POLYHYDROXYALKANOATES AS RESERVE MATERIALS FOR SURVIVAL DURING NUTRIENT STARVATION AND ENHANCED TOLERANCE TOWARDS OXIDATIVE STRESS

by

GOH LAY KOON

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Goh Lay Koon
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LIST OF SYMBOLS AND ABBREVIATIONS

%	percentage
°C	degree Celsius
3HA	3-hydroxyalkanoate
3HB	3-hydroxybutyrate
3HB-CoA	D(–)-3-hydroxybutyryl-coenzyme-A
3HHx	3-hydroxyhexanoate
3HV	3-hydroxyvalerate
3HV-CoA	D(–)-3-hydroxyvaleryl-coenzyme-A
4HB	4-hydroxybutyrate
AtoDA	acetoacetyl-CoA transferase
ATP	adenosine triphosphate
$CaCl_2$	calcium chloride
CFU	colonies forming unit
cm	centimetre
CME	caprylic acid methyl ester
CoA	coenzyme-A
$CoSO_4$	cobalt sulphate
$CuCl_2$	copper (II) chloride
$FeSO_4$	ferrum sulphate
FID	flame ionisation detector
g	gram
$\times g$	times gravity
GC	gas chromatography
h	hour
HCl	hydrochloric acid
H_2O_2	hydrogen peroxide
KH_2PO_4	potassium dihydrogen phosphate
K_2HPO_4	dipotassium hydrogen phosphate
kPa	kilopascal
L	litre

LB	Luria-Bertani
M	molar
MCL	medium-chain-length
mg	milligram
$MgSO_4$	magnesium sulphate
min	minute
mL	millilitre
mm	millimetre
$MnCl_2$	mangan chloride
mol%	mol percentage
µg	microgram
µL	microlitre
µm	micrometre
NA	nutrient agar
NaCl	sodium chloride
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NaOH	sodium hydroxide
NB	nutrient broth
nm	nanometre
$(NH_4)_2SO_4$	ammonium sulphate
O_2^-	superoxide anion
OD	optical density
OH•	hydroxyl radicals
OsO_4	osmium tetroxide
P(3HB)	poly(3-hydroxybutyrate)
P(3HB-*co*-3HV)	poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)
P(3HB-*co*-3HHx)	poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate)
P(3HB-*co*-4HB)	poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate)
P(3HV)	Poly(3-hydroxyvalerate)

PHA	polyhydroxyalkanoate
PhaP	phasin
PhaR	repressor of phasin
PhaZ	intracellular depolymerase
PHO	polyhydroxyoctanoate
ppGpp	guanosine tetraphosphate
ROS	reactive oxygen species
rpm	revolution per minute
RpoS	the effector molecule of the stringent response
SCL	short-chain-length
SPL	split injection unit
sp.	species
TEM	transmission electron microscope
TiO_2	titanium dioxide
UV	ultraviolet
UVA	ultraviolet A
vol	volume
W	watt
wt	weight
wt%	weight percentage
$ZnSO_4$	zinc sulfate
β	beta

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POLYHYDROXYALKANOATES AS RESERVE MATERIALS FOR SURVIVAL DURING NUTRIENT STARVATION AND ENHANCED TOLERANCE TOWARDS OXIDATIVE STRESS

ABSTRACT

Delftia acidovorans DS 17 was used to study the roles of polyhydroxyalkanoates (PHAs) in enhancing starvation survival and oxidative stress tolerance of the cells. *D. acidovorans* containing poly(3-hydroxybutyrate) [P(3HB)] or poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] with various 3HV compositions (11 mol%, 40 mol% and 94 mol%) were transferred into carbon-free mineral medium supplemented with 1% (wt/vol) ammonium sulphate. The bacterium was able to survive from nutrient starvation (carbon-free conditions with excess nitrogen) by mobilizing the intracellular PHAs. Surprisingly, *D. acidovorans* containing P(3HB-*co*-94 mol% 3HV) was also able to survive from starvation although the mobilization was not effective. In addition, this study showed that *D. acidovorans* containing PHAs was slightly more resistant to oxidative stress compared to cells without PHAs. In order to obtain further evidence, cell suspension of *Escherichia coli* pGEM-T::*phbCAB*$_{Cn}$ with and without P(3HB) granules were subjected to oxidative stress generated by activated titanium dioxide. *E. coli* pGEM-T::*phbCAB*$_{Cn}$ containing P(3HB) granules showed higher survival ratios as compared to cells without P(3HB) granules although mobilization of P(3HB) did not occur in the former cells. This study shows that, besides being a carbon and energy storage compound, the accumulation of PHA improves bacterial survival via multiple mechanisms.

POLIHIDROKSIALKANOAT SEBAGAI BAHAN SIMPANAN BAGI KETAHANAN SEMASA KEHABISAN NUTRIEN DAN PENINGKATAN TOLERANSI TERHADAP TEKANAN OKSIDATIF

ABSTRAK

Delftia acidovorans DS 17 digunakan untuk mengkaji peranan polihidroksialkanoat (PHA) dalam peningkatan kemandirian sel semasa kehabisan nutrien dan toleransi terhadap tekanan oksidatif. *D. acidovorans* yang mengandungi poli(3-hidroksibutirat) [P(3HB)] atau poli(3-hidroksibutirat-*ko*-3-hidroksivalerat) [P(3HB-*ko*-3HV)] dengan pelbagai komposisi 3HV (11 mol%, 40 mol% dan 94 mol%) dipindahkan ke dalam media mineral (tanpa sumber karbon) dengan 1% (b/i) ammonium sulfat. Bakteria ini berupaya untuk terus hidup semasa kehabisan nutrien (tanpa sumber carbon tetapi dengan kehadiran nitrogen) dengan memobilisasikan PHA intrasel. Akan tetapi, *D. acidovorans* yang mengandungi P(3HB-*ko*-94 mol% 3HV) juga berupaya untuk terus hidup walaupun proses mobilisasi tidak berkesan. Tambahan lagi, kajian ini menunjukkan *D. acidovorans* yang mengandungi PHA menunjukkan daya toleransi yang lebih tinggi terhadap tekanan oksidatif berbanding dengan sel yang tidak mengandungi PHA. Untuk mendapatkan penjelasan yang lebih jelas, ampaian *Escherichia coli* pGEM-T::$phbCAB_{Cn}$ dengan atau tanpa P(3HB) didedahkan kepada tekanan oksidatif yang dihasilkan oleh titanium dioksida yang aktif. *E. coli* pGEM-T::$phbCAB_{Cn}$ yang mengandungi granul P(3HB) menunjukkan nisbah kemandirian yang lebih tinggi berbanding dengan sel-sel tanpa granul P(3HB) walaupun proses mobilisasi P(3HB) tidak berlaku di dalam sel-sel ini. Selain bertindak sebagai karbon dan bahan simpanan tenaga, kajian

ini menunjukkan bahawa pengumpulan PHA meningkatkan kemandirian bakteria melalui pelbagai mekanisme.

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are accumulated as granules in the cytoplasm of numerous Gram positive and Gram negative bacteria when nitrogen is limited but carbon source is in excess (Yu, 2007; Khanna and Srivastava, 2005; Sudesh *et al.*, 2000). Poly(3-hydroxybutyrate) [P(3HB)] homopolymer is the most common type of polymer in PHAs family. Besides that, bacteria can also accumulate PHA copolymers. These water insoluble granules have long been described as carbon and energy reserve materials. During the absence of carbon source, mobilization (hydrolysis) of these previously accumulated storage materials is essential for continuous survival. P(3HB) will be broken down (during mobilization) to form acetyl-CoA (Sudesh *et al.*, 2000). The mobilized products are then oxidized via tricarboxylic acid cycle to synthesize adenosine triphosphate (ATP) which would enhance the survival and resistance of bacteria to stressful environmens (Ruiz *et al.*, 2001).

Previous studies on mobilization of P(3HB) had been done on several bacterial strains such as *Cupriavidus necator* (formerly known as *Wautersia eutropha*; *Ralstonia eutropha*) (Handrick *et al.*, 2000), *Legionella pneumophila* (James *et al.*, 1999) and some *Pseudomonas* sp. (Ayub *et al.*, 2004; Ruiz *et al.*, 2001). These studies showed that the mobilization of P(3HB) is important to improve survival during carbon starvation. Besides that, the consumption of accumulated P(3HB) also induced the synthesis of guanosine tetraphosphate (ppGpp) and RpoS (a starvation or stationary phase sigma factor) molecules and hence, improved the resistance towards stress agents such as ethanol and

heat (Ayub *et al.*, 2004; Ruiz *et al.*, 2001). A recent study by Zhao and coworkers proposed that the biosynthesis of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] with the presence of RpoS increased the survival of *Aeromonas hydrophila* under various stress conditions (Zhao *et al.*, 2007). This is because the biosynthesis and mobilization of PHA are active processes which proceed at the same time (Uchino *et al.*, 2007; Uchino and Saito, 2006; Yan *et al.*, 2000).

Study of PHAs mobilization is important especially for process optimization during the production of PHAs. However, little is known about mobilization of copolymers. On the other hand, although there are increasing number of studies on the advantages of P(3HB) mobilization for enhancing tolerance towards stress and starvation, studies on the contributions of PHA copolymers are still limited. Besides that, the role of unmobilized intracellular granules in conferring protection to cells remains unknown. Thus, the objectives of this study are:

(i) to study the effect of carbon sources as inducer for PHA biosynthesis

(ii) to investigate the mobilization effect of P(3HB) and P(3HB-*co*-3HV) with different 3HV compositions in *Delftia acidovorans*

(iii) to determine the effect of mobilization of P(3HB) an d P(3HB-*co*-3HV) on stress resistance in *D. acidovorans*

(iv) to determine the effect of P(3HB) accumulation on enhancement of tolerance towards oxidative stress in *Escherichia coli* pGEM-T::*phbCAB*$_{Cn}$.

In this study, *D. acidovorans* was chosen as it is able to synthesize P(3HB-*co*-3HV) with various 3HV compositions by controlling the concentrations of carbon sources provided to the cultures. In addition, *E. coli* pGEM-T::*phbCAB*$_{Cn}$ which harbors P(3HB) synthetic genes from *C. necator* was used in this study because this recombinant is unable to mobilize the previously synthesized granules (Uchino *et al.*, 2007; Wang and Lee, 1998; Lee, 1996). TiO_2 was used as source of oxidative stress as this chemical had been widely studied recently as a potential and safe bactericide (Fujishima and Zhang, 2006).

LITERATURE REVIEW

2.1 Bacterial inclusions

Bacteria are capable of forming various kinds of intracellular inclusions (Shively, 1974). Bacterial inclusions are dense aggregates of chemical compounds which is synthesized and stored in the bacterial cells. In general, these chemical reservoirs serve as energy-rich compounds and building blocks for bacteria. Some examples of bacterial inclusions are glycogen, polyphosphate, elemental sulfur, magnetosome and polyhydroxyalkanoates (PHAs).

2.1.1 Glycogen

Glycogen is a starch-like polymer which is made up of glucose monomers (Madigan *et al.*, 2000). This granule is synthesized by, for example, *Saccharomyces cerevisiae* not only as carbon and energy source but also responsible for stress protection such as heat shock and osmotic stress (Guillou *et al.*, 2004; Francois and Parrou, 2001; Parrou *et al.*, 1997). This is because accumulation of glycogen is, usually, induced by these stress agents. Glycogen granules can be observed under electron microscope but the sizes are usually smaller than PHAs granules (Madigan *et al.*, 2000).

2.1.2 Polyphosphate

Various bacteria and cyanobacteria are able to accumulate polyphosphate (Madigan *et al.*, 2000). Polyphosphate appears as dark, electron

dense granules under electron microscope (Schönborn *et al.*, 2001). Polyphosphate is a reserve of inorganic phosphate, usually a limiting nutrient in natural environments (Madigan *et al.*, 2000). Besides that, polyphosphate also serves as substitute for adenosine triphosphate (ATP), intracellular pH regulator and also component of cell capsule.

2.1.3 Elemental sulfur

Elemental sulfurs are granules accumulated in certain prokaryotes including purple sulfur bacterium (Friedrich *et al.*, 2001). Elemental sulfurs are synthesized from oxidization of sulfur components such as hydrogen sulfide and thiosulfate. These granules are then stored intracellulary. Oxidization of elemental sulfur is essential for attainment of energy by purple sulfur bacteria (Madigan *et al.*, 2000).

2.1.4 Magnetosome

Magnetosomes are intracellular crystals consisting of iron magnetite (Fe_3O_4) (Keim *et al.*, 2005; Schüler, 2002). Some prokaryotic cells, such as *Magnetospirillum gryphiswaldense*, are able to accumulate this storage material. The accumulation of magnetosomes enables these microbes to orient themselves in a magnetic field.

2.1.5 Polyhydroxyalkanoates (PHAs)

PHAs are one of the most common storage inclusions in bacteria. A wide variety of prokaryotes, including Gram-positive and Gram-negative bacteria, are able to synthesize PHAs from various carbon sources (Khanna and Srivastava, 2005; Sudesh *et al.*, 2000; Anderson and Dawes, 1990). Cyanobacteria are also able to accumulate PHA granules (Jau *et al.*, 2005; Sudesh *et al.*, 2002; Asada *et al.*, 1999). The occurrence, metabolism and roles of PHAs in bacteria are discussed in the following sub-topics.

2.2 Occurrence of PHAs

2.2.1 Chemical structure

Most of the PHAs that were discovered are 3-hydroxyalkanoates (3HA) (Steinbüchel and Valentin, 1995). There are close to 150 different constituents of PHAs which had been identified. Poly(3-hydroxybutyrate) [P(3HB)] is the most common member of PHAs (Tokiwa and Ugwu, 2007; Reddy *et al.*, 2003; Sudesh *et al.*, 2000).The general structure of PHA is as shown in Fig. 2.1.

In general, PHAs are divided into two groups according to the number of carbon atoms in the monomer units. They are short-chain-length (SCL) PHAs and medium-chain-length (MCL) PHAs (Yu, 2007; Sudesh *et al.*, 2000). Monomers of SCL PHAs contain 3-5 carbon atoms while that of MCL PHAs contain 6-14 carbon atoms.

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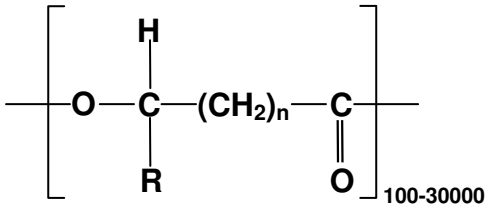

n=1	R=hydrogen	Poly(3-hydroxypropionate)
	R=methyl	Poly(3-hydroxybutyrate)
	R=ethyl	Poly(3-hydroxyvalerate)
	R=propyl	Poly(3-hydroxyhexanoate)
	R=pentyl	Poly(3-hydroxyoctanoate)
	R=nonyl	Poly(3-hydroxydodecanoate)
n=2	R=hydrogen	Poly(4-hydroxybutyrate)
n=3	R=hydrogen	Poly(5-hydroxyvalerate)

Fig. 2.1 General chemical structure of polyhydroxyalkanoates (Yu, 2007; Sudesh *et al.*, 2000; Lee, 1996; Doi, 1990).

2.2.2 Biosynthesis of PHAs

2.2.2.1 Conditions favorable for biosynthesis of PHAs

In natural environments, bacteria are always exposed to fluxes of nutrients including excess, sub-optimal amounts or even the complete absence of substrates (Dawes, 1985). When essential nutrients such as nitrogen, phosphorus, magnesium or oxygen are limited or absent, bacteria will collect the excess carbon sources from their environments and store them in the form of PHA granules (Yu, 2007; Egli and Zinn, 2003; Sudesh *et al.*, 2000; Anderson and Dawes, 1990).

2.2.2.2 Pathway of P(3HB) and P(3HB-*co*-3HV) biosynthesis

In general, P(3HB) are synthesized from acetyl coenzyme A (CoA) through a three-step enzymatic reaction as shown in Fig 2.2 (Yu, 2007; Sudesh *et al.*, 2000; Lee, 1996; Doi, 1990). The first step is a condensation reaction where β-ketothiolase catalyses the formation of acetoacetyl-CoA from two acetyl-CoA molecules. With the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH), this intermediate is then reduced by acetoacetyl-CoA reductase to form D(–)-3-hydroxybutyryl-CoA (3HB-CoA). Finally, PHA synthase catalyzes the polymerization of 3HB-CoA to form P(3HB).

Some bacteria can also be stimulated to synthesize copolymer of P(3HB) such as poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)], when co-substrates such as valerate or propionate are provided. When propionate is added as co-substrate, with the presence of acetyl-CoA synthase,

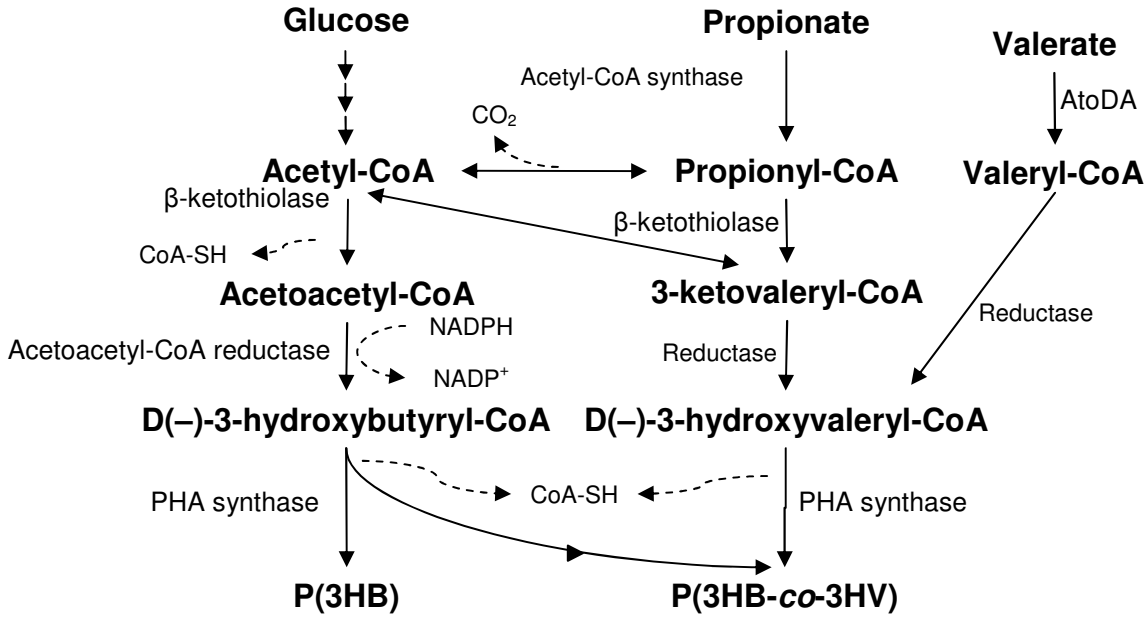

Fig. 2.2 Schematic representation of P(3HB) and P(3HB-*co*-3HV) biosynthesis from glucose, propionate and valerate (Lemos *et al.*, 2006; Sudesh *et al.*, 2000; Yim *et al.*, 1996; Doi, 1990).

the carbon source will be converted into propionyl-CoA (Lemos *et al.*, 2006; Yim *et al.*, 1996; Doi, 1990). Reaction of propionyl-CoA with acetyl-CoA will form 3-ketovaleryl-CoA. The product is then reduced by reductase to form D(–)-3-hydroxyvaleryl-CoA (3HV-CoA). 3HV-CoA and 3HB-CoA are then polymerized to form P(3HB-*co*-3HV). The presence of valerate is also able to induce synthesis of the copolymer. However, the enzymes involved in this reaction are unclear (Anderson and Dawes, 1990). Yim and coworkers suggested that acetoacetyl-CoA transferase (AtoDA), reductase and PHA synthase are involved in this pathway (Yim *et al.*, 1996). Valerate will be first transformed into valeryl-CoA (Lemos *et al.*, 2006; Yim *et al.*, 1996). The presence of AtoDA is needed for this reaction. Valeryl-CoA is then reduced by reductase to form 3HV-CoA. PHA synthase later catalyzes the polymerization of P(3HB-*co*-3HV) from 3HB-CoA and 3HV-CoA.

2.2.2.3 Model bacteria for synthesis of SCL PHAs

The best characterized bacterium regarding SCL PHA metabolism is *Cupriavidus necator* (formerly known as *Wautersia eutropha*; *Ralstonia eutropha*) (Yu, 2007; Sudesh *et al.*, 2000; Lee, 1996; Anderson and Dawes, 1990). This strain is widely studied as it is able to accumulate P(3HB) up to 80 wt% of the cell dry weight. Thus, *C. necator* is used in the production of P(3HB) in industrial scale. Besides P(3HB), Imperial Chemical Industry (ICI) commercially produced P(3HB-*co*-3HV) under the tradename Biopol® using this

strain (Luzier, 1992). This was done by feeding the bacteria with propionic acid or pentanoic acid.

In this study, *Delftia acidovorans* was chosen as this strain is able to synthesize P(3HB-*co*-3HV) with various compositions of 3-hydroxyvalerate (3HV) (Loo and Sudesh, 2007).

2.2.2.4 Biosynthesis of PHAs in natural conditions and in laboratory

In natural environments, bacteria accumulate low PHA contents ranging from 1 to 30 wt% of the cell dry weight (Brandl *et al.*, 1990). P(3HB) is the most common type of polymer that is found in the majority of PHA-producing bacteria. Foster and coworkers collected some samples of soil from rural, semi-rural and industrial areas and analyzed the existence of PHAs (Foster *et al.*, 2001). The results showed that the PHAs extracted from the samples consisted mainly of P(3HB). However, the authors also showed that a sample collected from an industrial area (polluted by some organic products) consisted of as much as 3.74 mg g^{-1} of P(3HB) and 0.26 mg g^{-1} of polyhydroxyoctanoate (PHO). Some previous study had also observed the natural occurrence of other PHAs such as P(3HB-*co*-3HV). For example, 3HV was detected in estuarine sediment as primary constituent of PHA besides 3HB (Findlay and White, 1983). Trace amount of 3HV was also detected in PHA extracted from *Bacillus megaterium* (Findlay and White, 1983). Besides that, *Agrobacterium* sp. was found to be able to synthesize P(3HB-*co*-3HV) with up to 6.1 mol% of 3HV when only glucose was present as carbon source (Lee *et*

al., 1995). *Rhodococcus* sp. NCIMB 40126 was also found to accumulate P(3HB-*co*-3HV) where the 3HV monomers contributed to the majority composition (75 mol%) when glucose was provided as sole carbon source (Haywood *et al*., 1991). The occurrence of copolymers from structurally unrelated carbon source also showed that copolymers could be synthesized in natural environments.

In laboratory, bacteria can be induced to synthesize not only P(3HB) homopolymer but also various kinds of copolymers, such as P(3HB-*co*-3HV) and poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)], up to higher contents compared to those in natural environments. Several cultivation strategies were developed to induce synthesis of higher content of PHAs (Khanna and Srivastava, 2007; Loo and Sudesh, 2007; Lee *et al*., 2004). Besides that, various kinds of genetically modified bacteria were also used to induce biosynthesis of desired polymers. The most extensively studied strain was recombinant *Escherichia coli* (Li *et al*., 2007). The originally non-PHA producer is inserted with PHA biosynthetic genes to induce synthesis of higher contents of SCL homo- and copolymer and also MCL PHA (Langenbach *et al*., 1997; Valentin and Dennis, 1997; Lee and Chang, 1995). Biosynthesis of terpolymer was also achieved by using recombinant bacteria (Bhubalan *et al*., 2008; Fukui *et al*., 1997). Besides that, recombinant strains are also used to synthesize SCL-MCL-PHA which is not a common ability found in wild type strains. For example, recombinant *C. necator* harboring PHA synthase gene of *Aeromonas caviae* is able to synthesize poly(3-hydroxybutyrate-*co*-3-

hydroxyhexanoate) [P(3HB-*co*-3HHx)] random copolymer (Loo *et al.*, 2005; Fukui and Doi, 1998).

2.2.3 Native PHA granules

PHAs are stored in cytoplasm of bacterial cells as discrete, water insoluble granules (Steinbüchel *et al.*, 1995). The native granules are in amorphous form where the highly mobile polymer chains are in a disordered conformation. PHAs are ideal storage materials as these polymers exert negligible osmotic pressure in the bacterial cells. PHAs can be observed under phase-contrast microscope and also transmission electron microscope (TEM). The granules appear as transparent granules having round/oval shape (Loo and Sudesh, 2007). The sizes of the granules vary from 100-800 nm depending on the type of granules (Loo and Sudesh, 2007; Sudesh *et al.*, 2000; Anderson and Dawes, 1990; Shively, 1974). As shown by Loo and Sudesh (Loo and Sudesh, 2007) (and also in this study), the size of P(3HB) granules in *D. acidovorans* appeared to be smaller than the size of P(3HB-*co*-3HV) granules with similar contents. This is because the density of the copolymer is lower than the homopolymer.

The PHA granule is surrounded mainly by proteins (Yu, 2007; Sudesh *et al.*, 2000; Anderson and Dawes, 1990). PHA biosynthetic proteins and PHA depolymerases are located on the surface of PHA granules and are responsible for the synthesis and mobilization of PHAs, respectively (Uchino *et al.*, 2007). The biosynthetic proteins are β-ketothiolase, acetoacetyl-CoA reductase and

PHA synthases. According to Uchino and coworkers (Uchino *et al.*, 2007), the PHA biosynthetic proteins and depolymerases are active even in the isolated native PHA granules. Some granule-associated proteins, including phasin (PhaP) and its repressor (PhaR), also exist on the surface of the granules (Dennis *et al.*, 2008; Neumann *et al.*, 2008; Sudesh *et al.*, 2004; Maehara *et al.*, 2002). PhaP is located on the surface of the granules to prevent the coalescence of individual granules and also to separate the hydrophobic granules from the cytoplasm (Steinbüchel *et al.*, 1995). Meanwhile, PhaR is the autoregulated repressor which negatively control the expression of *phaP* (Yamada *et al.*, 2007).

2.2.4 Some properties and applications of PHAs

PHAs are widely studied because of its properties such as nontoxic, biocompatible and biodegradable thermoplastic (Hazer and Steinbuchel, 2007; Reddy *et al.*, 2003; Sudesh *et al.*, 2000; Anderson and Dawes, 1990; Doi, 1990). PHAs are also potential substitute to non-degradable polypropylene (the petrochemical derived plastic) because of their similar properties. In addition, PHAs can be produced from renewable resources such as palm oil (Yu, 2007).

In the beginning, PHAs were used mainly for packaging purposes and materials for disposable items such as manufacturing of bags, containers, razors and disposable cups (Reddy *et al.*, 2003; Sudesh *et al.*, 2000; Anderson and Dawes, 1990).

Besides that, PHAs are used as absorbable biomaterials. In tissue engineering, some devices such as sutures and wound dressing were developed from various kinds of PHAs (Chen and Wu, 2005b; Sudesh, 2004; Zinn *et al.*, 2001). These polymers are also used as carriers for long term drugs and hormones (Chee *et al.*, 2008).

Recently, our group revealed the excellent oil-absorbing and oil-retaining properties of PHA film besides its nontoxic and biodegradable properties (Sudesh *et al.*, 2007). This makes it a suitable material for facial oil-blotting films. Thus, this finding has widened the application of PHA (other than traditional applications as packaging materials) to cosmetic purpose.

2.3 Utilization of PHAs by bacteria

2.3.1 Mobilization of PHAs

Deficiency of nutrients is a common occurrence in natural environments (Dawes, 1985; McSwain and Swank, 1977). This will lead to starvation of bacteria that inhabit the environments. Thus, it is very important for bacteria to develop survival ability during starvation to maintain the size of their population. For PHA-accumulating bacteria, the ability of so-called 'self-digestion' of PHAs is important to restore carbon in bacterial cells when extracellular carbon sources are not available (Merrick and Doudoroff, 1964). This activity is called mobilization (Handrick *et al.*, 2000). Mobilization is the intracellular degradation (hydrolysis) of endogenous PHA granules by the PHA-accumulating bacterium itself. This activity involves enzymes which are different from those responsible

for extracellular degradation (Merrick *et al.*, 1999). Extracellular degradation is the utilization of PHA granules which are released from other bacteria after cell lysis. The extracelular degradation enzymes are able to degrade denatured (crystalline) PHAs which are released from dead bacterial cells. In contrast, enzymes involved in mobilization can only hydrolyze native amorphous granules.

Fig. 2.3 shows the pathway of P(3HB) mobilization. During mobilization, intracellular PHA depolymerase catalyzes the breakdown of P(3HB) granules through thiolysis reaction (Uchino *et al.*, 2007). Besides that, previous study showed that 3HB-oligomer hydrolase is also involved in this reaction (Kobayashi *et al.*, 2005; Ueda *et al.*, 2002). The enzyme hydrolyses 3HB oligomers, including dimers, trimers, tetramers and pentamers, and releases monomers as products (Kobayashi *et al.*, 2005). It was also found that 3HB-oligomer hydrolase is able to degrade artificial amorphous P(3HB) granules. These enzymes exist on the surface of the granules from the time when the P(3HB) is accumulated (Uchino *et al.*, 2007). In general, researchers believe that the product of the thiolysis reaction is D(–)-3-hydroxybutyric acid (Kobayashi *et al.*, 2005; York *et al.*, 2003; Handrick *et al.*, 2000). However, in a recent finding, Uchino and coworkers suggested that instead of the free acid, the thiolysis reaction released 3HB-CoA as primary mobilization products (Uchino *et al.*, 2007). This 3HB-CoA could be converted back to P(3HB) when necessary without wasting any energy. Following the depolymerization, 3HB dehydrogenase catalyzes the formation of acetoacetate from the primary

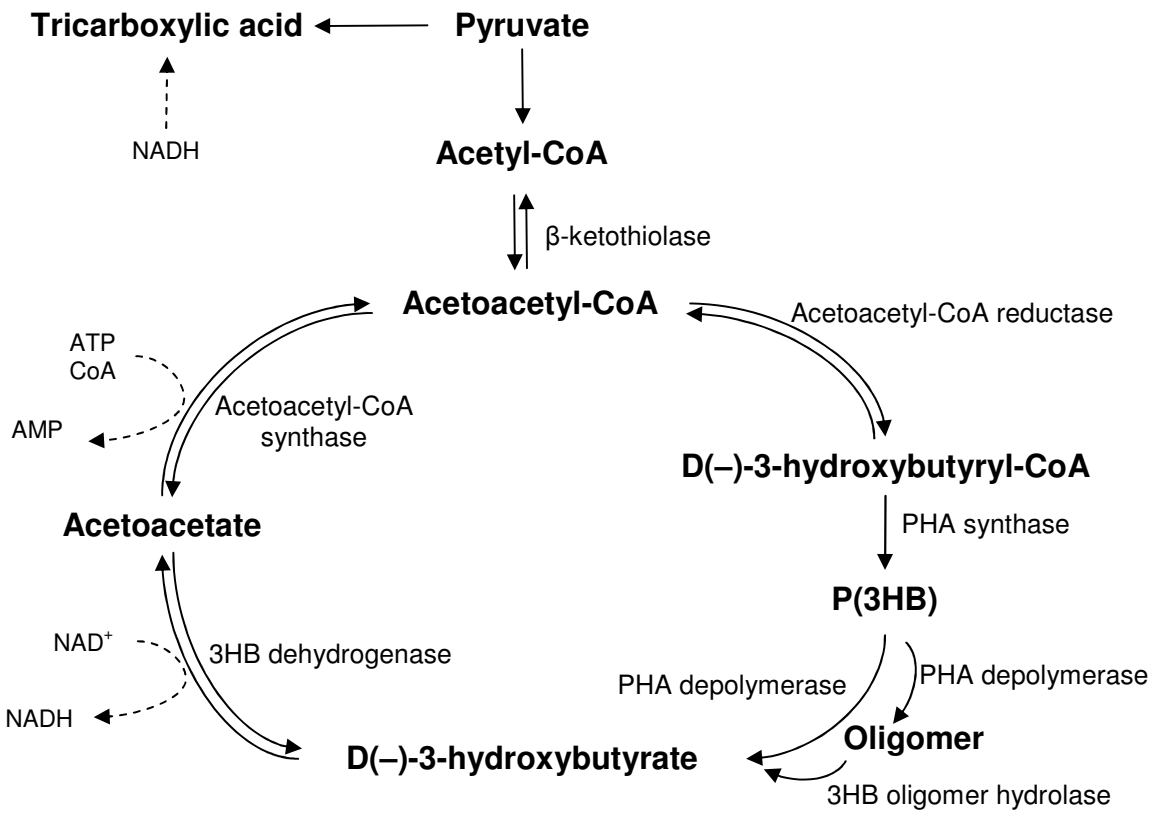

Fig. 2.3 P(3HB) cycle in bacteria (Saito and Kobayashi, 2002).

mobilization product (Nakada *et al.*, 1981). Nicotinamide adenine dinucleotide (NAD^+) is needed in this process. Subsequently, acetoacetate is converted to acetoacetyl-CoA with the presence of acetoacetyl-CoA synthase, ATP and CoA (Cai *et al.*, 2000). Finally, β-ketothiolase catalyzes the formation of acetyl-CoA from acetoacetyl-CoA (Segura *et al.*, 2000). The mobilized products are then oxidized via tricarboxylic acid cycle (Saito and Kobayashi, 2002; Sudesh *et al.*, 2000; Doi, 1990).

Uchino and coworkers suggested that the initiation of mobilization was highly dependant on the presence of key metabolites including CoA, acetyl-CoA, 3HB-CoA and NAD^+/NADH (Uchino *et al.*, 2007). The authors also showed that the presence of phasin protein is necessary for mobilization of P(3HB). When native P(3HB) granules which were isolated from recombinant *E. coli* harboring a combination of P(3HB) biosynthetic genes and intracellular depolymerase gene were added with CoA, no mobilization activity was observed. However, mobilization took place when CoA was added with native granules isolated from recombinant *E. coli* harboring a combination of P(3HB) biosynthetic genes, intracellular depolymerase gene and gene encoding phasin protein.

However, not much is known about the mobilization of polymers other than P(3HB). In 1999, Yoon and coworkers pointed out that the rate of the 3HV and 4HB mobilization was very slow as compared to 3HB (Yoon and Choi, 1999). Yan and coworkers also showed that mobilization of 3HB was much faster than the 3HV comonomer (Yan *et al.*, 2000).

2.3.2 Advantages of mobilization of PHAs to bacteria

Intracellular P(3HB) is an important carbon source to increase the survival of bacteria during nutrient starvation (Dawes, 1985). Starvation survival refers to the ability of bacteria to survive longer in the absence of nutrients (Morita, 1982). The P(3HB)-accumulating bacteria are able to invoke mobilization of reserved granules to enable them to survive from carbon starvation. For example, *C. necator* (López *et al*., 1995) *and Pseudomonas oleovorans* (Ruiz *et al*., 2001; Ruiz *et al*., 1999) showed increased starvation survival in low nutrient natural water by mobilizing the previously accumulated P(3HB). Mobilization of intracellular P(3HB) granules also increased the starvation survival of *Legionella pneumophila* (James *et al*., 1999). The bacterium survived from incubation in low-nutrient tap water for at least 600 days. Some nitrogen-fixing bacteria such as *Rhizobium tropici* (Povolo and Casella, 2004) and *Rhizobium leguminosarum* (Lodwig *et al*., 2005) also showed increased survival during carbon starvation by mobilizing P(3HB). Handrick and coworkers revealed that *C. necator* was able to use the mobilized products of P(3HB) for one or two cell divisions during absence of extracellular carbon source (Handrick *et al*., 2000). Previous study on *Spirillum* sp. indicated that starvation survival had direct relationship with the P(3HB) content (Matin *et al*., 1979). By increasing the polymer content from 10 to 18 wt%, the starvation survival of this bacterium increased as much as 50%. However, there were also some cases where bacteria with lower contents of polymer survived better than those with higher polymer contents (Dawes, 1985). This might be caused by

better regulation of mobilization of granules in the bacteria with lower contents of P(3HB).

Recently, there has been increasing efforts put on the study of mobilization of PHAs as many researchers found that mobilization of the polymers could increase the stress resistance of the polymer-accumulated strains. By mobilizing the previously accumulated P(3HB), *P. oleovorans* showed enhanced stress resistance (Ruiz *et al.*, 2001). The bacterium was starved for three days (to allow mobilization if possible) and followed by treatment with 20% ethanol or 47℃ heat treatment. After mobilizing the intracellular granules, the wild type strain showed higher resistance than P(3HB) depolymerase deficient mutant. The mutant strain was unable to mobilize accumulated P(3HB) during the three-days starvation period. A *Pseudomonas* sp. isolated from Antarctic environments also showed increased resistance to heat and oxidative stress by mobilizing the stored P(3HB) granules (Ayub *et al.*, 2004). Some earlier studies showed that mobilization of PHAs could increase the concentration of guanosine tetraphosphate (ppGpp, the effector molecule of the stringent response) and RpoS (a starvation or stationary phase sigma factor). Consequently, this increased the stress resistance of bacteria (Ruiz *et al.*, 2004; Ruiz *et al.*, 2001). A recent study proposed that RpoS was induced at the onset of PHA biosynthesis (Zhao *et al.*, 2007). This is because the synthesis and mobilization of PHAs are dynamic processes where both activities can proceed at the same time (Uchino *et al.*, 2007; Yan *et al.*, 2000).

Mobilization of P(3HB) granules are also important for sporulation process in *Bacillus* species (Anderson and Dawes, 1990; Dawes, 1985). López and coworkers showed that P(3HB) played an important role in germinations of spores in *B. megaterium* (López *et al.*, 1995).

Besides that, P(3HB) also plays an important role in encystment of the genus *Azotobacter* (Stevenson and Socolofsky, 1966). Encystment is the process of cysts forming under nutrient limitation conditions (Dawes, 1985). PHAs serve as carbon and energy source which encourage the extensive formation of cysts in *Azotobacter* (Stevenson and Socolofsky, 1966).

In a very special case, P(3HB) serves as an oxidizable substrate which provides respiratory protection to nitrogenase of *Azotobacteriaceae* (Anderson and Dawes, 1990; Dawes, 1985). The nitrogenase system of *Azotobacter* is very sensitive to oxygen (Wong and Burris, 1972). High concentration of oxygen is inhibitory to this strain. Senior and coworkers suggested that mobilization of P(3HB) could increase the respiratory activity, reduce the concentration of oxygen and thus, protect the nitrogenase system (Senior *et al.*, 1972).

Although there are extensive studies done on the roles of P(3HB), the roles of PHAs other than P(3HB) are still poorly understood (Anderson and Dawes, 1990). However, researchers had discovered some natural occurrences of copolymer such as P(3HB-*co*-3HV) (Foster *et al.*, 2001; Lee *et al.*, 1995; Haywood *et al.*, 1991; Findlay and White, 1983). Therefore, it is believed that these copolymers could be utilized by the PHA-accumulated strains for certain purposes. Recently, Zhao and coworkers revealed that the accumulation of

P(3HB-*co*-3HHx) is capable to enhance the resistance of the granules-accumulated cells against some environmental stress agents (Zhao *et al.*, 2007).

2.4 Importance of study on mobilization of PHAs

2.4.1 Prevention/decrement of mobilization activity during production of PHAs

Study of mobilization is important in order to understand the mechanism of this system. This may be beneficial for the decrement or prevention of the activity of mobilization during industrial production of PHAs. As there are more than one type of intracellular depolymerases (PhaZ) (York *et al.*, 2003), the characterization of all genes encoding these enzymes and construction of a truly null *phaZ* mutant may be difficult in the near future. Besides that, this may incur cost and effort. There were also evidences from various sources which indicated the simultaneous regulation of the synthesis and mobilization of PHAs (Uchino *et al.*, 2007; Yan *et al.*, 2000). Thus, it is important to understand and prevent the conditions favorable to mobilization of PHAs not only for scientific purposes but also for industrial production.

2.4.2 Synthesis of (*R*)-3-hydroxybutyric acid

(*R*)-3-hydroxybutyric acid [(*R*)-3HB] is the monomer of P(3HB). This chiral chemical can be used to synthesize fine chemicals such as antibiotics, aromatics, vitamins and pheromones (Tokiwa and Ugwu, 2007; Chen and Wu,

2005a). High yield of (*R*)-3HB can be obtained from natural PHA-producing bacteria under culture conditions which induce high activity of intracellular depolymerases (Lee and Lee, 2003). This can be done, for example, by increasing the ratio of nitrogen to carbon in the bacterial cultures (Handrick *et al.*, 2000). However, the activity of (*R*)-3HB dehydrogenase should be stopped. For this purpose, (*R*)-3HB dehydrogenase knock-out mutants are used (Tokiwa and Ugwu, 2007).

2.4.3 Bactericides and PHAs-accumulated bacteria

Recently, there has been increasing evidences which showed that mobilization of previously accumulated PHAs enabled the PHAs-accumulated bacteria to enhance their resistance towards some bactericides (Zhao *et al.*, 2007; Ruiz *et al.*, 2004; Ruiz *et al.*, 2001). It is very important to study the mobilization of PHAs and the effect on resistance towards bactericides. This is because wrong doses of bactericides, instead of killing the bacteria, may actually make the bacteria stronger (Boor, 2006; Christman *et al.*, 1985).

2.4.3.1 Ethanol

Ethanol is a common antimicrobial agent which is used against vegetative bacteria, viruses and also fungi (McDonnell and Russell, 1999). Previous study revealed that by mobilizing previously accumulated P(3HB), *P. oleovorans* showed enhanced stress resistance against 20% ethanol at 25 ℃ compared to PHA depolymerase-negative mutant (Ruiz *et al.*, 2001). A recent

study on *A. hydrophila* showed that P(3HB-*co*-3HHx) copolymer also enabled the bacterium to maintain its survival when stressed by 10% ethanol solution (Zhao *et al.*, 2007).

2.4.3.2 Hydrogen peroxide (H_2O_2)

H_2O_2 appears as pale-blue covalent liquid (Halliwell *et al.*, 2000). This chemical mixes readily in water. Although the pathways remaine unclear, H_2O_2 can easily cross cell membranes (Halliwell and Gutteridge, 1999). However, with the presence of P(3HB) or P(3HB-*co*-3HHx), *P. oleovorans* and *A. hydrophila*, respectively, showed increased tolerance towards H_2O_2 treatment (Zhao *et al.*, 2007; Ruiz *et al.*, 2004; Ruiz *et al.*, 2001).

2.4.3.3 Titanium dioxide (TiO_2)

TiO_2 was reported by Akira Fujishima and Kenichi Honda of the University of Tokyo in 1972 as a photocatalyst under ultraviolet A (UVA) (Hoffmann, 2001). The photocatalytic reaction is also called Honda-Fujishima effect. When irradiated under UVA (wavelength <400 nm), reactive oxygen species (ROS) will be generated (Fujishima and Zhang, 2006; Fujishima *et al.*, 2000). ROS such as hydroxyl radicals (OH•), superoxide anion (O_2^-) and H_2O_2 are able to break down organic compounds. TiO_2 is used as bactericide not only in water and air but also in food (Vohra *et al.*, 2006; Yew *et al.*, 2006; Kim *et al.*, 2003). Many studies were done on the bactericidal effect of TiO_2, for example, in *Bacillus cereus* (Luo *et al.*, 2008). This foodborn pathogen can cause serious