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Minireview

Production of Polyhydroxyalkanoates, a bacterial biodegradable polymer

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There has been considerable interest in the development and production of biodegradable polymer to solve the current problem of pollution caused by the continuous use of synthetic polymer of petroleum origin. Polyhydroxyalkanoates (PHAs) are known to be accumulated as intracellular inclusion in some bacteria. The materials properties exhibited by PHAs, ranging from stiff, brittle to rubber-like makes it a close substitute for the synthetic plastic. The high cost of PHAs production has restricted its applications. The possibility of producing this polymer commercially and at comparable cost has been the main focus in this area.

Key words: Polyhydroxyalkanoates, biodegradable polymer, bioplastic, poly(3-hydroxybutyrate), biosynthesis.

INTRODUCTION

Synthetic polymers (known as plastics) have become significant since the1940s, and since then they are replacing glass, wood and other constructional materials, and even metals in many industrial, domestic and environmental applications (Poirier et al., 1995; Cain, 1992; Lee, 1996; Lee et al., 1991). These widespread applications are not only due to their favourable mechanical and thermal properties but mainly due to the stability and durability (Rivard et al., 1995). On the other hand, plastic also play important role for many “short live” applications such as packaging and these represent the major part of plastic waste (Rivard et al., 1995; Poirier et al., 1995; Witt et al., 1997; Muller et al., 2001). Because of their persistence in our environment, several communities are now more sensitive to the impact of discarded plastic on the environment, including deleterious effects on wildlife and on the aesthetic qualities of cities and forest. The increased cost of solid waste disposal as well as the potential hazards from waste incineration such as dioxin emission from PVC, makes synthetic plastic a waste management problems.

Consequently, for the past two decades, there have been a growing public and scientific interest regarding the use and development of biopolymer (biodegradable polymers) materials as an ecologically useful alternative to plastics, which must still retain the desired physical and chemical properties of conventional synthetic plastics; thus offering a solution for the existing grave problem of plastic waste (Bichler et al., 1993; Lee, 1996; Amass et al., 1998; Muller et al., 2001). Biodegradable plastics are made from renewable resources and do not lead to the depletion of finite resources. A number of

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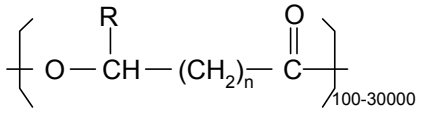
biodegradable plastic materials – mostly biodegradable polyesters, namely polyhydroxyalkanoates (PHAs), polylactides, aliphatic polyesters, polysaccharides, and the copolymer or blends of these, have been developed successfully over the last few years to meet specific demands in various fields and industries (Byrom, 1991; Chang, 1994; Lee and Chang, 1994; Lee, 1996). These materials offer solution to managing of waste and in some cases, good substitute for conventional plastic where mechanical properties are desired (Lee, 1996; Steinbüchel and Fuchtenbusch, 1998)

Among the candidates for biodegradable plastics, PHAs have been drawing much attention because of their similar material properties to conventional plastics and complete biodegradability (Hocking and Marchessault, 1994; Steinbüchel and Fuchtenbusch, 1998). There exist a number of literatures on PHAs (Steinbüchel, 1992; Steinbüchel et al., 1992; Lenz et al., 1994; Mergaert et al., 1992; Lee, 1996; Steinbüchel and Fuchtenbusch, 1998). The fact that it can be produced from renewable resources and its good processibility on equipment just like some polyolefins and other synthetic plastics (Hocking and Marchessault, 1994; Steinbüchel and Fuchtenbusch, 1998) make PHAs suitable for applications in several areas as a partial substitute for non biodegradable synthetic polymers. Although the high cost of production makes PHAs substantially more expensive than synthetic plastics (Steinbüchel and Fuchtenbusch, 1998), exploring it production from locally available and renewable carbon source such as horticultural agricultural waste, corn, cassava etc would be of economic interest considering the environmental gains that would result from PHA application. Here we review advances in PHA research, and present knowledge of synthesis in bacteria and plants is discussed.



n =1	R =	hydrogen	poly (-3-hydroxypropionate)
		methyl	poly (-3-hydroxybutyrate)
		ethyl	poly(3-horoxyvalerate)
		propyl	poly (-3-hydroxyhexanoate)
		pentyl	poly (-3-hydroxyoctanoate)
		nonyl	poly (-3-hydroxydodecanoate)
n = 2	R =	hydrogen	poly (-4-hydroxybutyrate)
n = 3	R =	hydrogen	poly (-5-hydroxyvalerate)

Figure 1. The general structure of polyhydroxyalkanoates.

BIOSYNTHETIC PATHWAYS OF PHA

Polyhydroxyalkanoates are polyesters of hydroxyalkanoates (HAs) with the general structural formula as shown in Figure 1. (Steinbüchel, 1991; Lee, 1996). It was first discovered in 1926 as constituent of the bacterium *Bacillus megaterium* (Lemoigne, 1926). Several bacteria synthesize and accumulate PHA as carbon and energy storage materials or as sink for redundant reducing power under the condition of limiting nutrients in the presence of excess carbon source (Steinbüchel, 1991; Byron, 1994; Yu, 2001; Du and Yu, 2002b). The stored PHA can be degraded by intracellular depolymerases and metabolized as carbon and energy source as soon as the supply of the limiting nutrient is restored. (Byrom, 1994). The majority of PHAs are composed of R(-)-3-hydroxyalkanoic acid monomers ranging from C3 to C14 carbon atoms with variety of saturated or unsaturated and straight- or branched chain containing aliphatic or aromatic side groups (Doi et al., 1992; DeSmet et al., 1983). The molecular weight of the polymers are in the range at 2×10^5 to 3×10^6 daltons, based on the type of microorganism and growth condition (Byron, 1994). PHAs are accumulated in the cells as discrete granules, the size and number per cell varies depending on the different species. According to Byrom (1994), about 8 to 13 granules per cell having diameter range of 0.2 to 0.5μm were observed in *Alcaligenes eutrophus*. The granules appear as highly refractive inclusion under electron microscopic observation. The Microorganisms accumulating PHA are easily identified by staining with Sudan black or Nile blue (Ostle and Holt, 1982; Schlegel et al., 1970).

Over 250 different bacteria, including gram-negative and gram-positive species, have been reported to accumulate various PHAs (Steinbüchel, 1991; Steinbüchel, 1992; Lenz et al., 1992). PHAs can be divided into two broad groups based on the number of carbon atoms in the monomer units; the short chain length polyhydroxyalkanoates PHAs $_{(SCL)}$, which consist of C_3-C_5 atoms, and medium chain length polyhydroxyalkanoates PHAs $_{(MCL)}$ consisting of C_6-C_{14} atoms. This grouping is due to the substrate specificity of the PHA synthesis that only accept 3-hydroxyalkanoates (3HAs) of a certain range of carbon length (Anderson and Dawes, 1990). The PHA synthetases of *A. eutrophus* can only polymerize 3HAs$_{(SCL)}$ while that of *Pseudomonas oleovorans* only polymerize 3HAs $_{(MCL)}$. For PHAs $_{(SCL)}$, the monomer units are oxidized at positions other than the third carbons while for PHAs$_{(MCL)}$, all the monomers units are oxidized at the third position except in few cases (Valentin et al., 1994). A lot of PHAs $_{(MCL)}$ containing various functional groups such as olefins, branched alkyls, halogens, aromatic and cyano have been reported (Fritzsche el al., 1990; Huijberts et al., 1992; Kim et al., 1992; Hazer et al., 1994). This flexibility of PHA biosynthesis makes it possible to design and produce related biopolymers having useful physical properties ranging from stiff and brittle plastic to rubbery polymers (Anderson and Dawes, 1990)

Although most of the PHA synthesis studied to date are specific for the synthesis of either PHA $_{(SCL)}$ or PHA $_{(MCL)}$.

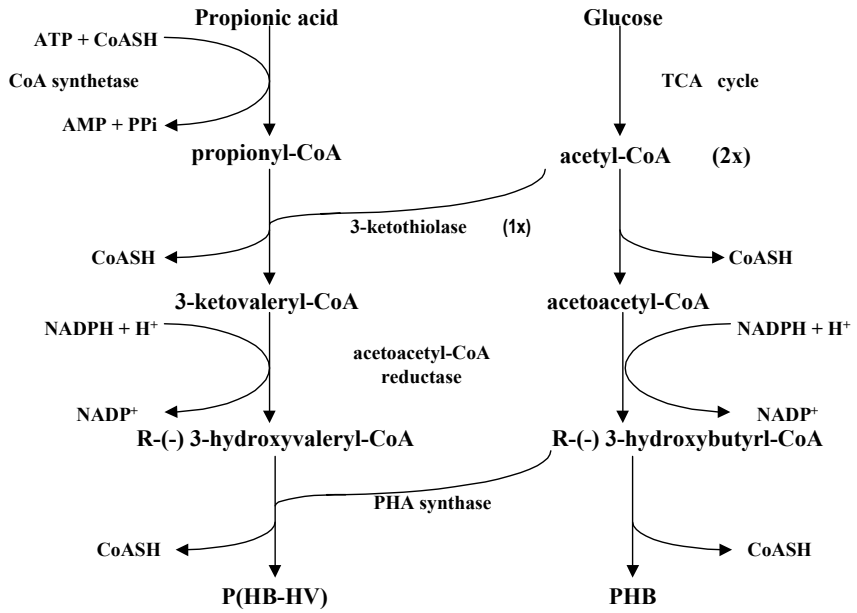


Figure 2. The biosynthetic pathway of PHB and P(HB-HV) in *Alcaligenes eutrophus*.

It has been found that at least six cases deviate form this study, details of which can be found elsewhere (Liebergesell et al. 1993; Valentin et al., 1994; Kobayashi et al 1994; Amos and McInerney, 1989; Abe et al., 1994). Four different pathways have been elucidated so far for the biosynthesis of PHA (Steinbüchel; 1991; Doi and Abe, 1990; Byrom, 1994; Poirier et al., 1995). In the bacterium *Alcaligenes eutrophus*, polyhydroxybutyrate (PHB), a thoroughly characterized PHA is synthesized from acetyl-CoA by a sequential action of three enzymes as shown in Figure 2. The first enzyme, in the pathway 3-ketothiolase promotes the condensation of two acetyl CoA moieties in a reversible manner to form acetoacetyl-CoA. This is followed by the action of acetoacetyl-CoA reductase which reduces acetoacetyl-CoA to R(-)-3-hydroxybutynl-CoA. The PHA synthase then polymerize the R(-)-3-hydroxybutyrl-CoA to form PHB (Poirier et al., 1995; Lee, 1996). *A. eutrophus* can accumulate PHB as inclusion up to 80% of the dry weight which the bacterial are cultivated in media containing excess carbon such as glucose, but limited in one essential nutrient, such as nitrogen or phosphate.

Varieties of PHAs with different C_3 to C_5 monomers have been produced in *A. eutrophus*, but the nature and proportion of these monomers are being influenced by the type and relative quantity of carbon sources supplied to the growth media (Steinbüchel, 1991; Doi et al.,1988; Steinbüchel et al., 1993). For example, it has been reported that addition of propionic acid or valeric acid to the growth media containing glucose leads to production of copolymer composed of 3-hydroxybutyrate a (3HB) and 3-hydroxyvalerate (3HV), the biosynthesis pathway of which is also shown in Figure 2 (Steinbüchel el al., 1993; Doi et al., 1988). Whereas if valeric acid is used as sole carbon source, the proportion of 3HV monomer in PHA produced in *A. eutrophus* of 90% has been reported. By substitution of *A. eutrophus* with *Chromobaterium violaceum* on the same substrate gave PHA with 100% 3HV units (Steinbüchel et al., 1993; Piorier et al., 1995). Apart from the production of PHB, other $PHAs_{(SCL)}$ synthesized in *A. eutrophus* are directly related to the structure of the carbon source used.

Thus addition of 4-hydroxybutyrate or 5-hydroxyvalerate in the growth media leads to synthesis of the corresponding CoA thioesters and incorporation into PHA by the PHA synthase. One few bacteria are known to synthesize $PHAs_{(SCL)}$ containing monomers other than 3HB when grown on simple sugar (Haywood et al., 1991; Williams et al., 1994). Some *pseudomonads* species have been reported to accumulate PHA copolymers containing MCL monomer. For example *Pseudomonas oleovorans* and *Pseudomonas putida*, when grown on alkanoic acid (Steinbüchel, 1991). The composition of PHA produced is usually related to the substrate used for growth mostly 2n carbon shorter than the substrate used (Poirier et al., 1995). The analysis of biosynthetic pathways of these species have been discussed extensively (Lageveen et al., 1988; Fritzsche et al., 1990; Lenz et al 1992; Haywood et al., 1990; Timm and Steinbüchel, 1990; Huijberts et al., 1992; Saito and Doi, 1993; Huijberts et al., 1994; Du and Yu, 2002a)

POLYHYDROXYALKANOATES AS BIODEGRABLE THERMOPLASTIC

Table 1 shows the physical properties of major PHAs and polypropylene. The homopolymer PHB is a stiff and

Table 1. Physical properties of various PHAs and polypropylene.

Property	PHB	P(HB-HN)[a]			Polypropylene
		3 mol %	14 mol %	25 mol %	
Melting point(^{0}c)	175	169	150	137	176
Glass-transition temp (^{0}C)	15	-	-	-1	-10
Crystalline (%)	80	-	-	40	70
Young's modulus	3.5	2.9	1.5	0.7	1.7
Tensile strength (MPa)	40	38	35	30	34.5
Elongation to Break (%)	6	-	-	-	400
Impact strength (v/m)	50	60	120	400	45

Data adapted from Lee (1996) and Poirier et al. (1995).
- data not available
[a] poly (-3-hydroxybutyrate-co-3yhdroxyvalerate)

relatively brittle thermoplastic. Most studies of the physical properties of bacteria PHAs have been with PHB and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (HB-*co*-HV). Polyhydroxybutyrate is 100% stereospecific with the asymmetric carbon atoms having D(-) configuration (Doi and Abe, 1990; Steinbüchel,1991) thus, highly crystalline. It melting point (175^{o}C) is just slightly lower than it degrading temperature (185^{o}C), this makes it processing by injection molding difficult. PHB has several useful properties such as moisture resistance, water insolubility and optical purity, this differentiate PHB from other currently available biodegradable plastics which are either water soluble or moisture sensitive. PHB also shows good oxygen impermeability (Lindsay, 1992; Holmes, 1988).

Attempts have been made to decrease the brittleness of PHB by incorporating comonomer such as 3-hydroxyvalerate or by blending PHB with other polymer (Holmes 1985; Holmes, 1988; Kumagai and Doi, 1992; Kunioka et al., 1989; Du et al., 2001a). The mechanical properties of PHB including Young's modulus (3.5GPa) and tensile strength (MPa) are similar to those of polypropylene (Table 1), but the elongation to break of PHB (6%) is significantly lower than that of polypropylene (400%). The PHB copolymer containing 3-hydroxyvalerate unit P(3HB-*co*-3HV) has been developed and has much improved mechanical properties (Lee, 1996); the polymer becomes tougher (increase in impact strength) and more flexible (decreases in young's modulus) as the fraction of 3-hydroxyvalerate unit increases. The elongation to break was also reported to increase as the comonomer fraction increases (Lee, 1996). Furthermore, the melting temperature decreases with increasing comonomer fraction without any change in the degradation temperature. This allows the thermal processing of the copolymer without thermal degradation. Therefore the material properties can be controlled by adjusting the fraction of 3-hydroxyvalerate during the fermentation.

PRODUCTION OF POLYMER

PHA are synthesized and intracellularly accumulated in most bacteria a under unfavourable growth condition such as limitation of nitrogen, phosphorus, oxygen or magnesium in the presence of excess supply of carbon source (Du et al., 2001a, Du and Yu, 2002a; Lee, 1996). Strategies are still being developed to simulate conditions for efficient production of PHAs. (Yu, 2001, Du et al., 2001b; Du and Yu, 2002b). Some bacteria such as *A. eutrophus*, *A. Latus* and mutant strain of *Azotobacter vinelandii* are known to accumulate PHA during growth in the absence of nutrient limitation. Several factors need to be considered in the selection of microorganism for the industrial production of PHA such as the ability of the cell to utilize an inexpensive carbon source, growth rate, polymer synthesis rate and the maximum extent of polymer accumulation of a particular cell based on the substrate. Some workers have derived equation that predicts the PHA yield on several carbon source (Yamane, 1992;Yamane, 1993; Yu and Wang, 2001) which could be used for the preliminary calculation of PHA yields.

In order to reduce the overall cost, it is important to produce PHA with high productivity and high yield. Several methods such as Fed-batch and continuous cultivations have been carried out to improve productivity (Lee, 1996; Du and Yu, 2002a; Du and Yu, 2002b; Du et al 2001b; Yu and Wang, 2001). Only three prominent PHAs [PHB, poly (3-hydroxybutyrate-co-3-hydroxyvalerate) and poly (3-hydroxyhexanoate-co-3-hydroxyoctanoate)] have been produced to a relatively high concentration with high productivity. Recently, workers have been exploring cultivation strategies involving inexpensive, renewable carbon substrates in order to reduce production cost and obtain high productivity (Lee, 1996; Byrom, 1992; Kim et al., 1994, Park and Damodarau, 1994; Ishizaki and Tanaka, 1991; Poirier et al., 1995; Doi et al., 1988). Recovery of PHA

should also be considered because it significantly affects the overall process economics.

The last stage of PHB production involves separating the polymer from the cells. To do this a solvent of aqueous extraction can be used. In the aqueous process, the cell walls are broken and the polymer is then extracted and purified. The aqueous process is less expensive, but the process reduces the polymer molecular weight. For example, solvent extraction can produce copolymer weights of 1 million, whereas typical molecular weights of aqueously extracted copolymer are in the range 600,000 (Luzier, 1992). But solvent extraction, according to Luzier(1992) present some safety concerns.

In the solvent extraction process, the solvent employed include chloroform, methylenechloride, propylene carbonate, and dichloroethane (Baptist, 1962; Lafferty et al., 1988; Ramsay et al., 1994). Lee (1996) reported that large amount of solvent is required due to high viscosity of PHA, this makes the method economically unattractive. Sodium hypochlorite is used for the aqueous process. Although the use of sodium hypochlorite significantly increased PHA degradation, polymer purity greater than 95% is achieved. Lee (1996) also reported aqueous enzymatic digestion method as an alternative to solvent extraction and washing with an anionic surfactant to solubilise non-PHA cellular materials. Since recovery of PHA contributes significantly to the overall economics, development of a process that allows the simple and efficient extraction of polymers will be well rewarded.

Due to the relative high cost of producing PHA, several researches have been considering the possibility of producing PHA as cheap as starch. Starch and lipids are two of the most industrially useful and versatile product harvested from crop plants. There has being some investigations on the possibility of producing PHB in transgenic plant (Poirier et al., 1995; Lee, 1996; Nawrath et al., 1994). It has been reported that the first enzymes of PHA synthesis, β-Ketothiolase, is found in the cytoplasm of some higher plants (Poirier et al., 1995), this means that only the reductase and the PHA synthase are required by the plant to synthesize PHA.

A small oil seed plant (transgenic plant) was engineered to harbour the *A. eutrophus* PHA biosynthesis genes, this was found to accumulate PHB granules of 0.2-0.5 μm diameter in the nucleus, vacuole and cytoplasm. The accumulation was 100 μg/g fresh weight (Lee, 1996; Poirier et al., 1995) but the transgenic plant growth was impaired, the reason attributable to the severe depletion of substrate from the mevalonate pathway (Nawrath et al., 1994; Lee, 1996). This problem was solved and the polymer accumulation was improved upon by further genetic manipulations in order to divert reduced carbon away from endogenous metabolic pathways and to regulate the tissue specificity and the timing of gene expression. Thus genetically engineered genes of *A. eutrophus* were successfully transferred to the plastids of *Arabidopsis thaliana*. The hybrid plant expressing the *A. eutrophus* PHA synthesis enzymes accumulated PHB up to 10 mg/g fresh weight, about 14% of dry weight (Lee,1996 and Poirier et al.,1995). These show that production of PHA by transgenic plant may be economically viable if efforts are concentrated at improving this process. It may be possible in the near future to see farmers growing plastics in their field and a new agricultural product “plastics fruit” may appear in our market (Pool, 1989; Lee, 1996).

THE POLYMER BIODEGRADABILITY

PHB, P(HB-HV) and other polyhydroxyalkanoates are viewed by microorganisms as an energy sources. P(HB-HV) biodegrades in microbially active environments (Luzier, 1992, Poirier et al., 1995 and Lee, 1996). Microorganisms colonize on the surface of the polymer and secrete enzymes which degrade P(HB-HV) into HB and HV units. These units are then used up by the cell as a carbon source for biomass growth. The rate of polymer biodegradation depends on a variety of factors, including surface area, microbial activity of the disposal environment, pH, temperature, moisture and the pressure of other nutrient materials. P(HB-HV) is water insoluble and is not affected by moisture, does not degrade under normal conditions of storage, and is stable indefinitely in air (Mergaert et al. 1993; Mergaert et al., 1994; Lee, 1996).

The end products of PHA degradation in aerobic environments are carbon dioxide and water, while methane is also produced in anaerobic conditions. The effect of different environments on the degradation rate of PHB and P(HB-HV) has been studied by several workers (Doi et al., 1994, 1992; Mergaert et al.,1992, 1993,1994). Degradation occurs most rapidly in anaerobic sewage and slowest in seawater. Lee (1996) showed that P(HB-HV) completely degraded after 6, 75 and 350 weeks in anaerobic sewage, soil and sea water, respectively.

PHAs have been drawing considerable and/or biocompatible plastics for a wide range of application (Holmes, 1985). The primary applications areas in which it features meet some market needs are:

(i) Packaging : P(HB-HV) could be used for films, blow-molded bottles and as a creating on paper; and
(ii) Medical : P(HB-HV) biocompatibility coupled with its slow hydrolytic degradation lead to potential in reconstructive surgery. According to Lee (1996) the degradation product of PHB, D(-)-3-hydroxybutyrate has been detected in relatively large amount in human blood plasma. Therefore, it is highly plausible that implanting PHB in mammalian tissues would not be toxic.
(iii) Disposable personal hygiene: P(HB-HV) could be used as the sole structural materials, it could also be

used as part of degradable composites. For example PHAs could be substituted for polypropylene/polyethylene as matrix material in some composite material to achieve desirable properties comparable to these of petro-chemical origin.

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