ACKLNOWLEDGEMENTS

I would like to extend my profound thankfulness and indebtedness to my supervisor, Assoc. Prof. Dr. K. Sudesh Kumar for accepting me to pursue my post-graduate study and for his resolute guidance, valuable advice, constructive suggestions, understanding, help and approachability throughout my project.

I would also like to express my deepest appreciation and sincere gratitude to my co-supervisor, Assoc. Prof. Dr. Mohd. Razip Samian for his valuable suggestion and generosity in providing assistance during the course of this study.

Very special thanks to all the members of lab 409, 414, 418, 406, 318, Muhamad Odeh, Pang Pei Kheng, Prof. Darah Ibrahim, Prof. Nazalan Najimudin, Dr. Amirul Al-Ashraf, Dr. Tengku Sifzizul Tengku Muhammad, staff of Electron Microscopy Unit, Biotechnology Lab and Microbiology Lab who have contributed directly or indirectly to the accomplishment of my study.

I express my appreciation to my parents and brother for their continuous understanding, moral support and unconditional love that I could not have done without.

Last but not least, I want to express my eternal gratitude to the Ministry of Education for providing me with a scholarship, Malaysian Toray Science

Foundation in supporting my project, Acidchem Ltd. and Unitata Ltd. for the kind provision of palm oil products.

Polyhydroxyalkanoate (PHA) biosynthesis from local mixed cultures and *Burkholderia* sp. USM (JCM15050)

ABSTRACT

Polyhydroxyalkanoate (PHA) production was conducted by mixed cultures obtained from various niches (rubber plantation soil, oil palm plantation soil, industrial aeration tank, industrial sedimentation tank, oil polluted wastewater and lake water) in Northern Peninsular Malaysia. Among the various types of monomers, 3-hydroxybutyrate (3HB) was the most wide-spread PHA among the microorganisms from all the samples. Most of the bacteria that capable of producing short-chain-length (SCL) PHA occurred in oil palm plantation soil, oil polluted waste water and lake water. While rubber plantation soil, industrial aeration tank and industrial sedimentation tank samples contained bacteria capable of synthesizing both SCL and medium-chain-length (MCL) PHA. Among the various PHA-producing bacteria isolated, one strain identified as *Burkholderia* sp. USM (JCM15050) from oil polluted wastewater sample showed the most efficient growth and PHA biosynthesis. *Burkholderia* sp. was capable of converting palm oil products and glycerol into P(3HB). Up to 70 wt% and 60 wt% of P(3HB) could be obtained when 0.5 % (v/v) crude palm kernel oil (CPKO) or glycerol was fed respectively. The isolate incorporated approximately 78 mol% of 3-hydroxyvalerate (3HV) monomer in two-stage cultivation when fed with sodium valerate as the sole carbon source. In order to investigate the PHA monomer supplying pathways in *Burkholderia* sp., a plasmid (pBBREE32d13) which carries

the PHA synthase gene (*phaC*$_{Ae}$) of *Aeromonas caviae* was transconjugated into this bacterium. The resulting recombinant *Burkholderia* sp. incorporated approximately 1 mol% of 3-hydroxyhexanoate (3HHx) monomer when CPKO or palm kernel acid oil (PKAO) was used as the sole carbon source. In addition, when the recombinant strain was cultivated in mixtures of CPKO and sodium valerate, PHA containing 69 mol% 3HB, 30 mol% 3HV and 1 mol% 3HHx monomers was produced. Batch feeding of carbon sources with 0.5 % (v/v) CPKO at 0 h, and 0.25 % (w/v) sodium valerate at 36 h could yield PHA content up to 86 wt% of the dry cell weight containing these three types of monomers. The 3HHx molar fraction could be increased up to 6 mol% by controlled feeding strategies. On the other hand, feeding of sodium propionate at 36 h could yield 76 wt% of PHA, with the 7 mol% of 3HHx composition was the highest obtained in this study. TEM images of wild type *Burkholderia* sp. revealed the presence of 2 – 7 PHA granules of variable sizes in each cell. In contrast, for the recombinant strain 5 – 13 granules per cell were observed. This study has successfully identified a suitable bacterium that can grow and produce various types of SCL-PHA from palm oil products and glycerol. In addition, the metabolic pathways of this bacterium were shown to be able to supply 3HHx monomer.

Biosintesis polihidroksialkanoat (PHA) oleh kultur campuran dan *Burkholderia* sp. USM (JCM15050)

ABSTRAK

Penghasilan polihidroksialkanoat (PHA) telah dikendalikan dengan menggunakan kultur campuran yang diperolehi dari pelbagai nic (tanah ladang getah, tanah ladang kelapa sawit, tangki pengudaraan industri, tangki pengenapan industri, air buangan yang dicemari minyak serta air kolam) di Utara Semenanjung Malaysia. Antara pelbagai jenis monomer, 3-hidroksibutirat (3HB) adalah monomer PHA yang paling biasa dihasilkan oleh mikroorganisma dari semua jenis sampel. Kebanyakan bakteria yang berupaya menghasilkan SCL-PHA (PHA dengan monomer rantai pendek) adalah didapati dari tanah ladang kelapa sawit, air buangan yang dicemari minyak dan air kolam. Manakala, tanah ladang getah, tangki pengudaraan industri dan tangki pengenapan industri didapati mengandungi bakteria yang berupaya menghasilkan kedua-dua jenis SCL- dan MCL-PHA (PHA dengan monomer rantai sederhana panjang). Antara bakteria yang dipencilkan, satu strain daripada air buangan yang dicemari minyak yang telah dikenalpasti sebagai *Burkholderia* sp. USM (JCM15050) didapati menunjukkan pertumbuhan dan biosintesis PHA yang paling baik. Bakteria ini mampu menukarkan pelbagai jenis produk minyak kelapa sawit dan gliserol kepada P(3HB) sehingga 70 % dan 60 % P(3HB) boleh diperolehi apabila 0.5 % (i/i) minyak isirong kelapa sawit mentah (CPKO) atau gliserol masing-masing ditambah ke dalam medium pengkulturan. *Burkholderia* sp. ini

didapati berupaya menghasilkan kira-kira 78 mol% monomer 3-hidroksivalerat (3HV) dari pengkulturan dua peringkat di mana sodium valerat digunakan sebagai sumber karbon tunggal. Demi mengkaji laluan metabolisme yang membekalkan monomer PHA dalam *Burkholderia* sp., plasmid pBBREE32d13 yang membawa gen PHA sintase (*phaC*$_{Ac}$) kepunyaan bakteria *Aeromonas caviae* telah ditranskonjugasi ke dalam *Burkholderia* sp. *Burkholderia* rekombinan ini didapati boleh menghasilkan kira-kira 1 mol% monomer 3-hidroksiheksanoat (3HHx) apabila CPKO atau minyak kernel asid kelapa sawit (PKAO) digunakan sebagai sumber karbon tunggal. Selain itu, sebanyak 69 mol% 3HB, 30 mol% 3HV dan 1 mol% 3HHx telah diperolehi apabila campuran CPKO dan sodium valerat digunakan. Sebanyak 0.25 % (b/i) sodium valerat ditambah pada jam ke-36 pengkulturan di dalam medium yang mengandungi 0.5 % (i/i) CPKO, menghasilkan kandungan PHA sebanyak 86 % daripada berat kering sel yang mengandungi ketiga-tiga jenis monomer. Komposisi 3HHx boleh ditingkatkan sehingga 6 mol% dengan menggunakan strategi pengawalan penyuapan. Monomer 3HHx sebanyak 7 mol% berjaya dihasilkan apabila sodium propionat ditambah ke dalam medium. Analisis TEM menunjukkan kira-kira 2 – 7 granul diperhatikan di dalam setiap sel *Burkholderia* sp. Manakala, kira-kira 5 – 13 granul per sel diperhatikan di dalam *Burkholderia* rekombinan. Kajian ini telah berjaya mengenalpasti bakteria yang berupaya tumbuh dan menghasilkan pelbagai jenis SCL-PHA dari produk minyak kelapa sawit dan gliserol. Tambahan lagi, laluan metabolisme bakteria ini menunjukkan ia berupaya membekalkan monomer 3HHx.

1.0 INTRODUCTION

Over the past decade, the intrinsic resistance of plastic materials to degradation neither chemically nor biologically has been increasingly regarded as a main source of environmental and waste management problems. Synthetic plastic materials that account for approximately 20 % of the volume of municipal solid waste have reduced the capacity of precious landfill sites (Stein, 1992). With the growing legislation in developed countries aimed to bar the usage of synthetic plastics in consumer products (Leaversuch, 1987), the development and production of biodegradable plastics are rapidly expanding.

Bacterially produced polyhydroxyalkanoate (PHA) is an intracellular reserve material which is accumulated as carbon and energy source for most of the bacteria usually under the conditions of limiting nutrient elements and the presence of excess carbon source. PHA possesses interesting characteristics almost similar to common synthetic plastics such as polypropylene (PP). PHA can be degraded enzymatically by extracellular depolymerase excreted by various PHA-degrading bacteria. Current attention on the environmental waste management problems has given a great impetus on the research of PHA as a suitable alternative for synthetic plastics.

However, the major drawback which is still hampering a greater application of PHA in our daily life is its high cost of production (Choi and Lee 1997; Salehizadeh and Van Loosdrecht, 2004). Among the substrates required,

carbon source is of primal concern of PHA production. Since cost is a critical issue in determining PHA as a successor over synthetic plastic in the near future, researchers are seeking for cheaper carbon sources which could generate higher production.

Recently, plant oils have been identified as cheap and renewable resources for the production of PHA. Plant oils are desirable as feedstock for producing PHA because their yield coefficients of PHA production are high. Approximately 1.0 g PHA / g soybean oil was obtained by Akiyama and co-workers (Akiyama *et al.*, 2003). In this study, various palm oil products have been screened to determine the most suitable carbon source for the production of bacterial PHA. Palm oil is readily available in Malaysia (Yacob *et al.*, 2006) and preliminary studies have indicated it to be suitable substrates for PHA (Majid *et al.*, 1994; Loo *et al.*, 2005; Zazali and Tan, 2005).

Another alternative approach for reducing the cost of PHA production is the application of mixed culture systems. This is because the use of PHA production in open mixed cultures required minimum process control, no requirement of sterility processing, and an improved use of waste materials (Salehizadeh and Van Loosdrecht, 2004). Thus, an open mixed cultures system could decrease the cost of PHA production and increase their potential in marketing (Patnaik, 2005). Previous studies with mixed culture systems indicate promising results in the production of PHA (Salehizadeh and Van Loosdrecht,

2004; Patnaik, 2005; Lemos *et al.*, 2006). Being a tropical country, Malaysia is rich in biodiversity of microorganisms from various ecosystems. Undeniably there are possibilities of identifying various microorganisms for the production of PHA.

1.1 Objectives of research and rationale behind this study

In recent years, development of mixed cultures has offered new advantages of combining different genetic characteristics of a variety of microorganisms into a single process. It excludes applying complex genetic knowledge. With to this idea, mixed cultures have attracted the attention of researchers especially in PHA synthesizing field. Production of PHA using mixed cultures offers an opportunity and demand to reduce costs that has been known to be the major factor in hampering the bulk production of PHA. Thus, it provides a better status to compete with synthetic plastics. To date, no studies have been carried out to survey the production of PHA by the microorganisms collected from various environmental locations. It is essential to understand the relationship between environmental diversity and the types of PHA produced by the mixed cultures of microorganisms. Therefore, based on the above rationale, the objectives of this study are listed as below:

1. To investigate the types of PHA produced from different environmental samples using various types of carbon sources. The knowledge obtained here would enable a more systematic search for a particular type of microorganism that can produce a specific type of PHA.

2. To isolate a pure culture from the selected mixed culture sample that could utilize palm oil efficiently and at the same time able to produce PHA. Palm oil is found to be the most readily available and moderately priced renewable carbon source in Malaysia. It was therefore believed that palm oil could reduce the cost of PHA production by contributing to higher yield.

3. To evaluate various palm oil products and by-products as well as special precursor compounds to determine the types of PHA produced by the isolate.

4. To investigate the PHA monomer supplying metabolic pathway of this isolate. For this, the PHA synthase gene of *Aeromonas caviae* (*phaC*$_{Ac}$) which is capable of polymerizing 3-hydroxyhexanoate (3HHx) monomer was introduced into the isolate. The analysis of PHA produced by the recombinant strain will enable the determination of active metabolic pathways for the supply of PHA monomers.

5. Ultrastructural studies on both wild type and recombinant strains were done to observe the morphological characteristics of intracellular PHA granules formation.

Overall, the scope of this research was to obtain an isolate capable of utilizing palm oil efficiently as well as synthesizing high amount of PHA. The findings will be of great importance to create an alternate solution for palm oil by-products which in turn make it a perfect candidate for commercial production of PHA.

2.0 LITERATURE REVIEW

2.1 Overview of the Malaysian oil palm industry

Malaysia is one of the world's major producers and exporters of palm oil. The total oil palm plantation in Malaysia has increased from 641,000 hectares in year 1975 to 4.2 million hectares in year 2006 (MPOB, 2006) Sabah remains as the state with the largest oil palm plantation accounting for 30 % of the total plantation area. The impressive increase in production of palm oil products is mainly credited to the increase in matured plantation areas, enhanced plantation and mill management, recovery in fresh fruit bunches yield per hectares and continued improvement in the oil extraction rate.

Overall, the palm oil exports have increased by 7.3 % to 14.23 million tonnes (year 2006) from 13.45 million tonnes in year 2005 (MPOB, 2006). China maintained as the largest importer followed by the European Union and India. The amount of palm oil exports to the United States of America (US) increased as the food manufactures switched to palm oil in anticipation of trans-fat labeling which came into effect since 1st January 2006 (MPOB, 2006). Overall, demand for palm oil looks promising because of the abolishment of palm oil import quota by China and because of the trans-fat labeling in the US (MPOB, 2006).

In year 2005, National Biofuel Policy was announced by the Malaysian Government to spur the development of the biofuel industry in Malaysia. The policy entails a strategy that includes the production of a biofuel blend comprising

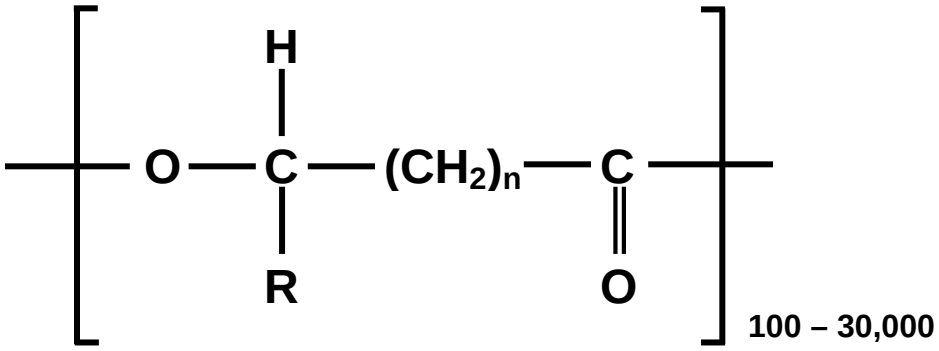
of 5 % processed palm oil with 95 % diesel. The outlook for palm oil industries in the future is likely to be positive with the setting up of biodiesel plants in Malaysia (MPOB, 2006).

2.2 Occurrence of polyhydroxyalkanoate (PHA) and its importance

Many bacterial strains possess inclusion bodies which have been classified into two major groups based on the presence or absence of a surrounding membrane (Shively, 1974). For example, protein, polyglucoside and polyphosphate storage granules are not enclosed by a membrane; and lipid deposits, such as PHA, sulphur globules and gas vacuoles are surrounded by a non-unit membrane (Sudesh *et al.*, 2000).

PHA is synthesized by microorganisms under conditions of nutrient limitation and in the presence of excess carbon and energy sources. It has been shown that the bacteria containing PHA storage materials would be able to survive during starvation period compared to those without PHA. The ability of the bacteria to survive is often said to be proportional to the initial PHA content and the length of starvation period, as this energy-reserved material slows down the cell autolysis and sequentially its mortality (Macrae and Wilkinson, 1958a). Since PHA is insoluble in water, it exerts negligible increase in osmotic pressure inside bacterial cytoplasm. Therefore, it is ideal as a storage compound (Doi, 1990; Sudesh *et al.*, 2000).

PHA, a group of organic polyoxoesters, is an optically active microbial polyester containing hydroxyacyl monomer units (Anderson and Dawes, 1990) with the general structure shown in Figure 2.1.



		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 = hydrogen	5-hydroxyvalerate
	R = methyl	5-hydroxyhexanoate
n = 4	R = hexyl	6-hydroxydodecanoate

Figure 2.1 General structure of PHA and some of its monomers (Sudesh *et al.*, 2000).

Poly(3-hydroxybutyrate), P(3HB) was first described by Lemoigne, a French scientist in year 1925 (Doi, 1990). Lemoigne had isolated and identified the material from *Bacillus megaterium*. Since then, various bacterial strains amongst archaebacteria (Doi, 1990), Gram positive (Williamson and Wilkinson, 1958; Findlay and White, 1983) and Gram negative bacteria (Forsyth *et al.*, 1958), photosynthetic bacterium (Hashimoto *et al.*, 1993; Hassan *et al.*, 1996; 1997; 1998), and even cyanobacteria (Jensen and Sicko, 1971; Jau *et al.*, 2005) have been identified to accumulate intracellular P(3HB) both aerobically and anaerobically. The recognition of the role of P(3HB) as a bacterial storage polymer that possesses a function almost similar to starch and glycogen was accepted worldwide in year 1973 (Dawes and Senior, 1973). Macrae and Wilkinson noticed that *B. megaterium* initiated the accumulation of P(3HB) homopolymer when the ratio of glucose to nitrogen in the culture medium was high (Macrae and Wilkinson, 1958b) and subsequent intracellular degradation of P(3HB) occurred in the absence of carbon and energy sources (Macrae and Wilkinson, 1958a). The opinion of 3HB monomer as the only constituent of forming this polymer changed after a year of its acceptance as bacterial storage materials (Steinbüchel and Valentin, 1995; Sudesh *et al.*, 2000).

Wallen and Rohwedder (1974) reported the discovery of other monomer constituents beside 3HB monomer from activated sewage sludge. Among the polymers extracted from the sludge, 3-hydroxyvalerate (3HV), 3-hydroxyhexanoate (3HHx) and 3-hydroxyheptanoate (3HHp) monomers existed

as the major and minor constituents respectively. In the year 1983, 3HHp was identified in *B. megaterium* (Findlay and White, 1983). Consequently in the same year, De Smet and co-workers (1983) identified a new monomer, 3-hydroxyoctanoate (3HO) with trace amount of 3HHx from *Pseudomonas oleovorans* when this bacterium was fed with structurally related substrate, *n*-octane. This investigation shows that, the production of HA monomers depend of the substrate fed.

To date, about 150 different monomer constituents have been found (Steinbüchel and Valentin, 1995; Steinbüchel, 2001) and hence, a general name, 'PHA' was designated for this family instead of HA, since it is chained up to form a biopolymer. Witholt and Kessler (1999) had documented the large variety of PHA monomers with straight, branched, saturated, unsaturated and also aromatic structures.

There are two major groups of PHA; short-chain-length (SCL) with five or less carbon atoms in a monomer, and medium-chain-length (MCL) with six to fourteen carbon atoms in a monomer. *Cupriavidus necator* (formerly known as *Alcaligenes eutrohus* or *Wautersia eutropha*) is a well studied bacterium capable of producing SCL and it has been identified to produce polymers consisting of 3HB, 3HV and 4HB monomers (Kunioka *et al.*, 1989; Doi, 1990; Saito *et al.*, 1996). *P. oleovorans* and *Pseudomonas putida* are known to synthesize MCL consisting of 3HO and 3-hydroxydecanoate (3HD) monomers as major

components. To date, only SCL-PHA possesses value impact and has been commercially produced up to 500 tonnes per year by Monsanto (Kellerhals *et al.*, 2000). MCL has yet to make a significant impact as a feasible choice due to the fact that this polymer is very expensive to produce in bulk amount as the yield is relatively low, thus hampering the development of its application (Lee *et al.*, 2000).

2.3 PHA from mixed culture samples

Many different types of microorganisms live together in natural ecosystems. In a complex ecosystem, many microenvironments exist to allow microorganisms to grow under their desired conditions. In more homogenous ecosystems like ocean and lake, the microbial community is less diverse. Under extreme conditions like hot spring water and Dead Sea, only a few microorganisms are able to grow (Stolp and Starr, 1981).

Direct isolation of bacteria from the natural environments is possible for many bacteria by using selective media. Selective media is defined as media which favors the growth of particular microorganisms and disfavours the others. Direct plating under selective conditions may yield a large number of microorganisms of the same metabolic type which is selectively favoured whereby the best adapted bacteria would develop with the best growth rate. On the other hand, when the substrate is present for a limited amount in the medium,

the microorganisms which are only able to grow on the substrate as well as producing PHA can survive and dominate in the system (Dionisi *et al.*, 2006).

The utilization of complex carbon sources in certain environments by a complex consortium of bacteria may result in accumulation of PHA with interesting and unexpected compositions. For example, samples taken from activated sludge consisted of 3HB, 3HV 3HHx, 3HHp, 3HO, 3-hydroxynonanoate (3HN), 3HD, 3-hydroxy-2-methylbutyrate (3H2MB), 3-hydroxy-2-methylvalerate (3H2MV), 3-hydroxy-6-methylheptanoate and 3-hydroxy-7-methyloctanoate monomers (Wallen and Rohwedder, 1974; Odham *et al.*, 1986; Satoh *et al.*, 1992; Steinbüchel *et al.*, 1994; Saito *et al.*, 1995). The role of PHA as metabolic intermediate in bacterial processes for activated sludge system that occurs under dynamic conditions has stimulated the idea of PHA production using mixed cultures (Van Loosdrecht *et al.*, 1997). Under anaerobic condition, microorganisms from activated sludge accumulate PHA to approximately 20 % of dry cell weight. Up to 62 % of PHA can be accumulated from microorganisms from activated sludge in microaerophilic-aerobic sludge process (Satoh *et al.*, 1998; Takabatake *et al.*, 2002). Saito and co-workers (1995) also reported that the activated sludge accumulated more PHA under aerobic conditions than anaerobic conditions. It was observed that when acetate was fed as carbon source, PHA was produced with the following monomer compositions; 87 % of 3HB, 11 % of 3HV, 2 % 3H2MV and 1 % 3H2MB. However, when this mixed culture was fed with propionate as carbon source, these subsequent monomer

compositions were obtained; 3 % of 3HB, 43 % of 3HV, 50 % 3H2MV and 6 % 3H2MB.

The most common PHA producers isolated from activated sludge are from the following genus; *Alcaligenes*, *Comamonas*, *Pseudomonas*, *Acinetobacter*, *Xanthobacter* and the newly isolated *Thauera* (Dionisi *et al.*, 2006). Some bacterial strains in mixed cultures have been known for their ability to store carbon sources anaerobically (Chech and Hartman, 1993). Polyphosphate-accumulating organisms and glycogen-accumulating organisms have also been identified.

2.4 Physical and chemical properties of PHA

2.4.1 Poly(3-hydroxybutyrate), P(3HB)

The P(3HB) polymer isolated from bacteria is of relatively high number average molecular weight, ranging from 10^5-10^6, and of very high crystallinity as well (Doi, 1990). P(3HB) is a stereoregular polyester. The absolute configurations of all the β-carbons in the polymer are identical, so the polymer is completely isotatic with its melting temperature around 180 °C, which is just slightly lower than its thermal degradation temperature (185 °C). Since the thermal degradation temperature is too close to the melting temperature, this makes the injection molding process difficult. P(3HB) has several useful properties such as moisture resistance and water insolubility. P(3HB) also shows good oxygen impermeability (Holmes, 1988; Lindsay, 1992). However, the elongation to break for P(3HB) is

only 5 %, which is apparently lower than that of PP (400 %), a widely used synthetic commodity plastic. Although P(3HB) is relatively brittle compared with PP, it is stronger in terms of stiffness.

Recombinant *Escherichia coli* harboring the PHA synthase gene (*PhaC*) from *C. necator* produced ultra-high-molecular weight P(3HB) homopolymer ranging from 3 X 10^6 to 1.1 X 10^7 (Kusaka *et al*., 1997). The mechanical properties of these ultra-high-molecular weight polymers were noticeably better compared to those produced by the wild-type bacterium with the elongation to break, Young's Modulus and tensile strength of 58%, 1.1 GPa and 62 MPa respectively (Kusaka *et al*., 1999).

P(3HB) occurs in the cell cytoplasm in the form of granules with diameters in the range of 0.3-1.0 μm (Doi, 1990) and it is known that P(3HB) polymers in the native granules are in mobile amorphous state (Barnard and Sanders, 1988; 1989). Kawaguchi and Doi (1990) reported that the crystallization of P(3HB) is started by the removal of a lipid component from native granules by various treatments, such as lipase, alkaline and acetone.

2.4.2 Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), P(3HB-*co*-3HV)

Introduction of 3HV into P(3HB) polymer chain influences the physical and mechanical properties of the resultant copolymer. The P(3HB-*co*-3HV) copolymer has been developed and has much improved mechanical properties compared to

the P(3HB) homopolymer. The polymer becomes tougher (increase in impact strength) and more flexible (decrease in Young's modulus) as the molar fraction of 3HV unit increases (Table 2.1). The elongation to break was also reported to increase as the 3HV fraction increases (Lee, 1996a). Furthermore, the melting temperature decreases with increasing 3HV fraction without any changes in the degradation temperature. This allows the thermal processing of the copolymer without thermal degradation. Luzier (1992) reported that the P(3HB-*co*-3HV) copolymer containing 20 mol% of 3HV monomer has a decreased Young's modulus from value 3.5 GPa to 0.8 GPa. The tensile strength of the copolymer also decreased from 43 MPa to 20 MPa and thus improving the elongation to break to 50 %. Therefore, the P(3HB-*co*-20 mol% 3HV) copolymer is less stiff and brittle but more elastic and easier to mould than P(3HB) homopolymer (Luzier, 1992).

Table 2.1 Comparison of polymer properties (Doi *et al.*, 1995; Saito *et al.*, 1996; Ojumu *et al.*, 2004; Khanna and Srivastava, 2005).

Properties	Melting point (°C)	Young modulus (GPa)	Tensile strength (MPa)	Elongation to break (%)	Crystallinity (%)	Glass transition temperature (°C)
P(3HB)	180	3.5	40	5	60	4
P(3HB-*co*-3HV)						
3 mol% 3HV	170	2.9	38	-	-	-
9 mol% 3HV	162	1.9	37	-	-	-
14 mol 3HV	150	1.5	35	-	-	-
20 mol% 3HV	145	1.2	32	-	-	-
25 mol% 3HV	137	0.7	30	-	40	– 1
P(3HB-*co*-4HB)						
10 mol% 4HB	159	-	24	242	-	-
16 mol% 4HB	130	-	26	444	45	– 7
64 mol% 4HB	50	30	17	591	15	– 35
78 mol% 4HB	49	-	42	1120	17	– 37
82 mol% 4HB	52	-	58	1320	18	– 39
90 mol% 4HB	50	100	65	1080	28	– 42
P(4HB)	53	149	104	1000	34	– 48
P(3HB-*co*-3HHx)						
5 mol% 3HHx	151	-	-	-	-	0
10 mol% 3HHx	127	-	21	400	-	– 1
15 mol% 3HHx	115	-	23	760	-	0
17 mol% 3HHx	120	-	20	850	-	– 2
19 mol% 3HHx	111	-	-	-	-	– 4
25 mol% 3HHx	52	-	-	-	-	– 4
Polypropylene	176	1.7	34.5	400	70	45
LDPE	130	0.2	10	620	-	– 30

2.4.3 Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate), P(3HB-*co*-4HB)

P(3HB-*co*-4HB) is another interesting PHA copolymer with great potential in the medical field. Increment of 4HB molar fraction in P(3HB-*co*-4HB) copolymers would lower the melting temperature (T_m) of the copolymer, from approximately 178 °C to 37 °C. In addition, the heat of fusion (ΔH_m) decreased with an increase in the 4HB molar fraction, indicating a decrease in the degree of crystallinity of P(3HB-*co*-4HB). Therefore, with an increase in the 4HB fraction, the copolymer properties change from highly crystalline to elastomeric (Saito and Doi, 1994; Kang *et al.*, 1995). The physical and mechanical properties of P(3HB-*co*-4HB) are summarized in Table 2.1. Pure P(4HB) homopolymer possesses good tensile strength and it is almost similar to ultrahigh molecular weight polyethylene (PE). Generally, when the composition of 4HB increases from 64 to 100 mol%, the tensile strength increases from 17 to 104 MPa, indicating that P(3HB-*co*-4HB) copolymer and P(4HB) homopolymer are very strong thermoplastic elastomers (Saito and Doi, 1994).

P(3HB-*co*-4HB) and P(4HB) are commercially important PHA that have potential applications in the medical and pharmaceutical fields as absorbable materials and as controlled drug delivery agents. Due to their high level of biocompatibility, they can be incorporated into human body (Zinn *et al.*, 2001). The incorporation of 4HB monomer into 3HB units results in a copolymer that could be hydrolyzed not only by P(3HB) depolymerases but also by lipases and

esterases since no alkyl side chains occur as pendant groups, attached to its backbone (Mukai *et al*., 1994; Saito *et al*., 1996).

2.4.4 Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate), P(3HB-*co*-3HHx)

Generally, PHA containing SCL such as P(3HB) is highly crystalline and have poor tensile strength (Doi, 1990). It is too brittle and breaks easily. In contrast, PHA containing MCL has much lower crystallinity and higher elasticity, which enables it to be used as a biodegradable rubber and elastomer. However it is too sticky to be processed conveniently. Therefore, combination of these both SCL and MCL would exhibit a better property of PHA. P(3HB-*co*-3HHx) is an interesting copolymer whereby its backbone consists of 3HB monomer and 3HHx monomer (an MCL monomer, which contains six carbon atoms). By combining both SCL-PHA and MCL-PHA, this copolymer has superior properties from both sides.

P(3HB-*co*-3HHx) copolymer was first found in *Aeromonas caviae* (Shimamura *et al.*, 1994; Doi *et al.*, 1995;). The T_m of P(3HB-*co*-3HHx) decreases from 178 °C to 120 °C once the 3HHx monomer fraction increases from 0 to 17 mol%. Whereas the ΔH_m and T_g also decrease with increasing 3HHx fraction, from 97 J/g to 34 J/g and from 4 °C to – 2 °C respectively (Kobayashi *et al.*, 1994; Shimamura *et al.*, 1994). In contrast, the elongation to break increases from 5 to 850 %. This result indicates that the P(3HB-*co*-3HHx) copolymer become soft and flexible with an increase in the 3HHx fraction.

2.4.5 MCL-PHA

Most SCL-PHA is highly crystalline materials and therefore possesses high modulus properties. Due to their intrinsic rigidity, these polymers only cover part of the applications for biodegradable materials (De Koning, 1995). Hence, a complementary type of PHA is needed to cover the rubbery applications. Gagnon and co-workers (1992) reported the existence of elastomeric PHA that contains longer branched carbon atoms with low crystallinity: the MCL-PHA.

Similar to P(3HB), MCL-PHA are fully isotatic (Lageveen *et al.*, 1988). This allows MCL-PHA to achieve some crystallinity (Preusting *et al.*, 1990). Showing a T_g below room temperature and with the crystals acting as physical cross-links, MCL-PHA consequently is thermoplastic elastomers (Gagnon *et al.*, 1992). However, if MCL-PHA is to be applied as elastomers, two major problems will have to be overcomed. First, MCL-PHA exhibits melting temperatures varying from 39 to 61 °C (Marchessault *et al.*, 1990; Preusting *et al.*, 1990; Gagnon *et al.*, 1992). Thus, they soften and lose their coherence at temperatures as low as 40 °C. The second problem is the low rate of crystallization which increases the time of crystallization to several days. This seriously limits the practicability of many processing techniques.

2.5 Role of renewable resources in PHA biosynthesis

The findings of other monomers besides 3HB has brought us a step forward into knowing the role of PHA synthase enzyme in polymerizing broad

range of substrates into a variety of monomers. This indicates carbon sources as a factor in determining the type of PHA constituents. Microorganisms are capable of producing PHA from various carbon sources ranging from inexpensive, complex waste effluents to plant oils (Fukui and Doi, 1998a), fatty acids (Eggink *et al.*, 1992), alkanes (Lageveen *et al.*, 1988) and as well as simple carbohydrates.

Wider application of PHA is obstructed mainly by their high production cost compared with the synthetic plastics (Byrom, 1987; Choi and Lee, 1997). Substantial efforts have been devoted to reduce the production cost through the development of bacterial strains, efficient fermentation and recovery processes (Lee, 1996b; Grothe *et al.*, 1999). The major cost in the PHA production is the cost of the substrate (Yamane, 1993). Selection of suitable carbon substrate is a critical factor that determines the overall performance of the bacterial fermentation and at the end it significantly influences the cost of the final product. Therefore, the simplest approach is to choose renewable, cheap and most readily available carbon substrates that could support the microbial growth and PHA production efficiently. Agricultural by-products such as beet molasses and alphéchin (wastewater from olive oil mills) (Pozo *et al*., 2002), sugars such as glucose and sucrose (Du *et al*., 2001; Borah *et al*., 2002), plant oils (Fukui and Doi, 1998a; Eggink *et al*., 1998; Kahar *et al.*, 2004), starch and even CO_2 (Jau *et al.*, 2005) are among the various attractive renewable resources.

2.5.1 Alpéchin

Alpéchin is the aqueous waste from olive oil extraction process. It is rich in sugars, phenolic compounds, potassium and phosphate ions, and it contains low quantities of nitrogen. Overall, the average chemical composition of alpéchin is water (83.4 %), organic matter (14.8 %) including 1-1.5 % of polyphenols, and minerals (1.8 %) (Moreno *et al.*, 1990). Recombinant *P. putida* KT2442 harboring plasmid pSK2665 showed the ability to grow in media with a high concentration of alpéchin (50 % v/v) and accumulate P(3HB) (Ribera *et al.*, 2001). Pozo and co-workers (2002) reported that *Azotobacter chroococcum* H23, an aerobic nitrogen-fixing bacteria was able to accumulate P(3HB-*co*-3HV) up to 80 wt% of dry cell weight in 0.12 % ammonium acetate mineral salts medium with 60 % alpéchin and valerate as precursor. García-Barrionuevo and co-workers (1993) suggested that growth of *A. chroococcum* H23 in alpéchin depends on the amount of phenolic acids or their polymer present in the waste. The future of alpéchin as carbon substrate in bacterial PHA production is promising. A definite reduction in the cost of PHA production could be observed since alpéchin has no economic value at present (Pozo *et al.*, 2002).

2.5.2 Plant oils

Plant oils such as soybean oil, palm oil, corn oil are desirable carbon sources for PHA production because they are relatively cheaper than most of the sugars. Although the production of PHA using sugars has been optimized to achieve high productivity, but the cost of production is still higher than the

‘acceptable’ level because these sugars contribute to low PHA yield (Lee and Choi 1999). Highest yield of PHA production from glucose has been reported to be roughly around 0.3 to 0.4 g of P(3HB) per g of glucose. On the contrary, plant oils are predicted to provide higher yield for both cell biomass and PHA production (0.6 to 0.8 g of PHA per g of oil) as they contain much more carbon content per weight (Akiyama *et al.*, 2003).

Study has been done by Kahar and co-workers (2004) using *C. necator* H16 and its recombinant strain (harboring PHA synthase gene from *A. caviae*) in using soybean oil as sole carbon source for synthesizing P(3HB) homopolymer and P(3HB-*co*-5 mol% 3HHx) copolymer. In both cases, the production of PHA from soybean oil was high, whereby up to 80 wt% of the dry cell weight was obtained (Fukui and Doi, 1998a; Kahar *et al.*, 2004). In particular, the incorporation of a small molar fraction of 3HHx monomer into 3HB alters the unfavorable original physical and thermal properties of P(3HB). The same phenomena also occurred when palm oil was used as the sole carbon source on recombinant *C. necator* H16 and the molar fractions of 3HHx monomer remained unchanged regardless of the type and concentration of palm oil products used (Loo *et al.*, 2005).

Besides *C. necator* H16, there are also several other bacteria that are known to produce PHA from plant oils, namely *Burkholderia cepacia* (Zazali and Tan, 2005) and *Comamonas testosteroni* (Thakor *et al.*, 2005). Due to the

absence of lipase activity in *P. putida*, plant oils in the form of triglycerides could not support both the cell growth and PHA production in *P. putida*. Therefore, an additional saponification step was needed to break down the triglycerides into free fatty acids, which can be assimilated by *P. putida* for growth and PHA production (Tan *et al.*, 1997). Kim and co-workers (1997) performed a two-step fed-batch cultivation using *P. putida* by supplying octanoic acid in the first step which resulted in good growth and could stimulate the biosynthesis of PHA containing MCL efficiently.

Comamonas testosteroni has been studied for its ability to synthesize PHA containing MCL from vegetable oils such as castor seed oil, coconut oil, mustard oil, cotton seed oil, groundnut oil, olive oil and sesame oil. This bacterium was shown to accumulate PHA up to 78.5 – 87.5 wt% of dry cell weight with major monomer compositions consisting of 3HO and 3HD (Thakor *et al.*, 2005).

Burkholderia cepacia (the new designation of *Pseudomonas cepacia*) was used by Ramsay's group to produce P(3HB) from fructose in batch fermentation (Ramsay *et al.*, 1989). Zazali and Tan (2005) have isolated *B. cepacia* from palm oil mill effluent (POME) and reported that this bacterium could produce P(3HB) more than 50 wt% of dry cell weight from palm oil compared to Ramsay's group which contributed only 47 wt% P(3HB) of the dry cell weight from fructose. However, this bacterium was only capable of producing P(3HB) homopolymer

from plant oil as the sole carbon source. In order to obtain a copolymer of P(3HB-*co*-3HV) from this bacterium, structurally related carbon sources such as valeric acid and propionic acid were needed as precursor for synthesizing this copolymer. Interestingly, the production of poly(3-hydroxybutyrate-*co*-3-hydroxy-4-pentenoate), P(3HB-*co*-3H4PE) by *B. cepacia* from unrelated carbon sources such as sucrose or gluconate amounting to 70 wt% of dry cell weight with 3 mol% of 3H4PE fraction was reported (Rodrigues *et al.*, 1995; 2000). The study indicated that the two types of PHA were synthesized by different PHA synthases.

2.5.3 Glycerol

Glycerol is a by-product from palm oil refining process, ethanol fermentation by *Saccharomyces cerevisiae*, soap-making industry and also lipolysis of triglycerides. It also emerges as a humectant as well as a plasticizer in certain applications such as oral-care products, tobacco, cosmetics, food and beverages. Glycerol is generated in bulk amount particularly from the co-product stream of biodiesel, and thus possesses the potential to be a new carbon source for PHA production by certain microorganisms (Madden *et al.*, 1999; Ashby *et al.*, 2005).

Past studies have shown that P(3HB) could be synthesized from glycerol by *C. necator* under appropriate growth conditions (Taidi *et al.*, 1994). Previous work by Ashby and co-workers (2005) showed a more efficient production system that included wild type *P. oleovorans* NRRL B-14682 and *Pseudomonas*