

POLY (β -HYDROXYALKANOATES): NATURAL BIOCOMPATIBLE AND BIODEGRADABLE POLYESTERS PRODUCED BY BACTERIA

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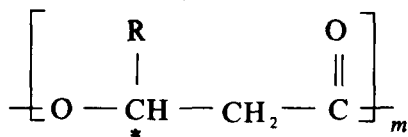
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ABSTRACT

A wide variety of different types of microorganisms are known to produce intracellular energy and carbon storage products, which have been generally described as being poly (β -hydroxybutyrate), PHB, but which are, more often than not, copolymers containing different alkyl groups at the β -position. Hence, PHB belongs to the family of poly (β -hydroxyalkanoates), PHA, all of which are usually formed as intracellular inclusions in bacteria under unbalanced growth conditions. Recently, it became of industrial interest to evaluate these PHA polyesters as natural biodegradable and biocompatible plastics for a wide range of possible applications, such as surgical sutures or packaging containers. For industrial applications, the controlled incorporation of repeating units with different chain lengths into a series of copolymers is desirable in order to produce polyesters with a range of material properties because physical and chemical characteristics depend strongly on the polymer composition. Such "tailor-made" copolymers can be produced under controlled growth conditions in that, if a defined mixture of substrates for a certain type of microorganisms is supplied, a well defined and reproducible copolymer is formed.

INTRODUCTION

A wide variety of different types of microorganisms are known to produce intracellular energy and carbon storage products which have been generally described as being poly (β -hydroxybutyrate), PHB^[1-4]. This particular polymer, however, belongs to the family of poly (β -hydroxyalkanoates), PHA, shown below, all of which are formed as intracellular inclusions under stressed growth conditions in the presence of an excess of a carbon or energy source. The signal to start producing these polymers is a limitation in the amount of nutrient or growth factor^[5-8].



In this structure R is an *n*-alkyl pendant group of variable chain length, including HB when R is methyl, HV or β -hydroxyvalerate when R is ethyl, and so on.

The spectrum of PHA producing microorganisms includes a wide variety of taxonomically different groups, and most of the organisms are capable of accumulating PHA in from 30 to 80% of their cellular dry weight. Under specific conditions, *Alcaligenes eutrophus* is known to contain as much as 96% PHA^[9]. It has also been demonstrated recently that the PHB biosynthetic pathway from *A. eutrophus* can be cloned and expressed in *E. Coli*^[10]. Polymer contents of up to 90% of the cellular dry weight were obtained from *E. Coli*.

Generally, environmental conditions and physiological capabilities of the bacteria control the amount of the storage polymer formed. Low concentrations or the total absence of a variety of different nutrients can induce or stimulate the formation of PHA, but usually nitrogen-limiting conditions are chosen for the experimental work because they are easily achieved by omitting ammonium from the growth medium. Beside the importance as a source of energy and carbon under conditions of starvation, the intracellular presence of PHA (or PHB in particular) seems to play a significant role in the survival of the microorganism under other conditions of environmental stress, such as when it is subjected to osmotic pressure, to desiccation, or to UV irradiation. In general, cells containing this polymer have a higher survival rate than PHA-free cells.

PHA is formed within the cell's cytoplasm as granular inclusions, which can be observed under the light microscope as refractile bodies^[4, 11, 12]. It is still an open question as to whether the polymer is or is not crystalline in the living cell. Electron microscopic observations have shown that the granules are surrounded by a membrane which does not show the typical bilayered structure of a biomembrane^[13, 14, 15], and it has been suggested that this membrane contains the PHA-synthetase or polymerase system^[16, 17]. Depending on the microorganisms, the PHA-depolymerase, which is responsible for the intracellular degradation of the polymer, may also be a part of this surrounding protein envelope, but some organisms also show the presence of a soluble, cytoplasmatic depolymerase. In any case, up to ten protein bands have been found for the membranes of purified PHA granules from *P. Oleovorans* when analyzed by polyacrylamide gel electrophoresis^[18], presumably indicating the complex nature of the polymerase system which is located at the transition zone between the aqueous cytoplasm and the hydrophobic PHA granule.

CHARACTERIZATION AND PROPERTIES OF PHA

PHB, which is the best-known member of the PHA series of polyesters, was discovered and initially described in 1925 by Lemoigne^[19], and during the late 1950's and early 1960's metabolic pathways in the synthesis and degradation of PHB in *Bacillus* and *Rhodospirillum* were investigated^[20, 21]. The first proposal for the use of PHB as a commercial plastic appeared in patents for the application of PHB as an absorbable suture^[22], but it was not until 1974 that the presence of other β -hydroxylalkanoate units than β -hydroxybutyrate in the PHA were found for bacteria in sewage sludge. Findlay and White proposed in 1983 to replace the term

PHB by the more general description PHA when it was realized that the presence of pure homopolymers of β -hydroxybutyrate as intracellular storage products was the exception rather than the rule.

All of the PHA's found to date are basically linear polyesters of β -hydroxy-alkanoic acids, and all of the units are believed to exist with only the [R]-configuration at their chiral center. That is, in polymer chemistry terms, all of these polymers are perfectly isotactic. High molecular weights of upto 2×10^6 are found in solvent-extracted polymer from different bacteria. This value corresponds to a degree of polymerization of approximately 20,000, and each PHA granule contains several thousands of polymer chains^[23].

In the crystal lattice the conformational structures of PHB and of copolymers of HB and HV are those of a right-handed 3_1 helix with a two-fold screw axis and a fiber repeat of either 0.556 or 0.596 nm^[24, 25]. The helix conformation is stabilized by carbonyl/methyl interactions and represents one of the few exceptions of a helix to be found in nature which does not depend upon hydrogen bondings for its formation and stability. However, it has been demonstrated recently by X-ray diffraction studies on oriented films, that PHA with long pendant groups shows the typical d-spacings of comb-like polymers such as polymethacrylates, poly(vinyl ethers), or poly(vinyl esters)^[26].

PHA extracted from sewage sludge, from estuarine^[27] and marine sediments^[28], and from compost heaps^[18] shows the presence of a variety of β -hydroxyalkanoate units indicating the presence of diverse microbial populations in these environments that are capable of producing different types of PHA. However, to date only a limited number of organisms is known to incorporate repeating units longer than β -hydroxyvalerate into their storage polyesters as shown by the compilation in Table 1.

Table 1 Compositions of various PHA found in specific microbes and in environmental samples

Bacterium	Repeating units present containing total number of carbon atoms indicated										Reference
	4	5	6	7	8	9	10	11	12		
<i>R. rubrum</i>	x	x	x	x						6	
<i>Rb. sphaeroides</i>	x	x								18	
<i>P. oleovorans</i>	x	x	x	x	x	x	x	x	x	6	
<i>A. eutrophus</i>	x	x								48	
<i>B. megaterium</i>	x	x		x	x					28	
<i>Aphanothece</i>	x	x								58	
<i>Microcoleus</i>	x	x								58	
Marine sediments	x	x	x	x	x					28	
Marine bacteria	x									59	
Sewage	x		x		x					59	
Sewage sludge	x	x	x	x						60	

The physical and mechanical properties of these copolymers such as stiffness, brittleness, melting point, glass transition temperature, or resistance to organic solvents can change considerably as a function of the monomer composition^[29]. Increasing the amount of HV in HB-HV copolymers reduces the melting point from 180 °C for the PHB homopolymer to approximately 75 °C for a copolymer containing about 30 to 40 mole% of HV units. In contrast, PHA isolated from *P. oleovorans*, which contains long-chain repeating units has a melting point below 60 °C and is soluble in acetone or ether, whereas PHB homopolymer is not^[30].

The amount of PHA formed can be determined by the digestion of cells containing the granules with hypochlorite and measuring the turbidity caused by the remaining polymer granules^[30], as was done in earlier studies. However, a much more accurate estimate of the PHA amount present is determined by gravimetric methods after extraction of the polymer with a solvent (such as chloroform or propylene carbonate) and precipitation of the polymer in ether^[31,32]. Another method for the quantitative determination of PHA is by UV spectrophotometric techniques after the acidic conversion of PHB to crotonic acid, which can be measured by UV spectroscopy from its absorption at 235 nm. However, this method is only useful for non-phototrophic organisms because the presence of pigments can interfere with the absorption of crotonic acid.

All of these methods do not allow for the determination of the monomer composition of the polymer, so for this purpose a quantitative determination of the types of monomers present other than HB itself must be used, such as gas chromatography, GC^[33], high pressure liquid chromatography, HPLC^[34], and nuclear magnetic resonance spectroscopy, NMR^[35]. For the chromatographic techniques, the polymer must be depolymerized to its equivalent monomer units by mild acidic or alkaline treatment, and the different repeating units are separated and identified. In contrast, the NMR method is, of course, non-destructive, and the polymer can easily be recovered.

Several methods have been applied to remove PHA quantitatively from bacterial cells, including solvent extraction^[36], hypochlorite treatment to destroy the cells^[37] followed by differential centrifugation^[38], and liquid/liquid extraction in an immiscible two-phase system^[39]. It has been observed in several studies that the technique applied for cell disruption and for a subsequent extraction of the PHA were crucial for the retention of certain physical and chemical properties of the polyester. The deleterious effect of hypochlorite on the molecular weight of the polymer has been demonstrated, and the highest PHA yields and molecular weights that have been obtained from *R. rubrum*, for example, were determined by extraction of the polymer with hot chloroform as shown by the results in Table 2. However, the relatively low polydispersity of two of the products in Table 2 indicates that, likely, a loss of the lower molecular weight fraction occurred during the extraction procedure. Mechanical destruction of whole cells using a French press also reduced the molecular weight significantly.

There is no report of the presence of any type of functional groups in PHA produced in nature by bacterial synthesis, although it has been demonstrated that *P. oleovorans* is capable of forming polyesters with unsaturated or alkene pendant

Table 2 Evaluation of different extraction and cell rupture methods for the PHA isolation from *Rhodospirillum rubrum*

	Chloroform Extraction	Lysozyme, Sonication	French Press	Hypochlorite
PHA content (% dry wt)	13.6	13.6	13.6	13.6
Isolated PHA (% dry wt)	12.5	9.7	7.7	12.1
Recovery (%)	92	71	57	89
$\overline{M}_w (\times 10^{-6})^b$	1.5	0.92	0.65	0.94
$\overline{M}_n (\times 10^{-6})^a$	1.1	0.36	0.46	0.37
$\overline{M}_w / \overline{M}_n$	1.4	2.6	1.4	2.5

^{a)} Numbering average molecular weight.

^{b)} Weight average molecular weight.

groups when α -olefins were used as growth substrates^[40]. In that case, the double bond was found to exist as a terminal group in the pendant side chain. Hence, the double bonds of this type of PHA could be used as a reactive group for the chemical modification of the polymers. It has also been shown that chlorine was incorporated into the storage polymer of *A. eutrophus* when either 3-chloropropionic or 5-chlorovaleric acid were used as a carbon source^[41,42,43]. The chloro group could also serve as a target for the chemical modification of PHA. Furthermore, quite recently, unusual copolymers containing HB and 4-hydroxybutyrate units were obtained, as well as copolymers of HB, HV and 5-hydroxyvalerate from *A. eutrophus* indicating an unusual flexibility of this organism in its ability to produce polymers which do not contain exclusively β -HA units^[44].

PHA FOR BIODEGRADABLE AND BIOCOMPATIBLE PLASTICS

It has become of considerable industrial interest and of environmental importance to evaluate PHA as polyesters for the use in either biodegradable and biocompatible plastics for a wide range of possible applications as indicated in Table 3.

Table 3 Practical applications of PHA^{a)}

Surgical pins, sutures, and swabs
Wound dressing
Bone replacement and plates
Stimulation of bone growth and healing by piezoelectric properties
Biodegradable carrier for long term dosage of drugs, veterinary medicines, herbicides, and insecticides
Packaging containers and films

^{a)} From References 48 and 50.

Early investigations of PHA granules by electron microscopy after freeze-etching showed that the polymer in the granule underwent a cold drawing process indicating the plastic nature of the polyester and suggesting that it could be processed as a conventional thermoplastic.

In addition to its potential as plastic material, PHA represents also a useful source for stereoregular compounds which can serve as chiral precursors for the chemical synthesis of optically active substances, particularly in the synthesis of certain drugs or insect pheromones^[45, 46, 47]. These substances are biologically active only in the correct stereochemical configuration.

An example of a useful PHA polyester is the one which is now being produced industrially, on a fairly large scale, by a subsidiary of ICI Ltd. in Great Britain using of *A. eutrophus*^[48]. Either PHB homopolymer or copolymers of HB and HV can be formed by these cells depending on the substrate or substrate mixtures used for growth and polymer production^[49]. These polymers are commercially available under the trade name "Biopol".

For industrial applications it is desirable to control the incorporation of different repeating units in a copolymer in order to produce polyesters with specific material characteristics because their physical and chemical characteristics depend strongly on copolymer composition. "Tailor-made" copolymers can be obtained for this purpose from bacteria subjected to the use of controlled growth conditions. If a defined mixture of nutrients for a certain type of microorganisms is supplied for growth, a defined and reproducible copolymer is formed.

Biochemically, there are two different ways to achieve polymer formation in microbes. In one method the growth substrate or carbon source can be metabolized to

Table 4 Chemical and physical properties of polypropylene (PP) and poly (β -hydroxybutyrate) (PHB)^{a)}

	PP	PHB
Melting point T_m [°C]	171 — 186	171 — 182
Glass transition temperature T_g [°C]	~ 15	5 — 10
Crystallinity [%]	65 — 70	65 — 80
Density [g/cm ³]	0.905 — 0.94	1.23 — 1.25
Molecular weight M_w ($\times 10^{-5}$)	2.2 — 7	1 — 8
Molecular weight distribution	5 — 12	2.2 — 3
Flexural modulus [GPa]	1.7	3.5 — 4
Tensile strength [MPa]	39	40
Extension to break [%]	400	6 — 8
UV resistance	poor	good
Solvent resistance	good	poor
Oxygen permeability [cm ³ /m ² /atm/d]	1700	45
Biodegradability	+	—
Approx. U.S. annual production [Mio. t]	1.8	ND ^{b)}

^{a)} From References 24, 48, 49, 50.

^{b)} Not determined.

form both the microbial biomass and the storage polymer simultaneously, as has been demonstrated by the formation of various PHA copolyesters in *R. rubrum* and *P. oleovorans*^[5,6]. During cell growth, however, part of the polymer-forming potential is lost because of the utilization of the substrate to maintain the cell's metabolism. Another possible way of industrial polymer production is a serial process where microorganisms are first grown on a carbon source to obtain a large amount of biomass, then the medium is depleted of an essential nutrient and a polymer-forming substrate is added. The latter is converted directly to polymers and essentially only little additional cell growth occurs. This approach is used by ICI Ltd. for the large scale PHA production by *A. eutrophus*^[48].

PHB homopolymer shows similarities in its physical properties and even in its molecular structure to the isotactic polypropylene, PP. For the latter, both polymers have a methyl pendant group attached to the main chain in a single configuration. Table 4 shows a comparison of several of the material properties of these two polymers^[50, 51]. The main difference between the two, however, is the biodegradability of PHB compared to the inability to degrade PP by a biological route. A further difference, which might be important in its practical application, is the density of the two plastic materials. Because of its high density, PHB does not float in an aquatic ecosystem, but PP does. Therefore, once discarded, plastic articles made from PHB will sink and could be degraded in some sediments by biogeochemical mechanisms.

BIODEGRADATION OF PHA

In general, a number of parameters can influence the rate of biodegradation and the lifetime of a plastic material in nature, including the type of environment, the presence of a microbial population and its microbial activity, the availability of water, the temperature, the section thickness of the plastic material, its surface texture, its porosity, and the presence of second components in the plastic, such as fillers or coloring agents. As examples, Table 5 shows the effect of different environments on the degradation rate of PHB homopolymer films^[49]. Two mechanisms are responsible for the degradation of PHA. Under sterile or aseptic conditions, PHA is degraded by a hydrolytic mechanisms, especially at high pH^[52]. This type of degradation is important for medical applications, such as for use of PHA in drug release carriers or surgical sutures. In contrast, in natural environments, the polymer can be degraded enzymatically by the action of a depolymerase or esterase.

Table 5 Degradation of PHB films in various environments^{a)}

Environment	Time for 100% Degradation Weeks ^{b)}
Seawater, 15 °C	350
Soil, 25 °C	75
Aerobic sewage	60
Anaerobic sewage	6

a) From Reference 49.
b) For a 1 mm thick film

Several microorganisms, such as *Alcaligenes faecalis*^[53], are known to degrade PHB by the secretion of an esterase to catalyze the hydrolysis of the polymer in order to generate organic compounds which can be used as a carbon source for growth. PHB can also be degraded by mixed bacterial cultures which can be obtained from soil.

In contrast to PHB, PHA's with longer chain length pendant groups should undergo slower biodegradation rates in environments such as sewage sludge or sediment for the following reasons: (1) low molecular weight carbon compounds are mineralized more rapidly, whereas more complex structures have much lower rates of mineralization^[56, 57] and (2) long-chain repeating units increase the hydrophobicity of the polymer, and a high hydrophobicity generally inhibits or prevents microbial growth on the polymer surface. Therefore, it is likely that PHA with long pendant groups are degraded more slowly than PHB, so that the lifetime of a plastic article made from PHA derived from *P. oleovorans*, for example, may be prolonged.

Table 6 Aerobic degradation of various types of PHA films by a mixed bacterial culture enriched from freshwater sediments

Polymer Sample	Chiral Center	Composition of Copolymers, HB/HV	M_w ^{a)}	Degradation ^{b)}
PHB	[R]	-	150,000	+
PHB	[R]	-	800,000	+
PHB	[R, S] stereoblock	-	ND	-
PHB	[R, S] atactic	-	ND	-
PHB	[S]	-	ND	-
P (HB/HV)	[R]	83/17	150,000	+
P (HB/HV)	[R]	80/20	ND	+
P (HB/HV)	[R]	70/30	ND	+
P (HH/HV) ^{c)}	ND	94/4	360,000	+
P (HN/HH) ^{d)}	ND	59/32	190,000	+

a) ND - not determined.

b) + indicates polymer degrades, - indicates no observable degradation.

c) Copolymer containing β -hydroxyheptanoate units, HH.

d) Copolymer containing β -hydroxynonanoate units, HN.

Table 6 shows the results of preliminary studies carried out in our laboratories on the biodegradation of PHA homo- and copolymers which differ in composition, stereochemistry and molecular weight. In general the initial molecular weight had no significant influence on the biodegradability of the polymer, and a high molecular weight PHB homopolymer was degraded as rapidly as a low molecular weight polymer. On the other hand, degradation was prevented when units with the [S]-configuration were present, indicating the stereoselectivity of the esterase enzyme. Copolymers of HB and HV were readily degraded independent of the amount of HV incorporated in the polymer, and a PHA with β -hydroxyheptanoate, HH, as the

major repeating unit was degraded as well. In contrast, no degradation of the polymer was detected when β -hydroxynonanoate, HN, was the main repeating unit. These results suggest that, by specific control of the unit present, the biodegradability and the degradation rates of the PHA polymers can be controlled.

The very wide range of microbes known to produce PHA allows the selection of organisms which are capable of forming specific polyesters under defined conditions. On an industrial scale, the use of photosynthetic bacteria, for instance, would harness sunlight as an energy source for the production of these polyesters. Because of their metabolic flexibility, organisms like *Pseudomonas* have the potential ability to produce a whole new variety of useful plastics all of which would be biodegradable.

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