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Biodegradable Polymer (Biopol®)

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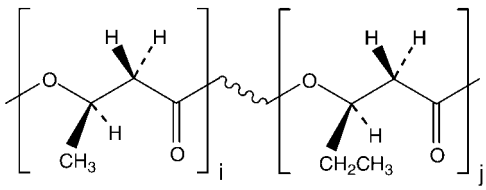
3HB	3-hydroxybutyrate
3HV	3-hydroyxvalerate
BN	boron nitride
CPA	cyclohexyl phosphonic acid
DBP	di-*n*-butyl phthalate
DSC	differential scanning calorimetry
GTA	glycerol triacetate
H_c	heat of crystallization
HEDP	1-hydroxyethylidene diphosphonic acid
IlvA	threonine deaminase
L/D	length/diameter
M_n	number average molecular weight
MCL	medium chain length
MVTR	moisture vapor transmission rate
NMR	nuclear magnetic resonance
OTR	oxygen transmission rate
PBA	polybutylene adipate
PCL	polycaprolactone
PDH	pyruvate dehydrogenase
PEE	pentaerythritol ethoxylate
PHA	poly(hydroxyalkanoate)
PhbA or BktB	β-ketothiolase
PhbB	acetoacetyl-CoA reductase
PhbC, PHB synthase; Poly(3HB)	poly(3-hydroxybutyrate)
Poly(3HB-*co*-3HV)	poly(3-hydroxybutyrate-*co*-3-hydroyxvalerate)

Poly(3HPE)	poly(3-hydroxy-4-pentenoic acid)
T_c	crystallization temperature
T_d	decomposition temperature
T_g	glass transition temperature
T_m	melting temperature
ZS	zinc stearate

1 Introduction

Poly(3-hydroxybutyrate) (Poly(3HB)) was first isolated and characterized in 1926 by Lemoigne at the Pasteur Institute in Paris (Lemoigne, 1926). Since then, this and other poly(3-hydroxyalkanoates) (PHAs) have been studied extensively by scientists who have come to the general conclusion that bacteria store Poly(3HB) as an energy reserve in much the same way that mammals accumulate fat. Although Poly(3HB) is the most common type of PHA, many different polymers and copolymers of this class are produced by a variety of organisms (Steinbüchel and Valentin, 1995). Beyond their biological curiosity, many PHAs have functional properties that are quite suitable for commercial applications. Whereas Poly(3HB) is somewhat brittle and thus limited in its commercial scope, a closely related copolymer, poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (Poly(3HB-*co*-3HV)), is more flexible because of reduced crystallinity and is suitable for many commercial applications (Structure. Indeed, this copolymer was commercially developed by ICI and was sold under the tradename Biopol®. The focus of this chapter is Biopol®. In it we will review all the pertinent literature on Biopol®, its properties, and those of other closely related PHAs. We also will discuss bacterial production of Biopol® and PHAs in general, recent learning on the Biopol® polymer science front, and efforts to produce this copolymer in transgenic plants.



2 Historical Outline

Poly(3HB) remained an academic curiosity until W. R. Grace in the US produced small quantities for commercial evaluation in the late 1950s and early 1960s. Several patents were issued as a result of that effort (Baptist, 1962a,b, 1965). Commercial interest lay dormant for over a decade until ICI began a research and development program. This project followed their single-cell protein animal feed project. Thus, ICI had the skills in place to run large-scale fermentation processes and polymer processing know-how was available in their plastics division (Holmes, 1984, 1985; Holmes et al., 1984). In the late 1980s, ICI began worldwide commercialization of a family of (Poly(3HB-*co*-3HV) copolymers with the tradename of Biopol®. In 1990, the agricultural and pharmaceutical businesses of ICI, including Biopol®, were spun-off as Zeneca Ltd. In 1996, Monsanto acquired the Biopol® business from Zeneca Ltd. Since this acquisition the emphasis at Monsanto was on producing Biopol® and related copolymers in plants,

and improving their properties for different end-use applications. This continued until Monsanto stopped their research program and commercial Biopol® business at the end of 1998.

3 Microbial Biosynthesis of PHAs

The specific enzymes involved in the synthesis and later utilization of the energy reserve PHA polymers is known to vary between microorganisms. The well-studied Poly(3HB) biosynthetic pathway in *Ralstonia eutropha* is shown in Figure 1. The pathway involves the condensation of two molecules of acetyl-CoA by β-ketothiolase to form acetoacetyl-CoA which is subsequently reduced by acetoacetyl-CoA reductase to form D-(–)-3-hydroxybutyryl-CoA (3HB). Monomeric 3HB is then polymerized to form Poly(3HB) by PHB synthase. The three enzymes that catalyze these reactions are encoded by genes organized as an operon in this organism designate PhbA, PhbB, and PhbC for the ketothiolase, reductase, and synthase, respectively (Slater et al., 1988). Like other PHAs, this polymer accumulates in discrete, membrane-bound granules in the bacterial cell (Williamson and Wilkinson, 1958; Merrick and Douboroff, 1961).

The percentage of Poly(3HB) in bacterial cells is normally low, from 1 to 30%, but under controlled fermentation conditions of carbon excess and nitrogen limitation, overproduction of polymer can be encouraged to produce yields of up to 80% of the dry cell weight (Dawes and Senior, 1973; Ward et al., 1977). Numerous microbiological species are known to be suitable for the production of PHAs (Anderson and Dawes, 1990). The microorganisms may be wild-type or mutated, or may have the necessary genetic material introduced into them using recombinant DNA techniques.

In *R. eutropha*, glucose is the common carbon source for the production of Poly-(3HB) (Holmes, 1985). However, other substrates such as methanol, sucrose, ethanol, and acetic acid can be used by microorganisms to produce the homopolymer (Suzuki, 1986). It is through various intracellular pathways that PHA-producing organisms like *R. eutropha* are able to convert these substrates to the precursor acetyl-CoA. As an example, a two-stage culture method has been reported in which *R. eutropha* was grown in an organic medium under heterotrophic conditions for exponential growth where the cells were cultivated for Poly-(3HB) accumulation under autotrophic conditions. The O_2 concentration in the substrate gas (H_2 + CO_2) was below the explosion limit of 6.9%. Through this process Poly(3HB) was obtained at a high production rate and concentration (Tanaka et al., 1994).

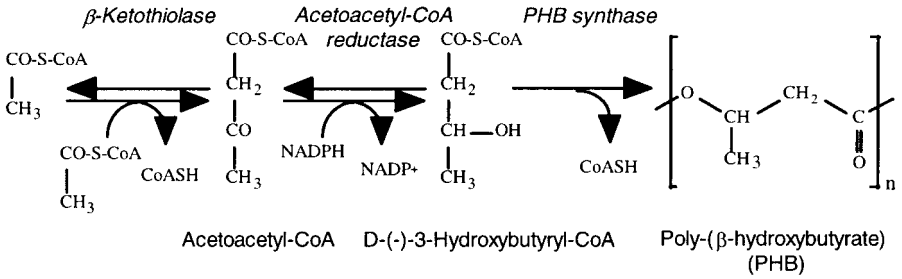


Fig. 1 Common three-step biochemical pathway to Poly(3HB) production as found in *R. eutropha*.

Interestingly, it is found that the Poly-(3HB) produced during exponential growth phase of *R. eutropha*, contains only carboxy chain ends; evidence of secondary hydroxyl is found only in polymers produced in the polymer accumulation phase (Madden et al., 1999). So, Poly(3HB) produced by fermentation always contains an excess of carboxy group in relation to the secondary hydroxyls. The number average molecular weight (M_n) of Poly(3HB) is frequently measured by end group analysis of the carboxy functionality. This is generally in excellent agreement with the true M_n measured by gel-permeation chromatography. However, M_n calculations based on the secondary hydroxyl are considerably higher due to the fact that not all the chains contain secondary hydroxyl end groups (Shah et al., 2000a). Therefore, M_n measured by secondary hydroxyls underestimates the actual number of chain ends and hence incorrectly leads to high M_n values.

By modifying the carbon source, *R. eutropha* can produce PHA copolymers. Pertinent to this chapter, Poly(3HB-*co*-3HV) or the Biopol® copolymer is typically produced using a combination of glucose and propionate in the growth media (Holmes, 1985). By adjusting the composition of the carbon sources, Poly(3HB-*co*-3HV) with up to 95 mol% 3HV content has been produced (Howells, 1982; Holmes, 1985; Doi, 1988). The fermentation process utilized by Zeneca and then Monsanto is based on descriptions written in various issued patents. In particular, Holmes et al. (1984) describe the use of the *R. eutropha* mutant NCIB 11599 grown under aerobic cultivation conditions to produce the Poly(3HB-*co*-3HV) copolymer when fed glucose and/or various amounts of propionic acid and other organic acids that lead to the 3HV component. The examples demonstrate that the copolymer can be produced up to 70% of the cell dry weight and with 3HV levels up to 30% of the monomer fraction.

Alternate feeding of glucose and propionic acid to the phosphate-depleted batch cultures of *R. eutropha* produced Poly(3HB-*co*-3HV) with thermal properties markedly different from random copolymers of similar monomer content. These polymers exhibited a single glass transition and a single melting peak that was significantly higher than expected for a random copolymer. The polymers were found, by solvent fractionation and nuclear magnetic resonance (NMR), to be a mixture of Poly(3HB) and a random copolymer, Poly(3HB-*co*-3HV). When compared with random copolymers of similar monomer content, the polymers produced by alternate substrate feeding displayed no improvement in mechanical properties and possessed similar aging characteristics (Madden et al., 1998).

The pathway for Poly(3HB-*co*-3HV) biosynthesis is essentially identical to that shown in Figure 1, where the steps to the 3HV component starts with the condensation of acetyl-CoA with propionyl-CoA to form 3-ketovaleryl-CoA. Interestingly, it was recently found that this condensation step for 3-ketovaleryl-CoA production requires a different β-ketothiolase than the PhbA ketothiolase because of the narrow substrate specificity of this latter enzyme (Slater et al., 1998). This finding had important implications for recombinant systems as will be described below. Poly(3HB-*co*-4 hydroxybutyrate) copolymers can also be produced by *R. eutropha* when fed on nitrogen-free cultures of either butyrolactone and butyric acid or 4-hydroxybutyrate and 4-chlorobutyric acid (Doi et al., 1988).

Outside of *R. eutropha*, using *Pseudomonas oleovorans* and a range of *n*-alkanoic acids, PHAs containing up to 12 carbon atoms have been produced (Brandl et al., 1988; Gross et al., 1989). Although all the PHAs

were heteropolymers containing up to six different monomers, the major monomer unit always had the same number of carbon atoms as the *n*-alkanoate substrate used. The introduction of functionalized chain-ends into PHA has also been reported (Hirt et al., 1996; Asrar et al., 1999; Madden et al., 1999; Shah et al., 2000a) and could facilitate subsequent modification, e.g. in the production of block copolymers. This has been accomplished through supplementation of the culture medium with various alcohols, diols, and polyols (Asrar et al., 1999; Madden et al., 1999; Shah et al., 2000a). A carboxyl group, belonging to the final 3HB monomer in a Poly(3HB) chain, is normally found at the chain terminus. However, this has been altered *in vivo* by the inclusion of hydroxy-containing compounds, which become incorporated at the polymer terminus via a chain transfer reaction in which the growing polymer chain is esterified to the hydroxy compound (Madden et al., 1999; Shah et al., 2000a). In addition to chain termination some glycols, e.g. ethylenglycol, are also found to be utilized as a carbon source for the production of Poly(3HB) (Shah, 2000b).

Escherichia coli does not normally synthesize PHAs since it lacks the PHA biosynthetic genes. However, it has been reported to accumulate Poly(3HB) levels approaching 80% of the dry cell weight when transformed with plasmids bearing the appropriate biosynthetic genes from *R. eutropha*. The major advantage of synthesizing Poly(3HB) in *E. coli* is that all the genetic engineering principles that apply to this organism can be utilized in optimizing the production of Poly(3HB) and other PHAs (Pouton and Akhtar, 1996). For example, high molecular weights, e.g. 4–5 MDa, can be achieved in this organism because *E. coli* does not contain PHA depolymerase enzymes (Sim et al., 1997). There are also potential advantages in terms of ease of extraction of the polymer and purity of the product (Pouton and Akhtar, 1996). Additionally, a Poly(3HB) synthesizing mutant of *E. coli* has been developed from which the polymer can be extracted by mild heat treatment rather than by chemical extraction techniques (Pool, 1989). This strain is thought to release the accumulated polymer through thermally induced cell lysis at a low temperature of 42 °C (Pouton and Akhtar, 1996).

4 Plant Production of Biopol®

The commercial production of Poly(3HB-*co*-3HV) has been done on a relatively small scale since it is not economically competitive with similar petrochemical-based polymers such as polyethylene and polypropylene. Therefore, efforts to produce PHAs with transgenic plants as the plastic factory has been pursued as an alternative (Poirier, 1999a; Poirier et al., 1995a). This route has the potential to be cost-competitive with traditional plastics because plant systems in theory only require CO_2, water, and sunlight for input. Of course, this is an oversimplification since other agriculture inputs are required to produce a crop (Gerngross and Slater, 2000). In addition, plant production of a copolymer is difficult to generate and control since the system relies on endogenous metabolic precursors in the plant. This is in contrast to the flexibility of supplementing the growth media with the required substrates in a microbial fermentation process. Nevertheless, efforts starting with the more simple Poly(3HB) homopolymer had shown promise that a plant production system could be viable. More specifically, Somerville and coworkers showed that Poly(3HB) levels of up to 14% of the plant dry weight could be produced in transgenic *Arabidopsis thaliana* leaves using

the *R. eutropha* enzymes (Poirier et al., 1992; Nawrath et al., 1994) with little effects on plant growth. This was true only if the Poly(3HB) biosynthetic enzymes were targeted to the plant chloroplast (Nawrath et al., 1994). Expression of these genes in the plant cytosol caused severe stunting and sterility (Poirier et al., 1992). The reasons for the differences between cytosolic versus plastid production are not entirely clear, but it may very well be due to the availability of acetyl-CoA where in plastids the production of this metabolite is robust (Nawrath et al., 1994).

The unavailability of direct metabolic precursors for the 3HV component of Poly(3HB-*co*-3HV) in a plant led us at Monsanto to devise a pathway that might be amenable to this biosynthesis (Slater et al., 1999). This is illustrated in Figure 2. Since the plastid was shown to be the preferred site for PHA biosynthesis, metabolic pathways present in this organelle were the focus. In the plastid, threonine is one product of the aspartate family of amino acids and is also the starting metabolite in the biosynthesis of isoleucine. The first committed step in isoleucine biosynthesis is the deamination of threonine to form α-ketobutyrate which is catalyzed by threonine deaminase. From there α-ketobutyrate is normally converted to 2-keto-2-hydroxybutyrate through a condensation reaction with pyruvate catalyzed by acetohydroxyacid synthase. This conversion step results in the maintenance of a very low steady-state concentration of α-ketobutyrate. It was hypothesized that significantly increasing the concentration of this 2-ketoacid in the plant plastid might allow a diversion of some of this carbon to form propionyl-CoA through the action of the plant plastid pyruvate dehydrogenase (PDH) complex. The normal function of this enzyme is in the oxidative decarboxylation of pyruvate to form acetyl-CoA, the direct precursor for fatty acid biosynthesis, and in the mitochondria the entry metabolite for the tricarboxylic acid cycle. α-Ketobutyrate is a one-carbon extended analogue of pyruvate, and we demonstrated that the plastid PDH complex can also catalyze the oxidative decarboxylation of

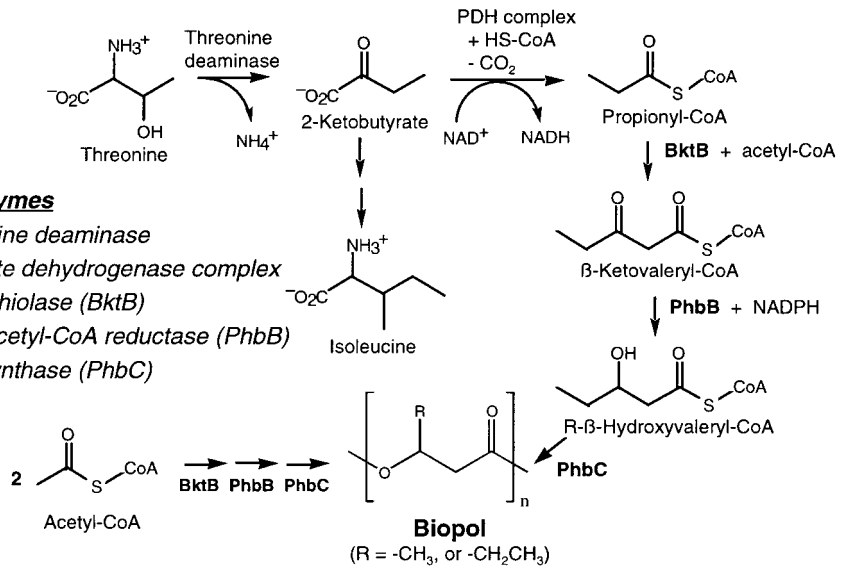


Fig. 2 Designed metabolic pathway for the production of Poly(3HB-*co*-3HV) in plant plastids.

this 2-ketoacid, although with less catalytic efficiency relative to pyruvate (Slater et al., 1999). The overexpression of threonine deaminase, either the wild-type or a deregulated form of the enzyme (biosynthetic threonine deaminase is normally feedback regulated by isoleucine in plant and microbial systems), was thought to be the means to significantly increase its concentration in the plant plastid. The PDH complex could then catalyze the formation of propionyl-CoA and following this step the normal PHA biosynthetic genes would carry reactions forward, in the same manner as in bacteria, to form the Biopol® copolymer as illustrated in Figure 2.

Transformation of plants, while now routine, nevertheless is time consuming and therefore not an efficient means to test a metabolic pathway in a recombinant system. Realizing this, we first used *E. coli* as the vehicle to see if the pathway shown in Figure 2 could be operative. The specific genes used in the initial transformation in *E. coli* were the *E. coli* threonine deaminase (*ilvA*), a wild-type or deregulated variant, and the three normal Poly(3HB) biosynthetic enzymes from *R. eutropha*, i.e. *phbA*, *phbB*, and *phbC*. The results from this experiment are given below in Table 1. As Table 1 shows, wild-type IlvA was expressed approximately 7-fold above the deregulated L481F mutant, but interestingly deregulation has a larger impact on the intracellular concentration of α-ketobutyrate. Unfortunately, no impact on the percent 3HV component in the polymer (each recombinant strain produced from 30 to 40% PHA based on cell dry weight) was evident, i.e. as constructed, the pathway was not operational. While it was apparent that a block in the pathway was not at the first step (i.e. threonine deaminase produces elevated levels of α-ketobutyrate), a series of enzyme substrate specificity assays were required to determine that the PhbA β-ketothiolase would not catalyze the formation of β-ketovaleryl-CoA. Fully realizing that *R. eutropha* produces the copolymer, our group identified and characterized a second β-ketothiolase designated BktB that is primarily responsible in this organism for the production β-ketovaleryl-CoA during the biosynthesis of Poly(3HB-*co*-3HV) (Slater et al., 1998). Reconstruction of recombinant *E. coli* strains substituting BktB for PhbA made a functional pathway for the production of Poly(3HB-*co*-3HV). Specifically, an 8% fraction of 3HV in the copolymer was produced by simply growing the organism on glucose (Valentin et al., 1999a). This is well within the range of 3HV for the typical composition of commercial Biopol®.

Success in demonstrating the pathway in *E. coli* led to the transformation of two plant systems: *Arabidopsis thaliana* for leaf expression and *Brassica napus* (rapeseed) for seed production (Slater et al., 1999). To direct biosynthesis in the plastids of these tissues the *agrobacterium* transformation cassettes

Tab. 1 Results from a test of the pathway shown in Figure 2 in transformed *E. coli* (the cells overexpressed four proteins, a wild-type or deregulated threonine deaminase, and the three *R. eutropha* Poly(3HB) biosynthetic enzymes PhbA (β-ketothiolase), PhbB (acetoacetyl-CoA reductase), and PhbC (PHB synthase))

***E. coli* construct**	***IlvA (μg/mg) no Ile***	***IlvA (μg/mg) + 0.10 mM Ille***	***Activity of no Ile (%)***	***α-Ketobutyrate (μM)***	***P(3HB-co-3HV) (% 3HV)***
phb ABC	< 0.04	< 0.04	–	13	1.3
phb ABC + wild-type IlvA	4.2	0.16	3.8	42	1.1
phb ABC + L481F IlvA	0.59	0.56	95	580	0.9

were constructed with a plastid transit peptide fused to an open reading frame for all genes, and all gene expression was driven by either the 35S promoter or *Lesquerella* hydroxylase promoter (Broun et al., 1998) for *Arabidopsis* or rapeseed, respectively. The results from this work showed copolymer production in both plants (Slater et al., 1999). Levels of Poly(3HB-*co*-3HV) were modest, however, relative to production previously reported for Poly(3HB) biosynthesis in *Arabidopsis* (Nawrath et al., 1994), and less than that seen for Poly(3HB) production in *Brassica* using a similar construct and transformation strategy (Houmiel et al., 1999). Specifically, copolymer levels were less than 3% of the plant tissue dry weight (*Arabidopsis*) or seed weight (rapeseed). It was also clear that at least for *Brassica* oilseeds, increasing polymer production was inversely proportional to the 3HV content in the polymer. The reasons for this appear to be metabolic channeling of increased α-ketobutyrate (produced by threonine deaminase), to both isoleucine and 2-aminobutyrate. The latter metabolite is the direct transamination product of the 2-ketoacid. The consequence is that more carbon is directed towards these non-target metabolites and, because of this, the steady-state levels of α-ketobutyrate, though measurably increased (approximately 20-fold), is not sufficient for robust conversion to propionyl-CoA by the plant plastid PDH complex. As such, additional engineering will be required to make the pathway operative as a commercial production system. Nevertheless, the results obtained from this work represent a complex feet of plant metabolic engineering with the introduction of four transgenes (not counting the selection marker) and the modification of two independent plant pathways (i.e. fatty acid and amino acid biosynthesis) to make a non-related product (Poirier, 1999b). Additional detail on this constructed pathway and the impact on plant metabolism is addressed in Volume 3a, Chapter 15 in this book series (Poirier and Gruys).

It is of interest to note that of the PHAs produced in transgenic plants from various research groups, that earlier reports noted a broad molecular weight distribution, ranging from 8 to 10, relative to bacterial PHA which typically produces narrower molecular weight distributions from 2 to 3 (Poirier et al., 1995b). However, Poly(3HB) and Poly(3HB-*co*-3HV) produced in *Arabidopsis* and canola at Monsanto resulted in a polymer that more typically resembles that seen in bacteria (Slater et al., 1999; Asrar et al., 2000a, 2001a). The reason for this difference in results from plant-produced PHA could well be due to the additional steps done at Monsanto to assure that the transgenic plants have a single loci (i.e. contain a single copy) for the individual PHA biosynthetic genes. This appears to be true for either a single loci that contains all the transgenes (a result from using a mutigene construct that has all the biosynthetic genes) or distinct loci for the individual transgenes (resulting from crossing homozygous plant lines that individually contain a portion of the pathway or cotransformation strategies using more than one plant vector).

If the technical hurdles towards the production of Biopol® in a plant system are overcome, a major challenge remains in the development of a process for the recovery of the copolymer from plant tissue. This is likely to be a major factor impacting the overall costs in a plant production system. As shown in the electron micrograph in Figure 3, the production of PHA, in this case Poly(3HB) in a *Brassica* seed plastid, will require separation from lipid, protein, and other cellular components. The characteristics of Poly(3HB-*co*-3HV), e.g. the crystalline nature and thermal properties (see

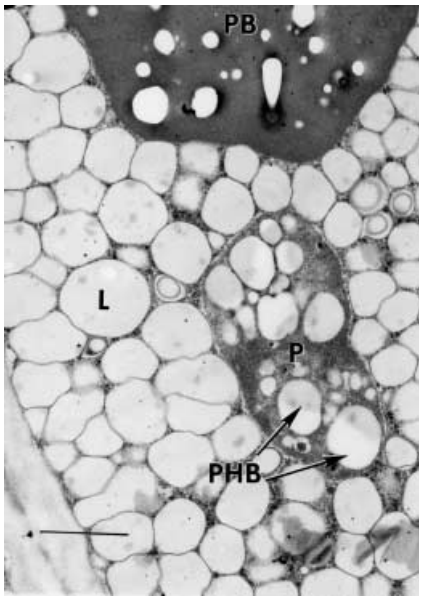


Fig. 3 Electron micrograph of a plastid from a *B. napus* seed producing Poly(3HB). Plastid (P), polymer (PHB), lipid bodies (L), and protein bodies (PB) are indicated. The bar indicates a 1 μm.

below), puts boundaries on the methods that can be utilized. In addition, the choice of crop and tissue type (i.e. seed or leaf) could have a large impact. With this in mind some possibilities include supercritical fluid extraction, selective non-halogenated solvent extraction of PHA from plant biomass, or routes that take advantage of density differences between PHA and biomass. It is worth noting that any type of solvent used in the process should already have regulatory approval as a synthetic food additive or flavoring agent. This is because a crop production system would likely be viable only if other byproducts such as oil, proteins, and carbohydrates can be recovered in addition to polymer. A current common laboratory method for PHA extraction which involves the use of chloroform is obviously not an option.

Recent studies by Mott et al. (Mott, 2000) took a preliminary look at processing of Poly(3HB) from wild-type (as an additive) and transgenic Poly(3HB) rapeseed, matching to some degree the conditions used in commercial oil extraction using hexane in conjunction with mechanical crushing. The desirable outcome would be that slight modifications to existing commercial extraction methods could separate PHA from oil and that residual meal would still be suitable for animal feed. This was done on an ultra scale-down level and, while qualitative in nature, nevertheless showed that transgenic seed required a higher mechanical force than non-transgenic seed to yield the same level of oil. The same group also studied separation of Poly(3HB) from protein meal (Hughes et al., 2000). For general methods of PHA extraction from various plant materials, a recent patent application described the use of PHA-poor solvents in separation processes (Kurdikar et al., 2000a). These solvents include linear and branched alcohols and esters that dissolve less than 1% (w/v) of the PHA at a temperature below the solvent boiling point. However, under conditions of elevated temperature and pressure, significant solvation of PHAs occurs in these solvents. Even when processed and mixed with excess biomass material, a clean PHA precipitate could be obtained by the use of agitation while the solution mixture was cooled. This precipitation occurred 20–30 °C higher than the temperature at which other biomass components precipitated out of solution. Other similar methods include solvation in a PHA-good solvent followed by recovery through solvent evaporation, or addition of a PHA-poor solvent to cause selective precipitation of PHA (Kurdikar et al., 2000b). From this it can be seen that while promising methods for the recovery of PHAs from plant biomass are described, it is apparent that optimization will be required when higher levels of transgenic PHA-containing plant material become available for further experimentation.

5
Properties of Biopol®

5.1
Morphology of Biopol® and other PHAs

As mentioned earlier, Poly(3HB) and other PHAs accumulate in discrete spherical granules in the cell cytoplasm. Granules have a diameter that ranges from 100 to 800 nm (Dawes, 1973; Ellar, 1973) and are enclosed in a unit membrane about 2–4 nm thick (Ellar, 1973). The granules are typically composed of about 98% polymer and the rest is protein and phospholipid. ^{13}C-NMR studies (Barnard and Sanders, 1989; Bonthrone et al., 1992) on live cells suggest that Poly(3HB) is predominantly in a mobile state, but not in solution, in the granules. Water was shown to be an integral part of the granule and appears to act as a plasticizer for the polymer, although the exact conformation within which water is included to plasticize the polymer is not entirely clear. It is now proposed that the enzymes involved in the PHA biosynthetic pathway operate only on a mobile hydrated material and that the solid granules characteristic of dried cells are an artifact of the drying process. The crystallization of the polymer is thought to be under kinetic control and is inhibited by the submicron size of the particles, and their protein and phospholipid coat (Bonthrone et al., 1992). A method for extraction of amorphous granules has been reported (Fuller et al., 1992). It has also been possible to produce kinetically stable amorphous granules *in vitro* from crystalline Poly(3HB) and other PHAs, using surfactant to stabilize the submicron size granules (Fuller et al., 1992). These studies may well have some future impact on the processing of Poly(3HB) and other PHAs into articles of commercial use (Pouton and Akhtar, 1996).

5.2
Composition and Molecular Structure of PHAs

Poly(3HB) is isotactic and similar to isotactic polypropylene as both have pendant methyl groups attached to the main chain in a single conformation (Brandl et al., 1988). Poly(3HB) is a compact right-handed helix with a 2-fold screw axis and a fiber repeat of 5.95 Å. The helix conformation is stabilized by carbonyl–methyl group interactions and represents one of the few exceptions of a helix found in nature which does not depend on hydrogen bonding for its formation and stability. Poly(3HB) can have a weight average molecular weight of 0.1–3 MDa, although for processing the molecular weights are usually in the range of 200 to 800 kDa. The polydispersity is in the range of 2.2–3 (Brandl et al., 1988). Since Poly(3HB) is made biologically as a stored energy source in cell walls, it can be separated from cell material in very high purity. The major impurities in Poly(3HB) are inorganic nitrogen, phosphorous, and sulfur-containing compounds which are present at concentrations less than 200 p.p.m. (Barham, 1984). There are no catalyst residues to be concerned about in the polymer.

Poly(3HB-*co*-3HV) copolymers have been shown to exhibit isodimorphism (Bluhm et al., 1986; Scandola et al., 1992) with the 3HB and 3HV units cocrystallizing. As the 3HV content of Poly(3HB-*co*-HV) is increased, the melting point decreases [from around 178 °C for Poly(3HB)] and passes through a minimum of around 75 °C at around 40 mol% 3HV content and increases to around 110 °C for Poly(3HB-*co*-3HV) containing 97 mol% 3HV. A change from the Poly(3HB) lattice to the Poly(3HV) lattice takes place at around 40% 3HB. At less than 40 mol% 3HV, 3HV units can crystallize in the Poly(3HB) lattice and at greater than 40 mol% 3HV, 3HB units can

crystallize in the Poly(3HV) lattice. Thus, isodimorphism makes it possible to achieve relatively high levels of crystallinity in these copolymers, retaining the useful hydrolysis and chemical resistance exhibited by Poly(3HB). Also the same nucleants can be used for Poly(3HB) and lower 3HV content Poly(3HB-*co*-3HV). Poly(3HB-*co*-3HV) satisfies the physical requirements for isodimorphism in that the two monomer units have approximately the same shape and occupy similar volumes, and the polymer chain conformations of both homopolymers are compatible with either crystalline lattice. Like Poly(3HB), the Poly(3HV) polymer chain has a 21 helix conformation, an orthorhombic unit cell and space group P_{212121} with a unit cell parameters $a = 9.32$ Å, $b = 10.02$ Å, and $c = 5.56$ Å (Yokouchi et al., 1974). NMR evidence indicates that at 3HV levels of 20–40 mol%, 3HV units are partially excluded from the Poly(3HB) lattice (Tokiwa et al., 1992). A recent study suggests that cocrystallization of the two monomer units certainly does occur but that the molar ratio of 3HV within crystals is approximately two-thirds of the total molar ratio present in the copolymer (Pouton and Akhtar, 1996).

5.3 Crystallization Behavior of Biopol®

The embrittlement of Poly(3HB) and its 3HV copolymers on storage at room temperature is not associated with relaxation of the amorphous regions but rather to the development of interlamellar secondary crystallization. The small crystallites produced underpin the amorphous regions and reduce the mobility of the chain segments thus raising the modulus and embrittling the material. Crystallizing the copolymer at high temperature leads to rejection of the 3HV units into the amorphous regions and reduces the extent of secondary crystallization which can develop at room temperature, such that these materials do not embrittle on storage. Dynamic mechanical and electrical thermal analysis were found to be useful techniques for measuring the mobility of the amorphous regions in partially crystalline samples, under conditions where differential scanning calorimetry (DSC) was unable to detect any glass transition (Biddlestone et al., 1996).

A patent (De Koning, 1994a) describes Poly(3HB) and high 3HB-containing PHAs in which aging had occurred and the original properties were restored by a heat treatment and subsequent aging is retarded. A patent has been filed on cooling the Polyester after preparation to less than 90 °C and then heating from 90 to 160 °C within 24 h of preparation to retard aging (Liggat and O'Brien, 1994a). Other patents were filed on PHA-plasticizer compositions (Liggat and O'Brien, 1994b) and Poly(3HB-*co*-3HV) copolymers (Liggat and O'Brien, 1994c) claiming to retard the aging process.

Tab. 2 Effect of 3HV content on the maximun crystalization rate

Polymer	*Maximum crystallization rate (mm s^{-1})*	T_c (°C)	T_m (°C)
P(3HB)	4.5	88	197
P(3HB-*co*-6%3HV)	1.4	80	186
P(3HB-*co*-12%3HV)	0.43	78	173
P(3HB-*co*-16%3HV)	0.23	70	167

5.4 Thermal Properties of Biopol®

Thermal degradation of Poly(3HB) occurs quite rapidly at temperatures above the melting point (T_m) of the polymer (171 °C). The significant benefit of the Poly(3HB-*co*-3HV) copolymers is their lower melting points that enable them to be melt processed at lower temperatures than Poly(3HB), generally in the range of around 140–180 °C, significantly reducing thermal degradation (Cox, 1994).

Poly(3HB-*co*-*x*%3HV) copolymers are also thermoplastics with melting points, T_ms, in the range of 75–170 °C (Poirer, 1995) depending on the value of *x* (the mol% 3HV). Increasing the amount of 3HV in the copolymer reduces the Tm from around 180 °C for Poly(3HB) to around 75 °C for a copolymer containing 30–40% 3HV. The heat distortion temperature of the copolymers decreases from 140 to 92 °C as the 3HV content increases. The temperature for the onset of decomposition, T_d, with fast heat is in the 232–244 °C range (Marchessault et al., 1988), but is lower with longer exposure. The β-substituted aliphatic polyesters are unstable at greater than 170 °C.

The thermal degradation mechanism of Poly(3HB) has been studied and is considered to be primarily a random chain scission process by a β-elimination reaction via a six-membered cyclic transition state (Grassie et al., 1984a–c; Kunioka and Doi, 1990). Activation energies between 109 ± 13 kJ mol^{-1} for Poly(3HB) and 126 ± 13 kJ mol^{-1} for Poly(3HB-*co*-3HV) copolymers have been reported (Mitomo and Ota, 1991). The major pyrolysis product of Poly(3HB) is crotonic acid produced by the β-elimination reaction (Billingham et al., 1978; Grassie et al., 1984a–c). Significant quantities of Poly(3HB) oligomers and small quantities of isocrotonic acid were also evolved on heating the polymer to around 300 °C. Secondary products such as propylene, ketene, CO_2, acetaldehyde, etc., were also formed by further decomposition of the primary products. Conventional thermal stabilizers have been shown to have little effect in preventing thermal degradation of Poly(3HB) (Billingham et al., 1978).

The thermal degradation mechanism of Poly(3HB-*co*-3HV) copolymers has been reported to be analogous to that for Poly(3HB), with a similar activation energy and degradation kinetics (Kunioka and Doi, 1990).

During thermal processing of Poly(3HB), both reactions, chain-end condensation leading to an increase in the molecular weight, as well as thermal degradation leading to a decrease in molecular weight, take place. Since each thermal degradation event produces a carboxyl and a crotonate chain end, the carboxyl concentration increases considerably with time and chain-end condensations are minimized leading to a drastic reduction in the molecular weight. It is found that an increase in the hydroxy-termination in Poly(3HB) leads to an increase in the thermal stability, most likely by prolonging the condensation and delaying the degradation reaction (Shah et al., 2000a).

5.5 Melt Rheology of Biopol® and its Impact on Processing

One of the key melt flow properties of a molten polymer is the extensional viscosity of the melt. 'Extensional thickening' and increase in the extensional viscosity above the linear viscoelastic limit is important to stabilize the polymer processing operations involving stretching of melts such as film blowing, fiber spinning, melt coating, etc. A polymer having low melt strength is unable to withstand the minimum strain required

to draw the polymer to the desired dimension and will exhibit instabilities such as breakage, sagging, or draw resonance.

Rheological characterization of Poly(3HB-*co*-3HV) indicates the viscosity decreases dramatically with increasing shear rate (shear thinning). An increase in the molecular weight results in an increase in shear viscosity and extensional viscosity. In the deformation range studied, the effect of molecular weight on the extensional viscosity is larger than on shear viscosity. Thus the increased molecular weight results in increased melt strength and enhanced stability of a bubble during blown film processing (Asrar and Pierre, 2000a).

5.6 Effect of Branching in Biopol®

The melt strength can be improved by branching. A PHA melted in the presence of a free radical initiator at a temperature above the decomposition temperature of the free radical initiator for a sufficient length of time undergoes crosslinking. The thermally induced decomposition of the free radical initiator results in the production of reactive radicals which produce interchain crosslink formation between PHA chains. The branched PHA compositions can be made by reactive extrusion, where the extrusion temperatures and residence times are sufficient for melting the PHA and for causing the decomposition of the peroxide. When producing branched PHAs by this method, there are competing reactions occurring in the melt. Thermal decomposition of the PHA results in a decrease in its molecular weight while the branching reaction produces an increase in its molecular weight. The choice of extruder temperature, free radical initiator, and initiator concentration can be optimized to give control of resulting molecular weight and degree of branching. The most effective concentrations of peroxide appear to be in the range of 0.05 – 0.1 up to 0.3 wt% (Asrar and D'Haene, 2000, 2001). At less than 0.05% there is no appreciable effect and at concentrations greater than 0.5 wt% the extruded materials become brittle. Branching also slows down the age-related embrittlement of articles produced from PHAs.

Figure 4 shows both the shear and the elongational viscosity of a linear and a branched Poly(3HB-*co*-3HV) prepared using 0.2 wt% DCP dicumyl peroxide as measured in a capillary rheometer. The data illustrate that the shear viscosities of both linear and branched polymers are almost equal, as these are mainly determined by the molecular weight of the polymer. Elongational viscosity is much larger for the branched material than for the linear polymer due to the existence of strain hardening in the elongational response of the branched product (D'Haene et al., 1999).

A novel approach for producing a branched bacterial polyester has recently been described. Poly(3HB) containing polyhydroxy chain ends, produced by batch fermentation in presence of pentaerythritol ethoxylate (PEE), using *R. eutropha*, are used as precursors for producing branched Poly(3HB) during thermal processing. Chain-end condensation and transesterification reactions during thermal processing of PEE terminated Poly(3HB) results in hyper-branched Poly(3HB). Hydrodynamic volume of Poly(3HB) produced in presence or absence of the PEE are found to be the same. However, the hydrodynamic volume of PEE-terminated Poly(3HB) increases considerably after its thermal processing, indicating the formation of star-branched Poly(3HB) (Asrar et al., 2000c, 2001b).

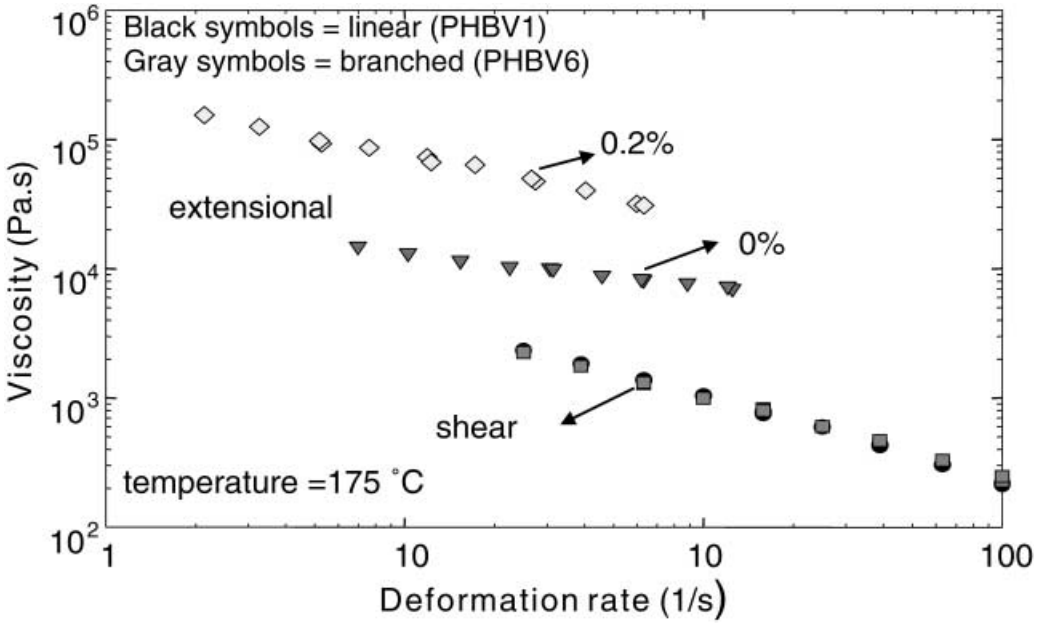


Fig. 4 Effect of branching on the shear viscosity (circles) and the elongational viscosity (triangles), as measured in a capillary rheometer. The elongational viscosity was determined from the entrance pressure drop using Cogswell's analysis (capillary data).

6 Processing Characteristics of PHAs

PHAs, as isolated from natural organisms, are difficult to process because of their relatively low decomposition temperatures (140–170 °C) near their melting points, low melt strength, their slow crystallization rates, etc. Because of thermal degradation in the melt, the melt flow index can change rapidly with time and volatile decomposition products must be handled safely. The slow crystallization rates lead to tacky products (e.g. fibers, films, molded articles) which adhere to themselves and the process equipment. Products produced by thermal processing undergo embrittlement with time (aging). Various approaches such as use of nucleating agents, plasticizers, branching, blends, etc., have been taken to overcome the processing and product difficulties and shortcomings.

6.1 Effect of Nucleating Agents

PHAs from biological sources can be of very high purity. As a result, Poly(3HB) melts may undergo homogeneous nucleation, but the nucleation density for Poly(3HB) is orders of magnitude less than for polyethylene and polypropylene. However, the growth rate, once nucleated, is similar to polypropylene, nylon 6 and PET. The low rate of nucleation in Poly(3HB) leads to development of large spherulites within the Poly(3HB). The large spherulites can significantly reduce the physical and mechanical properties of the polymers.

The overall rate of crystallization can be increased by the use of nucleating agents which promote crystallization of the molten or glassy resin by increasing the number of nuclei within the Poly(3HB). The increased

number of nuclei leads to smaller diameter spherulites, a more rapid loss of tackiness, and concurrent increase in mechanical strength and reduced cycle times in thermal processes. Potential nucleating agents can be tested using DSC. As the molten polymer is cooled at a constant rate, an exotherm is produced as the polymer crystallizes. The temperature range over which crystallization occurs, the area of the peak, and the peak sharpness give an indication of the crystallization behavior of the material. Addition of a nucleating agent generally causes an increase in the crystallization peak and the peak area. Effective nucleating agents include saccharin and particulates such as talc, micronized mica, boron nitride (BN), chalk, calcium hydroxyapatite, and calcium carbonate (Webb, 1998). BN is commonly used as the nucleant for Poly(3HB) and other PHAs.

Saccharin ($T_m = 226$ °C) can nucleate Poly(3HB) when added to the melt due to epitaxial growth on its surface (Barham, 1984). The nucleating effect of saccharin is due to adsorption of molecules onto the surface in what is close to their crystallographic form.

Technical grade NH_4Cl incorporated as an aqueous solution is found to be a more effective nucleating agent.

Poly(3HB) seed crystals were used to nucleate crystallization of Poly(3HB-*co*-12%3HV) ($T_m = 149.9$ °C). At 5% of Poly(3HB) seed crystals, the crystallization temperature (T_c) was 88.7 °C and heat of crystallization (H_c) was 45.7 J g^{-1}. At 1 p.h.r. of Poly(3HB) seed crystals, the T_c was 70.3 °C and H_c was 41.9 J g^{-1}. Without the Poly(3HB) seed crystals, no T_c was observed (Liggat, 1994d).

Cyclohexyl phosphonic acid (CPA) and zinc stearate (ZS) mixtures are more effective nucleating agents than BN for Poly(3HB-*co*-3HV) copolymers with high 3HV content. For example, when 0.10 p.h.r. CPA and 0.385 p.h.r. ZS were added to a Poly(3HB-*co*-22%3HV) polymer and compared with Poly(3HB-*co*-22%3HV) containing 1 p.h.r. BN at a T_c of 80 °C, the half-crystallization time and the half-heat of crystallization peak were similar for CPA/ZS and BN; 1.26 and 1.39 min and 19.85 and 19.26 J g^{-1}, respectively. At a T_c of 50 °C, the half-crystallization time for CPA/ZS was 0.40 versus 3.91 min for BN and the half-heat peak was 47.09 versus 11.73 J g^{-1}. Thus at lower T_cs, CPA/ZS yields significantly faster crystallization rates with improved energies of crystallization. With high 3HV containing polymers it should be possible to use lower mold temperatures and shorter cycle times with CPA/ZS. However, for low 3HV containing polymers, both types of nucleant yield similar crystallization rates (Herring and Webb, 1990).

More recently, organophosphorous compounds having at least two phosphonic acid groups such as 1-hydroxyethylidene diphosphonic acid (HEDP) have been shown to serve as nucleating agents and give products with excellent clarity. For example, a Poly(3HB-*co*-3%3HV) copolymer containing 0.2 p.h.r. HEDP showed a T_c of 78 °C and an H_c of 55 J g^{-1}. A combination of HEDP with Ca or Mg stearate has also been shown to be an effective nucleating agent. A Poly(3HB-*co*-8%3HV) copolymer containing 0.1 p.h.r. HEDP and 0.3 p.h.r. calcium or manganese stearate had a T_c of 72 °C and an H_c of 51 and 50 J g^{-1}, respectively, compared to a T_c of 56 °C and an H_c of 15 J g^{-1} for Poly(3HB-*co*-8%3HV) alone (Asrar and Pierre, 1999).

6.2 Effect of Plasticizers

The use of monomeric and polymeric plasticizers in PHAs to impart properties and impede loss of properties by secondary