ELUCIDATION OF PHA BIOSYNTHESIS REGULATORY ELEMENTS IN *Pseudomonas* sp. USM4-55

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ELUCIDATION OF PHA BIOSYNTHESIS REGULATORY ELEMENTS IN *Pseudomonas* sp. USM4-55

By

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LIST OF SYMBOLS AND ABBREVIATIONS

β	beta
BLAST	Basic Local Alignment Search Tool
bp	Base pair
C_T	Threshold cycle
dNTP	Deoxyribonucleic acid
et al.	*et alia* (and others)
ext	external
gm^R	Gentamycin resistant gene
hrs	hours
kDa	Kilo dalton
km^R	Kanamycin resistant gene
min	minutes
mRNA	Messenger ribonucleic acid
nm	Nanometer
PFAD	Palm fatty acid distillate
rpm	Revolution per minute
σ	sigma
sec	seconds
sm^R	Streptomycin resistant gene
sp.	species
Tn*5*	transposon
U	Unit
w/v	Weight per volume
v/v	Volume per volume

KAJIAN ELEMEN PENGAWALATUR BIOSINTESIS PHA DALAM *Pseudomonas* sp. USM4-55

ABSTRAK

PHA sintase ialah enzim penting dalam penghasilan bioplastik oleh bakteria. Untuk mengenalpasti pengawalatur bagi gen PHA sintase, perpustakaan mutan selitan bagi *Pseudomonas* sp. USM4-55 menggunakan mini-Tn*5* telah dijana. Binaan cantuman transkripsi mengandungi gen *lacZ* di dalam gen *phaC1* and *phaC2* dari *Pseudomonas* sp. USM4-55 telah digunakan dalam kajian ini. Lima mutan yang menunjukkan peningkatan pada ekspresi *phaC1* telah berjaya dipencilkan manakala tiada mutan *phaC2* dihasilkan. Selitan Tn*5*-mini ditemui pada gen yang mengkodkan protein berikut; anti-sigma faktor K, subunit protein translocase (SecB), protease bergantung ATP (Lon) dan PhaF (sejenis protein berkaitan granul PHA). Dengan menyelitkan gen gm^R, *secB* dan *lon* telah mengalami proses 'knockout' di dalam jenis liar *Pseudomonas* sp. USM4-55 dan terbitannya yang mengandungi cantuman transkripsi gen *lacZ* di dalam gen *phaC1* dan *phaC2* (*Pseudomonas* sp. USM LZC1 dan *Pseudomonas* sp. USM LZC2). Mutan $SecB^-$ dan Lon^- dalam *Pseudomonas* sp. USM LZC1 mempamerkan peningkatan ekspresi *lacZ* seperti mutan asal, menunjukkan gen ini memainkan peranan dalam pengawalaturan *phaC1*. Untuk mengkaji kadar ekspresi gen, PCR transkripsi berbalik kuantitatif-masa benar (qRT-PCR) telah dijalankan untuk mutan $SecB^-$ dan Lon^- dalam *Pseudomonas* sp. USM4-55. Tahap ekspresi gen *phaC* untuk strain ini meningkat pada fasa log manakala ia menurun pada fasa malar apabila dibandingkan dengan jenis liar. Melalui kajian fisiologi, strain $SecB^-$ menunjukkan peningkatan penghasilan PHA sebanyak 50% hingga 75% apabila dikultur di dalam medium tertakrif bersama 'Palm Fatty Acid Distillate' (PFAD) dan glukosa sebagai sumber karbon. Walaubagaimanapun, strain

Lon^{-} yang dikultur di dalam sumber karbon yang sama menunjukkan penurunan penghasilan PHA. Mutan Lon^{-} juga menunjukkan pengurangan bilangan sel pada fasa malar dalam medium tertakrif. Hasil kajian ini menunjukkan bahawa Lon memainkan peranan penting dalam pertumbuhan bakteria dan penghasilan PHA dalam *Pseudomonas* sp. USM4-55. Kesimpulannya, didapati SecB dan Lon protease terlibat dalam pengawalaturan sintesis PHA dalam *Pseudomonas* sp. USM4-55 melalui cara yang berbeza dan peranan sebenar mereka sebagai pengawalatur memerlukan kajian lanjut.

ELUCIDATION OF PHA BIOSYNTHESIS REGULATORY ELEMENTS IN *Pseudomonas* sp. USM4-55

ABSTRACT

PHA synthases are the key enzymes for bioplastic production in bacteria. To determine regulators of PHA synthase genes, a library of insertional mutants of *Pseudomonas* sp. USM4-55 by mini-Tn*5* was generated. Transcriptional fusion constructs containing the *lacZ* within *phaC1* and *phaC2* genes of *Pseudomonas* sp. USM4-55 were used in this study. Five *phaC1* over-expressed mutants were successfully isolated and none for *phaC2*. The mini-Tn*5* insertions were found to be located on genes encoding these proteins: anti-sigma factor K, a protein translocase subunit (SecB*)*, an ATP dependent protease (Lon) and PhaF (PHA granule associated protein). By inserting a gm^R gene, the *secB* and *lon* genes were knocked out in the wild type *Pseudomonas* sp. USM 4-55 as well as its derivatives which contained a transcriptionally-fused *lacZ* gene within the *phaC1* and *phaC2* genes (*Pseudomonas* sp. USM LZC1 and *Pseudomonas* sp. USM LZC2, respectively). The $SecB^-$ and Lon^- mutants in *Pseudomonas* sp. USM LZC1 showed an over-expression of *lacZ* as similarly shown by the original mutants indicating that these genes indeed play a role in *phaC1* regulation. To study gene expression, real time qRT-PCR was performed in the $SecB^-$ and Lon^- mutants of *Pseudomonas* sp. USM4-55. The *phaC* expression levels of these mutant strains were up regulated at log phase but were down regulated at stationary phase compared to the wild type. In physiological studies, the $SecB^-$ strain increased PHA production by 50% and 75% when grown in defined medium supplemented with palm fatty acid distillate (PFAD) and glucose, respectively, as a carbon source relative to the wild type. However, the Lon^- strain grown in these carbon sources showed reduced PHA production. The Lon^- mutant also exhibited a

reduced cell density at stationary phase in the minimal medium. This result implied that Lon played an essential role in bacterial growth and PHA accumulation in *Pseudomonas* sp. USM4-55. In conclusion, it was shown that the SecB and the Lon protease are involved in the regulation of PHA synthesis in *Pseudomonas* sp. USM4-55 in different ways and their exact regulatory role is yet to be elucidated.

CHAPTER 1: GENERAL INTRODUCTION

1.1 Research background

Plastic is one of the great inventions that play an important part in our lives due to its many attractive properties including stability and resistance to degradation. Since the mass production of plastics in the 1950s, people have been depending on it without realizing the adverse effects it has on the environment. While plastics are undeniably very useful in our modern lifestyle, the uncontrolled usage of this non-biodegradable polymer will contribute to various problems including exhausted landfill areas and environmental pollution. Plastics also pose a considerable threat by choking and starving wildlife. As a result, millions of pounds were spend for yearly clean-up operation organized in many countries (Barnes *et al.*, 2009). The growing awareness on the threat of this non-degradable polymer has prompted scientists to find its replacement. The increase in the human population to more than 7 billion recently further enhanced the need for renewable and biodegradable substances as a substitute to the non-biodegradable materials to sustain our future.

The emergence of biodegradable plastics was seen as a potential solution to the problem. Among the materials that caught attention are polyhydroxyalkanoate (PHA), polylactic acid (PLA) and polybutylene succinate (PBS), a synthetic bioplastic. These polymers draw interest due to their ability to be degraded under natural environment and are good candidates to replace the conventional plastics. Among these environmentally friendly plastics, PHA is the best candidate because this biopolymer is produced by bacteria which can feed on renewable substrate which is an alternative energy source to the depleting fossil resources.

The biggest obstacle in replacing conventional plastic with PHA is the high cost of large scale production which resulted in soaring cost of degradable plastics, therefore hindering the global use of this biopolymer. To overcome the problem, many studies have been conducted to understand the behavior of PHA producing bacteria and how they regulate the production and degradation of this bioplastic in the environment. Hopefully the findings will help improve the properties, increase the production and eventually reduced the cost.

The lack of understanding on the regulation of PHA produced by bacteria is one of the obstacles that hampered the research in optimizing PHA production. Few regulators such as PhaF, PhaR and PhaP that might play roles in PHA production were previously uncovered. However, other proteins that are not directly link to PHA metabolic pathway might also play important roles in regulating PHA production. Therefore, in this study, we aim to uncover more of these regulators.

One of the most studied PHA producing bacteria is from the genus *Pseudomonas*. These bacteria produce mainly mcl-PHAs (medium chain length PHAs) and some produce both mcl-PHAs and scl-PHAs (short chain length PHAs). One of the few bacteria that can produce both types of PHAs was isolated in a soil sample taken from an oil palm plantation in Felda Tasek Chini, Pahang (Few, 2001). This bacterium was named as *Pseudomonas* sp. USM4-55 and can accumulate a blend of PHB (poly-3-hydroxybutyrate, scl-PHA) and mcl-PHA homopolymer, and a small amount of random copolymer [P(3HB-*co*-3HA)] (Sudesh *et al.*, 2004). Several researches were performed to study the synthases in this bacterium. Mcl-PHA operon (*phaC1, phaZ* and *phaC2*) was cloned and

characterized (Baharuddin, 2002) while scl-PHA operon (*phbC, phbA* and *phbB*) was also cloned and characterized (Tan *et al*., 2010). The expression of *phaC1* and *phaC*2 genes were also studied in *E. coli* (Balqis, 2007). Quantitative reverse transcription real-time PCR (qRT-PCR) have revealed that these two synthases are transcribed independently of each other and are controlled by their own promoter (Tan, 2010).

Previously, a reporter system to measure the expression of each *phaC1* and *phaC2* by integrating a promoterless *lacZ* in the middle of each gene was constructed. The two resulting strains were named as *Pseudomonas* sp. USM LZC1 and *Pseudomonas* sp. USM LZC2 which contained the *phaC1::lacZ* and *phaC2::lacZ,* respectively (Kamariah, 2007). These reporter gene infused strains provide the best candidates to study *phaC* gene regulation by looking at the expression of *lacZ*. Apart from acting as a reporter, the integration of *lacZ* has also disrupted the PHA synthase genes. Thus, these mutant strains are excellent candidates to study the role of individual *phaC* genes.

In this research, the regulation of *phaC1* and *phaC2* genes in *Pseudomonas* sp. USM4-55 was investigated. This study focused on finding new regulators that might help in the regulation of the PHA synthase genes utilizing a reporter gene, *lacZ* as an indicator. In the first stage, random mutagenesis was employed using mini-Tn*5* which disrupted certain genes and affects the expression of *phaC*::*lacZ* transcriptional fusion. The mutant strains which showed altered expression of *lacZ* gene were subjected to β-galactosidase assay to verify the LacZ activity. Genomic DNA from the mutant strains were then subjected to DNA walking process, DNA sequencing and BLAST analysis to identify the genes involved in

altering the *phaC* expression. The second stage of the research is the construction of gene insertion mutants in *Pseudomonas* sp. USM4-55, *Pseudomonas* sp. USM LZC1 and *Pseudomonas* sp. USM LZC2. The genes identified in the first part of the study were mutated in the wild type strain and its derivatives through homologous recombination process utilizing suicide plasmids.

To characterize the wild type and mutant strains, growth population study was carried out by plotting growth curves of bacteria cultured in minimal medium. Gene expression level study was performed utilizing quantitative real-time PCR (qRT-PCR) method to compare the *phaC1* and *phaC2* gene expression. Finally, the wild type and mutant strains grown in minimal medium supplemented with either PFAD or glucose as a carbon source were subjected to gas chromatography (GC) analysis to compare PHA production. Formation of PHA granules was also observed utilizing transmission electron microscopy (TEM). The approach that was taken is outlined in Figure 1.1.

1.2 Research objectives

This research was done to fulfill the following objectives:

a) To determine regulators involved in the expression of *phaC1* and *phaC2* (transcriptionally fused to the reporter gene *lacZ*) via transposon mutagenesis.
b) To perform targeted disruption of genes uncovered by the transposon random mutagenesis in *Pseudomonas* sp. USM4-55 and its derivatives.
c) To evaluate the effect of mutations on the *phaC* genes expression via qRT-PCR.
d) To assess PHA accumulation in wild type and mutants by performing GC analysis.

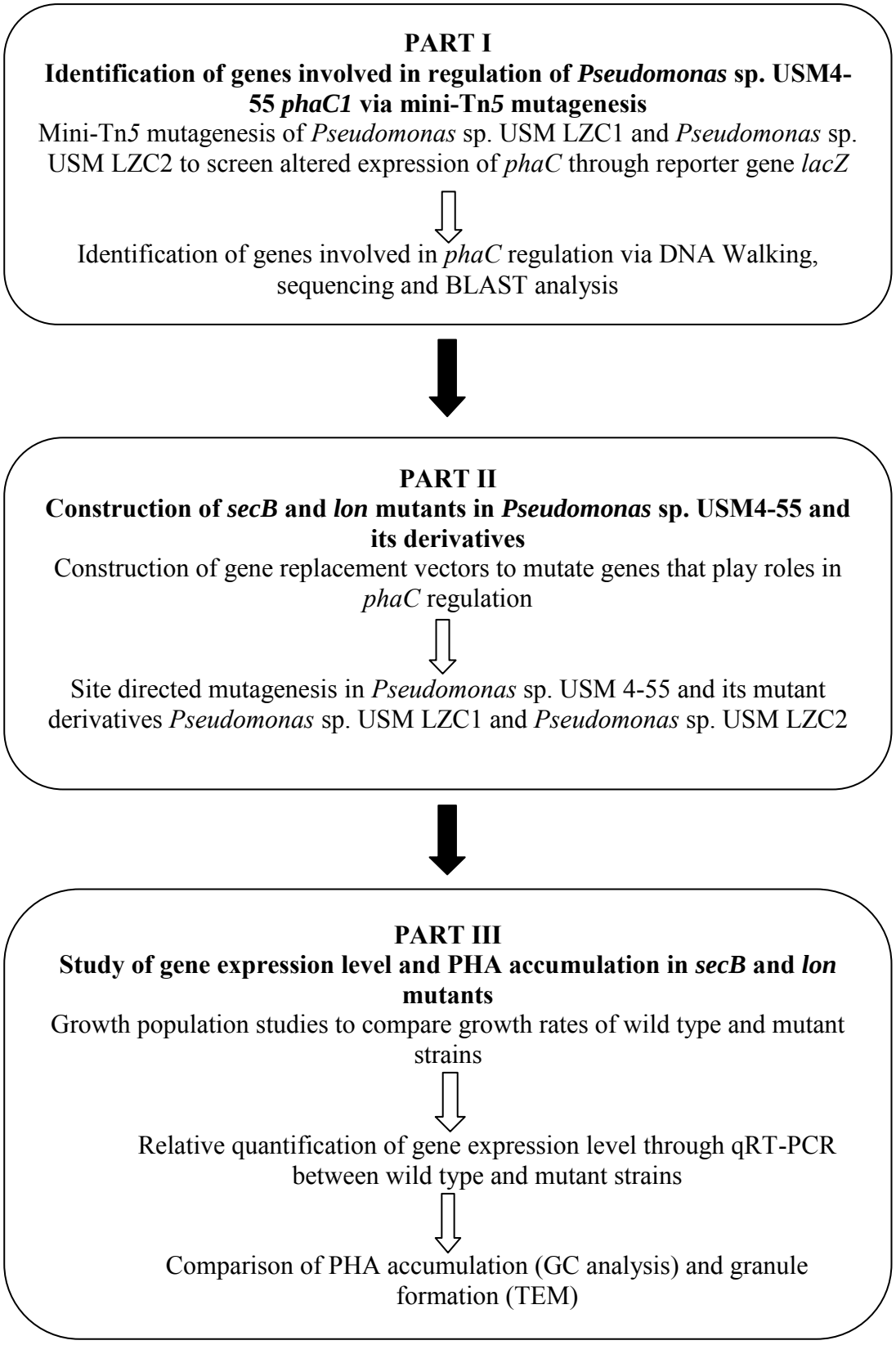


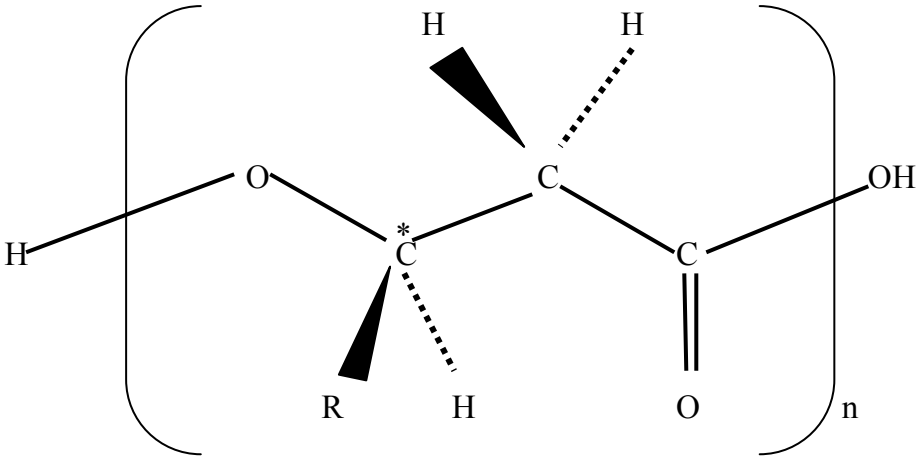
Figure 1.1 Outline and flow of thesis. Experimental approach to identify genes that play roles in the regulation of *phaC* and characterization of the mutant strains.

CHAPTER 2: LITERATURE REVIEW

2.1 Polyhydroxyalkanoates (PHAs)

Polyhydroxyalkanoates or PHAs are high molecular weight polymers synthesized by microorganisms as storage compounds during imbalanced conditions such as an excess in carbon source and limitation of other growth essential nutrients such as nitrogen or phosphorus (Anderson & Dawes, 1990). PHAs, accumulated by bacteria up to 90% of their dry cell weight, are deposited as amorphous water insoluble granules in the cytoplasm. They play a role as a sink for carbon and reducing equivalents (Madison & Huisman, 1999; Sudesh *et al.*, 2000). PHAs gained interest due to their biodegradable and biocompatible properties and hence are the best candidates to replace conventional plastics. When carbon starvation occurs, PHAs are mobilized (degraded) by PHA depolymerases (Jendrossek, 2001). Accumulation of PHAs also increases bacteria survival under stress conditions such as UV irradiation, salinity, thermal and oxidative stress, desiccation and osmotic shock (Castro-Sowinski *et al.*, 2010).

More than 150 different polyhydroxyalkanoic acids with different properties were discovered in more than 300 different microorganisms isolated from various sources including aerobic and anaerobic habitats (Kim do *et al.*, 2007). The most common chemical structure of PHA is shown in Figure 2.1. The side chain length and its functional group influence crystallinity, melting point and glass transition temperature of the polymer (Eggink *et al.*, 1995). The discovery of PHAs has prompted the need to study their structure, physical state and biological properties. The metabolic pathways involved in their biosynthesis and gene regulation are also of interest to many researchers.



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

Figure 2.1 Chemical structure of PHA. Adapted from (Zinn *et al.*, 2001) and (Ojumu *et al.*, 2004). All monomers have one chiral center (*) in the *R* position.

2.1.1 Properties of PHAs

PHAs are composed of 3-hydroxy fatty acid monomers which form linear, head to tail polyester. PHAs can exist as homopolymers, copolymers or a blend of both depending on the producers and growth substrates provided (Peoples & Sinskey, 1989). Once extracted from the cell, PHAs show crystalline and thermoplastic properties similar to polypropylenes or petrochemical-based plastic.

PHAs can be divided into a few classes according to the size of the monomers. PHAs containing up to 5 carbons are classified as short chain length PHAs (scl-PHAs) while PHAs with 6 to 14 carbons are classified as medium chain length PHAs (mcl-PHAs). Scl-PHAs have properties similar to conventional plastic albeit relatively stiff and brittle while mcl-PHAs are elastomers and sticky (Madison & Huisman, 1999). PHA copolymers, which composed primarily of HB with additional chain monomers such as HV, HH or HO, are more flexible and tough (Rai *et al.*, 2011). Homopolymers of scl-PHAs such as poly(3-hydroxybutyric acid) (PHB) have T_m values of 180°C and T_g values of 4°C. Copolymers of mcl-PHAs have T_m values of between 40 to 60°C and T_g values between -50 and -25°C. Table 2.1 demonstrates the properties of a variety of mcl-PHAs produced by pseudomonads.

Other than biodegradable, PHAs are biocompatible because their breakdown products are 3-hydroxyacids which are commonly found in animals. PHAs also exhibit piezoelectricity property which help stimulates bone growth and aid in wound healing. These characteristic of PHAs can be very useful in many medical applications for example implants, gauzes, suture filaments and as matrix material for slow release of drugs (Zinn *et al.*, 2001).

Table 2.1 Comparison of physical properties of various mcl-PHAs (adapted from Rai *et al.*, 2011).

Organism	Carbon source	M_W (kDa)	M_n (kDa)	PDI	T_g (°C)	T_m (°C)
P. oleovorans	n-hexane	333	182	1.8	-25.8	NO
	n-heptane	308	160	1.9	-30.8	38.9
	n-octane	178	99	1.8	-36.5	58.5
	n-nonane	240	131	1.8	-39.7	47.8
	n-decane	225	113	2.0	-38.4	47.6
	1-octene	242	101	2.4	-36.6	NO
P. putida GPO1	Octanoate 100%	286	118	2.4	-33.1	58.1
	Octanoate 75%	253	113	2.2	-39.5	44.5
	Octanoate 50%	290	156	1.9	-44.6	39.9
	Octanoate 25%	278	118	2.4	-47.4	NO
P. putida IPT046	Glucose and fructose	223	88	2.5	-39.7	56
P. putida	styrene	76.5	25.2	3.0	-41.7	38.1
P. aeruginosa	octanoic	316	191	1.7	ND	ND
	decanoic	251	121	2.1	ND	ND
	tetradecanoic	255	148	1.7	ND	ND
P. sp 61-3	glucose	176	40	4.4	-43	ND
P. putida	Oleic acid	135	40	2.8	-43.5	ND
	vegetable	168	65	2.7	-52	ND
P. oleovorans	Tallow free fatty acid	134	68	2.0	ND	ND
P. resinovorans	Tallow free fatty acid	157	79	2.0	ND	ND
	Tallow	192	90	2.1	ND	ND
P. citronellis	Tallow free fatty acid	125	55	2.3	ND	ND
P. putida	Tallow free fatty acid	141	71	2.0	ND	ND
P. oleovorans	octanoate	339	160	2.1	ND	ND

M_W = weight average molecular; M_n = Number average molecular weight; PDI = polydispersity index; T_g = Glass transition temperature; T_m = melting temperature; ND = not determined; NO = not observed.

2.1.2 Occurrence of PHAs

PHA granules were first observed in bacterial cells under the light microscopy as refractile bodies by Beijerinck in 1888 (reported in Chowdhurry, 1963). However, a French scientist Maurice Lemoigne was the first to chemically identify intracellular PHB granules in *Bacillus megaterium* (Braunegg *et al.*, 1998). Since then, various microorganisms such as archae and photosynthetic bacteria were found to accumulate PHB. For almost 50 years, PHB was the only known PHA until Wallen & Rohwedder (1974) discovered 3-hydroxyvalerate (3HV) and 3-hydroxyhexanoate (3HHx) in the chloroform extract of activated sludge.

PHA with longer side chains, 3-hydroxyheptanoate (3HHp) monomer was identified in *B. megaterium* (Findlay & White, 1983). Following this discovery, 3-hydroxyoctanoate (3HO) with 3HHx was discovered in *P. putida* GPo1 (De Smet *et al.*, 1983). Ten years later, a *Pseudomonas s*train GP4BH1 which accumulates a blend of PHB and mcl-PHA from various carbon sources was successfully isolated (Steinbüchel & Wiese, 1992). Another strain, *Pseudomonas* sp. 61-3 which produced a blend of PHB homopolymer and a random copolymer, P(3HB-*co*-3HA) consisting 3HA units of 4-12 carbon atoms was also isolated (Matsusaki *et al.*, 1998).

After the discovery of PHA production by bacteria, the research has shifted towards cloning and characterization of genes involved in PHA production. By the end of the 1980s the genes coding for enzymes in PHA production from *Cupriavidus necator* (formerly known as *Ralstonia eutropha*) were successfully cloned and expressed in *E. coli* (Peoples & Sinskey, 1989).

2.1.3 Biodegradation of PHAs

Unlike conventional plastics that can remain in the environment for decades, biodegradable plastics are easily degraded by a variety of bacteria within months. Degradation of PHAs occur in the absence of carbon and energy sources upon exposure to soil, compost or marine sediments (Macrae & Wilkinson, 1958). Biodegradation is dependent on factors such as microbial activities, composition of the polymer, temperature, moisture, pH and molecular weight (Boopathy, 2000). Bacteria colonize the surface of PHA polymers and degrade PHAs into carbon dioxide and water under aerobic environment and methane is produced under anaerobic environment. PHA degradation by bacteria was achieved by using their own secreted PHA hydrolases and PHA depolymerases which were found attached to the PHA granule surface (Gao *et al.*, 2001).

The types of monomer units were found to affect degradation. PHA copolymers containing the 4-hydroxybutyrate (4HB) monomers degrades more rapidly than homopolymer PHB or P(3HB-*co*-3HV) copolymers (Doi *et al.*, 1989). In *C. necator*, R-3-hydroxybutyric acid is the sole product of PHB hydrolysis but a mixture of dimers and monomers of the acid can be found in hydrolysis products of other organisms (Braunegg *et al.*, 1998). PHAs can be degraded within few months in anaerobic sewage or few years in seawater (Madison & Huisman, 1999). Lee (1996) showed that P(HB-*co*-HV) was absolutely degraded after 6, 75 and 350 weeks in anaerobic sewage, soil and seawater respectively (Lee, 1996). Apart from the environmental conditions, other factors such as composition, crystallinity, additive and surface area of PHAs can significantly affect the degradation rates.

2.1.4 Applications of PHAs

Currently, various types of PHAs are being studied for different applications ranging from medical to production of everyday use products due to their special properties of biocompatibility and biodegradability. PHAs can be used to manufacture bottles and fibres for biodegradable packaging. The molten PHA can be applied on papers and cardboards to form a water resistant layer (Hocking & Marchessault, 1994). Other than that, PHAs can also be used as a medium for slow discharge of drugs, hormones, herbicides, insecticides, flavours and fragrances. PHAs are also considered as sources for the synthesis of raw materials for paint production and enantiomerically pure chemicals (Steinbüchel & Fuchtenbusch, 1998).

Due to its high production cost, PHAs are currently more attractive for use in the medical field. The applications include implants like heart valves, stents, osteosynthetic materials and bone scaffold (Hazer & Steinbüchel, 2007). In tissue engineering, the cells are grown *in vitro* on biodegradable polymers to reconstruct tissues for implantation purposes (Zinn *et al.*, 2001). Yao and co-workers (2008) used phasins (PhaP) of *C. necator* as a tag by fusing it to the PHA nanoparticles and attached them to cell specific ligands. Flourescence microscopic examination showed that the PhaP-nanoparticles were taken up by the correct tissue, demonstrating the success of targeted drug delivery (Yao *et al.*, 2008). In agriculture, insecticides are incorporated into PHA granules and sown along with crops. By doing so, the release of insecticides into the environment can be controlled since PHA degrading bacteria can slowly degrade the PHA granules. However its application is still under study (Philip *et al.*, 2007).

2.2 Biosynthesis of PHAs

PHAs can be divided into two different classes based on the number of carbon atoms and their compositions. The first class of PHA is scl-PHAs which is represented by a classic PHB producer, *C. necator* (Peoples & Sinskey, 1989). The second class of PHA is mcl-PHAs, which is represented by pseudomonads (Rehm, 2007). Figure 2.2 summarizes the various major metabolic pathways involved in PHA biosynthesis.

2.2.1 Biosynthesis of scl-PHAs

Biosynthesis of PHB in *C. necator* is through pathway I when sugar is provided as the carbon source (Peoples & Sinskey, 1989). As shown in Figure 2.2, this pathway involved three enzymes, namely ß-ketothiolase, acetoacetyl-Coenzyme A (CoA) reductase and PHA synthase encoded by *phaA, phaB* and *phaC* respectively. The first step involves condensation of two acetyl-CoA to form acetoacetyl-CoA catalyzed by the enzyme ß-ketothiolase (PhaA). This is followed by a reduction of acetoacetyl-CoA to (*R*)-3-hydroxybutyryl-CoA by the NADPH-dependent acetoacetyl-CoA reductase (PhaB). The last step is catalyzes by the enzyme PHA synthase (PhaC) and it involves polymerization of (*R*)-3-hydroxybutyryl-CoA monomer into PHB with a release of a free CoA molecule. During normal growth condition, β-ketothiolase is inhibited by free CoA molecules coming out of the Krebs cycle. However, during imbalance nutrient condition, the entry of acetyl-CoA into the Krebs cycle is restricted. The excess acetyl-CoA is channelled into PHB biosynthesis and resulted in the accumulation of PHAs (Verlinden *et al.*, 2007).

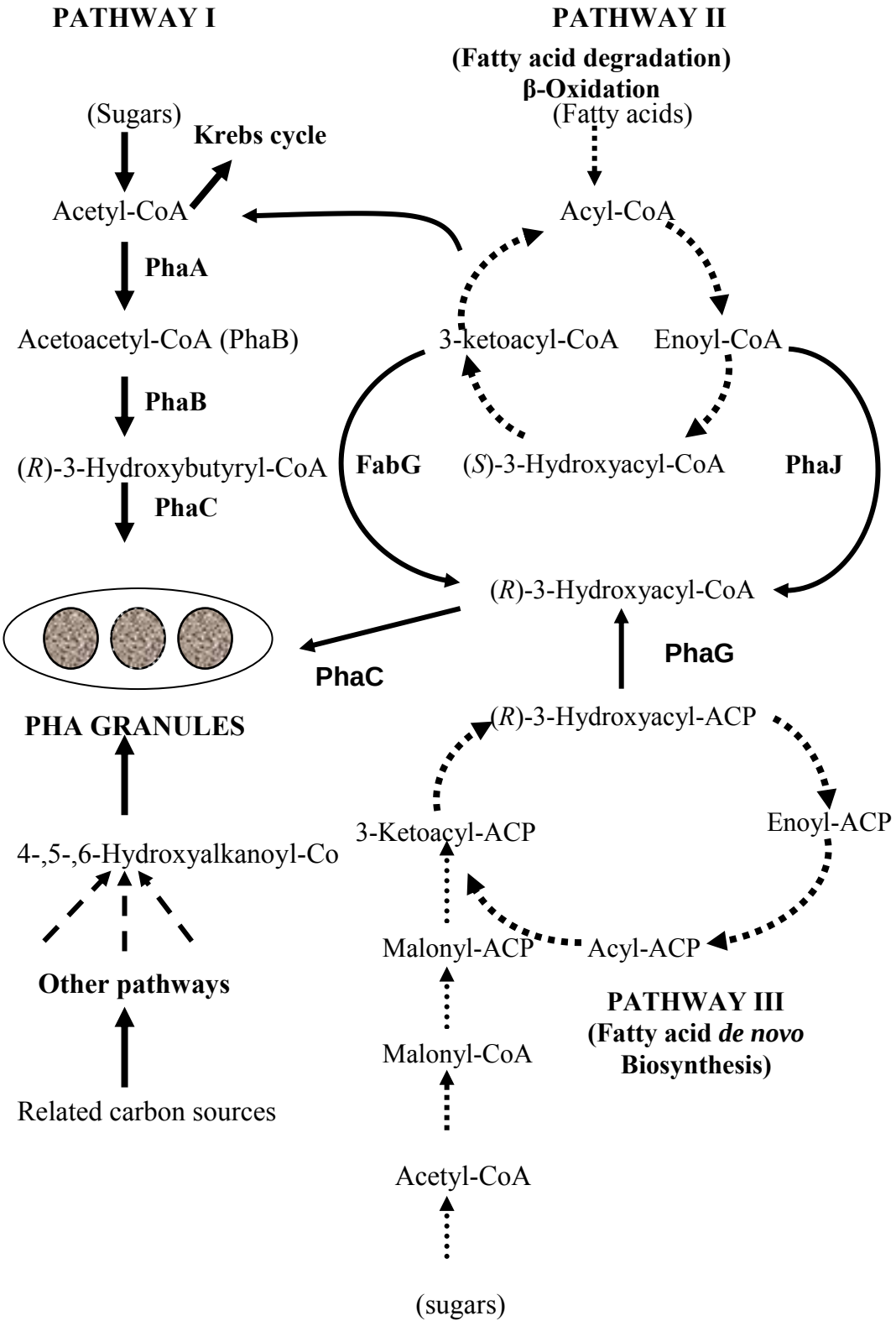


Figure 2.2: Major metabolic pathways that supply hydroxyalkanoate monomers for PHA biosynthesis. PhaA, β-ketothiolase; PhaB, NADPH-dependent acetoacetyl-CoA reductase; PhaC, PHA synthase; PhaG, 3-hydroxyacyl-ACP-CoA transferase; PhaJ (*R*)-enoyl-CoA hydratase; FabG, 3-ketoacyl-CoA reductase (adapted from Sudesh *et al.*, 2000).

2.2.2 Biosynthesis of mcl-PHAs

Pseudomonads belongs to the rRNA-homology-group I can synthesize mcl-PHAs from various alkanes, alkanols or alkanoates (Lageveen *et al.*, 1988). These bacteria can also synthesize PHA from unrelated carbon sources such as sugars which produce precursors that do not exhibit similar structure to the monomers (Anderson & Dawes, 1990). Biosynthesis of mcl-PHAs requires an additional enzyme to redirect 3-hydroxyacyl-CoA thioesters towards PHA synthase. From studies on *P. putida* KT2442, three different pathways were found to be involved in the synthesis of the 3-hydroxyal-CoA thioesters. These are fatty acid β-oxidation cycle, fatty acid *de novo* biosynthesis pathway and fatty acid chain elongation reaction. These pathways provide the intermediates for the production of mcl-PHAs.

2.2.2.1 Fatty acid β-oxidation cycle

When fatty acids are provided as the carbon sources, Pathway II which involves the fatty acid β-oxidation cycle generates substrates, 3-hydroxyacyl CoA that can be polymerised by the PHA synthase of pseudomonads such as *P. putida*. The length of monomers produced via this pathway is similar to the substrates or shortened by 2, 4 or 6 carbon atoms (Lageveen *et al.*, 1988). The intermediates of fatty acid β-oxidation cycle includes enoyl-CoA, 3-ketoacyl-CoA and (S)-3-hydroxyacyl-CoA which is used directly in mcl-PHA synthesis. In this pathway, fatty acids are activated by the acyl-CoA synthetase complex (FadD) forming the corresponding acyl-CoA thioesters. This molecule then enters the β-oxidation cycle where it is shortened by the removal of 2 carbon atoms in the form of acetyl

CoA. The shortened acyl-CoA is then reduced to trans-2, 3-enoyl-CoA catalyze by acyl-CoA dehydrogenase (FadE).

2.2.2.2 Fatty acid *de novo* biosynthesis pathway

The fatty acid *de novo* pathway is the most important pathway that produces monomers of 3-hydroxyacyl CoA for PHA synthesis as shown in pathway III in Figure 2.2. Simple carbon sources such as glucose, ethanol and acetate can be used as the starting materials (Huijberts *et al.*, 1994). *P. putida* and *P. aeruginosa* produce copolyesters with 3-hydroxydecanoic acid as the main constituent and other 3HAs (3-hydroxyalkanoates) as minor constituent from glucose via this pathway (Steinbüchel & Fuchtenbusch, 1998). The (*R*)-3-hydroxyacyl intermediates from the fatty acid biosynthetic pathway are transferred from their acyl carrier protein (ACP) to CoA form by acyl-ACP-CoA transacylase (PhaG) (Rehm *et al.*, 1998). This enzyme is the key connection between fatty acid synthesis and PHA biosynthesis and was identified in *P. putida.*

2.2.2.3 Chain elongation reaction pathway

The chain elongation pathway takes place during all types of PHA biosynthesis. In this pathway, acetyl-CoA molecules are condensed to 3-hydroxyacyl-CoA. Ketoacyl-CoA was formed by addition of acyl-CoA to acetyl-CoA. Ketoacyl-CoA was then converted to (*R*)-3-OH-acyl-CoA by the reaction of ketoacyl-CoA reductase (Hoffmann & Rehm, 2004). PHAs accumulated from sodium octanoate are generated via chain elongation reaction catalyzed by thiolase (Huijberts *et al.*, 1994).

2.3 PHA Granules

PHA granules are found as insoluble spherical inclusions in the cytoplasm with hydrophobic PHA polyester as the core. Embedded proteins such as PHA synthases, phasins, depolymerising enzymes and regulatory proteins are present on the lipid monolayer surface (Grage *et al.*, 2009). The size and molecular mass of PHA granules vary between organisms depending on carbon source provided, types of PHA synthase and the biosynthesis pathway involved. The sizes of the PHA molecules are about 50 to 1000 kDa with diameters of between 100 to 500 nm (Steinbüchel *et al.*, 1995). These granules consist of about 10^3 to 10^4 monomers (Luengo *et al.*, 2003). The number of granules per cell depends on the bacteria. For example, *C. necator* accumulates 8 to 12 granules per cell while *P. oleovorans* generates only one or two large granules per cell (Klinke *et al.*, 2000).

2.3.1 Structure of PHA granules

An electron microscopy study has shown that the core of PHA granules is surrounded by a 4 nm boundary layer, comprising a phospholipid monolayer (Mayer & Hoppert, 1997). Wide angle X-ray scattering showed that PHA granules are amorphous and treatments that removed lipid components can initiate crystallization (Kawaguchi & Doi, 1992). Analysis of PHA granules by atomic force microscopy (AFM) revealed an extra network layer with globular areas that incorporate structural proteins such as phasins (PhaP) (Dennis *et al.*, 2008). The AFM also showed porin-like structures in the surrounding membrane and these were suggested to provide a portal to the polymer core for PHA metabolism and depolymerisation to occur (Dennis *et al.*, 2003).

2.3.2 Formation of PHA granules

Two models for the PHA granule formation was described: the micelle model and the budding model as shown in Figure 2.3. Both models consider the location of the PHA synthase and phasin proteins on the surface of the granule. The micelle model supported PHA granule formation *in vitro* and without membranes. This model assumes that once the first PHA chains have been synthesized, the polymer molecules aggregate to form nascent small granules by hydrophobic interaction. PHA synthase binds to the surface of PHA granules and becomes insoluble while phasin and other PHA specific proteins (PhaZ and PhaR) are also attached to the growing surface of the granules (Tian *et al*., 2005).

The budding model presumes that PHA synthase is linked to the cytoplasmic membrane. At the early stage, granules are close to the inner cell membrane and are not distributed in the cytoplasm (Tian *et al.*, 2005). Electron microscopy studies showing membrane-like material surrounding PHA granules provided evidence for this model (Lundgren *et al.*, 1964). *In vivo* studies using GFP-labeled PHA synthase from *P. aeruginosa* also supported the budding model by localizing granule formation close to the cytoplasmic membrane at the cell poles (Peters & Rehm, 2005). The budding model as shown in *P. aeruginosa*, involves four steps. First, PHA synthase was attached to the inner surface of the cytoplasmic membrane. This was followed by the formation of oligomers that bind to the polymerase in the inner surface of the cytoplasmic membrane. Then, polymer elongation takes place between the phospholipid monolayer of the membrane. This environment is hydrophobic. Finally, granule associated proteins (phasins) direct the formation of the budding granules (Rehm, 2006).

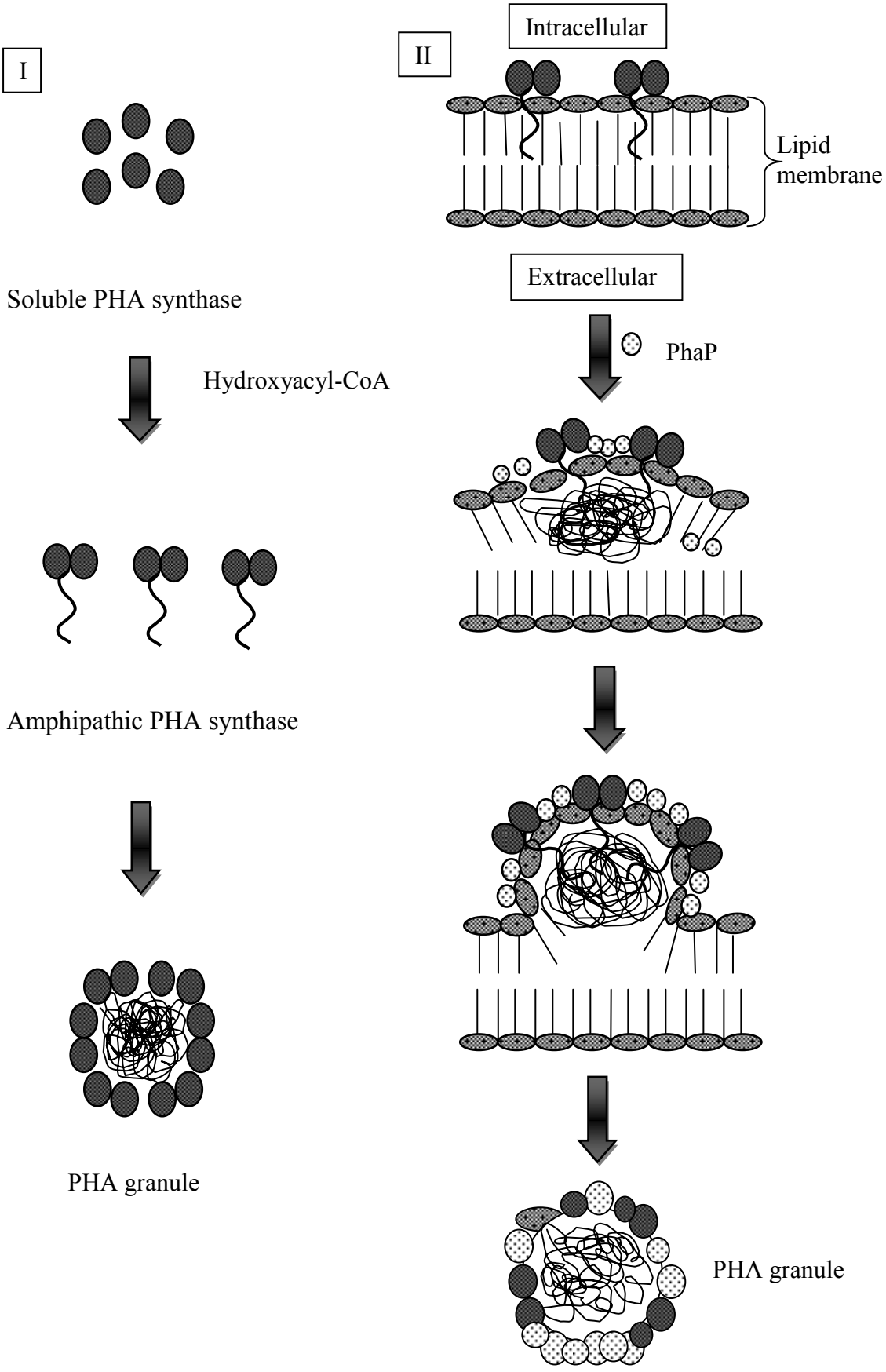


Figure 2.3 PHA granule formation models. (I) Micelle model representing *in vitro* formation in the absence of membranes and (II) Budding model representing granule formation at the cytoplasmic membrane (adapted from Tian *et al*., 2005).

2.3.2.1 Formation of PHA granules in *Pseudomonas* sp. USM4-55

Pseudomonas sp. USM4-55 is capable of producing a blend of PHB homopolymer and copolymer of mcl-PHAs that contains a small amount of PHB monomers. The copolymers are inseparable by solvent fractionation. The PHA granules of *Pseudomonas* sp. USM4-55 have been isolated and studied in detail (Sudesh *et al.*, 2004). In this bacterium, PHB and mcl-PHA exist in the same cell but are separated in different granules. The micelle and budding models cannot be used to explain why PHB chain and mcl-PHA growing from different PHA synthases are segregated into separate granules. Therefore, a different mechanism was adopted to describe the granule formation event in this bacterium (Sudesh *et al.*, 2004). Two mechanisms were proposed for the granule formation in *Pseudomonas* sp. USM4-55 as shown in Figure 2.4. In the first mechanism, two types of PHAs are segregated into different granules due to phasin proteins that can interact directly with the hydrophobic PHA chains (Sudesh *et al.*, 2004). The presence of mcl-PHA in a polymer chain of mostly 3HB monomers resulted in unstable granule (Sudesh *et al.*, 2002). Therefore segregation of PHB and mcl-PHA into separate granules is essential.

The second mechanism demonstrated that the formation of the PHA synthase complex is due to the observation of a lag phase in the polymerization reaction. Enzymes with the same substrate specificity will form a complex with each other and thus each of PHA synthase enzymes will generate one granule (Sudesh *et al.*, 2004). Mechanism II offers a straightforward description for the separation of PHB and mcl-PHA into different granules in *Pseudomonas* sp. USM4-55.

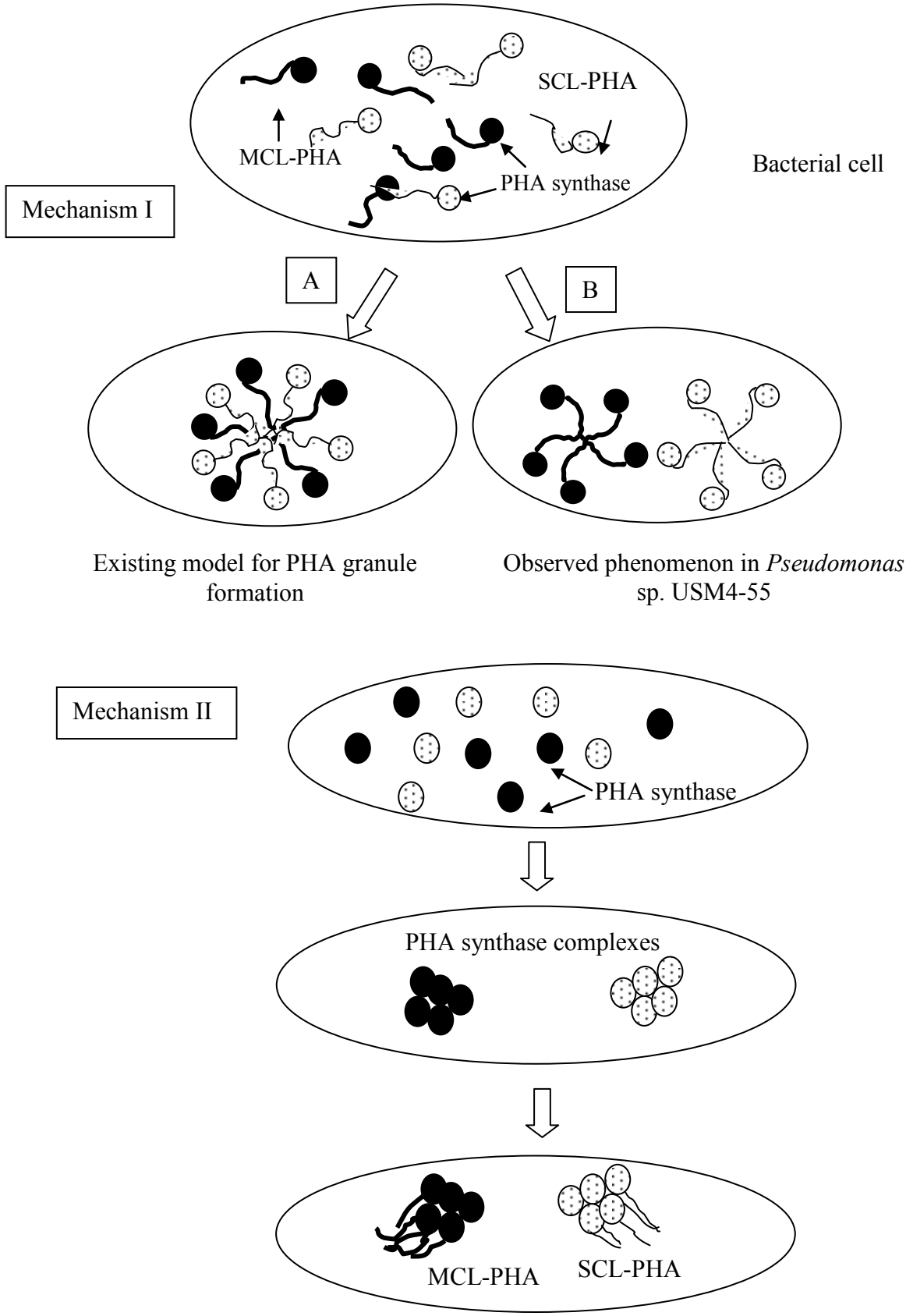


Figure 2.4 Predicted granule formation model in *Pseudomonas* sp. USM4-55 (adapted from Sudesh *et al.*, 2004). White spheres represent scl-PHA synthase while black spheres represent mcl-PHA synthase.

2.4 PHA synthases

PHA synthase is the key enzyme of PHA biosynthesis and determines the type of PHA synthesized. This enzyme catalyzes the polymerization of hydroxyacyl-CoA to PHA and free CoA. PHA synthase enzyme shows a wide range of substrate specificity and hence a variety of monomers can be polymerised.

PHA synthases from a variety of bacteria were cloned and the alignment of the primary structures showed an overall identity of 21-88% with 8 conserved amino acid residues (Amara & Rehm, 2003). All PHA synthases share a conserved cysteine at the catalytic active site to which the growing PHA is covalently bonded. These enzymes are mainly composed of variable loops (49.7%) and α-helical (39.9%) secondary structures, while 10.4% are predicted as β-sheet secondary structures (Cuff & Barton, 2000). PHA synthases are attached to the surfaces of PHA granules in PHA accumulating cells as shown by immunogold labelling in electron microscopy and *in vivo* analysis using PhaC-GFP fusion (Mayer *et al.*, 1996; McCool & Cannon, 1999).

2.4.1 Classification of PHA synthases

PHA synthases can be divided into four classes based on their primary structure, substrate specificity and subunit composition as shown in Table 2.2(A). Class I PHA synthases consist of one type of subunit (PhaC) with sizes of 61 kDa to 73 kDa. Synthases in this class prefer to utilize CoA thiosters of various (R)-3-hydroxy fatty acids comprising 3 to 5 carbon atoms (Qi & Rehm, 2001). The PHA synthase in *C. necator* is an example of a Class I synthase. However, *in vivo*

studies of *C. necator* PHA synthase in recombinant *E. coli* showed that this enzyme also accepts mcl 3-hydroxy fatty acid-CoA thioesters as substrate (Antonio *et al.*, 2000).

Class II PHA synthases also comprise of one type of subunit which utilizes CoA thioesters of various (*R*)-3-hydroxy fatty acids comprising 6 to 14 carbon atoms (Amara & Rehm, 2003). Class II synthases synthesize smaller PHAs with sizes up to 500 kDa while Class I synthases synthesize PHA bigger than 500 kDa (Rehm & Steinbüchel, 1999). The PHA synthases in *P. putida* is an example of Class II synthases.

Class III PHA synthases represent enzymes with two different subunits of PhaC and PhaE with a size of 40 kDa each. These synthases prefer CoA thioester of (R)-3-hydroxy fatty acids of 3 to 5 carbon atoms (Liebergesell *et al.*, 1992). The PHA synthase in *Allochromatium vinosum* is an example of the Class III synthase. PhaC subunit from *A. vinosum* exhibit low (21% to 28%) but significant sequence similarity with Class I and Class II synthases and shared highly conserved amino acid positions, albeit smaller than the other synthases. However, PhaE does not exhibit any significant homology with other synthases. Aside from *A. vinosum*, PHA synthase from phototrophic purple sulphur bacteria and cyanobacteria are also classified as Class III PHA synthases (Steinbüchel & Hein, 2001).

Class IV PHA synthase is represented by *B. megaterium.* This synthase resembles Class III synthases but PhaE is substituted by PhaR. PhaR has the size of 20 kDa. *B. megaterium* required both PhaC and PhaR for activity *in vivo* and *in vitro* (McCool & Cannon, 2001).

There are other synthases that do not belong in any class. *Thiocapsa pfennigii* synthase possesses two different subunits with strong similarity (85%) to PhaC from Class III synthases. This PHA synthase is characterized by broad substrate specificity comprising CoA thioesters of 3 to 14 carbon atoms. This synthase catalyzes the formation of copolyester PHB and P(3HHX) (Rehm, 2003).

2.4.1.1 PHA synthases in pseudomonads (Class II)

Class II PHA synthases (PhaC1 and PhaC2) are primarily found in pseudomonads and they prefer mcl-3HAs containing 6 to 14 carbons as substrates (Huisman *et al.*, 1991). The variation between *phaC1* and *phaC2* sequences may signify the structural and functional differences (Zhang *et al.*, 2001). A study on *phaC1* and *phaC2* knockout mutant strains in *P. mendocina* revealed that PhaC1 is the major enzyme for PHA synthesis while PhaC2 contribute to the PHA accumulation only when cells are cultivated in gluconate (Hein *et al.*, 2002). PhaC2 could not replace PhaC1 very effectively and resulted in a reduced ability of *phaC1* mutant strain to form PHA from gluconate and no PHA was accumulated from fatty acids. Disruption of *phaC2* did not show a serious effect. The same phenomenon was also demonstrated in *Pseudomonas* sp. USM4-55 and this lead to the conclusion that PhaC1 is the major PHA synthase in pseudomonads (Kamariah, 2007).

Few pseudomonads exhibit broader substrate specificity as shown by *Pseudomonas* sp. 61-3 and *Pseudomonas* sp. USM4-55 (Abe *et al.*, 1994; Sudesh *et al.*, 2004). These strains are able to polymerize both scl-PHAs and mcl-PHAs.