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Review

FUNCTIONAL POLYHYDROXYALKANOATES SYNTHESIZED BY MICROORGANISMS*

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Abstract Many bacteria have been found to synthesize a family of polyesters termed polyhydroxyalkanoate, abbreviated as PHA. Some interesting physical properties of PHAs such as piezoelectricity, non-linear optical activity, biocompatibility and biodegradability offer promising applications in areas such as degradable packaging, tissue engineering and drug delivery. Over 90 PHAs with various structure variations have been reported and the number is still increasing. The mechanical property of PHAs changes from brittle to flexible to elastic, depending on the side-chainlength of PHA. Many attempts have been made to produce PHAs as biodegradable plastics using various microorganisms obtained from screening natural environments, genetic engineering and mutation. Due to the high production cost, PHAs still can not compete with the non-degradable plastics, such as polyethylene and polypropylene. Various processes have been developed using low cost raw materials for fermentation and an inorganic extraction process for PHA purification. However, a super PHA production strain may play the most critical role for any large-scale PHA production. Our recent study showed that PHA synthesis is a common phenomenon among bacteria inhabiting various locations, especially oil-contaminated soils. This is very important for finding a suitable bacterial strain for PHA production. In fact, PHA production strains capable of rapid growth and rapid PHA synthesis on cheap molasses substrate have been found on molasses contaminated soils. A combination of novel properties and lower cost will allow easier commercialization of PHA for many applications.

Keywords Polyhydroxybutyrate, Polyhydroxyalkanoate, Biopolymer, Polyester

Polyhydroxyalkanoates (PHAs) are a family of intracellular biopolymers synthesized by many bacteria as intracellular carbon and energy storage granules (Figs. 1 and 2). It is generally believed that PHA synthesis is promoted by unbalanced growth[1~6]. The plastic-like properties and biodegradability of PHAs make them attractive as a potential replacement for non-degradable polyethylene and polypropylene. Many efforts have been made to produce PHA as environmentally degradable thermoplastics[7~10].

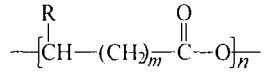

Fig. 1 General molecular structure of polyhydroxyalkanoates m = 1, 2, 3, yet m = 1 is most common, n can range from 100 to several thousands. R is variable. When m = 1, $R = CH_3$, the monomer structure is 3-hydroxybutyrate, when m = 1 and $R = C_3H_7$, it is a 3-hydroxyhexanoate monomer.

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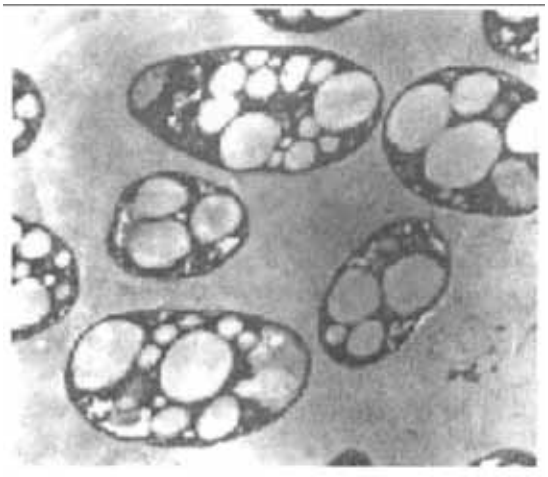

Fig. 2 PHA granules stored in cells of *Azotobacter vinelandii* UWD(10000*)[2]

Among the PHA family, poly-3-hydroxybutyrate (PHB) is the most common member; it belongs to the short chain length PHA (scl PHA) with its monomers containing 4~5 carbon atoms. Other PHA containing monomers consisting of 6~16 carbon atoms have been designated as medium chain length PHA (mcl PHA), poly(hydroxyoctanoate-*co*-hydroxydecanoate) or P(HO-*co*-HD) is a typical mcl PHA[3]. Copolyesters of PHA containing scl monomers such as 3-hydroxybutyrate (HB) and mcl monomers such as 3-hydroxyhexanoate (HHx) have been found to show a dramatic improvement in their mechanical properties compared with PHB[11]. However, only a few wild type microorganisms were reported to be able to produce HB and mcl HA containing PHA, regardless of copolymers or blend polymers although broad screening processes have been done[11~15].

Many structure variations of PHA have also been synthesized. Due to the small amount of these unconventional PHAs, only a few physical characterizations and only little application research have been carried out so far[16~19]. On reviewing the PHA research carried out to date, it is clear that work has been directed towards the design, biosynthesis, and properties of biodegradable and biocompatible materials. These materials can be explored for bioengineering new optical and other "smart" chiral materials[18].

Some of the recent advances in this area are reviewed in this paper. Much of this work has been done in our lab. We believe that interdisciplinary interaction and cooperation among biologists, polymer scientists and material scientists are the key for successful exploration of novel applications for these unique polyesters.

MICROBIAL SYNTHESIS OF POLYHYDROXYALKANOATES WITH DIVERSE MONOMER STRUCTURES

Over 90 PHAs with various monomer structures have been reported and the number is still increasing[3]. The most typical PHA is PHB which is a scl PHA, which includes also poly(hydroxybutyrate-*co*-hydroxyvalerate) or P(HB-*co*-HV). Scl PHA is normally brittle (Fig. 3). The most typical mcl PHA is P(HO-*co*-HD), which is elastic in nature (Fig. 3). Copolyester of hydroxybutyrate and hydroxyhexanoate, abbreviated as P(HB-*co*-HHx), combines the properties of scl- and mcl PHA and become a real thermoplastic (Table 1).

The monomer structures of PHA are normally very much dependent on substrates used for cell growth. For example, when *Pseudomonas aeruginosa* 44T1 was grown on euphorbia oil, various novel monomers were identified in the PHA (Fig. 4a)[17]. On a mixture of octanoate and *para*-cyanophenoxyhexanoate, a PHA copolymer consisting of related monomer structures were observed (Fig. 4b)[17]. On soybean oil, a unique monomer structure was synthesized by strain *Pseudomonas stutzeri* 1317 (Fig. 4d)[18]. *Pseudomonas oleovorans* grown on 1-heptanene or on a mixture of octane and 1-chlorooctane will synthesize monomers of PHA with unsaturated bonds and chlorine at the end of the side chains, respectively (Fig. 4e, 4f)[21, 22]. Various monomer

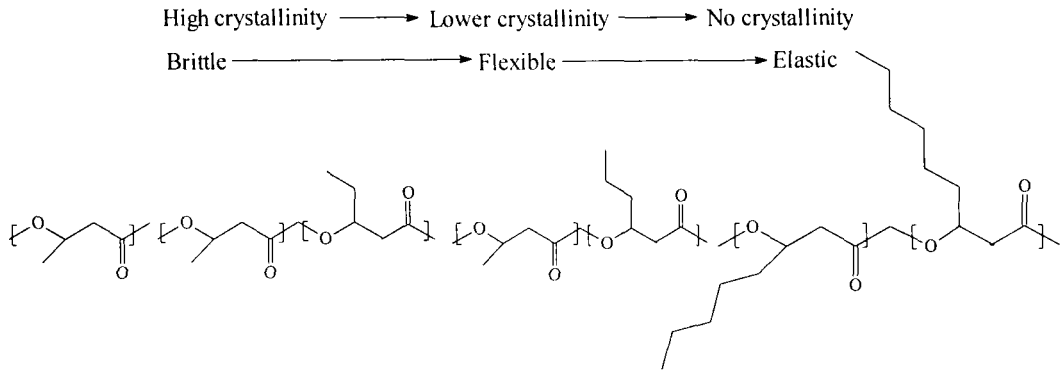

Fig. 3 Molecular structures of scl PHB and P(HB-*co*-HV), copolymer of scl HB and mcl HHx P(HB-*co*-HHx) and mcl P(HO-*co*-HD). Mechanical property of the above PHAs changes from brittle to flexible to elastic from left to right, while crystallinty decreased from left to right.

Table 1. Physical properties of various PHA in comparison with conventional plastics[3, 20]

Samples	T_m (℃)	T_g (℃)	Tensile strength (Mpa)	Elongation at break (%)
PHB	177	4	43	5
P(HB-*co*-10% HV)	150	-	25	20
P(HB-*co*-20% HV)	135	-	20	100
P(HB-*co*-10% HHx)	127	−1	21	400
P(HB-*co*-17% HHx)	120	−2	20	850
Polypropylene	170	-	34	400
Polystyrene	110	-	50	-
PET	262	-	56	7300
HDPE	135	-	29	-

HV: 3-hydroxyvalerate; HHx: 3-hydroxyhexanoate; PET: poly(ethylene teraphthalate); HDPE: high density polyethylene

structures of PHA were synthesized by *Pseudomonas putida* grown on a mixture of octanoate and paracyanophenoxyhexanoate, on phenoxyalkanoates, on a mixture of nonanoic acid and 10-undecynoic acid, and on a mixture of nonanoic acid and fluorinated acid cosubstrates, respectively. These synthesized PHAs have cyanophenoxyhexanoate, phenoxyalkanoate, carbon-carbon triple bonds and fluorinated groups at the end of their monomer chain (Fig. 4, structures c, g, h and i)[18, 23-26]. These functional groups are highly valuable for further chemical modification. There are many ways to add or replace functional groups on PHA side chains, and consequently, there are many possibilities to modify the physical properties of these PHA. Currently, research on these novel PHAs was limited by their amount that could be available for study. Considering that all the PHA monomers have chirality, they may demonstrate some interesting properties that have not been unveiled.

The PHA monomer structures described above are dependent on the structure of substrates available for the microorganisms. Therefore, it should be possible to design a PHA with desired functional groups if the substrates containing the functional groups are available. There are also many microorganisms that can synthesize PHAs with unconventional structures from simple substrates, such as the production of mcl PHAs from glucose and gluconate[15, 19]. The synthesis of unconventional PHA from unrelated substrates can open a way to produce PHAs with low cost.

PHYSICAL PROPERTIES OF POLYHYDROXYALKANOATES

The mechanical properties of PHAs range from brittle to flexible to elastic, depending on the side chain length of hydroxyalkanoates. Normally, this scl PHA such as PHB has high crystallinity and thus PHB is very brittle. However, copolymer of HB and 3-hydroxyvalerate (PHBV) has a better mechanical property compared with homopolymer PHB[3]. Copolymers of HB and 3-hydroxyhexanoate improve the mechanical property of PHBV

further[20], making them comparable with the conventional plastics such as polypropylene, polystyrene, poly(ethylene teraphthalate) (PET) and high density polyethylene (HDPE) (Table 1). Mcl PHAs, such as P(HO-*co*-HD), become a completely elastic substance (Fig. 3).

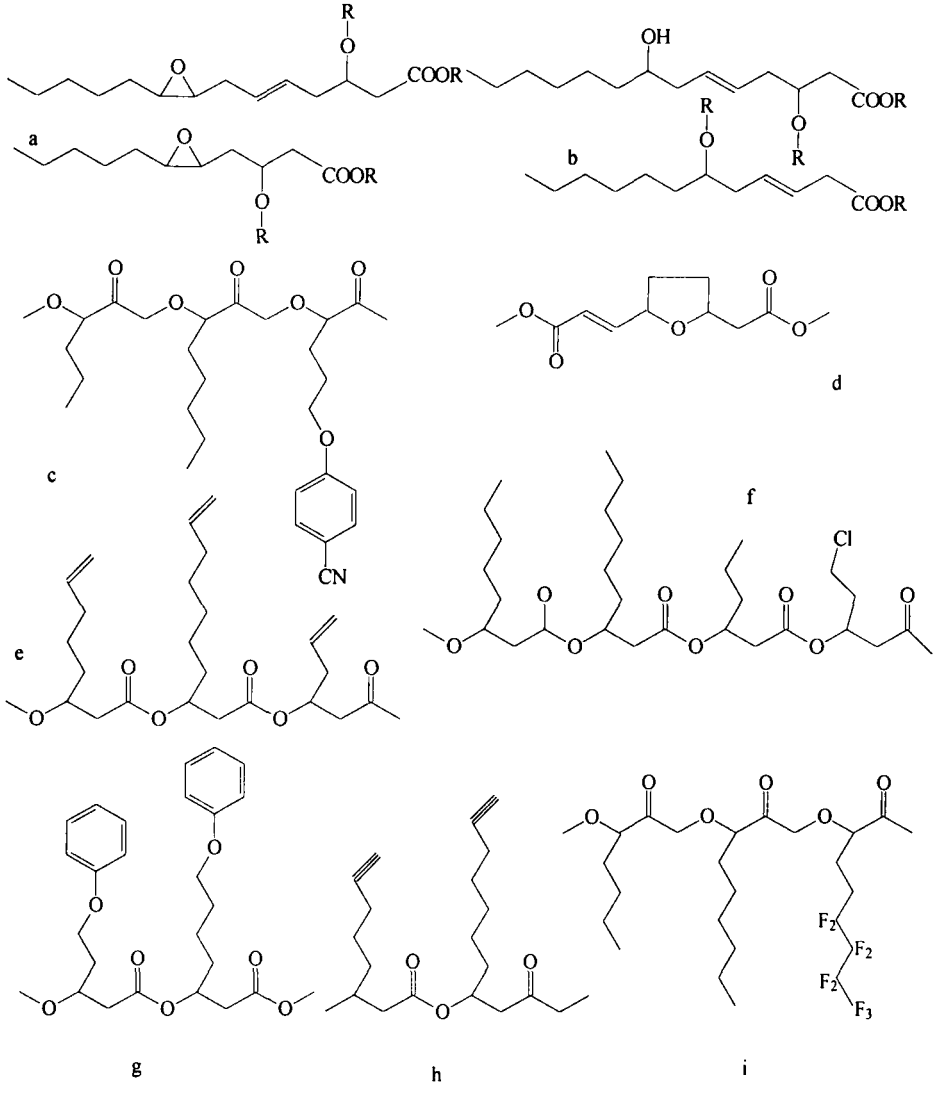

Fig. 4 PHAs monomer structures derived from vernolic acid identified in euphorbia oils (a)[17]; from ricinolic acid identified in castor oil (b)[17]; from a mixture of octanoate and *para*-cyanophenoxyhexanoate (c)[18]; from soybean oil (d)[19]; from 1-heptanene (e)[21]; from a mixture of octane and 1-chlorooctane (f)[22]; from phenoxyalkanoates (g)[23], from a mixture of nonanoic acid and 10-undecynoic acid (h)[24] and from a mixture of nonanoic acid and fluorinated acid cosubstrates (i)[25, 26].

The physical properties of mcl PHAs are unsuitable for application due to their low melting points and low glass transition temperatures[27, 28]. They are also elastic but lack the toughness required for applications. When the monomer chain length increases to 12 carbon atoms, the PHAs become liquids.

Besides the mechanical property, the most interesting applications of PHAs rely on their degradability. Various HA monomer structures affect PHA degradation rate. Studies have shown that PHB lost over 80% of its original dry weight after 28 days on incubation in active sludge while same PHA sample lost less than 5% of its original weight in tap water under the same conditions[29]. It has been reported that poly(3-hydroxybutyrate-*co*-4-

hydroxybutyrate) has a much faster degradation rate than homopolymer PHB[30]. Other interesting properties of PHAs including piezoelectricity[31], nonlinear optical activity and biocompatibility have also been studied[1, 3]. The lack of sufficient amounts of samples prevents the characterization of other unconventional PHAs described above. It is expected that structure modification will improve the high-value-added properties of these unique polyesters. The possibility of synthesizing PHAs with desired functional groups will open many opportunities to improve the functionality and novel properties of PHAs.

POLYHYDROXYALKANOATES PRODUCING BACTERIA ARE UBIQUITOUS

As it is difficult to carry out a large scale PHA analysis using the gas chromatographic technique developed previously[32], there has been no systematic study concerning the accumulation of PHAs by microorganisms grown in various geological locations, although many bacteria were known to accumulate PHAs from various substrates such as glucose, sucrose, *n*-alkyl, *n*-alcohols or *n*-organic acids[1, 3, 5, 33, 34]. Recently, our lab has developed a rapid noninvasive technique using FTIR, which allows us to detect PHA accumulation intracellularly within seconds[35]. If PHA contents are high in the cells, FTIR spectra of PHA can also differentiate the fine structures of PHB, mcl PHA and PHA consisting of HB and mcl HA monomers[35]. Among the PHAs, PHB appears to be the most common intracellular storage compound present in all locations. Our results have shown that mcl PHAs, as well as PHA consisting of HB and mcl HA are commonly synthesized by bacteria inhabiting oil-contaminated soils or waters[34]. Depending on the substrates, bacteria may or may not synthesize PHA.

Approximately 400 morphologically diverse strains were isolated from 20 soil samples collected from various locations. Our results showed that approximately 40% of the strains had PHA accumulation ability from at least one of the six carbon sources used in our screening study (unpublished results). Considering that many bacteria may be able to synthesize PHAs from other carbon sources beside the six ones employed in our study, it can be presumed that the actual number of PHA producing bacteria inhabiting the soil we sampled should exceed 40%. This result demonstrated that many bacteria possess the ability to synthesize PHA, provided a suitable substrate is available. This result contradicts with the generally believed argument that PHAs are synthesized when growth conditions are unbalanced, such as nitrogen, phosphate, oxygen limitation and oversupply of carbon sources[1]. Our broad screening outcome actually demonstrated that PHA accumulation is in fact substrate dependent, rather than limitation dependent.

Our broad screening results showed that quite a number of the strains have $\nu_{c=o}$ vibration bands around 1735 cm^{-1} which is characteristic for PHAs in FT-IR spectra when grown on glucose or molasses substrates demonstrating the possible existence of HB and mcl HA containing PHAs in these bacterial cells[34, 35]. As copolymers of HB and mcl HA have improved mechanical properties and processability, they are more interesting for industrial applications as biodegradable materials[11]. Among the 19 strains randomly picked for studies, 14 strains accumulated PHA containing HB and mcl HA monomers, although the PHAs were found to be blend polymers consisting of HB and mcl HA (unpublished data). It seems that these bacteria contain two PHA synthase systems that are responsible for synthesizing PHB and mcl PHA, respectively[36]. This result points out that the ecological system in soil embraces many bacteria that are capable of synthesizing PHB, mcl PHA and PHA consisting of HB and mcl PHA. The reason why no bacteria synthesizing HB and mcl HA containing copolymer inhabited the soil we sampled is still unclear. However, considering that only a small fraction of bacteria has been studied, it is logical to imagine that nature is inhabited by a rich community of various microbes that possess the ability to produce many PHAs with novel structures and interesting properties. A broades screening program is under way to find more interesting PHA producers.

SYNTHESIS OF POLYHYDROXYALKANOATES BY RECOMBINANT MICROORGANISMS AND TRANSGENIC PLANTS

As early as 1988, the genes for PHB synthesis were reported to be cloned from *Alcaligenes eutrophus* (now called *Ralstonia eutropha*) and they were successfully expressed in *Escherichia coli*[37, 38]. Since then, many

attempts have been made to improve the expression and stabilization of PHB genes in *E. coli* cells[39~43]. Much attention has also been focused on cloning of different strains and expression of other PHA genes in various hosts[44~48]. PHAs with various monomer structures including poly(3-hydroxybutyrate-*co*-3-polyhydroxyhexanoate), poly(3-hydroxyvalerate-*co*-3-hydroxyheptanoate), poly(hydroxybutyrate-*co*-hydroxyoctanoate)[49], mcl PHA, poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-4-hydroxyvalerate) and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) etc. have been synthesized by recombinant microorganisms[47, 48, 50, 51].

Recombinant DNA techniques allow us to modify or introduce new metabolic pathways to the microorganism hosts and thus broaden the utilizable substrate range, enhance PHA synthesis capacity and produce PHAs with novel monomer structures. It has been reported that recombinant *E. coli* produced 101 g/L of cell dry weight with 80% of PHB content within 39 h of fermentation which was carried out in glucose mineral medium. This is the most efficient PHB production reported so far[52].

Transgenic plants harboring the *Ralstonia eutropha* PHA synthesis genes have been developed, with the aim of ultimately reducing the price of PHA to close to that of the starch[53]. The PHB expression level is still very low at the present stage, yet with our understanding of plant biochemistry and genetics improving, the PHA expression level will also be increased. In the near future, however, PHA production probably has to be dependent on microbial fermentation.

POTENTIAL APPLICATIONS OF POLYHYDROXYALKANOATES

Although many possibilities have been tried to lower the production cost of PHAs, they are still not in a position to challenge conventional plastics such as polypropylene and polystyrene that cost only $1/kg[1, 4, 10, 54]. The success of transgenic plants that produce large quantity of PHA could eventually lower the cost of PHA to a level comparable to conventional plastics. For this goal, much research work is still needed to improve PHA gene expression levels in economically interesting plants, such as oilseeds and potatoes.

The synthesis of PHAs with novel monomer structures will open up another interesting research field. More and more bacterial strains, which have shown some unusual ability to synthesize various PHAs, have been isolated from various locations. Some strains have high PHA productivity and can produce unusual PHA structures from simple substrates such as glucose and sucrose[28, 29, 55~57]. By using unconventional precursors with specific functional groups, a series of PHAs with such functional groups that endow the PHAs with desirable properties have been produced. PHAs with enhanced piezoelectricity, nonlinear optical activity, biodegradability and biocompatibility will provide PHAs with the potential to challenge other functional polymers produced by conventional chemical synthesis.

In addition to the application as plastics, PHAs can also represent a potential source of chiral hydroxy acid feedstock for the fine chemical industry. In contract to the introduction of new polymers, the PHA hydroxy acids and the related derivatives can be readily integrated into existing fine chemical markets[58].

The long-term development for PHAs will be a promising and precursory thing profitable to many industries[1, 3, 4, 58]. Collaboration among microbiologists, molecular biologists, polymer scientists, material scientists and industry is the key for PHA exploration. Many new developments should be expected in the new century.

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