synthase based on subunits and substrate specificities

Class	Subunits	Species	Substrate
I	PhaC  ~60-73 kDa	*Cupriavidus necator*	$3HA_{SCL}$-CoA (C3-C5) $4HA_{SCL}$-CoA $5HA_{SCL}$-CoA $3MA_{SCL}$-CoA
II	PhaC  ~60-65 kDa	*Pseudomonas oleovorans*	$3HA_{SCL}$-CoA (-C5)
III	PhaC PhaE  ~40 kDa ~40 kDa	*Allochromatium vinosum*	$3HA_{SCL}$-CoA $3HA_{MCL}$-CoA (-C6-C8) 4HA-CoA 5HA-CoA
IV	PhaC PhaR  ~40 kDa ~20 kDa	*Bacillus megaterium*	$3HA_{SCL}$-CoA

(Source: Rehm, 2003)

five carbon atoms. This type of PHA synthase prefers CoA thioester of (*R*)-3-hydroxyfatty acids which comprise three to five carbon atoms. However, class I and III PHA synthases activities are found to be different *in vitro* compared to *in vivo* (Yuan *et al*., 2001).

Class IV PHA synthase also consists of two subunits such as PhaC-subunit and PhaR-subunit with approximately 40 kDA and 20 kDA molecular weights in sizes. This class IV PHA synthase is represented by *Bacillus megaterium*. This type of PHA synthase utilizes a similar substrate range as class III PHA synthase.

2.9 Molecular organization of PHA biosynthesis genes

PHA biosynthesis genes and genes for other proteins that are involved in the metabolism of PHA frequently appear as a cluster of genes in the bacterial genome (Rehm and Steinbüchel, 2002). Regarding PHA biosynthesis genes, the most well studied microorganism is *C. necator* (Steinbüchel and Schlegel, 1991). In *C. necator*, PHA biosynthesis genes that are involved include PHA synthase (*phaC*), β-ketothiolase (*phaA*), and NADPH-dependent acetoacetyl-CoA reductase (*phaB*) arranged in a *phaCAB* operon (Peoples and Sinskey, 1989a, Peoples and Sinskey, 1989b, Schubert *et al*., 1988, Slater *et al*., 1988).

Molecular organization of PHA synthase genes can be divided into four groups based on the PHA synthase classes as discussed earlier. Figure 2.5 shows the gene organization of PHA biosynthesis genes in representative bacteria from four different classes.

Class I PHA synthase represents by *C. necator* which consists of *phaC*, *phaA* and *phaB* arranged in a single operon. *Pseudomonas oleovorans* is represented

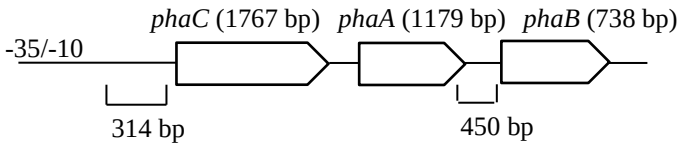
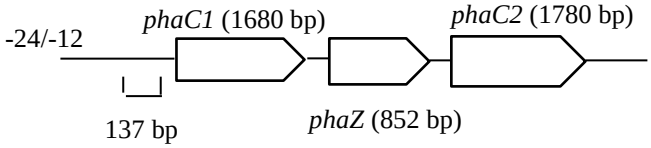
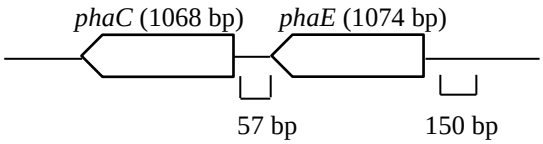
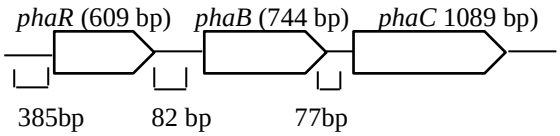
Type of PHA synthase	Molecular organization of PHA synthase
Class I *Cupriavidus necator*	
Class II *Pseudomonas oleovorans*	
Class III *Allochromatium vinosum*	
Class IV *Bacillus megaterium*	

Figure 2.5 Organization of PHA synthases genes

(Source: Rehm, 2003)

By class II synthase. This group synthase is having two *phaC* genes; *phaC1* and *phaC2*. The synthases genes are separated by *phaZ* which encodes for PHA depolymerase.

Meanwhile, for the class III synthase, *phaC* and *phaE* are found to be colocalized in a single operon (Liebergesell and Steinbüchel, 1993). For the class IV synthase, *phaC* and *phaR* is separated by *phaB* in a single operon. Based on the studies by McCool and Cannon, PhaC and PhaR are required for *in vivo* or *in vitro* activities of PHA synthase from *Bacillus megaterium* (McCool and Cannon, 2001).

2.10 Key enzymes of PHA biosynthesis

2.10.1 β-ketothiolase, PhaA

β-ketothiolase is a key regulatory enzyme that channels the direction of acetyl-CoA towards PHB biosynthesis (Oeding and Schlegel, 1973). Activity of β-ketothiolase is inhibited when the concentration of free coenzyme-A is high, thus biosynthesis of PHA is inhibited as well. The acetyl-CoA subsequently enters the tricarboxylic acid cycle to generate energy and to form amino acid under balanced conditions.

According to Pohlmann *et al*. (2006), 15 β-ketothiolases were found in the genome sequence of *C. necator* H16 including *phaA* and *bktB*. However, another 13 isologues of β-ketothiolase (*phaA*) involve in catabolic pathways based on neighbourhood genes found. The existence of the genes that encode for short-chain dehydrogenase or dehydrogenases of unknown substrate specificity and dioxygenases confirms that those clusters are involved in catabolic processes not in PHA biosynthetic pathways.

Slater *et al*. (1998) reported that β-ketothiolase (*bktB*) which is located approximately 4.5 kb downstream of *phaCAB*, is required to synthesise P(3HB-*co*-3HV) copolymer by *C. necator* when provided with appropriate carbon source. β-ketothiolase (*bktB*) is capable to form β-ketovaleryl-CoA that is driven to the accumulation β-hydroxyvalerates and the third *bktC* was also identified through genetic analysis.

2.10.2 NADPH-dependent acetoacetyl-CoA reductase, PhaBI

There are about 39 isologues of acetoacetyl-CoaA reductase PhaB found in genome *C. necator* as reported by Pohlmann *et al*. (2006). However, not all the reductases found are totally involved in PHA biosynthesis pathways. As about 17 of PhaB isologues are found on chromosome 1, while another 22 reductases are located on chromosome 2. Among three of isologues; *phaB1*, *phaB2* and *phaB3* only *phaB3* is located near to *phaP3* (encodes for phasin) that involve in PHA metabolism (Reinecke & Steinbuchel, 2009).

2.10.3 PHA synthase, PhaC

Two PHA synthases found in *C. necator*; PhaC1 and PhaC2 which are encoded by *phaC1* and *phaC2*, respectively. Both genes are located on the chromosome 1 but in different operon. *phaC2* is localized at the locus upstream to the *phaB2*, where *phaC1* located at the upstream of *phaCAB* operon. However, based on the findings from York *et al*. (2003) the second PHA synthase (PhaC2) is an inactive enzyme as it is unable to accumulate P(3HB) polymer although all the essential residues are presence.