UTILIZATION OF PALM OIL-BASED BY-PRODUCTS AND WASTE AS FEEDSTOCK FOR POLYHYDROXYALKANOATE BIOSYNTHESIS

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by

KEK YIK KANG

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

conditions. (A) High accumulation of P(3HB) contributing to about 65 wt% of DCW was attained using low urea concentration (5 mM). (B) Cells were actively dividing at high urea concentration (50 mM) and gas chromatography revealed intracellular P(3HB) content of 5 wt% at the end of cultivation. (C) and (D) Small and discrete P(3HB) granules found to be localized around the nucleoid region, which were observed near the center of the cells

LIST OF SYMBOLS AND ABBREVIATIONS

$(NH_2)_2CO$	Urea
$(NH_4)_2SO_4$	Diammonium sulphate
°C	Degree Celsius
3HB	3-hydroxybutyrate
3HHx	3-hydroxyhexanoate
3HO	3-hydroxyoctanoate
3HV	3-hydroxyvalerate
4HB	4-hydroxybutyrate
Å	Angstrom
A_{3HB}	The area below the 3HB peak from GC analysis
A_{3HHx}	The area below the 3HHx peak from GC analysis
A_{CME}	The area below the CME peak from GC analysis
ATCC	The American Type Culture Collection
C/N	Carbon to nitrogen
C16:0	Palmitic acid
C18:1	Oleic acid
C18:2	Linoleic acid
$CaCl_2$	Calcium chloride
CME	Caprylate methyl ester
CO	Fresh cooking oil
CO_2	Carbon dioxide
CoA-SH	Coenzyme-A

$CoCl_2 \cdot 6H_2O$	Cobalt (II) chloride hexahydrate
CPKO	Crude palm kernel oil
CPO	Crude palm oil
$CrCl_3 \cdot 6H_2O$	Chromium chloride hexahydrate
$CuSO_4 \cdot 5H_2O$	Copper sulfate pentahydrate
DCW	Dry cell weight
fadD	Acyl-CoA synthetase
$FeCl_3$	Iron (III) chloride
FFAs	Free fatty acids
FTIR	Fourier transform mid-infrared spectrometry
GC	Gas chromatography
h	Hour(s)
HA	Hydroxyalkanoate
HPLC	High performance liquid chromatography
HVLV	High-value low-volume
ICI	Imperial Chemical Industries
IR	Infrared
k_{3HB}	3HB monomer constant
k_{3HHx}	3HHx monomer constant
kDa	Kilo Dalton
KH_2PO_4	Potassium dihydrogen phosphate
LDPE	Low density polyethylene
LVHV	Low-value high-volume

mcl	Medium-chain-length
$MgSO_4 \cdot 7H_2O$	Magnesium sulphate heptahydrate
MM	Mineral medium
mM	Milimolar
mol%	Mole percent
MPa	Mega pascal
N	Normality
Na_2HPO_4	Sodium phosphate
Na_2SO_4	Sodium sulphate anhydrous
NAD(P)	Nicotinamide adenine dinucleotide (phosphate)
NAD(P)H	Reduced nicotinamide adenine dinucleotide (phosphate)
$NaNO_3$	Sodium nitrate
NH_4Cl	Ammonium chloride
$NH_4H_2SO_4$	Ammonium dihydrogen sulphate
NH_4NO_3	Ammonium nitrate
$NiCl_2 \cdot 6H_2O$	Nickel chloride hexahydrate
nm	Nanometer
NMR	Nuclear magnetic resonance
NR	Nutrient rich
O.D.	Optical density
OsO_4	Osmium tetroxide
P(3HB)	Poly(3-hydroxybutyrate)
P(3HB-*co*-3HHx)	Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate)

PAO	Palm acid oil
PFAD	Palm fatty acid distillate
PHA	Polyhydroxyalkanoate
phaC	PHA synthase
phaJ	Enoyl-CoA hydratase
phaP	Phasins
phaR	Regulator of phasin
phaZ	Intracellular PHA depolymerase
PKAO	Palm kernel acid oil
PP	Polypropylene
psi	Pounds per square inch
rpm	Revolutions per minute
scl	Short-chain-length
TCA	Tricarboxylic acid
TEM	Transmission electron microscopy
TES	Trace elements solution
UCO	Used cooking oil
v/v	Volume per volume
w/v	Weight per volume
wt%	Weight percent
x *g*	Times gravity
μm	Micrometer

Penggunaan produk sampingan dan bahan buangan berasaskan minyak kelapa sawit sebagai sumber karbon untuk penghasilan polihidroksialkanoat

ABSTRAK

Penggunaan produk sampingan dan bahan buangan yang berasaskan minyak kelapa sawit adalah bertujuan untuk mengurangkan kos keseluruhan penghasilan polihidroksialkanoat (PHA). Dalam kajian ini, produk sampingan dari proses penulenan minyak kelapa sawit seperti minyak asid kelapa sawit (PAO), minyak asid isirong kelapa sawit (PKAO) dan distilat asid lemak kelapa sawit (PFAD) serta minyak masak terpakai (UCO) telah dinilai sebagai sumber karbon untuk penghasilan poli(3-hidroksibutirat) [P(3HB)] dan poli(3-hidroksibutirat-*co*-3-hidroksiheksanoat) [P(3HB-*co*-3HHx)] dengan menggunakan bakteria *Cupriavidus necator* H16 dan transformannya. Parameter penting untuk pengkulturan yang mempengaruhi pertumbuhan *C. necator* H16 dan sintesis P(3HB) olehnya secara signifikan telah ditentukan dengan menggunakan 5 g/L PKAO. Kepekatan urea 4 mM (nisbah C/N ~38) telah menyumbang berat sel kering (BSK) dan jumlah P(3HB) yang paling tinggi iaitu 5.4 dan 3.2 g/L masing-masing. Kajian juga menunjukkan peningkatan isipadu kultur dari 50 ke 100 mL (dalam kelalang 250 mL) berjaya meningkatkan tahap penghasilan P(3HB) ke 77% (berat/berat) [(b/b)] daripada BSK, dengan kepekatan P(3HB) sebanyak 4.1 g/L. Biopenukaran UCO kepada P(3HB) oleh *C. necator*, kepekatan sumber nitrogen dan karbon yang paling sesuai untuk pertumbuhan sel dan sintesis P(3HB) telah ditentukan. BSK tertinggi yang tercapai adalah 25.4 g/L dengan kandungan P(3HB) sebanyak 71% (b/b) apabila 18 mM urea dan 20 g/L UCO (nisbah C/N ~30) digunakan. Di samping itu, kopolimer P(3HB-*co*-3HHx) dihasilkan oleh transforman *C.*

necator apabila PKAO, PAO, PFAD dan UCO digunakan sebagai sumber karbon tunggal. Dengan kandungan P(3HB-*co*-3HHx) sebanyak 50% (b/b), BSK yang lebih tinggi diperoleh dari UCO; 5 g/L berbanding hanya 3.8 g/L dari PKAO. Tambahan pula, manipulasi kepekatan sumber karbon didapati berupaya meningkatkan penghasilan P(3HB-*co*-3HHx) dari bahan samping/buangan tersebut secara signifikan. Dengan kepekatan PAO/PKAO/PFAD sebanyak 9 g/L, BSK dan kandungan kopolimer tertinggi yang tercapai adalah 5.7 g/L dan 70% (b/b) masing-masing. Sementara itu, BSK dan kepekatan P(3HB-*co*-3HHx) tertinggi yang tercapai adalah 8.4 dan 5.6 g/L apabila 10 g/L UCO digunakan sebagai sumber karbon tunggal. Profil masa penghasilan P(3HB-*co*-3HHx) oleh transforman *C. necator* yang dibekalkan dengan 10 g/L UCO dan 15 mM urea menunjukkan komposisi monomer 3HHx yang tetap di antara 3 – 5 mol% sepanjang 96 jam tempoh pertumbuhannya. Kajian ini menunjukkan bahawa bahan buangan/sampingan berasaskan minyak kelapa sawit berpotensi untuk digunakan sebagai sumber karbon untuk penghasilkan PHA.

Utilization of palm oil-based by-products and wastes as feedstock for polyhydroxyalkanoate biosynthesis

ABSTRACT

The utilization of by-products and wastes from the palm oil industry is aimed to reduce the overall production cost of polyhydroxyalkanoate (PHA). In this study, palm oil refining by-products; palm acid oil (PAO), palm kernel acid oil (PKAO), palm fatty acid distillate (PFAD), and palm oil-based used cooking oil (UCO) were evaluated as the carbon substrate for poly(3-hydroxybutyrate) [P(3HB)] and poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] synthesis using *Cupriavidus necator* H16 and its transformant. Important cultural parameters that significantly affected the cell biomass and P(3HB) synthesis of *C. necator* H16 grown on 5 g/L PKAO were identified. An initial urea concentration of 4 mM (C/N ratio~38) gave the highest DCW and total P(3HB); 5.4 and 3.2 g/L respectively. Elevation of culture volume from 50 to 100 mL (in 250 mL flask) further enhanced the P(3HB) production up to 77 wt% of DCW, whereby P(3HB) concentration of 4.1 g/L was obtained from 5 g/L PKAO. For the bioconversion of UCO to P(3HB) by *C. necator* H16, the most suitable nitrogen and carbon source concentration for cell growth and P(3HB) synthesis were determined. A total of 18 mM urea and 20.0 g/L UCO (C/N~30) contributed to the highest DCW (25.4 g/L) and intracellular P(3HB) content (71 wt%), which resulted in P(3HB) concentration of 18 g/L. On the other hand, P(3HB-*co*-3HHx) copolymer was synthesized when the transformant of *C. necator* mutant was grown on PAO, PKAO, PFAD or UCO as the sole carbon source. With an approximate P(3HB-*co*-3HHx) content of 50 wt%, a higher DCW could be obtained from UCO; 5 g/L than

those obtained from PKAO; 3.8 g/L. In addition, manipulation of the initial carbon source concentration was found to improve significantly the P(3HB-*co*-3HHx) yield from the wastes/by-products. At the initial PAO/PKAO/PFAD concentration of 9 g/L, the highest DCW and copolymer content were 5.7 g/L and 70 wt% respectively at 72 h of cultivation. In the case of using UCO as the carbon source, an initial concentration of 10 g/L contributed to 8.4 and 5.6 g/L of DCW and P(3HB-*co*-3HHx) concentration respectively and the corresponding copolymer content in the biomass was 67 wt%. Time profiles of *C. necator* transformant grown on 10 g/L UCO and 15 mM urea showed a constant 3HHx monomer fraction of 3 – 5 mol% within 96 h of cultivation. This study has demonstrated that the surplus palm oil-based wastes/by-products are potential carbon source for the production of PHA.

1.1 INTRODUCTION

Mankind relies extensively on petrochemical derived plastics and these conventional plastics have become indispensable materials in modern society over the past few decades. The worldwide production of synthetic plastic reaches 140 million tons per annum (Suriyamongkol *et al.*, 2007) and a significant portion of these materials after their usage lifetime, accumulates in our biosphere. Fossil fuel is the principal source providing power needed for various mankind activities. One fine example is the transformation of fossil fuel into common plastics such as polyethylene, polypropylene and polystyrene. The growing reliance on polyolefin polymers has raised a number of environmental and health concerns, i.e. depletion of finite fossil feedstock and the scarcity of space for plastic wastes disposal. In addition, the presence of non-degradable residues in the environment is also affecting the potential survival of a large number of marine species (Derraik, 2002).

To prevent these catastrophes of non-sustainable resources scarcity and environmental ruins, much effort has been dedicated for the development of alternative polymers that are biodegradable and can be produced from renewable resources (Grengross and Slater, 2000; Verlinden *et al.*, 2007; Narodoslawsky *et al.*, 2008). The demand for biodegradable materials is growing by 30% each year, slowly capturing the capital market solely dominated by petrochemical derived polymers (Leaversuch, 2002). Polyhydroxyalkanoate (PHA), a class of bacterial polyester which could be composed of enormous ranges of hydroxyalkanoate (HA) unit, is highlighted as an alternative because PHA can be derived from renewable resources and possesses

extensive spectra of physical and mechanical properties depending on the monomer compositions (Sudesh *et al.*, 2000; Philip *et al.*, 2007; Verlinden *et al.*, 2007).

However, at their present state of technological development, the production cost is still the major drawback of their substitution for the currently dominating conventional plastics. Therefore, the interest in the use of renewable vegetable oils and various agricultural or industrial by-products/waste streams for the bioconversion to PHA is growing throughout the world, with the aim to reduce the overall production cost of these biologically derived materials. Several reports have demonstrated that various triacylglycerol and other oily industrial by-products are superior carbon substrate for PHA synthesis by using wild type or recombinant PHA-accumulating bacteria (Kahar *et al.*, 2003; Taniguchi *et al.*, 2003; Ashby and Solaiman, 2008; Kek *et al.*, 2008; Lee *et al.*, 2008). Palm oil industry in Malaysia generates vast quantities of by-products annually from the refining processes. Palm acid oil (PAO), palm kernel acid oil (PKAO) and palm fatty acid distillate (PFAD) are amongst the palm oil by-products generated from chemical/physical refining processes. Besides, other oily waste such as used cooking/frying oil is constantly generated from food manufacturing and catering facilities. These surplus materials generally undergo further treatment before being used as ingredients in animal feed or before being used to make low quality soaps. In fact, they can be considered as an attractive and inexpensive raw material for bacterial fermentation towards biosynthesis of PHA in a cost effective manner.

1.2 Objectives of this study

i. To study the feasibility of palm acid oil, palm kernel acid oil, palm fatty acid distillate and used cooking oil as the sole carbon substrate for PHA production by *Cupriavidus necator* H16 and its transformant.

ii. To determine crucial cultural parameters for improvement of PHA yield from these palm oil-based by-products and waste.

iii. To investigate the P(3HB) granule initiation site in *C. necator* H16 grown on PKAO under nitrogen rich condition.

2.0 LITERATURE REVIEWS

2.1 Polyhydroxyalkanoate (PHA)

2.1.1 Discovery and physiology of PHA granules

The occurrence of microbial polyhydroxyalkanoate (PHA) has been known since 1926, where it was first discovered by a French scientist Maurice Lemoigne from Pasteur Institute, Paris (Lemoigne, 1926). The prototype of PHA family, poly(3-hydroxybutyrate) [P(3HB)] was observed in an aerobic spore-forming bacterium, i.e. *Bacillus megaterium*. P(3HB) was the sole identified member of PHA family for almost 50 years until a number of additional 3-hydroxy fatty acids in activated sludge samples were identified by Wallen and Rohwedder (1974). Polyesters with a longer side chain were then reported in 1983, whereby *Pseudomonas putida* GPo1 (previously known as *P. oleovorans* GPo1) growing on alkanes (de Smet *et al.*, 1983) produced a copolymer consisting of various medium-chain-length (mcl) monomer units. To date, approximately 150 different constituents of PHA have been identified (Steinbüchel and Valentin, 1995; Kim and Lenz, 2001; Steinbüchel and Hein, 2001). These polyesters are deposited as spherical water-insoluble granules in the cytoplasm when the bacterial cells are cultivated in the presence of excessive carbon supply and the growth is impaired by the lack of other essential nutrients. Depletion of nutrient such as nitrogen, phosphorus, sulphur or oxygen acts as a trigger for the metabolic shift from biomass synthesis to PHA accumulation, whereby its stimulatory effect is species-dependant (Madison and Huisman, 1999; Sudesh *et al.*, 2000; Luengo *et al.*, 2003).

This diverse group of bacterial storage compounds exists in two different physical states; isolated PHA granule and *in vivo* PHA granule. After the solvent

extraction of PHA granule from bacterial cells, the polyester exhibits thermoplastic and/or elastomeric properties depending on its monomer composition (Witholt and Kessler, 1999; Philip *et al.*, 2007). On the other hand, the *in vivo* or native PHA granules are in the amorphous state surrounded by phospholipids (Griebel *et al.*, 1968) and catalytic/non-catalytic proteins (Pötter and Steinbüchel, 2005; Jendrossek *et al.*, 2007; Grage *et al.*, 2009). Such granules can be isolated in the native form by glycerol density gradient centrifugation (Wieczorek *et al.*, 1996; Jendrossek, 2007). At the surface of PHA granules, four major types of proteins are bound: (i) PHA synthase (*phaC*), (ii) intracellular PHA depolymerase (*phaZ*), (iii) phasins (*phaP*) and (iv) regulator of phasin (*phaR*) (Gerngross *et al.*, 1993; Prieto *et al.*, 1999; Maehara *et al.*, 2002; York *et al.*, 2002; Rehm, 2006). Other additional proteins with unknown function were also reported (Klinke *et al.*, 2000; Pötter and Steinbüchel, 2005).

PHA synthase is the key enzyme of PHA biosynthesis which catalyzes the polymerization of (*R*)-3-hydroxyacyl-CoA substrates to PHA and hence determines the composition of the synthesized polyesters (Rehm, 2003). PHA synthase will be discussed in more detail in Section 2.5. Besides, phasins are predominant compounds in the interface of granule and considered as a class of structural proteins that play an important role in PHA biosynthesis. The existence of phasins in the phospholipid layer stabilizes the PHA granules, prevents the coalescence of the granules and also binding of cytosolic proteins to the hydrophobic granule surface (Steinbüchel *et al.*, 1995; Stubbe and Tian, 2003). Generally, the level of PhaP is parallel to the levels of P(3HB) in cell (York *et al.*, 2001;2002) and influences the number and size of PHA granules (Pötter *et al.*, 2002; Pötter and Steinbüchel, 2005). Studies have shown that the

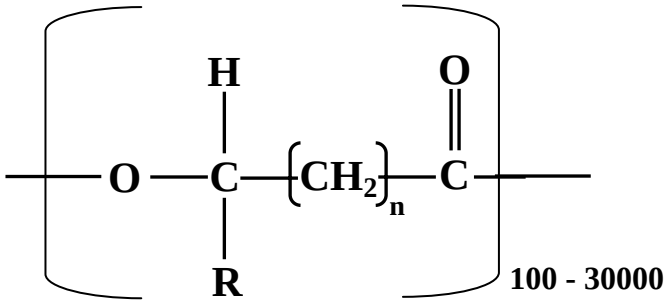
intracellular concentration of PhaP is inversely related to the number/size of the granule, since over expression of *phaP* resulted in the formation of many small granules while *phaP* mutant contained only a single PHA granule (Wieczorek *et al.*, 1995; Stubbe and Tian, 2003).

2.1.2 Types of PHA

P(3HB) is considered the parent member of PHA family, having the same three-carbon backbone structure but differing in the pendant R group at the beta or 3 position, which can be varied from methyl (C1) to tridecyl (C13) (Figure 2.1). In addition to saturated side groups, unsaturated, aromatic, halogenated and branched monomers have been also reported (Abe *et al.*, 1990; Doi and Abe, 1990; Fritzche *et al.*, 1990a; Fritzche *et al.*, 1990b; Hazer *et al.*, 1994; Choi and Yoon, 1994; Curley *et al.*, 1996; Song and Yoon, 1996). PHA is produced by the formation of ester bonds between the carboxyl groups of one monomer with the hydroxyl groups of the neighboring monomer. All monomer units of PHA are enantiomerically pure, in *R*-configuration and can be generally sub-divided into three groups according to the chain length of the monomers.

The first group of PHA, which can be represented by *C. necator*, is short-chain-length PHA (scl-PHA) containing monomer units ranging from 3 – 5 carbons in length. The second group of PHA, synthesized by a number of pseudomonads, is medium-chain-length PHA (mcl-PHA) containing monomers ranging from 6 – 16 carbons in length (Steinbüchel, 1991). The third group of PHA consists of both scl- and mcl-monomer units, which to date is only produced by microorganisms such as *Aeromonas caviae*, *A. hydrophila*, *Pseudomonas* sp. 61-3, *P. stuzteri* and *Nocardia corallina* that

possesses *phaC* with a broader substrate specificity (Hall *et al.*, 1998; Matsusaki *et al.*, 1998; Chen *et al.*, 2004).



Polymer

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)

Figure 2.1 General structure of PHA

Such variation in monomer composition translates into a wide spectrum of material properties, from firm and brittle plastics to softer plastics, elastomers, rubbers and glues which can certainly compete with fossil fuel-derived synthetic plastics (Steinbüchel and Valentine, 1995; Sudesh *et al.*, 2000; Philip *et al.*, 2007) if all these PHA can be produced in a cost effective manner.

The class of PHA synthesized by a respective bacterium depends strictly on the substrate specificity of its PHA synthase and the availability of the precursor, which is strongly influenced by the metabolic background of the microorganism (Rehm and Steinbüchel, 1999). Thus, PHA can be tailor made by numerous means, including

external substrate manipulation, pathway inhibitor addition, recombinant gene expression, genome manipulation and protein engineering of PHA biosynthetic enzymes (Aldor and Keasling, 2003).

2.1.3 Detection and quantification of PHA

Intracellular PHA granules can be determined by a number of methods. Without any staining procedure, PHA can be observed intracellularly as light-refracting granules under phase-contrast microscope (Figure 2.2). Rapid staining techniques using lipophilic reagents are commonly applied for the detection of PHA granules, particularly during the screening process for isolation of potential PHA-producing bacteria. Lipophilic dyes such as Sudan black B (Murray *et al.*, 1994), Nile blue A and Nile red (Ostle and Holt, 1982; Kitamura and Doi, 1994; Pierce and Schroth, 1994; Gorenflo *et al.*, 1999; Spiekermann *et al.*, 1999) are used for the staining of PHA granules in bacterial cell. The fluorescent staining of the granules using Nile Blue or Nile red is the most common detection technique *in vivo* (Ostle and Holt, 1982). After de-staining process, cells containing lipid inclusion bodies such as PHA granules are identified by the retention of the dye. Advantage of Nile red staining over using Nile blue is that the former can be applied directly into the growing culture without causing detrimental effect on the cells. However, all these dyes are not specific towards PHA granules and can bind to any lipid compounds (Alvarez *et al.*, 1997; Waltermann *et al.*, 2000).

Gas chromatography (GC) analysis involving simultaneous solvent extraction and hydrolytic esterification of PHA is generally carried out for the determination of intracellular PHA content (Braunegg *et al.*, 1978). GC analysis with propanolysis in

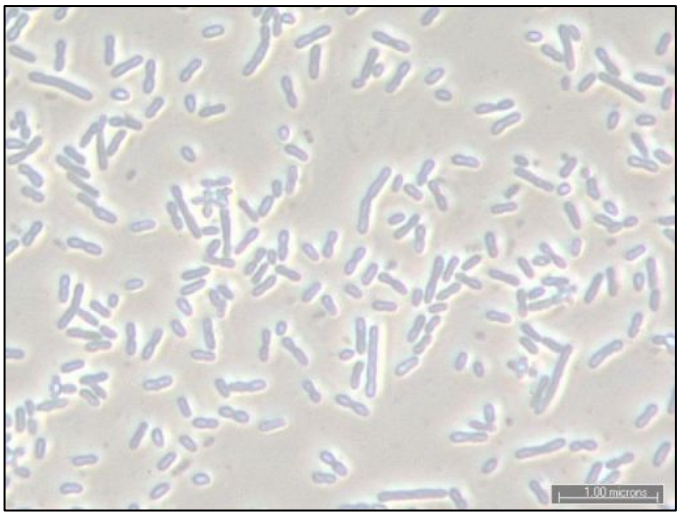


Figure 2.2 Under phase-contrast microscope, P(3HB) was observed intracellularly as light-refracting granules in *C. necator* H16. Cells were cultivated for 72 h at 30 °C by using crude palm kernel oil as the sole carbon source

hydrochloric acid rather than acidic methanolysis in sulphuric acid was also reported (Riis and Mai, 1988). Quantification of microbial PHA using GC method is rapid, sensitive, reproducible, and requires only small amount of samples for the analysis. Other techniques of analysis such as IR spectrometry at 5.75 Å (Juttner *et al.*, 1975), two-dimensional fluorescence spectroscopy, flow cytometry (Degelau *et al.*, 1995), HPLC (Karr *et al.*, 1983), ionic chromatography and enzymic determination (Hesselmann *et al.*, 1999) were also described. For on-line determination of PHA content in recombinant *E. coli* system, Fourier transform mid-infrared spectrometry (FTIR) and microcalorimetric technique (Jarute *et al.*, 2004; Ruan *et al.*, 2007) were also reported. For precise composition determination and structural elucidation of PHA, a variety of nuclear magnetic resonance (NMR) spectroscopy techniques also have been applied and the most commonly used are proton (^{1}H) and carbon-13 (^{13}C) NMR (Doi *et al.*, 1986a,b; Jacob *et al.*, 1986).

2.1.4 PHA granule: self-assembly and structure

Although it has been more than eight decades since the discovery of PHA in microorganism, the initiation site of PHA granule formation and its biogenesis mechanism remained unraveled. *In vitro* synthesis of PHA was first demonstrated by Gerngross and Martin (1995) and it was clearly showed that the synthase possesses all the features required for the formation of spherical PHA particles. Two models have been proposed for the *in vivo* PHA granule formation; micelle model (Ellar *et al.*, 1968; Gerngross *et al.*, 1994) and budding model (Stubbe and Tian, 2003), which both consider the defined location of the PHA synthase (Figure 2.3). In the micelle and budding models, *phaC* is converted into an amphipathic molecule upon polyester chain synthesis, and a self-assembly process occurs randomly in the cytosol and in the membrane, respectively. A micelle like structure was formed due to the aggregation of P(3HB)-linked synthase, whereby the synthase proteins residing on micelle surface acquire the 3-hydroxybutyrate-CoA substrate from the cytosol and lead to the growing insoluble P(3HB) chain within the micelle. On the other hand, the budding model would be analogous to that proposed for lipid body biogenesis. In this model, the PHA synthase and peripheral membrane bound proteins adhere to the inner face of the plasma membrane. As the PHA chain elongate and the phasins is produced, budding of a vesicle with a phospholipid layer could ensue and ultimately lead to granule formation. This model of granule formation was supported by the observation in recent works by Jendrossek (2005), Peters and Rehm (2005), and Schultheiss *et al.* (2005). Cell pole location of early synthesized granules was observed, either by N-terminal fusion of GFP reporter protein to PhaC, C-terminal fusion of a yellow fluorescent

protein to PhaP, or by using Nile red staining of PHA granules. Aside from these two models of PHA granule formation, Tian and co-workers noticed that emerging granule arose from the cell center of wild type *C. necator*, which localized at unknown mediation elements (Tian *et al.*, 2005a,b). They proposed that these mediation elements may act as nucleation sites for P(3HB) granule initiation.

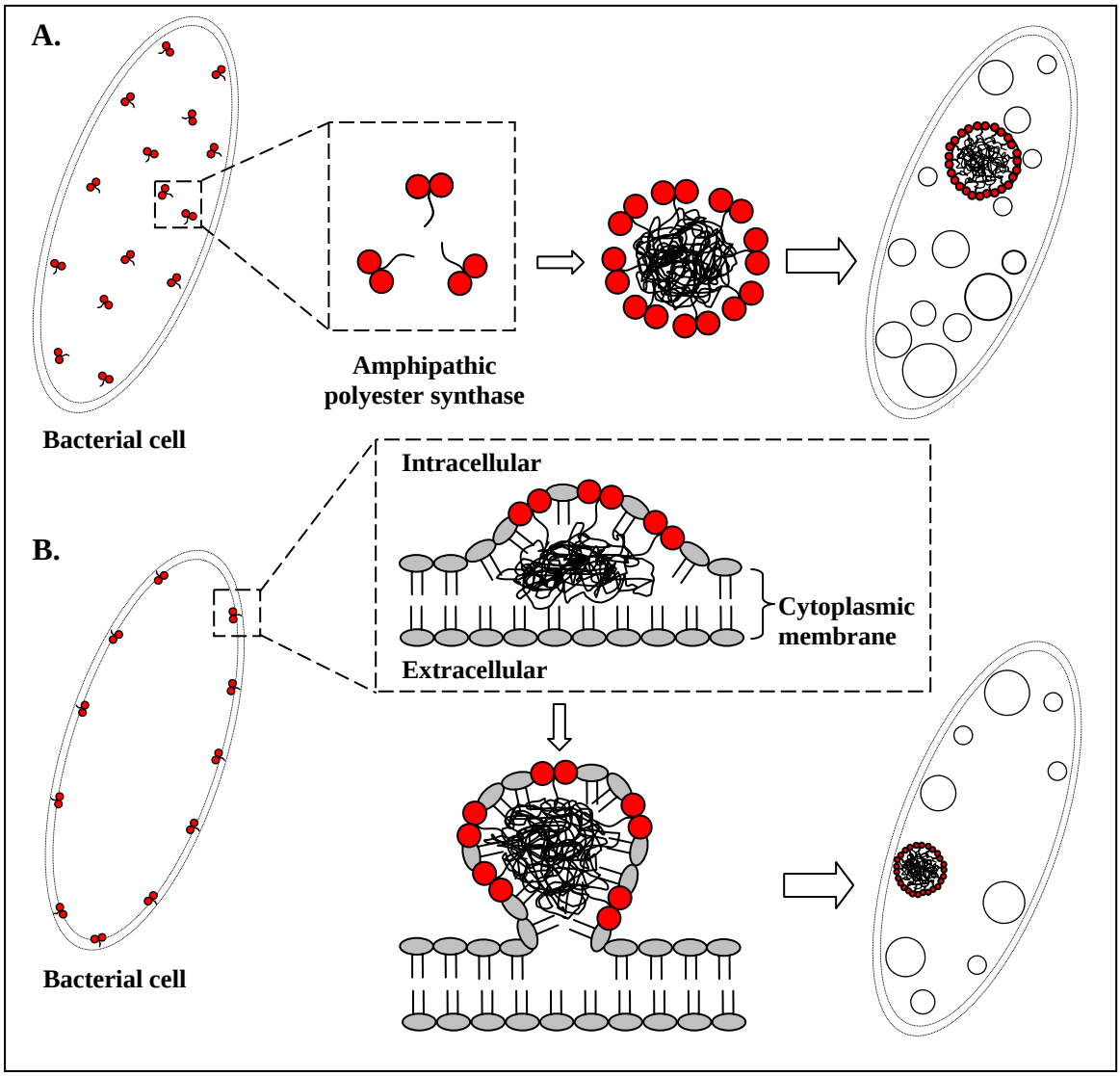


Figure 2.3 Models of PHA granule formation in bacteria. The granule self-assembly process occurs randomly in the cytosol for (A) micelle model whereas near the cytoplasmic membrane for (B) budding model. The irregular lines represent the elongation of polyester chains. ●, soluble polyester synthase; ●●, amphipathic polyester synthase; ⊤, phospholipid of cytoplasmic membrane; ◯, polyester inclusion

2.2 PHA properties and applications

2.2.1 Biodegradability

PHA, the storage materials of numerous microorganisms, is synthesized for eventual breakdown and utilization as carbon source when extracellular carbon source is no longer available. Complete biodegradability of PHA is the most distinguished feature as compared to its petroleum-derived counterpart, which is highly resistant to chemical and enzymatic degradation (Braunegg *et al.*, 1998; Kolybaba *et al.*, 2003). In nature, PHA granules that are liberated by the death and lyses of bacterial cells can be rapidly hydrolyzed to its monomer units by extracellular depolymerase and PHA hydrolases secreted by numerous bacteria and fungi (Jendrossek *et al.*, 1996). Under aerobic conditions, the final products of PHA biodegradation are water and carbon dioxide, whereas under anaerobic conditions, methane is produced as well (Urmeneta *et al.*, 1995). However, the activities of these depolymerase enzymes may vary depending on PHA composition, its physical form (amorphous or crystalline), the dimensions of the PHA sample and more importantly, the environmental conditions (Abe and Doi, 1998; Sudesh *et al.*, 2000; Tokiwa and Calabia, 2004).

For practical applications of biodegradable PHA, its lifetime control, which is strongly dependent on the monomer composition of the polyester, is among the important issue. Cloning and characterization of numerous extracellular depolymerase of different microorganisms, e.g. *P. lemoigne*, *Alcaligenes faecalis*, *Comamonas testosterone*, etc., have been reported (Jendrossek *et al.*, 1993; Kasuya *et al.*, 1994; Bachmann and Seebach, 1999; Schober *et al.*, 2000). These extracellular depolymerases share three common characteristics along the polypeptide chain including a catalytic

domain, a substrate binding domain and a linking region connecting these two domains (Lenz and Marchessault, 2005). Incorporation of additional monomer units, particularly with longer chain length, will significantly accelerate the biodegradation rate of the polyester (Sudesh *et al.*, 2000; Li *et al.*, 2007).

2.2.2 Thermal and mechanical properties

In addition to the biodegradability of PHA, other aspects related to processing are also crucial. Material properties such as thermal stability and viscosity are important for PHA processing by allowing the use of conventional technologies without large adaptations. The distinguishing features of a polymer are determined by its composition, the way in which the monomer units are linked together, the average molecular weight and molecular weight distribution of the polymer. Owing to the diversity of PHA family, their mechanical and thermal characteristics can be tailored to resemble elastic rubber or stiff crystalline plastic (Table 2.1). PHA can be processed by standard extrusion and molding techniques, to produce films, bottles, and other products in various shapes. P(3HB) is a highly crystalline material after it is extracted out from bacterial cells. This type of brittle and stiff polyesters has low stress resistance and a relatively high melting temperature (~170 °C), which is close to its decomposition temperature, thus making processing difficult. However, it was reported that P(3HB) possessing better mechanical properties could be produced by hot-drawing of ultra-high molecular weight P(3HB) from recombinant *E. coli* (Kusaka *et al.*, 1998; Aoyagi *et al.*, 2003). New techniques such as cold-drawing and two-step drawing were also shown to produce films that possess properties similar to common plastics from P(3HB) of relatively low molecular weight (Iwata *et al.*, 2003).

Table 2.1 Thermal, physical and mechanical properties of various PHA (Doi, 1990; Tsuge, 2002; Philip *et al.*, 2007)

Properties	**P(3HB)**	**P(3HB-*co*-20 mol% 3HV)**	**P(3HB-*co*-10 mol% 3HHx**	**P(3HB-*co*-16 mol% 4HB**	**PP**	**LDPE**
Melting temperature (°C)	177	145	127	150	176	130
Glass transition temperature (°C)	4	- 1	- 1	- 7	- 10	- 36
Crystallinity (%)	70	56	34	45	60	20 – 50
Elongation to break (%)	6	50	400	444	400	620
Tensile strength (MPa)	40	25	21	26	38	10

3HB, 3-hydroxybutyrate; 3HV, 3-hydroxyvalerate; 3HHx, 3-hydroxyhexanoate; 4HB, 4-hydroxybutyrate; PP, polypropylene; LDPE, low-density polyethylene

Incorporation of second monomer, 3HV unit, into P(3HB) polyester chain leads to a lower crystallinity and melting temperature besides producing a significant increase in its elongation to break (Table 2.1). However, cocrystallization (isodimorphism) of 3HB and 3HV monomer units in either P(3HB) or P(3HV) crystal lattices depend on the 3HV molar fraction (Bluhm *et al.*, 1986). The lack of flexibility of scl-PHA homo- or copolymers hinders their range of applications. Bacterial polyesters which are composed of both scl- and mcl-monomer units possess desired physical and mechanical properties, depending on the molar fraction of the different monomers in the copolymer (Matsusaki *et al.*, 2000). With the incorporation of small amount of 3HHx units, the copolymer melting point could be reduced to the level well below the thermal decomposition temperature and also shown to exhibit better biocompatibility and mechanical properties than P(3HB) homopolymer and scl-copolymer (Doi *et al.*, 1995; Chen *et al.*, 2001).

2.2.3 Applications

Based on the choice of microorganism, its class of polymerase, carbon feedstock and other cultural conditions, bacterial fermentation allows the synthesis of a wide spectra of PHA with various physico-chemical properties (Sudesh *et al.*, 2000; Aldor and Keasling, 2003; Philip *et al.*, 2007). Various PHA are suitable for low-value high-volume (LVHV) commodity applications, while others are suitable for specific high-value low-volume (HVLV) niche market applications, such as medical or pharmaceutical field applications (Philip *et al.*, 2007).

The flexibility of PHA material properties ensures its wide applications in the industrial sector; particularly for consumer packaging items such as bottles, food or cosmetic containers and wrapping. Due to the gas barrier property of P(3HB-*co*-3HV), applications for making food packaging materials and beverage bottles were also possible. Other target market for these biodegradable polyesters include paper or cardboard coating, disposable nonwoven fabrics and personal hygiene products like diaper back sheets and cotton swabs (Steel and Norton-Berry, 1986; Martini *et al.*, 1989a,b; Marchessault *et al.*, 1995; Gross and Kalra, 2002). For agricultural applications, PHA is frequently used as mulch film which is to modify soil temperature, prevent moisture loss and limit the growth of weed. Nodax^{TM}, a copolymer consisting of primarily 3HB monomer with a small quantity of mcl-monomers is used to manufacture agricultural film and also as a coating for controlled release of urea fertilizer, herbicides and insecticides (Philip *et al.*, 2007).

PHA also possesses crucial characteristics of scaffold material such as biocompatibility, support cell adhesion and cell growth, guide and organize cells and

biodegradability without generation of toxic compounds (Williams *et al.*, 1999). The biocompatibility of PHA; P(3HB), P(3HB-*co*-3HV), P(4HB), P(3HB-*co*-4HB), P(3HB-*co*-3HHx) and P(3HHx-*co*-3HO) have been tested in various host system (Valappil *et al.*, 2006). Tepha is company based in the U.S. that specializes in fabricating artery augments, vascular grafts, heart valves, sutures and microparticulate carrier using PHA (Williams and Martin, 2002).

PHA synthesis by bacterial fermentation using renewable resources has potentially large benefits in environmental aspects particularly. Nevertheless, it remains a relatively young technology that has to compete in an arena in which petroleum-based plastics has been a highly optimized industry for nearly a century. Hence, for the commercialization of PHA, much effort has to be dedicated to reduce the overall production cost by the development of better bacterial strains and more efficient fermentation as well as recovery processes (Lee, 1996; Khanna and Srivastava, 2004; Verlinden *et al.*, 2007).

2.3 Metabolic pathways

In bacterial cell, different carbon substrates are metabolized by many different pathways. The three most studied metabolic pathways involved in the biosynthesis of PHA are shown in Figure 2.4. In general, when sugar is used for bacterial fermentation, it is metabolized via pathway I to synthesize P(3HB) homopolymer; whereas copolymers with a longer chain length are produced from the intermediates of pathway II and III when fatty acids or sugars are supplemented as the carbon substrate (Tsuge, 2002; Aldor and Keasling, 2003; Steinbüchel and Lütke-Eversloh, 2003).

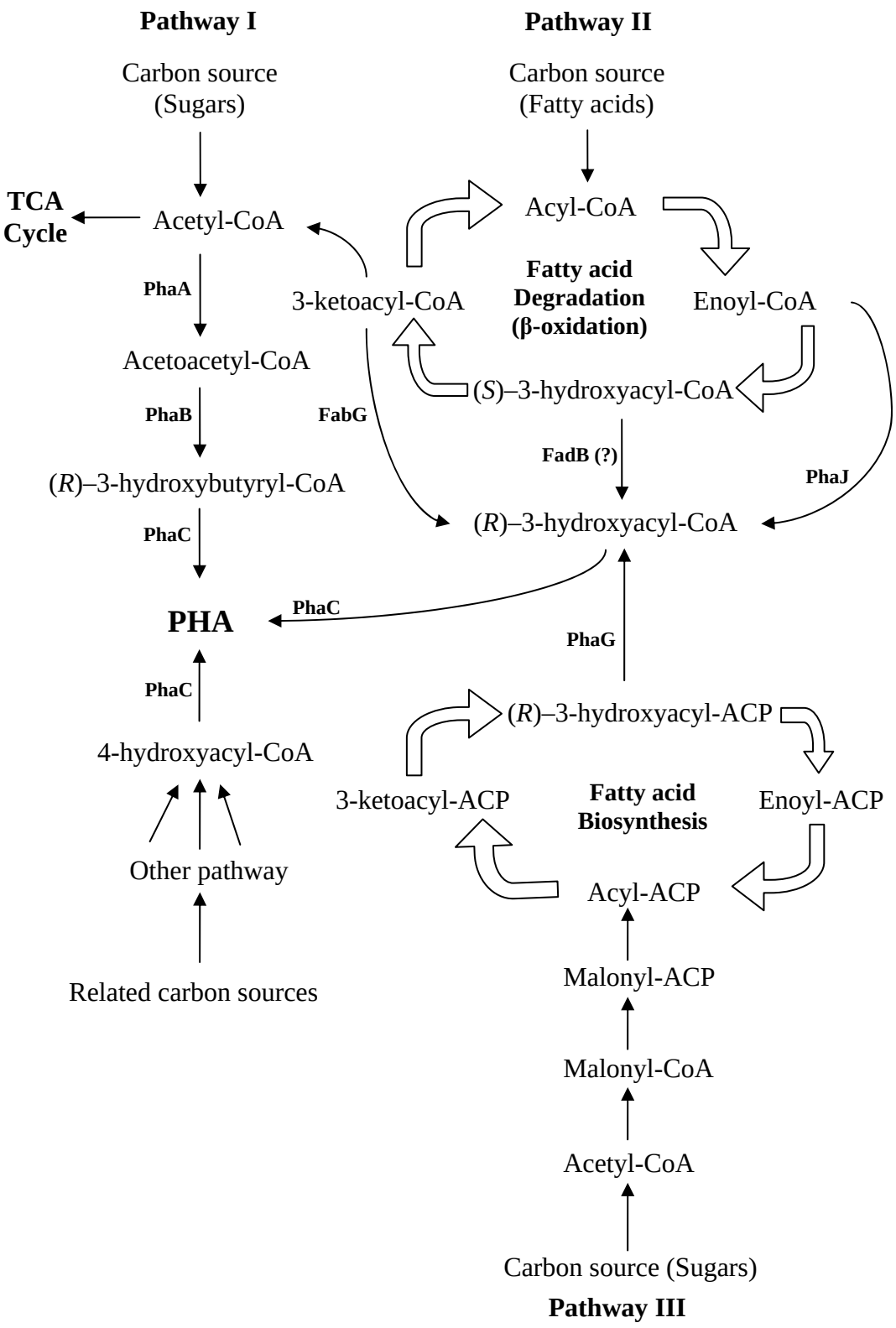


Figure 2.4 The major metabolic pathways involved in supplying monomer units for PHA synthesis

2.5.1 Short-chain-length PHA (scl-PHA)

P(3HB) homopolymer is the most widespread and thoroughly characterized member of PHA found in microorganism. The biosynthesis pathway of P(3HB) has been studied in detail in *C. necator* (Schubert *et al.*, 1988; Slater *et al.*, 1988; Peoples and Sinskey, 1989a,b) and most of our knowledge of P(3HB) synthesis has been obtained from this bacterium (Steinbüchel and Schlegel, 1991). The first enzyme of the P(3HB) synthesis pathway, β-ketothiolase catalyzes the dimerization of acetyl-CoA derived from the metabolism of sugar to acetoacetyl-CoA. The resulting acetoacetyl-CoA is subsequently reduced by NADPH-dependent (*R*)-specific acetoacetyl-CoA reductase to (*R*)-3-hydroxybutyryl-CoA, which is readily polymerized to P(3HB) by PHA synthase. Both NADPH- and NADH-dependent acetoacetyl-CoA reductase activities have been observed in *C. necator* cell extracts, only the former is involved in P(3HB) synthesis pathway (Haywood *et al.*, 1988). The biochemical pathways for the copolymer consists of another scl-monomer units; P(3HB-*co*-3HV) is essentially identical with the three-step pathway mentioned above. P(3HB) and P(3HB-*co*-3HV) biosynthetic pathway differ only in the initial metabolites (Figure 2.5). The first step of P(3HB-*co*-3HV) synthesis involves condensation of acetyl-CoA and propionyl-CoA to form β-ketovaleryl-CoA, which is catalyzed by a distinct 3-ketothiolase; BktB. This enzyme (BktB) has a higher specificity for propionyl-CoA than the 3-ketothiolase encoded by the *phaA* gene (Slater *et al.*, 1998). Reduction of 3-ketovaleryl-CoA to (*R*)-3-hydroxyvaleryl-CoA and subsequent polymerization to form P(3HB-*co*-3HV) are catalyzed by the same enzymes involved in P(3HB) synthesis (Poirier, 2001).

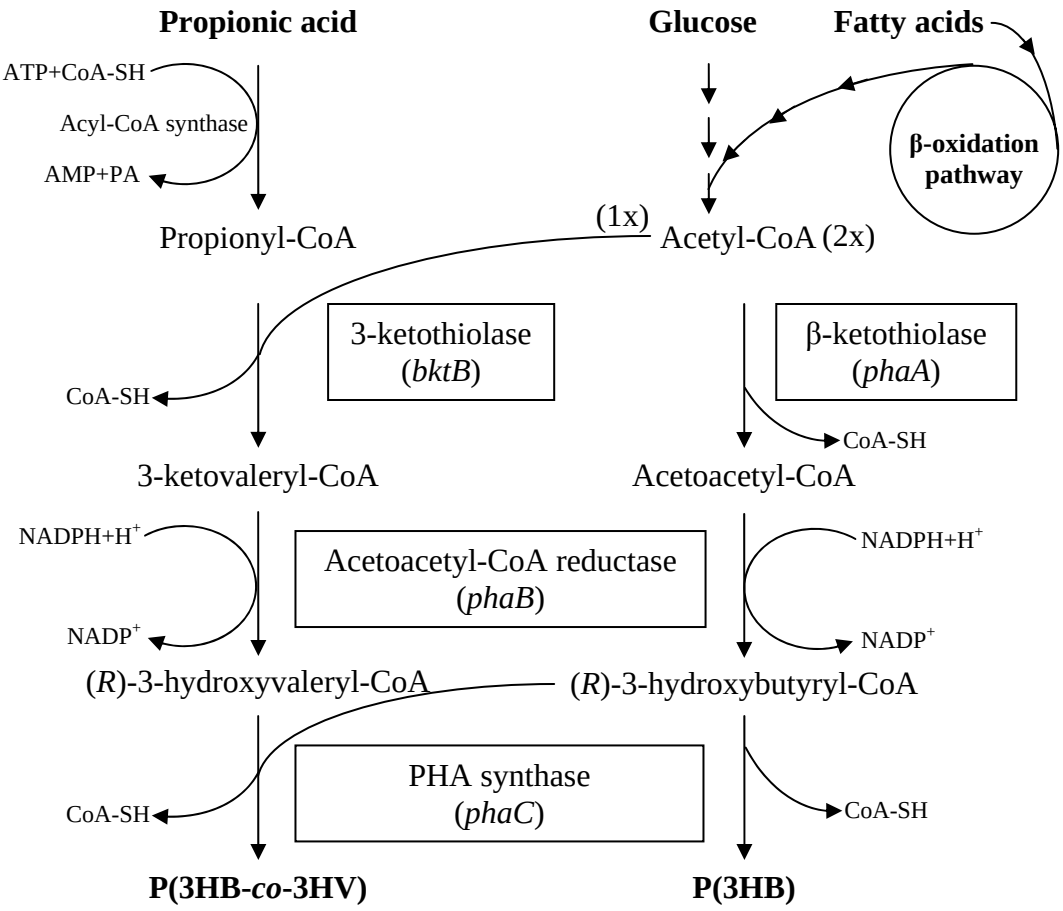


Figure 2.5 Biosynthesis pathways of P(3HB) and P(3HB-*co*-3HV) synthesis in *C. necator*. Formation of acetoacetyl-CoA and 3-ketovaleryl-CoA were catalyzed by *phaA* (β-ketothiolase) and *bktB* (3-ketothiolase) respectively. The subsequent reactions involve reduction by *phaB* (acetoacetyl-CoA reductase) and polymerization of (*R*)-3-hydroxybutyryl-CoA and (*R*)-3-hydroxyvaleryl-CoA monomers by *phaC* (PHA synthase) into respective polymer chains

2.5.2 Medium-chain-length PHA (mcl-PHA)

A number of mcl-PHA is synthesized by fluorescent pseudomonads of the rRNA homology group I when fatty acids or other aliphatic carbon sources are served as the carbon substrate. Analysis of the co-monomer composition of mcl-PHA shows their direct relationship with the structure of the growth substrate (Brandl *et al.*, 1988; Lageveen *et al.*, 1988; Huisman *et al.*, 1989); the length of the resulting monomer units

are of the same length as the carbon source or have been shortened by 2n (n≥0) carbon atoms. Hence, it was suggested that the mcl-PHA biosynthesis pathway is a direct branch of the fatty acid β-oxidation pathway (Lageveen *et al.*, 1988). After the transportation of fatty acids into the cell, they are activated by the acyl-CoA synthetase (*fadD* gene product) forming the corresponding acyl-CoA thioesters. These acyl-CoAs are catabolized via β-oxidation pathway by the removal of C2 (2 carbon atoms) units as acetyl-CoA per cycle. The pathway intermediates; trans-2-enoyl-CoA, (*S*)-3-hydroxyacyl-CoA and 3-ketoacyl-CoA with different number of carbon atom are supposed to be precursors for the mcl-PHA synthesis. However, these intermediates are not in the ultimate form favorable for the PHA synthase and thus an additional step is required for synthesis of the (*R*)-3-hydroxyacyl-CoA monomer for the synthesis of the mcl-PHA. Enzymes such as (*R*)-specific enoyl-CoA hydratase, hydroxyacyl-CoA epimerase and β-ketoacyl-CoA reductase have been postulated connecting the β-oxidation pathway with the mcl-PHA biosynthesis (Madison and Huisman, 1999; Luengo *et al.*, 2003; Rehm, 2007). In *A. caviae*, a gene encoding enoyl-CoA hydratase ($phaJ_{Ac}$) has been identified as an additional enzyme that indirectly contributes to PHA synthesis. Product of PhaJ catalyzes (*R*)-specific hydration of 2-enoyl-CoA for supplying (*R*)-3-hydroxyacyl-CoA monomer units for PHA synthesis (Fukui and Doi, 1997; Fukui *et al.*, 1998).

The second route for the biosynthesis of mcl-PHA in microorganism is through the use of intermediates of *de novo* fatty acid biosynthesis pathway. Carbohydrates or sugars can be used as the carbon substrate for the synthesis of mcl-PHA, but the composition of these PHA is not related to the carbon source. When pseudomonads of

rRNA homology group I are grown on sugars, mcl-PHA consisting primarily of C10 and C8 monomers is produced (Haywood *et al.*, 1990; Timm and Steinbüchel, 1990; Huijberts *et al.*, 1992). This indicates that the *de novo* fatty acid biosynthesis pathway is connected to PHA biosynthesis pathway whereby the PHA monomer units are derived from intermediates of fatty acid biosynthesis (Madison and Huisman, 1999). *Pseudomonas* strains such as *P. oleovorans*, *P. fragii*, *P. aeruginosa* and *P. putida* can synthesize mcl-PHA from related alkanoic acids present in the growth media. In contrast to *P. oleovorans*, *P. fragii*, *P. aeruginosa* and *P. putida* are capable of synthesizing similar type of mcl-PHA when grown on unrelated substrates, such as fructose, glucose or other substrates that require *de novo* fatty acid biosynthesis (Haywood *et al.*, 1990; Timm and Steinbüchel, 1990). Metabolic pathway involved in fatty acid biosynthesis generates many intermediates activated by acyl carrier protein (ACP), and thus, another new enzymatic activity is required for their conversion to (*R*)-3-hydroxyacyl-CoA (Rehm *et al.*, 1998; Park *et al.*, 2005). *phaG*, which encodes an (*R*)-3-hydroxydecanoyl-ACP:CoA transacylase, was found to be the key link between fatty acid synthesis and mcl-PHA biosynthesis. A *phaG* has been cloned from *P. putida* and this enzyme catalyzes the conversion of (*R*)-3-hydroxyacyl-ACP to its corresponding CoA derivatives (Rehm *et al.*, 1998; Hoffmann *et al.*, 2000a,b). This enzyme is only required when non-related carbon substrate which will ultimately be metabolized to acetyl-CoA; sugars, acetate, ethanol, etc., is supplemented as the carbon substrate for bacterial fermentation.

2.4 Carbon sources for PHA biosynthesis

PHA production by microbial fermentation is perfectly integrated into nature's closed cycle of carbon attributable to its renewable nature (Braunegg *et al.*, 2004; Sudesh and Iwata, 2008). PHA synthesis is based on renewable resources; agricultural products such as sugars and fatty acids are commonly used as the carbon and energy sources (Kadouri *et al.,* 2005; Solaiman *et al.,* 2006). These agricultural feedstocks are derived from carbon dioxide (CO_2) and water. After their bioconversion to biodegradable PHA and the usage periods, their final oxidative breakdown merely yields CO_2 and water.

2.4.1 Sugars

For conventional PHA production, sugars such as glucose, fructose and sucrose are the most common substrates supplemented to the bacterial culture as the main carbon source. The industrial-scale production of PHA using 200,000-L stirred fermentation vessels began in the 1970s by Imperial Chemical Industries (ICI, London, Great Britain). After the attempts using Methylotrophic bacteria and *Azotobacter*, *C. necator* finally became the production organism of choice for P(3HB) production by using fructose as the sole carbon source (Byrom, 1987). With co-feeding of propionate, a glucose-utilizing mutant strain of *C. necator* (ATCC 11599) was employed by Monsanto to synthesize P(3HB-*co*-3HV), a copolymer with a better flexibility and impact resistance, which was marketed under the trade name Biopol^{TM}. The first large scale production of P(3HB-*co*-3HHx) copolymer from glucose was performed using *Aeromonas hydrophila* 4AK4 in 20,000-L fermentor. Sucrose was also used as the sole

carbon source by Chemie Linz GmbH (Linz Australia) for the production of P(3HB) at up to 1000 kg per week by employing *Alcaligenes latus* (Hanggi, 1990; Hrabak, 1992).

2.4.2 Triglycerides

For an economically feasible production process for PHA synthesis, there have been considerable interests in using inexpensive carbon substrate as an alternative to sugars (Lee *et al.*, 1999; Tsuge, 2002; Solaiman *et al.*, 2006). Various plant oils and their derived fatty acids have emerged as superior candidates for PHA production by various scl- and mcl-PHA producers. PHA accumulation of more than 80 wt% of DCW was reported when palm kernel oil, corn oil, coconut oil and olive oil was supplemented as the sole carbon source. Besides, a range of commercially important vegetable oils were also found to be feasible for the bioconversion to scl-, mcl- or scl-mcl-PHA (Fukui and Doi, 1998; Solaiman *et al.*, 1999; Kahar *et al.*, 2004; Loo *et al*., 2005; Thakor *et al.*, 2005). Vegetable oils, which are composed of a much higher number of carbon atoms per weight as compared to sugars, gave a higher theoretical yield coefficient; over 1 g PHA per 1 g of vegetable oil used (Akiyama *et al*., 2003).

2.4.3 Industrial waste stream and by-products

The idea of using various agricultural and industrial waste streams or by-products as the fermentative substrate for PHA synthesis has piqued interest among researchers due to their availability and renewable merits. There have been several reports on the production of P(3HB) from waste or residual materials of agro-industrial sector by wild-type P(3HB) producers. Cheese whey, xylose, molasses and bagasse or starch hydrolysate are among the inexpensive carbon substrates used for biosynthesis of P(3HB) (Lee, 1998; Gouda *et al.*, 2001; Quillaguaman *et al.*, 2005; Chen *et al.*, 2006;

Nath *et al.*, 2008; Yu and Stahl, 2008). Other oily substrates such as tallow, waste frying oil and its derived fatty acids were also found to be feasible for the bioconversion into PHA (Cromwick *et al.*, 1996; Solaiman *et al.*, 1999; Taniguchi *et al.*, 2003; Fernandez *et al.*, 2005). Bioconversion of renewable and cheap waste or by-products can offer multiple benefits to the environment by diverting high pollution potential products to the synthesis of value-added green materials.

2.5 PHA synthases

PHA synthase, PhaC is the key enzyme of PHA biosynthesis which catalyzes the enantio-selective conversion of (*R*)-3-hydroxyacyl-CoA monomers into water-insoluble PHA while concomitantly releasing CoA-SH (Rehm, 2003). To date, more than 59 different PHA synthases have been sequenced and cloned (Rehm and Steinbüchel, 1999; Rehm and Steinbüchel, 2001; Rehm *et al.*, 2002). With respect to primary structure, subunit composition and substrate specificity, four major classes of PhaC have been proposed (Table 2.2). For the *phaC* products of Class I and Class II, they consist of only one subunit (PhaC) with molecular weight between 61 – 73 kDa (Qi and Rehm, 2001). Class I PhaC, which can be represented by *C. necator*, preferentially utilize CoA thioesters of various (*R*)-3-hydroxyalkanoic acids comprising 3 – 5 carbon atoms; whereas Class II PhaC (represented by *P. aeruginosa*) preferentially polymerize CoA thioesters of various (*R*)-3-hydroxyalkanoic acids comprising 6 – 14 carbon atoms (Ren *et al.*, 2000; Rehm, 2003). On the other hand, Class III PhaC consist of two different types of subunits; PhaC (~40 kDa) and PhaE (~40 kDa) can be found in PHA-producing strains such as *Allochromatium vinosum*, *Thiocystis violacea*, *Thiocapsa pfennigii* and *Synechocystis* sp. PCC 6803 (Sudesh *et*