PRODUCTION AND CHARACTERIZATION OF POLY(3-HYDROXYBUTYRATE-*co*-4-HYDROXYBUTYRATE) COPOLYMER

BY

RAHAYU BINTI AHMAD

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ABBREVIATIONS

C/N	Carbon/Nitrogen
3HA	3-hydroxyalkanoate
3HB-CoA	3-hydroxybutyryl-CoA
3HV	3-hydroxyvalerate
4HB	4-hyxroybutyrate
4HB-CoA	4-hydroxybutyryl-CoA
CME	Caprilic acid methyl ester
CPO	Crude palm oil
DO	Dissolved oxygen
DSC	Differential scanning calorimetry
FID	Flame ionization detector
g/L	weight/volume
GC	Gas chromatography
GPC	Gel permeation chromatography
HA	Hydroxyacyl
MCL	Medium-chain-length
M_n	Number-average molecular weight
MPa	Mega Pascal
MSM	Mineral salts medium
M_w	Weight-average molecular weight
NA	Nutrient agar
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NB	Nutrient broth
NMR	Nuclear magnetic resonance
OA	Oleic acid
P(3HB)	Poly(3-hydroxybutyrate)
P(3HB-*co*-3HHx)	Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate)
P(3HB-*co*-3HV)	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)

P(3HB-*co*-4HB)	Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate)
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
PKO	Palm kernel oil
PS	Palm stearin
Rpm	Revolution per minute
SCL	Short-chain-length
TCA	Tricarboxylic acid cycle
T_g	Glass transition temperature
T_m	Melting temperature
V	Vesawit
v/v	volume/volume
vvm	volume/volume/minute

PENGHASILAN DAN PENCIRIAN KOPOLIMER POLI(3-HIDROKSIBUTIRAT-*ko*-4-HIDROKSIBUTIRAT)

ABSTRAK

Pencilan bakteria tempatan, *Cupriavidus* sp. USMAA2-4 mempunyai kebolehan menghasilkan polimer biodegradasi telah digunakan dalam kajian ini untuk mengkaji penghasilan biopolimer melalui proses pengkulturan satu peringkat dan fermentasi sesuapan kelompok. Pelbagai sumber karbon berasaskan minyak kelapa sawit, asid lemak serta kombinasinya telah digunakan untuk meningkatkan berat kering sel bakteria *Cupriavidus* sp. USMAA2-4. Penggunaan asid oleik sebagai sumber karbon tunggal menghasilkan berat kering sel tertinggi iaitu 8.4 g/L. Kesan nisbah sumber karbon kepada nitrogen (C/N) terhadap pengumpulan polimer P(3HB) sebelum suapan γ-butyrolactone menunjukkan peningkatan komposisi 4HB yang signifikan iaitu daripada 9 hingga 55 mol% apabila nisbah C/N menurun daripada 20 ke 8. Komposisi monomer 4HB yang pelbagai daripada 4 ke 55 mol% diperolehi apabila karbon prrekursor bernombor genap seperti 1,4-butanadiol, 1,6-hexanadiol, 1,8-octanadiol, 1,10-decanadiol and 1,12-dodecanadiol digunakan. Strategi suapan kaedah sela masa berjaya meningkatkan berat kering sel sehingga 30 dan 31.1 g/L masing-masing berdasarkan 12 dan 6 jam sela masa suapan, tetapi komposisi 4HB gagal untuk dipelbagaikan. Suapan C/N 5 pada fasa pertumbuhan dan suapan pelbagai kepekatan γ-butirolakton pada fasa pengumpulan polimer menerusi kaedah 'DO-stat' telah berjaya menghasilkan komposisi 4HB yang pelbagai. P(3HB-*ko*-4HB) yang mempunyai komposisi 35-68 mol% berjaya dihasilkan dengan kaedah ini. Pencirian sifat fizikal dan mekanikal bagi kopolimer P(3HB-*ko*-4HB) telah dilakukan melalui spektroskopi resonans nukleus (NMR),

kromatografi penjerapan gel (GPC), ujian tensil dan kalorimetri pengimbasan pembezaan (DSC). Peningkatan komposisi 4HB telah merendahkan berat molekul purata-nombor kopolimer (M_n) iaitu dalam lingkungan 31 x 10^3 hingga 441 x 10^3 Dalton dengan indeks polidispersiti (M_w/M_n) antara 1.2 hingga 2.5. Analisis kerawakan polimer mengesahkan bahawa polimer yang dihasilkan melalui kaedah DO-stat adalah kopolimer campuran. Kekuatan tensil meningkat dari 21 kepada 33 Mpa dengan pemanjangan untuk putus mencecah 14% apabila komposisi 4HB meningkat. Walau bagaimanapun, kepelbagaian pecahan 4HB tidak menunjukkan sebarang perbezaan yang ketara pada suhu melebur (T_m) iaitu di antara 166 hingga 170 °C dengan suhu peralihan kaca (T_g) berada dalam lingkungan 5 hingga -45°C.

PRODUCTION AND CHARACTERIZATION OF POLY(3-HYDROXYBUTYRATE-*co*-4-HYDROXYBUTYRATE) COPOLYMER

ABSTRACT

A locally isolated *Cupriavidus* sp. USMAA2-4 possessing the ability to produce biodegradable polymer was used in this study to explore the production of biopolymer through one-stage cultivation process and fed-batch fermentation. Various palm oil-based carbon sources, fatty acids and its combination were used for producing high cell dry weight of *Cupriavidus* sp. USMAA2-4. Utilization of oleic acid solely yielding the highest cell dry weight with 8.4 g/L. Effects of carbon to nitrogen (C/N ratio) on the accumulation of P(3HB) before feeding of carbon precursor (γ-butyrolactone) exhibited significant increased of 4HB compositions from 9 to 55 mol% when C/N ratio decreased from 20-8. The monomer composition of 4HB also varied from 4 to 55 mol% when alkanediols with even carbon numbers such as 1,4-butanediol, 1,6-hexanediol, 1,8-octanediol, 1,10-decanediol and 1,12-dodecanediol were used. Feeding strategy of interval feeding time could increased the cell dry weight up to 30 and 31.1 g/L according to 12 and 6 h interval of feeding time respectively, but the 4HB compositions unable to be varied. Feeding of C/N 5 at growth phase and supplementing different concentrations of γ-butyrolactone in accumulation phase using DO-stat method had successfully produced various 4HB compositions ranging from 35 to 68 mol% with the cell dry weight increased up to 14.9 g/L. The physical and mechanical properties of P(3HB-*co*-4HB) copolymers were characterized by NMR spectroscopy, gel-permeation chromatography, tensile test and differential scanning calorimetry. Increase in the 4HB compositions lowered the molecular weight (M_n) of these copolymers which ranging

from 31 x 10^3 to 441 x 10^3 Dalton and the polidispersities (M_w/M_n) were between 1.2 to 2.5. The randomness analysis using NMR spectroscopy confirms that the polymers produced using DO-stat method were blend copolymer. Tensile strength increase from 21 to 33 MPa with the elogation to break up to 14% as the 4HB composition increase. However, no significant differences were observed in the melting temperature (T_m) (170-166°C) with glass transition temperature (T_g) ranging from 5 to -45°C.

1.0 INTRODUCTION

1.1 Discovery of bacterial polyesters

With the dawn of the 21st century, a global pandemic of the escalating problem of plastics disposal has landed upon us. Disposal of plastics, particularly used for packaging, is a serious problem confronting many countries. Therefore, the awareness of the need to consider the types of plastics, which are kinder to our environment, is those becoming critical view in our population. In response to the problem and harmful effects of the plastic wastes on the environment, there is considerable interest in the development of biodegradable plastics (Poirier *et al.*, 1995). The plastic materials have to be classified as biodegradable and are able to be synthesized from renewable sources.

A partial solution lays in using biodegradable plastics namely polyhydroxyalkanoates (PHAs) which can be produced through bacterial fermentation (van Wegen *et al.*, 2001). Polyhydroxyalkanoates (PHAs) are a family of biodegradable polymers that are being considered for usage in commercially manufactured plastics on an industrial scale. There are numerous types of prokaryotes that have the capability to accumulate PHAs as water-insoluble inclusions in the cytoplasm or simply known as PHA granules. These granules are produced inside the bacterial cells as a result of the activity of PHA synthases, which are considered as the key enzymes of PHA biosynthesis (Tsuge, 2002; Steinbüchel and Lütke-Eversloh, 2003). PHAs are actually common carbon and energy storage materials that are synthesized under unbalanced growth conditions (Pötter and Steinbüchel, 2006) and accumulated to levels 90% of the cell dry weight (Madison and Huisman, 1999). Various types of PHAs in the form of homopolyesters, copolyesters or polyesters blends can be produced by microorganisms (Steinbüchel, 2001). These diverse types of PHAs are due to the different carbon

precursors fed to the microorganisms, as well as the substrate specificity of PHA synthases which involved in the metabolic pathway in the cells (Sudesh *et al.*, 2000; Loo and Sudesh, 2007). Continuously, over 150 different hydroxyalkanoic acids have been identified as PHA constituents (Pötter and Steinbüchel, 2006; Steinbüchel and Lütke-Eversloh, 2003).

The fermentative production of PHAs is normally operated as a two-stage fed-batch process (Doi, 1990). In two-stage cultivation, cell biomass from nutrient broth is transferred to a second step in which the medium is usually nitrogen-limited or nitrogen-free (Sudesh and Doi, 2000). In the second step, suitable precursor carbon is added to promote the accumulation of desired polymer. Previous research has reported that two-stage cultivation is one of the effective methods for the production of P(3HB-*co*-4HB) by *Cupriavidus* sp. USMAA2-4 (Amirul *et al.*, 2004). P(3HB-*co*-4HB) accumulation is significantly promoted with the limitation of nitrogen and oxygen in the culture medium during the second stage of the cultivation process (Amirul *et al.*, 2004).

One of the major problems preventing the commercial application of PHAs is their high cost of production. From an economical point of view, the cost of carbon sources (mainly substrate) and cultivation stage may contribute significantly to the overall cost production of PHA (Lee *et al.*, 1995). Therefore much effort has been devoted to reduce the cost of PHA by improving and developing more efficient fermentation process and utilization of alternative carbon sources for PHA production. One-step and fed-batch cultivation process can be one of the ways to reduce the cost of producing PHA. Instead of reducing the cost of bacterial culture enrichment, one-stage cultivation process will also shorten the cultivation time. Fed-batch fermentation with a proper feeding strategy enables to increase the cell dry weight, PHA yields as well as the

composition of the copolymer (Choi and Lee, 1999; Poirier *et al.*, 1995). This study focused on the usage of alternative carbon sources and development of feeding strategies with various kind of monomer compositions particularly 4HB monomer. The production of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] copolymer was carried out through fed-batch fermentation process by locally isolated bacterium, *Cupriavidus* sp. USMAA2-4. The effort on the development of feeding strategy was mainly due to the issue of obtaining various types of 4HB monomer compositions, which is crucial in the view of medical and pharmaceutical application.

1.2 Objectives

1. To study the growth characteristics of *Cupriavidus* sp. USMAA2-4 and the production of P(3HB-*co*-4HB) copolymer in shake flask cultivation.
2. To produce P(3HB-*co*-4HB) copolymer with various 4HB composition through fed-batch fermentation.
3. To characterize the thermal, randomness, molecular weight and mechanical properties of P(3HB-*co*-4HB) with various 4HB compositions.

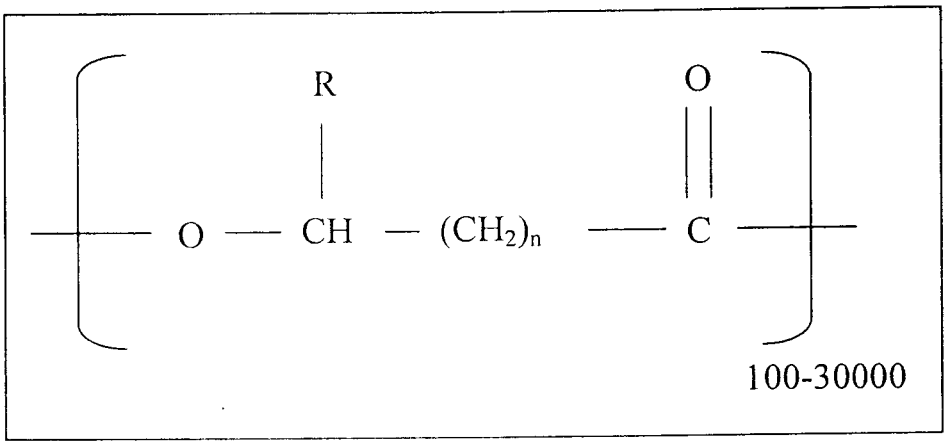
2.0 Literature review

2.1 Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHAs) are polyesters of various hydroxyalkanoates (HAs). They are a biodegradable and biocompatible thermoplastic which are synthesized by numerous microorganisms from almost all genera of the microbial kingdom (Steinbüchel, 2001). PHA are optically active microbial polyesters which contain hydroxyacyl (HA) monomer units (Anderson and Dawes, 1990). Many microorganisms synthesize PHAs as intracellular carbon and energy reserve materials (Anderson and Dawes, 1990). PHAs are usually accumulated within cells which experience the limitation of nutrients such as nitrogen, oxygen, and other essential elements while in the presence of excess carbon source (Anderson & Dawes, 1990; Steinbüchel & Füchtenbusch, 1998; Reddy *et al.*, 2003). Instead of being consumed for cellular growth, the excess carbon is taken into the cells and stored in the form of PHA granules.

PHA granule which exist as discrete inclusions of typically 0.2 ± 0.5 mm in diameter are localized in the cell cytoplasm and can be visualized with a phase contrast light microscope due to their high refractivity (Dawes and Senior, 1973). PHA possesses properties similar to various synthetic thermoplastics like polypropylene and hence can be used as a replacement of synthetic plastics. Most of the various PHAs that have been elucidated are mainly linear; head-to-tail polyesters composed of 3-hydroxy fatty acid monomers (Ojumu *et al.*, 2004). In the construction of these polymers, ester bonds are bonded between the carboxyl groups of one monomer with the hydroxyl group of the adjacent monomer (Anderson and Dawes, 1990). The hydroxyl-substituted carbon atom is found to be the (*R*) configuration (Madison and Huisman, 1999). PHA undergoes complete degradation to water and carbon dioxide under aerobic conditions and to

methane under anaerobic conditions by microorganisms in soil, sea, lake water and sewage (Jendrossek, 2001; Khanna and Srivastava, 2005a). Figure 2.1 shows the general structure of PHA.



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

Figure 2.1: The general chemical structure of monomer of PHAs (Lee, 1996a).

2.2 Types of PHA

There are various types of PHA produced as distinctly shown by their different structures and properties such as melting temperature, crystallinity and flexibility. All these characteristics depend on the taxanomic position and physiological-biochemical properties of the producing microorganisms, culture conditions, and type of carbon sources applied. To date, approximately 150 different constituents are known to occur either as homopolyesters or in combination as copolyesters (Steinbüchel and Valentin, 1995). Poly(3-hydroxybutyrate) [P(3HB)] which contains repeated unit of (*R*)-3HB is the most common type of PHA produced by bacteria in nature (Kimura *et al.*, 2008). Owing to its inherent properties of brittleness and thermal instability above melting point, this P(3HB) monomer units has several deficiencies as an engineering material (Kimura *et al.*, 2008). The incorporation of other monomer units makes this P(3HB) more useful with extravagant features and can be applied into many fields of application (Sudesh, 2000; Reddy *et al.*, 2003;).

Depending on the number of carbon atoms in the chain, PHAs can be classified into two groups namely short-chain-length PHA (SCL-PHA), which consists of 3-5 carbon atoms and medium-chain-length PHA (MCL-PHA), which consists of 6-14 carbon atoms (Anderson and Dawes, 1990). The difference in the length of carbon chain is mainly due to the substrate specificity of the PHA synthase that can accept 3HA of a certain range of carbon length (Anderson and Dawes, 1990; Brandl *et al.*, 1988). To modify the properties of P(3HB), a series of copolymers listed as poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] (Holmes, 1985; Doi *et al.*, 1988), poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] (Kunioka *et al.*, 1988), poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHX)] (Doi *et al.*,

1995) have been produced by a wide variety of bacteria with respect to specific carbon sources. Among these copolyesters, P(3HB-*co*-4HB) copolymer has shown a great potential for medical and pharmaceutical usage. The copolymers having various physical properties from being highly crystalline to elastic are due to the incorporation of various 4HB monomer compositions (Saito and Doi, 1994). As a result, high 4HB monomer composition in P(3HB-*co*-4HB) will yield very strong thermoelastomers which shows great promise for biomedical application (Williams and Martin, 2002; Martin and Williams, 2003).

2.3 Biosynthesis of PHA

In recent years, it has become evident that various metabolic pathways can contribute to the generation of hydroxyalkanoate monomers for the PHA biosynthesis (Taguchi *et al.*, 2002). Most of the bacteria in nature are able to synthesize PHA containing P(3HB) monomer units from various carbon sources which will be converted into acetyl-CoA (Steinbüchel and Lütke-Eversloh, 2003). Generally, the biosynthesis of PHA comprises two essential steps, which include the uptake and conversion of carbon sources into precursor molecules that are subsequently polymerized into monomer units by the PHA synthase enzyme (Taguchi *et al.*, 2002; Sudesh and Doi, 2000).

The biosynthesis of PHA by microorganisms is largely dependent on the type of carbon sources available. Carbon substrates utilized by bacteria range from inexpensive, complete waste effluents like beet/cane molasses (Page, 1992) to plant oils (Fukui and Doi, 1998) and its fatty acids (Tan *et al.*, 1997). The uptake and conversion is started when carbon sources supplied is assimilated into the cell and being metabolized into various intermediate compounds of which acetyl-coenzymeA (acetyl-CoA) is the

universal intermediate present in any organism (Sudesh and Doi, 2000). Acetyl-CoA is converted into P(3HB) by a sequence of three enzymatic reactions. 3-ketothiolase enzyme first catalyzes the reversible condensation of acetyl-CoA to acetoacetyl-CoA. The intermediate is then reduced to (*R*)-3-hydroxybutyryl-CoA by NADPH-linked acetoacetyl-CoA reductase. The P(3HB) is subsequently generated through the polymerization of (*R*)-3-hydroxybutyryl-CoA by P(3HB) synthase (Doi, 1990; Sudesh and Doi, 2000). Sudesh and co-workers (2000) also reported that instead of carbon sources supplied and metabolic pathways of the bacteria, other intermediate compounds can also be generated as intermediate substrates for the polymerizing enzyme, PHA synthase.

In one-step cultivation process of PHA production, the regulation of substrate concentration in growth phase is crucial in order to avoid the unbalanced conditions of the culture. During unbalanced conditions, the levels of NADH and acetyl-CoA increases resulting in the decreases of free coenzyme A. Activity of 3-ketothiolase is no longer inhibited by the coenzyme A and the synthesis of P(3HB) begins. Meanwhile, in balanced growth conditions, the acetyl-CoA are channeled to the tricarboxylic acid cycle (TCA cycle), causing the increases amount of the free coenzyme A. The presence of high coenzyme A inhibits the activity of 3-ketothiolase, thus stops the synthesis of P(3HB) (Doi, 1990; Loo and Sudesh, 2007). Figure 2.2 shows the metabolic pathway involved in the synthesis of P(3HB).

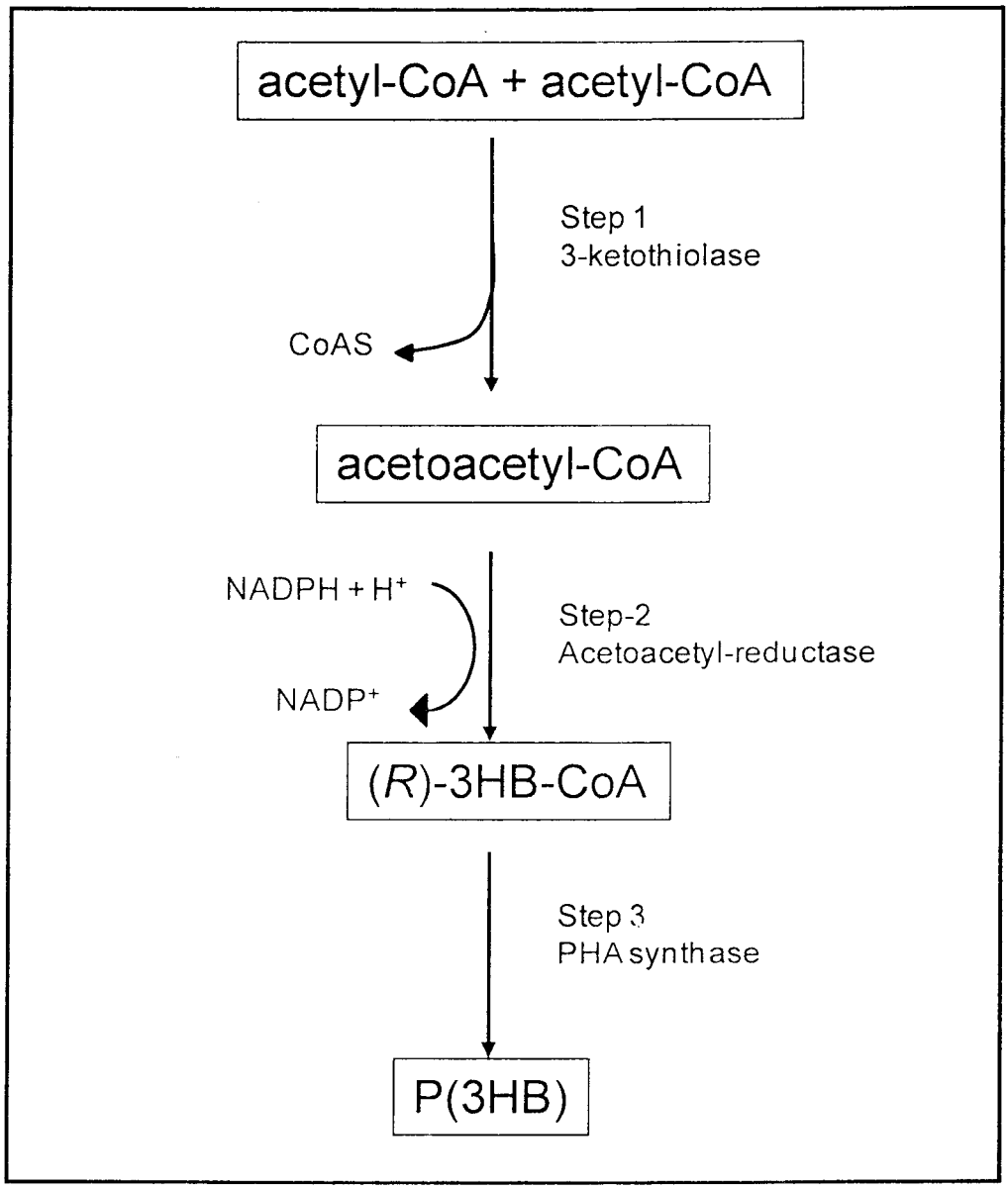


Figure 2.2: Metabolic pathway involved in the synthesis of P(3HB) (Doi, 1990).

2.4 PHA synthase

The key enzyme of PHA biosynthesis is PHA synthase which catalyzes committed steps of the various PHA biosynthetic pathways. All the PHA synthases that have been studied are based on their substrate specificity (Sudesh and Doi, 2000). To date, more than 60 PHA synthases genes (*pha*C) from eubacteria have been cloned and sequenced, and many more PHA synthase sequences have been revealed due to homology searches in prokaryotic genome sequence data banks (Rehm and Steinbüchel, 1999; Steinbüchel and Hein, 2001; Rehm and Steinbüchel, 2002). The natural substrate of this key enzyme is coenzyme A thioesters of (*R*)-hydroxyalkanoic with the hydroxyl group at position 3, 4, 5 or 6 of the acyl moiety, with a large variety of substituents (Steinbüchel and Valentin, 1995). Throughout the accumulation phase of PHA, PHA synthases are bound to the surface of the PHA granules (Haywood *et al.*, 1989) together with other proteins such as phasins (Pieper-Fürst *et al.*, 1994; Steinbüchel *et al.*, 1995) and specific regulator proteins which are probably the most important protein (York *et al.*, 2002; Pötter *et al.*, 2002).

PHA synthases can be classified into two groups and the first group represented by that of *Cupriavidus eutropha* (previously known as *Ralstonia eutropha*) which can polymerize only scl-(*R*)-3-hydroxyacyl-CoAs consisting of 3-5 carbon atom (Sudesh and Doi, 2000). This also includes in many cases of 4- and 5-hydroxyacyl-CoAs. Meanwhile, the second group of PHA synthases is represented by the pseudomonads and these PHA synthases are found to be efficient in polymerizing mcl-(*R*)-3-hydroxyacyl-CoAs containing 6-14 carbon atoms (Lavegeen *et al.*, 1988; Huisman *et al.*, 1989). Matsusaki and co-workers (1998) have revealed investigation at molecular level that *Psuedomonas* sp. 61-3 contains three different PHA synthases. Of which, only one synthesizes P(3HB)

homopolyesters while the other two are capable of incorporating 3HB as well as $3HA_{MCL}$ of up to 12 carbon atoms (Matsusaki *et al.*, 1998).

2.5 PHA biosynthetic pathway

The naturally occurring pathways of PHA biosynthesis are relying on three different pathways. Pathway I, is the most common pathway occurs in a wide range of bacteria which generates *R*-3HB monomers from acetyl-CoAs (Tsuge, 2002). In this pathway, PHA is synthesized in a three-step reaction starting with acetyl-CoA. The first step involves two molecules of acetyl-CoA which are condensed in a reaction catalyzed by 3-ketothiolase enzyme (PhaA) to form acetoacetyl-CoA. Then, the acetoacetyl-CoA generated is stereoselectively reduced to (*R*)-3-hydroxybutyryl-CoA by a NADPH-dependent acetoacetyl-CoA reductase (PhaB). Finally, the (*R*)-3-hydroxybutyryl-CoA monomers are polymerized by PHA synthase, releasing PHA and free CoA as end products. The (*R*)-3-hydroxybutyryl-CoA in the form of (*R*)-isomers only can be polymerized by PHA synthase (PhaC). Pathway II mainly generates mcl-(R)-3HA monomers from fatty acid β-oxidation intermediates and most of the *Pseudomonads* rely on this pathway. In this pathway, fatty acid is converted by some specific enzymes through β-oxidation to generate (*R*)-3-hydroxyacyl-CoAs. These metabolites are then used as substrates by the PHA synthases (PhaC) which catalyzes the committed step of mcl-PHA biosynthesis and finally end up in PHA polymer. As shown in Figure 2.3, (*R*)-specific enoyl-CoA hydratase (PhaJ) and (*R*)-3-hydroxyacyl-ACP-CoA transferase (PhaG) are capable of supplying (*R*)-3HA-CoA from trans-2-enoyl-CoA and (*R*)-3HA-ACP, respectively (Tsuge, 2002).

In pathway III, formation of PHA from sugars is linked to fatty acid biosynthesis intermediates. Here, sugar is oxidized to acetyl-CoA without involvement of the fatty acid β-oxidation pathway. Instead, the (*R*)-3-hydroxyacyl-acyl carrier protein (*R*)-3-hydroxyacyl-ACP intermediates of the fatty acid biosynthesis route are directed towards PHA biosynthesis by the transacylase reaction catalyzed by PhaG. The specific transacylase catalyzes the transfer of the (*R*)-3-hydroxyacyl moiety of the respective ACP thioester to CoA (Fukui *et al.*, 1998; Rehm *et al.*, 1998). Taguchi and co-workers (1999) have found several enzymes, having the ability to supply the monomers through β-oxidation pathway. The most significant interest is 3-ketoacyl-ACP reductase (FabG), which is a constituent of the fatty acid biosynthetic pathway. Here, it has been demonstrated that, instead of acyl-ACP, the FabG also accepts acyl-CoA as a substrate and capable of supplying mcl-PHA-(*R*)-3HA-CoA from fatty acid β-oxidation in *E. coli* (Taguchi *et al.*, 1999).

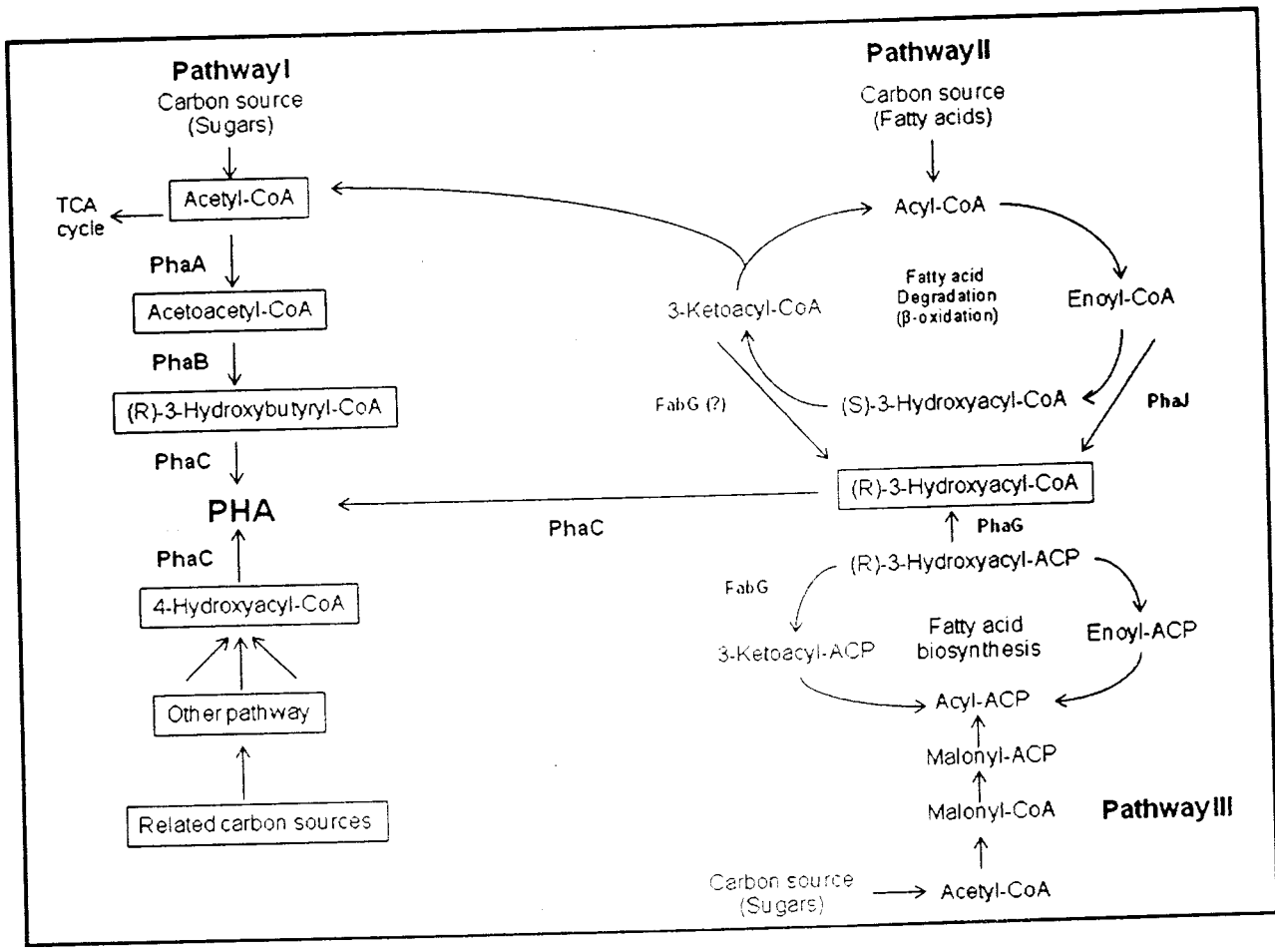


Figure 2.3: Metabolic pathways that supply various hydroxyalkanoate (HA) monomers for PHA biosynthesis. PhaA, 3-Ketothiolase; PhaB, NADPH-dependent acetoacetyl-CoA reductase; PhaC, PHA synthase; PhaG, 3-hydroxyacyl-ACP-CoA transferase; PhaJ, (*R*)-specific enoyl-CoA hydratase; FabG, 3-ketoacyl-ACP reductase (Tsuge, 2002).

2.6 Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)]

Microorganisms are able to accumulate various types of PHA in the form of homopolyesters, copolyesters, or polyester blends. It is reported that, more than 150 different monomer units are known to be incorporated into the polyester chain (Steinbüchel and Lütke-Eversloh, 2003). Accordingly, various copolyesters are expected when a bacterium is grown on mixtures of different precursors. One of these polyesters, poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] has been found to exhibit useful properties relative to other PHAs (Sudesh *et al.*, 2000). Figure 2.4 shows the chemical structure of copolymer P(3HB-*co*-4HB). Incorporation of 4HB monomers result in copolymers with various physical properties that range from being highly crystalline to elastic (Saito and Doi, 1994). To date, there are five wild-type of bacteria that have been identified as potential P(3HB-*co*-4HB) producer. These include *Cupriavidus eutropha* (previously known as *Ralstonia eutropha*) (Nakamura *et al.*, 1992), *Alcaligenes latus* (Hiramitsu *et al.*, 1993), *Comamonas acidovorans* (Lee *et al.*, 2004), *Comamonas testosteronii* (Renner *et al.*, 1996) and *Hydrogenophaga pseudoflava* (Choi *et al.*, 1999).

2.6.1 Properties and structures

Generally copolymer P(3HB-*co*-4HB) consists of two different monomers, 3-hydroxybutyrate and 4-hydroxybutyrate. It has been known that the composition of monomer has a great influence on the physical and mechanical properties of the copolymers which can be manipulated by varying the compositions. The incorporation of 4HB monomer units into the 3HB chain has greatly improved the thermal, crystalline

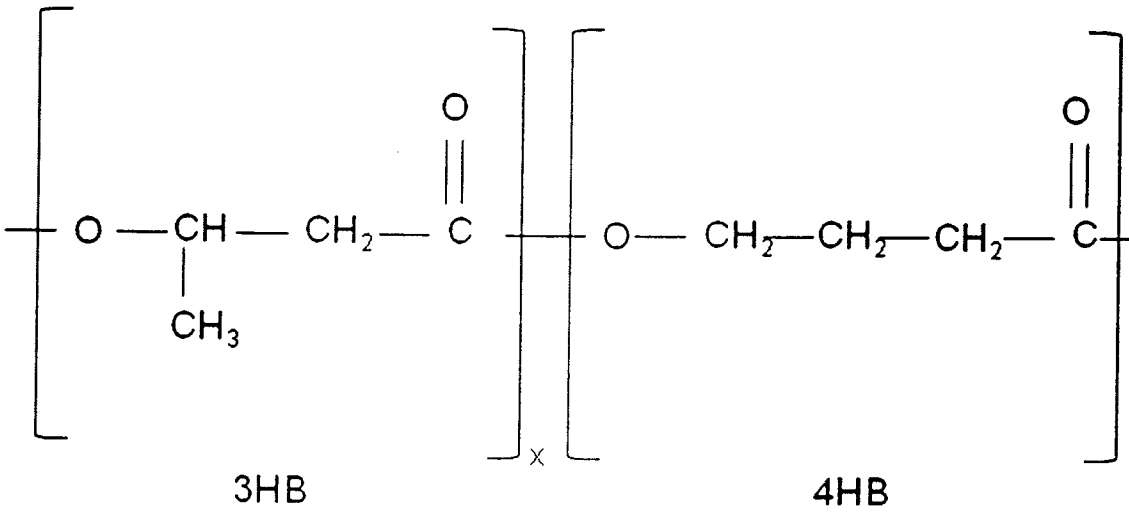


Figure 2.4: Chemical structure of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] (Abe and Doi, 2001)

and mechanical properties of the PHA (Saito and Doi, 1994; Rehm and Steinbüchel, 2002).

Among other members of PHA, P(3HB) is the most well-studied and compared with common commodity plastics. The homopolymer P(3HB) with the properties of high crystallinity, stiffness and brittleness have limited its application and the incorporation of second monomer can significantly enhance its useful properties. According to Doi (1990), P(3HB) isolated from bacteria exhibits 55-70% of crystallinity which is considered as high crystallinity. Previous research conducted by Saito and co-workers has proven that by incorporating 4HB monomer units into the P(3HB) chain has significantly decreased the crystallinity of the copolymer P(3HB-*co*-4HB) from 60%-14% as the 4HB content increases from 0 to 49 mol%. P(4HB) is identified as much more ductile (200 times higher elongation to break) than P(3HB) (Saito and Doi, 1994).

2.6.2 P(3HB-*co*-4HB)-producing microorganisms

A number of bacteria possessing PHA_{SCL} synthase are capable to incorporate 4-hydroxybutyric acid (4HB) into PHAs. A copolymer of (*R*)-3-hydroxybutyrate and 4-hydroxybutyrate, P(3HB-*co*-4HB) with the 4HB monomer compositions varies from 0 to 34 mol% is reported produced by *Cupriavidus eutropha* (previously known as *Alcaligenes eutrophus*) in nitrogen-limited medium containing either 4-hydroxybutyric acid, γ-butyrolactone or 1,4-butanediol as carbon sources (Kunioka *et al.*, 1988; Kunioka *et al.*, 1989; Doi *et al.*, 1990). The copolymer compositions produced are depending on the concentration of carbon substrates supplied in culture medium. Nakamura and co-workers (1992) have found that P(3HB-*co*-4HB) with high contents of 4HB unit (70-100 mol%) are accumulated in *C. eutrophus* cells using 4-hydroxybutyric acid as carbon

source in the presence of additives such as citrate and ammonium sulphate. Saito and Doi (1994) have reported that P(4HB) homopolymer has been produced by *Comamonas acidovorans* isolated from activated sludge by supplying 1,4-butanediol as carbon source. A wide range compositions of P(3HB-*co*-4HB) can also be produced by *Alcaligenes latus* using a mixture of 3-hydroxybutyric acid and 4-hydroxybutyric acids as carbon sources in one-stage cultivation process (Kang *et al.*, 1995). On the other hand, Amirul and co-workers (2008) reveals a finding of *Cupriavidus* sp. USMAA1020 isolated from sludge is able to produce copolymer P(3HB-*co*-4HB) with 53 mol% of 4HB monomer in two-stage cultivation process using γ-butyrolactone as sole carbon source.

2.6.3 Biosynthesis of copolymer P(3HB-*co*-4HB)

In most bacterial strains, the incorporation of 4HB monomers is strongly depends on the precursor substrates supplied in the medium (Steinbüchel and Lütke Eversloh, 2003). Previous research has demonstrated that normally bacteria possessing a PHA_{SCL} synthase have the capability to incorporate 4-hydroxybutyric acid (4HB) into PHAs (Steinbüchel and Lütke-Eversloh, 2003; Choi *et al.*, 1999). However, the incorporation of 4HB is only allowed when related carbon precursors are used as the substrates. After uptake by the cells, carbon precursors such as 1.4-butanediol, γ-butyrolactone or 4-chlorobutyric acid is first converted to 4-hydroxybutyric acid (Figure 2.5). The 4-hydroxybutyric acid is then converted to 4-hydroxybutyryl-CoA and this 4HB-CoA enzyme is further catabolized, resulting in the formation of 3HB monomer (Steinbüchel and Lütke-Eversloh, 2003). In most cases, as shown in Figure 2.6, the 4-hydroxybutyryl-CoA is also being converted into 3HB-CoA via acetyl-CoA, thus lead to the formation of 3HB monomer in *Hydrogenophaga pseudoflava* (Choi *et al.*, 1999).

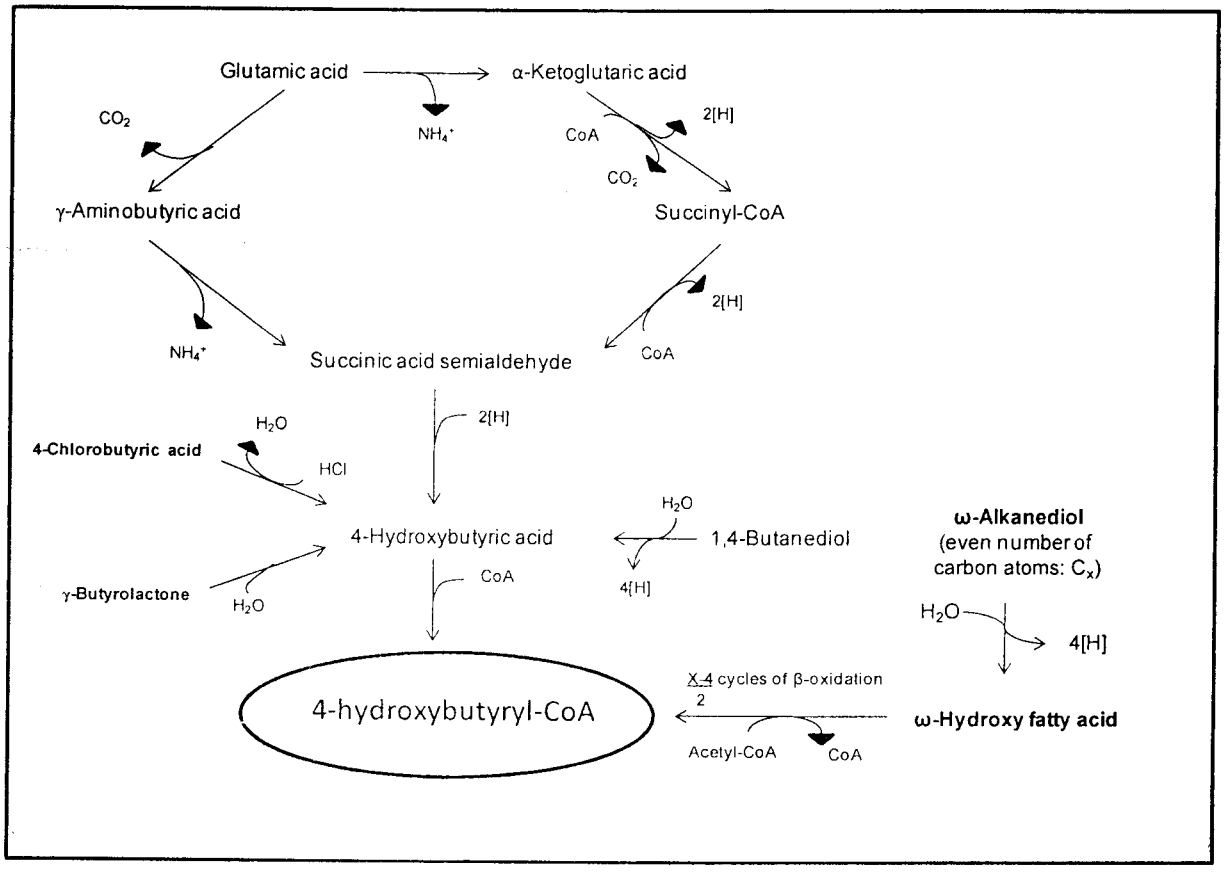


Figure 2.5: Sources of 4-hydroxybutyryl-CoA for biosynthesis of PHAs containing 4HB as constituent (Steinbüchel and Lütke-Eversloh, 2003).

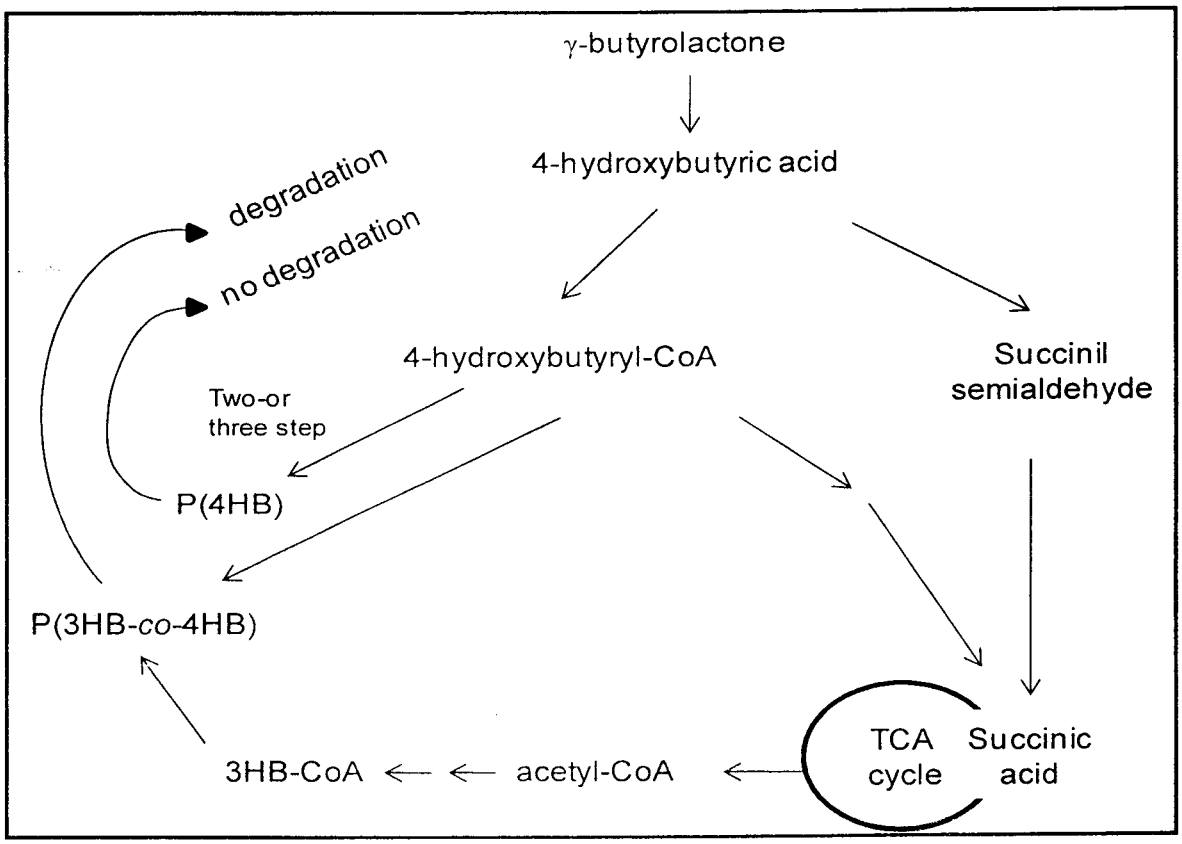


Figure 2.6: Putative metabolic pathways for PHA synthesis from γ-butyrolactone in *H. pseudoflava*. TCA, tricarboxylic acid (Choi *et al.*, 1999).

2.6.4 Substrate for P(3HB-*co*-4HB) production

A list of precursor substrates which related to the 4HB monomers unit have been recognized. The used of 4-hydroxybutyric acid as a precursor substrate has been extensively used in previous research in a wide range of microorganisms namely *Cupriavidus eutropha* (Kunioka *et al.*, 1988; Doi *et al.*, 1990), *Comamonas acidovorans* (Sudesh *et al.*, 1999), *Hydrogenophaga pseudoflava* (Choi *et al.*, 1999), and *Cupriavidus metallidurans* (formerly *R. metallidurans*) (Song *et al.*, 2000) and in many other bacteria possessing a PHA_{SCL} synthase.

Another suitable precursor substrates which have been described previously are γ-butyrolactone (Doi *et al.*, 1990; Kunioka *et al.*, 1988; Choi *et al.*, 1999; Valentin *et al.*, 1995), 1,4-butanediol (Kunioka *et al.*, 1988; Doi *et al.*, 1990) and other ω-alkanediols and 4-chlorobutyric acid (Choi *et al.*, 1999). As for γ-butyrolactone, the lactone is hydrolytically cleaved to 4-hydroxybutyric acid which catalyzed by esterases or lactonases. Esterases (Est A) which capable of cleaving γ-butyrolactone is recently cloned from *C. metallidurans* strain CH34 (Song *et al.*, 2000). The generated 4-hydroxybutyric acid is then converted to 4HB-CoA and also catabolized further, resulting in the incorporation of 3HB monomer units. The use of ω-alkanediols involves two-subsequent enzymatic reactions which oxidizes ω-alkanediol to 4-hydroxybutyric acid and is further converted to form 4HB-CoA. ω-alkanediols with a greater carbon chain length but an even number of carbon atoms are also suitable as precursor substrate (Doi *et al.*, 1990). Here, the greater carbon chain length of ω-alkanediols are first oxidized to the corresponding ω-hydroxyfatty acid, which is then converted into coenzyme A thioester and subjected to β-oxidation in order to form 4HB-CoA. Meanwhile, 4-chlorobutyric acid is probably converted to 4-hydroxybutyric acid by a

haloalkane dehalogenase which employs hydrolytic mechanism (Fetzner, 1998). The product is then converted to 4HB-CoA for further polymerization. As described above, it clearly indicates that the 4HB-CoA is the final intermediates from various precursor substrates before polymerized by PHA_{SCL} synthase. However, in most cases, bacteria are incorporating 3HB monomer unit into the cells, hence synthesizing P(3HB-*co*-4HB) copolyesters. The incorporation of 3HB comonomer usually resulted from the catabolism of 4-hydroxybutyric acid which forms intermediates from which 3-hydroxybutyryl-CoA is synthesized (Steinbüchel and Lütke Eversloh, 2003).

2.7 Fed-batch fermentation

Fed-batch fermentation has long been used to enhance the yield and productivities of many bioprocesses including production of PHA (Khanna and Srivstava, 2005b). Fed-batch fermentation is a strategy in between batch and continuous culture which normally initiated with batch culture and fed-continuously or sequentially without removing the culture medium (Stanbury and Whittaker, 1994). On the other hand, fed-batch fermentation also helps to induce desired nutrient limitations, which are often necessaray for high yield and productivity of the final products (Khanna and Srivastava, 2006). In fed-batch, depended on the feedback prameter, nutrients were usually fed in the reactor intermittently or constantly in response to dissolved oxygen (Yano *et al.*, 1978) or pH (Kim *et al.*, 2002). The main reason of choosing fed-batch cultivation is because it allows the concentration of the nutrients and the carbon source to be dynamically controlled in an optimal strategy within the liquid culture (Patnaik, 2006) and hence some of the biochemical parameters can be controlled as well (Du *et al.*, 2001).

The choice of fed-batch-fermentation arises from the mechanism of the PHA synthesis (Patnaik, 2006) and as reported in previous research, various feeding strategies had been implemented for high cell density, increased PHA productivity and increased the compositions of the desired product (Du *et al.*, 2001; Kim *et al.*, 2002; Kim *et al.*, 2005; Khanna and Srivastava, 2006; Patwardhan and Srivastava, 2004; Sun *et al.*, 2007 and Kulpreecha *et al.*, 2009). The fermentative process of PHA is normally operated as a two-stage fed-batch cultivation process (Doi, 1990). In most researches of PHA, studies have indicated that fed-batch cultivation is the most preferred method of production (Patnaik, 2006) since it allows the improvement of high cell concentration, the polymer content in the cells, and shortening of the cultivation time for cell growth and polymer accumulation (Du *et al.*, 2001). In addition, improvement in PHA production strategies i.e by feeding appropriate carbon and nitrogen sources at suitable concentrations or ratio (Kulpreecha *et al.*, 2009) can lead to the reduction of the final product cost thus implying wider use of PHAs in daily life (Li *et al.*, 2007).

2.7.1 Feeding strategies

Previously, many reports are available for the synthesis of P(3HB) by fed-batch fermentation and several feeding strategies have been reported for the polymer accumulation (Kim *et al.*, 1994; Choi and Lee, 1999; Grothe *et al.*, 1999; Du *et al.*, 2001). The most common feeding strategies to obtained maximum cell growth were exponential mode, in which the substrate flowrate is increased exponentially accompanying the exponential increase of cell biomass (Du *et al.*, 2001). In this condition, the substrate concentration in the medium is kept constant while the cell growth is not limited by the substrate concentration. In PHA production there are two

different conditions that need to take into account where non-limiting concentration of carbon source and limiting concentration of nitrogen are needed (Khanna and Srivastava, 2005b). Thus, others indirect estimation could be applied where nutrients are usually fed in the reactor intermittently or constantly in response to dissolved oxygen (DO) (Kim *et al.*, 2005) or pH (Kim *et al.*, 1992) as a feedback parameter.

Feed-back control strategies have been studied previously in fed-batch fermentation including dissolved oxygen (DO). In a bioreactor, DO is closely related to the substrate concentration so that when the substrate is exhausted and oxygen consumption decreases the DO level increases rapidly. This have been prove by previous study both for pure cultures of *Escerichia coli* (Lee *et al.*, 2000) and mixed, PHA producing cultures (Serafim *et al.*, 2004). Pulse feeding is a straightforward method of DO-control. However, this feeding mode can be modified into cascade mode by constantly maintaining the DO concentration in the medium. The feeding of the substrates solution is depending on the drop of stirrer speed as the indicator of the substrate depletion.

Previous studies have applied the DO-stat method when the dissolved oxygen (DO) concentration is maintained at above 20% of air saturation with the aeration rate of 1.5 L/L/min and by adjusting the agitation speed from 600 to 100 rpm min^{-1} in response to the oxygen requirement of the culture. This has been proven in research study conducted by Du and co-workers (2001) in the production of copolymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) by *Cupriavidus eutropha* (Du *et al.*, 2001). This study has demonstrated that by appling a fed-batch strategy with variation in propionic acid feeding rate and minimizing the propionic acid accumulation had improve the P(3HB-*co*-3HV) content, productivity with a desired HV mol fraction (16 mol%).

Besides, the production of copolymer P(3HB-*co*-4HB) using DO-stat feeding mode also have been reported by Kim and co-workers (2005). Through this DO-stat feeding strategy of fructose in the growth phase, it can possibly improve the cell concentration up to 38-48 g/L (Kim *et al.*, 2005). The feeding of γ-butyrolactone to fructose ratio in the second step feeding resulted in 13.8-24.4 g/L of PHA concentration and 2-25 mol% of 4HB fraction with 0.32-0.55 g/L/h of productivity.

On the other hand, the feeding of the substrate solution can also be operated intermittently. The intermittent feeding strategy of substrate was performed by feeding limiting substrates at a predetermined concentration at a certain interval of time. Son and co-workers (2000) have conducted fed-batch fermentation by intermittently feeding sufficient nitrogen source in an appropriate C/N ratio in the production of copolymer P(3HB-*co*-3HV) (Son *et al.*, 2000). A wide range of C/N ratio ranged from 4 to 200 have been employed in order to investigate the relationship between C/N ratio and the specific production rate and without nitrogen the ratio of γ-hydroxybutyric acid to total carbon sources was varied from 0-100 wt%. Carbon and nitrogen sources were fed at interval of 12 h so that the C/N ratio is maintained at an appropriate value throughout the cultivation.

2.7.2 Carbon-nitrogen ratio (C/N)

The typical elemental compositions for microbial cells are carbon, nitrogen, phosphorus, sulfur and magnesium (Wang and Lee, 1997). However, there are two chemical elements, which are extremely important especially in their relation of proportion to each other; they are carbon and nitrogen (Bailey and Ollis, 1986; Stanbury