CHAPTER TWO

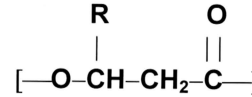
LITERATURE REVIEW

2.1 Microbial Polyester

Microbial polyesters are thermoplastics with biodegradable and biocompatible properties which have drawn much attention worldwide as alternative to petrochemical based plastics (Page,1995). The general class of microbial polyesters are termed poly-β-hydroxyalkanoates and the most common of its kind is poly-β-hydroxybutyrate (PHB). These polymers are composed of 3-hydroxy fatty aicds. They have the general structure as shown in Figure 1. PHAs are lipidic granules accumulated by numerous microorganisms when cells encounter unbalanced growth conditions in the presence of excessive carbon source. It functions as a storage of carbon and energy (Anderson and Dawes,1990).

PHAs can be divided into two groups by the number of carbon atom in the monomers. Short Chain Length PHA (SCL_{PHA}) consists of 3-5 carbon atoms and Medium Chain Length PHA (MCL_{PHA}) of 6-14 carbon atoms. The physical and mechanical properties such as brittleness, stiffness and melting temperature were reported to vary considerably depending on monomeric compositions. Therefore, the physical properties of the PHA can be regulated by varying the composition of the different individual monomers or copolymers.

PHB was first isolated from *Bacillus megaterium* by Lemoigne in 1925. Since then a wide variety of bacteria and cynobacteria were found to



R = hydrogen:	3-hydroxypropionate (3HP)
R = methyl:	3-hydroxybutyrate (3HB)
R = ethyl:	3-hydroxyvalerate (3HV)
R = propyl:	3-hydroxycaproate (3HC)
R = butyl:	3-hydroxyheptanoate (3HH)
R = pentyl:	3-hydroxyoctanoate (3HO)
R = hexyl:	3-hydroxynonanoate (3HN)
R = heptyl:	3-hydroxydecanoate(HD)
R = octyl:	3-hydroxyundecanoate (3HUD)
R = nonyl:	3-hydroxydodecanoate (3HDD)

Figure 1

Chemical structure of poly-β-hydroxyalkanoate
(Adapted from Doi,Y.1990).

accumulate PHB when growth was limited by depletion of esssential nutrients. *Alcaligenes eutrophus* has been studied in most detail due to its ability to accumulate large amount of PHB (ca. 80% w/w CDW). Doi *et al.*,1990 suggested that the biosynthesis and degradation of PHB in

Alcaligenes eutrophus occur via a cyclic process. It means that the biosynthesis and degradation occur simultaneously when growth was limited by absence of nitrogen source. In most bacteria, PHB is synthesised from acetyl coenzyme A (acetyl-CoA) by a sequence of three enzymatic reactions (Figure 2). The enzymes involved are ß-kethothiolase. NADPH dependent acetoacetyl-CoA reductase and PHB synthase. PHB synthase initiates the polymerisation of D-(-)-3-hydroxybutyryl-CoA to produce homopolymer of PHB.

Heteropolymers or microbial copolymers of PHA was then isolated from activated sewage sludge sample (Wallen and Rohwedder,1974). Through gas chromatography-mass spectroscopy analysis, it was found that the polymer contained four different monomers, namely 3-hydroxybutyrate, 3-hydroxyvalerate, 3-hydroxycaproate and 3-hydroxyheptanoate. After that a variety of PHA copolymers have been isolated from different environmental samples.

A series of PHAs with different monomeric compositions can be produced as it is determined by the specificity of the PHA polymerase system, the nature of substrate and the metabolic routes leading to PHA formation (Huijbert *et al.*, 1992). A copolymer of 3-hydroxybutyrate and 3-hydroxyvalerate, P(3HB-*co*-HV), has been produced commercially by *Alcaligenes eutrophus* from propionic acid and glucose by Imperial Chemical

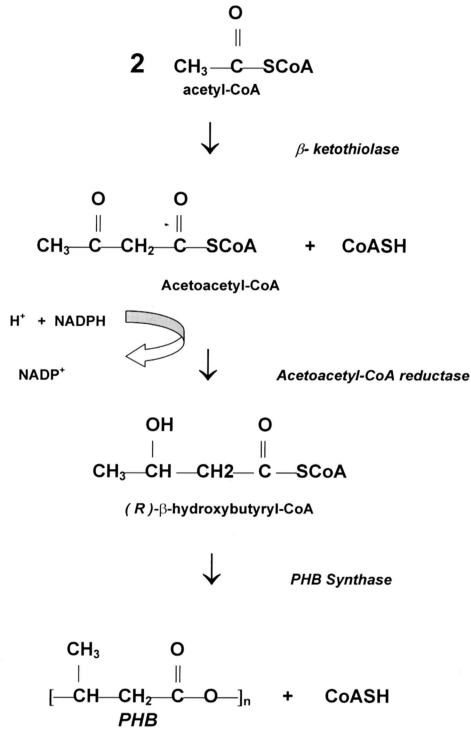


Figure 2

Biosynthetic pathway of poly-β-hydroxybutyrate
(Adapted from Griffin,1994).

Industries (ICI). The copolymer was produced in a controlled fermentation process where bacteria was fed with variety of carbon substrate. Doi *et al.*, 1988, found that copolymer of 3-and 4-hydroxybutyrate, P(3HB-*co*-4HB), could be produced by *A. eutrophus* from 4-hydroxybutyrate or γ-butyrolactone. PHA copolymers containing 3-hydroxyalkanoate from C_6 to C_{14} was identified to be produced by *Pseudomonas oleovorans* (Witholt *et al.*,1990).

2.2 PHA from Pseudomonads

Fluorescent Pseudomonads which belongs to rRNA homology group 1 (Stainer *et al.*,1966) are capable of synthesising and accumulating PHA. Generally, *Pseudomonas* strains do not accumulate PHB but they produce MCL_{PHA} during unbalanced growth on medium and long chain alkanols and fatty acids (Huismann *et al.*,1989). Besides, they generally produce PHA with higher molecular weights (Timm and Steinbuchel,1990). PHA having various MCL 3-hydroxyalkanoate were first detected in cells of *Pseudomonas oleovorans* ATCC 29347 grown on n-octane (de Smet *et al.*,1983).

Pseudomonas oleovorans is able to grow on MCL n-alkanes because it contains the catabolic OCT plasmid, which encodes an alkane hydroxylase complex. The enzyme complex oxidises *n*-alkane to terminal alkanols. Lageveen *et al.*, (1988) showed that monomer composition of

polyester is a reflection of the substrate used. *P. oleovorans* when grown on MCL, C_6 and C_{12} n-alkanes and 1-alkenes, produced PHAs contained both saturated and unsaturated monomers, which contained as many C atoms as did in substrate and monomers shorter by one or more C_2 units. The substrates are oxidised to corresponding fatty acids, which then undergo β-oxidation yielding shorter fatty acids. This has been shown that these beta oxidation pathway intermediates served as precursors to PHA biosynthesis. Huismann *et al.*, (1989) reported when LCL fatty acids (LCFA) are supplied in growth medium, these were taken up by *Pseudomonas oleovorans* and degraded via β-oxidation cycle until MCL intermediates are formed and incorporated into PHAs. This suggested that the incorporation of fatty acids derivatives was restricted to the specificity of a key enzyme in PHA biosynthesis.

Huijberts *et al.*, (1992) showed that *P. putida* KT2442 when grown on non related substrate (glucose, fructose and glycerol), PHA monomers are derived from intermediates of *de novo* fatty acids biosynthesis. Huijberts *et al.*,1994, demonstrated through physiological studies using ^{13}C-labelled substrates and specific inhibitors of fatty acids metabolic pathways, that ß-oxidation and fatty acids biosynthesis can function independently and simultaneously in PHA formation in *Pseudomonas* species. Thus, the precursors of PHA biosynthesis can be derived from intermediates of ß-oxidation cycle and fatty acids biosynthesis. Eggink, *et al.*, (1993) postulated

the relationship between β-oxidation of fatty acids and PHA formation in *P. putida* (Figure 3).

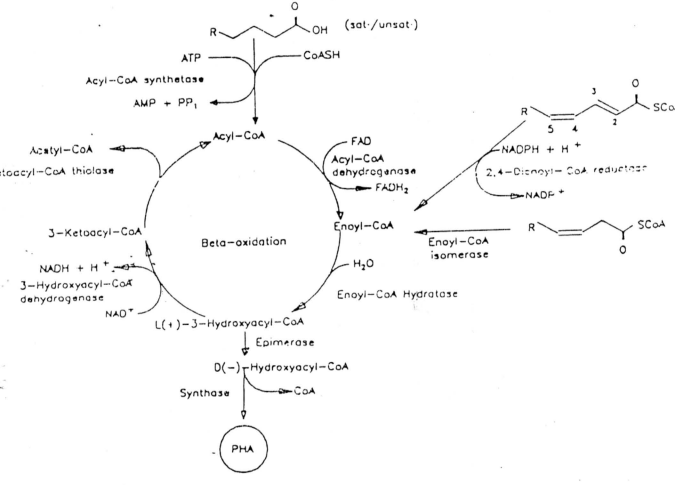


Figure 3

Biosynthetic pathway of poly-β-hydroxyalkanoate through β-oxidation (Adapted from Eggink *et al.*,1993).

2.3 Triglycerides as Carbon Substrate

The use of triglycerides as carbon substrate in PHA biosynthesis has been demonstrated with *Aeromonas caviae* which produce poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) when grown on olive oil (Shimamura *et al.*,1994), and with *Aeromonas* sp. from palm oil and fats (Kobayashi *et al.*,1994). *Alcaligenes* sp. AK201 was reported to produce P(3HB) and its copolymer P(3HB-*co*-HV) from n-alkanoic acids of carbon numbers ranging from C_2 to C_{22} (Akiyama *et al.*,1992) and from palm oil. (Abd. Majid *et al.*,1994). *Pseudomonas aeroginosa* produced relatively more complex polymers when grown on euphorbia or castor oil (Eggink *et al.*,1995), while the biosynthesis of PHA from triglycerides was also reported in Pseudomonas putida (Tan *et al.*,1997) and *Pseudomonas resinovorans* (Ashby *et al.*,1998).

Bacteria which are likely to utilise triglycerides for growth and PHA accumulation presumably have to meet two requirements. Firstly, the bacteria need to produce lipase enzyme that hydrolyses the triglycerides to liberate the fatty acids. Secondly, the bacteria have to be able to grow and accumulate PHA from these fatty acids. Lipases are hydrolases acting on carboxyl ester bonds present in acylglycerols to liberate organic acids and glycerols (Jaeger *et al.*,1994).

Due to the abundance of palm oil in Malaysia, it is viewed as a potential renewable resource for bacteria growth and PHA accumulation. Palm oil is extracted from the mesocarp layer of the palm fruit and is composed predominantly of palmitic C_{16} and oleic acid $C_{18:1}$. Another possible carbon source is palm kernel oil (PKO) which is extracted from the kernel of the oil palm fruit. It is composed predominantly of lauric acid C_{12} and myristic acid C_{14} (Table 1). Tan *et al*, (1997) had reported the utilisation of saponified palm kernel oil (SPKO) by *Pseudomonas putida* to produce MCL PHA composed of monomers which ranged from C_6 to C_{14}, with C_8 predominating. Unsaturated monomers were also detected. *Pseudomonas putida* lacks the lipase enzyme to hydrolyse the palm kernel oil, therefore the oil was saponified. During saponification, the glycerides are split by alcoholic alkali which results in release of free fatty acids.

Week *et al.*, (1969) suggested that bacterial growth on fatty acids requires the coordinated induction of the beta oxidation enzymes and fatty acids transport system. The transport, acylation and beta oxidation of medium chain fatty acids (MCFA) and long chain fatty acids (LCFA) are due to the fatty acids degradative (*fad*) system. In addition to fatty acids oxidative (FAO) enzyme, short chain fatty acids (SCFA) metabolism requires at least two degradative enzymes, which are encoded by the *ato A* and *ato B* genes (Nunn,1986). The activities of the five key enzymes of β-oxidation (palmityl-coenzyme A (Co-A) synthetase, acyl-CoA dehydrogenase, enoyl-CoA hydrase, β-hydroxyacyl-CoA dehydrogenase and thiolase) vary coordinately

Table 1

Fatty acids composition (%) of Palm Kernel Oil, Palm Oil, Palm Stearin and Palm Olein (Iftikhar, PORIM,1984).

Fatty Acid %	Palm Kernel Oil	Palm Oil	Palm Olein	Palm Stearin
Lauric (12:0)	48.0	0.1	0.7	0.3
Myristic (14:0)	15.3	1.0	1.6	1.5
Palmitic (16:0)	7.7	43.7	39.8	61.5
Stearic (18:0)	1.7	4.4	4.4	5.0
Oleic (18:1)	15.6	39.9	42.3	26.3
Linoleic (18:2)	2.7	10.3	11.9	6.2
Linolenic (18:3)	0.3	Traces	0.4	Traces

over a wide range of activity indicating that they are all under unit control. The ability of fatty acids to induce the enzymes of beta oxidation and support growth in *E. coli* is a function of its chain length. Fatty acids of carbon chain length C_{14} and longer induce the enzymes of fatty acids oxidation and readily support growth (Nunn,1986). For LCFA they are degraded via the β-oxidation route until medium chain length intermediates are formed and used as substrates for PHA synthesis. Schulz and Kunau (1987) reported that the degradation of unsaturated LCFA requires the involvement of auxiliary enzymes, namely NADPH-dependent dienoyl-CoA reductase. It is essential for degradation of LCFA with double bonds extending from odd numbered carbom atoms.

However, the growth of both Gram-positive and to a lesser extent Gram-negative bacteria is inhibited by fatty acids (Weeks *et al*,1969, Sheu and Freese, 1972). Fay and Farias , (1975) reported that the effect of fatty acids on *Escherichia coli* K12 was dependent on the source of the inoculum, the growth phase and the washing of the bacteria. The effect of 0.1% and 0.4% saturated fatty acids (C4 to C16) and oleic acid on exponentially growing *E. coli* K12/154 was tested in different culture medium. It was concluded that a given concentration of fatty acids had different effects depending on; the chain length of the added fatty acid and culture medium in which the bacteria were grown, the growth phase of the culture, the presence of an adaptation phase brought about by changing the culture medium to

another and washing of the bactera. An increase in fatty acid concentration (e.g. adding 0.4% C10) was found to produce death of culture.

It was shown that fatty acids inhibit the growth and oxygen consumption of *Bacillus substilis* in nutrient medium by inhibiting the transport of amino acids, keto acids etc., through the cellular mambrane. The effectiveness of inhibition increased with increasing fatty acids chain length (Freese, *et al.*,1973). In contrast to *E.coli* the inhibitory effect was exerted by short chain (C_2-C_6), while medium or long chain fatty acids (C_{10}-C_{18}) had no effect. Reason to this was that the lipopolysaccharide (LPS) layer of gram-negative bacteria protects them against the inhibition by medium and long chain fatty acids (Sheu and Freese,1973).

2.4 Fed-Batch Culture Feeding Strategies

Pseudomonas oleovorans is able to accumulate poly(3-hydroxyalkanoates) under conditions of excess n-alkanes, which serve as sole energy and carbon source, and limitation of an essential nutrient such as ammonium (Preusting *et al.*, 1992). Preusting *et al.*, (1992) reported the studies of PHA production by *P.oleovorans* in fed-batch cultures. The cells were first grown batchwise to a density of 6 g/l. After that nutrient solution containing $(NH_4)_2SO_4$ and $MgSO_4$ was fed. The limiting ammonium was added at a constant rate of 0.23 g per hour and resulted in biomass concentration of 37.1 g/l after 38 hours of cultivation. The overall PHA

productivity was 0.25 g PHA per liter medium per hour. Kellerhals *et al.*, (1999) described further improvement in the cultivation process done by Preusting *et al.*, (1992). In the fed-batch studies of better oxygen transfer capabilities a biomass of up to 90 g/l was obtained. Then by applying computer-controlled nitrogen feed in combination with various metal ions a biomass of 112 g/l was achieved. However, the PHA content of these cells decreased from 25% to 10 % as cell density increased. It was suggested that high cell density is not neccessarily accompanied by high PHA productivies, and may be due to depolymerase activity in prolonged cultivation time.

Suzuki *et al.*, (1990) demonstrated the characteristic differences between pH-stat model fed-batch cultures using a low limit and those a high limit and the advantages of the pH-stat modal fed-batch culture using a set point of high limit. Kim *et al.*, (1992) reported the production of PHB by pH-stat fed-batch culture of recombinant *Escherichia coli* strain harboring the PHB biosynthesis genes of *Alcaligenes eutrophus*. During the fed-batch operation nutrients containing glucose were added intermittently when glucose became depleted. This was indicated by a sharp rise of pH. When the pH becomes higher than 7.1, 50ml of feeding nutrient solution, which corresponded to 20g glucose + 5g yeast extract + 5g tryptone, was added. The final PHB concentration of 88.8g/l was obtained after 42 hours of cultivation.

Nishio *et al.*, (1977) studied a fed-batch culture controlled by pH signal, in which methanol-ammonia mixture was fed intermittently into the culture vessel to compensate for the methanol consumed by *Pseudomonas* AM-1 and *Klebseilla* sp. 101 and adjust pH to a predetermined level, in order to obtain high biomass production of 36g/l after 85 hours of cultivation.

Yano *et al.*, (1978) studied that during a batch culture of methanol utilising microorganisms, the dissolved oxygen tension increased abruptly when methanol in the medium became the growth limiting factor. In this case, dissolved oxygen (DO) was used as control indicator for feeding methanol in a fed batch culture. In the study, a batch culture resulted in cell density of 45g/l and fed-batch cuture of 85g/l. Kim *et al.,* (1993) studied two methods to maintain the glucose concentration, using Carbon Dioxide Evolution Rate (CER) obtained from mass spectrometer and using an on line glucose analyser. High concentration of PHB (121g/l) and total cells (164g/l) were obtained in 50 hours.

Suzuki *et al.*, (1986a) reported a high concentration of PHB (136g/l) and total cell concentration (206g/l), when methylotrophs were cultivated through an automatic fed-batch culture for 175 hours.In the study, other nutrients, nitrogen source and mineral ions were controlled to maintain their initial concentration during the growth phase. At cell concentration of 160g/l, feedings of nitrogen and mineral ions were stopped and only methanol was supplied successively to accumulate PHB.

Suzuki *et al.*, (1986b), noted that nitrogen source was necessary for PHB production phase in *Protomonas extorquens* cultured by fed-batch system. Feeding with small quantity of ammonia resulted in rapid increase in intracellular PHB content compared to without ammonia feeding. Excessive feeding however resulted in degradation of cellular PHB and reduction in PHB synthetic activity. Suzuki *et al.*, (1986c), reported the effect of the ratio of methanol to ammonia in the feeding solution. The result indicated that the maximum PHB production can be obtained in short time when C/N was automatically controlled. At 170 hours, PHB concentration reached 149g/l and cell density became 233g/l, i.e. the PHB content was 64% of the dry cell mass.