# Degradation of Natural and Artificial Poly[(*R*)-3-hydroxyalkanoate]s: From Biodegradation to Hydrolysis

**Philippe Guérin, Estelle Renard, and Valérie Langlois**

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**Abstract** Biodegradability of polymers has drawn much attention as a solution to problems concerning the global environment and biomedical technologies. Poly[(*R*)-3-hydroxybutyrate] and its copolymers are representative biodegradable polyesters which can be degraded in natural environments such as soil, sludge, freshwater, and seawater where many microorganisms utilize the degraded products as a carbon source. The ability to degrade poly[(*R*)-3-hydroxyalkanoate]s (PHAs) is widely distributed among fungi and bacteria and depends on the extracellular PHA depolymerases, which are carboxyesterases, and on the physical state of the polymer (amorphous or crystalline). Intracellular depolymerase systems lead to $CO_2$ and $H_2O$ when bacteria need energy or carbon sources. All polyesters are susceptible degradation by simple hydrolysis to some extent. The degradation rate is very dependent on the chemical structure and the crystallinity of the materials.

P. Guérin (✉), E. Renard, and V. Langlois
Institut de Chimie et des Matériaux Paris-Est, Université Paris-Est, 2–8, rue Henri Dunant, 94320, Thiais, France
e-mail: guerinp@icmpe.cnrs.fr

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One way to obtain more hydrophilic PHAs consists in the introduction of specific functions into the macromolecular side chains. The combination of bioconversion and organic chemistry allows modulation of the physical properties of these bacterial polyesters, such as solubility, hydrophilic–hydrophobic balance, and water stability, from the perspective of biomedical applications.

## 1 Introduction

A variety of polymers has been developed to obtain useful polymeric materials which can degrade in biological environments when living cells or microorganisms are present around the polymers and without harm to these environments. Biodegradation, in this case, is the process by which polymeric materials are broken down by the enzymes produced by living organisms. The term is often used in relation to ecology, waste management, and environmental remediation. Material can be degraded aerobically, with oxygen, or anaerobically, without oxygen. Novel biomedical technologies that have emerged, including tissue engineering, regenerative medicine, cellular therapy, and controlled drug delivery, require biodegradable biomaterials. A biomaterial can be defined as a material to interface with biological systems. The essential prerequisite for a biomaterial is biocompatibility, which is the ability of a material to perform with an appropriate tissue response in a specific application. In the case of biodegradable biomaterials, biocompatibility must be active over time. Some of the properties of biodegradable biomaterials can be summarized as follows: no sustained inflammatory or toxic response after implantation; chemical, physical, mechanical, and biological properties of the biomaterial must be related to the healing process; for biocompatibility, the intermediate and ultimate products of the degradation must be nontoxic and metabolized or/and cleared from the body. Biodegradation of polymeric biomaterials, in biomedical applications, involves cleavage of hydrolytically or enzymatically sensitive bonds. Depending on the mode of degradation, one can classify these materials as hydrolytically degradable polymers and enzymatically degradable polymers. Among the biodegradable plastics, four major aliphatic polyester families have been developed: poly(L-lactide) (PLLA) and its copolymers, poly(butylene succinate) (PBS), poly(ε-caprolactone) (PCL), and poly[(*R*)-3-hydroxyalkanoate]s (PHAs). They can be synthesized from petrochemicals (PBS, PCL) or from renewable resources (PLLA, PHAs).

## 2 Biodegradation of Bacterial Polyesters

PHAs have attracted much attention as environmentally compatible materials owing to their production from renewable carbon resources and their unique property of biodegradability. All PHAs contain monomer units with only the *R* configuration, owing to the stereospecificity of the synthesis enzymes. PHAs are stored by bacteria for eventual breakdown and utilization as a carbon source when extracellular carbon

is no longer available. There is an effective and rapid mechanism within the cell for the biodegradation of this high molecular weight polyester into simple organic compounds (Lenz and Marchessault 2005). PHAs can be rapidly hydrolyzed to the monomers by intracellular degradation enzymes or by extracellular depolymerase enzymes secreted by a wide variety of bacteria and fungi that can utilize these compounds after they have been liberated by the death and lyses of bacteria in which they are stored. Many different types of intracellular and extracellular polyester depolymerases have now been isolated and characterized. Considerable knowledge of the biochemical properties of the extracellular depolymerases has been accumulated in recent years (Jendrossek and Handrick 1996; Jendrossek and Handrick 2002).

The differentiation between extra- and intracellular degradation is necessary (Fig. 1) because PHAs in vivo and outside the bacteria are present in two different biophysical states. In intracellular native PHAs the polymer is in the amorphous state (highly mobile chains in a disordered conformation) covered by a protein and phospholipid surface layer. After extraction from the cells, the polyester chains tend to adopt ordered helical conformations and to develop a crystalline phase (denatured PHA). PHAs are high molecular weight water-insoluble polymeric materials and they cannot be transported through the cell wall. Therefore, bacteria and fungi excrete extracellular PHA depolymerases (Jendrossek 1998), which hydrolyze the material into the monomer and soluble oligomers. Low molecular weight degradation products are then transported in the degrading microorganisms and subsequently metabolized as carbon and energy sources; $H_2O$ and $CO_2$ can be the ultimate molecules of the biodegradation.

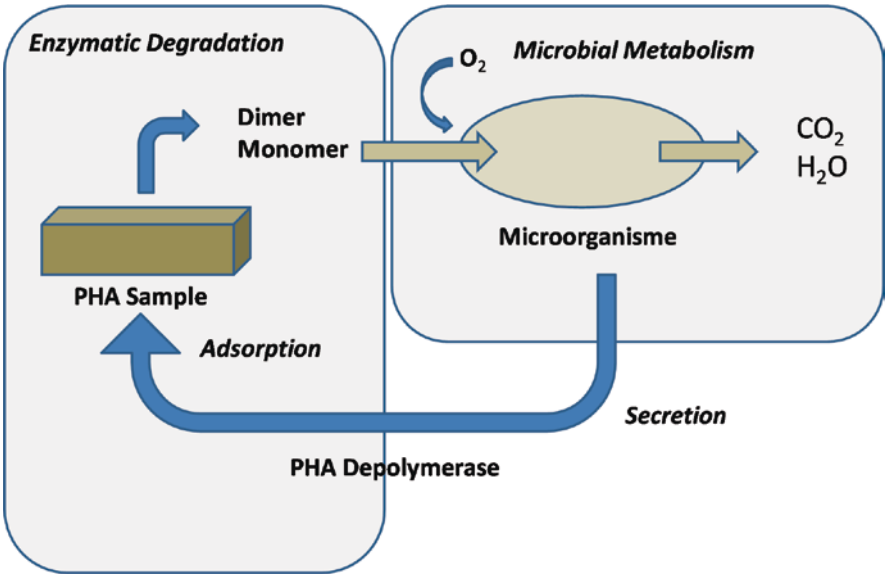


**Fig. 1** Extracellular degradation

## 2.1 Extracellular Degradation

The ability to degrade short-chain-length (scl) PHAs is widely distributed among bacteria and filamentous fungi and a large number of depolymerases have been purified and characterized (Jendrossek 2007). However, few reports concerning the degradation of medium-chain-length (mcl) PHAs have been documented (Kim et al. 2000a, b, 2002, 2003). The PHAs are water-insoluble but PHAs depolymerases are water-soluble. Consequently, the enzymatic degradation is a heterogeneous reaction.

### 2.1.1 Short-Chain-Length PHAs

Aerobic and anaerobic PHA-degrading microorganisms have been isolated from various ecosystems. Analysis of the primary structure of extracellular depolymerases revealed that the enzymes are composed of three functional domains: a catalytic (320–350 amino acid) domain at the N terminus, a substrate-binding domain (40–60 amino acids) at the C terminus, and a linker region (50–100 amino acids) connecting the two domains. The substrate-binding domain is responsible for the adsorption of the enzyme to the surface of water-insoluble polymer, which permits the catalytic domain to interact with polymer chains (Fukui et al. 1998). The catalytic domain contains a lipase-like catalytic triad (serine, aspartate, and histidine residues) (Brucato and Wong 1991), known as the lipase box (Jendrossek and Handrick 2002). Two types of catalytic domains have been identified, on the basis of the position of the lipase box. In type I, the lipase box is located in the middle of the primary structure. The sequential order of these active amino acids is histidine (oxyanion hole)–serine–aspartate–histidine from the N terminus to the C terminus. In type II, the lipase box is located at the N terminus of the structure. The order is serine–aspartate–histidine–histidine (oxyanion hole). It has recently been proposed that the substrate-binding domain has an additional function of disrupting the structure of the polymer (Murase et al. 2002a, b). The linker domain may function as a spacer that introduces a flexible region between the catalytic and substrate-binding domains to increase the hydrolytic efficiency of the catalytic domain (Nojiri and Saito 1997).

A recent study of a homologous protein to the linker domain, the fibronectin type III homology domain of cellobiohydrolase ChbA of *Clostridium thermocellum*, indicates that the linker domain may also exhibit a disruptive function against crystalline substrates, analogous to the substrate-binding domain (Kataeva et al. 2002) The substrate-binding and linker domains are divided into two and three types, respectively, on the basis of sequence similarity. Since these three functional domains are essential for the enzymatic degradation of water-insoluble polymers, it has been proposed that the degradation of the crystalline region of the polymer should proceed in three steps: adsorption of the enzyme to the polymer, nonhydrolytic disruption of the structure of the polymer, and hydrolysis (Murase et al. 2001a, b, c).

The mature form of the fungal poly[(*R*)-3-hydroxybutyrate] (PHB) depolymerase from *Penicillium funiculosum* (Fig. 2) (Hisano et al. 2006; Kasuya et al. 2007) is a

glycoprotein containing 319 amino acid residues (the optimum pH is 6.0 and the optimum pI is 6.5). *P. funiculosum* enzyme lacks a domain corresponding to a bacterial substrate-binding domain and displays only nonspecific and very weak binding to the PHB surface. This PHB depolymerase degrades (*R*)-3-hydroxybutyrate (3HB) dimer and 3HB trimer in addition PHB. The catalytic residues Ser39, Asp121, and His155 are located in a crevice formed on the surface. The inside of the crevice serves as a substrate-binding site, which is of sufficient length and width to permit the incorporation of a single chain of the polymer. A number of hydrophobic residues are located inside the crevice, providing a hydrophobic environment favorable for the binding of polymer chains. The structure of the 3HB trimer–enzyme complex has three subsites for binding each monomer unit of the polymer substrate. Two methyl groups (side chains) of units 1 and 2 of the trimer interact with hydrophobic pockets of the substrate-binding sites. The size and shape of the two hydrophobic pockets are such that they may be unable to accommodate side chains larger than an ethyl group, consistent with the observed substrate specificity of this enzyme toward poly[(*R*)-3-hydroxyvalerate] (PHV). Fungal enzyme eroded 100% PHV (Miyazaki et al. 2000). The spatial arrangement of the catalytic residues indicates that the mechanism of the depolymerase reaction may be similar to that of a lipase/serine esterase (Yamashita et al. 2006). Ser39 plays a central role in the catalytic reaction, participating in a nucleophilic attack on the carbonyl carbon atom of a bound PHB chain (Fig. 2). The His155–Asp121 hydrogen-bonding system may enhance the nucleophilicity of the hydroxyl group of Ser39.

The PHB-adsorption site contributes to the efficient binding affinity of the enzyme for PHB granules, which aids in the degradation of insoluble PHB granules without a substrate-binding domain. These hydrophobic interactions destabilize the hydrophobic intrachain and interchain interactions of the PHB chains. The adsorption site of the single-domain enzyme should introduce mechanical damage to ordered regions of PHB chains via hydrophobic interactions, thus increasing the mobility of PHB chains and creating a susceptible site for hydrolysis by the catalytic site of the enzyme, resulting in the efficient degradation of PHB.

The bacterial PHB depolymerase from *Ralstonia pickettii* T1 (PhaZafa) consists of three characteristic domains, i.e. catalytic, substrate-binding, and linker domains. The molecular weight of the PHB depolymerase is 47,000, and the optimum pH for enzyme reaction is 7.5. An active serine is part of the highly conserved pentapeptide [Gly-Leu-Ser$^{139}$-Ser-Gly], the lipase box (Tanio et al. 1982; Jendrossek et al. 1996). Depolymerase hydrolyzes bacterial PHB in monomers and dimers. A degradation mechanism of the lamellar crystal by the PHB depolymerase was proposed. The adsorption of the PHB depolymerase from *R. pickettii* T1 was found to change the molecular state of PHB and to generate concaves at the surface of the PHB crystal at the initial stage of enzymatic reaction (Numata et al. 2006, 2007). Atomic force microscopy observations revealed that the PHB depolymerase from *R. pickettii* T1 adsorbs homogeneously onto the surface of PHB single crystals. The PHB depolymerase from *R. pickettii* T1 is anchored to the substrate surface with the substrate-binding domain by strong affinity with PHB molecules (Numata et al. 2005). The absence of a substrate-binding domain leads to the decrease in binding affinity of the enzyme

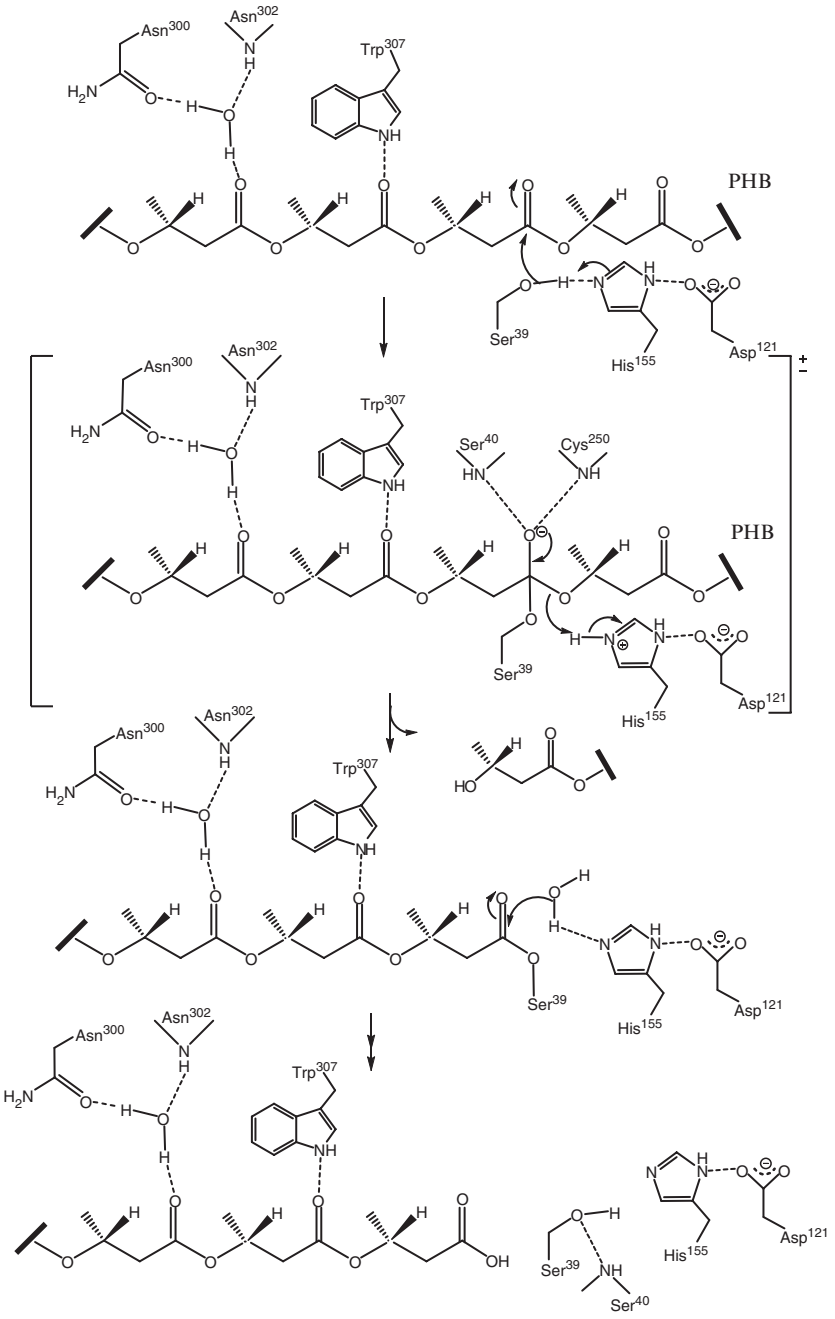


**Fig. 2** A proposed mechanism for the action of the poly[(*R*)-3-hydroxybutyrate] (PHB) depolymerase from *Penicillium funiculosum* (Hisano et al. 2006)

from *P. funiculosum* compared with that of the enzyme from *R. pickettii* T1. Feng et al. (2004) have studied the degradation rate of poly(3HB-*co*-3HV) (PHBV) with different (*R*)-3-hydroxyvalerate (3HV)-unit contents. The rate increased with the 3HV-unit content and reached the maximum value at about 40 mol% 3HV-unit content. The difference in the crystal structure influenced the enzymatic degradation. Absorption research on *R. pickettii T1* depolymerase mutant on PHA revealed that the absorption depended not only on hydrogen bonds between hydroxyl groups of serine in the enzyme and carbonyl groups in the PHB polymer, but also on hydrophobic interaction between hydrophobic residues in the enzyme and methyl groups in the PHB polymer (Hiraishi et al. 2006; Abe et al. 2005). The number of PHB depolymerase enzyme molecules adsorbed on each single crystal increased in the following order: poly(3HB-*co*-8 mol% 3HH) < PHB ≈ poly(3HB-*co*-6 mol% 3HV), where 3HH is (*R*)-3-hydroxyhexanoate. The 3HH units excluded from the crystalline region accumulate and concentrate on the surface of single crystals, resulting in the surface of poly(3HB-*co*-8 mol% 3HH) single crystals being extremely rough. Such surface roughness of the single crystal may hinder the formation of multiple adhesive interactions between the substrate-binding domain and polymer molecules (Numata et al. 2007). Li et al. (2007) have studied enzymatic degradation of microbial copolyesters of 3HB and mcl-PHA to better understand degradation mechanisms. A series of PHAs was produced: PHB, poly(HB-19 mol% HV), poly(HB-19 mol% HH), poly(HB-15 mol% mcl-HA), where HB is hydroxybutyrate, HV is hydroxyvalerate, HH is hydroxyhexanoate, and HA is hydroxyalkanoate. Crystallinities decreased in the order PHB > poly(HB-19 mol% HV) > poly(HB-19 mol% HH), > poly(HB-15 mol% mcl-HA), and the rate of degradation by *R. pickettii T1* depolymerase was in the order poly(HB-19 mol% HV) > PHB > poly(HB-19 mol% HH) > poly(HB-15 mol% mcl-HA). The PHA depolymerase attached first to the polymer, and started degradation from the amorphous phase, which is more easily accessible compared with the polymer crystalline regions during PHB enzymatic erosion. mcl-HAs in the copolyesters, which were more hydrophobic, could prevent the access of depolymerase to the short-length PHA regions which were crystalline, thus slowing down the degradation. If the side chain of the PHA monomer became sufficiently long, the long hydrophobic side chain could cover preferably the hydrophobic crevice, which captures the PHA chain in the enzyme, and thus prevent the approach of the main chain to the crevice, inhibiting the degradation.

*Pseudomonas lemoignei* is unique among PHA-degrading bacteria (Jendrossek et al. 1995; Nobes et al. 1998; Schöber et al. 2000) because of its high number of extracellular PHA depolymerases (at least six) and its ability to degrade the homopolyester PHV in addition to PHB. Most other PHB-degrading bacteria are not able to degrade PHV and usually have only one PHB depolymerase. Three PHA depolymerases are specific for PHB and copolymers of 3HB and 3HV with low 3HV content (PHB depolymerases A, B, and D). The activity of these enzymes with the homopolyester PHV is below 5% of the activity obtained with PHB as a substrate. None of the three PHB depolymerases is able to produce clearing zones on opaque PHV-granule-containing agar. The two remaining PHA depolymerases (PHB depolymerase C and PHV depolymerase) also degrade PHB, but are additionally able to

hydrolyze PHV, with about 15 and 30% activity compared with PHB hydrolysis. The sequential order of the catalytic amino acids of all six *P. lemoignei* PHA depolymerases is histidine (oxyanion), lipase box serine, aspartate, and histidine.

PHB-degrading bacterium *Pseudomonas stutzeri* (PhaZpst) was isolated from seawater and the biochemical properties of its PHB depolymerase were investigated (Uefuji et al. 1997). This depolymerase has three components and a few unique characteristics: two putative substrate-binding domains (SBDI and SBDII); the N-terminal amino acid sequence of the enzyme was different from those of other PHB depolymerases; 3HB monomer is the final PHB hydrolysis product (3% of dimer). In the case of PhaZafa the ultimate major product of PHB degradation is the dimer; the substrate recognition in the active site of PhaZpst for the hydrolysis of 3HB oligomers and PHB is different in specificity from that of PhaZafa (Hiraishi et al. 2000). Kinetic analysis of enzymatic hydrolysis was carried out by using several different types of water-soluble oligomers of 3HB (Ohura et al. 1999). The methyl ester of 3HB monomer [H(3HB)M] was not hydrolyzed by the enzyme, whereas the methyl ester of 3HB dimer [$H(3HB)_2M$] was hydrolyzed to yield a mixture of H(3HB)H, H(3HB)M, and $H(3HB)_2H$ as the hydrolysates, suggesting that the active site of the catalytic domain recognizes at least two monomeric units as substrates for the hydrolysis of ester bonds in a 3HB sequence. The hydrolysis rate of the 3HB trimer [$H(3HB)_3M$] was 2 orders of magnitude higher than the hydrolysis rates of the 3HB dimer derivatives and was almost the same as the rate for the 3HB tetramer [$H(3HB)_4M$], suggesting that the active site of the catalytic domain prefers to bind three 3HB units for the hydrolysis of a 3HB sequence.

### 2.1.2 Medium-Chain-Length PHAs

Most of the purified depolymerases are specific for PHB and/or other scl-PHAs. Microorganisms producing extracellular mcl-PHA depolymerases are relatively rare and most belong to the genus *Pseudomonas* (Ramsay et al. 1994). The first strain, which was identified as *Pseudomonas fluorescens* GK13, was isolated from activated sludge and was selected to study the degradation of poly[(*R*)-3-hydroxyoctanoate] [PHO] and poly[(*R*)-3-hydroxydecanoate-*co*-(*R*)-3-hydroxyoctanoate] [PHOHD] (Schirmer et al. 1993). After aerobic cultivation for 38 h for PHO or 5 days for [PHOHD], polymers were decomposed successively by 95 and 82%. The molecular mass of PHO depolymerase was about 50,000 Da and this enzyme consists of two identical polypeptide chains and is active in a pH range from 6.3 to 9.3.

A novel bacterial strain capable of growing in a medium containing a mcl-PHA as the sole carbon source was isolated from a soil sample. The isolate, which was identified as *Pseudomonas* sp. RY-1, secreted mcl-PHA depolymerase when cultivated on PHO or poly[(*R*)-3-hydroxynonanoate] [PHN] (Kim et al. 2000a, b, c). The organism also grew in the presence of poly[(*R*)-3-hydroxyheptanoate] and others mcl-PHAs bearing functional groups. The native molecular mass of the enzyme was estimated to be 115 kDa. The depolymerase appears to be constituted by four identical subunits, has an isoelectric point of 5.9 and a maximum activity

(more than 80%) over a pH range from 7.0 to 10.0 (most active at pH 8.5) at 35°C. The depolymerase was partially inactivated by EDTA but was not inhibited by usual scl-PHA depolymerase inhibitors such as dithiothreitol and phenylmethylsulfonyl fluoride (Mukai et al. 1992; Shiraki et al. 1995; Müller and Jendrossek 1993). These results suggest that serine residues, reduced thiol groups, or disulfide bonds are not essential in the active site of the depolymerase [in analogy with the PHO depolymerase of *P fluorescens* GK13].

Kim et al. (2003) have isolated a bacterial strain capable of degrading mcl-PHAs consisting of repeating units with a carbon chain length of $C_6$ to $C_{11}$. This organism, which was identified as *Streptomyces* sp. KJ-72 secreted mcl-PHA depolymerase into the culture fluid only when it was cultivated on mcl-PHAs. The enzyme consists of a unit with a molecular mass of 27.1 kDa and an isoelectric point of 4.7. The maximum activity was observed at pH 8.7 and 50°C. The enzyme was sensitive to *N*-bromosuccinimide and acetic anhydride, indicating the presence of tryptophan and lysine residues in the catalytic site. This enzyme is believed to be an esterase activity belonging to the serine hydrolase family. The purified depolymerase was shown to have an *exo*-type depolymerase activity producing the dimeric units of (*R*)-3-hydroxyheptanoate as the major and product from poly[(*R*)-3-hydroxyheptanoate] homopolyester with monomeric units of (*R*)-3-hydroxyheptanoate as the minor product [as in the case of PHO and PHO depolymerase from *P. fluorescens*, where the dimeric form of (*R*)-3-hydroxyoctanoate (3HO) was the major hydrolysis product]. Elbanna et al. (2004) selected the bacterial strain *Ps. Indica* K2 owing to its ability to hydrolyze mcl- and scl-PHAs. This *Pseudomonas* strain produces two different depolymerases, one for mcl-PHA and one for scl-PHA. The purified PHO depolymerase is relatively similar to those from S*treptomyces* sp. KJ-72. The showed that PHA depolymerases are specific for oxoester linkages and do not hydrolyze thioester bonds of polythioester copolymers and homopolymers.

The characteristics of an extracellular mcl-PHA depolymerase purified from the marine isolate *Pseudomonas luteola* M13-4 have been reported by Rhee et al. (2006). This enzyme is similar to *Pseudomonas alcaligenes* LB 19 (Kim et al. 2002) but is different from poly(HB-*co*-HV) (PHBV) depolymerase of *Xanthomonas sp* JS02 (Kim et al. 2000a, b, c); the molecular mass is 28 kDa, the isoelectric point is 6.0 with a reactivity optimum at pH 10 and 40°C. Serine residues seem to play an important role in the hydrolysis reaction by analogy with *P. alcaligenes* LB 19. PHO has been totally degraded; polyhydroxyhexanoate (PHH) and a copolymer of (PHB-*co*-60%HV) have been partially hydrolyzed.

### 2.1.3 Structure and Degradation of PHB and Copolymers

Enzymatic degradation of racemic PHB films with different stereoregularities (crystallinity from 33 to 8% for an isotactic dyad fraction [*i*] varying from 0.88 to 0.54) (Abe and Doi 1996) was carried out at 37°C at pH 7.4 in the presence of PHB depolymerase (1.0 μg) from *A. faecalis.* In the absence of depolymerase, no hydrolysis was observed for over 48 h for all films. The racemic PHB film with an isotactic

dyad fraction [*i*]=0.88 was eroded only during the initial stage of the enzymatic reaction, but no more weight loss of the film occurred after 10 h. This sample is a stereoblock copolymer of racemic PHB with sequences of predominantly *R*-units and predominantly *S*-units. The sequences enriched in *R*-units are hydrolyzed by the PHB depolymerase, but the *S*-enriched-unit sequences are not hydrolyzed. Then, the inactive poly[(*S*)-hydroxybutyrate] sequences may cover the surface of the film, inhibiting the degradation after the initial stage of enzymatic degradation. For [*i*] values of 0.54–0.74, the weight loss of the film increased proportionally with time over 48 h as a result of the action of depolymerase as in the case of [(*R*)-3-hydroxybutyrate], (enzymatic degradation products, monomer, dimer, trimer, and tetramer), whereas little erosion took place on the syndiotactic PHB film. PHB depolymerase adsorbed on the surface of predominantly isotactic PHB films, whereas little enzyme adsorption took place on the surface of syndiotactic PHB film, suggesting that the binding domain of PHB depolymerase has an affinity toward the isotactic crystalline phase. The highest rate of enzymatic hydrolysis was observed at an [*i*] value of 0.74. Syndiotactic PHB was hardly hydrolyzed by the enzyme. Scanning electron microscopy (SEM) of the surfaces of PHB films during the enzymatic hydrolysis (Fig. 3) showed that the center of spherulites consisted of less ordered lamellae which are more susceptible to enzymatic attack (Koyama and Doi 1997).

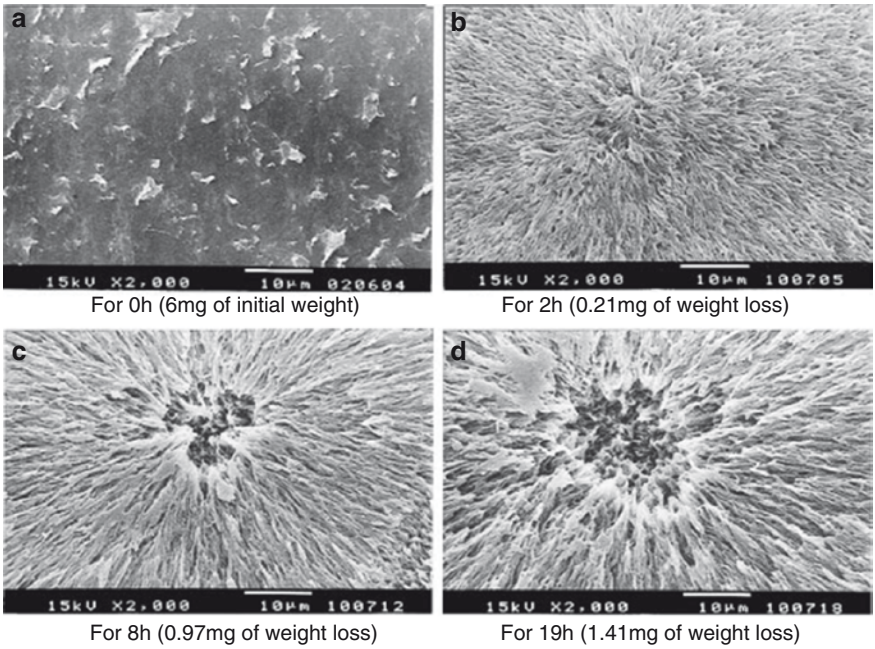


**Fig. 3** Scanning electron micrographs of the PHB films crystallized at 120°C for 168 h from the melt before and after enzymatic erosion (Koyama and Doi 1997)

It has been widely believed that the enzymatic hydrolysis first occurs in the amorphous phase, followed by the degradation in the crystalline phase. Extracellular PHB depolymerase can degrade only polymer chains in the surface layer of the film. The rate of enzymatic degradation of PHB and its copolymer films decreases with an increase in the crystallinity, crystal size, and lamellar thickness, whereas that the size of the spherulites has less effect on the degradation rate (Iwata et al. 1997a, b; Kasuya et al. 1999). The effect of solid-state structures on enzymatic degradability has been studied through the analysis of solution-cast films and melt-crystallized films with various degrees of crystallinity, lamellar crystal sizes, spherulite sizes, monomer composition, and sequence distribution (Tomasi et al. 1996; Tokiwa and Calabia 2004; Jendrossek and Handrick 2002).

Hydrolysis occurred on the surface of PHB films exposed to PHB depolymerases isolated from *R. pickettii* T1 and *P. stutzeri* (Yoshie et al. 2002), whereas no weight loss was observed for film exposed to the buffer without enzyme. The variation in the weight loss is explained by considering the dependence of the degradation rate on the enzyme concentration. The degradation rate increases steeply with the concentration of PHB depolymerase to the maximum value, followed by a gradual decrease. The bulk crystallinity was unchanged during the enzymatic hydrolysis. The hydrophobic substrate-binding domain of PHB depolymerase assists in increasing the mobility of PHB chains by adsorbing to crystal surfaces and generates the disordered chain-packing regions. The overall degradation rate is strongly dependent on the rate of enzymatic attack on the polymer chains by the catalytic domain, whereas the adsorption of enzyme molecules to the polymeric materials via the binding domain is necessary to initiate the enzymatic attack on the polymer chains by the catalytic domain. Though the chain scission may also be a cause of the increase of the chain mobility, the molecular weight was reported to remain unchanged during the enzymatic hydrolysis with PHB depolymerases for PHB films and PHB single crystals (Hocking et al. 1996). Sudesh et al. (2000) have purposed a model for the enzymatic degradation of PHB single crystals by PHB depolymerase (Fig. 4). The PHB depolymerase molecules bind on the entire surface of PHB single crystals. The resulting disordered chains can be compared to polymer chains in the amorphous phase and are facilely attacked by the active site of the enzyme. Many narrow cracks and small crystal fragments along the crystal long axis corresponding to the *a*-axis appear during the enzymatic hydrolysis in all cases (independent of both surface morphologies of single crystals and the nature of PHB).

The crystal structure of PHB is a orthorhombic form with unit cell parameters $a = 0.576$ nm, $b = 1.320$ nm, and $c$(fiber axis) $= 0.596$ nm, and space group $P2_12_12_1$ (Alper et al. 1963; Okamura and Marchessault 1967). The conformational analysis by intermolecular energy calculation has indicated that the PHB molecule has a left-handed $2_1$ helix conformation (Cornibert and Marchessault 1972; Yokouchi et al. 1973; Bruckner et al. 1988). The crystal structure of random copolymers of 3HB and 3HV has been investigated extensively (Bloembergen et al. 1986; Kamiya et al. 1991; VanderHart et al. 1995). A structural characteristic of poly(3HB-*co*-3HV) is isodimorphism, i.e., cocrystallization, of the two monomer units. In contrast, the cocrystallization of 3HB with 3HH or (*R*)-6-hydroxyhexanoate (6HH) does not occur,

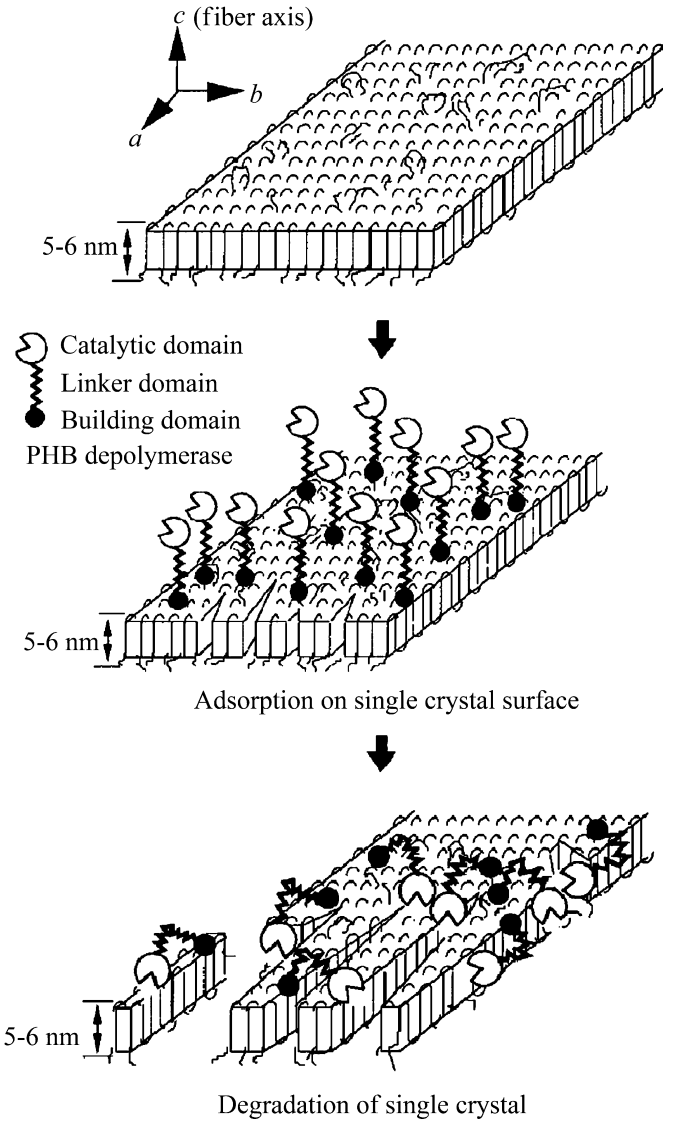


**Fig. 4** Enzymatic hydrolysis of PHB single crystals by PHB depolymerase (Iwata 1999)

and 3HH and 6HH units are excluded from the PHB crystalline phase of poly(3HB-*co*-3HH) and poly(3HB-*co*-6HH) (Abe et al. 1998). The single crystals of PHB have been used to elucidate the enzymatic degradation behavior of the crystalline phase in lamellar crystals (Hocking et al. 1996; Nobes et al. 1996; Iwata et al. 1997a, b). It has been demonstrated that PHB depolymerases may erode the lateral side at the edge and end of PHB single crystals along the crystallographic *a*-axis.

The thickness of the PHB single crystal remained unchanged during the enzymatic degradation, indicative of no erosion along the *c*-axis (Iwata et al. 1997a, b, 1999; Murase et al. 2002a, b). Recently, Murase et al. (2001a, b, c) proposed that a straight degradation pathways exist in PHB single crystals along the *a*- and *b*-axes, resulting in the formation of the slits and crevices during enzymatic degradation.

Iwata et al. (1999) have suggested that the hydrophobic substrate-binding domain of PHB depolymerase assists in increasing the mobility of PHB chains by adsorbing to crystal surfaces and generates the disordered chain-packing regions. This type of disordering may be another cause of the decrease of the attenuated total reflection/IR crystallinity of PHB films upon enzymatic hydrolysis. The overall degradation rate is strongly dependent on the rate of enzymatic attack on the polymer chains by the catalytic domain, whereas the adsorption of enzyme molecules to the polymeric materials via the binding domain is necessary to initiate the enzymatic attack on the polymer chains by the catalytic domain. Though the chain scission may also be a cause of the increase of the chain mobility and thus the decrease of the attenuated total reflection/IR crystallinity, the molecular weight was reported to remain unchanged during the enzymatic hydrolysis with PHB depolymerases for PHB films and PHB single crystals (Hocking et al. 1996).

Enzymatic degradation of PHB single crystals with PHB depolymerases from *Pseudomonas lemoignei* (Nobes et al. 1996) and *R. pickettii* T1 (Iwata et al. 1997b; Murase et al. 2001a) was observed with the transmission electron microscope and the atomic force microscope. These studies demonstrated that enzymatic hydrolysis progresses from the short ends of the lath-like-shaped PHB lamellar single crystals to form cracks along the long axis. The cracks lengthen during enzymatic treatment, whereas the hydrolysis from the lateral side of crystals hardly occurs. Therefore, the degradation process by PHB depolymerase converts a lath-shaped lamellar single crystal into narrower laths, and as a result, enzymatic action on PHB single crystals progressively enlarges the crystalline surface area.

## *2.2 Intracellular Degradation*

Intracellular degradation (often called *mobilization*) consists in enzymatic breakdown of polymers to monomers, which are then converted by D-hydroxybutyrate dehydrogenase into acetacetate. As a result of the dehydrogenase reaction, the latter is transferred to CoA, serving as a substrate for β-ketothiolase, which converts it into acetyl-CoA. Studies of intracellular degradation may be important in regard to mass production of microbial polyesters.

### 2.2.1 Short-Chain-Length PHB

The putative intracellular PHB depolymerase system from *Rhodospirillum rubrum* has been used to degrade native granules isolated from *Bacillus megaterium* KM

(Merrick and Doudoroff 1964). It consists of three components. The first component is a soluble heat-sensitive depolymerase. Efficient hydrolysis of native PHB granules in vitro by soluble PHB depolymerase required pretreatment of PHB granules with a heat-stable second component called the activator, ApdA. The activator was purified (Handrick et al. 2004b) and was resistant against various physical and chemical stresses, such as heat (up to 130°C), pH 1–12, dryness, and oxidation by $H_2O_2$. Analysis of the activation process in vitro showed that the activator acts on native PHB granules but not on the depolymerase. The effect of the activator could be mimicked by pretreatment of native PHB granules with trypsin or other proteases. Surface layer proteins of native PHB granules are the targets of the activator. Trypsin and the activator enable the PHB depolymerase to find and to bind to the polymer surface: trypsin by removing a portion of the proteins from the polymer surface, the activator by modifying the surface structure in a not yet understood manner presumably by interaction with phasins of the proteinous surface layer of native PHB (Steinbüchel et al. 1995; Handrick et al. 2004a, b). The third component of the intracellular depolymerase system is a dimer hydrolase responsible for hydrolysis of the primary degradation products of PHB (i.e., dimers and oligomers of 3HB) to 3HB. It turned out that ApdA in *R. rubrum* in vivo is a PHB-bound molecule with all the feature of a phasin. None of the depolymerases described in the literature (Gao et al. 2001; Handrick et al. 2000; Kobayashi et al. 2003, 2005; Jendrossek 2007; Saegusa et al. 2002; Tseng et al. 2006; York et al. 2003) require a cofactor such as ApdA in *R. rubrum,* which appears to be an exception.

PHB intracellular degradation systems are often very complex as in the case of *Wautersia eutropha* H16 (*Ralstonia eutropha*) (Uchino et al. 2008), which might have as many as nine (Pohlmann et al. 2006) PHB depolymerases/oligomer hydrolases (four classes of internal PHB depolymerases named PhaZa, PhaZb, PhaZc, and PhaZd). PhaZa1 (PhaZ1) is widely distributed among bacteria (York et al. 2003) and does not contain the lipase box pentapeptide (Gly-$X_1$-Ser-$X_2$-Gly) which all extracellular PHB depolymerases contain. Instead of the serine residue, PhaZ1 has a cysteine residue at the active site (Kobayashi and Saito 2003). PhaZb and PhaZc have the lipase box sequence at their active sites. PhaZb is a 78-kDa protein and is similar to the extracellular 3HB oligomer hydrolase of *R. pickettii* A1 (Zhang 1997). The PhaZa enzyme hydrolyzes PHB but is not active with 3HB oligomers. The PhZb enzyme efficiently hydrolyzes 3HB oligomers and degrades amorphous PHB at a lower rate. This enzyme type has been found only in members of the genus *Wautersia*, such as *W. eutropha* and *W. metallidurans*, so far. PhaZb efficiently hydrolyzed all linear and cyclic 3HB oligomers; it utilizes, both endo and exo modes of hydrolysis (Scherer et al. 2000). The localization of PhaZb in PHB granules is very important for PHB degradation in *R. eutropha*. Since the major products of the degradation of amorphous PHB by PhaZ1 are 3HB oligomers, PhaZb, which has a broad substrate specificity for 3HB oligomers of various lengths, probably has an important role in degrading the resulting 3HB oligomers to monomers. Colocalization of PhaZa and PhaZb in PHB granules ensures a rapid degradation of PHB in vivo. The PhaZc enzyme (molecular mass about 30 kDa) exhibits strong 3HB oligomer hydrolase activity compared with other PHB

depolymerases or 3HB oligomer hydrolases. Some enzymes (PhaZc in this study, $PhaZc_{Weu}$) hydrolyze the 3HB pentamer most efficiently and exhibit weak hydrolytic activity with amorphous PHB, but another PhaZc in *Acidovorax* sp. strain SA1 ($PhaZc_{Asp}$) (Sugiyama et al. 2002) hydrolyzes the 3HB dimer at a high rate with no PHB-hydrolyzing activity. Some of the enzymatic properties of PhaZc in vitro clearly suggest that the enzyme is a specific 3HB oligomer hydrolase, not a lipase or a nonspecific esterase, and it may function partly as a PHB depolymerase. Although the enzyme hydrolyzed 3HB oligomers efficiently, it also degraded artificial amorphous PHB, and a certain amount of PhaZc was found in native PHB inclusion bodies. The PHB depolymerase activity with artificial amorphous PHB observed in vivo does not necessarily mean that PhaZc has weak activity with native PHB in the cell; the amorphous PHB in native PHB inclusion bodies probably differs from the artificial amorphous PHB in terms of accessibility and degradability. Previously, it was shown that PhaZ1 and PhaZb synergistically hydrolyze PHB (Kobayashi et al. 2003). For these reasons, PhaZ1, PhaZb, and PhaZc may work together and hydrolyze PHB efficiently, as well as 3HB oligomers in the cell. The synthesis and degradation of PHB seem to occur simultaneously. Such a turnover of PHB has already been pointed out (Kawaguchi and Doi 1992). In view of economy, a quick turnover of PHB seems to be a disadvantage to bacteria. The steps for in vivo degradation of PHB appear to be as follows: (1) PhaZa1 or PhaZb makes several nicks in the chains of amorphous PHB molecules; (2) as a result, medium-sized 3HB oligomers that still bind to the granules owing to their hydrophobicity, some loosened 3HB ends of PHB chains protruding from the granules, and a small amount of 3HB monomer/short-chain 3HB-oligomers that diffuse from the granules are produced; (3) PhaZb degrades 3HB oligomers on PHB granules and the loosened ends of amorphous PHB chains to 3HB in an exo fashion; (4) finally, PhaZb localized in the cytosol hydrolyzes the diffused 3HB oligomers.

*Bacillus thuringiensis* is known to be a PHB producer (He et al. 2002). A gene that codes for a novel intracellular PHB depolymerase has now been identified in the genome of *B. thuringiensis* subsp. *israelensis* ATCC 35646 (Tseng et al. 2006). The *B. thuringiensis* PhaZ bears no significant similarity with any known intracellular or extracellular PHB depolymerase. That is also the case of a Gram-positive PHB-degrading bacterial strain which was isolated from compost (Takaku et al. 2006). This organism, identified as *B. megaterium* N-18-25-9, produced a clearing zone on opaque PHB agar, indicating the presence of extracellular PHB depolymerase. A PHB depolymerase gene, $PhaZ_{Bm}$, of *B. megaterium* N-18-25-9 was cloned and sequenced, and the recombinant gene product was purified from *Escherichia coli*. The N-terminal half region of $PhaZ_{Bm}$ shared significant homologies with a catalytic domain of other PHB depolymerases. Although the C-terminal half region of $PhaZ_{Bm}$ showed no significant similarity with those of other PHB depolymerases, that region was necessary for the PHB depolymerase activity. Therefore, this enzyme's domain structure is unique among extracellular PHB depolymerase domain structures. The maximum activity was observed at pH 9.0 at 65°C.

In contrast to other known intracellular PHB depolymerases, the *B. thuringiensis* and *B megaterium PhaZ* generate many more 3HB monomers as the hydrolytic

product. The amount of 3HB monomer released from hydrolysis of native PHB granules corresponds to approximately 42 and 34% of the total 3HB equivalents present in the native PHB granules of *B. megaterium* and *B. thuringiensis*. The amount of 3HB monomer released from hydrolysis of native PHB granules by the amorphous PHB-specific extracellular PHB depolymerase PhaZ7 of *Paucimonas lemoignei* corresponds to only 0.5–2.5% of the total 3HB equivalents present in the native PHB granules (Handrick et al. 2001). The intracellular PhaZb and PhaZc of *R. eutropha* H16 are actually 3HB oligomer hydrolases (Kobayashi et al. 2003, 2005). The intracellular PhaZd of *R. eutropha* H16 shows similarity with the type I catalytic domain of extracellular PHB depolymerases from bacteria such as *R. pickettii* T1 and *P. lemoignei* (Jendrossek and Handrick 2002) and produces various 3HB oligomers from amorphous PHB as hydrolytic products. 3HB monomer was rarely detected as a hydrolytic product (Abe et al. 2005).

Intracellular degradation of various PHA inclusions in *Hydrogenophaga pseudoflava* was investigated (Yoon et al. 1995; Yoon and Choi 1999). Two types of PHA copolymers and blend-type polymers were separately accumulated in cells for comparison. The substrates were (*R*)-3-hydroxybutyric acid (3HB), 4-hydroxybutyric acid (4HB), and (*R*)-3-hydroxyvaleric acid (3HV). The 3HB/4HB copolymer was degraded when the polymer contained a minimum level of 3HB units. With the cells containing a 3HB/4HB blend-type polymer, only PHB was degraded, whereas poly(4HB) [P(4HB)] was not degraded, indicating the totally inactive nature of the intracellular depolymerase against P(4HB). The concentration of the 4HB unit in a suspension containing cells with a blend-type 3HB/4HB polymer initially composed of 45 mol% 3HB/55 mol% 4HB was constant throughout the degradation period of 60 h, which indicated that the 4HB units in the blend-type polymer were not degraded. In contrast, the 3HB units in the cells disappeared according to the first-order degradation process. In contrast to the case of the 3HB/4HB blend-type polymer, the 4HB units in a copolymer composed of 41 mol% 3HB and 59 mol% 4HB were found to be degraded under the same incubation condition as in the blend-type polymer with two different first-order degradation rate constants ($k_{3HB} = 3k_{4HB}$). The dissimilarity in $k_1$ values for the two monomer units in the copolymer sample thus suggests the existence of two different types of polymer chains, 3HB-rich and 4HB-rich chains. Such probable microstructural heterogeneity, as in the 3HB–4HB copolymer, may be related with the different assimilation rates of the two monomer precursors, glucose and γ-butyrolactone, in PHA accumulation by the bacterium. The suggested microstructural heterogeneity in the 3HB–4HB copolymer was detected in detail at a molecular level by using high-resolution $^{13}C$ NMR spectroscopy. For the copolymers composed of 3HB and 4HB monomer units, the sequences of dyad, triad, and tetrad were assigned assuming a first-order Markovian random copolymerization in cells (Doi et al. 1988). According to the $^{13}C$ NMR analysis, the blend-type polymer is principally a mixture of PHB and P(4HB). Degradation experiments were carried out for two types of cells, one containing a copolymer composed of 57 mol% 3HB/43 mol% 3HV and the other a blend-type polymer composed of 55 mol% 3HB/45 mol% 3HV. In contrast to the case of the 3HB/4HB blend-type polymer, degradation of the second monomer 3HV unit in the 3HB/3HV blend-type polymer cells was observed. The degradation of the 3HV unit was

slower than that of the 3HB unit. The similar degradation rate constants for both monomer units in the 3HB–3HV copolymer indicate the almost complete random nature of the copolymer, containing no long blocked chains as well as being less heterogeneous than in the 3HB–4HB copolymer. In addition, the higher degradation rate of the 3HB-rich chains in the blend-type polymer may imply that the *H. pseudoflava* intracellular depolymerase is more specific to 3HB units than to 3HV units. Local sequence-specific degradation of 3HB–3HV polymers was also investigated by using 125 MHz $^{13}$C NMR spectroscopy. The sequence assignment was made according to the method of Doi and coworkers (Kunioka et al. 1989). For direct comparison of the relative degradation rate between 3HV units and 4HB units, they prepared two types of 3HV–4HB polymers: a blend-type polymer [PHV to P(4HB) ratio of 43:57, in terms of monomer mole ratio] and a poly(42 mol% 3HV-*co*-58 mol% 4HB) copolymer. Both polymers contained less than 1–2 mol% 3HB. The amount of the remaining 3HV unit was 85% for the copolymer and 47% for the blend-type polymer. This indicates that the introduction of the 4HB unit into the 3HV-containing polymer retarded the degradation of the polymer. No degradation of the 4HB unit was observed for the two polymers. This result clearly shows again that the intracellular depolymerase has no activity against the 4HB unit in P(4HB). The local sequence-dependent degradation suggests that the depolymerization step is the rate-determining one in intracellular PHA degradation. In other words, the depolymerization rate is governed by the specificity of the enzyme toward the local monomer sequence such as dyad, triad, and tetrad. *H. pseudoflava* intracellular PHA depolymerases are not the processive enzymes that cleave the polymer chain sequentially. In addition, from the lack of intracellular degradability of P(4HB) or 4HB-rich poly(3HB-*co*-4HB), it can be concluded that the presence of a chiral carbon in the ester backbone and/or the oxidation position located three bonds away from the carbonyl is essential for the hydrolysis reaction by the depolymerase. In conclusion, if any two different monomers are copolymerized in a cell and the $^{13}$C NMR signals of the copolymer synthesized exhibit splittings because of the neighboring monomer units, the relative specificity of the intracellular depolymerase against the local sequences could be determined by analyzing PHA before and after degradation using $^{13}$C NMR spectroscopy without purification of the enzyme and native substrates. On the basis of the magnitude of the first-order degradation rate constants, the relative substrate specificity of the depolymerase toward the constituting monomer units is in the order 3HB > 3HV > 4HB. The results of $^{13}$C NMR spectroscopy showed that the intracellular degradation depended on the local monomer sequence of the copolymers.

### 2.2.2 PHAs with Long Alkyl and/or Phenyl Substituents in the Side Chain

*Pseudomonas oleovorans* has been utilized to synthesize a large number of mcl-PHAs (Lenz and Marchessault 2005). Investigations concerning intracellular depolymerase functionality and location in *P. oleovorans* inclusions containing PHO granules isolated from *P. oleovorans* grown on octanoic acid (OA) showed that intracellular inclusion bodies represent a highly organized subcellular system

consisting of polyester, enzymatic and structural proteins, and phospholipids (Stuart et al. 1995). A protein species associated with the inner array of the double protein lattice was identified as a PHO depolymerase. Prior to complete utilization of the carbon substrate in the medium, the cells decrease the concentration of polymerase while increasing the concentration of depolymerase. This behavior would appear to represent the cell's response to an increasingly less permissive metabolic environment (Stuart et al. 1996). During normal fermentation, the OA is utilized by the organisms and as the sole carbon source disappears as the level of oxygen increases. As the OA concentration reached a value of approximately 3 mM, the dissolved oxygen began to increase and when the substrate concentration was 1 mM, the dissolved oxygen had increased to approximately 25%. Thus, the polymerase decreased from 20 to 12% and the depolymerase increased from 10 to 15%. The cells must prepare themselves to mobilize the stored intracellular reserves to survive once the carbon source in the medium is exhausted. In *Alcaligenes eutrophus*, polyester synthesis and degradation can proceed simultaneously (Doi 1990). Degradation by depolymerase in isolated PHO granules has indicated that the protein complex which encompasses the polymer can be released from purified granules by freeze-thawing. Moreover, the proteins released into the supernatant by this treatment were demonstrated to enhance the observed degradation activity when added to freshly isolated granules and to cause rapid degradation of an amorphous colloidal suspension of the polymer (Foster et al. 1996).

In vitro degradation of isolated *P. oleovorans* inclusion bodies has been carried out on polyesters prepared from nonanoic acid PHN, 3-hydroxy-5-phenylvaleric acid {poly[(*R*)-3-hydroxy-5-phenylvalerate], PHPV}, or a mixture of PHN and PHPV (Foster et al. 1999). The degradation rate which occurred in isolated PHN inclusion bodies, at pH 9 and 30°C was 0.92 mg $h^{-1}$. This observed rate is remarkably similar to the in vitro rates previously reported for PHO inclusion bodies. Isolated inclusion bodies containing PHPV also exhibited a similar degradation rate of 0.98 mg $h^{-1}$. The inclusion bodies containing a PHN/PHPV mixture degraded at a slower rate of 0.30 mg $h^{-1}$. This difference could be explained by difficulties in the isolation or PHN/PHPV inclusion bodies. Complications may have been caused by the unusual distribution of the polymers within the inclusion body. The intracellular depolymerase activities in in vitro studies are in contrast to those obtained through the in vivo degradation studies of PHN, PHPV, and PHV/PHPV inclusion bodies. In those cases, the in vivo degradation of PHN inclusion bodies proceeded significantly faster than that of their PHPV counterparts. Furthermore, PHN degradation occurred preferentially in PHN/PHPV inclusion bodies. The in vivo degradation of the (*R*)-3-hydroxy-5-phenylvalerate (HPV) component began after nearly complete utilization of the PHN reserves (Curley et al. 1997). The presence of the aromatic group could be a significant factor (Foster et al. 1995).

There are a number of reasons why differences between in vitro and in vivo degradation rates should occur. First, the in vivo degradation rate may be limited compared with the in vitro rate owing to the variety of enzyme(s) or the lack of substrate specificity for polymer utilization in the former compared with the single depolymerase in the in vitro assays. Second, the in vitro assay was performed at a

previously determined optimum of pH 9 (250 mM) with additional magnesium, and changing to a more neutral pH and the absence of additional magnesium may both have significantly reduced the in vitro PHA degradation rate (Foster et al. 1996).

The comparison of the in vivo and in vitro degradation of *P. oleovorans* inclusion bodies containing PHN, PHPV, and PHN and PHPV supports the suggestion that the activity of the intracellular depolymerase is not a limiting factor in the metabolism of these PHAs. However, in vitro studies this depolymerase readily degraded both PHN and PHPV, whereas in vivo, PHN was degraded preferentially. It is reasonable to suggest, therefore, that other processes in the PHA metabolism in *P. oleovorans* function to limit the PHPV degradation rates in vivo. Quite possibly, the presence of the aromatic group in the units of the polymer is a significant factor. Certainly evidence from other studies examining the degradation of PHN and PHPV by extracellular enzymes would support this suggestion (Foster et al. 1995). In these extracellular depolymerase studies the growth rate of *Pseudomonas maculicola* was apparently limited by the presence of PHPV.

mcl-PHA intracellular depolymerization systems are still not well elucidated, compared with knowledge of the scl-PHA intracellular enzyme-coding genes. Recent important progress has been established by de Eugenio et al. (2007, 2008a, b). The phaZ gene depolymerase from *Pseudomonas putida* KT2442 has been biochemically characterized. They have demonstrated that phaZ is an intracellular depolymerase that is located in PHA granules and that hydrolyzes specifically mcl-PHAs containing aliphatic and aromatic monomers. The enzyme behaves as a serine hydrolase. This study constitutes the first biochemical characterization of PhaZ and de Eugenio has proposed this enzyme as the paradigmatic representative of intracellular *endo*/*exo*-mcl-PHA depolymerases.

From a set of mixed carbon sources, 3-hydroxy-5-phenylvaleric acid and OA, polyhydroxyalkanoic acid (PHA) was accumulated in the two separated pseudomonads *P. putida* BM01 and *Pseudomonas citronellolis* (ATCC 13674) to investigate any structural difference between the two PHA accumulated under a similar culture condition using a one-step culture technique (Chung et al. 2001). The solvent fractionation analysis showed that the PHA synthesized by *P. putida* was separated into two fractions, an HPV-rich PHA fraction in the precipitate phase and a 3HO-rich PHA fraction in the solution phase, whereas the PHA produced by *P. citronellolis* exhibited a rather little compositional separation into the two phases. The structural heterogeneity present in the *P. putida* PHA was also confirmed by a first-order degradation kinetics analysis of the PHA in the cells. Two different first-order degradation rate constants ($k_1$), 0.087 and 0.015 $h^{-1}$ for 3HO and 3HPV units, respectively, were observed in a polymer system over the first 20 h of degradation. In the later degradation period, the disappearance rate of the 3HO unit was calculated to be 0.020 h, comparable to $k_1 = 0.015\ h^{-1}$ for the HPV unit. The initial faster disappearance of the 3HO unit was considered to be due to the degradation of PHO, whereas the later slower one was due to the degradation of the 3HO units incorporated along with HPV units into the copolymers chains. A $k_1$ value of 0.083 $h^{-1}$, almost the same as for the 3HO unit in the *P. putida* PHA, was obtained for the PHO accumulated in *P. putida* BM01 grown on OA as the only carbon source. In addition, the $k_1$ value of 0.015 $h^{-1}$

for the HPV unit in the *P. putida* PHA, was also close to the 0.019 $h^{-1}$ for the PHPV homopolymer accumulated in *P. putida* BM01 grown on 3-hydroxy-5-phenylvaleric acid and butyric acid. In contrast, the $k_1$ values for the *P. citronellolis* PHA were determined to be 0.035 and 0.029 $h^{-1}$ for the 3HO and HPV units, respectively; thus, these two relatively similar values imply a random copolymer nature of the *P. citronellolis* PHA. In addition, the faster degradation PHO than PHPV by the intracellular *P. putida* PHA depolymerase indicates that the enzyme is more specific against the aliphatic PHA than the aromatic PHA.

## 2.3 Degradation of PHAs Under Aqueous Conditions

In the biomedical field, degradation by simple hydrolysis is possible. For this reason, PHAs and copolymers have been used to develop devices including sutures, meniscus repair devices, screws, bone plates, and wound dressings (Cheng and Wu 2005). They have also emerged as alternative candidates which offer the potential of releasing entrapped drugs by surface erosion. These applications have been limited to slow degradation and high stability in tissue (Aoyagi et al. 2002; Tsuji and Suzuyoshshi 2002). Hydrolytic degradation of PHB in vitro proceeds to the monomer D-(−)-3-hydroxybutyric acid. This acid is a normal constituent of blood. The in vitro degradation proceeds relatively slowly when compared with poly(lactic acid–glycolic acid) polymer systems (Majid 1988; Pouton et al. 1988). The rate of mass loss from films of PHBV copolymers appeared to be rapider. But the gravimetric data on PHBV polymers obtained by Holland (1987) the increase of mass loss is due to lower crystallinity.

For the development of a gastrointestinal patch, the in vitro degradation of PHB solvent-cast films was examined by Freier et al. (2002). The molecular weight of pure PHB decreased by half after PHB had been in buffer solution (pH 7.4 37°C) for 1 year. An acceleration in molecular weight decrease was observed by blending with atactic PHB. Leaching of a water-soluble additive led to a slight acceleration of PHB degradability and a deceleration with the addition of a hydrophobic plasticizer. The PHB degradation rate was accelerated about threefold by addition of pancreatin. A PHB/atactic blend patch was prepared for repair of a bowel defect in Wistar rats. Twenty-six weeks after implantation, material remnants were found in only one for four animals. The bowel defects were closed in all cases. The patch material was resistant to the intestinal secretions for a sufficiently long time, but then finally degraded completely.

PHB, PHBV and poly(3HB–4HB) were used in the preparation of biodegradable, implantable rods for the local delivery of antibiotics in chronic osteomyelitis therapy (Tagmurlu et al. 1999). PHBV microspheres for the sustained release of the anticancer drug 5-fluorouracil were tested (Khang et al. 2001).

In a natural environment, it is difficult to separate biodegradation and simple hydrolysis. For example, Wang et al. (2004) investigated the degradation and the biodegradability of novel thermoplastics poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyhexanoate] (PHBHH). Films made of PHBHH were subjected to degradation in

activated sludge and compared with PHB and Ecoflex®, a biodegradable product from BASF. The average molecular weight of PHH was greatly reduced from 200,000 to about 120,000 after 12 degradation days and the surface became more porous, indicating that the surface morphology may have a relationship with polymer hydrolysis, involving both types of degradation. After 18 days of degradation, 40% of poly(HB-*co*-12% HH) and 20% of PHB were degraded, whereas Ecoflex® only lost 5% of its weight. SEM revealed that the surface of Ecoflex® was much smoother than that of poly(HB-*co*-12% HH) and PHB. At the same time, PHBHH degradation in a simplified system containing 0.1 g $L^{-1}$ lipase in phosphate-buffered saline was found to be affected by its HH content. It was found that poly(HB-*co*-12% HH) was degraded faster than PHB, poly(HB-*co*-5% HH), and poly(HB-*co*-20% HH). SEM results revealed that poly(HB-*co*-12% HH) films had the most porous surface after degradation. Before degradation, PHB and PHBHH films had holes on the surface, whereas Ecoflex® was smoother with fewer holes. Holes on the surface allowed bacteria and water molecules to come into contact with the surface and the polymer around the holes started to degraded. The holes became bigger and bigger and more bacteria and water molecules filled the big holes, leading to further degradation. On a smooth surface, this process will be much more difficult.

Scanning electron micrographs of PHB after enzymatic and hydrolytic degradation were shown by Doi et al. (1990). After an enzymatic degradation, the sample weight decreased by 32%. The surface was apparently blemished by depolymerase, whereas no change took place in the inside of the film. The hydrolytic degradation of PHB films occurred throughout the whole polymer matrix and the molecular weights at all the films decreased with time in 0.01 M phosphate buffer at 55 °C and pH 7.4. The molecular weight loss could be explained by a random chain scission of the ester groups. The weight of the PHB film was unchanged for 48 days, whereas the $M_n$ decreased from 768,000 to 245,000. The surface of the film was unchanged as the film thickness increased from 65 to 75 μm for 48 days, suggesting that water permeated the polymer matrix during the hydrolytic degradation.

The degradation behavior (Marois et al. 1999a, b, 2000) of the mcl-PHA PHO has been examined on solution-cast PHO films during hydrolysis incubation. PHO cast films underwent a simple hydrolysis degradation process characterized by water absorption, gradual molecular weight decrease, and negligible mass loss after 24 months of incubation. As in all PHAs cases, the degradation occurred in the amorphous zone, followed by an attack in the crystalline domain. The process is very slow (owing to the presence of long hydrophobic pendant chains); after a 2-year incubation period, the weight loss remained lower than 1%. $M_w$ and $M_n$ both slowly decreased with incubation time, reaching approximately 30% of the initial molecular weight. In the absence of the release of soluble low molecular weight oligomers, it has been concluded that the hydrolytic process is homogenous. In vivo biocompatibility and degradation studies of PHO have been carried out in the rat for evaluation of it as a sealant for polyester arterial prostheses. The degradation process was controlled by a random hydrolytic reaction and by a local enzymatic attack by macrophages and giant cells.

In vitro degradation studies on PHB films and PHO in buffer showed very slow degradation (Marois et al. 1999a, b; Doi 1990). Hydrolytic degradation of PHAs is generally a slow process taking several months. This is due to the very high crystallinity or the hydrophobic nature of long alkyl pendant chains. The PHB degradation can be increased by the addition of polymers or plasticizers. Amorphous or hydrophilic additives lead to higher water absorption and accelerate hydrolysis. The water content was found to be higher in PHB/poly(DL-lactic acid) (PLA) than in a PHB/PCL blend (Zhang et al. 1995). In contrast, a reduced degradation rate was observed with the addition of a hydrophobic plasticizer (triethyl citrate or butyryltrihexyl citrate) (Frier et al. 2002). Renard et al. (2004) have examined the hydrolytic degradation of films of PHO, PHBV/PLA, and poly(ethylene glycol) (PEG) blends. Degradation of polymers for periods of 7–160 days (pH 10 and 37°C) was studied and the results showed that the presence of a second component, whatever its chemical nature, is sufficient to perturb the crystallization behavior of highly PHBH and increase hydrolytic degradation. As for PHO, both blends, [PHBV (750 g mol$^{-1}$) 50/50] and [PHBV/PEG(2,100 g mol$^{-1}$) 50/50], examined are immiscible. In the case of PHO blended with oligomers (PLA50 and PEG), weight loss occurred rapidly upon incubation in the buffer. The weight losses were assigned to the release of water-soluble PEG or hydrolysis of short PLA chains. The weight losses strictly corresponded to the oligomer contents in the blends. After the oligomers had been removed from the polymeric matrices, no significant weight loss was noted after 100 days. In the case of high molecular weight PLA50 (42,500 g mol$^{-1}$), the blend was resistant to hydrolytic degradation. This polymer remained trapped in the immiscible blend. Water absorption in polymer material was the most important factor influencing the hydrolytic process, and in the case of PHO the matrix was very hydrophobic and water penetration was limited.

## 3 Chemical Modification of Bacterial Polyesters: Hydrophilicity, Hydrolysis, Wettability

The intrinsic hydrophobic properties of natural PHAs and their difficulty to degrade in water under normal conditions, or in the human body, restrict their biomedical and biological applications. Chemical modifications represent an alternative route to introduce functional groups which cannot be introduced by bioconversion.

### *3.1 Introduction of Polar Groups*

Some bacteria are able to synthesize polyesters with reactive pendant double bonds which can be chemically modified. These new artificial bacterial polyesters are of great interest because they can be used for further modifications. The combination of bioconversion and organic chemistry allows one to modulate more precisely the physical

properties of these bacterial polymers, such as solubility, hydrophilic–hydrophobic balance, bioavailability, and wettability of surfaces. *P. putida* GPo1 has been largely used because of its high capability to grow on octanoate/10-undecenoic acid mixtures and produce unsaturated polymer (Lageveen et al. 1988; Fritzsche et al. 1990; Kim et al. 1995). The proportion of these unsaturated monomer units in the bacterial polyester is directly related to the nutrient composition (Bear et al. 1997). It was therefore possible to prepare copolyesters of poly[(*R*)-3-hydroxyoctanoate-*co*-(*R*)-3-hydroxyundecenoate] $P(HO)_{100-x}U_x$] with 0–100% (*R*)-3-hydroxyundecenoate by varying the proportion of undecenoic acid.

### 3.1.1 Introduction of Hydroxy Groups

Two methods have been investigated to increase the hydrophilicity of PHAs by hydroxylation of unsaturated pendant groups by oxidation with $KMnO_4$ (Lee et al. 2000) and by hydroboration–oxydation of alkenes (Renard et al. 2005). The transformation of the olefin groups in PHOU into hydroxyl groups caused a slight increase (10°C) in the glass-transition temperatures and the loss of crystallinity. The polar hydroxyl groups formed intramolecular interactions that affected backbone mobility. The presence of repeating units containing pendant hydroxyl groups in a proportion of 25 mol% caused an increase in the hydrophilicity of these new PHAs because they were soluble in polar solvents such as ethanol. Besides, these reactive PHAs can be used to bind bioactive molecules or to prepare novel graft copolymers with desired properties (Timbart 2005).

### 3.1.2 Introduction of Carboxy Groups

The presence of this functional group improved the hydrophilicity and the hydrolysis of corresponding polymers which could be used in a drug delivery system. Moreover carboxylic groups are of greatest importance to bind bioactive molecules, hydrolyzable, hydrophilic oligomers, or targeting proteins. The chemical modification was first carried on $P(HO)_{90}U_{10}$ with $KMnO_4$ as the oxidation reagent, in the presence of $KHCO_3$ (Lee et al. 2000). But 50% of the unsaturated groups were not transformed and the molecular weights were significantly reduced. The oxidation reaction conditions were modified using $KMnO_4$ and 18-crown-6-ether as a phase transfer and dissociation agent for $KMnO_4$. $P(HO)_{75}U_{25}$ was totally oxidized in 16 h (Bear et al. 2001; Renard et al. 2003a, b; Kurth et al. 2002). The presence of 25% polar groups in $P(HO)_{75}$–$COOH_{25}$ modified the hydrophobicity/hydrophilicity balance. This amorphous sticky material ($T_g = -19$°C) polymer is now soluble in methanol, acetone, and in some acetone/water mixtures [acetone/water (v/v) 85:15]. Weight loss of $P(HO)_{75}$–$COOH_{25}$ has been studied in a buffer solution (pH 10) and at room temperature. The weight loss decreased continuously, reaching 100% after about 2.5 h.

Diblock copolymers have been synthesized based on elastomeric PHO or $P(HO)_{75}$–$COOH_{25}$ as a soft segment and a more crystalline PCL segment (Timbart

et al. 2004; Timbart 2005) . Copolymers were prepared by combining in a first step the preparation of $P(HO)_{75}U_{25}$ oligomers ($4{,}800 < M_n < 20{,}500$), having a hydroxyl end group, and in a second step the controlled coordination–insertion ring-opening polymerization of ε-caprolactone (CL). Films have been immersed in a buffer solution at pH 7.3 (37°C). The degradation is observed for both polymers, but with a different rate owing to the presence of hydrophobic PCL sequences. PHO films are known to be very hydrophobic and to not degrade at pH 10 at 37°C. The oxidized PHO, noted P(HO–COOH), was actually the only mcl-PHA easily hydrolyzable (a few hours at pH 10). The presence of carboxylate groups increased the hydrolytic degradation. Timbart et al. (2007) have compared the hydrolytic comportment of novel diblock copolyesters poly(HO-*b*-CL) and poly[(HO–COOH)-*b*-CL] with natural P(3HO) and P(HO–COOH) at pH 7.3 and 37°C to study the effect of the presence of carboxylate groups. The hydrolytic degradation of the P(HO–COOH) is presented in Fig. 5. Results concerning degradation of the diblock copolymers are presented in Fig. 6. The weight loss of the P(HO–COOH) film is about 4% after 42 days of hydrolysis and reached 75% after 105 days. The molar masses of the supernatant were nearly the same during this period (10,000 and 7,000 g mol$^{-1}$). As expected, the copolymer poly(HO-*b*-CL) was not degraded because the two blocks were not hydrolyzable in these conditions. On the other hand, the copolymer poly[(HO–COOH)-*b*-CL] and P(HO–COOH) were degraded because of the presence of carboxylate groups which catalyzed the hydrolytic degradation. The presence of the PCL block limited the hydrolysis rate, and the molecular weights decreased after 15 days more rapidly for P(HO–COOH) than for poly[(HO–COOH)-*b*-CL].

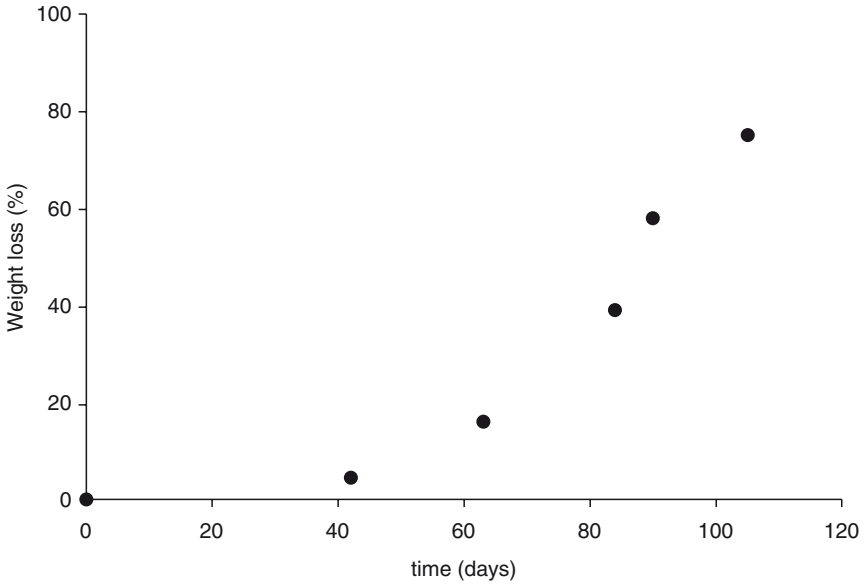


**Fig. 5** Weight loss of oxidized poly[(*R*)-3-hydroxyoctanoate] [P(HO–COOH)] film during the hydrolytic degradation at pH 7.3 at 37°C (Timbart 2007)

When the chain scission is completely random, a linear relationship between the number of chain scissions and the time is predicted as described in the following equation:

$$N_{(t)} = k_d P_{n(0)} t, \text{ with } N_{(t)} = \frac{M_{n(0)}}{M_{n(t)}} - 1,$$

where $k_d$ is the rate constant of hydrolytic degradation, $P_{n(0)}$ is the number-average degree of polymerization at time 0, $M_{n(0)}$ is the molecular weight at time 0, and $M_{n(t)}$ is the molecular weight at time $t$. Linear relationships were obtained for P(HO–COOH) and poly[(HO–COOH)-*b*-CL], demonstrating the random process of chain scission for polymers containing carboxylic functions in their side chains. The rate constant of hydrolytic degradation of P(HO–COOH) is calculated to be $13.8 \times 10^{-5}\,\text{day}^{-1}$, whereas for the diblock copolymer poly(HO–COOH-*b*-CL), the $k_d$ value is smaller, $1.84 \times 10^{-5}\,\text{day}^{-1}$).

## 3.2 Synthesis of Cationic PHA

To produce a water-soluble PHA, Sparks and Scholz (2008) first prepared the copolymer poly(3-hydroxyoctanoate-*co*-3-hydroxy-10-epoxyundecenoate) ($M_n$ 100,000), according the method of Bear et al. (1997). Epoxidation of PHOUs with *m*-chloroperbenzoic acid resulted in quantitative conversions of the unsaturated groups into epoxy groups. Diethanolamine was used to modify the epoxide groups to give side chains terminated with the corresponding tertiary amine, resulting in the polymer poly(3-hydroxyoctanoate)-*co*-(3-hydroxy-11-(bis(2-hydroxyethyl) amino)-10-hydroxyundecanoate) ($M_n$ 20,000). This polymer was water-soluble at a pH below its p$K_a$ owing to protonation of the nitrogen atom.

## 3.3 Graft Copolymers from PHAs and Their Behavior in Aqueous Media

Graft copolymers allowed the expansion of the spectrum of material properties, controlled by their chemical composition, and the length of the different segments. They can be prepared by three main methods (Nguyen 2008): "grafting onto," "grafting from," or "macromonomer technique." The grafting onto method consists in the covalent coupling of pendant reactive sites with end groups of oligomers, polymers, or copolymer segments. In the grafting from method, pendant functional sites of the macromolecular chain are activated and lateral chains are grown by polymerization of a second monomer. The third method involves copolymerization of a low molecular weight monomer with a macromonomer.

The hydrophilic PEG was selected for its good biocompatibility and its hydrophilicity. The synthesis of grafted PHAs has been carried out by direct esterification of poly($HO_{75}$–$COOH_{25}$), noted PHOD, with PEG (Renard et al. 2003a, b). The grafting

reaction proceeds by direct condensation between carboxylic groups of PHOD and hydroxyl terminal groups of PEG. Dicyclohexylcarbodiimide was used for the activation of carboxylic functions with 4-dimethylaminopyridin as a catalyst.

Monofunctional oligomers of PEG ($M_n = 350$ g mol$^{-1}$) were selected to avoid cross-linking side reactions. The percentage of reacted carboxylic groups was about 75 and the presence of residual carboxylic acid groups on the surface can be advantageously used to covalently conjugate bioactive molecules by esterification or amidification reactions for targeting applications.

To study the behavior of PHOD-*g*-PEG in aqueous media, the grafting of PHOD was carried out with three monomethylated PEG with, respectively, $M_n$ of 350, 750, and 2,000 g mol$^{-1}$. The maximum grafting percentage is about 50 for PEG350 and only 20 for PEG2000. This result can be explained by the steric hindrance with increasing chain length, and by a decrease of the hydroxyl terminal groups. PHOD-*g*-PEG is an interesting candidate for preparation of particles; their stability was essentially dependent on the COOH amount and on the hydrophobic–hydrophilic balance. A certain number of COOH groups are located in the core of the particles. The architecture is not compact, promoting water and salt penetration inside the particles. PEG appears to be a protective agent against the alteration promoted by salts. PEG units in graft PHOD act as a surfactant and contribute to the prevention of particle coalescence. Another parameter is the lower COOH number (6–9%), which contributes to a better stability. The introduction of hydrophilic side groups modifies the solubility parameters of the polymer. The solubility of the graft polymer in acetone/water mixtures is gradually increased with the PEG chain length [PHOD-*g*-PEG2000 in acetone/water 30:70 (v/v).

The hydrolytic degradation of graft PHOD films at 37°C and pH 7.24 was followed over approximately 7 months (Domenek et al. 2007). The weight loss was recorded and compared with the weight loss of the nongrafted polymer PHOD under identical conditions. The PHOD sample was degraded into soluble fragments after 3 months, whereas the graft films remained stable during the same period of time. The results are shown in Fig. 7.

The rapid weight loss of the polymer films during the initial 2 weeks is correlated to the length of PEG and with the unreacted carboxylate groups. After this first period, the film weight remains approximately constant for 200 days. The graft polymer is much more resistant to hydrolysis, at physiological pH, than PHOD. The graft groups of PEG, although hydrophilic, do not favor the penetration of water towards the main chain and thus are capable of stabilizing the functionalized polymer. The unreacted carboxylate groups should be altered by the PEG grafts to act against hydrolysis.

## *3.4 Amphiphilic Block Copolymers*

Amphiphilic block copolymers have attracted great interest in biomaterials applications. From micelle formation to thermally induced gelation, these amphiphilic block copolymers find interesting applications in drug delivery and tissue engineering. Poly(ethylene

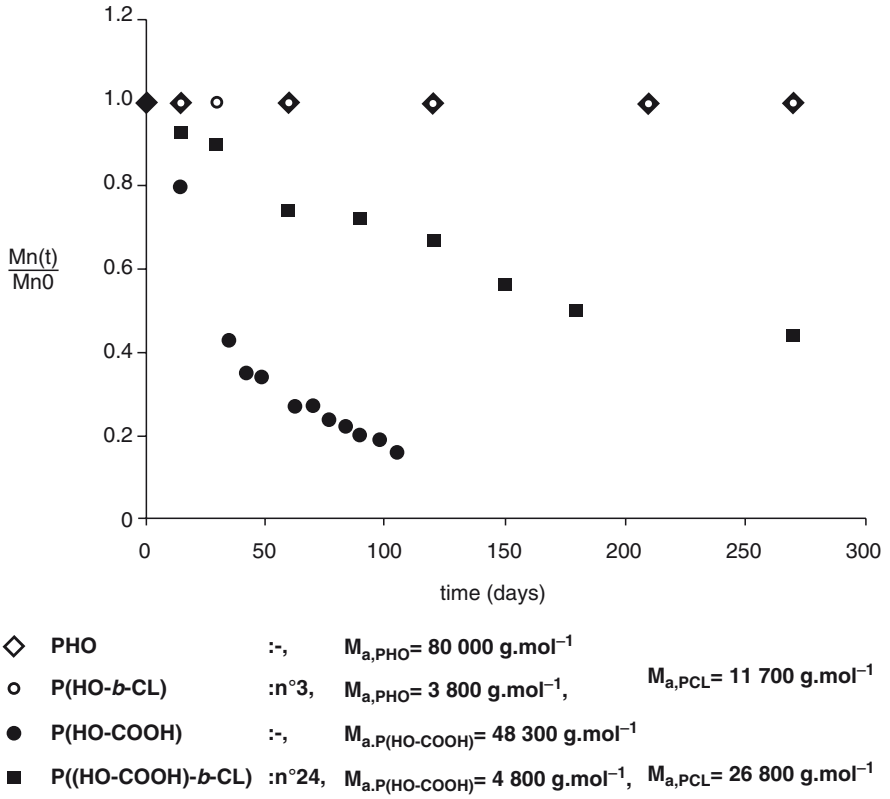


**Fig. 6** Hydrolytic degradation of different copolymers at pH 7.3 at 37°C (Timbart 2007)

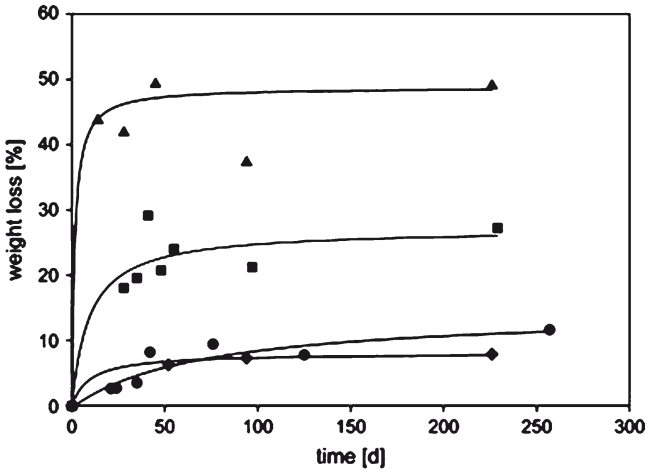


**Fig. 7** Weight loss of different poly($HO_{75}$–$COOH_{25}$) (PHOD)-*g*-poly(ethylene glycol) (PEG) films in phosphate buffer (pH 7.24, 37°C) with time: *circles* PHOD-*g*-PEG350 (grafting 52%), *squares* PHOD-*g*-PEG350 (grafting 28%), *diamonds* PHOD-*g*-PEG750 (grafting 29%), *triangles* PHOD-*g*-PEG2000 (grafting 19%)

oxide) (PEG), as a hydrophilic and biocompatible polyether, is widely used in biomedical research and applications. Amphiphilic ABA triblock copolymers consisting of PEG as the hydrophilic segments are particularly interesting. Recently, with biodegradability as a desirable feature, more attention has been focused on incorporating biodegradable segments as the hydrophobic block together with the hydrophilic PEG block.

### 3.4.1 PEG–PHB–PEG Copolymers

For synthesis of the PEG–PHB–PEG (EHE) triblock copolymers, (Li et al. 2003a, b) high molecular weight PHB was converted into telechelic hydroxyl-terminated PHB (PHB-diol) with lower molecular weights by transesterification with ethylene glycol in the presence of dibutyltin dilaurate (Hirt et al. 1996). The PHB-diol was then allowed to react with methoxy-PEG-monocarboxylic acid using dicyclohexylcarbodiimide to give the EHE triblock copolymers. Two series of triblock copolymers with PEG block lengths of $M_n$ 1,820 or 4,740 were synthesized. Each series of triblock copolymers has middle PHB block lengths ranging from a few hundred to more than 5,000, corresponding to PHB contents ranging from 8 to 59% in weight.

The crystallinity of the PHB block in the copolymers increases as compared with that of the pure PHB precursor, presumably being caused by the presence of the soft PEG block. In contrast, the crystallinity of the PEG block in the copolymers decreases as compared with that of the PEG precursor because of the presence of a hard PHB block, which restricted the crystallization of the PEG blocks. Triblock copolymers with lower PHB contents such as EHE(20-05-20), EHE(50-08-50), and EHE(50-38-50) are water-soluble (the numbers in parentheses show the indicative molecular mass of the respective block in 100 g mol$^{-1}$). Amphiphilic triblock copolymers can self-assemble to form micelles in an aqueous medium, and these micelles contain dense cores of the insoluble blocks, surrounded by diffuse outer shells formed by the soluble blocks. Li et al. (2005a, b) prepared a series of water-soluble EHE triblock copolymers and studied the micelle formation of the copolymers. Inclusion complexes of biodegradable amphiphilic EHE triblock copolymers with α-cyclodextrin or γ-cyclodextrin were prepared from an aqueous medium. The formation of inclusion complexes led to an increase in the thermal stability of both cyclodextrins and the triblock copolymers (Li et al. 2003b).

### 3.4.2 Poly(PHB/PEG urethane)s

Poly(ester urethane)s with PHB as the hard and hydrophobic segment and PEG as the soft and hydrophilic segment were synthesized from PHB-diol and PEG using 1,6-hexamethylene diisocyanate as a nontoxic coupling reagent (Li et al. 2005a). The bulk hydrophilicity and swelling property of poly(PHB/PEG urethane)s (PHE) were measured by water uptake. In general, the test films reach the equilibrium weight after 15 min of immersion in deionized water at 37°C. The equilibrium water uptake of natural-source PHB is only 5% owing to its hydrophobic nature. In contrast, for the poly(PHB/PEG urethane)s with identical PHB segments ($M_n$ = 1,740), the equilibrium

water uptake increases from 55% for PHE(17–20) to 575% for PHE(17–80) (PEG content more than 80 wt%), with increasing PEG segment length from 2,000 to 8,000.

### 3.4.3 PHB/PEG Diblock Copolymers

Catalyzed transesterification in the melt was used to produce diblock copolymers of PHB and monomethoxy PEG (mPEG) in a one-step process (Ravenelle et al. and Marchessault 2002, 2003). The rapid one-step synthesis described combines chain-cleaving and chain-coupling reactions, i.e., pyrolysis and transesterification in the absence of solvent. Bacterial PHB of high molecular weight is depolymerized by consecutive and partly simultaneous reactions: pyrolysis and transesterification. The formation of diblocks is accomplished by the nucleophilic attack from the hydroxyl end group of the mPEG catalyzed by bis(2-ethylhexanoate) tin. The resulting diblock copolymers are amphiphilic and self-assemble into sterically stabilized colloidal suspensions of PHB crystalline lamellae.

PHB/mPEG diblock copolymers do not generally self-assemble spontaneously in water because of the hydrophobicity and crystallization propensity of the PHB block; that is, the PHB block has no appreciable mobility in water to self-assemble. Once formed by evaporation of a solvent common to both blocks from a water suspension (oil-in-water emulsion), self-assembled structures are not in equilibrium with free chains because the PHB core is in a folded chain lamella arrangement, as was observed from X-ray diffraction. They are considered as “dead” or “frozen” organizations (Cameron et al. 1999). This is similar to systems where the core polymer block would be below its glass-transition temperature or where, after the self-assembly, it would have been cross-linked or a polymerizable function on one of the blocks would be polymerized after the self-assembly. The PHB folded chain lamella is sterically stabilized by the water-soluble mPEG segments.

## *3.5 Wettability of Surfaces*

The intrinsic hydrophobic properties of PHAs restrict their applications as cell colonizing materials. The surfaces of PHB and PHBV are quite inert and hydrophobic and have no physiological activity. This is unfavorable for adhered cell growth. Therefore, as for many polymer surfaces, the cytocompatibility should be improved by either chemical modification with functional groups or modification of the surface topography. Both parameters play an important role in the interaction between a biomaterial surface and cells, as illustrated by many reports focusing on this topic. Functionalization of the polymer is needed for tissue engineering (Tesema et al. 2005; Grondahl et al. 2005) or antibacterial activity of biomaterials (Hu et al. 2003; Yu et al. 2006). Effective chemical modifications include changes in chemical group functionality, surface charge, hydrophilicity, and wettability. Investigation of surface modification can be achieved by means of various chemical or physical processes, including graft polymerization using oxygen plasma treatment

(Kang et al. 2001), UV-induced photografting (Ma et al. 2002), γ-irradiation (Grondahl et al. 2005; Mitomo et al. 1995), and ozone treatment (Hu 2003; Yu et al. 2006).

The graft copolymerization of 2-hydroxyethyl methacrylate (HEMA) onto PHBHV films has been investigated (Renard et al. 2007). The graft copolymerization was conducted in aqueous media using benzoyl peroxide (BPO) as the chemical initiator. The reaction gives free poly(2-hydroxyethyl methacrylate) (PHEMA) and graft PHEMA; only approximately 5% of the monomer participates in graft polymerization and the remainder is homopolymerized. This trend is generally observed with 2,2′-azobis(isobutyronitrile), which is well known to be unable to abstract hydrogen from a polymer. BPO was insoluble in the reaction solvent (water); these heterogeneous conditions may explain the difficulty for the initiator to abstract a hydrogen atom from PHBV and migrate into the film. The hypothesis of the macroradical involvement in the reaction may be proved by performing a reaction without monomer. The radicals produced by hydrogen abstraction due to an initiation reaction will be involved in secondary reactions which can lead to a degradation of the polymer backbone or cross-linking. The molecular weight was studied as a function of the initiator concentration. The PHBV film was stirred with BPO in water at the reaction temperature (80°C) for 2 h. All samples were soluble in $CHCl_3$, indicating that no cross-linking reaction occurred. As expected $M_n$ and $M_w$ decreased with increasing concentration of the BPO, resulting in degradation of the macromolecular chains. These results suggest that BPO is able to abstract hydrogen from PHBV even in heterogeneous conditions. The molecular weights are sufficiently high to maintain good mechanical properties. Characterization of the grafted PHBV films assumed that the graft copolymerization not only occurred on the film surface but also took place in the film bulk. Introduction of hydroxyl groups obviously improved the wettability of the graft films. This way of surface modification of PHBV can be used for other monomer-containing chemical functions. This provides an opportunity to adapt the grafting strategy to a chosen wettability and functionality, therefore potentially improving their ability for cellular interaction.

The water contact angles were investigated to subjectively compare the hydrophobicity of native PHBV and PHEMA-grafted PHBV. The unreacted PHBV has a contact angle around 75°. This value shows that the unmodified PHBHV is hydrophobic. The contact angle decreased with increased grafting, indicating enhancement of the surface hydrophilicity. The grafting reaction occurs in the bulk of the film and becomes more prominent at the surface with an increase of the HEMA content in the feed. Consequently, the bulk of the graft films appears to be a mixture of PHBV-*g*-PHEMA and free PHBV. Saturation of the surface appears to be faster with thin films and high HEMA concentration.

## 4 Concluding Remarks and Future Perspectives

The development of PHAs has gained more importance owing to the relatively new concerns of our societies: ecology, sustainable development, biomedical engineering. These bacterial polyesters are synthesized from renewable resources, are biodegradable

to water and carbon dioxide, and are considered as environmentally friendly alternatives to petrol-based polymers. Different sources (natural isolates, recombinant bacteria, plants) and other methods are being investigated to exert more control over the quality, quantity and economics of PHB production. An extensive amount of knowledge on the biochemistry of PHA synthesis and on its biodegradation has been accumulated during the last two decades. Numerous genes encoding enzymes involved in the formation of PHA and in PHA degradation (PHA depolymerases) have been cloned and characterized from many microorganisms. A few PHAs, such as PHB and copolymers of 3HB and 3HV, are already produced industrially on a scale of a few hundred tons per year by several plants all over the world. Presumably, several other PHAs will enter the commercial market in the near future. But chemists and biologists will have to complete the work of Mother Nature. Concerning PHAs materials, it will be difficult to find in the soil the appropriate bacterium able to synthesize an adequate polymer with specific properties for a given application. Genetic and structural studies will be necessary to improve our understanding of the mechanism of action of these enzymes and aid us in improving and selecting better candidates for increased and diversified productions. Novel pathways for the synthesis of PHA will be provided and methods of synthesizing recombinant PHA monomer synthases will be also provided. These recombinant PHA synthases can be derived from multifunctional fatty acid synthases and generate hydroxyacyl acids capable of polymerization by a PHA synthase. *E. coli*, as the best known bacterium, is an ideal host for the production of PHAs. It is suitable as a heterologous expression host for foreign genes that can be easily manipulated and improved by means of recombinant DNA methodologies or metabolic engineering. In addition, high-cell-density cultivation strategies for numerous *E. coli* strains are well established. Metabolic pathways of PHAs in *E. coli*, including $PHA_{scl}$ and $PHA_{mcl}$, were set up 10 years ago).

However, the production of PHAs employing recombinant *E. coli* was restrained on both the laboratory scale and the industrial scale owing to low efficiency and high cost.

In the same manner, chemical modifications will be a strategy to enlarge the spectrum of degradable materials.

Concerning hydrolysis of PHAs by PHA depolymerase systems, the situation is also very complex owing to the existence of extracellular and intracellular depolymerases corresponding to two different morphologies of PHAs. Moreover, these enzymes are specific to one type of polymer structure and their activity and efficiency are very versatile. Biodegradation is not simple compared with pure hydrolysis. Research in this area is sill at the beginning and it will be some time before industrial or household applications are realized.

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