DEVELOPMENT OF AN ANIMAL MODEL TO RECOVER POLY(3-HYDROXYBUTYRATE) GRANULES FROM DRIED CELLS OF *Cupriavidus necator* H16

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

3HB	- 3-hydroxybutyrate
3HHx	- 3-hydroxyhexanoate
3HV	- 3-hydroxyvalerate
ANOVA	- One-way analysis of variance
ALP	- Alkaline phosphatase
ALT	- Alanine aminotransaminase
AST	- Aspartate aminotransferase
CDW	- Cell dry weight
CFU	- Colony forming unit
$CHCl_3$	- Chloroform
CH_3COOH	- Acetic acid
CME	- Caprylate methyl ester
CPKO	- Crude palm kernel oil
DFP	- Dark-coloured faecal pellets
DO	- Dissolved oxygen
DSC	- Differential scanning calorimetry
GC	- Gas chromatography
GPC	- Gel permeation chromatography
^{1}H-NMR	- Proton nuclear magnetic resonance
HCl	- Hydrochloric acid
HPLC	- High performance liquid chromatography
HSD	- Honestly Significant Difference
MCL	- Medium-chain-length

MM	- Mineral medium
M_n	- Number average molar mass
M_w	- Weight average molar mass
M_w/M_n	- Polydispersity index
NaOH	- Sodium hydroxide
NaOCl	- Sodium hypochlorite
NPCM	- Non-polymeric cellular materials
NR	- Nutrient-rich
OD	- Optical density
P(3HB)-*co*-(HHx)	- Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate)
P(3HB)-*co*-(HV)	- Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)
P(3HB)	- Poly(3-hydroxybutyrate)
PBS	- Polybutylene succinate
PHA	- Polyhydroxyalkanoate
PhaA	- 3-ketothiolase
PhaB	- NADPH-dependent acetoacetyl-CoA reductase
PhaC	- PHA synthase
*pha*C	- PHA synthase gene
PLA	- Polylactic acid
PP	- Polypropylene
rpm	- Revolution per minute
SCL	- Short-chain-length
SCP	- Single cell protein
SDBS	- Sodium dodecylbenzene sulfonate
SDS	- Sodium dodecyl sulfate

SEM	- Scanning electron microscope
T_c	- Crystallization temperature
T_g	- Glass transition temperature
T_m	- Melting temperature
TEM	- Transmission electron microscope
TGA	- Thermogravimetric analysis
UV-vis	- Ultraviolet-visible
v/v	- Volume per volume
wt.%	- weight percent
WCW	- Wet cell weight
WFP	- White-coloured faecal pellets

PEMBANGUNAN MODEL HAIWAN UNTUK PEROLEHAN GRANUL POLI(3-HIDROKSIBUTIRAT) DARIPADA SEL KERING *Cupriavidus necator* H16

ABSTRAK

Kepentingan untuk membangun satu proses perolehan polihidroksialkanoat (PHA) daripada sel bakteria yang mesra alam, dan cekap telah mendorong penyelidikan ini. Salah satu objektif kajian ini adalah untuk menilai kebajikan dan toleransi model haiwan, Sprague Dawley diberikan sel kering sebagai sumber diet tunggal. *C. necator* H16 telah dikulturkan dengan menggunakan media mineral yang mengandungi minyak isirung kelapa sawit mentah (CPKO) sebagai sumber karbon tunggal. Sel bacteria yang mengandungi 37 wt% poli(3-hidroksibutirat), P(3HB) telah digunakan sebagai sumber protein. Dengan mengambil kira aspek maklumat nutrisi sel *C. necator* yang telah didokumenkan, sel kering telah diberikan sebagai sumber makanan kepada model haiwan (Sprague Dawley). Kumpulan yang mengandungi 6 ekor haiwan bagi setiap jantina telah diberi makan sel kering C. *necator* H16 untuk selang kajian selama 7, 14 dan 28 hari. Tiada kadar kematian yang direkodkan dan haiwan yang diuji didapati boleh bertahan dengan baik terhadap keseluruhan sel diet. Berbeza dengan haiwan kawalan, haiwan ujian menunjukkan kadar penambahan berat badan haiwan ujian pada kadar yang sangat rendah. Ini disebabkan oleh fakta bahawa bahagian khasiat sebenar makanan sel yang dimakan oleh haiwan ujian adalah lebih kurang daripada separuh mengikut berat berbanding dengan makanan yang diberikan kepada haiwan kawalan. Analisis hematologi dan

serum biokimia tidak mendedahkan sebarang keabnormalan yang ketara. Pemeriksaan anatomi secara kasar bagi organ-organ yang penting tidak mempamerkan sebarang tanda toksifikasi manakala keputusan analisis histologi terhadap tisu buah pinggang dan hati adalah menggalakkan. Objektif yang lain adalah untuk mencirikan P(3HB) yang diasingkan dengan menggunakan satu kaedah perolehan biologi yang baru yang dibangun melibatkan model haiwan. Haiwan ujian menghasilkan pelet najis yang berwarna putih yang dijangkakan adalah granul P(3HB). Pelet-pelet tersebut didapati mengandungi kira-kira 89 wt% P(3HB) dan mempunyai jisim molekul sekitar 930 ± 40 kg /mol. Granul P(3HB) yang diperoleh secara biologi mempunyai jisim molekul yang sama berbanding dengan P(3HB) yang diekstrak menggunakan kloroform [950± 100 kg /mol]. Penulenan selanjutnya granul P(3HB) yang diperoleh secara biologi dengan menggunakan surfaktan yang berkepekatan rendah didapati tidak menyebabkan perbezaan yang besar dalam jisim molekul (770-820 kg / mol) dan taburannya. Ciri-ciri rheologi polimer-polimer ini juga telah dikaji pada suhu 180 °C dan frekuensi 10 Hz dengan amplitud ketegangan malar 2%. Granul P(3HB) yang ditulenkan dan P(3HB) yang diekstrak dengan kloroform menunjukkan sifat pergantungan kepada masa yang sama di mana kelikatan dinamik berkurangan dengan penambahan masa dan ini menjadi petunjuk kepada degradasi haba polimer oleh pemutusan rantaian. Proses perolehan biologi boleh membentuk asas kepada makanan sinergetik bergabung dan proses penulenan untuk mengekstrak granul PHA dari sel bakteria tanpa melibatkan penggunaan meluas pelarut dan bahan kimia kuat.

DEVELOPMENT OF AN ANIMAL MODEL TO RECOVER POLY(3-HYDROXYBUTYRATE) GRANULES FROM DRIED CELLS OF *Cupriavidus necator* H16

ABSTRACT

The importance of developing an environmentally friendly and efficient recovery process of polyhydroxyalkanoates (PHAs) from bacterial cells motivated this research. One of the objectives of this study was to evaluate the welfare and tolerability of the animal model, Sprague Dawley given lyophilized cells of *Cupriavidus necator* H16 as sole diet source. *C. necator* H16 was cultured using a mineral medium containing crude palm kernel oil (CPKO) as the sole carbon source. The bacterial cells containing about 37 wt% of poly(3-hydroxybutyrate), P(3HB) were used as a source of protein. By taking into account the well-documented nutritive aspects of *C. necator* cells, the lyophilized cells were given as feed source to animal model (Sprague Dawley). Groups of 6 animals each for both sexes were fed with lyophilized cells of *C. necator* H16 for study intervals of 7, 14 and 28 days. No mortality was observed and the test animals were found to tolerate well with the whole cell diet. In comparison with the control animals, the test group showed poor weight gain. This is due to the fact that the actual nutritious portion of the feed received by test animals was less than half by weight as compared with the feed consumed by control animals. Haematology and serum biochemistry analyses did not reveal any significant abnormalities. Gross anatomical examinations of vital organs did not produce any sign of toxicity while results of histological examinations of

kidney and liver tissues were encouraging. The other objective was to characterize the P(3HB) isolated using a newly developed biological recovery method involving animal model. The test animals readily produced faecal pellets that were whitish in color, as would be expected of P(3HB) granules. The pellets were determined to contain about 89 wt% P(3HB) and possessed molecular mass of around 930 ± 40 kg/mol. The P(3HB) granules recovered biologically possessed similar molecular mass compared to chloroform extracted P(3HB) [950 ± 100 kg/mol]. Further purification of biologically recovered P(3HB) granules using low concentration of surfactants did not induce any big differences in the molecular mass (770-820 kg/mol) and their distribution. The rheological properties of these polymers were also investigated at 180 °C and frequency of 10 Hz with constant strain amplitude of 2%. Both purified P(3HB) and chloroform extracted P(3HB) showed similar time dependency behaviour in which the dynamic viscosity decreased with increasing time, this being an indication of the thermal degradation of the polymers by chain scission. Biological recovery process may form the basis for a combined synergetic feed and purification process to extract PHA granules from bacterial cells without extensive use of solvents and strong chemicals.

1.0 INTRODUCTION

The versatility of plastic materials in terms of mechanical properties and durability has been manipulated by mankind to enhance quality of life without realizing they have become increasingly ubiquitous. An unprecedented expansion in the production with increasing demands for plastics in tandem with growing human population begins to create vast plastics waste stream in the environment. The global plastic production has witnessed an incredible rise from 1.7 million tonnes in 1950 to 265 million tonnes in 2010 (Plastics Europe, 2011). It is clear from this figure that the long term deleterious environmental impacts caused by plastics were entirely overlooked and this in turn poses greater difficulties for plastic waste disposal. This situation has triggered global attention towards the biopolymers as the next generation of sustainable materials for certain applications.

The most extensively studied thermoplastic biopolymers are the polyhydroxyalkanoate (PHA) and polylactic acid (PLA). PHA is a lipid inclusion synthesized by many microorganisms as a form of storage material (Anderson and Dawes, 1990). PHAs are attractive due to its renewable, biodegradable nature with zero toxic waste and recyclable into organic waste. PHAs are extensively researched not only for biological curiosity, mainly as many PHAs have functional properties useful for commercial exploitations. To date, there are about 150 types of monomers that can yield a range of copolymers with tunable properties which are suitable for various applications ranging from stiff packaging goods to highly elastic materials for coatings (Corre et al., 2012).

In spite of these interesting properties, the market acceptance of PHAs is still scarce due to its high production cost. Since PHAs are microbial plastics, the overall process economic are dependent on the fermentation strategies as well as the

downstream recovery and purification technologies with the latter significantly contributing to its high cost. Various cultivation strategies and use of waste materials as carbon sources have been investigated to lower the production cost. Sugars have been shown to be an effective feedstock for PHA production in Brazil, especially when the PHA production is integrated to the sugarcane-processing factory (Koller et al., 2009). On the other hand, it has been demonstrated that vegetable oils are also potential feedstock for PHA production (Kahar et al., 2004; Lee et al., 2008; Loo et al., 2005; Ng et al., 2010; Sudesh et al., 2011) in countries like Malaysia where palm oil is produced in very large scales. High levels of PHA accumulation have been achieved using crude palm kernel oil. The yield of PHA from vegetable oils is at least two times the yield of PHA from sugars (Akiyama et al., 2003). Besides, by exploiting the high-density cell culture technologies, bacterial cells could be grown up to 150 g/L of cell dry weight with PHA content up to 80% wt. (Akiyama et al., 2003; Riedel et al., 2012). Nevertheless, the price of PHA hinges largely on downstream processing.

There are a few PHA recovery methods that are currently available. Solvent extraction is the mostly adopted method to recover PHA from the cell biomass. Although it is possible to obtain PHA of high purity that undergoes limited degradation during the recovery process, the use of hazardous chlorinated solvents is of great concern. Therefore, solvents with lower environmental impact have been investigated to substitute those toxic and volatile solvents (Fiorese et al., 2009; Wampfler et al., 2010). Yet, several other factors discourage the use of solvents such as high capital and operational costs (Yu and Chen, 2006). Enzymatic digestion is developed as an alternative to solvent extraction. Since, it involved a complex process and also expensive, chemical based-PHA recovery has been extensively

investigated by using various types of surfactants and chemicals. This method resulted in 50% reduction of overall cost when compared to solvent extraction (Yu, 2009). However, the common problem associated with chemical digestion is the isolation of PHA with low purity. Other techniques like mechanical disruption, supercritical fluid, cell fragility and flotation have been attempted in the effort to reduce the downstream costs (Jacquel et al., 2008).

The extraction and purification of the PHA from the cell biomass still remaining as a challenging task though a number of PHA recovery methods already available. One of the challenges in the PHA production is the isolation of polymer of high purity from bacterial biomass without undergoing severe reduction in the molecular mass while avoiding the use hazardous chemicals. This research was intrigued by the serious need for an environmentally friendly and efficient recovery process of PHAs from bacterial cells.

In recent decades, the bacterium *C. necator* H16 has been well known among researchers mainly for its ability to synthesize PHA. However, the initial interest in this wild type strain was not for its PHA synthesizing ability, but for its nutritive values (Waslien and Calloway, 1969). In the 1970s, much work was devoted to utilizing *C. necator* H16 as a source of single cell protein (SCP) for animal feed owing to its high protein content and quality. It was proposed that the bacterium would form an excellent supplement to cereal grain that is lack in lysine content.

Though the nutritive aspects of the cells were found promising to be developed as SCP, an obstacle was encountered where the *C. necator* cells also accumulated P(3HB) (Waslien and Calloway, 1969). Most of the P(3HB) granules consumed by animals were excreted without being absorbed by the digestive system. As P(3HB) does not possess any nutritive value, attempts were made to suppress its

synthesis and accumulation in bacterial cells. However, such attempts were not successful as the efficiency of biomass production was affected when the accumulation of the biopolymer was inhibited (Schlegel and Lafferty, 1971).

There has been a considerable resurgence of interest in recent years to use PHA as a component of animal feed to increase the metabolizable energy content (Boon et al., 2010; Forni et al., 1999a; 1999c; Peoples et al., 2001). To improve digestibility, the PHA is pretreated with sodium hydroxide (NaOH) before being fed to animals (Forni et al., 1999b). Patent literature describes the use of animal feed containing whole bacterial cells with PHA for modulation of the gut flora by delivering short-chain fatty acids such as 3-hydroxybutyric acids (Boon et al., 2010; Ushida and Kuriyama, 2006). Defoirdt *et al*. (2009) reviewed the possible application of PHA as new biocontrol (bacteriostatic) agents for animal production.

It is evident from previous studies that PHAs are not toxic and have been used in animal feed (Forni et al., 1999a; 1999b; 1999c; Peoples et al., 2001; Waslien and Calloway, 1969). The *C. necator* H16 cells containing P(3HB) found to be tolerated by animals. Simultaneously, these studies also reported the poor digestibility of PHAs by monogastric animals. Therefore, it is anticipated that a new biological recovery process for PHA from bacterial cells could be developed by employing the cells as animal feed.

In this study, the lyophilized cells of *C. necator* H16 containing P(3HB) were given as sole diet source to the laboratory rats, Sprague Dawley for study intervals of 1, 2 and 4 weeks and the welfare and tolerability of these animals were evaluated. The test animals readily produced faecal pellets that were whitish in color, as would be expected of P(3HB) granules. The undigested P(3HB) granules obtained from the faecal pellets and chloroform extracted P(3HB) were subjected to various standard

analyses for PHA. Rheological measurements were performed with untreated P(3HB) granules which indicated the importance of further purifying those P(3HB) granules. The rheological behaviour of purified granules using mild chemical solutions were compared with chloroform extracted P(3HB) which was considered as a reference sample. The bacterial cells were also digested using various chemicals such as acid, alkali and surfactants to recover PHA. Finally, the yield and purity of P(3HB) recovered biologically were compared with the values obtained using chemical digestion and solvent extraction methods.

1.1 Research objectives

The main objective of this study was to develop a new biological recovery process of PHA that form the basis for a combined synergetic feed and purification process of PHA granules from the lyophilized cells of *C. necator* H16 without extensive use of solvents and strong chemicals which involves a series of objectives as follow:

1) To evaluate the welfare and tolerability of the animal model, Sprague Dawley given lyophilized cells of *C. necator* H16 as sole diet source.

2) To characterize the P(3HB) isolated using a newly developed biological recovery method involving animal model, Sprague Dawley.

3) To compare the PHA recovery efficiency of a newly developed biological recovery method with solvent extraction and chemical digestion methods.

2.0 LITERATURE REVIEW

2.1 Biobased and biodegradable plastics

Biobased polymers can be defined as organic macromolecules synthesized from renewable resources by chemical and/or biological methods. Depending on the mode of synthesis, biobased polymers are classified into the following groups: (i) Biosynthetic polymers which are produced by microorganisms such as polyhydroxyalkanoate (PHA); (ii) Bio-chemosynthetic polymers which involved chemical polymerization of monomers derived from organic resources such as polylactic acid (PLA) and polybutylene succinate (PBS); (iii) Modified natural polymers such as starch and cellulose However, it is important to note that not all biobased polymers are biodegradable (Naik et al., 2008; Sudesh and Iwata, 2008). Biodegradable polymers can be returned to the environment in the form of their basic building blocks hydrolytically or enzymatically (Nair and Laurencin, 2007; Narayan, 2001).

Among various biobased and biodegradable polymers, PHA is attractive due to its excellent properties over the rest. To date, approximately 150 different monomers of PHA have been identified which can yield copolymers exhibit a wide range of properties having potential for commercial exploitations (Sudesh and Iwata, 2008).

2.2 Polyhydroxyalkanoate (PHA)

PHA constitutes a large family of biopolyesters synthesized by numerous microorganisms as intracellular carbon and energy storage compounds under unfavorable growth conditions of limiting essential nutrients in the presence of abundant carbon source (Anderson and Dawes, 1990; Braunegg et al., 1978). Gram

positive and gram negative bacteria as well as some Archaea are known to accumulate PHA (Reddy et al., 2003; Rehm, 2003). The low solubility and high molecular weight nature of PHA have enabled the bacteria to accumulate from some to about 12 granules per cell without significantly affecting the osmotic pressure of the cell. The storage of PHA has been shown to enhance the environmental persistence of the host during starvation. In addition, PHA plays a major role as endogenous carbon and energy source for the formation of spore in some *Bacillus* species and encystment of azotobacters (Anderson and Dawes, 1990; Madison and Huisman, 1999; Sudesh et al., 2000).

PHA granules can be stained specifically with Sudan Black B or light fluorescent dyes such as Nile blue and Nile red (Kitamura and Doi, 1994; Ostle and Holt, 1982; Spiekermann et al., 1999). The average size of the PHA granules is approximately 0.2 to 0.5 μm in diameter. The high refractivity of PHA granules allows it to be visualized under phase contract light microscope. PHA inclusions appear as electron-dense bodies when thin sections of PHA containing cells are examined by transmission electron microscope (TEM) (Sudesh et al., 2000). Atomic force microscope (AFM) is exploited by the curiosity to understand in more detail the surface architecture of PHA granules. This is because the procedures involved in preparation of PHA granules for characterization using TEM such as staining and dehydration are rather destructive; therefore conclusive observations could not be drawn. Atomic force microscopy analysis has shown the presence of a protein monolayer on the surface of PHA granules (Sudesh et al., 2002; Sudesh et al., 2004). Figure 2.1 shows the morphology of PHA granules when observed using various microscopy techniques.

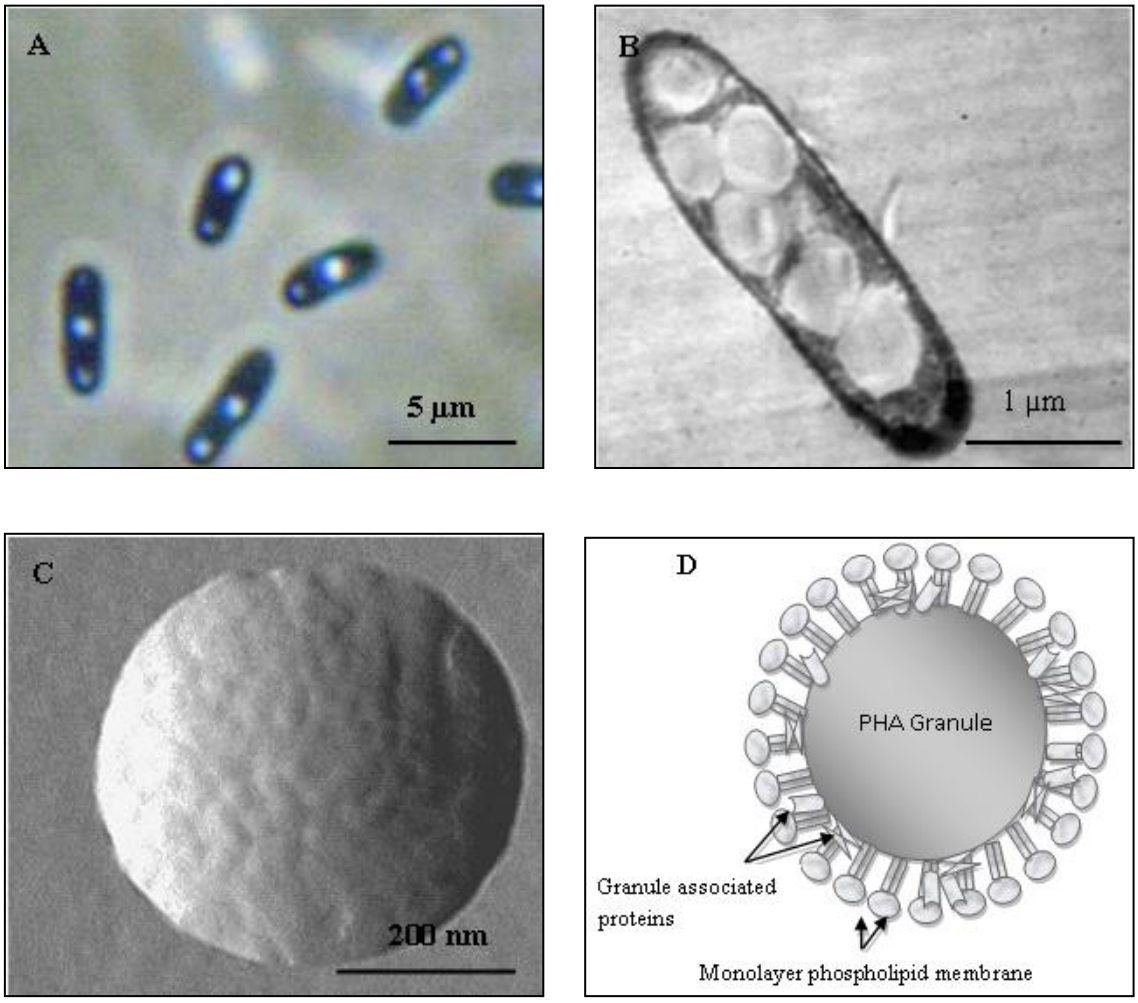
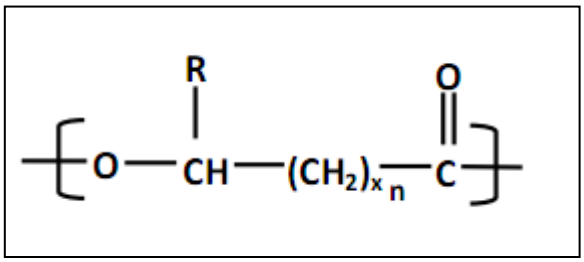


Figure 2.1: Morphology of PHA granules in the bacterial cells observed under (A) Phase contrast and (B) Transmission Electron Microscope. (C) Atomic force microscope deflection image showing the presence of globular particles on the granule surface. (D) A model representing the native PHA granule with a protein monolayer on the surface (Not drawn according to actual scale)

More than 150 different constituents of PHA have been discovered (Steinbüchel and Lütke-Eversloh, 2003) since the identification of poly(3-hydroxybutyrate) [P(3HB)] in *Bacillus megaterium* by Lemoigne (1926). The various PHA monomers can be classified based on the number of carbon atoms synthesized in a variety of configurations as short-chain length PHA (scl-PHA) and medium-chain length PHA (mcl-PHA). Scl-PHA comprised of monomers having 5 or less carbon atoms which include the most widely studied homopolymer, P(3HB) and 3-hydroxyvalerate. The mcl-PHA consists of monomers having 6 to 14 carbon atoms. These include 3-hydroxyhexanoate, 3-hydroxyoctanoate and 3-hydroxydodecanoate. The general structures of PHA are summarized in Figure 2.2. PHA with an extensive range of physical and chemical properties that have potential application in various areas possible to be synthesized due to high number of monomers and variable monomeric compositions (Jendrossek and Handrick, 2002; Steinbüchel and Lütke-Eversloh, 2003). Chemical modification reactions and the engineering of the PHA biosynthetic pathways have further diversified the PHA family (Hazer and Steinbüchel, 2007). The monomer units of microbial polyesters are all in the *R (-)* configuration due to the stereospecificity of PHA synthase which is essential for the biodegradability and biocompatibility of the material (Zinn and Hany, 2005). These water insoluble PHA are thermoplastic and/or elastomeric, piezoelectric and exhibit a high degree of polymerization with molecular weight ranges from 2×10^5 to 3×10^6 daltons (Akaraonye et al., 2010; Zinn and Hany, 2005).



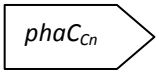
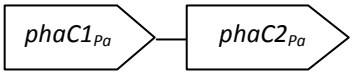
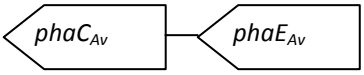
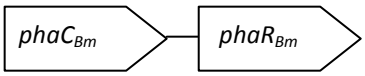
Number of repeating units, n	Alkyl group, R	Polymer type
1	Hydrogen	Poly(3-hydroxypropionate)
	Methyl	Poly(3-hydroxybutyrate)
	Ethyl	Poly(3-hydroxyvalerate)
	Propyl	Poly(3-hydroxyhexanoate)
	Pentyl	Poly(3-hydroxyoctanoate)
	Nonyl	Poly(3-hydroxydodecanoate)
2	Hydrogen	Poly(4-hydroxybutyrate)
3	Hydrogen	Poly(5-hydroxyvalerate)

Figure 2.2: General structures of polyhydroxyalkanaoates (Lee, 1996).

2.3 PHA biosynthesis

PHA synthases are the key enzymes responsible for PHA biosynthesis and catalyze the polymerization of *(R)*-3-hydroxyacyl-CoA substrates to polyesters with the concomitant release of CoA. More than 59 different PHA synthases have been sequenced and cloned from 46 different bacteria (Rehm, 2003). Four major classes of PHA synthases have been distinguished (Table 2.1) based on their primary structures, substrate specificities and subunit composition (Pötter and Steinbüchel, 2006; Rehm, 2003). Class I and class II PHA synthases comprise of only one type of subunit (PhaC) with an average molecular masses between 60 to 73 kDa which are

Table 2.1: Four classes of polyesters synthases (Pötter and Steinbüchel, 2006)

Class	Gene structure	Subunits	Substrate	Representative species
I		~60 – 73 kDa	3HA$_{scl}$-CoA (~C3-C5)	*Cupriavidus necator*
II		~60 – 65 kDa	3HA$_{mcl}$-CoA (~C5-C14)	*Pseudomonas aeruginosa*
III		PhaC ~ 40 kDa PhaE ~ 40 kDa	3HA$_{scl}$-CoA (~C3-C5) 3HA$_{mcl}$-CoA (~C5-C8)	*Allochromatium vinosum*
IV		PhaC ~ 40 kDa PhaR ~ 22 kDa	3HA$_{scl}$-CoA (~C3-C5)	*Bacillus megaterium*

represented by *Cupriavidus necator* and *Pseudomonas aeruginosa* respectively. Class I PHA synthases preferentially polymerize various scl-HA-CoA monomers comprising three to five carbon atoms to form either homopolymer or copolymer of scl-PHA. However, PHA synthases of *Aeromonas caviae* that belong to this class of enzymes do also incorporate 3HHx monomers. PHA synthases from class II specifically react with mcl-HA-CoA monomers consisting of 6 to 14 carbon atoms. On the other hand, class III and class IV PHA synthases possess two different subunits. Class III synthases represented by *Allochromatium vinosum* are made up of PhaC and PHA synthase expression protein (PhaE) with molecular mass of approximately 40 kDa for both subunits respectively. They accept scl-HA-CoA monomers while some in addition have preference towards the mcl-HA-CoA monomers. Class IV PHA synthases has been characterized in *Bacillus megaterium* resembles class III PHA synthases, but PhaE is replaced by PhaR. They exhibit a

similar substrate range as class III PHA synthases (Jendrossek, 2009; Pötter and Steinbüchel, 2006; Rehm, 2003; Rehm and Steinbüchel, 1999).

There are three well known PHA biosynthetic pathways (Figure 2.3) with route for P(3HB) synthesis in *C. necator* H16 is one of the simplest and extensively studied. This pathway involves three successive enzymatic reactions. Firstly, *β*-ketothiolase (PhaA) condenses two molecules of acetyl-CoA to form acetoacetyl-CoA. An NADPH-dependent acetoacetyl-CoA reductase (PhaB) then catalyzes the conversion of acetoacetyl-CoA to *(R)*-3-hydroxybutyryl-CoA (3HB-CoA). The final step is the polymerisation of *(R)*-3-hydroxybutyryl-CoA to P(3HB) catalysed by PHA synthase (PhaC). It is known that PHA synthase in *C. necator* H16 only active towards scl-HA monomers. Nevertheless, the incorporation of 4- and 5-HA monomers besides the more common 3-HA clarified that the position of the oxidized carbon in the monomer is apparently not a crucial factor (Philip et al., 2007; Sudesh et al., 2000).

The production of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] can be demonstrated when propionic acid is fed as additional carbon source to *C. necator* H16. Biosynthesis of this copolymer is determined by the availability of 3-hydroxyvaleryl-CoA (3HV-CoA). Propionic acid 3HV-CoA is first converted to propionyl-CoA while acetyl-CoA is generated from TCA cycle. Latter, 3-ketothiolase mediates the condensation of acetyl-CoA and propionyl-CoA into acetoacetyl-CoA and 3-ketovaleryl-CoA, respectively. The condensation products are subsequently reduced to (3HB-CoA) and 3HV-CoA by NADPH-dependent acetoacetyl-CoA reductase. Finally, PHA synthase polymerizes both monomers to P(3HB-*co*-3HV) (Suriyamongkol et al., 2007). Propionic acid is widely used as

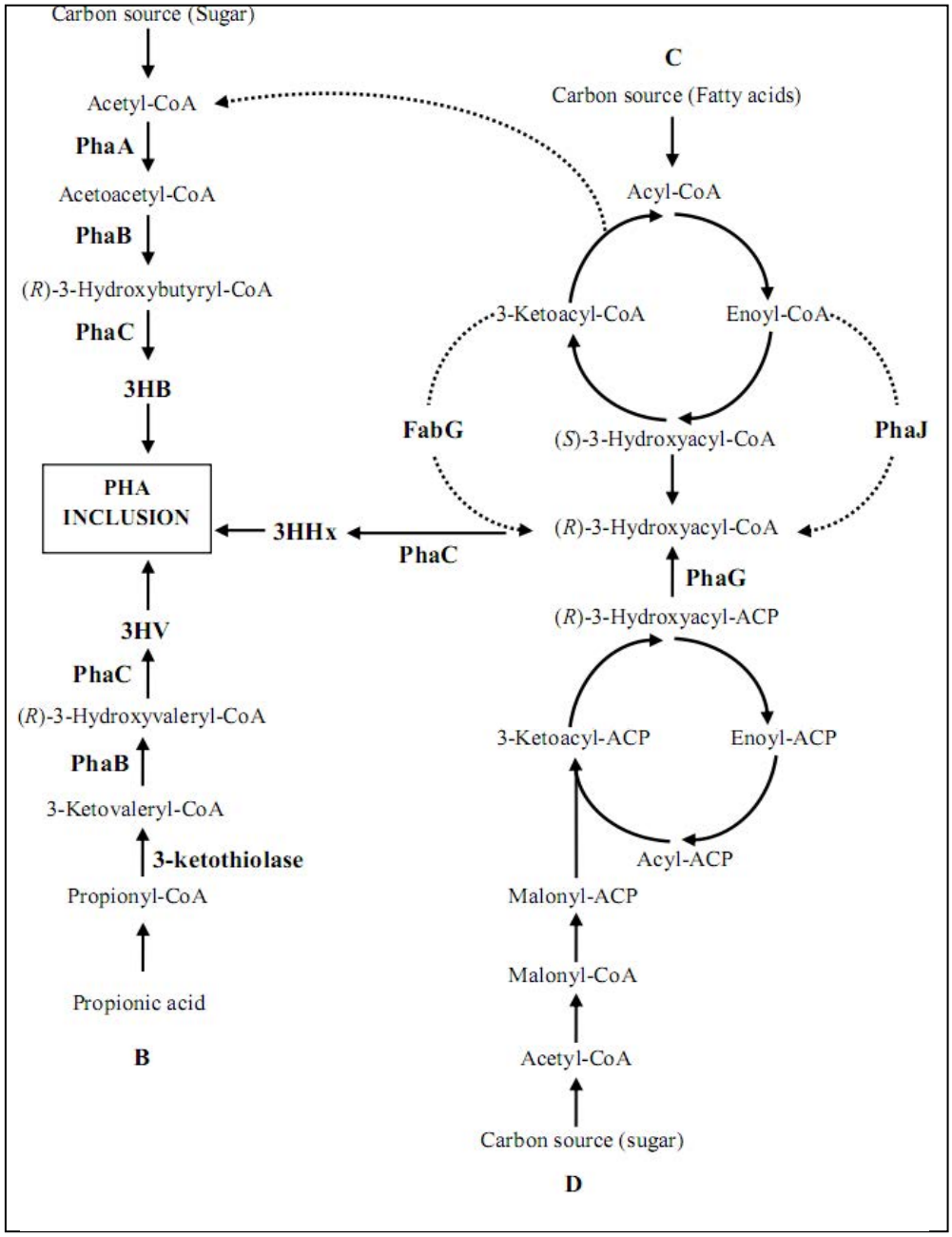


Figure 2.3: Biosynthesis pathway of (A) P(3HB); (B) P(3HB)-*co*-(3HV); (C) P(3HB)-*co*-(3HHx) via fatty acid *β*–oxidation and (D) P(3HB)-*co*-(3HHx) via fatty acid *de novo* synthesis. PhaA, *β*-ketothiolase; PhaB, NADPH dependent acetoacetyl-CoA reductase; PhaC, PHA synthase; PhaG, 3-hydroxyl-ACP-CoA transferase; PhaJ, (*R*)-enoyl-CoA hydratase; FabG, 3-ketoacyl-CoA reductase (Sudesh et al., 2000).

precursor substrate for the P(3HB-*co*-3HV) biosynthesis despite being expensive and possessing high level of toxicity. Thus, propionic acid should be fed in relatively low concentrations to the medium to prevent the toxicity effects. In addition, propionic acid is not only converted to 3HV, it is also catabolized into intermediate products of pyruvic acid or succinyl-CoA. Besides propionic acid, aliphatic fatty acids comprised of higher carbon chain length with odd number carbon atoms such as valeric acid, heptanoic acid and nonanoic acid also can be classified as propiogenic substrates (Steinbüchel and Lütke-Eversloh, 2003).

Aeromonas caviae (Doi et al., 1995) can naturally synthesize poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] when fed with fatty acids with even number of carbons. PHA synthase and (*R*)-specific enoyl-CoA hydratase (PhaJ) are enzymes responsible for the production of P(3HB-*co*-3HHx) in this bacteria. The precursors for P(3HB-*co*-3HHx) synthesis can be supplied via fatty acid *de novo* and *β*-oxidation pathway similar to that mcl-PHA. The PhaJ enzyme is important for the conversion of enoyl-CoA to (*R*)-3-hydroxyacyl-CoAs in *β*-oxidation pathway. Initially, fatty acids will be converted by thiokinase into respective acyl-CoA thioesters which are then oxidized via *β*-oxidation pathway to acetyl-CoA and acyl-CoA comprising two less carbon atoms than when entered the first cycle. In the case of fatty acids with even carbon number, the cycles will continue until the original acyl-CoA is fully converted to acetyl-CoA. For the fatty acids with odd carbon number, acetyl-CoA plus propionyl-CoA are the final products of *β*-oxidation pathway (Steinbüchel and Lütke-Eversloh, 2003).

2.4 Poly(3-hydroxybutyrate) [P(3HB)]

Among the all known PHAs, P(3HB) is the best characterized and most extensively studied. High-resolution ^{13}C nuclear magnetic resonance (NMR) spectroscopy has revealed that P(3HB) exists predominantly in a highly mobile amorphous state within the cells. Isolation of the granules could trigger irreversible conversion to the crystalline state (Amor et al., 1991; Barnard and Sanders, 1989). The molecular weight of P(3HB) synthesized is in the range of 200,000 to 3,000,000 Da depending on the producer and growth conditions (Hinrichsen, 1994). P(3HB) is a relatively stiff and brittle material with melting temperature around 180°C. The processing window of this polymer is very narrow as it is thermally unstable in the melt with significant degradation above its melting point (Abe et al., 1994). Incorporation of second monomer such as 3HV into P(3HB) chain reduces the crystallinity and melting temperature with marked increase in its elongation to break (Table 2.2).

Table 2.2: Properties of PHAs and polypropylene (Tsuge, 2002)

Property	P(3HB)	P(3HB-*co*-3HV)*	Polypropylene
Melting temperature (°C)	177	145	176
Glass transition temperature (°C)	4	–1	–10
Crystallinity (%)	60	56	50-70
Tensile strength (MPa)	43	20	38
Elongation to break (%)	5	50	400

*20 mol% of 3HV

2.5 Recovery techniques for the isolation and purification of PHA from microbial cells

2.5.1 Solvent extraction

Solvent extraction is the most extensively adopted method to recover PHA from the cell biomass. This method is also used routinely in the laboratory because of its simplicity and rapidity. Two main steps are involved, first is the modification of cell membrane permeability thus allowing release and solubilization of PHA. This is then followed by non-solvent precipitation (Jacquel et al., 2008). Extraction of PHA with solvents such as chlorinated hydrocarbons, i.e., chloroform, 1,2-dichloroethane (Ramsay et al., 1994) or some cyclic carbonates like ethylene carbonate and 1,2-propylene carbonate (Lafferty and Heinzle, 1979) is common. Lower chain ketone such as acetone is the most prominent solvent especially for the extraction of mcl-PHA (Jiang et al., 2006). Precipitation of PHA is commonly induced by non-solvent such as methanol and ethanol (Ramsay et al., 1994). Solvent extraction has undoubted advantages over the other extraction methods of PHA in terms of efficiency. This method is also able to remove bacterial endotoxin and causes negligible degradation to the polymers (Jacquel et al., 2008). So, it is possible to obtain very pure PHA with high molecular weights. Unfortunately, large-scale application of solvent extraction is generally viewed as a method that is not environmentally friendly. In addition, several other factors discourage the use of solvents such as high capital and operational costs. Another problem is the high viscosity of the extracted polymer solution when the P(3HB) concentration exceeds 5% (w/v). The viscosity of the solution interferes with cell debris removal resulting in lengthy separation process. Besides, there is a possibility that solvent extraction may disrupt the unique nascent state of the P(3HB) granules that may be useful in

certain applications. In case of accidents, the potential release of a large amount of highly toxic and volatile solvents to the environment is also of great concern (Jacquel et al., 2008; Tamer et al., 1998; Yu, 2009; Yu and Chen, 2006). Therefore, 1,2-propylene carbonate has been proposed as an alternative to halogenated solvents in the recovery process of PHA (Fiorese et al., 2009; McChalicher et al., 2010). Higher boiling point (240°C) of 1,2-propylene carbonate prevents the evaporation to the environment at lower temperatures and allows its reusability for several cycles of purification. This could reduce the solvent consumption and therefore it is viewed as economically advantageous. Besides, 1,2-propylene carbonate is considered safe due to its low toxicity. It is widely used in many applications including cosmetics (McChalicher et al., 2010). Fiorese et al. (2009) reported a maximum PHA yield of 95% and a purity of 84% when extracted from the *C. necator* H16 cells at 130°C for 30 min without involving any pretreatment. This is comparable to the values obtained from chloroform extraction (94% yield and 98% purity). For the extraction of mcl-PHA methyl *tert*-butyl ether (MTBE) has been evaluated and the extractability was found to be promising as in the case with chlorinated-solvents (Wampfler et al., 2010). Wampfler et al. (2010) claimed that MTBE would have lower environmental impact if the recovery of PHA as well as the production and recycling of MTBE could be carried out in closed facilities.

2.5.2 Digestion methods

While solvent extraction techniques involve the solubilization of the PHA granules, digestion methods involve the solubilization of the cellular materials surrounding the PHA granules. Digestion methods are well established approaches developed as an alternative to solvent extraction and can be classified into either

chemical digestion or enzymatic digestion. Because of the ready availability of various chemicals with known properties many studies have been directed towards the development of chemical digestion methods compared to enzymatic digestion.

Various chemical digestion methods have been evaluated for the recovery PHA from cellular biomass. The approach mainly utilizes sodium hypochlorite or surfactants. The important features of sodium hypochlorite such as strong oxidizing properties and non-selectivity can be manipulated to digest NPCM and facilitate PHA recovery (Yu and Chen, 2006). A range of surfactants has been evaluated such as sodium dodecyl sulfate (SDS), Triton X-100, palmitoyl carnitine , betaine and among them, SDS showed good performance. However, the quality of PHA obtained using either surfactant or sodium hypochlorite alone was not good enough. Therefore, a combination of surfactant-sodium hypochlorite was used (Yu, 2009). Isolation of PHA granules by surfactant digestion exhibited lower degree of purity but had slightly higher molecular weight than sodium hypochlorite digestion. In contrast, PHA of higher purity was obtained using sodium hypochlorite but with severe degradation of molecular weight up to 50% (Ramsay et al., 1990). Sequential surfactant-hypochlorite treatment promoted better and rapid recovery of PHA (Dong and Sun, 2000; Ramsay et al., 1990) and resulted in 50% reduction of overall cost when compared to solvent extraction (Yu, 2009). Yet, the low operating cost (Jacquel et al., 2008) and technical simplicity of this process are not complemented by the complex and unresolved problems caused by surfactant in wastewater treatment and relatively high cost of chemical agents such as SDS and sodium hypochlorite (Yu, 2009).

Hahn and co-researchers established a separation process that took advantage of both differential digestion and solvent extraction (Hahn et al., 1993; Hahn et al.,

1994). The hydrophobicity of P(3HB) and hydrophilicity of lyophilized cells accounted for the development of dispersions of a sodium hypochlorite solution and chloroform. A study by Valappil et al. (2007) described that the high molecular weight of P(3HB) could be retained by using this method. The main limitation is the use of large quantity of solvent that would raise the recovery cost. Surfactant-chelate digestion system was also explored to improve cell disruption and to increase the rate of PHA release (Chen et al., 1999). Use of recycled wastewater was proposed later as this system produced large volume of wastewater (Chen et al., 2001). Surfactant-chelate digestion (Triton X-100 and ethylenediaminetetraacetic acid [EDTA]) could isolate PHA with 90% purity from enzymatically hydrolyzed cells of *S. meliloti* (Lakshman and Shamala, 2006). Another method is the selective dissolution of non-PHA cell mass by protons to enhance PHA recovery (Yu, 2009; Yu and Chen, 2006). This method is comprised of few steps, but the most important is the solubilization of NPCM in an acidic solution to release partially crystallized PHA granules and later subjecting the suspension to decolorization in a bleaching solution. This method was claimed to lower the recovery cost by using cheaper chemicals with higher recovery efficiency. However, the process parameters have to be controlled stringently if the molecular weight is to be maintained at a minimum of 50% the original molecular weight. The P(3HB) granules recovered by this method was reported to be highly crystalline.

Recovery process of PHA using enzymatic digestion involves a rather complex procedure. Solubilization of cell components other than PHA typically consists of heat treatment, enzymatic hydrolysis and surfactant washing (Holmes and Lim, 1990). To date, various types of enzymes, especially proteases, have been evaluated for their efficiency in causing cell lysis (Yasotha et al., 2006). Lakshman

and Shamala (2006) used *Microbispora* sp. culture, which was identified to secrete protease, in the fermented broth of *S. meliloti* containing 50% of PHA to induce hydrolysis. The culture was introduced to the thermally (80°C for 10 min) inactivated biomass of *S. meliloti* and incubated for 72 h. The *S. meliloti* cells were hydrolyzed by the protease resulting in the release of the intracellular components together with the PHA granules. The culture containing the lysed cells was then subjected to a simple filtration process and PHA of 94% purity was recovered using chloroform extraction. In contrast, PHA with only 66% purity was isolated from the undigested cells by using chloroform extraction. Similar study was conducted recently with a different strain by Divyashree *et al*. (2009). *Microbispora sp*. culture mixed with *Bacillus flexus* and subjected to separation by aqueous two phase system (ATPS) resulted in PHA with 92% purity. The enzyme technique is attractive because of its mild operation conditions (Kapritchkoff et al., 2006; Middelberg, 1995). Because enzymes are very specific with respect to the reactions they catalyze, recovery of PHA with good quality could be expected. Nevertheless, the high cost of enzymes and complexity of the recovery process outweigh its advantages (Kapritchkoff et al., 2006).

2.5.3 Mechanical disruption

Mechanical cell disruption is widely used to liberate intracellular protein (Harrison, 1991). The concept has been tested to recover PHA from bacterial cells (Tamer et al., 1998). Among the various mechanical disruption methods, bead milling and high-pressure homogenization dominate the large scale cell disruption in pharmaceutical and biotechnology industries (Bury et al., 2001). Unlike other recovery methods, mechanical disruption is favored mainly due to economic

advantageous and because it causes mild damage to the products (Tamer et al., 1998). Mechanical disruption of cells does not involve any chemicals so it minimizes environmental pollution (Jacquel et al., 2008) and contamination to the products (Tamer et al., 1998). In general, the drawbacks of mechanical disruption method are, high capital investment cost, long processing time and difficulty in scaling up (Balasundaram and Harrison, 2008; Park et al., 2007).

The principle of bead mills is based on the shearing action and energy transfer from beads to cells in the contact zones. The key parameters that affect the disruption process are the bead loading and bead diameter (Middelberg, 1995). Tamer et al. (1998) reported that with bead diameter of 512 μm and 2800 rpm agitation speed, complete disruption of the *Alcaligenes latus* cells was achieved after eight passes when the loading was 85% compared to loading of 75% that required more than 16 passes to release most of the cellular protein. The extent of cell disruption also depends on numerous parameters such as residence time distribution (RTD), shear forces, type of microorganisms, cell concentration, feed rate of the suspension, agitator speed, geometry of the grinding chamber and design of the stirrer (Doucha and Lívanský, 2008). Bead mills disruption was recommended for PHA recovery as it requires less power supply, not susceptible to blockages and different diameter of beads did not significantly affect the disruption rate although micronization of P(3HB) is possible with smaller bead size (Tamer et al., 1998). The major concern is that large number of factors has to be considered to establish a good bead mill disruption system (Doucha and Lívanský, 2008).

With high pressure homogenization, disruption of cell suspension occurs under high pressure through an adjustable, restricted orifice discharge valve (Geciova et al., 2002). Process parameters such as operating pressure, number of passes,

suspension temperature and homogenizer valve design must be carefully scrutinized for efficient disruption (Diels and Michiels, 2006; Kelly and Muske, 2004). It was reported that less efficient recovery of P(3HB) from *A. latus* was obtained with homogenizer compared to bead mill disruption due to severe micronization (Tamer et al., 1998). Nevertheless, P(3HB) with 95% purity and 98% yield was recovered from 5% (w/v) SDS pretreated *Methylobacterium* sp. V49 cells subjected to homogenization at an operating pressure 400 kg cm^{-2} after two cycles (Ghatnekar et al., 2002). Process parameters are not the only factors that influence the cell disruption but microbial physiological parameters, namely type and growth phase of the microorganisms as well as cell concentration also affect the disruption efficiency. Generally, Gram-positive bacteria are more difficult to be disrupted compared to Gram-negative bacteria (Diels and Michiels, 2006). Among the drawbacks associated with high pressure homogenization include the possibility of thermal degradation of desired products (Ghatnekar et al., 2002) and formation of fine cellular debris that would interfere with the further downstream processing of PHA granules (Kelly and Muske, 2004).

2.5.4 Supercritical fluid (SCF)

Supercritical fluids (SCF) have emerged as a potential extraction technique in the areas of PHA recovery (Hejazi et al., 2003; Khosravi-Darani and Mozafari, 2009; Khosravi-Darani et al., 2003). The unique physicochemical properties of SCF such as high density and low viscosities proposed them as suitable extraction solvents (Hejazi et al., 2003). The advantages offered by SCF have led to its popularity. Supercritical-carbon dioxide (SC-CO_2) is the most predominantly used SCF due to its low toxicity and reactivity, moderate critical temperature and pressure (31°C and

73 atm), availability, low cost, and non-flammability (Hejazi et al., 2003). By using this method, P(3HB) recovery of 89% from *C. necator* H16 at 200 atm, 40°C and 0.2 mL of methanol was reported (Hejazi et al., 2003). Although, the recovery obtained are comparable to other methods, it has to be highlighted that SC-CO_2 efficiency in bacterial cell disruption is very much dependent on the process parameters such as operating pressure, temperature, type of modifier as well as culture cultivation time (Khosravi-Darani et al., 2004). High temperature and pressure significantly influence the physiological properties of cell membrane that prevent the biopolymer from being extracted. The addition of modifier plays an important role in increasing the polarity of the CO_2 therefore suitable modifier should be selected to enhance the cell wall permeability. Matured cells are difficult to be disrupted compared to those in early-exponential phase as new proteins would be synthesized increasing the cell resistance to disruption. In order to make the process more economically viable, Khosravi-Darani *et al*. (2004) investigated on a series of pretreatment to improve the SC-CO_2 disruption. They found that with 1% (v/v) toluene as a modifier, 200 bar of pressure, 30°C temperature and two times SC-CO_2 pressure release, up to 81% P(3HB) recovery could be achieved by using wet cells of *C. necator H16.* To further improve the purity of the P(3HB), chemical pretreatment with 0.4% (w/w) sodium hydroxide (NaOH) was employed. The proposed recovery process of PHA is more economical as the costly freeze drying step could be avoided.

2.5.5 Cell fragility

Increase in osmotic fragility during the accumulation of PHA are well documented with some microorganisms such as *Azotobacter vinelandii* UWD and recombinant *E. coli* (Choi and Lee, 1999b; Page and Cornish, 1993). The cell wall

strength of these microorganisms could be compromised by modifying the composition of the growth medium. Cell fragility mechanism is not only restricted to Gram negative microorganisms but could be also exploited for Gram positive microorganisms (Divyashree and Shamala, 2010). Page and Cornish (1993) demonstrated that the addition of fish peptone to the cultivation medium of *A. vinelandii* UWD led to the formation of large, pleomorphic, osmotically sensitive cells while high molecular weight P(3HB) synthesis was enhanced. About 92% of P(3HB) could be quickly extracted from the fragile cells with 1 N aqueous NH_3 (pH 11.4) at 45°C for 10 min. The same phenomenon was observed with *B. flexus* (Divyashree and Shamala, 2010). The cells grown in the inorganic salts medium suffered from the absence of diaminopimelic acid (DAP) and decreased concentrations of other amino acids. DAP is an important component that formed cross bridge in the peptidoglycan and have a great influence on the structural stability of the cell wall. Up to 86-100% PHA recovery was obtained using hot chloroform or mild alkaline hydrolysis with cells cultivated in inorganic medium while only 32-56% of PHA was able to be extracted from cells grown in organic medium supplemented with peptone or yeast. Simple recovery method with mild extraction conditions could be developed based on cell fragility. However, it is necessary to balance the cell wall softening and cell wall integrity (Page and Cornish, 1993) in order to promote microbial growth with higher PHA content.

2.5.6 Flotation

Ibrahim and Steinbüchel (2009) investigated the recovery of P(3HB) from a recently isolated bacterium, *Zobellella denitrificans* MW1. Simple extraction with various organic solvents followed by self-flotation of cell debris was tested. The cells