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Natural Functions and Applications

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Editor

Plastics from Bacteria

Natural Functions and Applications

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Preface

Due to the possibility that petroleum supplies will be exhausted in the next decades to come, more and more attention has been paid to the production of bacterial plastics including polyhydroxyalkanoates (PHA), polylactic acid (PLA), poly(butylene succinate) (PBS), biopolyethylene (PE), poly(trimethylene terephthalate) (PTT), and poly(p-phenylene) (PPP). These are well-studied polymers containing at least one monomer synthesized via bacterial transformation.

Among them, PHA, PLA and PBS are well known for their biodegradability, whereas PE, PTT and PPP are probably less biodegradable or are less studied in terms of their biodegradability. Over the past years, their properties and applications have been studied in detail and products have been developed. Physical and chemical modifications to reduce their cost or to improve their properties have been conducted.

PHA is the only biopolyester family completely synthesized by biological means. They have been investigated by microbiologists, molecular biologists, biochemists, chemical engineers, chemists, polymer experts, and medical researchers for many years. PHA applications as bioplastics, fine chemicals, implant biomaterials, medicines, and biofuels have been developed. Companies have been established for or involved in PHA related R&D as well as large scale production. It has become clear that PHA and its related technologies form an industrial value chain in fermentation, materials, feeds, and energy to medical fields.

In this monograph, *Dr. Yaacov Okon* and his lab focus their attention on PHA as energy and intracellular carbon storage compounds that can be mobilized and used when carbon is a limiting resource. They describe the phenomena of intracellular accumulation of PHA, which enhances the survival of several bacterial species under environmental stress conditions imposed in water and soil, such as UV-irradiation, salinity, thermal and oxidative stress, desiccation, and osmotic shock. The ability to endure these stresses is linked to a cascade of events concomitant with PHA degradation and the expression of the genes involved in protection against damaging agents.

Dr. Sang Yup Lee reviews the strategies for the metabolic engineering of PHA producers, genomic and proteomic studies performed to understand the PHA biosynthesis in the context of whole cell metabolism and to develop further engineered strains. Finally, he suggests strategies for systems metabolic engineering of PHA

producers, which will make it possible to produce PHA with higher efficiencies and to develop tailor-made PHAs by systems-level optimization of metabolic network and establishment of novel pathways.

Drs. Martin Koller and Gerhart Braunegg suggest that facilities for the production of biopolymers, biofuels and biochemicals should be integrated into existing production lines, where the feedstock directly accrue as waste streams, to save costs on transportation. They point out that the utilization of waste streams for production of value-added products not only enhances the economics of such products, but also provides the industry with a strategy to overcome disposal problems.

Dr. José M. Luengo found that unusual polyhydroxyalkanoates (UnPHAs) constitute a particular group of polyoxo(thio)esters belonging to the PHA family, which contain uncommon monomers. He classifies the UnPHAs into four classes and discusses some of their characteristics and biotechnological applications.

Despite some successes in PHA production by plants, *Drs. Yves Poirier and Stevens Brumbley* believe production of PHA in crops and plants remains a challenging project. The challenges for the future are to succeed in the synthesis of PHA co-polymer with a narrow range of monomer composition, at levels that do not compromise plant productivity, and to find methods for efficient and economical extraction of polymers from plants. These goals will undoubtedly require a deeper understanding of plant biochemical pathways and advances in biorefinery.

Dr. Manfred Zinn focuses on the production of medium-chain-length poly[(R)-3-hydroxyalkanoates] (mcl-PHAs) in pseudomonads. He reviews the biosynthesis of mcl-PHA in high cell density cultures that is economically very important..

Dr. Isao Noda has been working on the family of PHA called Nodax™ that consists of *(R)*-3-hydroxyalkanoate comonomer units with medium size chain side groups and *(R)*-3-hydroxybutyrate. Because of the unique design of their molecular structure, the Nodax™ class PHA copolymers have a set of useful attributes, including polyolefin-like thermo-mechanical properties, polyester-like physicochemical properties, and interesting biological properties. Therefore, a broad range of industrial and consumer product applications are anticipated.

Drs Tadahisa Iwata and Toshihisa Tanaka succeeded in obtaining strong fibers, using two new ways of drawing techniques, from microbial PHA polyesters produced by both wild-type and recombinant bacteria. The improvement in the mechanical properties of the fibers is due not only to the orientation of molecular chains but also to the generation of a planar zigzag conformation. They present the processing, mechanical properties, molecular and highly-ordered structure, enzymatic degradation, and bioabsorption of strong fibers and nanofibers produced from microbial polyesters.

Drs. Philippe Guérin, Estelle Renard and Valérie Langlois found that all polyesters are susceptible to degradation by simple hydrolysis to some extent. The degradation rate is highly dependent on the chemical structure and material crystallinity. One way to obtain more hydrophilic PHA is to introduce specific functions in the macromolecular side chains. The combination of bioconversion and organic chemistry allows modulating the physical properties of these bacterial polyesters as solubility,

hydrophilic/hydrophobic balance, and water stability in the perspective of biomedical applications.

Drs. K. Jim Jem, Johan F. van der Pol and Sicco de Vos calculated lactic acid derivatives back to the equivalent amount of original lactic acid, and they concluded that the total global market volume in 2008 is estimated at around 260,000 metric tons of lactic acid (calculated at 100% concentration) for traditional applications (excluding PLA). They claim that today more than 95% of lactic acid produced is derived from biological sources (e.g. sucrose or glucose from starch) by microbial fermentation, which typically produces the L(+) form of lactic acid.

Besides lactic acid, *Dr Jun Xu* opines that increasing demand on biodegradable poly(butylene succinate) (PBS) will open a new market for succinic acid produced via microbial fermentation. He reviews the synthesis of succinic acid, PBS polymerization, crystalline structure, thermal and mechanical properties, and biodegradability.

Polyethylene is an important engineering material. It has been traditionally produced through the ethylene polymerization process. Ethylene can be produced through steam cracking of ethane, steam cracking of naphtha or heavy oils, or ethanol dehydration. With the increase in oil prices, bio-ethylene, produced through ethanol dehydration, is a more important production route for ethylene. Based on the ethanol dehydration chemistry principle, Dr. He Huang describes the research and development progress on catalysts and the process of ethanol dehydration to form ethylene.

Poly(trimethylene terephthalate) (PTT) fiber, as a new type of polyester, has been characterized by much better resilience and stress/recovery properties than poly(ethylene terephthalate) (PET) and poly(butylene terephthalate) (PBT). *Dr Dehua Liu* proved that PTT is highly suitable for uses in fiber, carpet, textile, film, and engineering thermoplastic applications. With this in mind, his lab has developed highly efficient fermentation technology based on glycerol for 1,3-propandiol (PDO) which is a monomer of PTT.

Benzene *cis*-diols, namely, *cis*-3,5-cyclohexadien-1,2-diols abbreviated as DHCD, can be used for synthesis of poly(*para*-phenylene) (PPP), which is a material with high thermal stability and electricity conducting ability when doped. Several types of bacterial dioxygenases, that can catalyze the conversion of aromatic compounds to their corresponding *cis*-diols, which can be polymerized to form PPP, are discussed.

With the support of the above experts, we are able to offer the readers up-to-date information on the bacterial plastics. We are grateful to the authors who have contributed these excellent chapters. Our thanks also go to Springer for publishing this monograph, especially to Jutta Lindenborn for all his/her effort in helping us.

Finally, I (George Guo-Qiang Chen) would also like to thank my wife Sherry Xuanming Xu and daughter Jenny Jiani Chen for supporting my effort to bring out this monograph.

Beijing
and Münster

George Guo-Qiang Chen
Alexander Steinbüchel

Contents

Introduction of Bacterial Plastics PHA, PLA, PBS, PE, PTT, and PPP

Guo-Qiang Chen

Contents

Abstract Polyhydroxyalkanoates (PHA), poly(lactic acid) (PLA), poly(butylene succinate) (PBS), polyethylene (PE), poly(trimethylene terephthalate) (PTT), and poly(*p*-phenylene) (PPP) are the best studied polymers containing at least one monomer synthesized via bacterial transformation. Among them, PHA, PLA, and PBS are well known for their biodegradability, whereas PE, PTT and PPP are probably less biodegradable or have been less studied in terms of their biodegradability. Over the past few years, their properties and applications have been studied in detail, and products have been developed. Physical and chemical modifications to reduce their cost or improve their properties have been conducted. Throughout this book, you will find more a detailed description of these bacterial plastics.

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1 Introduction

Over the past few years, bio-based plastics have been developed rapidly owing to rising petroleum prices and many environmental concerns related to plastic pollution. Increasingly, reduction of carbon dioxide emissions has become another reason for promoting bio-based plastics amid the worldwide financial tsunami.

Generally speaking, bio-based plastics include starch-based plastics, protein (soybean protein) based plastics, and cellulose-blended plastics. They can also be blended with conventional plastics such as polyethylene (PE), polypropylene (PP), and poly(vinyl alcohol). However, such bio-based plastics are only partially biodegradable. The residual petroleum-based plastics remain as broken pieces, creating additional pollution. In addition, these plastics have intrinsic thermal and mechanical weaknesses, and they are now discouraged for applications.

To produce bio-based plastics completely resembling conventional plastics, bacteria are employed to make the building blocks for plastic polymers from renewable sources, including starch, cellulose, fatty acids, and whatever bacteria can consume for growth.

So far, the following building blocks can be produced microbially for polymerization purposes: hydroxyalkanoic acids with many structural variations, lactic acid, succinic acid, (*R*)-3-hydroxypropionic acid, bioethylene produced from dehydration of bioethanol, 1,3-propanediol, and *cis*-3,5-cyclohexadiene-1,2-diols from microbial transformation of benzene and other chemicals. They have been successfully used for making various bacterial plastics.

In this chapter, we will give an overview of these bacterial plastics.

2 Monomers of Bacterial Plastics Synthesized by Microorganisms

Six types of monomers produced by microbial fermentation are the most common bio-based polymer building blocks (Fig. 1). Among them, hydroxyalkanoates have rich structural variations, *n* can be 0–5, and R can be alkyl to benzyl. However,

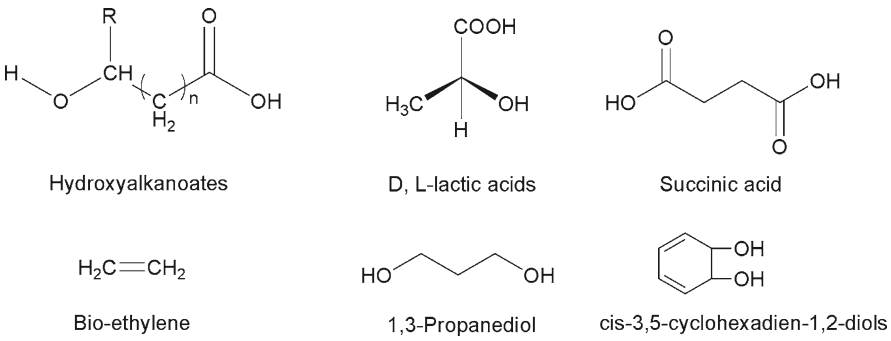

Fig. 1 Most common bio-based polymer building blocks for bacterial plastics

hydroxyalkanoates normally do not appear as monomers alone; they mostly exist as polyhydroxyalkanoates (PHA). Unless specially required, monomer hyrdoxyalkanoic acids will not be produced by microorganisms. However, with use of genetic engineering and low- or high-pH incubation, various hydroxyalkanoates can be produced (Chen and Wu 2005a)

3 Polymerization of the Bacterial Plastics

Except for polymerization of hydroxyalkanoates, which is conducted in vivo, all other monomers are polymerized in vitro by chemical reactions, leading to the formation of PHA, poly(lactic acid) (PLA), poly(butylene succinate) (PBS), PE, poly(trimethylene terephthalate) (PTT), and poly(*p*-phenylene) (PPP) (Fig. 2).

4 Comparison of Bacterial Plastics

Although these plastics are bio-based, their properties are very similar to those of traditional petroleum-based plastics. Like PE based on bioethanol (leading to bioethylene), they are exactly the same as petroleum-based PE (Table 1)

4.1 Thermal Properties and Mechanical Properties

PHA have the most diverse structural varieties, resulting in the most variable melting temperature (T_m), glass-transition temperature (T_g), and thermodegradation temperature ($T_{d(5\%)}$), ranging between 60 and 177, −50 and 4, and 227 and 256°C, respectively (Steinbüchel 1991; Doi et al. 1995; Wang et al. 2009; Spyros and Marchessault 1996; Galegoa et al. 2000). The mechanical properties include a very flexible Young's modulus, an elongation at break ranging from 2 to 1,000%, and a tensile strength of 17–104 MPa (Table 1).

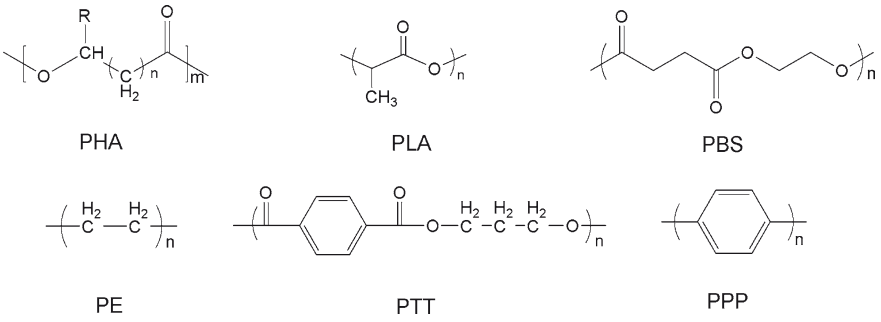

Fig. 2 Most common bio-based polymer molecular structures

Table 1 Comparison of various properties of bacterial plastics

Bacterial plastics	Biosynthesized monomers	Polymerization approach	$M_w \times 10^4$	Polydispersity	T_m (°C)	T_g (°C)	$T_{d(5\%)}$ (°C)	Mechanical properties			References
								Young's modulus (MPa)	Elongation at break (%)	Tensile strength (MPa)	
PHA	Hydroxyalkanoates	Biological	10–1000	1.2–6.0	60–177	−50–4	227–256	Flexible	2–1000	17–104	See text
PLA	D,L-Lactic acids	Chemical	5–50	1.8–2.6	175	60	339	384–481	5.2–2.4	49.6–61.6	See text
PBS	Succinic acid	Chemical	3–20	2.0–6.3	115.8–146.5	−36.6 to −33	353	268.0	175.2	24.8	See text
PE	Bioethylene	Chemical	10–600	2.1–6.8	136.4	33.4.3	371	102	297.7	22–29	See text
PTT	1,3-Propanediol	Chemical	3.8	2	227.55	42.6	364	727.88	159.48	49.24	See text
PPP	*cis*-3,5-Cyclohexadiene-1,2-diols	Chemical	0.4–33	1.5–3.6	ND	173–232	380	ND	ND	ND	See text

M_w molecular weight measured by gel permeation chromatography, $T_{d(5\%)}$ temperature at 5% weight loss determined by thermogravimetric analysis, T_g glass-transition temperature, T_m melting temperature, *PHA* polyhydroxyalkanoates, *PLA* poly(lactic acid), *PBS* poly(butylene succinate), *PE* polyethylene, *PTT* poly(trimethylene terephthalate), *PPP* poly(*p*-phenylene), *ND* not detectable

In comparison, the low-cost PLA is brittle with an elongation at break of 5.2–2.4%, yet its tensile strength is the highest among these bacterial plastics, ranging from 49.6 to 61.6 MPa, together with a Young's modulus of 384–481 MPa (Suyatma et al. 2004; Zenkiewicz et al. 2009; Aji et al. 2005; Zhang and Sun 2004; Maiardo et al. 2003; Ljungberg and Wesslen 2002; Table 1). A T_g of 60°C has been a weak point for PLA application as articles made from PLA change shape at this temperature. In this case, a more diversified PHA seem to be more useful.

PBS has T_m and T_g ranging between 116 and 147, −33 and 37, and 227 and 256°C, respectively, depending on the copolymer composition ratios. $T_{d(5\%)}$ of PBS is constant at 353°C (Jin et al. 2000a; Shibata et al. 2006; Kim et al. 2001; Velmathi et al. 2005; Gan et al. 2001). Young's modulus, the elongation at break, and the tensile strength of PBS are around 268 MPa, 175%, and 25 MPa, respectively. In addition, PBS is quite thermostable, with a $T_{d(5\%)}$ of 353°C. This is a quite flexible material with considerable strength for many applications (Table 1).

PE based on bioethanol has the same characteristics as petroleum-based PE, it also has a high elongation at break of 298%, with a T_g of 33.4°C. The bio-PE can be used in the same way as petroleum-based PE (Zhao et al. 2005; Yeh et al. 2000; Liu et al. 2002; Luyta and Geethamma 2007; Wei et al. 2006).

Two half-bio-based and half-petroleum-based polymers are PTT and PPP. PTT is a reasonable elastic material (159% elongation at break; Chiu and Ting 2007; Pisitsak and Magaraphan 2009), with the highest Young's modulus among all bacterial plastics mentioned here, whereas PPP is a very brittle polymer without a T_m. Owing to its brittle property, one cannot obtain the Young's modulus, elongation at break, and tensile strength of PPP. PPP, like other electricity-conducting polymers, is difficult to process. Modification to change the PPP structure is needed to increase its application potential (Li et al. 2008a, b; Ballard et al. 1988).

4.2 Molecular Weights

PHA have the most diverse structural varieties, resulting in the most variable molecular weights, ranging from 10×10^4 to 10×10^6, with a polydispersity of 1.2–6.0 (Table 1; Steinbüchel 1991; Doi et al. 1995; Wang et al. 2009; Spyros and Marchessault 1996; Galegoa et al. 2000). In contrast, it was not so easy to get a high M_w for PLA: normally, PLA has M_w ranging from 5×10^4 to 50×10^4, with a polydispersity of 1.8–2.6 (Suyatma et al. 2004; Zenkiewicz et al. 2009; Aji et al. 2005; Zhang and Sun 2004; Maiardo et al. 2003; Ljungberg and Wesslen 2002). With PBS, M_w of 3×10^4–20×10^4 have been achieved with a wide polydispersity of 2.0–6.3 (Jin et al. 2000a; Shibata et al. 2006; Kim et al. 2001; Velmathi et al. 2005; Gan et al. 2001). For bio-PE, the same M_w can be obtained as for petroleum-based PE since their polymerization processes are exactly the same (Zhao et al. 2005; Yeh et al. 2000; Liu et al. 2002; Luyta and Geethamma 2007; Wei et al. 2006).

The synthesis of PTT with a low M_w of 3.8×10^4 to achieve the above-mentioned manageable thermal and mechanical properties has been reported (Table 1; Chiu and Ting 2007; Pisitsak and Magaraphan 2009). Therefore, it may not be necessary to make PTT with a super-high M_w. In addition, since PPP has a highly crystallized structure, M_w cannot affect its thermal and mechanical properties much (Li et al. 2008a, b; Ballard et al. 1988).

4.3 Biodegradability

Enzymes and microorganisms such as bacteria and fungi are involved in the degradation of both natural and synthetic plastics (Gu et al. 2000; Table 2). The biodegradation of bacterial plastics proceeds actively under different soil conditions according to their different properties. PHA is one of the natural plastics. Microorganisms which can produce and store PHA under nutrient-limited conditions can normally degrade and metabolize it when the carbon or energy source is limited (Williams and Peoples 1996; Table 2). The biodegraded product of poly(3-hydroxybutyrate) is (*R*)-3-hydroxybutyric acid (Doi et al. 1992), whereas extracellular degradation of poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyvalerate] yields both (*R*)-3-hydroxybutyrate and (*R*)-3-hydroxyvalerate (Luzier 1992). The degradable PHA monomers are watersoluble and small enough to passively diffuse through the cell wall. They can also be metabolized by β-oxidation and the tricarboxylic acid cycle of many organisms to produce carbon dioxide and water under aerobic conditions (Scott 1999; Sun et al. 2007).

Abiotic hydrolysis is the most important reaction for initiating the environmental degradation of synthetic polymers (Göpferich 1997) such as PE (Gu 2003), PTT (Heidary and Gordon 1994), PLA, and their copolymers (Hiltunen et al. 1997; Nakayama et al. 1996). The degradation of most synthetic plastics in nature is slower than that of natural polyesters. This process involves environmental factors, followed by the action of microorganisms in their surroundings (Albertsson et al. 1994; Cruz-Pinto et al. 1994).

PLA is fully biodegradable under the composting condition in a large-scale operation with temperatures of 60°C and above (Pranamuda and Tokiwa 1999). PBS is hydro-biodegradable and begins to biodegrade via a hydrolysis mechanism. Hydrolysis occurs at the ester linkages, which reduce the polymer molecular weights, allowing for further degradation by many more microorganisms (Aamer et al. 2008). Biodegradation of PE occurs via two mechanisms: hydro-biodegradation and oxo-biodegradation (Bonhomme et al. 2003).

4.4 Structural and Property Modification

Bacterial plastics have some weaknesses that need to be addressed. Modification of their structures can normally bring about the expected results. Chemical and physical modifications are commonly adopted to improve their properties (Table 3).

Table 2 Microorganisms reported to degrade the bacterial plastics and some applications of bacterial plastics

Bacterial plastics	Biodegradability		Applications	
	Microorganisms to biodegrade	References	Applications	References
PHA	*Pseudomonas lemoignei*; *Alcaligenes faecalis*; *Schlegelella*; *Thermodepolymerans*; *Clostridium botulinum*; *Clostridium acetobutylicum*	Jendrossek et al. (1995), Kita et al. (1997), Romen et al. (2004), Abou-Zeid et al. (2001)	Packaging materials, agricultural applications, medical devices and disposable personal hygiene products, as a material for tissue engineering scaffolds and for controlled drug release carriers	Chen and Wu (2005a, b), Philip et al. (2007)
PLA	*Fusarium moniliforme*; *Penicillium roquefort*; *Amycolatopsis* sp.; *Bacillus brevis*; *Rhizopus delemer*	Torres et al. (1996), Pranamuda et al. (1997), Pranamuda and Tokiwa (1999), Tomita et al. (1999), Fukuzaki et al. (1989)	Packaging and paper coatings; other possible markets include sustained release systems for pesticides and fertilizers, mulch films, and compost bags	Plastics recycling-Economic and Ecological Options (2006)
PBS	*Alcaligenes faecalis*; *Pseudomonas stutzeri*; *Comamonas acidovorans*	Kasuya et al. (1999)	Packaging materials, dishware, fibers, agricultural film materials, medical materials	Liu et al. (2009), Bhatia et al. (2007), Lee and Wang (2006)
PE	*Brevibacillus borstelensis*; *Rhodococcus rubber*; *Penicillium simplicissimum* YK	Hadad et al. (2005), Sivan et al. (2006), Gilan et al. (2004), Yamada-Onodera et al. (2001)	Plastic bags, milk and water bottles, food packaging film, toys, irrigation and drainage pipes, motor oil bottles	Vona et al. (1965), Aamer et al. (2008)
PTT	*Trichosporum*; *Arthrobacter*; *Aspergillus niger*; *Rhizopus delemar*	Huang and Byrne (1980), Witt et al. (1995), Nagata et al. (1997), Müller et al. (2001)	Carpet fiber, textiles, films, engineering thermoplastics, electronic connectors, wire harnesses	Yang et al. (2002), Chen et al. (2004), Chuah et al. (2001)
PPP	No relative data	None	Light-emitting diodes and polymer lasers in the military industry	Mitschke and Bäuerle (2000), Li et al. (2008a, b), McGehee and Heeger (2000)

Table 3 Structural and property modification of the bacterial plastics

Bacterial plastics	Structural and property modification			
	Chemical modification	References	Physical modification	References
PHA	Block copolymerization and grafting reactions, chlorination, cross-linking, epoxidation, and hydroxyl and carboxylic acid functionalization of the PHA	Chen et al. (2009), Wu et al. (2008), Li et al. (2003), Loh et al. (2007), Baki and Steinbüchel (2007)	Blends with natural fibers, PLA, PCL, and difference types of PHA, even with inorganic particles, such as bioglass and tricalcium phosphate	Avella et al. (2000), Urakami et al. (2000), Misra et al. (2006), Luo et al. (2007)
PLA	PLA-based block copolymers include diblock, triblock, and multiblock copolymers	Jeon et al. (2003), Chen et al. (2003), Pospiech et al. (2005)	Blend with starch, other polyesters, and low molecular weight plasticizers such as glycerol, sorbitol, and triethyl citrate	Cargill (2007)
PBS	Copolymerization has been used to improve its properties, such as poly(ethylene terephthalate-*co*-1, 4-butylene succinate) block copolymers	Darwin et al. (2003), Jin et al. (2000b, c, d, 2001)	Generally blended with other polyesters and compounds, such as PLA, PEO, protein, starch (TPS), and adipate copolymers	Aamer et al. (2008), Qiua et al. (2003), Harada et al. (2007), Li et al. (2008a, b)
PE	Incorporation of polar segments into PE backbone make PE biodegradable and self-assemble, such as PE-*b*-PEO copolymer and polystyrene-*b*-PE-*b*-poly(ε-caprolactone)	Inoue and Matyjaszewski (2004), Sun et al. (2004), Balsamo et al. (1998)	Biodegradable and engineering plastics added to modify its crystallinity and mechanical properties, such as starch, polycarbonate, and PET	Zhong and Yang (2004), Pan et al. (2006)
PTT	Incorporation of other adipic acids, such as sebacic acid, trimethylene isophthalate, *p*-cetoxybenzoic acid, and ethylene glycol units	Seo et al. (2006), Chen et al. (2007), Wei et al. (2006), Ou (2002)	Blended with polyesters to develop high-performance materials, including crystalline engineering thermoplastic polymers, amorphous engineering thermoplastic polymers, and thermoplastic elastomers	Nadkarni and Rath (2002), Run et al. (2007), Krutphan and Supaphol (2005), Jafari et al. (2005), Yavari et al. (2005)
PPP	Copolymers with partial substitution, for example, the random copolymer with unsubstituted phenylene, unsubstituted bromobenzene boronic acids, and unsubstituted phenylenes	Chaturvedi et al. (1992), Jing et al. (1994), Bao and Yu (1995), Savenkova et al. (2000)	Blending the violet–blue-emitting copolymer poly(*N*-vinyl carbazole)	Birgerson et al. (1996a), Birgerson et al. (1996b), Salaneck (1997), Edwards et al. (1997), Huang et al. (1996)

PCL poly(ε-caprolactone), *PEO* poly(ethylene oxide), *TPS* theromplastic starch

4.4.1 Chemical Modification

A number of studies were made to produce structural alterations along the chain of bacterial plastics to improve the properties, among which copolymerization has been of primary interest (Aamer et al. 2008; Baki and Steinbüchel 2007; Hossein et al. 2008; Grimsdale and Müllen 2006; Table 3).

PHA chemical modification can be done via block copolymerization and grafting reactions, chlorination, cross-linking, epoxidation, hydroxyl and carboxylic acid functionalization, etc. (Chen et al. 2009; Wu et al. 2008; Li et al. 2003; Loh et al. 2007). A common approach to confer toughness to PLA is the use of a flexible monomer or macromolecules for copolymerization with lactide to form PLA-based random or block copolymers. Reported PLA-based block copolymers include diblock, triblock, and multiblock copolymers, such as poly(L-lactic acid) (PLLA)–polycaprolactone (Jeon et al. 2003), poly(ethylene glycol)–PLLA (Chen et al. 2003), poly(trimethylene carbonate)–PLLA (Tohru et al. 2003), and PLA-PBS-PLA.

PBS is one of the members of the family of biodegradable aliphatic polyesters. A number of copolymerization attempts have been made to improve its properties (Darwin et al. 2003; Jin et al. 2000b, c, d, 2001). For example, Jin et al. (2000b, c) introduced phenyl units into the side chain of PBS, leading to better biodegradability of the copolyesters. Jung et al. (1999) successfully synthesized new PBS copolyesters containing alicyclic 1,4-cyclohexanedimethanol.

Wilt et al. (1994a, b) copolymerized terephthalic acid with aliphatic diols and diacids. Incorporation of trimethylene isophthalate (Seo et al. 2006), *p*-cetoxybenzoic acid (Ou 2002), and ethylene glycol (Chen et al. 2007; Wei et al. 2006) into the macromolecular structure of PTT improved the mechanical or thermal performances of PTT.

One way to modify PPP is to prepare copolymers with only partial substitution. Jing et al. (1994) prepared a random copolymer (Chaturvedi et al. 1992) with 33% of unsubstituted phenylene units by copolymerization of the substituted and unsubstituted bromobenzene boronic acids. Copolymers (Tanigaki et al. 1996) with alternating substituted and unsubstituted phenylenes were obtained by Stille (Bao and Yu 1995; Savenkova et al. 2000) or Suzuki coupling, both resulting in reduction of brittleness of PPP.

4.4.2 Physical Modification

The advantage of blending bacterial plastics with other polymers is to offset the relatively high cost and to further improve the physical properties, tailoring the plastic to a specific performance–cost profile (Table 3).

Blending PHA with high or low molecular weight molecules helps improve their material properties. It also helps to reduce production costs (Savenkova et al. 2000). PHA blended with natural fibers were found to have some improvements in their mechanical properties (Avella et al. 2000; Urakami et al. 2000). PHA composites

containing inorganic phases help enhance the mechanical properties of PHA, affecting the degradation rate and bioactivity (Misra et al. 2006). PLA is often blended with starch to increase biodegradability and to reduce costs. However, the brittleness of the starch–PLA blend is a major drawback in many applications. To remedy this limitation, a number of low molecular weight plasticizers, including glycerol, sorbitol, and triethyl citrate, have been used (Cargill 2007). PBS is generally blended with other compounds, such as starch (themoplastic starch) and adipate copolymers [to form poly(butylene succinate-*co*-butylene adipate)], to make its use economical. PBS has excellent mechanical properties and can be applied in a range of end applications via conventional melt processing techniques (Aamer et al. 2008).

Readily biodegradable compounds such as starch are added to a PE matrix to enhance the degradation of the carbon–carbon backbone of PE (Griffin 1977, Pan et al. 2006). The biodegradability of starch–PE blends and chemically modified samples of blends has been investigated (Johnson et al. 1993; Bikiaris et al. 1998). Similar to PE, PTT that are blended with polyesters to develop high-performance materials chiefly include crystalline engineering thermoplastic polymers (ETPs), amorphous ETPs, and thermoplastic elastomers (Nadkarni and Rath 2002; Run et al. 2007; Krutphan and Supaphol 2005; Jafari et al. 2005; Yavari et al. 2005). Also, blending is a useful method for tuning the emission from substituted PPP. Salaneck and coworkers found that blending a violet–blue-emitting copolymer with a blue–green emitter (Birgerson et al. 1996a, b; Salaneck 1997). Edwards et al. (1997) reported that blending with poly(*N*-vinyl carbazole) produced a redshift in the emission wavelength, with the maximum moving (Huang et al. 1996).

4.5 Applications

Global interest in bacterial plastics is very high because of their uses as packaging materials (Chen 2009), in medical devices (Chen and Wu 2005b), in disposable personal hygiene products, and as agricultural mulching films as a substitute for synthetic polymers such as PP and PE (Ojumu et al. 2004; Lee 1996; Table 2). PHA have been exploited as bioplastics, fine chemicals, implant biomaterials, medicines, biofuels, and even for improving the robustness of industrial microorganisms and regulating bacterial metabolism (Chen 2009; Takaku et al. 2006). The applications for PLA are mainly as thermoformed products such as drink cups, take-away food trays, containers, and planter boxes. The PLA material has good rigidity, allowing it to partially replace polystyrene and PET in some applications. Applications include mulch film, packaging film, bags, and ‘flushable’ hygiene products (Aamer et al. 2008).

PTT has been targeted for use as carpet fibers. Its combination of stain resistance and resilience with basic polyester features extends its use to other markets, including textiles, films, and now engineering thermoplastics (Chuah 2004; Chen et al. 2004). Bio-PE will mainly penetrate the existing petro-PE market for use as plastic

bags, milk and water bottles, food packaging film, toys, irrigation and drainage pipes, and motor oil bottles (Aamer et al. 2008).

PPP is one of the most important classes of conjugated polymers and has been the subject of extensive research, particularly as active materials for light-emitting diodes (Mitschke and Bäuerle 2000; Li et al. 2008a, b) and polymer lasers (McGehee and Heeger 2000).

5 Conclusion and Future Perspectives

The marketing potential for PHA, PLA, PBS, PE, PTT, and PPP and possibly others that come from reactions involving bacteria is getting more and more mature. Throughout this book, you will learn many more details about their production and applications. In the future, cost and effectiveness will also be a very important factor deciding the marketing success of these plastics.

Blending of the above-mentioned bacterial plastics 1:1 or 1:2 may bring about more improvements in their properties. Involvement of starch or cellulose that can bring down the cost of the bacterial plastics should be an area to which attention should be paid. The rapid expansion of PLA applications has shown how important cost is for the success of a bio-based polymer. In the end, it is not easy to convince customers to pay more for environmental reasons if they have a cheaper choice.

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Contents

Abstract Polyhydroxyalkanoates (PHA) produced by many bacteria have been investigated by microbiologists, molecular biologists, biochemists, chemical engineers, chemists, polymer experts, and medical researchers over the past many years. Applications of PHA as bioplastics, fine chemicals, implant biomaterials, medicines, and biofuels have been developed. Companies have been established or involved in PHA-related R&D as well as large-scale production. PHA synthesis has been found to improve the robustness of non-PHA-producing microorganisms and to regulate bacterial metabolism, leading to yield improvement for some bacterial fermentation products. In addition, amphiphilic proteins related to PHA synthesis including PhaP, PhaZ, and PhaC have been found to be useful for achieving protein

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purification and even specific drug targeting. It has become clear that PHA and its related technologies are forming an industrial value chain ranging from fermentation, materials, and energy to medical fields.

1 Introduction

Polyhydroxyalkanoates (PHA), a family of biopolyesters with diverse structures, are the only bioplastics completely synthesized by microorganisms. PHA can be synthesized by over 30% of soil-inhabiting bacteria (Wu et al. 2000). Many bacteria in activated sludge, in high seas, and in extreme environments are also capable of making PHA. In the last 10 years, PHA have been developed rapidly to find applications in various fields (Fig. 1) (Chen 2009a).

PHA have rich properties depending on the structures (Figs. 2, 3). Homopolymers, random copolymers, and block copolymers of PHA can be produced depending on the bacterial species and growth conditions. With over 150 different PHA monomers being reported, PHA with flexible thermal and mechanical properties have been developed (He et al. 1999). Such diversity has allowed the development of various applications, including environmentally friendly biodegradable plastics for packaging purposes, fibers, biodegradable and biocompatible implants, and controlled drug

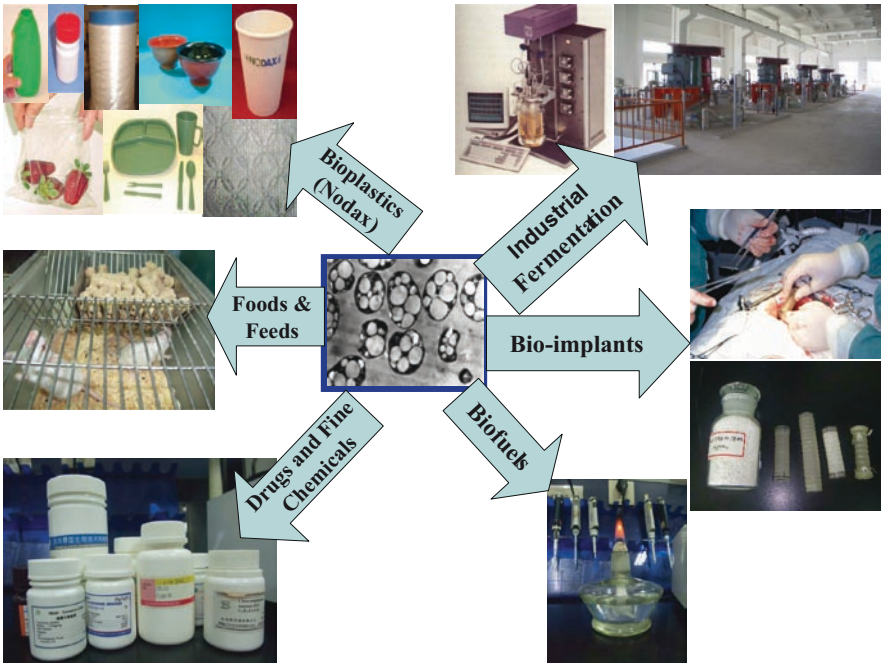

Fig. 1 Applications of polyhydroxyalkanoates (PHA) in various fields (Chen 2009a)

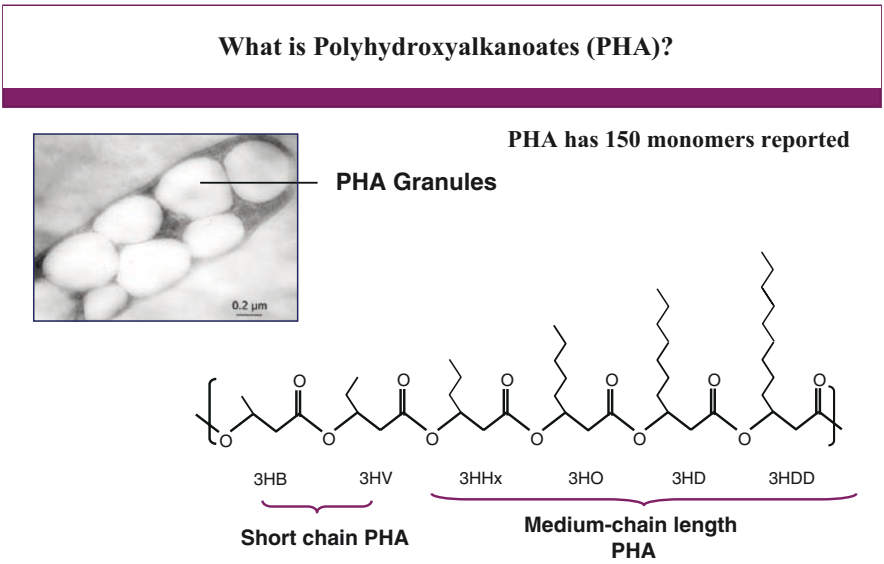

Fig. 2 Common PHA monomer structures. Short-chain-length monomers: 3-hydroxybutyrate (3HB), 3-hydroxyvalerate (3HV). Medium-chain-length monomers: 3-hydroxyhexanoate (3HHx), 3-hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD), 3-hydroxydodecanoate (3HDD)

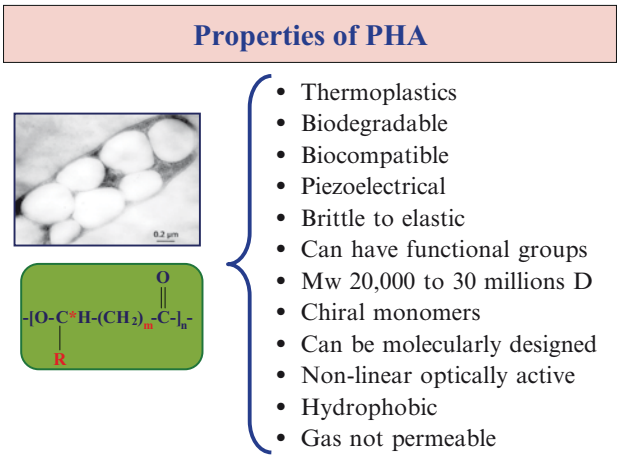

Fig. 3 Common properties of PHA

release carriers (Chen 2009a), PHA monomers can also be used to develop biofuels, drugs, or chiral intermediates. Oligomers of PHA were reported to be nutrients for animals (Tasaki et al. 1999).

Owing to these developments, microbial PHA has formed an industrial value chain ranging from industrial fermentation, materials, medicine, and biofuels to fine chemicals. More and more applications are the subject of intensive research. Globally, more than 20 companies have been established to commercialize these developments (Chen 2009a; Fig. 1).

In this chapter, we will discuss the above-mentioned aspects of PHA.

2 Biosynthesis of PHA

PHA can be synthesized either by chemical means or by biological approaches (Kemnitzer et al. 1993; He et al. 1999). Biosynthesis of PHA leads to much a higher molecular weight compared with that achieved with chemical methods. However, biosynthesis of PHA does not allow much control over the monomer structures in the PHA polymers; the specificity of PHA polymerase (or PHA synthase) will influence the monomers incorporated into the polymers (Chen et al. 2004). Since biosynthesis of PHA is conducted by microorganisms grown in an aqueous solution containing sustainable resources such as starch, glucose, sucrose, fatty acids, and even nutrients in waste water under 30–37 °C and atmosphere pressure, it is considered as more environmentally friendly and sustainable, especially when petroleum as a nonsustainable resource is being depleted quickly, and plastics or fuels based on petroleum show the same trend.

2.1 Biochemistry and Molecular Biology of PHA Synthesis

PHA biosynthesis has been well studied over the past many years. Acetyl-CoA is the key component to supply the 3-hydroxyalkanoyl-CoA of different lengths as substrates for PHA synthases of various specificities (Fig. 4, Table 1). In addition, 3-hydroxyalkanoyl-CoA can also be supplied from β-oxidation of fatty acids of different chain lengths (Fig. 4). Many genes encoding various enzymes are directly or indirectly involved in PHA synthesis (Table 1).

So far, biosynthesis of PHA can be summarized in eight pathways (Fig. 4, Table 1). The first pathway involves the three key enzymes β-ketothiolase, NADPH-dependent acetoacetyl-CoA reductase, and PHA synthase encoded by genes *phaA*, *phaB*, and *phaC*, respectively. *Ralstonia eutropha* is the representative of this pathway. An associated pathway involving PHA degradation catalyzed by PHA depolymerase, dimer hydrolase, 3-hydroxybutyrate dehydrogenase, and acetoacetyl-CoA synthase helps regulate PHA synthesis and degradation. The associated pathway was found in strains of *Aeromonas hydrophila*, *Pseudomonas stutzeri*, *R. eutropha*, and *Pseudomonas oleovorans* (Sudesh et al. 2000).

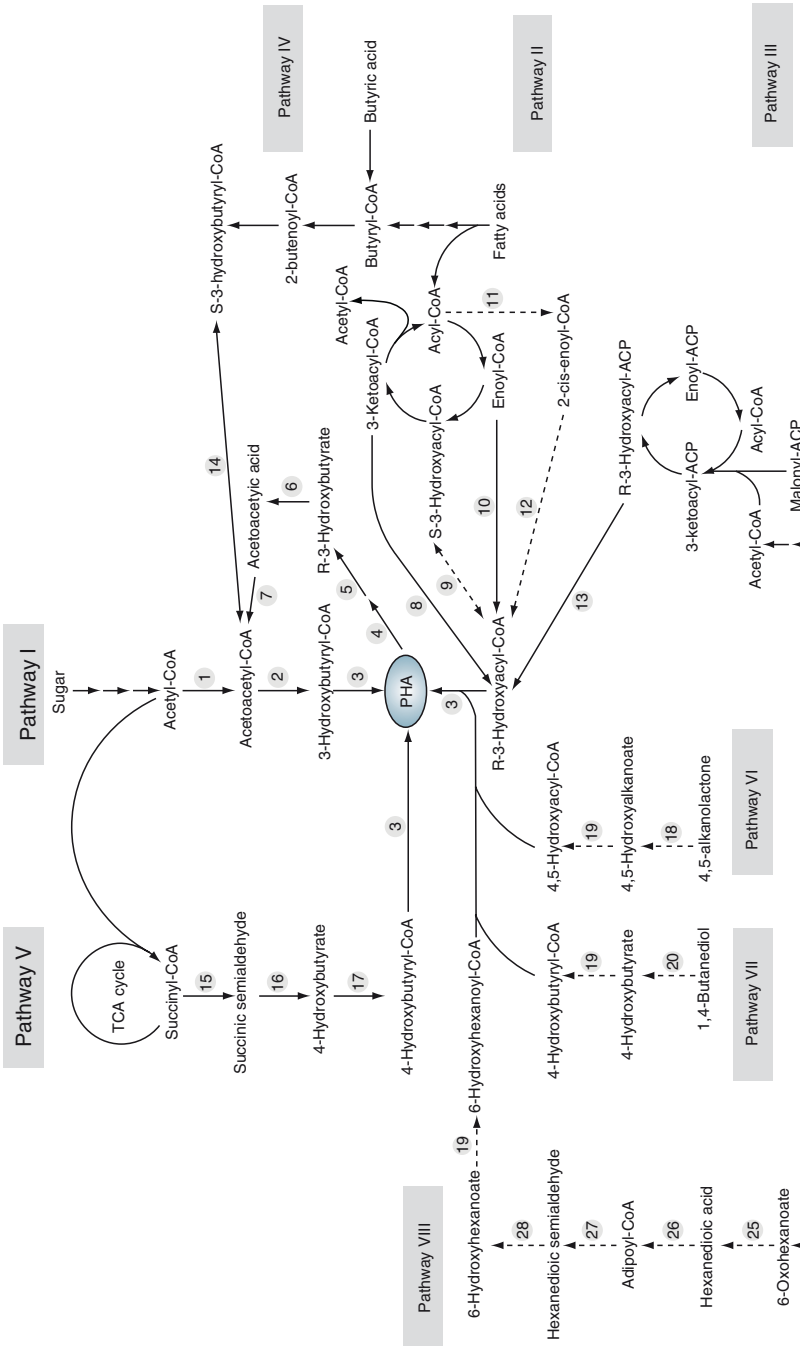

Fig. 4 PHA biosynthesis pathways. *Numbers* representing detailed enzymes (or genes) involved in the PHA synthesis can be obtained from Table 1

Table 1 Synthesis pathways for polyhydroxyalkanoates (*PHA*) and the enzymes involved

No.	Pathway	Abbreviation	Enzyme	Species	Reference
1	Pathway I	PhaA	β-Ketothiolase	*Ralstonia eutropha*	Sudesh et al. (2000)
2		PhaB	NADPH dependent acetoacetyl-CoA reductase		
3		PhaC	PHA synthase		
4	Associated way	PhaZ	PHA depolymerase	*Aeromonas hydrophila* 4AK4	Sudesh et al. (2000)
5			Dimer hydrolase	*Pseudomonas stutzeri* 1317	
6			(*R*)-3-Hydroxybutyrate dehydrogenase	*R. eutropha*	
7			Acetoacetyl-CoA synthetase	*Pesudomonas oleovorans*	
8	Pathway II	FabG	3-Ketoacyl-CoA reductase	*Pesudomonas putida* KT2442, *A. hydrophila* 4AK4, *Pesudomonas aeruginosa*	Sudesh et al. (2000), Mittendorf et al. (1998)
9			Epimerase		
10		PhaJ	(*R*)-Enoyl-CoA hydratase/enoyl-CoA hydratase I		
11			Acyl-CoA oxidase, putative		
12			Enoyl-CoA hydratase I, putative		
13	Pathway III	PhaG FabD	3-Hydroxyacyl-ACP-CoA transferaseMalonyl-CoA-ACP transacylase	*Pseudomonas mendocina*, recombinant *Escherichia coli*	Sudesh et al. (2000), Zheng et al. (2005), Taguchi et al. (1999)
14	Pathway IV		NADH-dependent acetoacetyl-CoA reductase	*Rhizobium* (Cicer) sp. CC 1192	Chohan and Copeland (1998)
15		SucD	Succinic semialdehyde dehydrogenase	*Clostridium kluyveri*	Valentin and Dennis (1997)
16	Pathway V	4hbD	4-Hydroxybutyrate dehydrogenase		
17		OrfZ	4-Hydroxybutyrate-CoA:CoA transferase		
18	Pathway VI		Lactonase, putative	Mutants and recombinant of *Alcaligenes eutrophus*	Valentin and Steinbüchel (1995)
19			Hydroxyacyl-CoA synthase, putative		
20	Pathway VII		Alcohol dehydrogenase, putative	*A. hydrophila 4AK4*	Xie and Chen (2008)
21	Pathway VIII	ChnA	Cyclohexanol dehydrogenase	*Acinetobacter* sp. SE19, *Brevibacterium epidermidis* HCU	Brzostowicz et al. (2002)
22		ChnB	Cyclohexanone monooxygenases		
23		ChnC	Caprolactone hydrolase		
24		ChnD	6-Hydroxyhexanoate dehydrogenase		
25		ChnE	6-Oxohexanoate dehydrogenase		
26			Semialdehyde dehydrogenase, putative		
27			6-Hydroxyhexanoate dehydrogenase, putative		
28			Hydroxyacyl-CoA synthase, putative		

The second PHA synthesis pathway (pathway II) is related to fatty acid uptake by microorganisms. After fatty acid β-oxidation, acyl-CoA enters the PHA monomer synthesis process. Enzymes including 3-ketoacyl-CoA reductase, epimerase, (*R*)-enoyl-CoA hydratase/enoyl-CoA hydratase I, acyl-CoA oxidase (putative), and enoyl-CoA hydratase I (putative) were found to be involved in supplying the PHA precursor 3-hydroxyacyl-CoA for PHA synthesis. *Pseudomonas putida*, *Pesudomonas aeruginosa*, and *A. hydrophila* are able to use pathway II to synthesize medium-chain-length (mcl) PHA or copolymers of (*R*)-3-hydroxybutyrate (R3HB) and (*R*)-3-hydroxyhexanoate (PHBHHx).

Pathway III involves 3-hydroxyacyl-ACP-CoA transferase (PhaG) and malonyl-CoA-ACP transacylase (FabD), which help supply 3-hydroxyacyl-ACP to form PHA monomer 3-hydroxyacyl-CoA, leading to PHA formation under the action of PHA synthase (Sudesh et al. 2000; Zheng et al. 2005; Taguchi et al. 1999).

Pathway IV uses NADH-dependent acetoacetyl-CoA reductase to oxidize (*S*)-(+)-3-hydroxybutyryl-CoA. A high ratio of NADPH to NADP+ could enhance the delivery of the reductant to nitrogenase in *Rhizobium* (Cicer) sp. strain CC 1192 (Chohan and Copeland 1998). This could also favor the reduction of acetoacetyl-CoA for poly[(*R*)-3-hydroxybutyrate] (PHB) synthesis.

Pathway V uses succinic semialdehyde dehydrogenase (SucD), 4-hydroxybutyrate dehydrogenase (4hbD), and 4-hydroxybutyrate-CoA:CoA transferase (OrfZ) to synthesize 4-hydroxybutyryl-CoA for forming 4-hydroxybutyrate-containing PHA. Pathway V was reported in *Clostridium kluyveri* (Valentin and Dennis 1997).

Pathway VI employs putative lactonase and hydroxyacyl-CoA synthase to turn 4,5-alkanolactone into 4,5-hydroxyacyl-CoA for PHA synthesis (Valentin and Steinbüchel 1995). Pathway VII is based on the putative alcohol dehydrogenase found in *A. hydrophila* 4AK4. In pathway VII, 1,4-butanediol is oxidized to 4-hydroxybutyrate, then to 4-hydroxybutyryl-CoA for 4-hydroxybutyrate-containing PHA synthesis (Xie and Chen 2008). Pathway VIII turns 6-hydroxy-hexanoate into 6-hydroxyhexanoate-containing PHA under the actions of eight enzymes (Table 1).

2.2 Prokaryotic PHA

Most PHA have been produced by prokaryotic microorganisms, including bacteria and archaea, although transgenic plants were reported to produce PHA (see Poirier and Brumbley 2009). Still, oligomers of PHA were reported to be discovered in eukaryotes, including many tissues and blood of human and animals (Reusch 1989). The functions of prokaryotic PHA were found to be related to carbon and energy storage as well as enhanced survival under environmental stress conditions (Castro-Sowinski et al. 2009). We humans exploit the fast growth of prokaryotes for our benefit to mass-produce PHA for applications as both bioplastics and biofuels. So far, all applications related to PHA are prokaryotic ones.

2.2.1 Homopolymer PHA

PHB was the first homopolymer PHA to be discovered. There have been very few studies related to other non-PHB homopolymers, including poly(4-hydroxybutyrate) (P4HB) (Steinbüchel et al. 1994), poly[(*R*)-3-hydroxyvalerate)] (PHV) (Steinbüchel and Schmack, 1995), poly[(*R*)-3-hydroxy-*co*-(*R*)-5-phenylvaleric acid] (Anderson et al. 1990), poly[(*R*)-3-hydroxyhexanoate] (Anderson et al. 1990), poly[(*R*)-3-hydroxyheptanoate] (Anderson et al. 1990; Chung et al. 1999; Wang and Chen 2009), poly[(*R*)-3-hydroxyoctanoate] (PHO) (Anderson et al. 1990), and poly[(*R*)-3-hydroxynonanoate] (Anderson et al. 1990; Chung et al. 1999). Many of these have not yet been fully characterized. Recently, the author's laboratory succeeded in producing poly[(*R*)-3-hydroxyundecanoate] and poly[(*R*)-3-hydroxydecanoate] (unpublished results). PHA homopolymers ranging from four to ten carbon atoms in length (or called C_4–C_{10} PHA homopolymers) have been produced. More homopolymers should be developed in the future.

Among these homopolymers, PHV can form solution-grown single crystals with a unique crystal and lamellar structure; this is very attractive for crystallography studies (Iwata and Doi 2000).

2.2.2 Copolymer PHA

In most cases, bacteria produce PHB. Also in many cases, short-chain-length (scl) PHA copolymers are synthesized consisting of C_3 and C_5, including poly[(*R*)-3-hydroxypropionate-*co*-(*R*)-3-hydroxybutyrate] (Shimamura et al. 1994), poly[(*R*)-3-hydroxybutyrate-*co*-4-hydroxybutyrate] (Saito et al. 1996), poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyvalerate] (PHBV) (Alderete et al. 1993), and poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyvalerate-*co*-4-hydroxybutyrate] (Zhao and Chen 2007). Many *Pseudomonas* spp. are able to accumulate mcl PHA copolymers containing C_6–C_{12} monomers. Typical mcl PHA are poly[(*R*)-3-hydroxyhexanoate-*co*-(*R*)-3-hydroxyoctanoate-*co*-(*R*)-3-hydroxydecanoate] and poly[(*R*)-3-hydroxyhexanoate-*co*-(*R*)-3-hydroxyl-octanoate-*co*-(*R*)-3-hydroxydecanoate-(*R*)-3-hydroxydodecanoate](Lageveen et al. 1988). Recently, the author's laboratory succeeded in producing poly[(*R*)-3-hydroxydecanoate-(*R*)-3-hydroxydodecanoate)] (unpublished results).

Copolymers of scl and mcl PHA possess useful and flexible mechanical properties; they are the preferred materials for application development. A successful example is the PHBHHx that was produced on an industrial scale (Chen 2009b). US-based Procter & Gamble has trademarked scl and mcl PHA copolymers of C_4 and C_6–C_{12} as Nodax™ (Noda et al. 2009).

2.2.3 Block Copolymer PHA

Pederson et al. (2006) synthesized PHA-containing block copolymers in *Cupriavidus necator* (also called *R. eutropha*) using periodic substrate addition. PHB segments

were formed during fructose utilization. Pulse feeds of pentanoic acid resulted in the synthesis of (*R*)-3-hydroxyvalerate (3HV) monomers, forming PHBV random copolymer. A combination of characterization techniques applied to the polymer batches strongly suggests the presence of block copolymers. Analysis of thermodynamically stable polymer samples obtained by fractionation by differential scanning calorimetry and nuclear magnetic resonance spectroscopy indicates that approximately 30% of the total polymer sample exhibits melting characteristics and nearest-neighbor statistics indicative of block copolymers. Rheology experiments indicate additional mesophase transitions only found in block copolymer materials. In addition, dynamic mechanical analysis shows extension of the rubbery plateaus in block copolymer samples, and uniaxial extension tests result in differences in mechanical properties (modulus and elongation at failure) expected of similarly prepared block copolymer and single polymer type materials.

McChalicher and Srienc (2007) showed that films consisting of block copolymers retained more elasticity over time with respect to films of similar random copolymers of comparable composition. Two PHBV films containing either 8 or 29% 3HV exhibited a quick transition to brittle behavior, decreasing to less than 20% elongation at fracture within a few days after annealing. Conversely, the block copolymer samples had higher than 100% elongation at fracture a full 3 months after annealing. Because block copolymers covalently link polymers that would otherwise form thermodynamically separate phases, the rates and degrees of crystallization of the block copolymers are less than those of the random copolymer samples. These differences translate into materials that extend the property space of biologically synthesized scl PHA.

Wu et al (2008) succeeded in producing PHB–poly(D,L-lactide) (PLA)–poly (ε-caprolactone) triblock copolymers using a low molecular weight methyl-PHB oligomer precursor as the macroinitiator through ring-opening polymerization with D,L-lactide and ε-caprolactone. The triblock copolymers exhibited flexible properties with good biocompatibility.

2.3 Eukaryotic PHA

PHB has been found to be a ubiquitous component of the cellular membranes of plants and animals (Reusch et al. 1992). The investigation of PHB distribution in human plasma using chemical and immunological methods found that PHB concentrations were highly variable: total plasma PHB ranged from 0.60 to 18.2 mg l^{-1}, with a mean of 3.5 mg l^{-1}, in a random group of 24 blood donors.

In plasma separated by density-gradient ultracentrifugation, lipoproteins constituted 20–30% of total plasma PHB, 6–14% was very low density lipoproteins (VLDL), 8–16% was low-density lipoproteins (LDL), and less than 3% was high density lipoproteins (HDL; Reusch et al. 1992). The majority of plasma PHB (70–80%) was found in protein fractions of density greater than 1.22 g ml^{-1}. Western blot analysis of the high-density fractions with anti-PHB F(ab')2 identified

albumin as the major PHB-binding protein. The affinity of albumin for PHB was confirmed by in vitro studies which demonstrated transfer of ^{14}C-PHB from chloroform into aqueous solutions of human and bovine serum albumins. PHB was less tightly bound to LDL than to other plasma components; the polymer could be isolated from LDL by extraction with chloroform, or by digestion with alkaline hypochlorite, but it could not similarly be recovered from VLDL or albumin. The wide concentration range of PHB in plasma, its presence in VLDL and LDL and its absence in HDL, coupled with its physical properties suggest it may have important physiological effects.

PHB of 130–170 monomer units is usually associated with other macromolecules by multiple coordinate bonds, or by hydrogen bonding and hydrophobic interactions (Reusch 1992). This conserved PHB has been isolated from the plasma membranes of bacteria, from a variety of plant tissues, and from the plasma membranes, mitochondria, and microsomes of animal cells.

PHB synthesis using genetic engineering approaches was reported in some plants, including switchgrass (Somleva et al. 2008), sugarcane (Purnell et al. 2007), sugar beet (Menzel et al. 2003), tobacco (Lossl et al. 2005), flax (Wrobel et al. 2004), *Arabidopsis thaliana* (Kourtz et al. 2005), rape, and corn (Poirier 2002).

3 Microbial Synthesis of PHA Monomers

Various enantiomerically pure (*R*)-3-hydroxyalkanoic acids (RHA) can be conveniently prepared by depolymerizing the biosynthesized PHA. De Roo et al. (2002) produced the chiral RHA and RHA methyl esters via hydrolytic degradation of PHA synthesized by pseudomonads. They first hydrolyzed the recovered PHA by acid methanolysis and then distilled the RHA methyl ester mixture into several fractions. Subsequently, the RHA methyl esters were saponified to yield the corresponding RHA with final yields of the RHA up to 92.8% (w/w).

3.1 PHA Monomers Produced by Microorganisms

Lee et al. (1999) demonstrated that R3HB could be efficiently produced via in vivo depolymerization by providing the appropriate environmental conditions. In their study with the strain *Alcaligenes latus*, they found that lowering the pH to 3–4 induced the highest activity of intracellular PHB depolymerase and blocked the reutilization of R3HB by the cells. Ren et al. (2005) suspended PHA-containing *P. putida* cells in phosphate buffer at different pH. When the pH was 11, the degradation and monomer release was the best. Under this condition, (*R*)-3-hydroxyoctanoic acid and (*R*)-3-hydroxyhexanoic acid were degraded with an efficiency of over 90% (w/w) in 9 h.

To produce extracellular chiral (*R*)-3-hydroxyacyl acids (3HA) by fermentation, a novel pathway was constructed by expressing *tesB* gene encoding thioesterase II into *P. putida* KTOY01, which was a PHA synthesis operon knockout mutant. A 0.35 $g l^{-1}$ 3HA mixture consisting of (*R*)-3-hydroxyhexanoate (3HHx), (*R*)-3-hydroxyoctanoate, (*R*)-3-hydroxydecanoate (3HD), and (*R*)-3-hydroxydodecanoate (3HDD) was produced in shake-flask study using dodecanoate as the sole carbon source. Additional knockout of *fadA* and *fadB* genes encoding (*R*)-3-ketoacyl-CoA thiolase and (*R*)-3-hydroxyacyl-CoA dehydrogenase in *P. putida* KTOY01 led to the weakening of the β-oxidation pathway. The *fadBA* and PHA synthesis operon knockout mutant *P. putida* KTOY07 expressing *tesB* gene produced 2.44 $g l^{-1}$ 3HA, significantly more than that of the β-oxidation intact mutant. The 3HA mixture contained 90% 3HDD as a dominant component. A fed-batch fermentation process carried out in a 6-l automatic fermentor produced 7.27 $g l^{-1}$ extracellular 3HA containing 96 mol% fraction of 3HDD after 28 h of growth. For the first time it became possible to produce 3HDD-dominant 3HA monomers (Chung et al. 2009).

3.2 The Application of PHA Monomers for Synthesis of Other Polyesters

Finally, the diverse PHA monomers are a rich pool for novel polymer synthesis (Taguchi et al. 2008; Rieth et al. 2002). Copolymerization of PHA monomers with commercially available polymer monomers will generate limitless new copolymers. This is an area that has not yet started to attract attention, possibly owing to the high cost of PHA monomer production. However, copolymer of lactide and 3-hydroxybutyrate (3HB) has recently been reported, signifying the start of the PHA monomer-based new polymer era.

4 Application of PHA

4.1 PHA as Packaging Materials

PHA were initially used to make everyday articles such as shampoo bottles and packaging materials by Wella (Germany) (Weiner 1997). PHA were also developed as packaging films mainly for uses as shopping bags, containers and paper coatings, disposable items such as razors, utensils, diapers, feminine hygiene products, cosmetic containers, and cups as well as medical surgical garments, upholstery, carpet, packaging, compostable bags and lids, or tubs for thermoformed articles by Proctor & Gamble, Biomers, Metabolix, and several other companies (Clarinval and Halleux 2005; Mikova and Chodak 2006).

PHB fibers with high tensile strength were prepared by stretching the fibers after isothermal crystallization near the glass-transition temperature (Tanaka et al. 2007).

Increasing the time for isothermal crystallization of PHB fibers resulted in a decrease in the maximum draw ratio. Yet the tensile strength of PHA fibers increased remarkably when the isothermal crystallization time was prolonged to more than 24 h. The tensile strength of low molecular weight drawn fibers was higher than that of high molecular weight fibers. PHB fibers stretched after isothermal crystallization had the oriented α-form crystal with the 2(1) helix conformation and the β-form with the planar zigzag conformation.

Vogel et al. (2007) attempted to use reactive extrusion with peroxide as a comfortable pathway for improvement of the crystallization of PHB in a melt spinning process. They succeeded in improving the crystallization in the spinline and of the inhibition of the secondary crystallization in the fibers. Those processes overcame the brittleness of PHA and created very strong fibers with promising applications.

4.2 PHA as Biomedical Implant Materials

Only several PHA, including PHB, PHBV, P4HB, PHBHHx, and PHO, are available in sufficient quantities for application research (Hrabak 1992; Byrom 1992; Chen et al. 2001). This is why most of the application research, including tissue engineering and controlled drug release, is based on the above-mentioned PHA.

Over the past 20 years, PHA and its composites have been used to develope devices including sutures, suture fasteners, meniscus repair devices, rivets, tacks, staples, screws (including interference screws), bone plates and bone plating systems, surgical mesh, repair patches, slings, cardiovascular patches, orthopedic pins (including bone filling augmentation material), adhesion barriers (Dai et al. 2009), stents, guided tissue repair/regeneration devices, articular cartilage repair devices (Wang et al. 2008a, b), nerve guides (Bian et al. 2009), tendon repair devices, atrial septal defect repair devices, pericardial patches, bulking and filling agents, vein valves, bone marrow scaffolds, meniscus regeneration devices, ligament and tendon grafts, ocular cell implants, spinal fusion cages, skin substitutes, dural substitutes, bone graft substitutes, bone dowels, wound dressings, and hemostats (Chen and Wu 2005). The changing PHA compositions also allow favorable mechanical properties, biocompatibility, and degradation times within desirable time frames under specific physiological conditions (Abe et al. 1995; Chen and Wu 2005).

In another study (Cheng et al. 2006a) it was shown that 3HB (0.02 g ml^{-1}) promoted cell proliferation in cultured L929 cells plated at high cell density (1×10^5 cells/well) but not at lower cell densities. Although 3HB did not affect cell cycle progression, it significantly inhibited cell death. 3HB treatment prevented necrosis, reducing cell membrane permeability 4 h following serum withdrawal from the medium, and for all subsequent time points. 3HB that promotes proliferation of L929 cells in high-density cultures by preventing apoptotic and necrotic cell death makes biodegradable polymers containing hydroxybutyrate, such as PHBHHx, attractive candidates for tissue engineering applications, especially those requiring the regeneration of large numbers of cells.

Cheng et al. (2006b) found that PHBHHx microparticles (0.005–0.10 g l^{-1}) promoted murine fibroblast L929 cell proliferation and elevated intracellular calcium concentrations. Ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid inhibited PHBHHx-microparticle-induced cell proliferation by chelating the extracellular Ca^{2+} and blocking the PHBHHx particle-induced intracellular Ca^{2+} concentration increase. Transwell experiments demonstrated that PHBHHx microparticles stimulated fibroblast proliferation when separated from cells by a 0.4-μm filter as effectively as when applied directly to cells. Since the PHBHHx microparticles had a diameter of 75 μm, the stimulatory effect of PHBHHx particles on cell growth was attributed to degradation products smaller than 0.4 μm in diameter. The trophic effect of these microparticles is consistent with our previous reports demonstrating good biocompatibility for PHBHHx.

Oligo[(*R*)-3-hydroxybutyrate]s (OHBs; less than 14 kDa) existing in various organisms. They can form complexes with inorganic polyphosphates, nucleic acids, and proteins. OHBs are also the degradation products of PHB in vivo. Sun et al. (2007) prepared OHB (M_n 2,000), oligo[(*R*)-3-hydroxybutyrate-*co*-4-hydroxybutyrate] (O3HB4HB, M_n 2,100, 6 mol% 4-hydroxybutyrate), oligo[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyhexanoate] (OHBHHx, M_n 2,800, 12 mol% 3HHx), and mcl oligo[(*R*)-3-hydroxyalkanoate]s (M_n 2,400, 71.2 mol% 3HD) via methanolysis of corresponding PHA polymers. The cells grew well in low-concentration (5 mg l^{-1}) liposomes containing the oligomers. Different cytotoxicity was exhibited after more oligomers (more than 20 mg l^{-1}) had been transported into the cells. The inhibition was decreased stepwise from OHB to OHD, as the monomer chain length increased. Compared with OHBHHx and OHD treatment, more cells arrested in G_0/G_1 phase, and died, probably induced by OHB and O3HB4HB. However, the cell death can be suppressed by R3HB released from the oligomers. It can be concluded that the more flexible chain combined with R3HB units had better biocompatibility and bioabsorbability. This can be a guide to select and develop new tissue engineering materials.

Besides, Ca^{2+} influx was also observed under a confocal laser scanning microscope in cells after transfection with oligomers–liposomes. It was presumed that not only OHB but also other OHA can form calcium channels in phospholipid bilayers, and can be incorporated into plasma membranes and had Ca^{2+} transport activity.

With successful approval of P4HB as an implant biomaterial by the FDA (http://www.tepha.com), more PHA-based biomaterials are expected to go into clinical trials soon. With the diversity of PHA materials, one can expect the PHA to become a family of bioimplant materials with rich applications.

4.3 PHA as Drug Delivery Carriers

Homopolymers and copolymers of lactate and glycolate are widely used in commercially available sustained release products for drug delivery. However, lactate and glycolate copolymers are degraded by bulk hydrolysis; hence, drug release cannot be

fully controlled (Pouton and Akhtar 1996). In the early 1990s, PHA became candidates for use as drug carriers owing to their biodegradability, biocompatibility, and degradation by surface erosion (Gould et al. 1987). PHA used as a drug carrier was reviewed in 1989 by Koosha et al (1989). The potential of matrices produced by direct compression of PHBV for oral administration has been proven with the benefits of simplified processing over alternative sustained release technologies (Gould et al. 1987). Increasing the polymer molecular mass caused an increased rate of sulfamethizole release from irregularly shaped PHB microparticles (Brophy and Deasy 1986). When the in vitro release and the in vivo release of the anticancer agent lomustine from PHB and PLA microspheres as potential carriers for drug targeting were compared, it was found that drug was released from the PHB microspheres faster (Bissery et al. 1985). Incorporation of ethyl esters or butyl esters of fatty acids into the PHB microspheres increased the rate of drug release (Kubota et al. 1988).

So far only PHB and PHBV have been studied for controlled drug release. It is expected that other PHA family members with diverse properties will bring more controlled release properties for the drug release field. This is still an area remaining to be exploited.

PHA granule binding protein PhaP is able to bind to hydrophobic polymers (Wang et al. 2008b). A receptor-mediated drug-specific delivery system was developed in this study based on PhaP (Fig. 5). The system consists of PHA nanoparticles, PhaP, and ligands fused to PhaP. The PHA nanoparticles were used to package mostly hydrophobic drugs, PhaP fused with ligands produced by overexpression of their corresponding genes in *Pichia pastoris* or *Escherichia coli* was able to attach to hydrophobic PHA nanoparticle. At the end, the ligands were able to pull the PhaP–PHA nanoparticles to the targeted cells with receptors recognized by the ligands. It was found in this study that the receptor-mediated drug-specific delivery

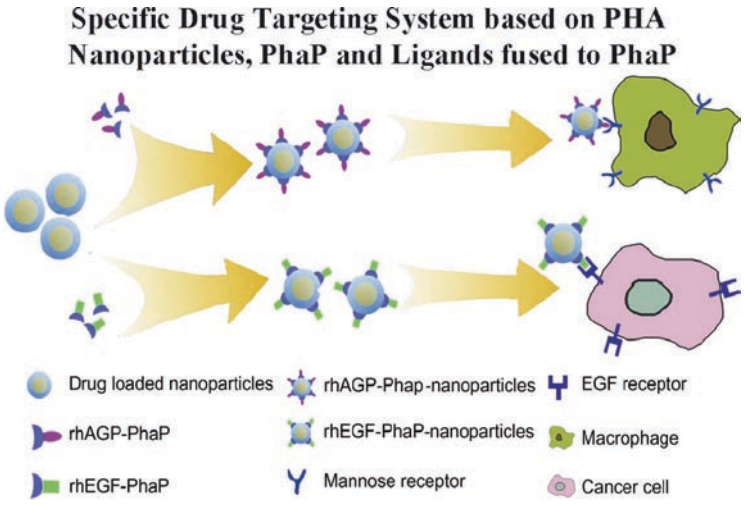

Fig. 5 PHA- and phasing-based specific drug delivery systems (Wang et al. 2008b)

system ligand–PhaP–PHA nanoparticles was taken up by macrophages, hepatocellular carcinoma cell BEL7402 in vitro, and hepatocellular carcinoma cells in vivo, respectively, when the ligands were mannosylated human α_1-acid glycoprotein and human epidermal growth factor (hEGF), respectively, which were able to bind to receptors of macrophages or hepatocellular carcinoma cells. The system was clearly visible in the targeted cells and organs under fluorescence microscopy when rhodamine B isothiocyanate (RBITC) was used as a delivery model drug owing to the specific targeting effect created by specific ligand and receptor binding. The delivery system of hEGF–PhaP–nanoparticles carrying RBITC was found to be endocytosed by the tumor cells in an xenograft tumorous model mouse. Thus, the ligand–PhaP–PHA specific drug delivery system was proven effective both in vitro and in vivo (Yao et al. 2008).

4.4 PHA as Biofuels

Recently, Zhang et al (2009) showed that 3-hydroxybutyrate methyl ester (3HBME) and mcl 3-hydroxyalkanoate methyl ester (3HAME) obtained from esterification of PHB and mcl PHA could be used as biofuels (Fig. 6). They investigated the combustion heats of 3HBME, 3HAME, ethanol, *n*-propanol, *n*-butanol, 0# diesel,

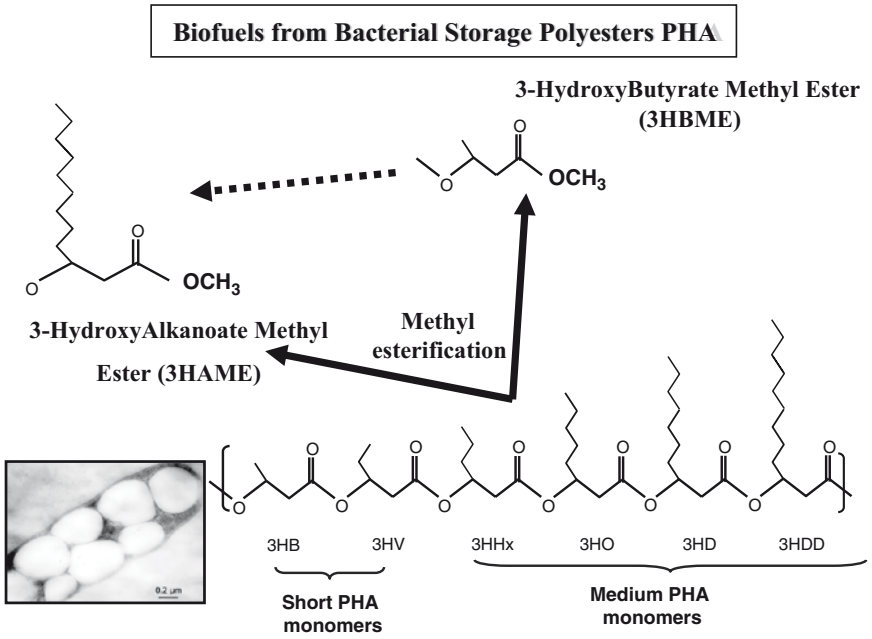

Fig. 6 PHA-based biofuels derived from methyl esterification of various PHA monomers (Zhang et al. 2009)

90# gasoline, and 3HBME-based and 3HAME-based blended fuels and found that 3HBME and 3HAME had combustion heats of 20 and 30 kJ g^{-1}, respectively, comparable to the combustion heat of 27 kJ g^{-1} of ethanol. Addition of 10% 3HBME or 3HAME enhanced the combustion heat of ethanol to 30 and 35 kJ g^{-1}, respectively. The addition of 3HBME or 3HAME to *n*-propanol and *n*-butanol led to a slight reduction of their combustion heats. The combustion heats of the blended fuels 3HBME/diesel or 3HBME/gasoline and of 3HAME/diesel or 3HAME/gasoline were lower than that of the pure diesel or gasoline. It was roughly estimated that the production cost of PHA-based biofuels should be around US $1,200/ton.

4.5 PHA Monomers as Drugs

Sodium salts of D-3-hydroxybutyrate (D-3HB), DL-3-hydroxybutyrate (DL-3HB), and 3HBME are derivatives of 3HB, a body ketone that is produced in vivo in animals, including human. D-3HB is the most common degradation product of microbial PHA that have been investigated for tissue engineering applications. 3HB and its derivatives (collectively called 3HB derivatives) were reported to have an effect on cell apoptosis and the cytosolic Ca^{2+} concentration of mouse glial cells (Xiao et al. 2007). The percentage of cells undergoing apoptosis decreased significantly in the presence of 3HB and its derivatives, as evidenced by flow cytometry. The in vitro study on the cytosolic Ca^{2+} concentration demonstrated that 3HB derivatives elevated dramatically the cytosolic Ca^{2+} concentration. Both the extracellular and the intracellular Ca^{2+} contributed as sources of such Ca^{2+} concentration elevation. The effect of 3HB derivatives on cytosolic Ca^{2+} concentration could be reduced by nitredipine, an L-type voltage-dependent calcium channel antagonist. In comparison, 3HBME worked more efficiently than D-3HB and DL-3HB did as 3HBME is most efficient in permeation into the cells. All the results indicated that 3HB derivatives had an inhibitory effect on cell apoptosis which is mediated by signaling pathways related to the elevation of cytosolic Ca^{2+} concentration. This positive effect helps explain the biocompatibility observed for PHA; it also points to the possibility of 3HB derivatives regardless of chirality becoming effective neural protective agents.

Learning and memory require energy-demanding cellular processes and can be enhanced when the brain is supplemented with metabolic substrates. It was found that neuroglial cell metabolic activity was significantly elevated when neuroglial cells were cultured in the presence of the PHB degradation product 3HB and derivatives. We demonstrated that the receptor for 3HB, namely, protein upregulated in macrophages by interferon-γ (PUMA-G), was expressed in brain and upregulated in mice treated with 3HBME. We also affirmed increased expression of connexin 36 protein and phosphorylated extracellular-signal-regulated kinase 2 (ERK2) in brain tissues following 3HBME treatment, although these differences were not statistically significant. Mice treated with 3HBME performed significantly ($p < 0.05$) better in the Morris water maze than either the negative controls (no treatment) or the positive controls (acetyl-L-carnitine treatment). Moreover, 3HBME was observed to

enhance gap junctional intercellular communication between neurons. Thus, 3HB and its derivatives enhance learning and memory, possibly through a signaling pathway requiring PUMA-G that increases protein synthesis and gap junctional intercellular communication (Zou et al. 2009).

5 Conclusion and Future Perspectives

The development of PHA into a branch of bulk chemical industry will address at least three issues: shortage of petroleum for plastic materials, reduction of CO_2 emissions, and environmental protection. It is related to the sustainable development of the chemical and material industries. The newly developed PHA-based biofuels open up a new area for development that avoids argument on food versus fuel and fuel versus land. However, much more work needs to be done to reduce the cost of PHA production so that PHA-based biofuel can be added to the existing bio-based fuels, including ethanol, propanol, butanol, biodiesel, hydrogen, and methane gas (Fig. 7).

High-value-added PHA applications should be developed simultaneously, especially the implant biomaterials that have begun to be recognized by the FDA. In addition, chiral monomers should be further exploited for medical usages (Fig. 7). So far, only 3HB and its derivatives have been studied and have revealed obvious therapeutic efforts, more monomers should be tested for medical efficacy.

The PHA surface binding proteins, including PhaP, PhaZ, and PhaC, can be developed into a protein purification system or specific drug delivery tools. More applications based on these proteins should be developed.

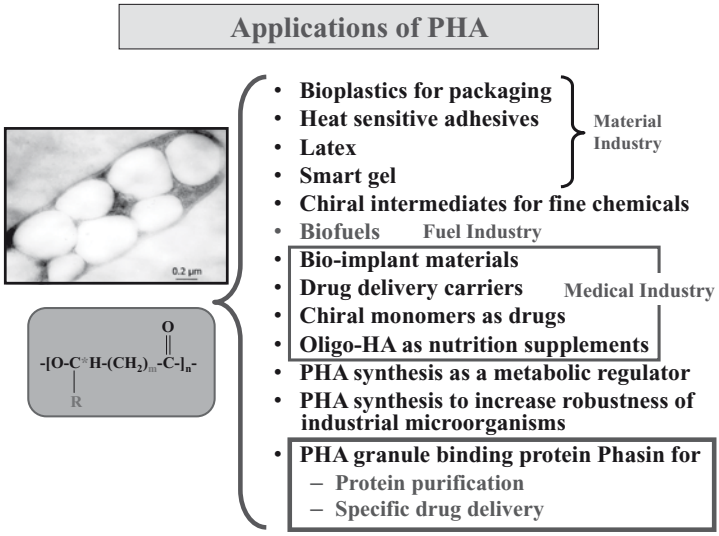

Fig. 7 PHA has been developed into an industrial value chain

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Natural Functions of Bacterial Polyhydroxyalkanoates

Susana Castro-Sowinski, Saul Burdman, Ofra Matan, and Yaacov Okon

Contents

Abstract Polyhydroxyalkanoates (PHAs) are energy- and intracellular carbon-storage compounds that can be mobilized and used when carbon is a limiting resource. Intracellular accumulation of PHA enhances the survival of several bacterial species under environmental stress conditions imposed in water and soil, such as UV irradiation, salinity, thermal and oxidative stress, desiccation, and osmotic shock. The ability to endure these stresses is linked to a cascade of events concomitant with PHA degradation and the expression of genes involved in protection against

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damaging agents. PHA synthesis involves enzymatic and transcriptional regulation, where the RpoS central stationary phase regulator sigma factor has been shown to be implicated. The energy generated during PHA degradation can also be used to drive various important energy-consuming pathways. In addition to its relevance for the plastic industry, PHA has important applications for agriculture, as those related to the production of reliable commercial inoculants, and in controlled release of insecticides when incorporated into degradable PHA granules.

1 Introduction

A wide variety of taxonomically different groups of microorganisms (Bacteria and Archaea domains) produce intracellular homopolymers or copolymers containing different alkyl groups at the β position, described as polyhydroxyalkanoates (PHAs). These polymers are used as energy- and carbon-storage compounds (Anderson and Dawes 1990). PHAs are structurally simple macromolecules that accumulate inside discrete granules to levels that can be as high as 90% of the cell dry weight. They are generally believed to play a role as a sink for carbon and reducing equivalents when other nutrient supplies are limiting resources, and when the bacterial population is not growing exponentially in batch culture (Senior and Dawes 1973; Williams and Peoples 1996; Madison and Huisman 1999). These molecules exhibit material features that are similar to those of some common plastics such as polypropylene (Williams and Peoples 1996; Madison and Huisman 1999). In this chapter we will mainly focus on the ecological significance of PHAs.

Since the identification and characterization of the enzymes involved in PHA synthesis, hundreds of genes from a wide range of prokaryotes have been cloned or identified as putative PHA biosynthesis genes. Microorganisms use different pathways for synthesis of PHA. One of the best studied PHAs is poly[(*R*)-3-hydroxybutyrate] (PHB). Among the enzymes involved in PHB synthesis, β-ketothiolase (PhaA) and acetoacetyl-CoA reductase (PhaB) are involved in general lipid metabolism. In contrast, a third enzyme, PHA synthase (PhaC), is exclusively involved in the biosynthesis of this polymer, being responsible for the polymerization of β-hydroxyalkanoyl-CoA monomers into poly(β-hydroxyalkanoate) (Pötter and Steinbüchel 2005; Philip et al. 2007). In addition to these enzymes, a noncatalytic group of proteins called phasins (PhaPs) are important for granule organization. PhaPs have been found associated with the surface of the granules, being involved in their stabilization and coalescence, and their absence has a significant effect on polymer synthesis (Pötter and Steinbüchel 2005).

The PHA degradation pathway as described in most bacteria studied begins with the depolymerization of PHA to D-3-hydroxybutyrate monomers by PHA depolymerase (encoded by *phaZ*). Extracellular and intracellular PHA degradation have been described (Jendrossek and Handrick 2002) for utilization of PHAs present in the environment or accumulated in PHA granules, respectively (Tanio et al. 1982; Saegusa et al. 2001; Jendrossek and Handrick 2002; Pötter and Steinbüchel 2005).

2 The Role of PHA in Cell Survival Under Stress

PHAs have attracted attention as environmentally friendly polymers owing to their biodegradability, thermoplastic properties, and biocompatibility (Philip et al. 2007). Consequently, many resources have been invested in the isolation of microorganisms capable of synthesizing PHAs with different desirable industrial properties and from different sources. The function of PHAs as intracellular carbon-storage compounds has been the subject of most of the research in assigning a role for these polymers (Macrae and Wilkinson 1958; Sierra and Gibbons 1962; Hippe 1967; Hippe and Schlegel 1967). However, with the identification of new PHA-synthesizing microorganisms and the investigation of the role that PHAs play in bacterial fitness, it became evident that this polymer is more than just an intracellular carbon-storage compound that can be mobilized and used when carbon becomes a limiting resource. It is actually known that intracellular accumulation of PHAs enhances the survival of several bacteria under environmental stress conditions imposed in water and in the soil (Kadouri et al. 2005; Zhao et al. 2007). In other words, PHAs endow bacteria that are able to synthesize them with an ecological advantage. The roles played by PHAs in bacterial environmental fitness are summarized in Table 1.

Table 1 The role of polyhydroxyalkanoates in bacterial environmental fitness

Features	Selected references
Cell survival under stressful low-nutrient conditions	Tal and Okon (1985), Anderson and Dawes (1990), James et al. (1999), Hai et al. (2001)
Cell survival under nutrient limitation in water, soil, rhizosphere, and phyllosphere	Okon and Itzigsohn (1992), López et al. (1995), Ruiz et al. (1999)
Cell survival in inoculant carriers	Fallik and Okon (1996), Dobbelaere et al. (2001), Kadouri et al. (2003b)
Establishment of inoculum in soil and plant surfaces	Kadouri et al. (2002, 2003b)
Energy source and flow for cell motility, chemotaxis, aerotaxis, and biological nitrogen fixation	Tal and Okon (1985), Cevallos et al. (1996), Willis and Walker (1998), Kadouri et al. (2002), Vassileva and Ignatov (2002), Trainer and Charles (2006), Wang et al. (2007)
Sporulation, cyst formation, and germination	Kominek and Halvorson (1965), López et al. (1995), Segura et al. (2003), Valappil et al. (2007)
Control of exopolysaccharide production	Encarnación et al. (2002), Kadouri et al. (2002, 2003a, b), Aneja et al. (2004), Wang et al. (2007)
Endurance under environmental stress: heat and cold, UV irradiation, desiccation, osmotic and solvent stress, osmotic shock, ethanol, and H_2O_2	Tal and Okon (1985), Asada et al. (1999), Kadouri et al. (2003a, b), Ayub et al. (2004), Arora et al. (2006), Villanueva et al. (2007), Zhao et al. (2007), Raiger-Iustman and Ruiz (2008), Trautwein et al. (2008)
Balanced use of available energy and distribution of carbon resources	Dawes (1986), Povolo and Casella (2000), Rothermich et al. (2000), Babel et al. (2001), Philip et al. (2007)

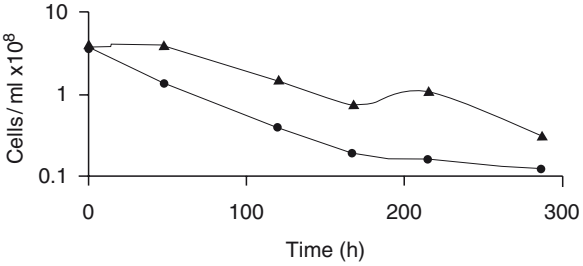

Fig. 1 The effect of poly[(*R*)-3-hydroxybutyrate] (PHB) on survival capability of starved bacteria. Cells of *Azospirillum brasilense* Sp7 (*filled triangles*) and *phaC* mutant (*filled circles*) were grown on a medium with a high carbon to nitrogen ratio for 24 h and transferred to phosphate buffer, where they were incubated for 12 days. Bacterial density was determined using dilution plating (Reproduced from Kadouri et al. 2002, with kind permission from the American Society for Microbiology)

Under certain circumstances, free-living bacterial cells with a high content of PHAs may survive longer than those that lack PHA or have a low PHA content, either because they are protected from adverse factors, or because they can utilize their reserve material longer and more efficiently than those bacteria that produce low contents of PHA or lack this ability at all (Dawes and Senior 1973; Matin et al. 1979; Kadouri et al. 2002). For example, the PHB-producing bacterium *Azospirillum brasilense* showed increased survival upon starvation in phosphate buffer as compared with its non-PHB-producing mutant (*phaC* minus mutant) (Fig. 1). As in *A. brasilense*, in *Sinorhizobium meliloti*, other plant-associated bacteria, and in the PHB-accumulating *Pseudomonas* sp. isolated from an Antarctic environment, PHA content was also shown to correlate positively with increased survival rates after exposure to adverse conditions such as salinity, thermal and oxidative stress, UV irradiation, desiccation, and osmotic pressure (Tal and Okon 1985; Ayub et al. 2004; Arora et al. 2006).

Proteomics-based research coupled with chemical determination of PHA content revealed that the denitrifying proteobacterium *Aromatoleum aromaticum* accumulates PHB during growth in the presence of the pollutant solvents toluene and ethylbenzene (Trautwein et al. 2008). The authors suggested that PHB formation in this bacterium is not induced by an imbalanced nutrient supply but rather by stress due to impaired coupling of alkylbenzene catabolism and denitrification. It was proposed that PHB could serve as a sink for reducing equivalents, ensuring continuous alkylbenzene degradation, and/or a type of hydrophobic trap for aromatic compounds (Trautwein et al. 2008).

Legionella pneumophila also accumulates PHA. The persistence of this bacterium in the environment is aided by its ability to adapt to a variety of different ecological niches, as intracellular parasites of amebae, as free-living members of complex biofilm communities, or as planktonic cells. In this bacterium, PHA accumulation in granules supports long-term survival in the culturable state under starvation, and accumulated PHA serves as an energy-reserve material to promote persistence of legionellae in stressful low-nutrient environments outside the amebic host (James et al. 1999).

Evidence has been provided suggesting that spore formation and germination, as well as cyst production, may be related to PHA biosynthesis and utilization. In *Bacillus cereus* and in *Clostridium botulinum*, PHA is accumulated maximally just prior to the formation of spores and is degraded during the process of sporulation (Valappil et al. 2007). In these cases, PHA may serve as a carbon and energy source for sporulation (Kominek and Halvorson 1965; Nakata 1965; Emeruwa and Hawirko 1973). López et al. (1995) observed that in a PHA negative mutant of *B. megaterium*, in contrast to the wild type, sporulation occurred immediately after exposure to river water, and survival of vegetative cells was clearly compromised, suggesting that in an oligotrophic environment cells depleted of an intracellular carbon source may be committed to earlier sporulation than normal cells. A heat shock was required for germination of PHA negative mutant spores, suggesting that PHA or its degradation products are involved in this process (López et al. 1995).

In *Azotobacter vinelandii*, PHA is utilized as a carbon and energy source during encystment (Lin and Sadoff 1968; Segura et al. 2003). Mutations in the *phaB* and *phaC* genes in *A. vinelandii* had no impact on encystment or on cyst viability under laboratory conditions; however, the possibility that under natural conditions PHA metabolism does have such effects cannot be ruled out (Segura et al. 2003).

Most investigations on prokaryotic PHA have been performed on proteobacteria, but cyanobacteria (Hein et al. 1998; Asada et al. 1999; Hai et al. 2001) and various members of the Archaea domain (Hezayen et al. 2000, 2002; Han et al. 2007; Lu et al. 2008) are PHA-producing organisms. Cyanobacteria can accumulate PHA (mainly PHB) under photoautotrophic or mixotrophic growth conditions in the presence of acetate; however, the relative PHA content is significantly lower than in other prokaryotes (Asada et al. 1999). Under conditions of nitrogen and sulfur starvation, and during light irradiation and recovery of vegetative growth by addition of nitrate, cyanobacteria accumulate PHA as storage products of fixed carbon (Asada et al. 1999; Hai et al. 2001).

The data gathered in these various studies suggest a complex role for PHA in stress alleviation. The PHA granules may offer protection against UV irradiation, by protecting DNA from damage, and increase bacterial resistance to oxidative, thermal, and osmotic shock, among others.

3 Molecular Evidence Supporting a Role for PHA Synthesis in Stress Endurance

The role of PHA in bacterial cell protection using molecular approaches has been assessed in several studies. For instance, with the aim of evaluating how bacterial inoculants of *A. brasilense* can be improved in important parameters (e.g., quality, longevity, reliability, efficacy), wild-type and mutant strains were challenged for their resistance to physical and chemical stresses. The ability of *phaC* and *phaZ* mutants of *A. brasilense* to survive, tolerate, or alleviate various stresses, such as UV irradiation, heat and osmotic shock, desiccation, and oxidative stress, was significantly impaired

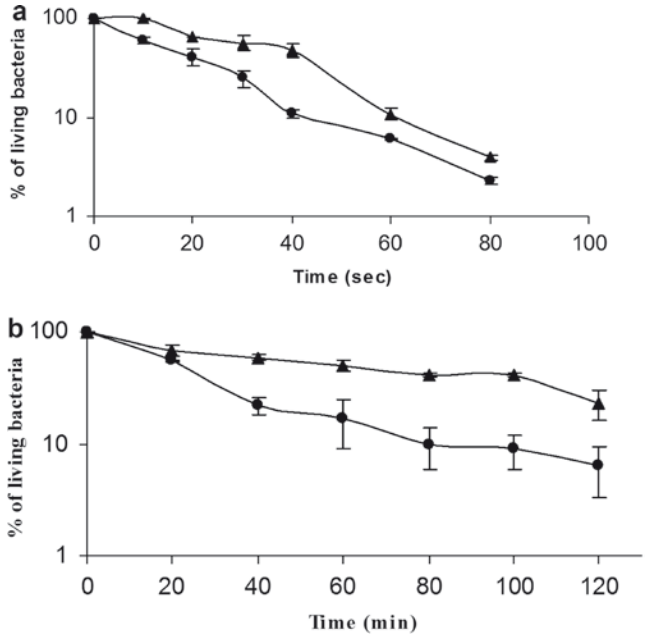

Fig. 2 Effect of heat (**a**) and UV irradiation (**b**) on the survival rate of *A. brasilense* 7030 (*filled triangles*) and of the *phaZ* mutant (*filled circles*). The initial number of cells for each experiment was 3×10^8. Each value represents the mean and the standard error of three replicates from one representative experiment. Each experiment was done three times and yielded similar results (Reproduced from Kadouri et al. 2003a, with permission of Springer-Verlag)

as compared with that of the wild type (Kadouri et al. 2002, 2003a, b; Figs. 2, 3). In addition, PHA accumulation supported cell multiplication in the absence of an exogenous carbon source in *A. brasilense* (Kadouri et al. 2002), in a similar manner as for *Cupriavidus necator* (formerly *Ralstonia eutropha*; Handrick et al. 2000). Interestingly, PHA was shown to maintain nitrogenase activity and aerotaxis, two physiological features that are extremely energy consuming (Tal and Okon 1985).

Aeromonas hydrophila is a heterotrophic bacterium found in warm climates, and in fresh, salty, marine, estuarine, chlorinated, and unchlorinated water. In addition, it is resistant to refrigeration and cold temperatures. This bacterium produces a PHA copolyester consisting of (*R*)-3-hydroxybutyrate and (*R*)-3-hydroxyhexanoate (PHBHHx; Chen et al. 2001). To understand the relationship between enhanced survival ability and PHA accumulation of *A. hydrophila*, the physiological behaviors of a wild type and a *phaC* mutant were compared. The ability of the *phaC* mutant to survive UV irradiation, heat and cold treatment, ethanol, osmotic pressure, and oxidative stress was significantly impaired as compared with that of the wild type. Thus, PHBHHx synthesis and accumulation in *A. hydrophila* is another example of positive correlation between resistance to environmental stresses and PHA accumulation (Zhao et al. 2007).

Studies done by Ruiz et al. (2004) demonstrated the association between PHA depolymerization and stress tolerance in *Pseudomonas oleovorans*.

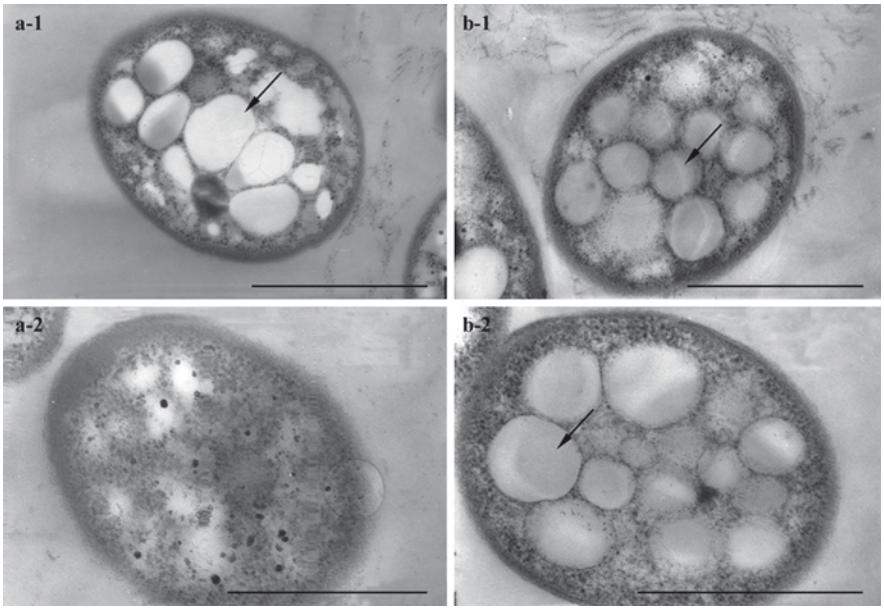

Fig. 3 Electron micrographs of thin sections of *A. brasilense* 7030 (**a**) and the *phaZ* mutant (**b**), grown for 48 h in high carbon to nitrogen medium, and of *A. brasilense* 7030 (**c**) and the *phaZ* mutant (**d**) following a 72-h starvation period in phosphate buffer. *Arrows* indicates PHB granules, *bars*, 1 μm. Thirty sections of each strain were examined and showed identical findings (Reproduced from Kadouri et al. (2003a), with permission of Springer-Verlag)

Experiments carried out during early stationary phase cultures (a carbon starvation condition that provokes a rapid PHA degradation) of *P. oleovorans* and its *phaZ* minus mutant showed that the mutant strain was more sensitive to heat and oxidative shocks than the wild type. In *P. putida*, impaired survival and resistance to oxidative stress of an *rpoS* mutant was shown under conditions inducing PHA accumulation (Raiger-Iustman and Ruiz 2008).

Altogether, the above-mentioned studies with different bacteria showed that PHA mutants affected in both anabolic and catabolic PHA pathways are affected in their tolerance to diverse stress conditions, suggesting that stress endurance can be traced to a normal functioning of the PHA cycle, and not exclusively to the presence of the polymer.

4 Regulation of PHA Synthesis

The mechanisms by which PHA favors stress alleviation are not yet fully understood. However, it is known that the PHA metabolism is regulated at both enzymatic and transcriptional levels, by cofactor inhibition and availability of metabolites, and by specific and global transcriptional regulatory factors, respectively (Kessler and Witholt 2001).

At the enzymatic level, it has been shown that, in PHA-producing bacteria, the intracellular levels of acetyl-CoA and free CoA, and both high intracellular level of NAD(P)H and high ratio of NAD(P)H/NAD(P) play a central role in the regulation of PHA synthesis (Haywood et al. 1988; Lee et al. 1995; Kessler and Witholt 2001).

An early work on *C. necator* suggested an association between PHA utilization and both respiration and oxidative phosphorylation (Hippe and Schlegel 1967). The effector guanosine tetraphosphate (ppGpp) was shown to increase messenger RNA translation of the sigma factor σ^s encoded by *rpoS* (Gentry et al. 1993; Brown et al. 2002), which is involved in PHA synthesis (see below). In *P. oleovorans*, it was found that the rise in ATP and ppGpp levels was concomitant with PHA degradation (Ruiz et al. 2001). This phenomenon was only observed in the wild-type strain but not in a PHA depolymerase-deficient mutant unable to degrade the polymer (Ruiz et al. 2001).

As stated above, evidence from recent years indicates that the central stationary phase regulator RpoS is involved in PHA metabolism. In *Escherichia coli*, RpoS controls the general stress response, inducing the expression of genes involved in protection against viability loss in nutrient-poor environments, such as those inducing PHA synthesis in several microorganisms. The half-life of RpoS is related to the cell nutrient status. The proteolysis of RpoS is mediated by the ClpXP protease. During starvation, aberrant misfolded proteins compete for ClpXP, reducing RpoS degradation (Ferenci 2007).

The synthesis of PHA and its regulation in *A. vinelandi* have been recently reviewed by Galindo et al. (2007). An extracellular signal is detected by the two-component global regulatory system formed by the histidine sensor kinase GacS and the response regulator GacA, activating *rpoS* transcription. At the transcriptional level, *rpoS* expression appears to be modulated by the GacSA system and by the intracellular levels of ppGpp. During stationary phase, RpoS stimulates the transcription of the *phaBAC* operon, through the pB2 promoter, and the transcriptional activator *phaR*, though its pR2 promoter. Consequently, PhaR activates the transcription of the *phaBAC* operon through the pB1 promoter. In contrast, during exponential phase there is no PHA production because PhaA activity is inhibited by the allosteric control produced by the acetyl-CoA to CoA ratio, and by low levels of transcription of *phaBAC* due to the lack of RpoS.

An enhanced expression of *rpoS* in *A. hydrophila* has been linked to the enhanced resistance to environmental stress conferred by PHBHHx (Zhao et al. 2007). In *P. oleovorans*, the increase of the intracellular concentration of RpoS during PHA depolymerization was related to an enhanced cross-tolerance to different stress agents (Ruiz et al. 2004). Interestingly, under PHA accumulation and nonaccumulation conditions, an *rpoS* mutant of *P. putida* had similar and lower survival under oxidative stress, respectively, as compared with the wild-type strain (Raiger-Iustman and Ruiz 2008).

The relevance of additional sigma factors in regulation of the PHA metabolism has to be considered since in *P. aeruginosa* PAO1, PHA accumulation from gluconate was found to require a functional RpoN sigma factor, whereas PHA accumulation in cells growing on fatty acids was only reduced in the absence of RpoN (Timm and Steinbüchel 1992). In addition, RpoS has not been documented in *Azospirillum* species.

5 PHA in Soil and in Plant–Microbe Interactions

Soil is a heterogeneous, discontinuous, and structured environment with a high diversity of microhabitats in which conditions can change rapidly (Postma et al. 1989). Thus, bacteria in soil have to cope with fluctuating – in time and space – biotic and abiotic stresses (van Elsas and van Overbeek 1993). One strategy by which bacteria can improve their establishment, proliferation, and survival in competitive niches such as soil and the rhizosphere is the accumulation and degradation of PHA (Okon and Itzigsohn 1992; Kadouri et al. 2005). In general, conditions of suboptimal growth are conducive to the production of PHAs (Madison and Huisman 1999).

Supporting data for PHA production in telluric environments were provided by Wang and Bakken (1998), who screened 63 soil bacteria for PHA production. They concluded that strains capable of producing PHA were not necessarily superior to those that lack this ability. Instead, survival ability was strain-specific and depended on the growth conditions prior to starvation. In this study, most PHA-producing bacteria were found to belong to the pseudomonad, coryneform, and bacillus groups. In addition to *Pseudomonas* and *Bacillus*, Arshad et al. (2007) reported the isolation of soil PHA-producing bacteria belonging to the genera *Citrobacter*, *Enterobacter*, *Klebsiella*, and *Escherichia*, all of them enterobacteria. Among symbiotic bacteria and plant growth-promoting rhizobacteria (PGPR), PHA production has been reported in members of the genera *Rhizobium*, *Azospirillum*, *Herbaspirillum*, and *Azotobacter* (Itzigsohn et al. 1995; Catalán et al. 2005; Trainer and Charles 2006).

Many PHA-producing *Bacillus* strains have been isolated from soil (Wang and Bakken 1998; Yilmaz et al. 2005; Arshad et al. 2007). In a recent proteomic analysis, Luo et al. (2007) reported that the soil bacterium *B. cereus* increased its fatty acid metabolism when grown in a medium prepared from oak forest soil. This increased fatty acid catabolism was reflected in changes in membrane structure and accumulation of PHA. In agreement with these findings, PhaR, which is required for PhaC activity, was one of the most upregulated proteins (Luo et al. 2007). In another study, it was shown that survival rate and the total cell number (including vegetative cells and spores) of the soil PHA-accumulating bacterium *B. megaterium* were higher than those of PHA negative mutants (López et al. 1998).

The nature of the carbon compounds found in the soil affects the growth rates of microorganisms and their root colonization ability (Chen et al. 1996; Simons et al. 1996; Jjemba and Alexander 1999). The relationship between PHA metabolism and plant root colonization is not obvious. Among PGPR, the free-living, Gram-negative, nitrogen-fixing bacteria belonging to the genus *Azospirillum* are well-established models for deciphering traits important for survival, colonization, and effectiveness (Okon and Vanderleyden 1997). One such trait appears to be the secretion of plant-growth-promoting substances (e.g., auxins, gibberellins, and cytokinins) that lead to an increase of the root surface area, promoting water and mineral uptake (Dobbelaere and Okon 2007; Steenhoudt and Vanderleyden 2000). PHA accumulation in *A. brasilense* is likely an important trait for root colonization of this bacterium (Tal and Okon 1985; Tal et al. 1990a). In support of this assumption, it was demonstrated that under certain conditions, including high carbon-to-nitrogen ratio or

low-oxygen partial pressure, *A. brasilense* cells accumulate above 75% of their dry weight exclusively as PHB (Nur et al. 1981; Tal and Okon 1985; Paul et al. 1990; Tal et al. 1990a, b; Itzigsohn et al. 1995).

Studies carried out with wild-type and *phaC* mutant strains of *A. brasilense*, under sterile and nonsterile conditions in soil showed that both root colonization and plant growth promotion were not affected in the mutant (Kadouri et al. 2002). The lack of influence of this mutation on these parameters may stem from the optimal plant growth conditions as well as from the relatively high inoculum level used in that study. It is still to be assessed whether the impaired stress resistance and physiological changes observed in cells with a disrupted PHA metabolism have negative implications in root colonization and plant growth promotion in the field.

Rhizobia are characterized by a free-living stage in the soil and by a symbiotic stage in the interaction with leguminous roots. The establishment of the symbiotic relationship involves a bidirectional signal exchange between the bacteria and the host plant, which leads to the formation of nitrogen-fixing root nodules. Results from studies performed to evaluate the relationship between PHA metabolism and the efficiency of the rhizobia–legume interaction have been diverse, and it seems that they vary not only because of differences between the various bacteria–host systems, but also because of differences in the experimental conditions among the studies. For instance, single strain inoculation experiments with *phaC* mutants of *S. meliloti* and *Rhizobium leguminosarum* bv. *viciae* on alfalfa and pea plants, respectively, suggested that both symbiotic systems are not affected by PHA formation (Povolo et al. 1994; Lodwig et al. 2005). On the other hand, the *S. meliloti–Medicago truncatula* system was severely impaired by the lack of PHA formation ability by the bacterium, as plants inoculated with the *S. meliloti phaC* mutant showed lower rates of nitrogen fixation, lower numbers of nodules, and reduced shoot dry weight as compared with plants inoculated with the wild-type strain (Wang et al. 2007).

In a study done by Willis and Walker (1998), coinoculation experiments of alfalfa with wild-type and *phaC* mutant strains of *S. meliloti* indicated that the wild-type strain outnumbered the PHA mutant by more than 200 times. This result indicates that the *phaC* mutant was less competitive, and that PHA production may provide an advantage to the bacterium during root invasion or nodule initiation. Wang et al. (2007) assessed the symbiotic efficiency of an *S. meliloti* double mutant impaired in *phaP1* and *phaP2*, which encode the PHA granule-associated phasins that regulate PHA synthesis and granule formation. Plants inoculated with this mutant exhibited a reduced shoot dry weight compared with those inoculated with the wild type, but there was no corresponding reduction in nitrogen-fixation activity. Thus, it appears that in the alfalfa–*S. meliloti* system, PHA production by the bacterium does not play a significant role after the establishment of nodules. Moreover, bacterial phasins seem to be involved in a metabolic regulatory response and/or to influence assimilation of fixed nitrogen rather than nitrogen-fixation activity (Wang et al. 2007).

Interestingly, it has been reported that common bean plants inoculated with a *phaC* mutant of *R. etli* show an increased nitrogen-fixation capacity and enhanced growth in comparison with plants inoculated with the wild-type bacterium (Cevallos et al. 1996). An important difference between *S. meliloti* and *R. etli* is that in the former,

bacteroids occupy indeterminate nodules, whereas in the latter, bacteroids occupy determinate nodules. Both bacteria produce granules of PHA (in the form of PHB) during the initial stage of invasion. However, in *S. meliloti* the PHB granules disappear during differentiation into the bacteroid state, and bacteroids occupying the alfalfa indeterminate nodules do not accumulate PHA after the establishment of the symbiosis (Lodwig et al. 2005). It is possible that in the case of S. *meliloti*, the intracellular PHA supports cell division and growth during root infection and invasion (Trainer and Charles 2006). In contrast, bacteroids of determinate nodules, such as those induced by *R. etli* on common bean, accumulate high levels of PHA during symbiosis. In this case, PHA could support nitrogen fixation under conditions of reduced carbon availability, and PHA accumulation and nitrogen fixation would compete for energy and reductant sources as well as for photosynthates (Cevallos et al. 1996; Trainer and Charles 2006). The relationship between carbon storage and nitrogen fixation is complex. For example, in free-living *Bradyrhizobium japonicum*, *R. leguminosarum*, and *S. meliloti*, at the same time as PHA is accumulated, there is production of glycogen as an additional storage compound (Lodwig et al. 2005).

Azorhizobium caulinodans, as *R. etli*, accumulates PHA in both free-living and symbiotic stages, but an *A. caulinodans phaC* mutant was totally devoid of nitrogenase activity ex plant, and induced nodules devoid of bacteria (Mandon et al. 1998). Interestingly, nitrogenase activity of the mutant was partially restored by constitutive expression of the *nifA* gene. Mandon et al. (1998) suggested that PHA is required for maintaining the reducing power of the cell, and that *nifA* expression mediates adaptation of nitrogen fixation to the levels of carbon and reducing equivalents available in the nodule. Vassileva and Ignatov (2002) studied the relationship between PHA formation and nitrogenase activity in the *Galega orientalis–R. galegae* system. They reported high nitrogen-fixation activity in parallel to PHA degradation when low concentrations of plant growth promoters and polyamine modulators were applied.

In summary, the fact that PHA production is a widespread trait supports the assumption that PHA accumulation plays a central role in survival, especially when bacteria are faced with starvation. In PHA-producing bacteria, PHA is a major determinant for overcoming periods of carbon and energy starvation, and may represent a basic feature for so-called environmental bacteria. However, the ability to produce PHA is apparently not absolute for improved survival ability during stress, as PHA was shown to enhance the survival of some, but not all bacteria tested, which likely rely on alternative strategies (Wang and Bakken 1998).

6 Relevance of PHA in Microbial Communities

Most microorganisms on Earth are organized into microbial biofilms and microbial mat communities. PHA production is very relevant in these kinds of microbial organization as these are niches where microbes have to cope with moderate physical and chemical stresses, and frequently have to adapt to changing conditions.

Biofilms are sessile microbial communities embedded within a matrix and attached to a solid surface. On one hand, surface-associated multicellular communities are generally advantageous over individual planktonic cells, especially in regard to protection against unfavorable environmental conditions. On the other hand, planktonic populations can quickly reach and colonize new niches. The shift between sessile and planktonic lifestyles depends upon the integration of many environmental cues. Several biofilm-producing bacteria have also been reported to produce PHA. For instance, it was recently reported that PHA accumulation in *P. aeruginosa* biofilms occurs in a spatially/temporally regulated way, and that it is in competition with alginate biosynthesis, playing an important role in stress tolerance and biofilm formation (Pham et al. 2004; Campisano et al. 2008).

Mats have been described as large microbial communities composed by a multilayered sheet of bacteria, archaea, and diatoms, which are characterized by both seasonal and diel fluctuations (e.g., flooding and desiccation, diel fluctuations of temperature, light, pH, oxygen, sulfide, and nutrients, among others). A culture-independent strategy for the detection of PHA-producing bacteria from a polluted marine microbial mat was adopted by López-Cortés et al. (2008). The authors showed a higher PHA-producing microbial diversity in a marine microbial mat exposed to environmental stress by organic pollution from a cannery of marine fish (nutrient imbalance) as compared with a pristine site. Because PHA synthesis is linked to lipid metabolism, PHA producers are more competitive in environments with high concentrations of fatty acids such as active sludge and microbial mats. Also, Villanueva et al. (2007) suggested that during diel fluctuations, heterotrophic microorganisms from phototrophic mats accumulate PHA, using as a precursor the excess of carbon that is generated and excreted by photosynthetic microorganisms, reflecting that changes in PHA levels depend on the time of day. Interestingly, the isolation of PHA-producing strains from mats with potential industrial applications has been successful, positioning mats as an excellent source for such microbes (Berlanga et al. 2006; Simon-Colin et al. 2008).

7 Utilization of the Energy Obtained from PHA for Environmental Cues

In addition to being a source of storage compounds and contributing to stress endurance, PHAs can serve as sources of NADH and ultimately ATP. Under diverse environmental conditions, the ability to generate energy from PHAs can be used to drive various important energy-consuming pathways, as discussed in the folowing.

7.1 Chemotaxis

Chemotaxis is the ability bacteria have to sense gradients of compounds and to drive motility toward the most appropriate niche, and is an important trait in

plant–microbe interaction. *A. brasilense* exhibits strong chemotaxis toward different attractants such as fructose, malate, and sweet corn seed exudates, and it was shown that this chemotaxis is significantly stronger in the wild type than in a *phaC* mutant strain (Kadouri et al. 2003b). It is possible that the reducing power produced during PHA degradation energizes the chemotactic process in the environment, where sources of reducing power are low. In *A. brasilense*, PHA oxidation involves a specific NADH-dependent dehydrogenase, which competes for tricarboxylic acid (TCA) cycle intermediates in the electron transport system (Tal et al. 1990a, b). When PHA accumulation is disrupted, more resources are available for the TCA cycle, resulting in an increased motility in the *phaC* mutant as compared with the wild type. In contrast, an *A. brasilense phaZ* mutant was shown to have motility similar to that of the wild type (Kadouri et al. 2003a). *phaZ* encodes a poly(β-hydroxybutyrate) depolymerase; thus, it is likely that, in contrast to the *phaC* mutant, the *phaZ* mutant is unable to generate excess reducing power (Kadouri et al. 2003a).

The redox state of the rhizosphere is one of the most important parameters for maintaining this ecological system. Thus, the energy taxis, driven by PHA catabolism, toward metabolizable substrates in plant root exudates may play a major role in plant–microbe interactions. On the other hand, rhizobia are positively chemotactic toward a variety of amino acids, dicarboxylic acids, sugars, and nodulation-gene-inducing flavonoids secreted by the roots of their hosts. Rhizobial mutants defective in motility or chemotaxis are impaired in their ability to compete for sites of nodule initiation in the host root (Caetano-Anollés et al. 1988). If as suggested for *A. brasilense*, PHA catabolism in *S. meliloti* is also involved in energy supply for chemotaxis, it could at least partially explain why the *S. meliloti phaC* mutant strain is less competitive than the wild type (Willis and Walker 1998).

7.2 Exopolysaccharide Production

The roles of exopolysaccharide (EPS) in bacteria are dependent on their natural environment. Most of the functions assigned to EPS are related to a protective role: the highly hydrated EPS layer with which bacteria are capable of surrounding themselves provides a shield against desiccation and predation (Kumar et al. 2007). Many bacteria produce and live within a matrix of EPS in their natural environment, for example, in soil. EPS contributes anchoring cells to different substrates, protecting them against phagocytosis, masking antibody recognition, and preventing lysis by other bacteria (Deaker et al. 2004). EPS also plays an important role in plant–bacteria interactions. In rhizobia, EPS is required for success in the different stages of the establishment of the nitrogen-fixing symbiosis, including root colonization, host recognition, infection thread formation, and nodule invasion. In protective roles, EPS is important for evasion of plant immune responses and protection from reactive oxygen species (Gonzalez et al. 1996; Cooper 2007). In azospirilla, EPS is known to be involved in cell aggregation and in root adhesion (Burdman et al. 2000a). Burdman et al. (2000b) and Bahat-Samet et al. (2004)

showed that the arabinose content of *A. brasilense* EPS plays a role in cell aggregation and Mora et al. (2008) identified an outer-membrane protein with lectin activity that specifically binds to the EPS produced by *A. brasilense* during aggregation conditions. In *A. brasilense,* several studies support EPS and PHA production as well as cell aggregation being interdependent phenomena (Burdman et al. 1998; Kadouri et al. 2002, 2003a, b; Aneja et al. 2004; Wang et al. 2007).

In the *phaC* mutant of *A. brasilense*, a considerable increase in excreted EPS was detected over the wild-type strain when grown under a medium characterized by a high carbon-to-nitrogen ratio. In such a mutant, EPS production may act as a sink for carbon and reducing equivalents which are diverted from the blocked PHA synthesis pathway. The *phaC* mutant was more aggregative, and exhibited a significantly increased ability to adhere to roots relative to the wild type (Kadouri et al. 2002, 2003b). In contrast, EPS production and cell aggregation capability in the wild-type strain were higher than in the *phaZ* mutant under the same growth conditions (Kadouri et al. 2003a). Burdman et al. (2000a) suggested that, in addition to PHB accumulation, cell aggregation could increase survival of *Azospirillum* cells under diverse stress conditions. Cell aggregation as well as a functional PHA metabolism may also be important during root colonization where cell aggregation is commonly observed (Kadouri et al. 2005).

In contrast to the findings with *A. brasilense*, in *S. meliloti* it has been shown that the inability to synthesize PHA is strongly associated with reduced production of EPS (Aneja et al. 2004; Wang et al. 2007). Interestingly, the *phaP1*/*phaP2* double mutant of *S. meliloti*, which as stated above is impaired in PHA production, produces more EPS and glycogen than does the wild-type strain (Wang et al. 2007). In *R. etli*, an *aniA* mutant strain exhibited a significant decrease in PHA accumulation, and a significant increase in EPS formation (Encarnación et al. 2002). The *aniA* gene encodes a transcriptional factor involved in the expression of genes that are important in partitioning the carbon flow in the bacterial cell, such as the ones stimulated under low-oxygen conditions and channeling of excess carbon into PHA and glycogen biosynthesis (Povolo and Casella 2000).

7.3 PHA as a Carbon and Energy Source for "Environmental Bacteria"

PHAs have attracted significant industrial interest because they are natural biodegradable thermoplastics, and they do not require special environmental conditions to be degraded. Beyond this, their biodegradability means that PHAs can be used as carbon and energy sources to support bacterial growth in different environments. When PHA-accumulating microorganisms cease dividing and undergo lysis, the polymer is released to the environment and it turns out to be readily metabolized by other microorganisms. PHAs can be degraded by the action of either intracellular or extracellular depolymerases (i-PhaZ and e-PhaZ, respectively) produced by PHA-degrading bacteria, algae, and fungi.

Two types of PHA polymers have been described: (1) native PHA granules containing lipids and proteins that are rapidly hydrolyzed by i-PhaZs and (2) denatured PHA granules, which are crystalline and hardly hydrolyzed by i-PhaZs but are efficiently degraded by ubiquitous e-PhaZs into water-soluble products (Tokiwa and Calabia 2004). Several environmental bacteria, algae, and fungi are able to "attack" PHA granules and to solubilize the PHA polymer. The polymer is then degraded by e-PhaZs, producing oligomers, which in some cases can be further degraded by hydrolases into monomers. The breakdown products can be utilized as a carbon and energy source by these organisms (Philip et al. 2007).

On the basis of the size of the PHA polymer that can be degraded, e-PhaZs are divided into two groups: short-chain-length PHA (SCL-PHA) and medium-chain-length PHA (MCL-PHA) depolymerases. The majority of PHA-degrading microbes produce only one type of e-PhaZ. Only a few bacterial species have been reported to produce both kinds of depolymerases, thus being able to degrade both SCL-PHA and MCL-PHA (Kim et al. 2007). The rate of PHA degradation is dependent on environmental conditions including temperature, pH, moisture, and nutrient supply, as well as on properties related the PHA substrate, such as monomer composition, crystallinity, additives, and surface area (Philip et al. 2007). *Pseudomonas* and *Stenotrophomonas* are the predominant MCL-PHA degraders in soil and marine environments (Kim et al. 2007). However, also microorganisms from the families *Pseudonocardiaceae*, *Micromonosporaceae, Thermomonosporaceae*, *Streptosporangiaceae*, and *Streptomycetacease* have been reported to degrade PHA in the environment (Philip et al. 2007).

8 Phylogenetic Aspects of PHA Metabolism and Their Relationship with the Environment

Systematic phylogenetic analyses of genes involved in PHA biosynthesis and degradation were recently carried out by Kadouri et al. (2005) and Kalia et al. (2007). From 253 sequenced genomes, Kalia et al. (2007) identified 71 and 111 complete *phaA* and *phaB* sequences, respectively. The PhaA and PhaB phylogenetic trees showed 12 and 16 cases of discrepancy, respectively, as compared with 16S ribosomal DNA (rDNA) phylogenies. These inconsistencies might be explained by horizontal gene transfer (HGT). The presence of the *phaC* gene was detected in 72 genomes belonging to 40 genera from different taxonomical groups such as *Actinobacteria*, *Cyanobacteria*, *Firmicutes*, and *Proteobacteria* (Kalia et al. 2007). Analysis of the PhaC phylogenetic tree revealed quite a few significant deviations as compared with the 16S rDNA. Similarly, a PhaC phylogenetic tree built with 67 homologous proteins from *Proteobacteria* by Kadouri et al. (2005) showed that the tree was congruent with the 16S rDNA data and clustered according to the phylogenetic taxa, suggesting the existence of genotypic clusters that correspond to traditional species designations. In addition, this PhaC tree topology was in agreement with previous analyses reported by Steinbüchel and Hein (2001) and Rehm (2003).

The mechanism and regulation of PHA mobilization is poorly understood, but as mentioned, a clear distinction exists between intracellular and extracellular PHA degradation by means of PhaZ enzymes (Jendrossek and Handrick 2002). Kadouri et al. (2005) analyzed the phylogeny of the i-PhaZ. Most clusters of the i-PhaZ phylogenetic tree were highly incongruent with those of the 16S rDNA tree. Interestingly, sequencing of the genome of *C. necator* (formerly *R. eutropha*) H16 revealed this bacterium possesses six different PhaZs (Pohlmann et al. 2006), and two copies of PhaZ were detected in the genomes of *R. metallidurans* and *Burkholderia fungorum* (Kadouri et al. 2005). The multiplication of genes encoding PHA depolymerases in a genome may reflect the diversity of the PHAs that a given microorganism is able to produce and utilize. The incongruence observed in PhaZ phylogenetic tress suggests that these genes have likely been subjected to HGT. In addition, the multiplication of these genes in some bacterial genomes possibly reflects duplication events that lead to parallel evolution of different genes to increase the versatility of the organism for PHA utilization. Thus, although the process of PHA synthesis seems to be highly conserved, it appears that a variety of options for PHA utilization have been laterally acquired and/or developed in parallel by several microorganisms.

Kalia et al. (2007) also revealed for the first time the presence of all three PHA biosynthesis genes (*phaA*, *phaB*, and *phaC*) in some cyanobacteria and *Firmicutes*. Prior to this, different combinations of *phaA*, *phaB*, and/or *phaC* were partially detected among a few members of Archaea, actinobacteria, and cyanobacteria (Kalia et al. 2007).

In conclusion, on the basis of the highly frequent appearance of *phaCAB* clusters and the relatively high congruence between PhaA, PhaB, and PhaC phylogenetic trees with 16S rDNA trees, it appears that the acquisition of PHA biosynthesis genes is an ancient event, at least in *Proteobacteria*. However, it is becoming evident that these genes have been spread among microorganisms by HGT, thus leading to the acquisition of the PHA accumulation trait by other groups of bacteria. Interestingly, it was recently reported that the *pha* gene cluster of a *Pseudomonas* isolate from the Antarctic, which produces high amounts of PHB, is located in a genomic island within a large genetic element of approximately 32.3 kb (Ayub et al. 2007). GC content, phylogeny inference, and codon usage analyses showed that in this bacterium the *phaBCA* operon itself has a complex mosaic structure and indicated that the *phaB* and *phaC* genes were acquired by HGT, probably derived from *Burkholderiales* (Ayub et al. 2007).

Some natural and anthropogenic activities are dramatically affecting the environment. As a result, we are witnesses to an increase in the intensity of extreme weather events, desertification, reduction of the ozone layer, and acidification of the oceans among other phenomena of concern. Supraorganism systems (namely, populations of micro- and macroorganisms, organized into trophic chains and capable of biotic cycling) have to adapt to the new situations. Considering that PHA accumulation is involved in bacterial cell survival and stress endurance, and that PHA genes have been subjected to HGT, it is reasonable to hypothesize that PHA accumulation and degradation is presently an evolutionarily valuable trait that microcommunities can exploit to deal with such environmental changes.

9 PHA Applications in Agriculture

Bacterial inoculants are commercial formulations for agricultural uses containing PGPR that can be applied to the seeds or to the soil during planting. During production, transportation, and storage of inoculants, bacteria should respond to (and survive) several stress factors, such as acidity, desiccation, chemical pesticides, and nonoptimal temperatures (Rebah et al. 2007). In other words, inoculants that have the capability to support survival rates of bacterial cells are highly desirable. Thus, appropriate materials for carrying microbes must offer special properties, such as chemical and physical uniformity, high water holding capacity, and lack of toxicity, and they must be environmentally friendly. Commercial inoculants are available as solid – in powder from peat or in granular form – or as liquid formulations (Stephens and Rask 2000; Rebah et al. 2007).

Many microbial inoculants are based on solid peat formulations owing the protective properties of this material. Recently, Albareda et al. (2008) evaluated six carriers (bagasse, cork compost, attapulgite, sepiolite, perlite, and amorphous silica) as alternatives to peat. Cork compost and perlite gave as good results as peat in terms of support of *B. japonicum* and *S. meliloti* growth, maintaining a long survival of inoculated strains, as well as survival on soybean seeds. Most of the research done in this field aims at developing new carriers or improving carrier properties by adding elements such as nutrients or other synthetic products that can prolong survival (López et al. 1998).

The vast amount of information gathered especially on azospirilla and rhizobia throughout the years suggests that for an inoculant to be successful, i.e., to provide efficient root colonization and/or invasion, not only the type of carrier material is important for bacterial survival within the carrier itself, but also the metabolic state of the cells, including their capability to use intercellular storage materials such as PHB. This knowledge originating from studies showing that although the carriers may vary, plant growth promotion effects are more consistent with *A. brasilense* inoculants containing cells with high amount of PHB (Fallik and Okon 1996). In support of these studies, field experiments carried out in Mexico with maize and wheat revealed that increasing crop yields were obtained using peat inoculants prepared with PHB-rich *Azospirillum* cells (Dobbelaere et al. 2001). Additionally, experiments done with an *A. brasilense phaC* mutant showed that among different inoculant carriers (peat, sianic sand, and perlite), peat sustained the highest survival rates, whereas the lowest survival rates were observed in perlite. Importantly, although variations between carriers were very large, in all carriers the wild-type strain survived better than the mutant. It was thus concluded that the production of PHA is of critical importance for improving the shelf life, efficiency, and reliability of commercial inoculants (Kadouri et al. 2003b).

In other agricultural aspects, it has been shown that relevant agricultural substances, such as insecticides, can be incorporated into PHA granules. If spread in the environment, PHA-degrading bacteria can slowly degrade the PHA granule, leading to controlled release of the insecticide. This pioneer idea was proposed for the first time by Holmes (1985) and was recently supported by Philip et al. (2007).

However, to date, few studies have been performed on this subject, and despite its potential, this PHA application still seems to be far from commercial utilization.

10 Conclusions

PHAs have attracted the attention of many research groups as they are environmentally friendly polymers. It is becoming evident that PHA production is a widespread trait among microorganisms, suggesting that, among other strategies, PHA production plays a central role in survival under environmental stress conditions, such as those imposed in water and soil (Table 1). Despite significant advances in recent years, research is needed to understand the molecular mechanisms of regulation of both PHA accumulation and degradation, and how these processes enhance bacterial survival and fitness. Advances in this research area in the future could, for instance, benefit the industry of bacterial inoculants used for plant protection or plant growth promotion, as in these cases the capabilities of the microorganisms to establish, survive, and proliferate in the target niche is of utmost importance.

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Towards Systems Metabolic Engineering of PHA Producers

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Contents

Abstract Polyhydroxyalkanoates (PHAs) are natural polyesters that accumulate in numerous microorganisms as a carbon- and energy-storage material under the nutrient-limiting condition in the presence of an excess carbon source. PHAs are considered to be one of the potential alternatives to petrochemically derived plastics owing to their versatile material properties. Over the past few decades, extensive detailed biochemical, molecular-biological, and metabolic studies related to PHA

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biosynthesis have been carried out. Advances in our knowledge of PHA biosynthesis led to the development of engineered strains and fermentation processes for the production of PHAs with high efficiency. Even though the traditional metabolic engineering based on our rational thinking and trial-and-error type approaches allowed development of improved strains, further enhancement in the performance is expected through systems metabolic engineering, which is metabolic engineering integrated with systems-biological approaches. In this chapter, the strategies taken for the metabolic engineering of PHA producers are briefly reviewed. Then, genomic and proteomic studies performed to understand the PHA biosynthesis in the context of whole cell metabolism as well as to develop further engineered strains are reviewed. Finally, the strategies for the systems metabolic engineering of PHA producers are suggested; these will make it possible to produce PHAs with higher efficiencies and to develop tailor-made PHAs by systems-level optimization of the metabolic network and establishment of novel pathways.

1 Introduction

As our concerns regarding the environmental problems represented by global warming are increasing, there has recently been much interest in developing bio-based processes for the production of chemicals, fuels, and materials from renewable resources. Polymers which we use everyday are no exception. Among many types of biopolymers, polyhydroxyalkanoates (PHAs) have been considered to be good alternatives to petrochemically derived polymers. PHAs are natural polyesters that accumulate in numerous microorganisms as a carbon- and energy-storage material under the nutrient-limiting condition in the presence of an excess carbon source (Anderson and Dawes 1990; Lee 1996). Since poly[(*R*)-3-hydroxybutyrate], P(3HB), homopolymer was discovered in *Bacillus megaterium* by Maurice Lemoigne in 1926, more than 150 kinds of (*R*)-hydroxyalkanoic acid monomers have been found to be incorporated into bacterial PHAs (Steinbüchel and Valentin 1995). This variety of monomers allows PHAs to have diverse material properties and broadly classifies PHAs into one of three types: thermoplastics, which are short-chain-length (SCL) PHAs consisting of C_3–C_5 monomers; elastomers, which are medium-chain-length (MCL) PHAs consisting of C_6–C_{16} monomers; and their copolymer (SCL–MCL) PHAs, which show properties of both of them and notably low density polyethylene like properties (Fig. 1). The monomer composition of PHAs depends highly on the metabolic capability of host microorganisms and on the substrate specificity of PHA synthase, and subsequently determines the physicochemical properties of PHAs (Lee 1996; Rehm 2003). This implies that tailor-made PHA production using microorganisms can be optimized by metabolic engineering and key enzyme engineering, and can be finally maximized by optimized fermentation. Recently, the genome sequences of the most common PHA producers, *Cupriavidus necator* H16 (formerly, *Ralstonia eutropha* H16) and *Pseudomonas putida* KT2440, were reported by Pohlmann et al. (2006) and Nelson et al. (2002), respectively.

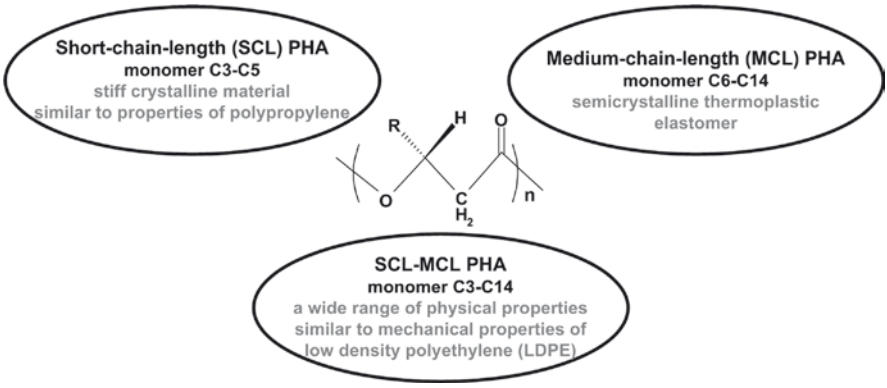

Fig. 1 General structure and three types of polyhydroxyalkanoates (PHAs), including short-chain-length (SCL) PHAs, medium-chain-length (MCL) PHAs, and SCL–MCL PHAs. Their characteristics are also summarized

Thus, PHA biosynthesis has also entered the genomics era, and strain development for novel PHA production and enhanced PHA production can be performed through systems metabolic engineering.

2 Traditional Metabolic Engineering of PHA Producers

Numerous bacteria accumulate PHAs under unfavorable growth conditions, such as the limitation of nitrogen, phosphorous, magnesium, or oxygen, in the presence of an excess carbon source. They share a common metabolism to generate core intermediates for PHA monomers from a carbon substrate, but the different characteristics of the preferred metabolism led to the generation of different monomers for PHAs (Anderson and Dawes 1990; Lee 1996). There have also been extensive studies on metabolic engineering of non-PHA-producing bacteria to make them efficiently produce PHAs to high levels (Fidler and Dennis 1992; Lee and Chang 1995; Lee et al. 1999).

2.1 Natural PHA Producers and Metabolic Engineering

Generally, many naturally PHA producing bacteria, including *C. necator* (Peoples and Sinskey 1989; Schubert et al. 1988; Slater et al. 1988; Doi et al. 1992), *Alcaligenes latus* (Braunegg and Bogensberger 1985; Hangii 1990; Hrabak 1992; Yamane et al 1996), *Pseudomonads* (Gross et al. 1989; Fritzsche et al. 1990; Huisman et al. 1991; Timm and Steinbüchel 1992; Timm et al. 1994), *Aeromonas* (Doi et al. 1995; Lee et al. 2000), *Rhodococcus ruber* (Pieper and Steinbüchel 1992), and *Syntrophomonas*

wolfei (McInerney et al. 1992), can synthesize a wide range of PHAs depending on the carbon source and the cultivation condition employed. Among them, *C. necator* and *P. putida* have been most widely used for PHA production.

C. necator has been studied extensively because of its great ability to accumulate P(3HB), up to 80–90 wt%, from simple carbon sources (Repaske and Mayer 1976; Heinzle and Lafferty 1980; Kim et al. 1994a). By applying nitrogen or phosphorous limitation, *C. necator* can accumulate a large amount of P(3HB) from a simple carbon source such as fructose or glucose. When both glucose and propionate are added during the polymer accumulation phase, poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyvalerate], P(3HB-*co*-3HV), can be produced in *C. necator*. Depending on the ratio of glucose to propionate fed into the medium, the (*R*)-3-hydroxyvalerate (3HV) fraction of this copolymer can be varied from 0 to 30 mol%, and a polymer content of about 75 wt% is obtained (Byrom 1992; Kim et al. 1994b). When 22 $g\,l^{-1}$ propionate was used as the sole carbon source during the polymer synthesis phase, the 3HV fraction reached up to 43 mol%, whereas the polymer content was only 35 wt% in *C. necator* (Doi et al. 1986). *C. necator* possesses class I PHA synthase, which preferentially utilizes and incorporates SCL (*R*)-3-hydroxyalkanoates into PHAs. However, it has been proved that *C. necator* has the capability to accumulate MCL PHAs consisting of (*R*)-3-hydroxyhexanoate (3HHx) and (*R*)-3-hydroxyoctanoate (3HO) as well as SCL PHAs when a β-oxidation inhibitor, sodium acrylate, is added and sodium octanoate is used as a carbon source (Green et al. 2002).

Although *C. necator* belongs to the naturally PHA producing bacteria, a number of engineered *C. necator* strains have been developed to improve PHA productivity as well as to control monomer compositions of PHAs. By introducing the genes encoding crotonyl-coenzyme A (CoA) reductase from *Streptomyces cinnamonensis*, and PHA synthase and (*R*)-specific enoyl-CoA hydratase from *Aeromonas caviae* into a *C. necator* PHA-negative mutant strain, the engineered strain was able to produce poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyhexanoate], P(3HB-*co*-3HHx), having 1.5 mol% 3HHx monomer up to 48 wt% from fructose (Fukui et al. 2002). Production of up to 87 wt% P(3HB-*co*-3HHx) having a constant level of 5 mol% 3HHx was achieved by cultivating a *C. necator* PHA-negative mutant strain harboring the *A. caviae* PHA synthase gene on palm kernel oil (Loo et al. 2005). Moreover, the 3HHx fraction could be altered in the range 0–5.1 mol% when several *A. caviae phaC* (*phaC*$_{Ac}$) mutant genes were introduced into the *C. necator* PHA-negative mutant strain, which resulted in the production of various P(3HB-*co*-3HHx) having different thermal properties of interest (Tsuge et al. 2004).

In pseudomonads belonging to the ribosomal RNA homology group I, the de novo fatty acid biosynthesis and degradation pathways generate intermediates including enoyl-CoA, 3-ketoacyl-CoA, (*S*)-3-hydroxyacyl-CoA, 3-ketoacyl-CoA, and 3-hydroxyacyl-acyl carrier protein (ACP), which can eventually serve as the precursors of MCL PHAs (Lee 1996; Madison and Huisman 1999; Witholt and Kessler 1999; Park and Lee 2003). Various enzymes, including enoyl-CoA hydratase (Fiedler et al. 2002; Fukui and Doi 1998), 3-ketoacyl-ACP reductase (Park et al. 2002; Ren et al. 2000; Taguchi et al. 1999), epimerase (Madison and Huisman 1999), and 3-hydroxyacyl-ACP:CoA transacylase (Rehm et al. 1998;

Hoffmann et al. 2000), play important roles in connecting the fatty acid metabolism and PHA biosynthesis. Since a novel metabolic link between fatty acid biosynthesis and PHA biosynthesis by the 3-hydroxydecanoyl-ACP:CoA transacylase (PhaG) was identified in *P. putida* (Rehm et al. 1998), there have been several reports showing that most pseudomonads use PhaG to accumulate PHAs containing MCL units from unrelated carbon sources such as sugar, except for *P. oleovorans* and *P. fragi* (Hoffmann et al. 2000; Fiedler et al. 2000). It was found that the expression system of the *P. oleovorans phaG* gene ($phaG_{Po}$) did not function properly in *P. oleovorans*. Reverse transcriptase polymerase chain reaction (PCR) of $phaG_{Po}$ obviously showed that $phaG_{Po}$ was not transcribed even when gluconate was used as a carbon source. However, the *P. oleovorans phaG* gene was expressed well using the *lac* promoter, which enabled *P. oleovorans* to accumulate MCL PHA up to 74 wt% (Hoffmann et al. 2000). Another non-PHA-producing *Pseudomonas* strain, *P. fragi*, equipped with the *phaC1* gene from *P. aeruginosa* and the *phaG* gene from *P. putida*, was able to accumulate 14 wt% MCL PHA composed mainly of about 60 mol% (*R*)-3-hydroxydecanoate, and additional constituents of 2 mol% 3HHx, 21 mol% 3HO, 11 mol% (*R*)-3-hydroxydodecanoate (3HDD), 4 mol% (*R*)-3-hydroxydodecenoate, and 1 mol% (*R*)-3-hydroxytetradecanoate (3HTD), when cultivated on gluconate as the sole carbon source (Fiedler et al. 2000).

It was reported that *P. putida* CA-3 was able to accumulate MCL PHA from the aromatic hydrocarbon styrene as the sole carbon source under nitrogen limitation through styrene degradation, de novo fatty acid biosynthesis, and β-oxidation pathways (O'Leary et al. 2001, 2005; Ward and O'Connor 2005). Recently, the *fadBA* mutant strain of *P. putida* KT2442 led to the enhanced production of MCL PHA having a dominant 3HDD or 3HTD fraction (up to 50 mol%) with higher crystallinity and tensile strength than typical MCL PHA, showing improved properties for various applications (Ouyang et al. 2007; Liu and Chen 2007).

2.2 Engineering of Non-PHA Producers

Although natural PHA producers have the ability to accumulate PHAs during their life cycle, they often have relatively low growth rate and low optimal growth temperature, and also possess PHA degradation pathways, which are disadvantageous to PHA production. On the other hand, *Escherichia coli*, a non-PHA-accumulating bacterium, is capable of neither PHA synthesis nor its degradation. *E. coli* grows fast at a relatively high temperature, and is easy to lyse, which allows a shorter cycle time for PHA production and cost savings associated with the cooling of the fermentor and the PHA purification process (Madison and Huisman 1999). Furthermore, *E. coli* has been intensively studied for PHA production because it was able to be metabolically engineered to synthesize PHAs thanks to versatile tools for genetic engineering (Fig. 2).

The plasmid-based expression of many genes for PHA synthesis in *E. coli* resulted in the production of various types of PHAs. The recombinant *E. coli* har-

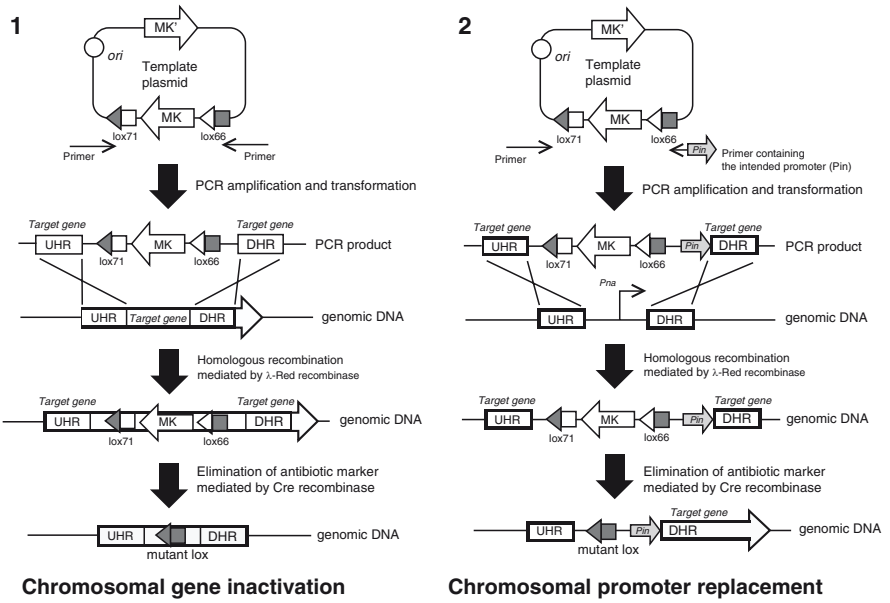

Fig. 2 PCR-mediated homologous recombination using the λ-Red recombinase as a typical *Escherichia coli* chromosomal manipulation method (Datsenko and Wanner 2000; Palmeros et al. 2000; Yuan et al. 2006). **1** Chromosomal gene inactivation, **2** chromosomal promoter replacement. *ori* replication origin, *MK* antibiotic resistance marker, *UHR* upper homologous region, *DHR* down homologous region, *Pna* native promoter

boring the *A. latus* PHA biosynthesis genes ($phaCAB_{Al}$) was able to produce the highest amount of P(3HB) compared with *C. necator* H16 wild-type strain and recombinant *E. coli* harboring *C. necator* H16 PHA biosynthesis genes ($phaCAB_{Cn}$) (Genser et al. 1998; Choi et al. 1998). The P(3HB) concentration and productivity obtained from the recombinant *E. coli* harboring the $phaCAB_{Al}$ genes were as high as 141.6 $g\,l^{-1}$ and 4.63 $g\,l^{-1}\,h^{-1}$, respectively (Choi et al. 1998). More interestingly, a filamentation-suppressed recombinant *E. coli* harboring the $phaCAB_{Al}$ genes and the *E. coli ftsZ* gene encoding the earliest acting cell division protein, FtsZ, accumulated P(3HB) up to 82.4 wt%, which was higher than that obtained with the recombinant *E. coli* harboring only the $phaCAB_{Al}$ genes (Choi and Lee 1999a).

In addition to P(3HB) homopolymer, P(3HB-*co*-3HV) copolymer could be produced in recombinant *E. coli* (Slater et al. 1992; Yim et al. 1996; Choi and Lee 1999b; Wong et al. 2007). When the PHA biosynthesis genes from *C. necator* ($phaCAB_{Cn}$) were introduced into *E. coli* LS5218 (*fadR atoC*(Con)), which can constitutively express the enzymes involved in the utilization of short-chain (C_4–C_6) fatty acids, P(3HB-*co*-3HV) could be produced from glucose and propionate as carbon sources. The P(3HB-*co*-3HV) content and the 3HV monomer fraction could be varied depending on the concentrations of glucose and propionate in the medium (Slater et al. 1992). Also, the recombinant *E. coli* XL1-Blue strain equipped with the $phaCAB_{Cn}$

genes was able to produce P(3HB-*co*-3HV) from glucose and propionate. Moreover, it was indicated that induction with acetate and/or oleate as well as propionate for the activation of propionate metabolism increased the PHA concentration and the 3HV fraction (Yim et al. 1996). P(3HB-*co*-3HV) could also be produced in recombinant *E. coli* harboring the *Salmonella enterica prpE* gene encoding propionyl-CoA synthetase and the $phaCAB_{Cn}$ genes. The 3HV monomer fraction in the copolymer varied from 5 to 18 mol%, depending on the expression level of PrpE, which was under the control of the isopropyl β-D-1-thiogalactopyranoside inducible *tac* promoter (Wong et al. 2007).

Production of MCL PHAs was first investigated in recombinant *E. coli* by Langenbach et al (1997). A number of strategies have been developed to improve MCL PHA productivity by providing PHA precursors from the fatty acid β-oxidation pathway (Fig. 3; Park et al. 2004). The β-oxidation pathway has been engineered by the overexpression of enoyl-CoA hydratase (Fiedler et al. 2002; Fukui and Doi 1998) or 3-ketoacyl-ACP reductase (Park et al. 2002; Ren et al. 2000;

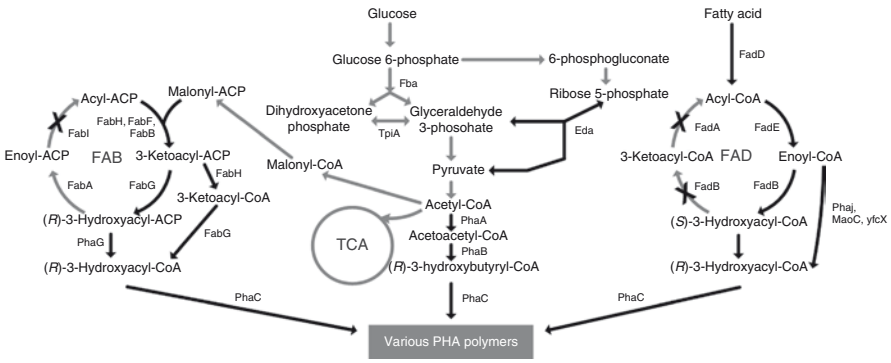

Fig. 3 Rational metabolic engineering for PHA production in *E. coli*. *Black arrows* and *crosses* represent genetic manipulations of overexpression and inhibition, respectively, for the enhancement of targeted monomers. The enzymes related to PHA production are shown. The enzymes are listed below along with the representative sources of the foreign enzymes: FabA, β-hydroxyl- acyl carrier protein (ACP) dehydrase; FabB, β-ketoacyl-ACP synthase/malonyl-ACP decarboxylase; FabF, β-ketoacyl-ACP synthase; FabG, β-ketoacyl-ACP reductase (Park et al. 2002); FabH, β-ketoacyl-ACP synthase/acetyl coenzyme A (CoA):ACP transacylase (Taguchi et al. 1999; Nomura et al. 2004); FabI, enoyl-ACP reductase (Rehm et al. 2001); FadA, β-ketoacyl-CoA thiolase (Park et al. 2003); FadB, multifunctional enzyme encoding hydratase and dehydrogenase activities (Park et al. 2003); FadD, fatty acyl-CoA synthetase (Park et al. 2003); FadE, acyl-CoA dehydrogenase (Park et al. 2003); MaoC, enoyl-CoA hydratase (Park and Lee 2003); YfcX, enoyl-CoA hydratase (Snell et al. 2002); Fba, fructose bisphosphate aldolase (Han et al. 2001); TpiA, triosephosphate isomerase (Han et al. 2001); Eda, 2-keto-3-deoxy-6-phosphogluconate aldolase (Hong et al. 2003; Han et al. 2001); PhaA, β-keththiolase from *Cupriavidus necator* (Lee 1996); PhaB, acetoacetyl-CoA reductase from *C. necator* (Lee 1996); PhaG, (*R*)-3-hydroxydecanoyl-CoA:ACP transacylase from *Pseudomonas putida* (Rehm et al. 1998); PhaJ, enoyl-CoA hydratase from *Aeromonads* (Fukui and Doi 1998); PhaC, PHA synthase from types I, II, III, and IV (Rehm 2003). *TCA* tricarboxylic acid cycle, *FAB* de novo fatty acid biosynthesis pathway, *FAD* fatty acid β-oxidation pathway

Taguchi et al. 1999), and/or by the deletion of FadB or FadA (Langenbach et al. 1997; Snell et al. 2002).

It has been reported that *E. coli* possesses enzymes belonging to the crotonase superfamily, including YfcX, PaaF, PaaG, and YgfG, all of which are highly homologous to FadB. A number of recent studies reported increasing the enoyl-CoA hydratase activity by the overexpression of one of these enzymes in *E. coli* (Haller et al. 2000; Snell et al. 2002; Fiedler et al. 2002; Park and Lee 2003, 2004). The *E. coli fadB yfcX* mutant strain harboring only the *P. oleovorans phaC1* gene ($phaC1_{Po}$) was not able to produce MCL PHA when grown in the presence of fatty acids. However, introduction of the *yfcX* gene along with the $phaC1_{Po}$ gene into an *E. coli fadB yfcX* mutant strain restored the capability of producing MCL PHA in this strain. Moreover, the PHA content increased significantly up to 27.7 wt%, which is 3 times more than that obtained with the *E. coli fadB* mutant strain (Snell et al. 2002). Furthermore, *E. coli* FadB homologous enzymes, such as PaaG, PaaF, BhbD, SceH, and YdbU, identified by a protein database search, were recently taken into consideration in an *E. coli* W3110 *fadB* mutant, WB101 strain (Park and Lee 2004). It is noticeable that no PHA accumulation was observed when each of these genes was coexpressed with the *Pseudomonas* sp. 61-3 *phaC2* gene ($phaC2_{Ps61\text{-}3}$) in *E. coli* W3110. The probable reason is that *E. coli* W3110 wild-type strain possesses a completely functional fatty acid β-oxidation pathway and does not use these enzymes to produce PHAs. On the other hand, coexpression of these genes along with the $phaC2_{Ps61\text{-}3}$ gene in *E. coli* WB101 led to MCL PHA accumulation. When the *paaG, paaF*, and *ydbU* genes were individually coexpressed with the $phaC2_{Ps61\text{-}3}$ gene in *E. coli* WB101, the PHA concentrations obtained were 0.37, 0.25, and 0.33 $g\,l^{-1}$, respectively, from 2 $g\,l^{-1}$ sodium decanoate, which was higher than that obtained (0.16 $g\,l^{-1}$) with *E. coli* WB101 expressing only the $phaC2_{Ps61\text{-}3}$ (Park and Lee 2004).

E. coli MaoC, which is homologous to *P. aeruginosa* (*R*)-specific enoyl-CoA hydratase (PhaJ1), was identified and found to be important for PHA synthesis in *E. coli* WB101. When the $phaC2_{Ps61\text{-}3}$ gene was introduced, *E. coli fadB maoC* mutant produced 43% less MCL PHA from decanoate, compared with *E. coli fadB* mutant WB101. The plasmid-based expression of the *maoC* gene was able to restore the PHA biosynthetic capability. Also, *E. coli* W3110 wild-type strain harboring the *maoC* gene and the $phaC2_{Ps61\text{-}3}$ gene was able to produce MCL PHA from decanoate. This study suggested that MaoC is an enoyl-CoA hydratase supplying (*R*)-3-hydroxyacyl-CoA, (*R*)-3HA-CoA, from the fatty acid β-oxidation pathway to the PHA synthesis pathway in *E. coli* (Park and Lee 2003).

The *P. aeruginosa phaJ1* and *phaJ2* genes ($phaJ1_{Pa}$ and $phaJ2_{Pa}$) have also been studied in *E. coli* for PHA production. Six recombinant *E. coli* LS5218 strains equipped with a PHA synthase gene ($phaC_{Ac}$ or $phaC1_{Ps61\text{-}3}$) alone or its combination with a hydratase gene ($phaJ1_{Pa}$ or $phaJ2_{Pa}$) were compared with one another for PHA production. Among them, four strains harboring both PHA synthase and hydratase genes accumulated much more PHA (up to 29 wt%) than the two strains harboring the PHA synthase gene alone. This result proved that both the $phaJ1_{Pa}$ gene and the $phaJ2_{Pa}$ gene play an important role in supplying the monomer (*R*)-3HA-CoAs for PHA synthesis in *E. coli* LS5218 (Tsuge et al. 2000).

The de novo fatty acid biosynthesis pathway has also attracted a great deal of attention as a means to direct PHA precursors in a desired way. The 3-hydroxydecanoyl-ACP:CoA transferase encoded by the *phaG* gene plays a role in bridging the fatty acid metabolism and PHA biosynthesis. Unlike the *Pseudomonas* fatty acid biosynthetic pathway, the *E. coli* fatty acid biosynthetic pathway does not efficiently provide MCL PHA precursors from unrelated carbon sources, such as glucose and gluconate, resulting in poor MCL PHA production in *E. coli.* Expression of the *phaG* gene from several *Pseudomonas* strains alone was not able to support (*R*)-3HA-CoAs in recombinant *E. coli* from unrelated carbon sources (Rehm et al. 1998). The strategy of chemical inhibition of fatty acid biosynthesis, which works well in some microorganisms, did not allow more efficient MCL PHA production in recombinant *E. coli.* When cerulenin, which specifically inhibits FabB (β-ketoacyl-ACP synthase I) and FabF (β-ketoacyl-ACP synthase II), was added to the culture of recombinant *E. coli* harboring the *P. putida phaG* gene and the *P. aeruginosa phaC1* gene, MCL PHA was not synthesized from gluconate. However, when FabI (enoyl-ACP reductase) was inhibited by a specific inhibitor, triclosan, a small amount (2–3 wt%) of MCL PHA could be synthesized in the same recombinant *E. coli* from gluconate (Rehm et al. 2001). Instead of PhaG in *Pseudomonas*, *E. coli* has thioesterase I (TesA) as a major link between the fatty acid metabolism and PHA biosynthesis. By expression of the *P. oleovorans phaC2* gene and the *E. coli tesA* gene in *E. coli*, MCL PHA could be synthesized with a content of 2.3 wt% from gluconate (Klinke et al. 1999).

The *E. coli fabH* gene encoding 3-ketoacyl-ACP synthase III and the *E. coli* and *Pseudomonas* sp. 61-3 *fabD* genes encoding malonyl-CoA:ACP transacylase were found to have 3-hydroxybutyryl-ACP:CoA transferase activity (Taguchi et al. 1999). The *E. coli* FabH with a limited substrate specificity (C_2–C_4) was engineered to have a broader substrate specificity (C_4–C_{10}). As a result, recombinant *E. coli* strain harboring the mutant *fabH* gene and $phaC1_{Ps61\text{-}3}$ was able to induce the production of monomers of C_4–C_{10}, and subsequently to produce PHA copolymers containing SCL and MCL units up to 2.6 wt% from glucose as a carbon source. This study indicated that the composition of PHA copolymers could be controlled by the activity of monomer-supplying enzymes present in the fatty acid metabolism (Nomura et al. 2004).

3 Systems-Biological Approach for PHA Production

The traditional metabolic engineering strategies described so far have worked out quite nicely in enhancing PHA production or producing novel PHAs. Recent advances in systems-biological studies are providing us with the tools that can be used to further improve the performance of PHA producers by optimizing the metabolic, regulatory, and signaling networks at the systems level. Some examples of employing omics and systems-biological tools in improving the strains are described in this section. Also, general strategies of performing systems metabolic engineering are described (Lee et al. 2005; Park et al. 2008).

3.1 Systems Metabolic Engineering for Strain Improvement

Traditional strain improvement was performed by random, laborious, and time-consuming procedures to develop the mutant strain overproducing the target bioproducts. However, these might cause unwanted alteration of the cellular metabolism, which makes it difficult to make further improvements when the conditions change (Lee et al. 2005). Rational metabolic engineering has been found to be a successful strategy for improving the ability of a microorganism to overproduce the desired bioproducts through modification of the cellular metabolism by using various recombinant DNA technologies and rational target identification (Lee and Papoutsakis 1999).

As the complete genome sequences of an increasing number of organisms are becoming available, postgenome research (the "omics" studies) has rapidly been advancing. Comparative analysis of genomes provides a powerful way to identify the genes which need to be introduced, deleted, and/or modified to develop the desired strain (Ohnishi et al. 2002; Rückert et al. 2003; Lee et al. 2005). In addition, *in silico* analysis including the construction of an *in silico* genome-scale metabolic model and metabolic simulation are able to predict the effects of genetic and/or environmental perturbations on cellular metabolism, which is also useful for the design of strategies for strain development (Wiechert 2002; Ishii et al. 2004; Lee et al. 2005; Park et al. 2008). Transcriptomics allows the simultaneous monitoring of relative messenger RNA expression levels in multiple samples by using high-throughput DNA microarrays. By comparing transcriptome profiles among different strains, time points, and culture conditions, one can further engineer potential target genes and regulatory circuits (Choi et al. 2003; Ohnishi et al. 2003; Tummala et al. 2003). Proteomics allows analysis of all the proteins of the cell or its parts by using two-dimensional gel electrophoresis (2DGE) or chromatography coupled with various mass spectrometry (MS) methods (Han and Lee 2006). Considering that most cellular metabolic activities are directly or indirectly regulated by proteins, proteome profiles give us valuable clues to further understand and engineer cellular metabolism (Han et al. 2001, 2003; Kabir and Shimizu 2003). Metabolomics allows quantitative analysis of cellular metabolites and metabolic intermediates by using chromatography coupled with MS or nuclear magnetic resonance. Fluxomics allows analysis of metabolic flux profiles based on flux and isotopomer balances. Comparative analysis of metabolic flux profiles under various genetic and environmental conditions allows us to understand the physiological status of the cells (Stephanopoulos 2004; Wittmann and Heinzle 2001). There have been several successful studies on strain improvement based on omics analyses (Yoon et al. 2003; Askenazi et al. 2003; Lee et al. 2003, 2005; Park et al. 2008).

Systems biotechnology makes it possible to comprehensively collect global cellular information, including omics data, and to integrate these data through metabolic, signaling, and regulatory networks, followed by the construction of computational models of the biological system (Lee et al. 2005; Park et al. 2008). This allows us to understand the cellular metabolism at a global scale and rationally engineer the strain for the enhanced production of the desired bioproducts (Fig. 4).

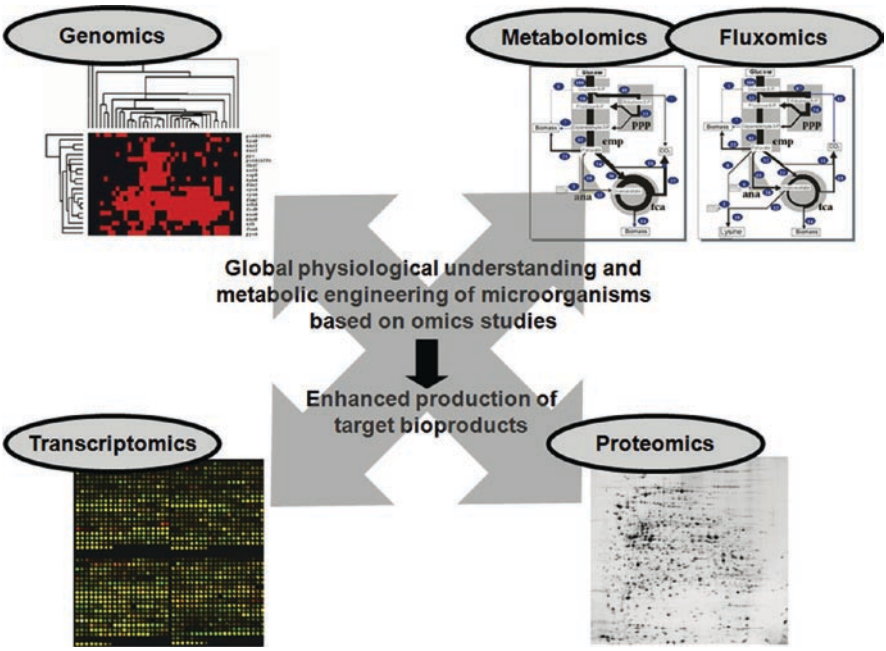

Fig. 4 Strain improvement based on the omics studies. Various omics analyses can be used to decipher the cellular physiological status in a comparative manner, which can subsequently be used to identify gene and pathway targets to be engineered

3.2 Metabolic Engineering Based on Omics Studies

Along with natural PHA producers, *E. coli*, a non-PHA-producing bacterium, has been metabolically engineered to produce PHAs. During the last few decades, various strategies, including host strain selection, change of plasmid copy number, filamentation suppression, use of PHA biosynthesis genes from different sources, and plasmid stabilization, have been employed (Park and Lee 2005). Also, the inherent metabolic pathways of *E. coli* were engineered to increase the availability of precursors for polymer synthesis.

There have been many successful cases of the development of metabolically engineered *E. coli* strains for the production of P(3HB), which is one of the best characterized PHAs. P(3HB) synthesis is initiated by condensation of two acetyl-CoA molecules into acetoacetyl-CoA, subsequently followed by reduction to 3-hydroxybutyryl-CoA using NADPH as a cofactor, and finally 3-hydroxybutyryl-CoA is incorporated into the growing chain of P(3HB) (Lee 1996). Because the P(3HB) synthesis pathway competes with inherent metabolic pathways needing acetyl-CoA, it is very important to increase the acetyl-CoA pool available for the P(3HB) synthesis reaction, resulting in increased P(3HB) yield and productivity.

Glucose is metabolized to generate acetyl-CoA and NADPH through the glycolytic and pentose phosphate pathways. The increase of the NADPH pool, required for the reduction reaction as mentioned earlier, was accomplished by overexpressing the *zwf* and *gnd* genes which encode glucose 6-phosphate and 6-phosphogluconate dehydrogenase, respectively (Lim et al. 2002). As a result, the NADPH to NADP+ ratio increased sixfold, and the P(3HB) content increased from 23 to 41%. But, the increase of P(3HB) content was actually due to the decreased cell concentration, which implies active cellular status is also critical to ultimately achieve a high concentration of P(3HB).

Integrated cellular responses of metabolically engineered *E. coli* to the accumulation of P(3HB) in the early stationary phase have been studied intensively by proteome analysis using 2DGE (Han et al. 2001). Out of 20 proteins showing the altered expression levels during the P(3HB) accumulation phase, 13 proteins were identified by MS. Among them, three heat shock proteins (GroEL, GroES, and DnaK) were remarkably upregulated in P(3HB)-accumulating cells, which indicated that the cells are under stress owing to the intracellular P(3HB) accumulation. Protein encoded by the *yfiD* gene, which was known to be expressed at low pH (Blankenhorn et al. 1999), was greatly induced with the accumulation of P(3HB). In the presence of glucose, EF-Tu, which is one of the most abundant cytosolic proteins and plays a key role in protein biosynthesis, decreased drastically, which retarded the protein biosynthesis process. Also, the levels of fructose bisphosphate aldolase (Fba), triosephosphate isomerase (TpiA), and 2-oxo-3-deoxy-6-phosphogluconate aldolase/2-keto-4-hydroxyglutarate aldolase (Eda) were increased in P(3HB)-accumulating cells. The increased expression of Fba and TpiA might be due to the fact that *E. coli* modified its metabolic fluxes to increase the glyceraldehyde 3-phosphate pool, which is subsequently used for P(3HB) synthesis. On the other hand, Eda catalyzes the final reaction of the Entner–Doudoroff (ED) pathway to supplement glyceraldehyde 3-phosphate and pyruvate, and finally to increase the acetyl-CoA pool. NADPH is simultaneously provided from the ED pathway. It could be concluded that cellular demand for the large amounts of acetyl-CoA and NADPH for P(3HB) biosynthesis caused the increased synthesis of two enzymes of the glycolytic pathway and one enzyme of the ED pathway (Fig. 3). In addition, the accumulation of P(3HB) in *E. coli* acted as a stress on the cells, which reduced the cellular metabolic activity and induced the expression of various protective proteins such as chaperones. On the basis of these results, a fermentation strategy should be developed in such a way that cells do not synthesize P(3HB) too early during the cultivation (Han et al. 2001).

P(3HB)-granule-associated proteome in recombinant *E. coli* harboring the *phaCAB* genes was also analyzed (Han et al. 2006). It was found that five proteins (EF-Tu, PhbA, IbpA, IbpB, and YbeD) out of seven spots identified were related to function of translation, heat-stress responses, and P(3HB) biosynthesis. Among them, IbpA and IbpB were already known to bind to the inclusion bodies of recombinant proteins in *E .coli* (Han et al. 2004). With use of immunoblotting and immunoelectron microscopy, it was found that IbpA and IbpB seem to have the function of phasins in *E. coli,* which affect the morphology of the granules and prevent other proteins

from binding to P(3HB) granules. In natural PHA producers, phasins are known as amphipathic proteins (12–28 kDa) which dominantly bind to the surface of PHA granules and form a layer on PHAs. Since there are no known phasins in *E. coli*, unlike in natural PHA producers, P(3HB) production in *E. coli* may cause more problems related to the direct exposure of hydrophobic P(3HB) granules to cytosolic proteins. In a previous study, during the P(3HB) accumulation, a heat-shock-like response was observed with increased levels of GroEL, GroES, and DnaK (Han et al. 2001). In this study, IbpA and IpbB were the major proteins on the surface of P(3HB) granules. Most of the P(3HB) granules accumulated in *E. coli* XL1-Blue harboring the *phaCAB* genes were smooth and round. Interestingly, the P(3HB) granules in *E. coli* XL1-Blue *ibpAB* mutant (XIB101) harboring *phaCAB* genes were significantly distorted and shrunken with wrinkles. The original shapes that were observed in the wild-type strain could be restored when the *ibpAB* genes were reintroduced into the XIB101 strain. Thus, IbpA and IpbB were found to be important in recombinant *E. coli* producing P(3HB) by stabilizing the interface between the hydrophobic P(3HB) granules and the hydrophilic cytoplasm. Furthermore, IbpA and IbpB act like phasins in the recombinant *E. coli*, affecting the morphology of the P(3HB) granules, and reducing the amount of cytosolic proteins bound to the P(3HB) granules (Han et al. 2006).

The *in silico* metabolic network of *E. coli* was constructed and was used to simulate the distribution of metabolic fluxes in the wild-type *E. coli* and recombinant *E. coli* producing P(3HB) (Hong et al. 2003). The acetyl-CoA flux into the tricarboxylic acid cycle, which competes with the P(3HB) biosynthesis pathway, decreased significantly during P(3HB) production. It was notable to find from *in silico* analysis that the ED pathway flux increased significantly under P(3HB)-accumulating conditions. To prove the role of the ED pathway in P(3HB) production, *E. coli eda* mutant strain (KEDA) was examined as a host strain for the production of P(3HB). The P(3HB) content obtained with *E. coli* KEDA harboring the *phaCAB* genes was lower than that obtained with its parent strain *E. coli* KS272 harboring the *phaCAB* genes. The reduced P(3HB) biosynthetic capacity of *E. coli* KEDA harboring the *phaCAB* genes could be restored by coexpression of the *E. coli eda* gene. Thus, the ED pathway was found to play an important role in P(3HB) synthesis in recombinant *E. coli* as predicted by metabolic flux analysis, which agreed well with the result of the aforementioned proteomic analysis of P(3HB)-producing *E. coli* (Fig. 3; Han et al. 2001; Hong et al. 2003).

Natural PHA producers have advantages in that they have inherent metabolic and regulation systems for PHA synthesis, which makes them attractive as a powerful platform for PHA production. Recently, the systematic analysis of *C. necator*, one of the most widely used PHA producers, was performed. The transcriptional analysis of the *phaA, phaB, phaC, phaP, phaR, phaZ1a, phaZ1b*, and *phaZ1c* genes related to P(3HB) homeostasis in *C. necator* H16 was carried out during three-stage cultivation [cell growth, P(3HB) biosynthesis, and P(3HB) utilization stages] by employing reverse transcriptase quantitative PCR and western blotting (Lawrence et al. 2005). It was known that these genes somehow correlate with P(3HB) formation and P(3HB) utilization in cells. However, how these genes work together in harmony and how they depend on P(3HB) granule assembly and breakdown are still

poorly understood. Therefore, it is necessary to construct a detailed general model of the dynamic granule-forming regime. A model having three distinct patterns of transcription observed during the cell growth, and P(3HB) biosynthesis and utilization phases was suggested (Lawrence et al. 2005). The first one is the transcriptional pattern of a group of five genes (*phaR*, *phaA*, *phaB*, *phaC*, and *phaZ1a*) which showed the same transcriptional trend, decreasing during the period of ammonium consumption and keeping a steady transcriptional state after the absence of ammonium (Lawrence et al. 2005). The second one is the transcriptional pattern of two coupled genes, *phaP* and *phaR*. Phasins (PhaP) are commonly known as stabilizing proteins which predominantly bind to the surface of P(3HB) granules in *C. necator*. The transcript level of the *phaP* gene was observed to increase sixfold within 1 h of the shift to P(3HB) production and rapidly decrease during the utilization phase. Moreover, very little transcript of the *phaP* gene was observed in the absence of P(3HB) synthase. Hence, these observations suggested that PhaP was coupled to P(3HB) biosynthesis, as previously proposed in several studies (York et al. 2001; Potter et al. 2002). On the other hand, it has been well established that PhaR is subject to autoregulation, and negatively regulates the accumulation of PhaP, which was also shown at the transcriptional level (York et al. 2002; Potter et al. 2002; Lawrence et al. 2005). If the *phaR* gene were regulated in the same manner as the *phaP* gene, transcript levels similar to those of the *phaP* gene should be observed; however, that is not the case. In addition, the transcript levels of the *phaR* gene did not depend on the P(3HB) accumulation. Thus, it could be concluded that the *phaR* gene is autoregulated, differently from the regulation of the *phaP* gene (Lawrence et al. 2005). The transcript level of the *phaZ1b* gene was classified as the third pattern. The transcription of the *phaZ1b* gene increased sharply, more than tenfold at 1–2 h after the onset of P(3HB) production, and remained at a high level during the P(3HB) production phase. It was undetectable in the P(3HB) utilization phase. However, even in the absence of PHA synthase, the *phaZ1b* gene showed a transcript level similar to that seen during the P(3HB) production phase, indicating that it was not involved in the catabolism of P(3HB), which did not match the expectation for the expression of an intracellular depolymerase (Lawrence et al. 2005).

Proteomic analysis using 2DGE and the genome sequence of *C. necator* H16 enabled the detection and identification of proteins that were differentially expressed during the different phases of P(3HB) metabolism. Samples from three different phases, including the exponential cell growth phase, the stationary growth phase for P(3HB) biosynthesis, and the P(3HB) utilization phase, were analyzed. Among several proteins changing quantitatively during the time course of cultivation, flagellin, which is a major protein of bacterial flagella, was identified. The flagella formation in *C. necator* changed significantly depending on the life cycle, nutritional supply, and, especially, P(3HB) metabolism. Cells were strongly flagellated in the exponential cell growth phase and lost flagella during the transition to the stationary phase. Then, in the stationary phase for P(3HB) biosynthesis (an excess of the extracellular carbon source; nutrient limitation), flagellation of the cells stagnated. In the P(3HB) utilization phase (addition of a nitrogen source to the cells that

were carbon-deprived, but filled with P(3HB); carbon limitation), cells significantly increased the degradation of their flagella, or stopped flagellin synthesis while normal degradation continued. Interestingly, in contrast to the wild-type cells, the phasin (PhaP)-negative mutant cells remained flagellated under the P(3HB) utilization phase, which suggested the assumption that a linkage between the loss of phasins with P(3HB) accumulation and flagellation. Moreover, *C. necator* H16 wild-type and the phasin-negative mutants were not flagellated when cultivated in complex and nutrient-rich medium, where cells were not exposed to nutritional limitation and obviously did not require much motility (Raberg et al. 2008).

3.3 Future of Systems Metabolic Engineering for PHA Production

As already described, there have been only several reports on the systems-level analysis and systems metabolic engineering of PHA producers. It is expected that an increasing number of studies will be performed to improve the PHA production system. In general, the following objectives will be pursued: utilization of inexpensive carbon sources, high PHA concentration, high PHA productivity, high PHA yield, and production of novel PHAs and tailor-made PHAs having desired properties. Examples of systems metabolic engineering for the enhanced production of amino acids recently reported can serve as guidelines for the approaches to be taken for the strain development (Park et al. 2007; Lee et al. 2007). Not only the metabolic network but also the regulatory circuits can be optimized together to achieve the desired level of performance. Furthermore, all steps for PHA production, from the upstream process (strain development) to the midstream process (fermentation and other unit operation) to the downstream process (recovery), should be optimized. Considering these processes all together, termed "systems biotechnology" (Lee et al. 2005), one can rationally engineer the strain for the production of desired PHAs with high efficiency (Fig. 5).

4 Concluding Remarks and Future Perspectives

Over the past few decades, PHA production in various microorganisms has been considerably improved by metabolic engineering. Recently, systems-level analysis of metabolic, signaling, and regulatory networks makes it possible to comprehensively understand the global physiological processes of the cells accumulating PHAs. Through systems-biological studies including omics and computational studies, new targets and strategies for the improvement of PHA production can be developed, followed by the construction of a new metabolic system for novel PHAs with desired

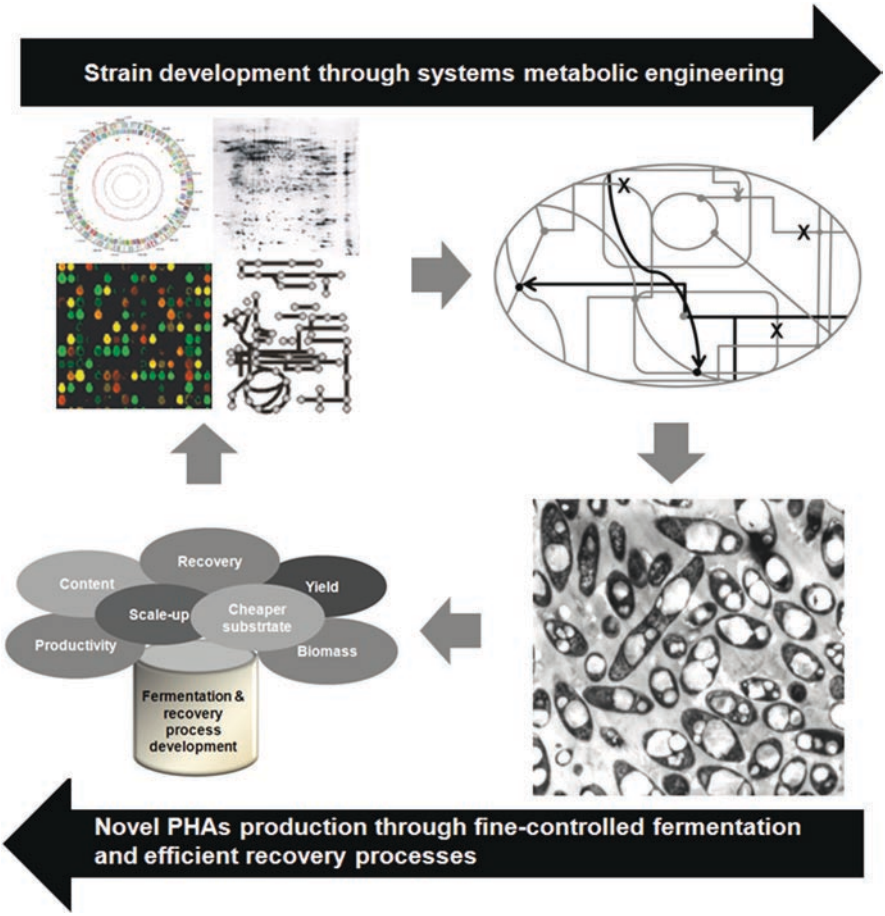

Fig. 5 Systems biotechnology for the development of an efficient PHA production system. Optimization of all steps, from the upstream process (strain development) and the midstream process (fermentation) to the downstream process (recovery), is required for the economic production of PHAs having desired characteristics

monomer compositions and molecular mass. The constructed strains should be further metabolically engineered to produce PHAs to a sufficiently high concentration with high productivity and yield from the most inexpensive carbon source through fine-controlled fermentation. Systems biotechnology is the strategy of choice for the development of a PHA production system that allows efficient and economically competitive production of polymers that can replace the petroleum-derived polymers without leaving a carbon footprint. It relies on successful systems metabolic engineering of microorganisms for the optimal performance by integrated analysis of midstream and downstream processes as well.

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Microbial PHA Production from Waste Raw Materials

Martin Koller, Aid Atlić, Miguel Dias, Angelika Reiterer, and Gerhart Braunegg

Contents

Abstract The application of biotechnological processes for industrial production can be regarded as promising for sustainable development, although for a range of products, biotechnological production strategies have not yet passed the test of economic viability. This is often caused by the cost of the raw materials. Here, a viable solution strategy is identified by the utilization of a broad range of waste and surplus materials that can be upgraded to the role of feedstocks for the biomediated production of desired end products such as polyhydroxyalkanoate biopolymers. The selection of the appropriate waste stream as a feedstock for biotechnological purposes mainly depends on the global region where the production plant will be constructed. To save costs for transportation, facilities for the production of biopolymers, biofuels and biochemicals

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should be integrated into existing production lines, where the feedstocks directly accrue as waste streams. In Europe and North America, surplus whey from the dairy industry is available in large quantities, whereas huge amounts of non-wood lignocellulosic materials from rice, corn and sugar cane plants are found in many different countries worldwide. The enormously increasing production of biofuels provides a range of by-products such as glycerol and low-quality fatty acid esters from biodiesel production or distillery residues from bioethanol factories. The utilization of waste streams for production of value-added products not only enhances the economics of such products, but also provides industry with a strategy to overcome disposal problems.

1 Introduction

1.1 General

To ensure the safe distribution of goods worldwide, it is beyond dispute that there is an increasing need for polymeric compounds acting as packaging materials. The contemporary utilization of restricted fossil resources for the production of polymers provokes prevailing worldwide problems such as the greenhouse effect and global warming. This is caused by the fact that these materials are utilized only during a relatively short time span. After that, they are often incinerated, elevating the atmospheric carbon dioxide concentration that contributes to the said heating effects. By incineration of plastic waste, the energy that is chemically stored therein is recovered as thermal energy. Braunegg et al. (2004) compared the heating value of plastic waste (approximately 42 MJ kg^{-1}) with the heating values of pit coal (approximately 28 MJ kg^{-1}), fuel oil (approximately 41 MJ kg^{-1}) and natural gas (45 MJ kg^{-1}). These heating values are rather high and, regarded simply from the energetic point of view, incineration of plastic waste is of interest. But it cannot be overlooked that the main problem arising from incineration is the same as for energy recovery from fuel oil, petrol, gasoline, natural gas and coal: carbon that was fixed over millions of years and that within this time was not part of the natural carbon cycle is converted to carbon dioxide that eventually accumulates in the atmosphere. When incineration is planned, strict emission standards have to be obeyed not only for carbon dioxide, but also for highly toxic compounds such as dioxins and HCl deriving from poly(vinyl chloride) (Ojumu et al. 2004). Furthermore, the optimum energy recovery has to be evaluated. This includes the definition of the composition of the plastic waste (Braunegg et al. 2004). Additionally, a Swiss study showed that the capacities of the incinerators currently in use are insufficient to handle the tremendous amount of plastic waste (Haenggi 1995).

Besides, more and more waste of highly resistant plastics that are not incinerated is piled up every year. Recycling systems demand a certain degree of purity of the plastics to be reutilized, as well as a sorting accuracy; otherwise they do not function sufficiently for a satisfactory solution of the problem. In addition, the collection costs are fairly high, and recycling has a negative impact on the mechanical properties of the materials, such as an increase in brittleness (Braunegg et al. 2004).

The environmental necessity to stop this negative development by switching to alternative strategies independent of fossil resources nowadays is generally undisputed. Already in 1992, the United Nations 'Rio Declaration on Environment and Development' explicitly specified the political intention and willingness of most countries to forcefully support the development of bio-based and biocompatible materials. Literally, Principle 4 of the 'Rio Declaration' states that: 'In order to achieve sustainable development, environmental protection shall constitute an integral part of the development process and cannot be considered in isolation from it.' With the tools of life cycle assessment (LCA) and cleaner production studies, much effort is contemporarily devoted to quantifying the environmental impact and feasibility of processes for production of polymeric materials (Sudesh and Iwata 2008).

In addition to the ecological considerations, the price of crude oil rocketed to a new all-time high of US $147 per barrel in 2008. Data for remaining amounts of fossil oil in Earth's interior are changing quickly owing to advanced methods for tracing and discharging of mineral oils. Nevertheless, one day fossil feedstocks will finally be depleted. This causes an increasing awareness of the industrial branches involved for the exigency of fostering novel production techniques based on renewable resources and embedded into nature's closed cycles. With this 'white biotechnology', sustainable production of fine chemicals, bulk chemicals, polymers and fuels is achieved by the action of living organisms or parts thereof such as enzymes.

1.2 The Increasing Interest in Polyhydroxyalkanoate Biopolyesters

Today, increasing industrial interest exists in the biotechnological production of polyhydroxyalkanoates (PHAs) from renewable resources to arrive at bio-based and biodegradable polymeric materials that can act as alternatives for common plastics derived from petrol (Braunegg et al. 1998, 2004; Brandl et al. 1990; Haenggi 1995; Luzier 1992; Steinbüchel and Valentin 1995). Depending on their exact composition, PHAs can be classified as thermoplasts or elastomers (Baptist 1963, 1965; King 1982). Classically, such polyesters are produced by numerous prokaryotic strains from renewable resources such as carbohydrates, lipids, alcohols and organic acids (e.g. lactic acid) under unfavourable growth conditions owing to an imbalance in the nutrient supply. In general, PHA accumulation is favoured by adequate availability of a suitable carbon source and a limiting supply with macrocomponents such as nitrogen, phosphate and dissolved oxygen or microcomponents such as magnesium, sulphate, iron, potassium, manganese, copper, sodium cobalt, tin and calcium (Kim and Lenz 2001; Helm et al. 2008). On an industrial scale, the switch from balanced microbial growth to PHA accumulation is normally done by phosphates and/or nitrogen limitation. Only recently, the influence of the microelements iron, sulphate and potassium as growth-limiting factors on PHA quality was investigated in detail and revealed the production of ultrahigh molecular masses of PHA, when potassium limitation occurred (Helm et al. 2008). Before, the biosynthesis of extremely high

molecular masses of PHA was merely reported for recombinant strains of *Escherichia coli* lacking PHA depolymerase enzymes (Kahar et al. 2004a, b).

Also, under balanced growth conditions, detectable amounts of PHAs stemming from the condensation of acetyl-CoA units from the central metabolism are found in most cases. This partially growth associated PHA accumulation was described for most PHA producers investigated, such as *Cupriavidus necator*, *Haloferax mediterranei*, *Azotobacter vinelandii, Hydrogenophaga pseudoflava* and *Pseudomonas hydrogenovora*. In some microbial strains PHA accumulation can also appear in parallel to biomass production. This 'growth-associated' PHA accumulation is known for *Alcaligenes latus*, *Methylobacterium* sp. ZP24 (Nath et al. 2008), *Bacillus mycoides* RLJ B-017 (Borah et al. 2002) and *recombinant E. coli*. Additionally, a hyperproduction of PHA after a period of carbon starvation was described for *Pseudomonas* 2F (Braunegg et al. 1999, 2002).

For PHA-producing microbial cells, PHAs serve as reserve materials for carbon and energy. Under conditions of starvation, these reserve materials can be mobilized, thus providing the cell with an advantage for survival. Figure 1 shows electron-microscopic pictures of *C. necator* DSM 545 harbouring different intracellular amounts of PHA at different magnifications.

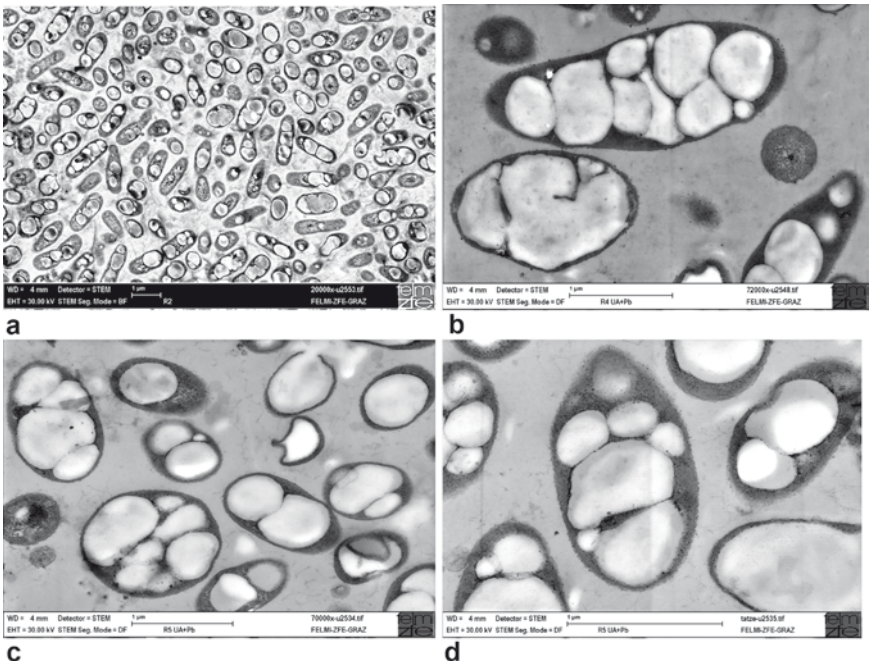

Fig. 1 Electron-microscopic pictures of polyhydroxyalkanoate (PHA)-rich *Cupriavidus necator* DSM 545 cells cultivated in a continuous fermentation process on glucose. Magnification: ×20,000 (**a**), ×72,000 (**b**), ×70,000 (**c**) and ×150,000 (**d**). Percentages of PHA in cell mass: 48% (**a**), 65% (**b**) and 69% (**c**, **d**). (The pictures were kindly provided by Elisabeth Ingolić, FELMI-ZFE-Graz)

If items made of PHAs are composted, they are completely degraded to water and carbon dioxide as the final products of their oxidative breakdown. Here it has to be emphasized that these final oxidation products are the basic materials for the photosynthetic regeneration of carbohydrates by green plants. This demonstrates that, in contrast to petrol-based plastics, PHAs are embedded into the natural closed cycle of carbon. The range of applications for PHAs is not limited to simple packaging materials, but encompasses commodity items, materials for agro-industrial purposes and pharmaceutical and medical applications. The major advantageous characteristics of PHAs can be summarized as follows:

Biodegradability. PHAs do not contribute to an increase of the landfill crisis owing to their biodegradability; in contrast to conventional plastics, they can be composted after use.

Bio-based nature and independence from fossil fuels. Because PHAs are produced from renewable resources, they are independent of the availability of mineral oils as feedstocks; under the precondition that the generation of energy for the PHA production process itself is also based on renewable resources, the independence from fossil fuels is valid for the entire production process (see also Sect. 2.7).

Carbon dioxide release. Carbon dioxide that is released as the final mineralization product of biopolymers originates from the renewable carbon source for their biosynthesis. Photosynthetic fixation of the released carbon dioxide by plants generates renewable carbon sources again. Thus, the carbon flux in the synthesis and degradation of biopolymers is balanced. PHAs therefore do not contribute to global warming.

Biocompatibility. In special fields of application (especially for medical purposes), PHAs are superb compared with conventional plastics owing to their biocompatibility. The ideal biocompatibility of PHAs is underlined by the natural occurrence of (*R*)-3-hydroxybutyric acid (3HB) and its low molecular weight oligomers and polymers in human blood and tissue (Agus et al. 2006; Steinbüchel and Hein 2001; Steinbüchel and Lütke-Eversloh 2003; Zinn et al. 2001). Table 1 provides an overview of the PHA-producing genera that have been reported in the literature.

Different applications require different material properties of the biopolyester. These properties can be triggered by fine-tuning of the composition of the PHA during the biosynthesis. The most common representative of PHAs, namely the homopolyester poly[(*R*)-3-hydroxybutyrate] (PHB), features a high degree of crystallinity and restricted processability of this material. The small difference between the decomposition temperature (typically around 270°C) and the high melting point (typically around 180°C) provides a quite small window of processability for melt extrusion technology. This can be changed by interrupting the PHB matrix through incorporation of alternative building blocks such as (*R*)-3-hydroxyvalerate (3HV) or the achiral building blocks 4-hydroxybutyrate (4HB) and 5-hydroxyvalerate (5HV). Such short-chain-length (scl) PHAs feature the characteristics of thermoplasts such as polypropylene. This is especially true for PHB and its copolyesters

Table 1 Polyhydroxyalkanoate (PHA)-accumulating genera of prokaryotic micro-organisms

Acidovorax	*Erwinia*	*Oscillatoria*[a]
Acinetobacter	*Escherichia* (rec.!)[a]	*Paracoccus*
Actinobacillus	*Ferrobacillus*	*Paucispirillum*
Actinomycetes	*Gamphospheria*	*Pedomicrobium*
Aeromonas	*Gloeocapsa*[a]	*Photobacterium*
Alcaligenes[a,b]	*Gloeothece*[a]	*Protomonas*
Allochromatium	*Haemophilus*	*Pseudomonas*[a,b]
Anabaena[b]	*Halobacterium*[a,c]	*Ralstonia*[a,b]
Aphanothece[a]	*Haloarcula*[a,b,c]	*Rhizobium*[a,b]
Aquaspirillum	*Haloferax*[a,b,c]	*Rhodobacter*
Asticcaulus	*Halomonas*[a]	*Rhodococcus*[b]
Azomonas	*Haloquadratum*[c]	*Rhodopseudomonas*
Azospirillum	*Haloterrigena*[c]	*Rhodospirillum*[b]
Azotobacter[a,b]	*Hydrogenophaga*[a,b]	*Rubrivivax*
Bacillus[a,b]	*Hyphomicrobium*	*Saccharophagus*
Beggiatoa	*Klebsiella* (rec.!)	*Shinorhizobium*
Beijerinckia[b]	*Lamprocystis*	*Sphaerotilus*[a]
Beneckea	*Lampropedia*	*Spirillum*
Brachymonas	*Leptothrix*	*Spirulina*[a]
Bradyrhizobium	*Methanomonas*	*Staphylococcus*
Burkholderia[a]	*Methylobacterium*[b]	*Stella*
Caryophanon	*Methylosinus*	*Streptomyces*
Caulobacter	*Methylocystis*	*Synechococcus*[a]
Chloroflexus	*Methylomonas*	*Syntrophomonas*
Chlorogloea[a]	*Methylovibrio*	*Thiobacillus*
Chromatium	*Micrococcus*	*Thiococcus*
Chromobacterium	*Microcoleus*	*Thiocystis*
Clostridium	*Microcystis*	*Thiodictyon*
Comamonas[a,b]	*Microlunatus*[b]	*Thiopedia*
Corynebacterium[b]	*Moraxella*	*Thiosphaera*
Cupriavidus[a,b]	*Mycoplana*[a]	*Variovorax*[a,b]
Cyanobacterium[b]	*Nitrobacter*	*Vibrio*
Defluviicoccus[b]	*Nitrococcus*	*Wautersia*[a,b] (today *Cupriavidus*)
Derxia[b]	*Nocardia*[a,b]	*Xanthobacter*
Delftia[a,b]	*Nostoc*	*Zoogloea*[a]
Ectothiorhodospira	*Oceanospirillum*	

[a]Detailed knowledge of the growth and production kinetics available
[b]Accumulation of copolyesters known
[c]Archaea

with low amounts of 3HV, 4HB or 5HV. The incorporation of different building blocks into the polyester chains normally requires expensive cosubstrates (precursors) and therefore constitutes a second cost factor of major importance. These precursors do not only contribute to the production cost, but are also often toxic for the production strain. Therefore, the dose has to be carefully controlled during cultivation (Son and Lee 1996). Here, a potential solution might be the utilization of such production strains that are able to produce special building blocks such as

Table 2 Characteristics of representative PHAs (Khanna and Srivastava 2005a; Williams and Martin 2002)

	PHB	Poly (3HB-*co*-3% 3HV)	Poly (3HB-*co*-20% 3HV)	Poly (4HB)	Poly (3HB-*co*-3% 4HB	Poly (3HB-*co*-16% 4HB)	Poly (3HB-*co*-64% 4HB	Poly (3HO-*co*-12% 3HH)
Melting temperature (°C)	177	170	145	60	166	152	50	61
Glass-transition temperature (°C)	4	–	−1	−50	–	−8	–	−35
Tensile strength (MPa)	40	38	32	104	28	26	17	9
Young's modulus (GPa)	3.5	2.9	1.2	149	–	ND	30	0.008
Elongation at break (%)	6	–	50	1,000	45	444	591	380

PHB poly[(*R*)-3-hydroxybutyrate], *3HB* 3-hydroxybutyrate, *3HV* 3-hydroxyvalerate, *4HB* 4-hydroxybutyrate, *3HH* 3-hydroxyhexanoate, *3HO* 3-hydroxyoctanoate, *ND* not determined

3HV from unrelated carbon sources such as simple sugars. Such organisms are found among osmophilic archaea (Rodriguez-Valera and Lillo 1992), some species of *Rhodococcus* and *Nocardia* (Alvarez et al. 1997; Steinbüchel and Lütke-Eversloh 2003; Valentin and Dennis 1996), some pseudomonades (Son and Lee 1996) and special non-sulphur cyanobacteria (Liebergesell et al. 1991). Further enhancement of cost-efficiency is possible by the utilization of highly robust production strains such as *H. mediterranei* that require minimum sterility demands during their cultivation and are accessible to simple methods of downstream processing for isolation of PHAs from the microbial biomass (Garcia Lillo and Rodriguez Valera 1990; Rodriguez-Valera and Lillo 1992).

PHAs containing (*R*)-3-hydroxyalkanoates longer than 3HV, so-called medium-chain-length (mcl) PHAs, constitute elastomers with lower melting points, low glass-transition temperatures and low crystallinity in comparison with scl-PHAs. Table 2 shows the characteristics of representative PHAs with different compositions.

1.3 Value-Added Utilization of 'Waste PHAs'

After their utilization as plastic items, PHAs can not only be composted, but can also be easily depolymerized to a valuable source of optically pure *R*-(−)-configured bifunctional hydroxy acids which are of interest as synthons for chiral high-value chemicals such as vitamins, antibiotics, pheromones and aromatics (Ren et al. 2005). Some of these acids also exhibit important biological properties such as antimicrobial and antiviral activity (Ruth et al. 2007).

The production of such enantiopure compounds, possessing a market value higher than that of the polymer itself, via chemical methods is complex and not economical. Classic PHA depolymerization methods via acidic alcoholysis after the

isolation from bacterial biomass are rather complex and highly solvent demanding (Seebach and Zueger 1982; Seebach et al. 1992; Ren et al. 2005). Therefore, efficient methods were developed for in vivo depolymerization of intracellular PHA. This can be achieved by triggering the activities of the enzymes involved in intracellular PHA catabolism by influencing the process conditions. After PHA is synthesized by the micro-organisms, the polyester is rapidly hydrolysed by increasing activity of PHA depolymerase. This can be accomplished by lowering the pH value to a range of 3–4. Under these pH conditions, the activity of (*R*)-(−)-3HB dehydrogenase is zero, hence the unwanted reaction of (*R*)-(−)-3HB towards acetoacetate is totally blocked, and (*R*)-(−)-3HB can be collected in a pure form. Alternatively, (*R*)-(−)-3HB can be released into the medium by PHA-rich cells when the external carbon source is depleted. Here, the subsequent metabolization of (*R*)-(−)-3HB towards acetoacetate can be avoided by interruption of the oxygen supply (Lee et al. 1999).

Considering the fact that more than 140 chiral building blocks are reported to be potential PHA constituents, the range of available synthons for organochemistry is huge. Lee and colleagues have already isolated a broad range of enantiopure PHA building blocks via the methods described, such as monomers from scl-PHAs, odd- and even-numbered mcl-PHA constituents and even components with pendent aromatic groups. The authors described high yields for the production of scl hydroxyalkanoates (over 90%), but rather low efficiency for the production of mcl hydroxyalkanoates. Recently (*R*)-3-hydroxy-6-heptenoic acid, (*R*)-3-hydroxy-8-nonenoic acid and (*R*)-3-hydroxy-10-undecenoic acid were isolated from *Pseudomonas putida* harbouring mcl-PHA. These processes turned out to be very effective owing to the fact that the investigators found a pH optimum of 11 for the PHA depolymerase of *P. putida*, differing from the pH optima of PHA depolymerases of scl-PHA producers that work under acidic conditions (Ren et al. 2005).

In addition, bio-based ‘green solvents’ can be generated from PHAs after their utilization via pyrolysis. This way, unsaturated compounds such as crotonic acid and 2-pentenoic acid needed for synthesis of lactones are generated.

1.4 The Need for Cheap Substrates and Their Occurrence

The application of biotechnological processes for industrial production can be regarded as promising for sustainable development, although for a range of products biotechnological production strategies have not yet passed the test of economic viability. This is often caused by the cost of the raw materials. Here, a viable solution strategy can be identified in the utilization of a broad range of waste and surplus materials that can be upgraded to the role of feedstocks for the biomediated production of desired end products. Such materials are mainly produced in agriculture and industrial branches that are closely related to agriculture (Braunegg et al. 1998; Solaiman et al. 2006; Khardenavis et al. 2007; Khanna and Srivastava 2005a). Especially the economics of PHA production is determined to a great extent (up to 50% of the entire production costs) by the cost of the raw materials. This is caused

by the fact that PHA accumulation occurs under aerobic conditions, resulting in high losses of the carbon substrate by intracellular respiration. Hence, only a maximum amount of less than 50% of the carbon source is directed towards biomass and PHA formation. The utilization of waste materials upgraded to the role of starting materials for PHA biosynthesis constitutes a viable strategy for cost-efficient biopolymer production and helps industry to overcome disposal problems.

For many regions in the world, the industrial-scale realization of value-added conversion of low-cost agricultural feedstocks can provide a certain degree of geopolitical independence. The selection of the appropriate waste stream as a feedstock for biotechnological purposes mainly depends on the global region where a production plant will be constructed. To save transportation costs, facilities for the production of biopolymers, biofuels and biochemicals should be integrated into existing production lines, where the feedstocks directly accrue as waste streams. The availability of a convertible substrate all year round has to be ensured. This creates problems such as the suitability of storage of these materials, especially for lignocellulosic materials during the off-season, when no harvest takes place.

In Europe, huge amounts of surplus whey are available in the dairy industry, providing lactose for the production of lactic acid, poly(lactic acid) (PLA), PHAs and bioethanol. Caused by new legislative situations, the increasing production of biodiesel in Europe generates enormous amounts of its major side stream, namely glycerol, a starting material for the production of PHA and lactic acid. For the production of catalytically active biomass of particular microbial production strains, different waste streams show high potential as precious sources for nitrogen required for the formation of biomass constituents. Here, meat and bone meal (MBM) from the slaughtering and rendering industries as well as several grass and silage residues show excellent results for the cultivation of microbes capable of the production of, e.g., PHA.

In other areas of the world, waste from the sugar industry (molasses), starch, waste lipids, alcohols such as methanol (Bourque et al. 1995) and especially lignocellulosic feedstocks are available in quantities that are appropriate for industrial process demands.

Waste lipids are available from a variety of sources: waste cooking oil, different plant oils, lipids of MBM or waste water from olive oil and palm oil production. In all cases, the triacylglycerides can be directly utilized as a carbon source, or after hydrolysation to glycerol and fatty acids, or after transesterification towards biodiesel and glycerol.

Lignocellulosic material (consisting of lignin, cellulosic and hemicellulosic fibres) and cellulosic material provide the feedstocks of highest quantity. Industrial branches generating the major shares of this waste are the wood-processing, paper and agriculture industries. Nowadays, plenty of effort is dedicated worldwide to develop biorefinery plants for the conversion of lignocellulose and cellulose waste to starting materials for biotechnological production of bioethanol, biopolymers and a range of fine chemicals. The optimization of methods for digestion of lignocellulose and the development of effective biocatalysts for the breakdown of cellulose and hemicellulose into microbially convertible sugars (hexoses and pentoses) are the prerequisite for an efficient biotechnological conversion of these promising raw materials into desired end products.

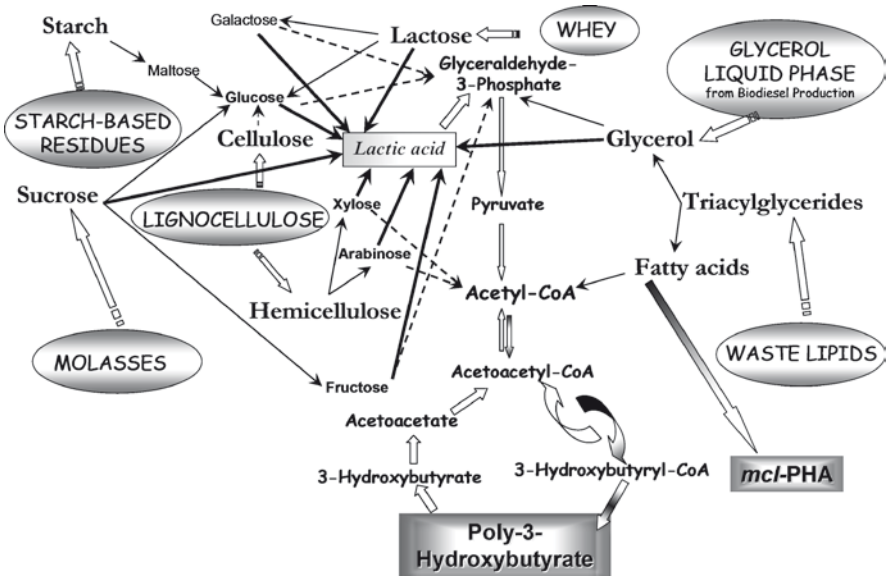

Fig. 2 The basic routes and the central metabolism for PHA production based on different substrates from the major global waste streams. The possibility of generating lactic acid as a stable intermediate for PHA production is also indicated (*bold black arrows*). *Dashed arrows* indicate intermediate metabolic steps

The integration of biopolymer production into an existing sugar cane mill has been realized on a pilot scale by the company PHBISA in Brazil, where the saccharose obtained is converted to bioethanol and partly to PHA. In this scenario, the energy required for bioethanol and biopolymer production is generated by burning surplus biomass, namely bagasse. The fusel oil fraction of the bioethanol distillation is applied as an extraction solvent for PHA isolation from microbial biomass (Nonato et al. 2001).

Figure 2 provides a scheme of the basic metabolic routes for PHA production based on different substrates.

1.5 Seasonal Availability of Waste Streams

In contrast to processes based on purified substrates, the utilization of waste materials for PHA production confronts us with the question of availability of the feedstock during an entire production year. In general, one has to distinguish between waste streams that accrue all through the year in more or less constant quantities, and others with an availability strongly fluctuating with time. The factor ‘seasonal availability’ is crucial for the planning and design of the PHA production facilities to be integrated into existing industrial plants.

An example for the first case is found in the utilization of permanently accruing surplus whey from the dairy industry. The sizes of PHA production facilities to be integrated into the dairy process lines can easily be harmonized with the expected amounts of arising whey and the production facilities are charged to capacity all through the year. Here, the biotechnological conversion of whey to final products such as PHA substitutes the disposal of this material in combination with value creation (see Sect. 2.4). Further, classic processing steps for whey (e.g. production of dry whey or lactose) are energy-demanding and show a low cost–performance ratio.

The situation changes fundamentally in the case of residues that accrue seasonally after harvest and processing of special agricultural crops. In this case, long periods without formation of the waste stream are interrupted by only one or a few time peaks a year, where large amounts of the material accrue. In such cases, the suitability of the raw material for low-cost storage is of major importance. Otherwise large-scale PHA production plants would have to be constructed to convert a huge amount of the waste material within a short time frame. Such large facilities, on the one hand, are expensive to build and, on the other hand, only operate at full capacity for very limited time periods. A solution to this problem is identified in the conversion of perishable materials into stable intermediates that can easily be stored without major energy requirements such as cooling, heating and drying. Lactic acid constitutes a prime example of such stable intermediates (see Sect. 2.8).

1.6 By-Products of Waste Streams

In addition to their main components, complex waste streams can contain additional substances that make them advantageous in direct comparison with pure and expensive substrates. For example, the permeate of surplus whey from the dairy industry provides the production strain in bioprocesses not only with a rich source of the carbohydrate lactose, but also with minor components such as minerals and protein residues that have positive impacts on the microbial cultivation. Additionally, beneficial growth components such as vitamins and biotin are reported to be available from complex, unrefined resources such as molasses (reviewed by Purushothaman et al. 2001).

On the other hand, such complex waste streams can also contain non-fermentable components and even compounds that have inhibiting effects on growth and production kinetics of microbes (Solaiman et al. 2006). Among such compounds are phenols, aldehydes and different heavy metals (Purushothaman et al. 2001; Silva et al. 2004). For certain microbial strains, methanol residues in the raw glycerol phase from biodiesel production have to be removed prior to the application of this substrate, e.g. for PHA production (Braunegg et al. 2007; see Fig. 4). Additionally, upstream processing of complex waste streams prior to their utilization as a substrate can create toxic compounds. For example, Maillard products easily occur during thermal sterilization of reducing carbohydrates together with free amino groups of protein residues. Acidic hydrolysis of different dimeric and polymeric substances in various waste streams often causes the formation of side products that make the hydrolysate unfeasible for application in a desired bioprocess.

2 Available Waste Streams in Different Global Regions

2.1 Cheap Nitrogen Sources for Production of Active Biomass

A crucial cost factor in typically phosphate-limited production processes for PHAs is the cost of complex nitrogen sources. It was found that supplementation of a defined production medium with small amounts of a complex nitrogen source such as tryptone could enhance PHB production by recombinant *E. coli* (Lee and Chang 1994). Examples of complex nitrogen sources that have been investigated are yeast extract (Chen et al. 2006), fish peptone (Page and Cornish 1993), meat extract, casamino acids, corn steep liquor (Purushothaman et al. 2001; Koller et al. 2005b), soybean hydrolysate and cotton seed hydrolysate (Lee 1998). The major advantage of using complex nitrogen sources is a possible shortening of the adaptation phase (lag phase) at the beginning of the fermentation process. This is caused by the availability of complete amino acids and peptides in the complex nitrogen source which can easily by converted by the cells to synthesize their own proteinaceous material. Hence, a higher concentration of catalytically active biomass that is able to accumulate PHA is produced in a shorter time, resulting in an increase of the volumetric productivity.

In the northern hemisphere, a considerable agricultural area of green grassland can be found. The green biomass is a convenient source of green grass juice as a primary product from biorefinery processes. Because of the current changes in the agricultural structure in many countries, characterized by a decrease of grassland utilization for production of cattle feed, the biorefinery process deals with new innovative utilization pathways for green biomass, not only for grass fibres, but also for grass juices. In a project for developing a biorefinery process, Koller et al. (2005b) investigated the influences of different grass juices on microbial growth and PHA production using *C. necator*, a stable organism that is known to accumulate PHA with high productivity. In laboratory-scale bioreactor cultivations, silage juice turned out to be a very promising cosubstrate in respect to its price, product quantity and moderate positive impact on growth. All cultivation parameters investigated show that silage juice has explicitly superior impacts on the process when compared with green grass juice.

Instead of expensive complex nitrogen sources such as yeast extract and casamino acids, cheaper products such as MBM hydrolysate can be successfully applied in PHA production processes (Koller et al. 2005a). Severe problems have arisen during the last decade in the EU because of bovine spongiform encephalopathy. At the summit of the crisis, the disease infected 3,500 head of cattle every week in the UK. This inspired several scientists at Graz University of Technology, Austria, to contemplate alternative methods for safe utilization of MBM. To evaluate biotechnological fields of application, MBM that was proven to be free of prions was subjected to alkaline hydrolysis (NaOH) and further neutralized with acid, yielding a highly saline hydrolysate. The hydrolysed MBM still contains about 78% of the nitrogen included in MBM prior to hydrolysis (Neto 2006).

Owing to the high salt demand of the strain *H. mediterranei*, chemically hydrolysed MBM constitutes a very suitable source of nitrogen and phosphate especially for this

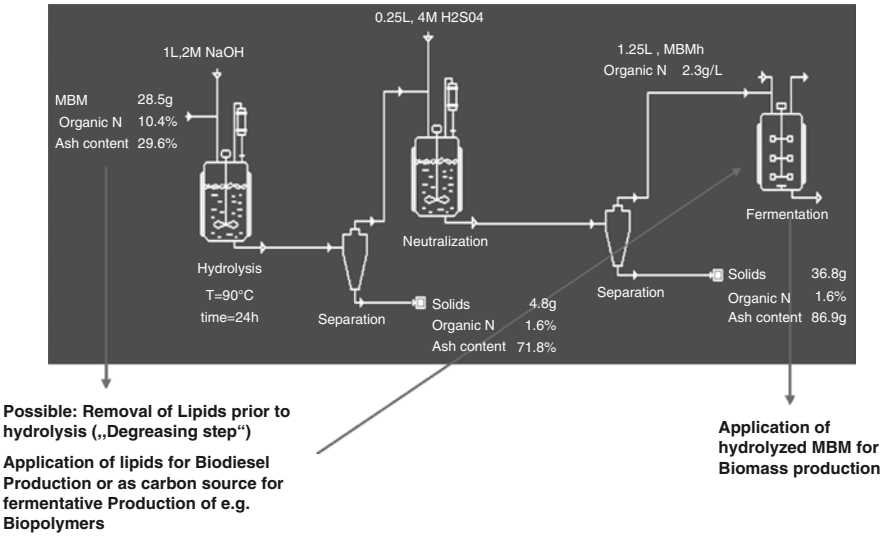

Fig. 3 Production and application of hydrolysed meat and bone meal (Neto 2006)

organism. On a bioreactor scale, *H. mediterranei* was cultivated with a maximum specific growth rate of 0.11 l h^{-1} when supplemented with MBM as a complex nitrogen and phosphate source together with glycerol liquid phase (GLP) as a carbon substrate; a PHA copolyester was produced with a maximum specific productivity of 0.10 g g^{-1} h^{-1} and a share of 75% PHA in cell mass (Koller et al. 2005a). Figure 3 shows a process sheet for the conversion of MBM towards a convertible substrate for biopolyester production.

2.2 Waste Lipids

Several waste lipids of different origin can be applied as substrates for biotechnological processes such as PHA production:

- Waste cooking oil and restaurant greases are waste products available in large amounts.
- Tallow from the slaughtering and rendering industries constitutes another inexpensive source of triacylglycerides.
- From the MBM hydrolysis process that can provide a useful nitrogen source for PHA-producing organisms (see above), about 11% of lipids remain as surplus material after the degreasing step.
- In PHA production, biomass has to be degreased before isolation of PHA if high product purities are required. Also here, typically 2–4% of lipids are removed from the cells.

Pseudomonas aeruginosa 42A2 was used by Fernandez et al. (2005) for PHA production from oily wastes such as residual waste cooking oil and other lipid wastes; the organism accumulated up to 54.6% of PHA. The authors investigated the influence of $k_L a$ and temperature on PHA productivity and monomer composition.

The production of PHA using residual oil from biotechnological rhamnose production as a carbon source for growth of *C. necator* H16 (the nomenclature in the article was '*Ralstonia eutropha*') and *P. oleovorans* was described by Füchtenbusch et al. (2000). The strains accumulated PHA at 41.3 and 38.9%, respectively, of the cell dry mass when they were cultivated in defined media with oil from the rhamnose production as the sole carbon source. The accumulated PHA isolated from *C. necator* was identified as PHB homopolyester, whereas the PHA isolated from *P. oleovorans* consisted, typically for this type of PHA-accumulating organism, of (*R*)-3-hydroxyhexanoic acid, (*R*)-3-hydroxyoctanoic acid, (*R*)-3-hydroxydecanoic acid and (*R*)-3-hydroxydodecanoic acid. Approximately 20–25% of the carbon components of the residual oil were converted into PHA. Up to 80% of cell dry mass of PHB homopolyester from different plant oils was produced by *C. necator* DSM 545 (Fukui and Doi 1998).

Tallow is one of the cheapest fats available in large amounts. The production of PHA from tallow was demonstrated for *Pseudomonas resinovorans*. Although the raw material is inexpensive, the process is not profitable owing to the low amounts of PHA produced (approximately 15% of cell dry mass, Cromwick et al. 1996). Taniguchi et al. (2003) reported that waste plant oils as well as waste tallow are assimilated and successfully converted to PHA with relatively high yield by *Ralstonia eutropha* (today known as *C. necator*). Waste sesame oil and virgin sesame oil were investigated and compared with fructose regarding utilization. Interestingly, much higher molecular weights ($M_w = 1.3 \times 10^6$) are reported for the polyester produced from fructose than for the oil-derived polymers ($M_w = 5 \times 10^5$–6×10^5). In all cases, the PHA accumulation was quite high, up to 80% of the cell dry weight. Further experiments in this study using the same organism reported PHA production from soybean, rapeseed, corn and palm oil as well as from tallow lard. Here, copolyesters containing small amounts of 3HV were found in those cases where lard or tallow was provided as a substrate, most probably caused by the availability of propionyl-CoA as the final product of the β-oxidation of odd-numbered fatty acids that are found in animal lipids.

The first production of poly(3HB-*co*-3HV), P(3HB-*co*-3HV), from olive oils by *Aeromonas caviae* was described by Doi et al. (1995). Here, the polyester content in the cells was still rather low (6–12%). The feasibility of using olive oil mill effluents as a substrate in biodegradable polymer production was studied by Dionisi et al. (2005), where olive oil mill effluents were anaerobically fermented at various concentrations combined with different pretreatments and without pretreatment to obtain volatile fatty acids (VFAs) such as acetate, propionate, butyrate, isobutyrate and valerate, which were used as substrates for PHA production. Olive oil mill effluents were also tested for PHA production by using a mixed culture from an aerobic sequencing batch reactor where olive oil mill effluents were centrifuged and tested with or without fermentation. The best results with regard to PHA production were obtained with

fermented olive oil mill effluents because of the higher VFA concentration. Garcia Ribera et al. (2001) studied the use of the olive mill waste waters produced from olive oil extraction, namely alpechin, which constitutes a severe environmental problem because of its high concentration of precarious phenolic compounds and its toxicity for many other micro-organisms. *P. putida* KT2442 was capable of growing in alpechin. The transformation with a plasmid harbouring *C. necator* PHB synthesis allows the organism to grow at high alpechin concentrations under PHA accumulation. Further studies on alpechin were carried out using *Azotobacter chroococcum* H23. With use of defined media with alpechin contents of 60%, considerable amounts of accumulated PHA were reported (González-López et al. 1996; Pozo et al. 2002).

Loo et al. (2005) studied the suitability of palm kernel oil, crude palm oil and palm acid oil as substrates for scl-PHA synthesis by *R. eutropha* PHB-4 harbouring the PHA synthase gene of *A. caviae*. The copolymer of 3HB and (*R*)-3-hydroxyhexanoate was synthesized at yields ranging from 1.5 to 3.7 $g l^{-1}$, containing 5% of (*R*)-3-hydroxyhexanoate in the polyester. Alias and Tan (2005) isolated a Gram-negative bacterium FLP1 from palm oil mill effluent (POME) by using a culture-enrichment technique and identified the organism as closely related to *Burkholderia cepacia*. When this strain was grown on crude palm oil and palm kernel oil, PHB homopolyester was produced. In contrast to the findings with animal lipids, no copolyester production was observed owing to the absence of odd-numbered fatty acids containing triacylglycerides in POME; the supplementation of odd-numbered fatty acids was needed for the incorporation of 3HV building blocks. Salmiati et al. (2007) produced PHA from organic wastes by mixed bacterial cultures by anaerobic–aerobic fermentation systems using POME as a carbon source. The production was carried out by a two-step process of acidogenesis (production of VFA) and acid polymerization. PHA production was carried out using mixed culture in aerobic bioreactor. The maximum PHA content was observed at 40% of the cell dried weight. Crude palm oil is a substrate of interest for *Erwinia* sp. USMI-20. Studies done by Majid et al. (1999) show that, with use of this strain, 46% PHB of cell mass was achieved after 48 h of cultivation.

2.3 Waste Streams from Biofuel Production

Caused by new legislative situations that require 5.75% biofuel penetration of the entire fuel market in the EU by December 2010, the increasing production of biodiesel via alkaline alcoholysis of different lipids is rapidly increasing (Bozbas 2008; Vasudevan and Briggs 2008). This generates enormous amounts of its major side stream, namely glycerol. As a 'three-carbon platform', glycerol acts as a starting material for the production of PHA, lactic acid and a broad range of chemicals and chemical intermediates. Per kilogram of biodiesel, more than 100 g of glycerol accrues as a by-product in the so-called liquid phase (GLP). Biodiesel is mainly used as an ecologically benign fuel that is gaining more and more interest owing to its better emission qualities than diesel and its independence from the availability of petrol.

The production of biodiesel normally is accomplished from different lipids such as food-grade rape seed oil, or palm oil. Considering the fact that in 1900 the first engine of Rudolph Diesel used vegetable oil as a fuel, the idea of food-oil-based lipids as fuels is definitely not new (Knothe 2001). Today, the utilization of food-grade raw materials for combustion is neither economically feasible nor acceptable from an ethical point of view. As an alternative, lipid wastes such as used cooking oil, restaurant greases and soapstocks are precious feedstocks for cost-efficient biodiesel production (Vasudevan and Briggs 2008).

Together with rising costs for petrol-based fuels, the production of biofuels is increasing enormously in many areas of the world, consequently decreasing the price of the by-product glycerol. All over Europe, the total production of biodiesel was reported to be 4.6×10^6 tons for 2006; this amount constitutes about 77% of the global biodiesel production in 2006. In the USA, 7.5×10^5 tons were produced in the same year (reported by Canakci and Sanli 2008). Just to illustrate the enormous increase in biodiesel production, it should be mentioned that only in Austria, the amount of biodiesel increased from 121,665 to 241,381 tons from 2006 to 2007, corresponding to an increase of 98% and to a production of about 24,000 tons of glycerol.

Applying glycerol as a raw material for other compounds not only improves the economics of biodiesel production, but is also ecologically reasonable. Numerous microbial strains exist that accept raw GLP without prior purification such as degreasing or demethanolization (see Fig. 4) as a substrate for production of PHA biopolyesters and lactic acid. Further, glycerol can be converted biotechnologically to 1,3-propanediol, an important compound for further organochemical synthesis. The utilization of GLP leads to an enormous cost advantage compared with

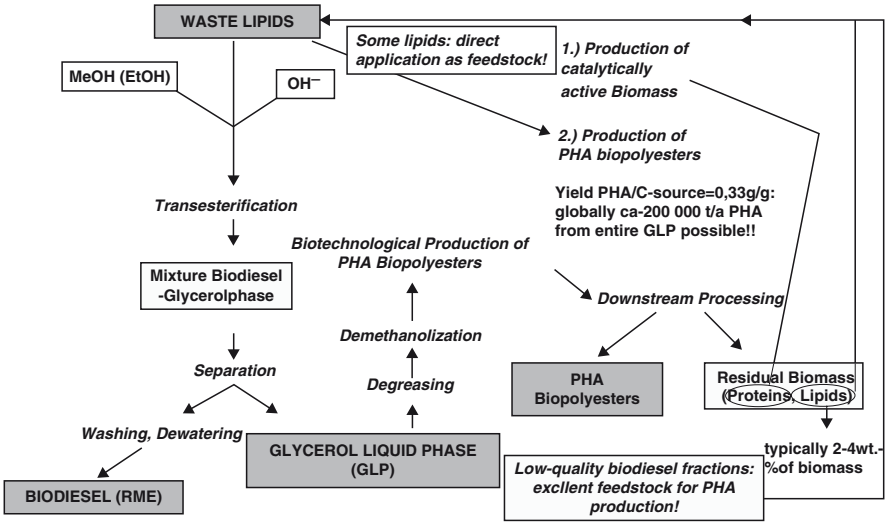

Fig. 4 From waste lipids to biofuels (biodiesel, RME) to PHA biopolymers, including the direct route of PHA production from lipids

commercially available pure glycerol. Via chemical catalysis, glycerol can easily be converted to acrolein, an intermediate in the production of several compounds such as methionine. Figure 4 shows the process lines from waste lipids to biofuels and PHA biopolyesters.

On a 10 l laboratory bioreactor scale, the highly osmophilic organism *H. mediterranei* was able to grow on GLP at a specific growth rate of 0.06 $1\,h^{-1}$ and produced PHA (76% of cell mass) at a specific rate of 0.08 $g\,g^{-1}\,h^{-1}$. The yield for PHA from glycerol was calculated as 0.23 $g\,g^{-1}$, resulting in a final concentration of 16.2 $g\,l^{-1}$ PHA (Koller et al. 2005b). With GLP as a carbon source, the polyester produced by *H. mediterranei* showed a weight-average molecular mass, M_w, of only 250,000, whereas on whey sugars $M_w = 700{,}000$ was found (Koller et al. 2005b). Other investigations with GLP as a substrate for PHA production have been described on a shaking flask scale or in small bioreactors using the eubacterial strain *Methylobacterium rhodesianum* MB 126 and its capsule-deficient mutant MB 126-J (Bormann and Roth 1999), *R. eutropha* DSM 11348 (Bormann and Roth 1999), *Pseudomonas oleovorans* NRRL B-14682 (Ashby et al. 2004; Ashby et al. 2005) and *Pseudomonas corrugata* 388 (Ashby et al. 2005). With use of *M. rhodesianum* MB 126 and *R. eutropha* DSM 11348, PHB was produced at volumetric productivities of 0.22 and 0.39 $g\,l\,h^{-1}$, respectively. *P. oleovorans* NRRL B-14682 converted glycerol into the PHB homopolyester. *P. corrugata* 388 accumulated mcl-PHA from glycerol consisting of (*R*)-3-hydroxyoctanoic acid, (*R*)-3-hydroxydecanoic acid, (*R*)-3-hydroxydodecanoic acid, (*R*)-3-hydroxydodecenonic acid and traces of C_6 and C_{14} (*R*)-3-hydroxyacids.

Similar to the results with *H. mediterranei*, Ashby et al. (2005) reported the decrease of molecular masses of PHA from *P. oleovorans* when glycerol is used as a carbon source. When glycerol or glycols are present in the medium, these substances cause termination of chain propagation by covalent linking at the carboxyl terminus of the polyester ('endcapping'; Madden et al. 1999; Ashby et al. 2002). This certainly is of major importance for the final product quality. If GLP is applied as a raw material for PHA biosynthesis, one has to consider if the molecular masses are sufficiently high for a required processing step such as melt extrusion. For certain special fields of application, low molecular mass PHA could be desired, i.e. for utilization as softeners.

2.4 Surplus Whey from the Dairy Industry

Whey from the dairy and cheese industries constitutes a waste and surplus material in many regions of the world. The reported amounts of whey that are produced globally vary from 1.15×10^8 tons per year (Peters 2006) to 1.40×10^8 tons per year (Audic et al. 2003). It is not only a cheap raw material, but also causes a disposal problem for the dairy industry owing to its high biochemical oxygen demand (40,000–60,000 ppm) and chemical oxygen demand (50,000–80,000 ppm) (Kim et al. 1995). Lactose, the major carbohydrate in whey, can serve as a substrate for growth

and product formation in numerous biotechnological processes. In the literature, the production of bioethanol (Zafar and Owais 2006), antibiotics (e.g. the bacteriocin nisin; Hickmann Flôres and Monte Alegre 2001), yeasts for yeast extract production (de Palma Revillion et al. 2003), surfactants (sophorolipids; Daniel et al. 1999), single-cell protein (Schultz et al. 2006), and biopolymers such as PHAs and PLA (Kim et al. 1995) has been described.

Cheese whey is a surplus product in the dairy industry. From the feedstock milk, casein is precipitated enzymatically or by acidification. This so-called transformation results in the generation of curd cheese (casein fraction) and full-fat whey (liquid fraction). The major part of the lipids is removed by skimming, leaving skimmed whey. The sweet skimmed whey undergoes a concentration step, where 80% of water is removed. This whey concentrate is separated via ultrafiltration into whey permeate (lactose fraction) and whey retentate (protein fraction with considerable lactose residues). Whereas special proteins of the retentate fraction are of interest owing to the importance of lactoferrin and lactoferricin for the pharmaceutical industry (Tomita et al. 2002), whey permeate (containing 81% of the total lactose originally included in the feedstock milk) can be used as a carbon source for biotechnological production of PHAs. Table 3 summarizes the composition of sweet whey, fermented whey, whey permeate and whey retentate.

Figure 5 illustrates the process line from the feedstock milk via whey towards PHA biopolyester production and provides a rough estimation of the theoretically possible PHA production from the entire whey lactose that is produced worldwide.

The utilization of surplus whey combines an economic advantage with ecological enhancement by converting the pollutant whey into valuable products. Biotechnological production of PHAs from different sugars via condensation of acetyl-CoA units stemming from hexose catabolism is well described (Sudesh et al. 2000), but only a limited number of micro-organisms directly convert lactose into PHAs. The principal possibility of direct conversion of whey lactose towards PHA using different wild-type bacterial strains was first investigated on shaking flask scale, where especially *H. pseudoflava* turned out to be a promising candidate for PHA production from whey (Povolo and Casella 2003). Recombinant *E. coli* strains harbouring PHA synthesis genes were well studied for directly converting lactose to PHAs, and are of interest owing to the high volumetric productivities found (Lee 1997; Schubert et al. 1988; Wong and Lee 1998). Moreover, also the direct utilization of whey permeate as a carbon source for growth and PHA production with recombinant *E. coli* strains has been well investigated (Ahn et al. 2000; Ahn et al. 2001; Kim 2000). The best results for PHA biosynthesis on whey by recombinant *E. coli*, namely a final PHA

Table 3 Composition of different types of whey (Braunegg et al. 2007)

Compound (%, w/w)	Sweet whey	Fermented whey	Whey permeate	Whey retentate
Lactose	4.7–4.9	4.5–4.9	23	14
Lactic acid	Traces	0.5	–	–
Proteins	0.75–1.1	0.45	0.75	13
Lipids	0.15–0.2	Traces	–	3–4
Inorganic compounds	~7	6–7	~27	~7

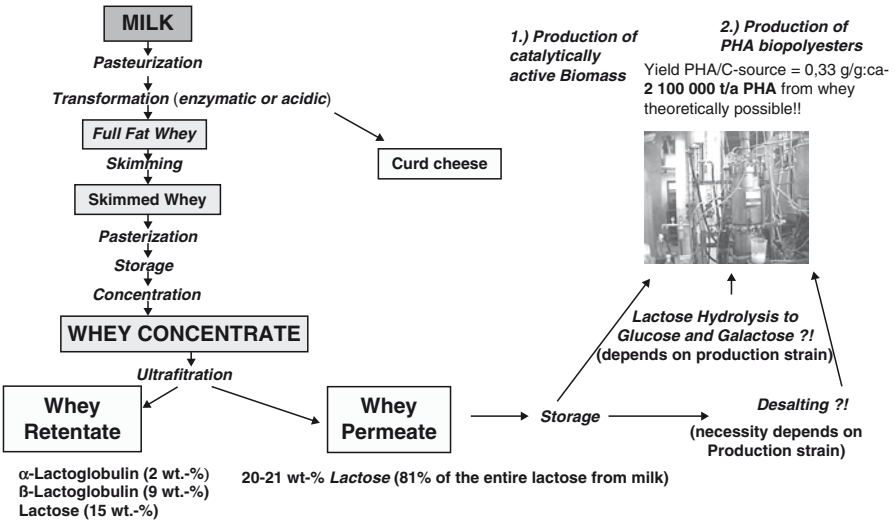

Fig. 5 From the feedstock milk to whey to the biopolyester, including a rough estimation of the material balances. The necessity for hydrolysis and/or desalting of the raw material whey depends on the PHA production strain (see the text)

concentration of 168 $g\,l^{-1}$ and a volumetric PHA productivity of 4.6 $g\,l^{-1}\,h^{-1}$, were reported by Ahn et al. (2001). To overcome the problems arising from the continual addition of the whey feed in fed-batch cultures, i.e. an increasing volume in the bioreactor, the authors employed a cell-recycle system.

If β-galactosidase activity of a production strain is not sufficiently high, lactose can be hydrolysed enzymatically or chemically to glucose and galactose. Compared with lactose, these monosaccharides are converted by a much higher number of organisms.

A third, more complex possibility arises from the anaerobic conversion of lactose to lactic acid in a first process step using lactobacilli capable of producing lactic acid with high yields (more than 0.9 g of lactic acid per gram of carbon source). In a subsequent aerobic cultivation, lactic acid is metabolized to acetyl-CoA and further to PHAs by numerous strains, e.g. most common PHA producers such as *C. necator*, *A. latus* and *A. vinelandii*. Alternatively, lactic can be converted to PLA, if wanted. Hence, for PHA production from whey, the decision whether to apply whey lactose, hydrolysed whey lactose or a first-step fermentation towards lactic acid mainly depends on the production strain (Fig. 6).

In a recent study, Koller et al. (2007a, b) compared the utilization of whey as a carbon source for three wild-type PHA-producing microbial strains, *H. mediterranei*, *P. hydrogenovora* and *H. pseudoflava*, in laboratory-scale bioreactors. Among these organisms, *H. mediterranei* turned out to be the most promising candidate for eventual industrial-scale PHA production starting from whey. This is due to the strain's high robustness and stability; the risk of microbial contamination during cultivation is restricted to an absolute minimum; thus, a lot of energy can be saved

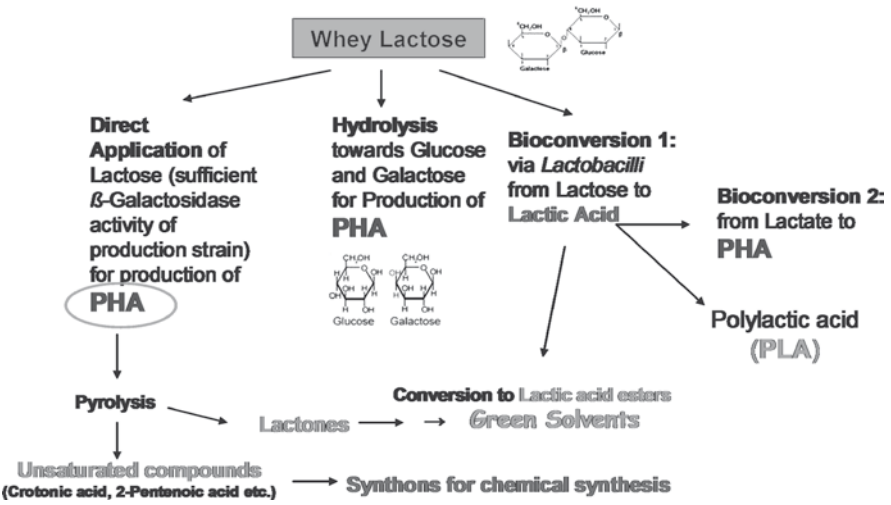

Fig. 6 Three different routes from whey lactose to PHA; alternative products such as poly(lactic acid) and follow-up products of PHA are included

by the lower sterility demands. Additionally the strain produces a poly(3HB-*-co*-8–10% 3HV) copolyester directly from the 3HV-unrelated carbon source whereby the normally high costs for propionic acid or valeric acid as precursors can be saved. The strain grew well on hydrolysed whey permeate with a maximum specific growth rate of 0.11 $l\,h^{-1}$. PHA was accumulated at a maximum specific production rate of 0.08 $g\,g^{-1}\,h^{-1}$. The yield of conversion of whey sugars to PHA was calculated as 0.33 $g\,g^{-1}$. The partial conversion of whey sugars to 3HV units and the excellent polymer characteristics (low melting temperature, high molecular masses within a narrow distribution) together with a viable cheap and simple downstream processing (Garcia Lillo and Rodriguez Valera 1990; Munoz-Escalona et al. 1994) render the strain especially interesting. The calculated production price amounted to €2.82 per kilogram of PHA. The recycling of the highly saline side streams has to be tested and optimized. Additionally, high salinity imposes special material demands on the bioreactor equipment and the probes (Hezayen et al. 2000). *P. hydrogenovora* features the disadvantage of low final polymer contents, productivities and product yields owing to redirection of the carbon flux towards unwanted by-products such as organic acid (Koller et al. 2008). *H. pseudoflava* produces biopolyesters of rather good quality (high molecular masses and low polydispersities) directly from whey lactose at acceptable specific production rates (12.5 mg PHA $g^{-1}\,l^{-1}\,h^{-1}$) and yields (0.2 g PHA g^{-1} whey lactose), but is not competitive with *H. mediterranei* in terms of strain stability and robustness (Koller et al. 2007a, b).

Yellore and Desai (1998) isolated a *Methylobacterium* sp. ZP24 that grew on cheese whey and produced PHB at a final concentration of 1.1 $g\,l^{-1}$ polymer. This work was extended later on a bioreactor scale, where Nath et al. (2008) cultivated this organism on cheese whey. In batch mode, final PHB concentrations of 2.07 $g\,l^{-1}$

with 67% of PHB in biomass and 0.06 $g\,l^{-1}\,h^{-1}$ of volumetric PHB productivity were reported. From a *Bacillus megaterium* strain isolated from the sludge of a sewage treatment plant, the production of a rather low amount of 26% PHB in cell dry mass from lactose was reported on a shaking flask scale (Omar et al. 2001).

Owing to the complex nature of the regulatory mechanism in PHA production, mathematical modelling of the processes, based on experimental results, becomes more and more interesting. This is especially true for multisubstrate carbon sources such as whey. Recently, mathematical models for production of PHB from whey and sucrose by *A. vinelandii* were developed using kinetic patterns from batch experiments. Various differential equations for formation rates of cell mass and PHA, and substrate utilization rates as functions of initial cell concentration, actual cell concentration and stationary cell concentrations, helped to predict the behaviour of the system (Dhanasekar and Viruthagiri 2005). For the biosynthesis of poly(3HB-*co*-3HV-*co*-4HB) terpolyesters from whey plus cosubstrates by *H. mediterranei*, Koller et al. (2006) compiled a formal kinetic model as well as a low structured model for PHB synthesis from whey by *P. hydrogenovora*. On the basis of the simple substrate glucose, similar models for PHB production by *C. necator* where compiled by Khanna and Srivastava (2005b) to provide the prerequisites for model-based fed-batch fermentations under desired nutrient conditions.

2.5 Lignocellulosic Wastes

Lignocellulosic materials, consisting of lignin (complex polyphenolic structure), cellulosic (β-D-1,4-glucan) and hemicellulosic (D-arabinose, D-xylose, D-mannose, D-glucose, D-galactose and sugar alcohols) fibres, constitute the most abundant renewable resources on our planet. Sixty percent of all plant biomass consists of lignocellulose. The composition of lignocellulosic biomass differs in terms of the shares of lignin (10–25%), cellulose (30–60%) and hemicellulose (25–35%) (Kumar et al. 2008; Peters 2006; Tengerdy and Szakacs 2003).

Industrial branches generating the major shares of this waste are the agro-industry, the wood-processing industry and the paper industry. Regarding the non-wood lignocellulosic biomass only, the amount produced annually is about 2.5×10^9 tons. An estimation done by the FAO indicates that about 7×10^8 tons of different crops such as pulse crops, oil seed crops plantation crops are produced annually worldwide (Rajaram and Verma 1990). As an example, the yearly cultivation of 6×10^8 tons of palm biomass generates a waste stream that contains about 90% of the entire palm plant. It consists of empty fruit bunches, fibres, POME, etc. (Kumar et al. 2008).

In the last decade, increasing efforts have been dedicated worldwide to realizing the implementation of biorefinery plants for the conversion of lignocellulosic and cellulosic waste to starting materials for the biotechnological production of bio-ethanol, biopolymers and a range of fine chemicals. Whereas the material recovery from the lignin fraction is limited to the production of adhesives, sulphur-free fuels and some aromatics, cellulose and hemicellulose provide a rich source of

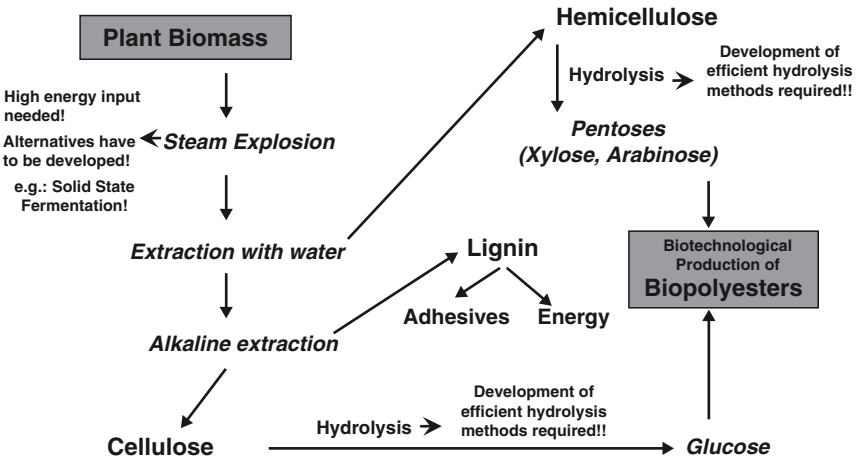

Fig. 7 Conversion of lignocellulosic plant biomass to biopolyesters. The necessity for enhanced hydrolysis methods and improved upstream processing for digestion of the plant biomass are indicated (Petschacher 2001)

carbohydrates that can be converted biotechnologically into a variety of final products. Figure 7 provides the classic scheme for the conversion of lignocellulose from plant biomass to PHA biopolyesters (Petschacher 2001).

The major obstacle for the utilization of lignocellulose is the high stability of this material. Lignocellulose was created by nature to provide plants with the required robustness and stability against hydrolytic attack, put into effect chemically by a high density of ester and ether bonds between the lignin and the carbohydrate fraction in plant biomass. The optimization of digestion methods for lignocellulose and the development of effective biocatalysts for the breakdown of cellulose and hemicellulose into sugars (hexoses and pentoses) accepted by the microbes are the prerequisite for any efficient biotechnological conversion of such raw materials into desired end products.

Currently, enzymatic saccharification and chemical hydrolysis are the main conventional methods for breaking these materials into smaller units. Although chemical hydrolysis using diluted acids is fast and easy to perform, it is hampered by its non-selectivity and the high temperature requirement. This can lead to the formation of inhibitory by-products that can negatively influence the subsequent biotechnological conversion of the hydrolysis products. Enzymatic methods used on a larger scale feature a rather low efficiency owing to end-product inhibition and adsorption phenomena leading to deactivation. Here, highly efficient systems of free or immobilized enzymes are needed to overcome this problem. To optimize the entire process chain of fast and efficient conversion of lignocellulosic biomass, several routes have to be followed and improved: (a) favouring ‘one-pot operation’ to avoid the necessity of separation steps, (b) utilization of all lignocellulose components, (c) high hydrolysis rates, (d) reduction of side products and (e) controlled

conversions to ensure selectivity. Classic energy-demanding digestion steps such as steam explosion for disruption of lignocellulose should be avoided to make the whole process energetically more profitable. An alternative to this process step might be solid-state fermentation (SSF). At the moment plenty of effort is being put worldwide into investigating the most promising lignocellulosic fungi and developing novel solid-state bioreactors for the production of a broad range of interesting bioproducts. SSF appears especially suitable for conversion of plant biomass because it is advantageous in terms of energy demand and it is suitable for on-site operation in agricultural facilities. The engineering aspects of SSF must still be further developed, especially regarding the biocatalysts and the controlled conversion of lignocellulose during SSF. From the engineering point of view, the structure of the lignocellulosic substrate, its change during fermentation, its heat conductivity and moisture content and the achievable O_2 mass transfer are the main points to be considered in reactor design (Tengerdy and Szakacs 2003).

The selection of appropriate production strains for PHA biosynthesis from lignocellulose-derived substrates mainly depends on the conversion rates of hexoses and pentoses by the organism. If the different sugars are not used in parallel and with similar rates, the bioprocess to be developed will be rather complicated. Sugars that are not accepted as a substrate by the strain or that are utilized considerably more slowly than others can pile up in the fermentation broth and may then cause inhibitory effects that are very likely to negatively influence growth and production kinetics and yields.

The number of bacterial strains that use pentoses beside hexoses is rather limited; therefore, a multistep hydrolysis might be needed, followed by separation of the different sugars. In the case of lignocellulose hydrolysate, pentoses are mainly represented by arabinose and xylose deriving from the hemicellulose fraction, whereas the hexose glucose is generated from the cellulose fraction.

Depending mainly on the global region of their occurrence, a vast number of highly interesting sources of lignocellulosic materials can be found, e.g. rice straw, corn straw and bagasse. These materials have favourable compositions with rather low amounts of lignin in the range of 10% (w/w) and a high percentage of carbohydrates. To implement the concepts of ‘refineries’ starting from lignocellulosic waste, the utilization of the entire plant has to be aspired to. Rice husks, e.g., contain high amounts of silicon dioxide that can be utilized for production of silicon (Kalapathy et al. 2002).

The conversions of lignocellulose waste into bioindustrial raw materials is a prerequisite for the development of sustainable process technologies worldwide, and it seems to be a chance for future industrial development of many tropical and subtropical countries. In many countries rice straw and other plant parts (e.g. dry dead leaves of sugar cane) are still directly burned in the fields, severely contributing to air pollution. This phenomenon is of increasing importance, e.g., in Egypt, where the burning of these materials results in the formation of the so-called mysterious black clouds in the sky above the Nile delta (Kenawy 2008). The controlled burning of these wastes in boilers for generation of energy (steam and electricity) appears reasonable for all processes in which energy costs constitute the bottleneck

for the entire process economics. A prime example for this can be found at the company PHBISA in Brazil, where, starting from sugar cane, sucrose, bioethanol and PHA biopolyesters are produced. The energy required for these processes is totally supplied from burning of bagasse that accrues in high amounts as a residue from the sugar cane plant.

The first study on PHA biosynthesis from the pentoses xylose and arabinose stemming from the hemicellulose fraction of poplar wood was done by Bertrand et al. (1990) with *Pseudomonas pseudoflava* ATCC 33668 (today known as *H. pseudoflava*). The authors reported significantly lower specific PHA production rates for the pentoses compared with glucose. This effect was interpreted by higher substrate utilization for maintenance of energy supply by the organisms when they are cultivated on pentoses instead of hexoses. The production of PHB from xylose by *Pseudomonas cepacia* ATCC 17759 was first investigated by Young and colleagues and was compared with PHB production from glucose and lactose. In all cases, excellent specific growth rates were achieved (0.3 $1h^{-1}$ on glucose and lactose, 0.1 $1h^{-1}$ on xylose), but rather low specific PHB production rates (below 0.02 $g\,g^{-1}\,h^{-1}$) and product yields (below 0.15 $g\,g^{-1}$) were reported (Young et al. 1994). Additionally, *P. cepacia* ATCC 17759 was examined for PHB production by Ramsay et al. (1995). The strain was found to be able to grow and produce PHB from xylose up to 60% of the cell dry mass under nitrogen-limiting conditions. The growth and PHB production kinetics were similar to those obtained when the same organism was cultivated on fructose. Cost estimations done by the authors showed that the price for hydrolysed hemicellulose as a substrate was in a range similar to that for cane molasses and half the price of using glucose.

Keenan et al. (2006) reported PHA biosynthesis in shaking flask cultures of *B. cepacia* grown on 2.2% (w/v) xylose, plus various concentrations of added laevulinic acid. Both substrates were derived from forest biomass, laevulinic acid from cellulose and xylose from the hemicellulose fraction. The concentrations achieved for different P(3HB-*co*-3HV) copolyesters with 3HV shares in the polyester from 1.0 to 61% (mol/mol) ranged from 1.3 to 4.2 $g\,l^{-1}$.

2.6 Starch

The advantage of starch as a carbon source is that its price is lower than that of glucose. Choi and Lee (1999) estimated that on a production scale of 100,000 tons of PHB per year, production costs would decrease from US $4.91 to 3.72 kg^{-1} if hydrolysed corn starch (US $0.22 kg^{-1}) were used instead of glucose (US $0.49 kg^{-1}). Most processes for PHA production based on starch require the conversion of starch to easily convertible substrates such as glucose by enzymatic or chemical hydrolysis (Chen et al. 2006; Huang et al. 2006). Alternatively, VFAs can be produced as fermentation substrates by acidogenesis (Yu et al. 2002). The production of P(3HB-*co*-3HV) by *H. mediterranei* on extruded starch in a pH-stat fed-batch mode was recently described by Chen et al. (2006). Here, an exogenous source of α-amylases was used.

The strain produced copolyester with a 3HV content of approximately 10% (mol/mol) and superb thermal characteristics. This is in excellent compliance with the results found when the strain was cultivated on whey or GLP (Koller et al. 2005b). Additionally, Huang et al. (2006) working with the same strain reported a PHA concentration of 77.8 $g\,l^{-1}$, corresponding to 55.6% (w/w) PHA in the dry cell mass, when the medium contained extruded rice bran and corn starch in a ratio of 1:8.

Recently, the newly isolated organism *Bacillus cereus* CFR06 was tested for PHA production from various carbon sources such as xylose, arabinose, hexoses and starch (Halami 2008). The authors described the strain as halo- and thermotolerant and non-pathogenic. In the case of starch as the sole carbon source, nearly 50% of PHB in the dry biomass was found after cultivating the strain in shaking flasks for 72 h. The authors stated that owing to its desirable high stability, this organism could be interesting for industrial-scale PHA production. A certain disadvantage is seen in the spore formulation of the strain that occurs as a competing reaction. The creation of non-spore-forming mutants appears reasonable in this case.

Haas and colleagues used saccharified waste potato starch as a carbon source for PHB production by *R. eutropha* NCIMB 11599 under phosphate-limited conditions. The researchers achieved 179 $g\,l^{-1}$ biomass, 94 $g\,l^{-1}$ PHB and reported the yield of total biomass from starch as 0.46 $g\,g^{-1}$, the yield for PHB from starch as 0.22 $g\,g^{-1}$, and the volumetric PHB productivity as 1.47 $g\,l^{-1}\,h^{-1}$. Residual maltose accumulated in the fed-batch reactor but caused no noticeable inhibition (Haas et al. 2008).

Maltose as the major product after hydrolysis of starch was utilized by Braunegg et al. (1999) for cultivation of three different strains of *A. latus* (DSM 1122, 1123 and 1124) in 10-l bioreactors. Compared with the results on glucose, specific rates for growth and product formation were lower using starch hydrolysate, but the yields for production of biomass and PHA were comparable.

Rusendi and Sheppard (1995) described the enzymatic utilization of potato processing wastes for utilization as a substrate for PHB production. The amylase for starch hydrolysis was provided by barley malt. The hydrolysate, containing about 200 $g\,l^{-1}$ glucose, was supplemented by other nutritional components needed for the cultivation of the production strain *Alcaligenes eutrophus*. In batch cultures, a concentration of 5 $g\,l^{-1}$ PHB was obtained, corresponding to 77% of cell dry mass.

2.7 Materials from the Sugar Industry

A different approach is provided by the utilization of carbon sources that have a considerable market value and do not constitute waste materials, but are produced in a process integrating the fabrication of the carbon substrate and PHA. This has been implemented on a pilot scale by the company PHB Industrial in the state of Sao Paulo, Brazil. Starting from sugar cane, the company produces saccharose and ethanol. The waste streams from the sugar production (bagasse) and the bioethanol production (fusel alcohols) are used for running the PHA production and making it economically competitive.

Starting from sugar cane, one needs about 3 kg of sucrose to produce 1 kg PHB using *C. necator* DSM 545 as a production strain. The electrical power needed is generated by high-pressure steam from burning bagasse, the major by-product of the sugar production. Low-pressure steam, which is additionally needed for heating and sterilization, is also provided from bagasse combustion (Nonato et al. 2001).

After fermentation and biomass separation, recovery constitutes another cost factor not to be neglected, especially in large-scale production. The PHA production process described is embedded in an ethanol production plant and can therefore resort to the fusel alcohols, mainly isopentyl alcohol, from the distillery step. The application of the fusel alcohols as extracting solvents unites two important points. These compounds normally constitute a waste stream without any market value. When they are used as extraction solvents, the costs for alternative solvents are saved. Furthermore, these extraction solvents are less harmful to handle than the classic extraction solvent chloroform (Nonato et al. 2001). Owing to the autarkic energy supply and the in-house availability of the carbon source saccharose, the production costs per kilogram of PHB are estimated as less than US $3 (Choi and Lee 1997; Nonato et al. 2001; Squio and Aragao 2004).

Recently, a ‘cradle to gate’ LCA study was carried out for the Brazilian PHA production process in comparison with petrochemical plastics. The study encompassed the net carbon dioxide production and all major categories of the production cycles. As a conclusion, PHB from the ‘Brazilian process’ turned out to be superior to polypropylene and polyethylene in all major LCA categories (Harding et al. 2007).

On the basis of an amount of 2.16×10^6 tons sugar cane crushed per milling season in an average sugar mill, the mass and energy balance for an integrated process annually producing 180,000 tons of commercialized cane sugar, 52,575 m^3 ethanol and 10,000 tons of PHB is visualized in Fig. 8 (values from Nonato et al. 2001).

Considering the fact that much more bagasse is available than needed for the energy production, further value creation might be possible. As an example, hemicellulose and cellulose fractions of bagasse can be hydrolysed and utilized by suitable microbial strains for PHA biosynthesis. This was successfully demonstrated by Silva et al. (2004) for strains of *B. cepacia* and *Burkholderia sacchari*. On a laboratory scale, hydrolysed bagasse was investigated as a carbon source for PHA biosynthesis, comparing its performance as a substrate in direct comparison with pure xylose plus glucose. The authors reported excellent results on a bioreactor scale using *B. sacchari* IPT 101 on hydrolysed bagasse; 62% of PHA cell dry mass was reached at a yield of 0.39 $g\,g^{-1}$; these results were considerably better than those obtained with pure sugars, but lower cell densities were achieved on hydrolysed bagasse than on pure sugars. Felipe et al. (1997) mentioned the formation of toxic compounds that are formed during acidic hydrolysis of bagasse such as furfural and 4-Hydroxy-5-methyl-3 (2 H)-furanone (HMF). Procedures have to be developed for removal of these components. Silva et al. (2004) compared different processes for detoxification of acidically hydrolysed sugar cane bagasse. Treatment with active charcoal turned out to be a feasible and effective method to significantly lower the concentration of HMF and furfural. Additionally, powdered bagasse might also be an interesting filler material for PHA-based composites.

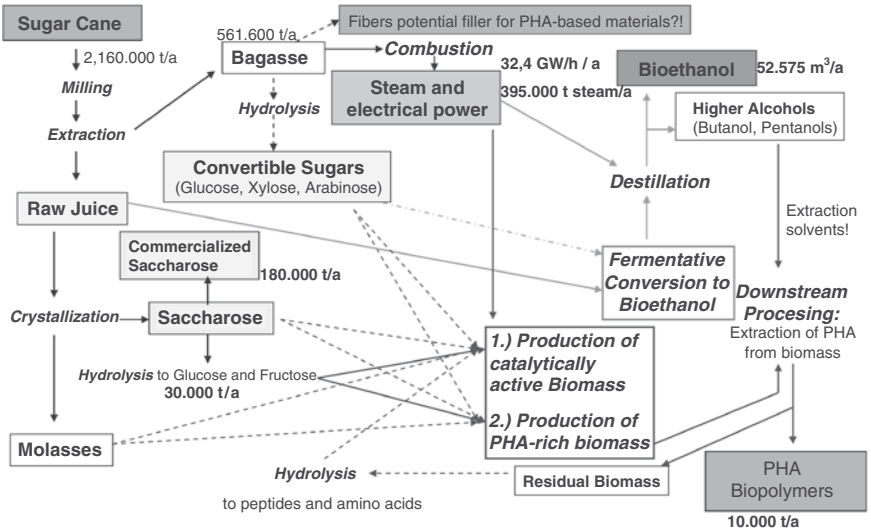

Fig. 8 PHBISA, Brazil: Integration of biofuel and biopolymer production in the sugar cane industry: actual (*solid arrows*) and potential (*dashed arrows*) utilization of the waste streams. The scheme provides a mass balance of the annually produced amounts of commercialized sucrose, bioethanol and PHA starting from 2,160,000 tons sugar cane per milling season (values based on Nonato et al. 2001)

Rocha and colleagues used the mutant strain *B. sacchari* IPT 189 for production of P(3HB-*co*-3HV) with a broad range of 3HV content from sucrose and propionic acid. The authors calculated a production price per kilogram of PHB and P(3HB-*co*-3HV) of below US $1, but took only into account the cost of the carbon sources and did not consider the running costs of the plant.

Molasses, a common waste material from the sugar industry, sell at about 33–50% of the price of glucose (Zhang et al. 1994). They contain sucrose as the major carbohydrate beside other sugars and additional growth promoters such as vitamins and biotin. The investigation of molasses as substrates for PHA production was done by the group of Page (1992). The authors reported a production of 2.5 $g l^{-1}$ PHB on a shaking flask scale after 24 h of cultivation using a mutant strain of *A. vinelandii* UWD on a molasses-based medium. The production of PHB from molasses by sucrose-utilizing recombinant organisms, namely *E. coli*, *Klebsiella oxytoca* and *Klebsiella aerogenes*, was investigated by Zhang et al. (1994). The authors described the accumulation of about 3 $g l^{-1}$ PHB on a shaking flask scale after 37 h of cultivation, corresponding to approximately 50% of PHB in cell mass. Years later, these results were enhanced by an Indian group that combined the utilization of molasses with the addition of the complex substrate corn-steep liquor. The organism utilized, *Azotobacter beijerinckii*, produced 3.7 $g l^{-1}$ PHB after 24 h of cultivation. Additionally, the study encompassed detailed investigations of the beneficial effect of minor compounds such as metals contained in untreated molasses, but not in refined sugar, on microbial growth and PHB production (Purushothaman et al. 2001).

Omar and colleagues described the PHB production by *B. megaterium* on a range of substrates such as date syrup, beet molasses and the corresponding pure carbohydrates in defined media. In each case, the authors noticed positive impacts of the inexpensive, complex substrates due to additional compounds included in these feedstocks. The background to this study is the enormous number of date palm trees cultivated in countries such as Saudi Arabia, where the amount of dates surmounts 500,000 tons annually (Yilmaz and Beyatli 2005). On a 10-l bioreactor scale, Braunegg et al. (1999) reported the production of PHB from green syrup and sugar beet molasses by three different strains of *A. latus* (DSM 1122, 1123 and 1124). The results were compared with those obtained with the pure sugars glucose and sucrose. Although specific rates for growth and product formation were significantly lower when complex substrates where used, the production yields of biomass and PHB from the carbon source were in the same range for pure and complex substrates.

2.8 *Lactic Acid as a Versatile Intermediate Towards Follow-Up Products*

Starting from many of the waste streams discussed already, lactic acid is accessible as a product of the anaerobic fermentation by lactobacilli or lactococci (Drumright et al. 2000; Tsuge et al. 2001). Especially the conversion of whey lactose, cassava starch or molasses into lactic acid is a well-known process (Wee et al. 2006). When homofermentative lactic acid bacteria are used, lactic acid is produced from the substrate at excellent conversion yields exceeding 0.9 g lactic acid per gram substrate (Wee et al. 2006). Using whey lactose, Kim et al. (1995) even reported yields of more than 0.98 g lactic acid per gram of lactose consumed by the organism *Lactobacillus* sp. RKY2.

Lactic acid is known to have numerous fields of industrial application, such as foods, cosmetics, pharmaceuticals, textiles and chemicals (Wee et al. 2006). Via simple chemistry, lactic acid can be converted into lactic acid esters that constitute so-called green solvents. In the area of polymers, lactic acid opens the route for the chemical production of PLA and the biotechnological production of PHA (see Fig. 6).

For biotechnological purposes, the production of lactic acid from different waste streams in a first fermentation process by lactobacilli appears very reasonable. This is due to the fact that, caused by the low-pH conditions, lactic acid can easily be stored without major conservation requirements. Hence, this compound features an excellent stable intermediate for a versatile range of follow-up products. In contrast to the transportation of lignocellulosic waste or whey, the transportation of lactic acid in a concentrated form to production facilities where it will be converted might be economically reasonable in many cases. Whereas the production of lactic acid itself is dependent on the seasonal availability of the waste materials, production facilities for the subsequent conversion of lactic acid to final products such as PHAs can be supplied from lactic acid stocks independent of the season.

The application of lactic acid, together with the 3HV precursor valeric acid as a substrate for P(3HB-*co*-3HV) production was successfully demonstrated.

Using the production strain *C. necator* DSM 545, a final concentration of poly(3HB-*co*-8.6% 3HV) of 12.7 $g\,l^{-1}$ after 62 h of cultivation was described on a 10-l bioreactor scale. The organism achieved a maximum specific growth rate of 0.14 $1\,h^{-1}$ (Braunegg et al. 2002).

3 Concluding Remarks and Future Perspectives

The selection of the appropriate substrate firstly depends on the intended location of the PHA production plant, and on the resource quantities available. Moreover, the suitability of the raw material for low-cost storage is of high importance because such resources very often accrue seasonally (e.g. straw, sugar cane, corn). Beside the carbon source, the availability of cheap complex nitrogen sources for effective and fast biomass production is advantageous. Adequate materials can be found in certain waste streams from agriculture such as MBM, grass juices and corn steep liquor. Wherever possible, industrial PHA production should be integrated into the existing structure of the waste or raw material generating industrial unit to minimize production costs owing to synergisms. A prime example for this strategy is the integration of PHA production into a sugar mill combined with ethanol production in Brazil. In this case the in-house product sucrose acts as the carbon source, the total process energy (steam and electricity) is generated from surplus bagasse, and the extraction solvent isopentyl alcohol is available from the ethanol distillation unit.

Owing to the enormous amounts available annually, lignocellulose-based wastes are likely to become the most important raw materials for future biotechnological production of polymers, chemicals and fuels. To make substantial progress in this field, increased know-how is required in the areas of microbiology, enzyme technology and chemical engineering to overcome still-existing bottlenecks in the efficient conversion of these feedstocks, especially during the upstream processing.

In future, it will be indispensable to create databases for agricultural feedstocks and their side streams to document the range of variability in composition and quality. Such databases can be obtained through a long-term monitoring and documentation of raw materials from different origins. Furthermore, it is desirable if the bioprocess itself is not sensitive against a certain variability of the feedstock quality.

Beside the raw material costs and the fermentation process itself, downstream processing is a decisive cost-determining factor in biopolymer production. Depending on the PHA-producing micro-organisms, a broad range of possibilities are available for PHA recovery and purification such as extraction or degradation of non-PHA biomass after harvesting the bacterial cells. Research in this field is quite far developed in terms of minimizing the required amounts of solvents and other cost-intensive and/or hazardous compounds.

Uniting the potential enhancements of each process step, one can definitely make substantial progress towards a cost-efficient technology. This appears possible by the selection of a production strain which is capable of synthesizing high-value copolyesters from low-cost raw materials without the need of a cosubstrate supply together

with minor sterility precautions and a cheap and convenient isolation method. In any case, the development of really efficient biopolymer production processes starting from diverse waste streams needs the narrow co-operation of experts from different scientific fields. Chemical engineers, microbiologists, enzymologists, polymer scientists, genetic engineers and experts in the fields of LCA and cleaner production have to concentrate their special expertise and know-how to close the existing gaps between promising data from the laboratory scale and industrial realization.

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Industrial Production of PHA

Guo-Qiang Chen

Contents

Abstract Many years of research efforts have led to the large-scale production of poly[(*R*)-3-hydroxybutyrate], copolymers of (*R*)-3-hydroxybutyrate and (*R*)-3-hydroxyvalerate, copolymers of (*R*)-3-hydroxybutyrate and 4-hydroxybutyrate, as well as copolymers of (*R*)-3-hydroxybutyrate and (*R*)-3-hydroxyhexanoate. A small amount of medium-chain-length polyhydroxyalkanoate (PHA) has also been reported to have been produced. Worldwide, 24 companies are known to be engaged in PHA production and applications. Beginning from 2009, more than 10,000 PHA will be available on the market, and new applications are expected to be developed when large amounts of PHA are available for exploitation. In the future, the large scale of PHA applications will be dependent on the production cost. Therefore, it is very urgent to develop low-cost PHA production technology, including those of continuous and nonsterile processes based on mixed cultures and mixed substrates.

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1 Introduction

Although many polyhydroxyalkanoates (PHA) have been found, only four of them have been produced on a large scale for commercial exploitation. These are poly[(*R*)-3-hydroxybutyrate] (PHB), poly[(*R*)-3-hydroxybutyrate-*co*-(R)-3-hydroxyvalerate] (PHBV), poly[(*R*)-3-hydroxybutyrate-*co*-4-hydroxybutyrate] (P3HB4HB), and poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyhexanoate] (PHBHHx). Small-scale production of medium-chain-length (mcl) PHA was also conducted. Generally, PHA production contains several steps, including fermentation, separation of biomass from the broth, biomass drying, PHA extraction, PHA drying, and packaging (Fig. 1). There is still a lot to improve for the production of these unique polyesters. Globally there are 24 companies known to have engaged in PHA R&D as well as production (Table 1; Chen 2009); some of them have stopped their PHA activities, mainly in the 1990s, owing to the low petroleum price. PHA has experienced another gold rush since the oil price increased to over US $100 per barrel in early 2003. Although the recent financial tsunami is slowing PHA progress a bit, most still believe that petroleum is not an inexhaustible resource and an alternative must be found. PHA production and application are related to green chemistry, reduced CO_2 emissions, environmental protection, and sustainable developments; these explain why many companies are still exploiting them even though PHA is not economically competitive with petroleum-based plastics using present technology.

Boston-based Metabolix has been in the PHA commercialization process for the longest time (Fig. 2; http://www.metabolix.com). The company has received many awards from various organizations for its environmental protection efforts; it has more than 500 owned and licensed patents and applications worldwide.

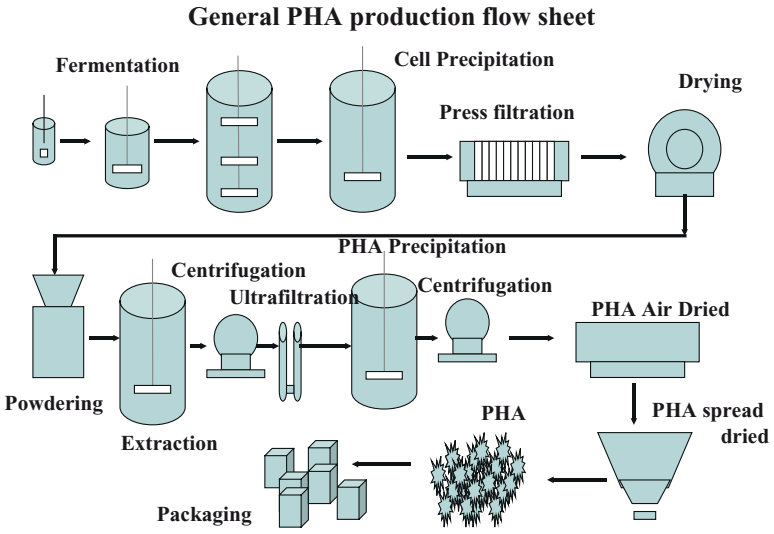

Fig. 1 General polyhydroxyalkanoate production and extraction process

Table 1 Worldwide polyhydroxyalkanoate (*PHA*) producing and research companies (Chen 2009)

Company	Types of PHA	Production scale (tons/year)	Period	Applications
ICI, UK	PHBV	300	1980–1990	Packaging
Chemie Linz, Austria	PHB	20–100	1980s	Packaging and drug delivery
BTF, Austria	PHB	20–100	1990s	Packaging and drug delivery
Biomers, Germany	PHB	Unknown	1990s to present	Packaging and drug delivery
BASF, Germany	PHB, PHBV	Pilot scale	1980–2005	Blending with Ecoflex
Metabolix, USA	Several PHA	Unknown	1980 to present	Packaging
Tepha, USA	Several PHA	PHA medical implants	1990 to present	Medical bioimplants
ADM, USA (with Metabolix)	Several PHA	50,000	2005 to present	Raw materials
P&G, USA	Several PHA	Contract manufacture	1980–2005	Packaging
Monsanto, USA	PHB, PHBV	Plant PHA production	1990s	Raw materials
Meredian, USA	Several PHA	10,000	2007 to present	Raw materials
Kaneka, Japan (with P&G)	Several PHA	Unknown	1990 to present	Packaging
Mitsubishi, Japan	PHB	10	1990s	Packaging
Biocycles, Brazil	PHB	100	1990 to present	Raw materials
Bio-On, Italy	PHA (unclear)	10,000	2008 to present	Raw materials
Zhejiang Tian An, China	PHBV	2,000	1990 to present	Raw materials
Jiangmen Biotech Center, China	PHBHHx	Unknown	1990s	Raw materials
Yikeman, Shandong, China	PHA (unclear)	3,000	2008 to present	Raw materials
Tianjin Northern Food, China	PHB	Pilot scale	1990s	Raw materials
Shantou Lianyi Biotech, China	Several PHA	Pilot scale	1990–2005	Packaging and medicals
Jiangsu Nan Tian, China	PHB	Pilot scale	1990 to present	Raw materials
Shenzhen O'Bioer, China	Several PHA	Unknown	2004 to present	Unclear
Tianjin Green Bioscience (+DSM)	P3HB4HB	10,000	2004 to present	Raw materials and packaging
Shandong Lukang, China	Several PHA	Pilot scale	2005 to present	Raw materials and medicals

PHBV poly[(*R*)-3-hydroxybutyrate-*co*-(R)-3-hydroxyvalerate], *PHB* poly[(*R*)-3-hydroxybutyrate], *PHBHHx* poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyhexanoate], *P3HB4HB* poly[(*R*)-3-hydroxybutyrate-*co*-4-hydroxybutyrate]

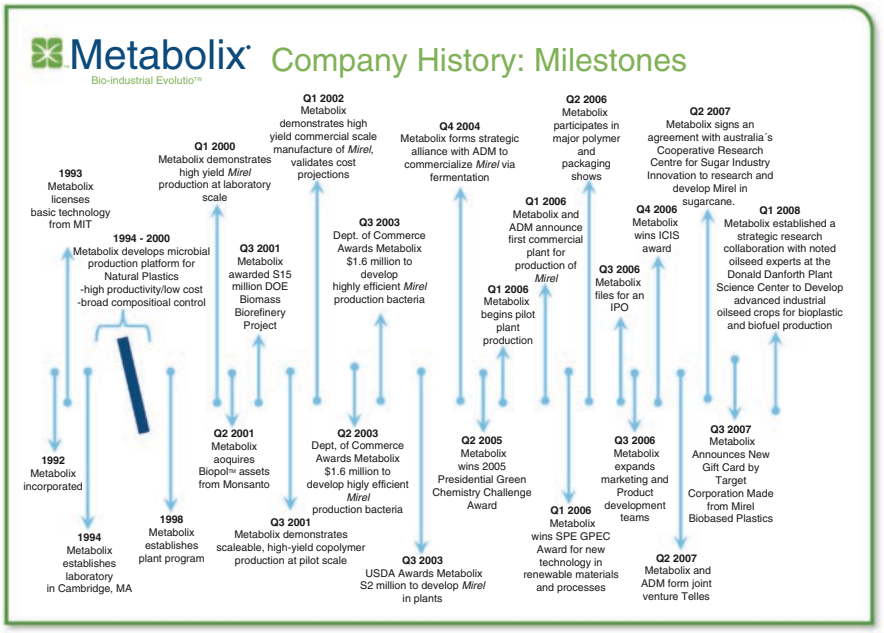

Fig. 2 Metabolix company history and milestones (adapted from http://www.metabolix.com)

In 2004, Metabolix formed a strategic alliance with ADM to commercialize PHA using the large fermentation capacity of ADM. Metabolix markets its PHA under the commercial name Mirel, and this turned into new gift card in 2007. In 2006, the company successfully filed an IPO in the USA. It is expected that the joint venture of ADM and Metabolix will soon produce 50,000 tons of Mirel using the new production facility under construction.

2 Industrial Production of PHB

2.1 PHB Produced by Chemie Linz, Austria, Using Alcaligenes latus

A. latus is one of the strains that satisfy the requirement for industrial PHB production (Hanggi 1990; Chen et al. 1991). The strain grows rapidly in sucrose, glucose, and molasses. PHB accumulation can be as high as over 90% of the cell dry weight (CDW; Chen 1989). There was even an attempt to produce PHB from waste materials using *A. latus* (Chen 1989).

Chemie Linz, Austria (later btf Austria), produced PHB in a quantity of 1,000 kg per week in a 15-m^3 fermentor using *A. latus* DSM 1124 (Hrabak 1992).

The cells were grown in a mineral medium containing sucrose as a carbon source. The PHB produced by *A. latus* was used to make sample cups, bottles, and syringes for application trials. The PHB production and processing technology are now owned by Biomer, Germany. Different products, including combs, pens, and bullets, have been made from PHB produced by *A. latus*.

2.2 PHB Produced by PHB Industrial Usina da Pedra-Acucare Alcool Brazil Using Bhurkolderia sp.

PHB and related copolymers can be advantageously produced when the production is integrated into a sugarcane mill (Nonato et al. 2001). In this favorable scenario, the energy necessary for the production process is provided by biomass. Carbon dioxide emissions to the environment are photosynthetically assimilated by the sugarcane crop and waste is recycled to the cane fields. The polymer can be produced at low cost considering the availability of a low-price carbon source and energy (Nonato et al. 2001). In 1995, the Brazilian sugar mill Copersucar assembled a pilot-scale PHB production plant. The goal of this pilot plant was to produce enough PHB to supply the market for tests and trials. Also, this pilot plant was intended as a training facility for future operators, and it is currently providing data for scale-up and economic evaluation of the process.

Copersucar managed to produce 120–150 $g l^{-1}$ CDW containing 65–70% PHB with a productivity of 1.44 kg PHB $m^{-3} h^{-1}$ and a PHB yield of 3.1 kg sucrose per kilogram of PHB.

Nonato et al. (2001) pointed out that large-scale production of PHB in sugarcane mills (in this case, 10,000 tons per year) presents a successful opportunity for expanding the sugar industry. The total amount of sugar diverted to PHB synthesis would account for a low percentage (17%) of the total sugar produced by the (average) mill into which the plant is integrated. This fact ensures that PHB production would not affect sugar stocks, nor would it have any significant impact on sugar prices. It is expected that the PHB production capacity could be increased approximately 2–3 times for the same mill if the market for biodegradable resins increases. Expansion will be possible by optimizing the energy consumption in PHB production, by the use of cane leaves and wastes as a new source of primary fuel at the mill, and mainly by optimization steps at the power plant, the sugar factory, and the distillery. When considering the prospects for the near future, one must mention that the annual production of sugar and ethanol in the south-central region of Brazil, from approximately 265×10^6 tons of sugarcane, is 16.9×10^6 tons and $11.6 \times 10^6 m^3$, respectively.

There exists a significant amount of available arable land now occupied by low-grade pastures that could be used for sugarcane production if market demand increased. This capacity could allow for the rapid growth of PHB production to cope with the market needs, should the demand for biodegradable resins match the projections.

2.3 PHB Produced by Tianjin Northern Food and Lantian Group China Using Ralstonia eutropha and Recombinant Escherichia coli, Respectively

R. eutropha was used to conduct PHB production research in a 1-m^3 fermentor under the joint action of the Institute of Microbiology affiliated with the Chinese Academy of Sciences and Tianjin Northern Food, China. Growth was carried out for 48 h in a glucose mineral medium. At the end of the cell growth, the cell density reached 160 $g\,l^{-1}$. The cells produced 80% PHB in their dry weight. Most surprisingly, the strain grown to such a high density did not require oxygen-enriched air. This was perhaps the highest cell density for PHB production achieved in pilot-scale production (unpublished results). On the other hand, Lantian Group (Jiangsu, China) used recombinant *E. coli* to produce PHB to a density as high as 168 $g\,l^{-1}$ in a 10-m^3 fermentor (Yu et al. 2003).

3 Industrial Production of PHBV

The strain is able to grow on glucose and produce the copolymer PHBV to a density as high as 70–80 $g\,l^{-1}$ after over 70 h of growth (Byrom 1992). Shampoo bottles were produced from PHBV (trademarked as Biopol) and were available in supermarkets in Europe. However, owing to economic reasons, the Biopol products did not succeed and the PHBV patents were sold to Monsanto and further to Metabolix.

NingBo TianAn, China, in collaboration with the Institute of Microbiology affiliated with the Chinese Academy of Sciences, has developed a model process that can produce PHBV in high efficiency. Without supply of pure oxygen, *R. eutropha* grew to a density of 160 $g\,l^{-1}$ CDW within 48 h in a 1,000-l fermentor. The cells accumulated 80% PHBV with a production efficiency of 2.5 $g\,h^{-1}\,l^{-1}$. The hydroxyvalerate content in the copolymer ranged from 8 to 10%. This process can significantly reduce the production cost for PHBV.

Only by achieving a high growth rate, a high PHBV production efficiency, and high cell and PHBV densities can the polymers become economically competitive. We assume that PHBV or other PHA will become cost-effective after extensive improvement of the fermentation process and the downstream process.

4 Industrial Production of P3HB4HB

R. eutropha and recombinant *E. coli* are used by Tianjin Green Bioscience, China, and Metabolix, USA, to produce P3HB4HB (Table 1). With the addition of 1,4-butanediol in different amounts, 4-hydroxybutyrate can be accumulated to 5–40 mol% in

the copolymer, thus generating copolymers with various thermal and mechanical properties for various applications. Tianjin Green Bioscience and Metabolix are building facilities with capacities of 10,000 and 50,000 tons of P3HB4HB, respectively. Both are scheduled to complete the capacity buildup in 2009. By then, P3HB4HB will be the PHA available in the greatest quantity on the market. At the same time, both companies are working to develop various bulk applications.

5 Industrial Production of PHBHHx

Recently, Tsinghua University in Beijing, China, in collaboration with the Guangdong Jiangmen Center for Biotech Development, China, KAIST, Korea, and Procter & Gamble, USA, succeeded in producing PHBHHx using *Aeromonas hydrophila* grown in a 20-m^3 fermentor (Chen et al. 2001). The PHBHHx production was carried out on glucose and lauric acid for about 60 h. The CDW reached 50 $g\,l^{-1}$; only 50% of PHBHHx was produced in the CDW. The extraction of PHBHHx was a very complicated process involving the use of ethyl acetate and hexane, which increased the polymer production cost dramatically. PHBHHx produced by Jiangmen, China, is now being exploited for application in the areas of flushables, nonwovens, binders, films, flexible packaging, thermoformed articles, coated paper, synthesis paper, coating systems, and medical devices (http://www.nodax.com). Copolymers consisting of hydroxybutyrate and mcl hydroxyalkanoate have been trademarked by P&G as Nodax. Many PHBHHx-based packaging products have been produced (Fig. 3).

The current production cost for PHBHHx is still too high for real commercial application. However, many efforts have been made to improve the production process for PHBHHx, including the downstream process technology. Most efforts have been focused on increasing cell density and simplifying the downstream process. A better production strain able to utilize glucose will be one of the most important issues for reducing the PHBHHx production costs.

Akiyama et al. (2003)simulated large-scale fermentative production of PHBHHx with 5 mol% (*R*)-3-hydroxyhexanoate (HHx) [P(3HB-*co*-5 mol% 3HHx)] from soybean oil as the sole carbon source using a recombinant strain of *Wautersia eutropha* harboring a PHA synthase gene from *Aeromonas caviae*. The annual production of 5,000 tons of P(3HB-*co*-5 mol% 3HHx) is estimated to cost from US $3.5 to 4.5 kg^{-1}, depending on the presumed production performances. Similar-scale production of PHB from glucose is estimated to cost US $3.8–4.2 kg^{-1}. In contrast to the comparable production costs for P(3HB-*co*-5 mol% 3HHx) and PHB, the life cycle inventories of energy consumption and carbon dioxide emissions favor the former product over the latter, reflecting smaller inventories and higher production yields of soybean oil compared with glucose. The life cycle inventories of energy consumption and carbon dioxide emissions of bio-based polymers are markedly lower than those of typical petrochemical polymers.

Fig. 3 Packaging products made from poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyhexanoate] (Nodax™) (photos from Isao Noda of P&G, USA)

5.1 Metabolic Engineering for PHBHHx Production

A. hydrophila 4AK4 produced PHBHHx with a stable HHx content ranging from 10 to 15% regardless of the growth conditions (Chen et al. 2001). *A. hydrophila* coded as CGMCC 0911 isolated from lake water was found to be able to synthesize PHBHHx consisting of 4–6 mol% HHx. The wild-type bacterium accumulated 49% PHBHHx containing 6 mol% HHx in terms of CDW when grown on lauric acid for 48 h. When *A. hydrophila* CGMCC 0911 expressed the acyl-CoA dehydrogenase gene (*yafH*) of *E. coli*, the recombinant strain accumulated 47% PHBHHx with 17.4 mol% HHx (Lu et al. 2004). It was also found that the presence of changing glucose concentration in the culture changed the HHx content both in wild-type and in recombinant *A. hydrophila* CGMCC 0911, from 8.8 to 35.6 mol% HHx. When the glucose concentration exceeded 10 g l^{-1}, cell growth, PHA content, and the mole percentages of HHx in PHBHHx were significantly reduced. Therefore, we could manipulate the PHBHHx contents by changing the strain's metabolic pathways or by changing the growth conditions.

An attempt was also made to manipulate PHBHHx production in *E. coli* (Lu et al. 2003). The acyl-CoA dehydrogenase gene (*yafH*) of *E. coli* was expressed together

with the PHA synthase gene ($phaC_{Ac}$) and the (*R*)-enoyl-CoA hydratase gene ($phaJ_{Ac}$) from *A. caviae*. The expression plasmids were introduced into *E. coli* JM109, DH5alpha, and XL1-blue, respectively. Compared with the strains harboring only $phaC_{Ac}$ and $phaJ_{Ac}$, all recombinant *E. coli* harboring *yafH*, $phaC_{Ac,}$ and $phaJ_{Ac}$ accumulated at least 4 times more PHBHHx. The CDWs produced by all recombinants containing *yafH* were also considerably higher than those without *yafH*. It appeared that the overexpression of the acyl-CoA dehydrogenase gene (*yafH*) enhanced the supply of enoyl-CoA, which is the substrate of (*R*)-enoyl-CoA hydratase. With the enhanced precursor supply, the recombinants accumulated more PHBHHx.

Genes *phbA* and *phbB* encoding β-ketothiolase and acetoacetyl-CoA reductase in *R. eutropha* were introduced into *A. hydrophila* 4AK4 (Qiu et al. 2004). When gluconate was used as a cosubstrate of dodecanoate, the recombinant produced PHBHHx containing 3–12 mol% 3HHx, depending on the gluconate concentration in the medium. The *Vitreoscilla* hemoglobin gene, *vgb*, was also introduced into the above-mentioned recombinant, resulting in improved PHBHHx content from 38 to 48 wt% in a shake-flask study. Fermentor studies also showed that increased gluconate concentration in a medium containing dodecanoate promoted the recombinant harboring *phbA* and *phbB* to incorporate more poly[(*R*)-3-hydroxybutyrate] units into PHBHHx, resulting in a reduced 3HHx fraction. Recombinant *A. hydrophila* harboring *phbA, phbB*, and *vgb* genes demonstrated better PHBHHx productivity and higher conversion efficiency from dodecanoate to PHBHHx than those of the recombinant without *vgb* in a fermentation study. Combined with the robust growth property and simple growth requirement, *A. hydrophila* 4AK4 appeared to be a useful organism for metabolic engineering toward enhanced production of PHBHHx.

6 Industrial Production of mcl Copolymers of (*R*)-3-Hydroxyalkanoates

Pseudomonas oleovorans forms mcl PHA most effectively at growth rates below the maximum specific growth rate (Weusthuis et al. 2002; Brandl et al. 1988). Under adequate conditions, PHA accumulates at rates in inclusion bodies in cells up to levels higher than half of the cell mass, which is a time-consuming process (Jung et al. 2001). For PHA production, Jung et al. (2001) developed a two-stage continuous cultivation system with two fermenters connected in series as a potentially useful system. It offers production of cells at a specific growth rate in a first compartment under conditions that lead cells to generate PHA at higher rates in a second compartment, with a relatively long residence time. Transient-state experiments allowed investigation of two different media of various nutrient concentrations, namely dilution 1 and dilution 2, over a wide dilution rate range at high resolution in time-saving experiments. With all culture parameters optimized, a volumetric PHA productivity of 1.06 $g l^{-1} h^{-1}$ was obtained. Under these conditions, *P. oleovorans* contained 63% (dry weight) PHA in the effluent of the second fermenter. This is the highest PHA productivity and PHA content reported thus far for *P. oleovorans* cultures grown on alkanes.

7 Conclusion and Future Perspectives

PHA industrial production is the result of many years of efforts by scientists and engineers working in the field. The critical point for large-scale applications of PHA as bulk materials is the cost. Currently, worldwide efforts are focusing on lowering the PHA production cost. These include the development of the following technologies (Table 2): high cell density growth within a short period of time, controllable lysis of PHA-containing cells, large PHA granules for easy separation,

Table 2 Future development for PHA production technology and applications (Chen 2009)

Development	Technology employed and other issues
To lower PHA production cost	
High cell density growth within a short period of time	Manipulate quorum-sensing pathways. Process control, pathway manipulation
Controllable lysis of PHA-containing cells	Genes related to cell lysis, e.g., lamda lysis factor and lysozyme
Large PHA granules for easy separation	Deletion of phasin led to large in vivo PHA granules
Super-high PHA content in cell dry weight	Delete PhaZ or overexpression of PHA synthesis genes, including PhaF
Microaerobic PHA production	Employ an anaerobic promoter and/or facultative anaerobic bacteria or other technology, including synthetic biology, that turns aerobic processes into microaerobic processes
Simple carbon sources for scl and mcl copolymers	Application of low specificity PHA synthase, and construction of pathways to supply mcl PHA monomers from non-fatty acid oxidation pathways
Enhanced substrates to increase PHA transformation efficiency	Delete pathways that affect PHA synthesis, or employ a minimum genome containing cells with an inserted PHA pathway
Inorganic extraction and purification	Apply to over 90% of PHA containing cells
Mixed cultures without sterilization	Employ a feast and famine selection process to find a robust PHA production strain
Continuous fermentation	The use of robust PHA production strains, better under a nonsterilization condition
Controllable PHA molecular weight	The manipulation of the N-terminus of PHA synthase
The use of PHA monomer methyl esters as a biofuel or fuel additives	The development of low-cost technology for production of low-cost PHA biofuels or fuel additives
Plants as PHA production machines	Plant molecular biology
PHA with special properties	
Novel PHA with unique properties	The uses of low specificity PHA synthases for production of PHA with functional groups for chemical modifications, and PHA with controllable compositions, including block PHA copolymers
Controllable PHA compositions	
Ultrahigh PHA molecular weights	The use of special strain and mutated PHA synthases
Block copolymerization of PHA	The making of PHA diols and the block copolymerization with other polymers

(continued)

Table 2 (continued)

Development	Technology employed and other issues
PHA monomers as building blocks for new polymers	Copolymerization of PHA monomers with other monomers, including lactides, for formation of novel copolymers with new properties
To develop low-cost PHA applications	
New PHA processing technology	Cost-effective processing of PHA as plastic packaging materials
PHA blending with low-cost materials	PHA blending with starch, cellulose, etc.
High-value-added applications	
PHA as bioimplant materials	Further improve the in vivo controllable degradation of PHA implants, seek FDA approval for clinical applications
PHA as tissue engineering materials	Develop 3D scaffolds as tissue engineering matrices
PHA monomers and oligomers as nutritional and energy supplements	Understand the mechanism behind the Ca^{2+} stimulation effect of oligomers and monomers of PHA
PHA monomers as drugs	Study of other non-3HB monomers as drug candidates
PHA monomers as fine chemicals	Chiral monomers should be exploited as intermediates for chiral synthesis
PHA as mart materials	Tailor-made PHA as shape-memory or temperature-sensitive gels

scl short chain length, *mcl* medium chain length, *3HB* poly[(*R*)-3-hydroxybutyrate]

super-high PHA content in the CDW; microaerobic PHA production, simple carbon sources for formation of short-chain-length and mcl PHA copolymers, enhanced substrates for increased PHA transformation efficiency, inorganic extraction and purification, mixed cultures without sterilization, continuous fermentation, and controllable PHA molecular weight (Chen 2009). If the PHA production cost can be brought down to that of conventional plastics, PHA application could be as wide as that of plastics.

At the same time, both low- and high-value-added applications should be developed to widen the value of PHA (Table 2). For low-value-added applications, PHA is mainly a target for environmentally friendly packaging purposes. For this application, a large amount of PHA is needed. For high-value-added applications, PHA can be exploited as bioimplant materials, tissue engineering materials (Chen and Wu 2005a), and smart materials Monomers and oligomers of PHA can be used as nutritional and energy supplements for animals or even humans, and they can also be used as drugs or fine chemicals (Yao et al. 2008; Chen and Wu 2005b).

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Unusual PHA Biosynthesis

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Contents

Abstract Unusual polyhydroxyalkanoates (UnPHAs) constitute a particular group of polyoxo(thio)esters belonging to the PHA family, which are tailored with uncommon monomers. Thus, unusual PHAs include (1) polyhydroxyalkanoates (PHAs) of microbial origin that have been synthesized either from natural monomers bearing different chemical functions, or from chemical derivatives of the natural ones and (2) PHAs obtained either by chemical synthesis or by physical modifications of naturally occurring polymers. Regarding their chemical structure, UnPHAs can be grouped in four different classes. Class 1 includes PHAs whose lateral chains contain double or triple bounds or/and different functional groups (methyl, methoxy, ethoxy, acetoxy, hydroxyl, epoxy, carbonyl, cyano, phenyl, nitrophenyl, phenoxy, cyanophenoxy, benzoyl, halogen atoms, etc.). Classes 2 and

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3 have been established regarding the nature of the PHA backbone; whereas class 2 includes PHAs in which the length of the monomer participating in the oxoester linkage has been modified (the hydroxyl group to be esterified is not located at C-3), class 3 groups those polymers in which some oxoester linkages have been replaced by thioester functions (thioester-containing PHAs). Finally, class 4 includes those PHAs that have been manipulated chemically or physically. In this chapter we shall describe the chemical structure of unusual PHAs belonging to these four classes; we shall analyse their biosynthetic particularities (if any), and we shall discuss some of their characteristics and biotechnological applications.

1 Introduction

Plastics are the most widely used synthetic polymers (Lee 1996; Steinbüchel and Füchstenbusch 1998; Di Lorenzo and Silvestre 1999; Scott 1999, 2000). They are basic constituents of most of the materials surrounding us, and it is well-nigh impossible to imagine contemporary human life without these products (Madison and Huisman 1999; Kalia et al. 2003, 2007). Their abundance, together with the exponential growth of the human population, has led to the accumulation of a huge amount of contaminants manufactured with plastics in different areas of our planet, even in specially protected areas, leading to severe waste management problems. Thus, mountains, coastal areas (Fig. 1), rivers, lakes and even desert areas are usually contaminated with conventional plastics (those of petrochemical origin) because these compounds are resistant to decomposition (they take decades to become fully

Fig. 1 Fully contaminated area on the coast in the southwest of Spain

mineralized – recalcitrant products; Scott 1997; Witt et al. 2001; De Lorenzo 2008). In sum, the presence of these compounds in aerial, aquatic or terrestrial ecosystems is having a high impact on the fine equilibrium that controls the dynamic relationships among the different populations belonging to these habitats (Scott 1994, 1997, 2000; Witt et al. 2001). Moreover, their negative influence in the environment is aggravated because during the processes of elaboration (chemical synthesis) and degradation highly toxic molecules are usually generated (Lee et al. 1991; Swift 1993; Lee 1996; Scott 2000; Witt et al. 2001). Furthermore, taking into account that most of these polymers are of petrochemical origin (fossil fuel derivatives), and that such reserves are finite, we urgently need alternative materials to substitute them. Additionally, the current political limitations imposed by different governments on the chemical industry to combat the greenhouse effect have increased interest in the development of new materials that can replace traditional plastics (Anderson and Dawes 1990; Witholt and Kessler 1999; Luengo et al. 2003; Lenz and Marchessault 2005; Prieto et al. 2007; Yu and Chen 2008). For these reasons, researchers have focused their endeavours on the development of biomaterials (natural products that are synthesized and catabolized by different organisms and that have broad biotechnological applications) to generate fully biodegradable compounds with potential industrial applicability (Angelova and Hunkeler 1999; Scott 2000; Kim and Lenz 2001; Kim and Rhee 2003; Abou-Zeid et al. 2004). Starch, cellulose, polypropiolactone, poly(ε-caprolactone), poly(L-lactide) (PLA), polyethylene (PE); poly(butylene succinate), poly(ethylene succinate), poly(*p*-phenylene) and polyhydroxyalkanoates, particularly PHAs, are currently being tested as substitutes for plastics (Anderson and Dawes 1990; Huisman et al. 1991; Eggink et al. 1995; Lee 1996; Mittendorf et al. 1998; Madison and Huisman 1999; Pötter et al. 2004; Stubbe et al. 2005; Lenz and Marchessault 2005; Prieto et al. 2007; Wang and Yu 2007; Wang et al. 2007).

One of the most important limitations that have hampered the use of PHAs as commodity materials is the high production cost of PHAs in comparison with conventional plastics (Byrom 1987; Choi and Lee 1997; Witholt and Kessler 1999; Salehizadeh and Van Loosdrecht 2004; Sudesh et al. 2007). Accordingly, the design of different strategies focused on reducing the cost of PHA production has attracted the interest of scientists. Among these approaches, the more interesting ones have been (1) the isolation of genetically manipulated strains (collection of mutants or recombinant strains) able to achieve both a high substrate conversion rate and a close packing of PHA granules inside the host cell (Langenbach et al. 1997; Qi et al. 1997; García et al. 1999; Olivera et al. 2001a, b; Aldor and Keasling 2003; Ren et al. 2005a, b; Sandoval et al. 2007; Kalia et al. 2007; Ouyang et al. 2007; Tsuge et al. 2003; Nomura et al. 2005; Taguchi et al. 2003; Nikel et al. 2006; Sujatha and Shenbagarathai 2006); (2) study of the physical and chemical parameters that affect the fermentation process to improve both the rate of growth and the yield of PHA production (Hong et al. 2000; Ahn et al. 2000; Durner et al. 2001; Lee and Choi 2001; Chanprateep et al. 2002; Park et al. 2002; Lawrence et al. 2005); (3) analysis of the conditions required for the yields of recovery during the purification process to be increased (De Koning et al. 1997; De Koning and Witholt 1997; Sun et al. 2007); (4) the selection of cheaper substrates (or industrial wastes)

that can be used as PHA precursors (Braunegg et al. 1998; Kellerhals et al. 1999; Lee et al. 1999b; Kim et al. 2000; Kocer et al. 2003; Li et al. 2007); and (5) the use of yeasts (Breuer et al. 2002; Poirier et al. 2002; Terentiev et al. 2004; Zhang et al. 2006) or transgenic plants (van der Leij and Witholt 1995; Williams and Peoples 1996; Poirier et al. 1995; Poirier 1999, 2002; Snell and Peoples 2002; Sudesh et al. 2002) for the mass production of these polyesters. Moreover, clarification of the role played by the different proteins involved in the synthesis of PHAs has allowed the reproduction of the main enzymatic steps in vitro, leading to the production of new or modified polymers (usually high molecular weight polymers) through quite effective polymerization reactions (Qi et al. 2000; Su et al. 2000; Stubbe et al. 2005; Gorke et al. 2007).

Additionally, the chemical synthesis of PHAs as well as the development of homogeneous or heterogeneous blends have contributed to the collection of new polymers with improved or modified properties (Arkin and Hazer 2002; Yu et al. 2006; Hazer and Steinbüchel 2007).

In this review we shall describe the structure of PHAs with unusual composition, we shall discuss the specificity of the enzymes involved in their polymerization [using as models (*R*)-3-hydroxy-*n*-phenylalkanoates] and, finally, we shall revise their biotechnological applications.

2 Naturally Occurring PHAs

PHAs are polyoxoesters (see Fig. 2) produced by a plethora of micro-organisms (some Archaea and certain Gram-positive and Gram-negative bacteria; Steinbüchel and Füchstenbusch 1998; Madison and Huisman 1999; Rothermich et al. 2000;

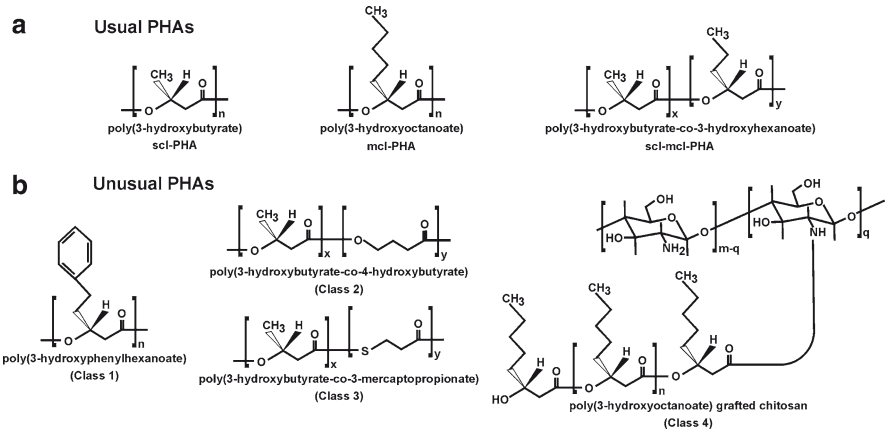

Fig. 2 Chemical structure of different types of usual (**a**) and unusual (**b**) polyhydroxyalkanoates (PHAs)

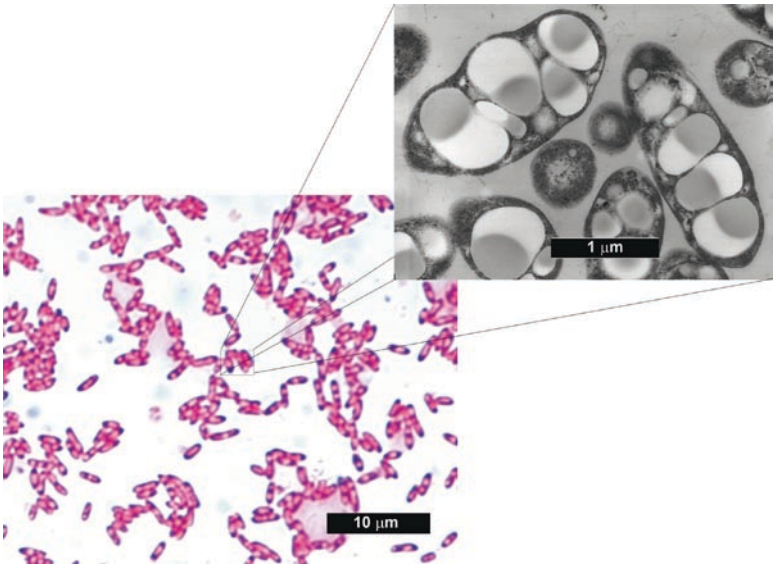

Fig. 3 Optical and electronic photographs of a PHA-overproducer mutant of *Pseudomonas putida* U cultured in a chemically defined medium containing 8-phenyloctanoic acid (10 mM) as a poly(3-hydroxyphenylalkanoate) precursor, and L-glutamate (10 mM) as the carbon source

Stubbe et al. 2005; Luengo et al. 2003; Prieto et al. 2007; Valappil et al. 2007) when cultured under different nutrient and environmental conditions (usually under N, P, S, O or Mg limitation or when an excess of polymer precursors exists; Schlegel et al. 1961). Conversely, other microbes (i.e. *Pseudomonas putida*, *Sphaerotilus natans*, *Bacillus mycoides*, *Azotobacter vinelandii*, *Alcaligenes latus*) accumulate PHAs even in the presence of high concentrations of nutrients (Page and Knosp 1989; Hänggi 1990; Huisman et al. 1992; Takeda et al. 1995; Wang and Lee 1997; García et al. 1999; Borah et al. 2002), suggesting the existence of differences in the global regulatory mechanisms triggering PHA accumulation (Kessler and Witholt 2001).

PHA producers accumulate these polyesters intracellularly as mobile, amorphous, liquid granules that can be observed as light-refracting deposits (Fig. 3) or as electrolucent bodies that, when overproduced, can dramatically alter both the size and the morphology of these bacteria (Stockdale et al. 1968; Jurasek et al. 2001; Luengo et al. 2003, 2004; Peters and Rehm 2005; Peters et al. 2007; Sandoval et al. 2007; Figs. 4, 5). Although the main function of these storage polymers is to serve as an energy source (Anderson et al. 1990; Sudesh et al. 2007), they also have other important physiological functions. PHA accumulation seems to be involved in other secondary functions such as stress tolerance, biofilm formation and the maintenance of the redox state (Pham et al. 2004; Ayub et al. 2009). It is to be expected that new functions for these interesting polymers will be reported in the near future. Further information about this issue can be found in Castro-Sowinski et al. (2009).

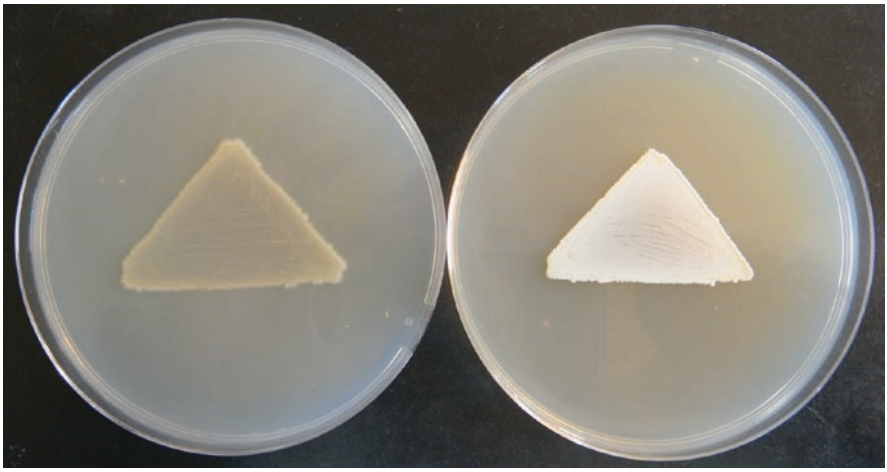

Fig. 4 Morphological aspect of cultures of *P. putida* U (*left*) and a PHA-overproducer mutant (*right*) when grown on a chemically defined solid medium containing 4-hydroxyphenylacetic acid as the carbon source and 7-phenylheptanoic acid as the source of PHA monomers

2.1 Classification of Naturally Occurring PHAs

Because of the broad chemical variability of PHAs (more than 150 different PHAs have been described; Steinbüchel and Valentin 1995), different criteria based on (1) their frequency of appearance, (2) chemical structure and monomer size (side-chain length), (3) biosynthetic origin, (4) types of monomers present in the polymer and (5) types of polyesters accumulated by the producer microbe have been applied to group them (Luengo et al. 2003).

According to their frequency of appearance in nature, PHAs can be classified as *common PHAs* and *uncommon PHAs*. Common PHAs are those PHAs usually found as storage materials in many different microbes. This group includes polymers tailored from monomers [i.e. (*R*)-3-hydroxypropionate, (*R*)-3-hydroxybutyrate; (*R*)-3-hydroxyvalerate; (*R*)-3-hydroxyhexanoate, (*R*)-3-hydroxyoctanoate; (*R*)-3-hydroxydecanoate and (*R*)-3-hydroxydodecanoate, or combinations thereof) which are obtained from different carbon sources (sugars, alkanes, aliphatic fatty acids, triacylglycerols, etc.) through general pathways (usually fatty acid synthesis and fatty acid β-oxidation) involving the synthesis of (*R*)-3-hydroxyacyl-CoAs. In a final step, these thioesters are polymerized to poly[(*R*)-3-hydroxyalkanoate]s, with a CoA molecule being released for each monomer residue incorporated into the polymer (Steinbüchel and Füchstenbusch 1998; Rehm et al. 1998; de Roo et al. 2000; Olivera et al. 2001a, b; Kessler and Witholt 2001; Luengo et al. 2003, 2004; Velázquez et al. 2007).

In contrast, uncommon PHAs are those polyesters rarely found in nature and that are constituted (1) by natural monomers with an unusual chemical structure (i.e. 4-hydroxyalkanoic acids, 5-hydroxyalkanoic acids and 6-hydroxyalkanoic acids)

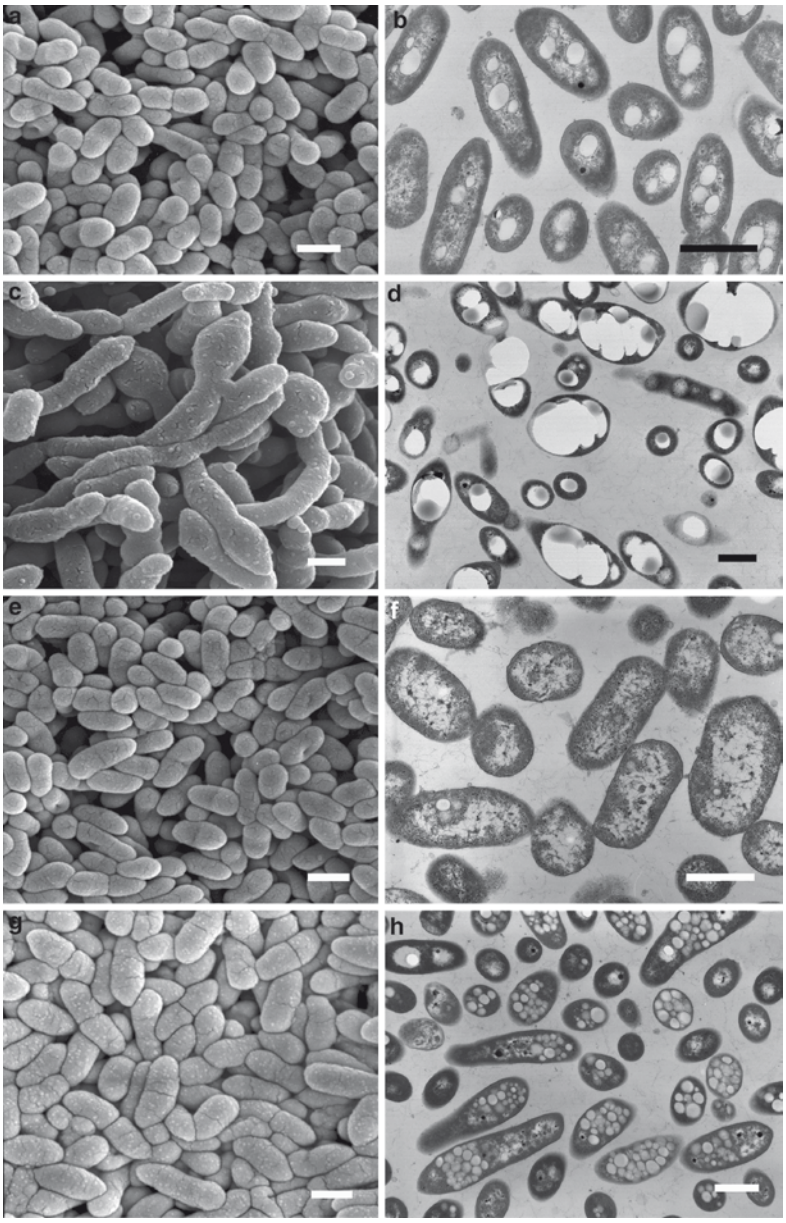

Fig. 5 Scanning (**a**, **c**, **e**, **g**) and transmission (**b**, **d**, **f**, **h**) microphotographs of *P. putida* U (**a**, **b**), *P. putida* U Δ*fadB* (**c**, **d**), *P. putida* U Δ*fadB phaZ* (**e**, **f**) and *P. putida* U Δ*pha phaC1* (**g**, **h**) cultured in a chemically defined medium containing 4-hydroxyphenylacetic acid as the carbon source and 7-phenylheptanoic acid as the source of PHA monomers. The *bar* represents 1 μm. *P. putida* U Δ*fadB* is a PHA-overproducer mutant of *P. putida* U in which the β-oxidation genes have been deleted, *P. putida* U Δ*fadB phaZ* is a strain derived from *P. putida* U Δ*fadB* that overexpresses the depolymerase gene (*phaZ*) and *P. putida* U Δ*pha phaC1* is a mutant in which the *pha* cluster was deleted and that expressed the gene encoding the polymerase C1 (*phaC1*) in *trans*

that are synthesized by a microbial species or even by only a few strains belonging to a particular species (Saito and Doi 1994; Schmack et al. 1998; Choi et al. 1999; Amirul et al. 2008) or (2) by unnatural monomers (generally obtained by chemical synthesis – xenobiotics) that can be taken up by the PHA-producer microbe, activated to their CoA thioesters, and used as substrates by the PHA polymerases. This second group includes PHAs whose lateral chains contain double or triple bonds or/and different functional groups (Doi et al. 1987; Fritzsche et al. 1990a, b, c; Eggink et al. 1995; Kim et al. 1998; García et al. 1999; Lee et al. 1999a, 2000a; Imamura et al. 2001; Kang et al. 2001; Kim et al. 2001a; Luengo et al. 2003; Trainer and Charles 2006).

According to the monomer size (PHA side-chain length), two different types of PHAs can also be distinguished. One of them, short-chain-length (scl) PHAs (also referred as poly[(*R*)-3-hydroxybutyrate], PHB) consists of poly[(*R*)-hydroxyalkanoate]s containing monomers whose carbon length ranges from C_3 to C_5. These types of polyester are produced by different bacteria (*A. latus*, *Bacillus cereus*, *Pseudomonas pseudoflava – Hydrogenophaga pseudoflava*, *Pseudomonas cepacia*, *Micrococcus halodenitrificans*, *Azotobacter* sp., *Rhodospirillum rubrum*, *Ectothiorhodospira shaposhnikovii* and *Cupriavidus necator)*, with *C. necator* (formerly known as *Wautersia eutropha*, *Ralstonia eutropha* or *Alcaligenes eutrophus*) being the paradigmatic example (Nakamura et al. 1991; Bear et al. 1997; Ballistreri et al. 1999; Babel et al. 2001; Pettinari et al. 2001; Zhang et al. 2004; Lenz and Marchessault 2005; Stubbe et al. 2005; Hazer and Steinbüchel 2007). The second type, called medium-chain-length (mcl) PHAs, are mainly produced by fluorescent pseudomonads (Timm and Steinbüchel 1990). These polymers are tailored from aliphatic (*R*)-hydroxyalkanoates whose carbon length ranges between six and 14 carbon atoms and are synthesized when the producer microbe is cultured in media containing *n*-alkanoates or their precursors (i.e. alkanes; Gross et al. 1989; Madison and Huisman 1999; Witholt and Kessler 1999). Very often, before they are polymerized, *n*-alkanoates are β-oxidized to monomers containing two fewer carbon atoms.

In relation to the biosynthetic origin, PHAs can be included in three different groups: (1) *natural PHAs* – those produced by micro-organisms from general metabolites (i.e. PHBs and PHAs); (2) *semisynthetic PHAs* – those that contain monomers that cannot be synthesized by the producer microbe and that must therefore be supplied to the culture broth (i.e. PHAs containing aromatic or other functionalized monomers) and (3) *synthetic PHAs* – polyesters that are obtained by full chemical synthesis or by chemical modification of natural or semisynthetic PHAs (i.e. synthetic thermoplastic polymers; Luengo et al. 2003; Foster 2007). The two first groups – natural and semisynthetic PHAs – are produced by different microbes using the biosynthetic enzymes encoded by the genes belonging to either the cluster *phaCBAPZ* (PHB) or the cluster *phaC1ZC2DFI* (PHA) (Fig. 6; Kraak et al. 1997; Madison and Huisman 1999; Ohura et al. 1999; Prieto et al. 1999; Klinke et al. 2000; Steinbüchel and Hein 2001; Luengo et al. 2004; Stubbe et al. 2005; Prieto et al. 2007; Sandoval et al. 2007). In contrast, the third group (synthetic PHAs) cannot be biosynthesized because their monomers (or at least some of them) either are toxic or cannot be taken up from the culture by the PHA-producer microbe.

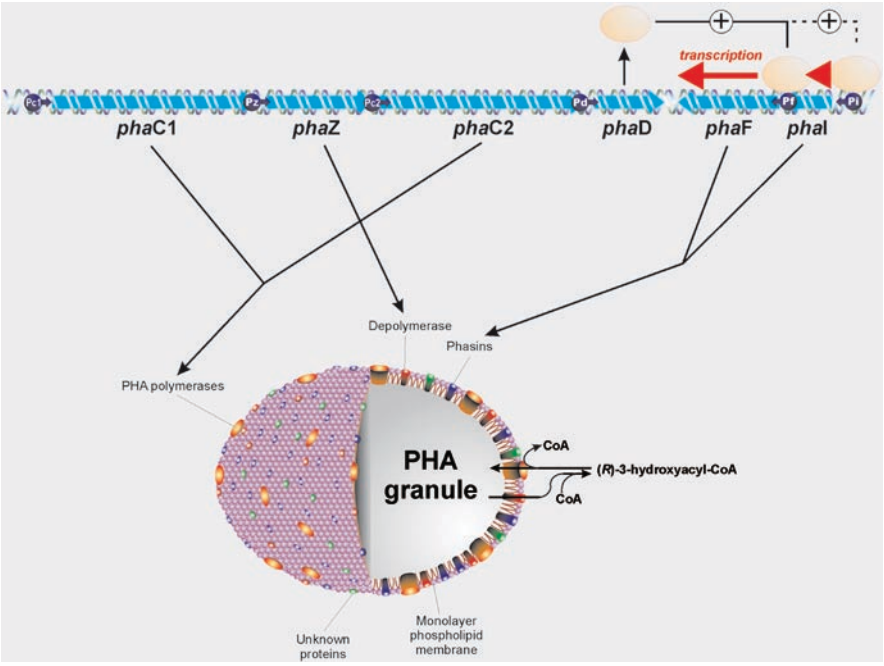

Fig. 6 Genetic organization of the cluster *phaC1ZC2DFI* involved in the synthesis of medium-chain-length PHAs. The putative functions of some medium-chain-length PHA proteins are indicated

Depending on the *types of monomers found in the polymer*, PHAs are classified as *homopolymers* (only one type of monomer is present in the polymer) or *copolymers* (so-called heteropolymers) if they are built from different monomers (Madison and Huisman 1999; Olivera et al. 2001a; Luengo et al. 2003). It is worth noting that many PHAs characteristics, including physical and mechanical properties, depend on the molar percentage of the monomers present in each polymer; such characteristics are also quite different when homopolymers tailored with different monomers are compared (Matsusaki et al. 2000; Chen et al. 2006). scl-PHAs (C_3–C_5 PHBs) are thermoplastics with a high degree of crystallinity forming stiff crystalline materials that are brittle and cannot be extended without breakage. However, mcl-PHAs are elastic or sticky materials with a low degree of crystallinity and a low melting temperature (Nakamura et al. 1991; De Koning 1995; Kim et al. 2001a; Zinn et al. 2001; Lenz and Marchessault 2005).

Although most PHA-synthesizing bacteria accumulate either scl-PHAs or mcl-PHAs, microbial copolymers consisting of scl and/or mcl hydroxyalkanoates (hybrid PHAs, see below) have been reported in some bacterial strains, in particular genetically engineered microbes (Schmack et al. 1998; Steinbüchel and Hein 2001; Lu et al. 2004; Chen et al. 2006; Wei et al. 2009).

2.2 General Properties and Biotechnological Applications of Naturally Occurring PHAs

From a biotechnological point of view, PHAs have two important properties conferring them a considerable advantage with respect to other synthetic products: they are *biodegradable* and *biocompatible* polymers. The term 'biodegradable' is applied to any polymer that is readily degraded to CO_2, water and biomass, suggesting that they can be assimilated by many microbial species, thereby preventing their environmental accumulation (Matavulj and Molitoris 1992; Foster et al. 1995; Jendrossek and Handrick 2002; Witt et al. 2001; Kim and Rhee 2003; Tokiwa and Calabia 2004). The second property (biocompatibility) indicates that PHAs do not cause a toxic effect when applied to a broad range of hosts. PHAs are immunologically inert and are only slowly degraded in human tissues (Verlinden et al. 2007). Furthermore, traces of PHB have been found within mammalian cells membranes (Reusch 2000), and its precursor [(*R*)-3-hydroxybutyrate] is present in the blood in the millimolar concentration range (Wiggam et al. 1997). All these reasons would justify the use of PHBs as biomaterials for medical devices (Zinn et al. 2001; Williams and Martin 2002; Foster 2007; Ji et al. 2008). However, their high crystallinity, brittle nature and the fact that they are fairly rigid materials that start to be degraded (ester pyrolysis) at a temperature slightly above the melting point have prevented their use (De Koning 1995; Kim and Lenz 2001; Lenz and Marchessault 2005). Conversely, mcl-PHAs (which are semicrystalline thermoplastic elastomers) seem to be better biomaterials for biomedical applications (Zinn et al. 2001). Unfortunately, PHAs containing monomers with a carbon length ranging from six to ten carbon atoms have low transition temperatures and lower crystallinity than PHBs (Lenz and Marchessault 2005).

Taking into account the advantages and the limitations of both kind of polymers, different approaches based on biochemical, genetic and bioprocess innovation have attempted to obtain hybrid scl–mcl copolymers {scl C_4–C_5/C_6 copolymers, poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyvalerate]/poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyhexanoate; Fig. 2} to generate new PHAs with different or improved physiochemical properties and broader biotechnological applications (Lee et al. 2000b; Lu et al. 2004; Ramsay et al. 1990; Hänggi 1995; Lenz and Marchessault 2005; Wei et al. 2009).

Different copolymers of PHB have been produced by following some of these procedures. The manipulation of bioprocessing systems when different bacteria (*A. latus*, *B. cereus*, *P. pseudoflava – H. pseudoflava*, *P. cepacia*, *M. halodenitrificans*, *Azotobacter* sp. and *C. necator*) were cultured, under nitrogen-limited conditions, in media containing glucose (or sucrose in the case of *A. latus*) and propionic acid (or other propionigenic carbon sources) led to the production of PHBs containing random units of (*R*)-3-hydroxyvalerate (Ramsay et al. 1990). These copolymers have reduced crystallinity and a low melting point, leading to improved flexibility, strength and easier processing (Findlay and White 1983). Furthermore, scl–mcl copolymers composed mostly of hydroxybutyrate (HB) monomers with a small amount of hydroxyhexanoate (HH) monomers have properties similar to those of

polypropylene (Abe and Doi 2002). This scl–mcl-PHA copolymer [P(HB–HH)] is a tough and flexible material (Lee et al. 2000a).

Genetic engineering has also contributed to the production of scl–mcl-PHA copolymers (Aldor and Keasling 2003). The expression of *pha* genes in *Escherichia coli* or in microbes other than natural producers has contributed to increasing the yields of the polymer synthesized as well as the monomer composition of the PHA produced by the wild type (Ahn et al. 2000; Lee et al. 2001; Park et al. 2001; van Wegen et al. 2001). Other genetic approaches that have also led to the collection of PHA-overproducer strains are those based on the isolation of mutants in which (1) the genes encoding β-oxidation enzymes (*fadA* and/or *fadB*), (2) the genes encoding enzymes belonging to the glyoxylic shunt or (3) the different *phaZ* (encoding scl-PHAs and mcl-PHAs depolymerases) have been deleted (García et al. 1999; Stubbe et al. 2005; Madison and Huisman 1999; Jendrossek and Handrick 2002; Nikel et al. 2006; Ouyang et al. 2007; Sandoval et al. 2007). Additionally, transformation of these mutants with the genes belonging to the *pha* cluster (in an isolated fashion or in tandem) have allowed the synthesis of a broad number of different polymers with new properties and interesting characteristics (Langenbach et al. 1997; Qi et al. 1997; Hang et al. 2002; Arias et al. 2008). PHA copolymers containing (*R*)-3-hydroxyhexanoic, (*R*)-3-hydroxyoctanoic and (*R*)-3-hydroxydecanoic acids were synthesized by recombinant mutants of *E. coli* (*fadB*$^{-}$) expressing the genes *phaC1* and *phaC2* from *Pseudomonas aeruginosa* and *Burkholderia caryophylli*, respectively. Moreover, when *E. coli* was transformed with genetic constructions carrying the *hbcT* gene from *Clostridium kluyveri* (encoding a 4-hydroxybutyric acid-CoA transferase) and the *phaC* gene from *C. necator*, different polymers were accumulated even though the genes *phaA* (encoding the β-ketothiolase which synthesizes acetoacetyl-CoA from acetyl-CoA) and *phaB* (encoding a NADPH oxidoreductase) were absent (Hein et al. 1997). Other authors have shown that the expression of the gene encoding a 3-ketoacyl–acyl carrier protein reductase (*fabG*) enhances the production of a PHA copolymer in a recombinant strain of *E. coli* JM109 (Nomura et al. 2005).

Moreover, technological advances in fermentation strategies and the application of new feeding programmes have also contributed to optimizing the yields of copolymer production in different microbes as well as to the production of other copolymers with new or modified structures (Ahn et al. 2000; Lee et al. 2001; Park et al. 2001; van Wegen et al. 2001; Oliveira et al. 2004; Henneke et al. 2005) and quite different physicochemical properties (ranging from brittle and crystalline polymers to flexible and elastomeric ones; Abraham et al. 2001; Olivera et al. 2001a; Steinbüchel 2001).

3 Unusual PHAs

As indicated already, over the past few decades many efforts has been made to obtain new microbial PHAs. The use of unusual sources of PHA precursors as well as the applications of genetic, biochemical, chemical or physical approaches for the

production of novel polyesters have allowed unconventional PHAs [henceforth indicated as unusual PHAs (UnPHAs)] to be obtained that could offer a real alternative to traditional plastics.

The term UnPHAs includes many different PHAs belonging to the different types indicated earlier (uncommon scl-/mcl-PHAs, semisynthetic/synthetic PHAs and homopolymer/copolymer PHAs) as well as others that have been obtained by physical modifications of the natural ones (i.e. blended polymers).

In sum, UnPHAs constitutes a particular group of polyoxo(thio)esters belonging to the PHA family which are tailored with uncommon monomers. UnPHAs include (1) PHAs of microbial origin that have been synthesized either from natural monomers bearing different chemical functions, or from chemical derivatives of the natural ones and (2) PHAs obtained either by chemical synthesis or by physical modifications of naturally occurring polymers. Regarding their chemical structure, UnPHAs can be grouped in four different classes. Class 1 includes PHAs whose lateral chains contain double or triple bounds or/and different functional groups (methyl, methoxy, ethoxy, acetoxy, hydroxyl, epoxy, carbonyl, cyano, phenyl, nitrophenyl, phenoxy, cyanophenoxy, benzoyl, halogen atoms, etc.). Classes 2 and 3 have been established regarding the nature of the PHA backbone; whereas class 2 includes PHAs in which the length of the monomer participating in the oxoester linkage has been modified (the hydroxyl group to be esterified is not located at C-3), class 3 groups those polymers in which some oxoester linkages have been replaced by thioester functions (thioester-containing PHAs). Finally, class 4 includes those PHAs that have been manipulated chemically or physically.

3.1 UnPHAs Synthesized by Micro-Organisms

The milestone in the obtaining biologically produced PHAs came from the single observation that by changing the carbon feedstock, one can tailor new PHA. Thus, the metabolic flexibility of certain microbes and the non-specificity of the enzymes involved in the polymerization of PHA monomers have been exploited in attempts to obtain new plastics that cannot be found in nature.

Most natural UnPHAs (polymers belonging to classes 1, 2 and 3) are PHAs belonging to the mcl-PHA group. However, a few groups of bacteria (*Burkholderia cepacia*, *C. necator, Cupriavidus* sp., *Comamonas acidovorans*, *A. latus*, *H. pseudoflava*, and some recombinant strains – *E. coli* and *P. putida*) simultaneously accumulate PHB and different scl-UnPHAs {a homopolymer of poly[(*R*)-3-hydroxy-4-pentenoate] in *B. cepacia*; poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-mercaptobutyrate] in *C. necator*; polyesters containing 4-hydroxyvalerate in some *E. coli* and *P. putida* recombinant strains; and poly[(*R*)-3-hydroxybutyrate-*co*-4-hydroxybutyrate] in the other bacteria} (Saito and Doi 1994; Saito et al. 1996; Valentin and Dennis 1997; Schmack et al. 1998; Choi et al. 1999; Valentin et al. 1999; de Andrade Rodrigues et al. 2000; Rodrigues et al. 2000; Gorenflo et al. 2001; Lütke-Eversloh et al. 2001a, b; Lee et al. 2004; Kocer et al. 2003; Kim et al. 2005b; Amirul et al. 2008).

The existence of a scarce number of scl-PHAs containing unusual monomers (scl-UnPHAs) suggests that the enzyme responsible for PHB polymerization (PhaC) has a narrower substrate specificity than others (PhaC1 and PhaC2, see later) involved in the polymerization of mcl-PHAs. Accordingly, henceforth when speaking of UnPHAs we shall be referring to PHAs in which the aliphatic part of the chain (without the functional group) ranges between six and 12 carbon atoms.

3.1.1 UnPHAs Belonging to Class 1

UnPHAs bearing double or triple bound as well as others containing different functional groups in the side chains (see before) are synthesized by some organisms, mainly pseudomonads belonging to the ribosomal RNA I group, when cultured in media containing substrates that can be used either as UnPHA monomers or as metabolic precursors of these monomers (Steinbüchel and Valentin 1995). These special kinds of PHAs are very interesting since the presence of unsaturations or/and functional groups in their monomers usually modifies the physical properties and the characteristics of the polymers. Furthermore, some of these functional groups can be modified chemically, broadening the number of UnPHAs that can be synthesized biologically (Hazer and Steinbüchel 2007).

Two kinds of UnPHAs can be obtained depending on the whether the source of the monomer can support bacterial growth or not. The use of UnPHA precursors that can serve as an energy source has usually led to the production of fully functionalized homopolymers or copolymers (García et al. 1999). However, when such precursors are added to the culture broth together with molecules that can be also used as a source of monomers (i.e. *n*-alkanoic acids), only copolymers containing a lower percentage of functionalized monomers are synthesized (Abraham et al. 2001; Olivera et al. 2001a; Lee et al. 2001; Park et al. 2001; van Wegen et al. 2001; Henneke et al. 2005).

Conversely, the use of UnPHA precursors that cannot be degraded (there is no catabolic pathway for their assimilation) always requires the presence of an additional carbon source to support microbial growth. In such cases, taking into account that these special monomers have to be polymerized intracellularly, the producer microbe must necessarily contain (1) the transport systems required to ensure their being taken up from the culture, (2) the acyl-CoA ligases needed to activate these molecules to CoA thioesters and, finally, (3) a β-oxidation pathway that will generate the hydroxyacyl-CoA derivatives used as substrates by the PHA polymerases.

UnPHAs Containing Unsaturated or Functionalized Monomers

Unsaturated PHAs

UnPHAs containing unsaturated monomers have been obtained from different bacteria (*P. aeruginosa*, *P. putida*, *Pseudomonas oleovorans* and *Pseudomonas resinovorans*) when cultured in media containing *n*-alkenes, *n*-alkynes, unsaturated

fatty acids (undecenoic, undecynoic, oleic or linoleic acids), triglycerides and oils from different origins (animal or plant; Ashby and Foglia 1998).

The first description of the existence of PHAs bearing unsaturated monomers was made from a culture of *P. oleovorans* grown on either 1-alkenes or in minimal medium containing 7-octenoic acid, 10-undecenoic acid or mixtures of 10-undecenoic acid and either nonanoic acid or octanoic acid (Lageveen et al. 1988). These cultures accumulated random PHAs containing repeating units with terminal alkene substituents at position 3. Furthermore, PHAs obtained from *P. oleovorans* or *P. putida* grown with 10-undecenoic acid as the sole carbon source contain different olefinic monomers [(*R*)-3-hydroxy-10-undecenoate, (*R*)-3-hydroxy-8-nonenoate or (*R*)-3-hydroxy-6-heptenoate] at their terminal positions (Kim et al. 1995b, 2000). When *P. putida* was cultured in a medium containing oleic acid, PHAs bearing an unsaturated monomer [(*R*)-3-hydroxy-5-*cis*-tetradecenoate] were obtained. However, when the carbon source was linoleic acid, PHAs containing (*R*)-3-hydroxy-6-*cis*-dodecenoate and (*R*)-3-hydroxy-5-*cis*-8-*cis*-tetradecadienoate were obtained (De Waard et al. 1993). Similar polymers were obtained from *P. aeruginosa* when cultured in media containing oleic acid. In this case, the polymer contains (*R*)-3-hydroxy-*cis*-5-tetradecenoate as a repeating unit (Ballistreri et al. 2001).

The accumulation of highly unsaturated PHAs has also been reported in *P. resinovorans* when cultured in media containing soybean oil. In this case, PHAs containing several olefinic monomers [(*R*)-3-hydroxydecenoate, (*R*)-3-hydroxydodecadienoate, (*R*)-3-hydroxytetradecatrienoate and (*R*)-3-hydroxyhexadecadienoate] were accumulated. In contrast, when the same strain was cultured in media containing coconut oil, only saturated PHAs were synthesized (Ashby and Foglia 1998). These data suggest that the PHA composition (repeat units) is directly related (at least in this strain) to the source of the monomers present in the culture, and they indicate that the monomers are generated throughout β-oxidation. However, when *P. putida* KT2442 was grown in a medium containing glucose, fructose or glycerol as the sole carbon source, a PHA similar to that isolated from *P. resinovorans* cultured in soybean oil (see above) was found. This UnPHA contained (*R*)-3-hydroxydecanoate as the main monomer; however, another six additional monomers [(*R*)-3-hydroxyhexanoate, (*R*)-3-hydroxyoctanoate, (*R*)-3-hydroxydodecanoate, (*R*)-3-hydroxydodecenoate, (*R*)-3-hydroxytetradecanoate and (*R*)-3-hydroxytetradecenoate] were also found. These data suggest that in *P. putida* KT2442, unlike what was observed in *P. resinovorans*, the monomers polymerized, both saturated and unsaturated, are not obtained through β-oxidation but through de novo fatty acid biosynthesis (Huijberts et al. 1992).

It is worth noting that although saturated mcl-PHAs are mainly built from monomers whose carbon length ranges from C_6 to C_{14}, when unsaturated monomers are polymerized, the UnPHAs synthesized usually contain monomers with a longer carbon chain length (from C_6 to C_{16}).

Interestingly, the existence of UnPHAs containing unsaturated fatty acids bearing triple bonds as monomers has also been reported. Cultures of *P. putida* and *P. oleovorans* grown in media containing mixtures of nonanoic and 10-undecynoic acid synthesize a soft polymer having repeat units of (*R*)-3-hydroxy-8-nonynoate and (*R*)-3-hydroxy-10-undecynoate (Kim et al. 1998, 2000).

The presence of unsaturations on the polymer side chains is important since this affects several physicochemical characteristics as well as certain properties of the polymers. Thus, an increase in the degree of unsaturation implies a decrease of the melting temperature, a lower enthalpy of fusion and a lower glass-transition temperature (Ashby and Foglia 1998; Kim et al. 1998).

PHAs Containing Functionalized Monomers

In this section we describe some UnPHAs that are built from monomers containing halogen atoms, those bearing methyl, methoxy, ethoxy, hydroxyl, epoxy, acetoxy, carbonyl, cyano, phenyl, nitrophenyl, phenoxy, thiophenoxy or cyanophenoxy groups and PHAs containing alicyclic monomers.

Halogenated PHAs. Several halogenated PHAs were obtained when certain bacterial species (*P. oleovorans, P. putida*) were cultured in media containing chlorinated, brominated or fluorinated compounds. A copolyester containing (*R*)-3-hydroxyoctanoate, (*R*)-3-hydroxy-8-chlorooctanoate, (*R*)-3-hydroxyhexanoate and (*R*)-3-hydroxy-6-chlorohexanoate was synthesized when a culture of *P. oleovorans* was fed with octane and 1-chlorooctane as carbon sources (Doi and Abe 1990). Additionally, PHAs containing brominated monomers were produced by *P. oleovorans* when it was cultured in media containing mixtures of *n*-alkanoic acids (octanoic and nonanoic acids) and ω-bromoalkanoic acids (Kim et al. 1992).

Furthermore, when *P. oleovorans* was cultivated on a medium containing mixtures of nonane and 1-fluorononane, a copolymer containing up to 24 mol% of (*R*)-3-hydroxy-9-fluorononanoic acid was isolated (Abe et al. 1990). Similar results were obtained when the same strain was grown in a medium containing 1-fluorononane and gluconate as carbon sources (Hori et al. 1994). It has been also reported that *P. oleovorans* and *P. putida* grown in mixtures of different alkanoic and fluoroalkanoic acids (nonanoic acid and 6,6,6-trifluorohexanoic, 6,6,7,7,8,8,8-heptafluorooctanoic, 6,6,7,7,8,8,9,9,9-nonafluorononanoic or 6,6,7,7,8,8,9,9,10,10,11,11,11-tridecafluoroundecanoic acid) accumulate PHA copolymers containing (*R*)-3-hydroxypolyfluoroalkanoates as monomers. The incorporation of fluorinated units into PHAs modifies some characteristics of the halogenated polymer (higher melting temperatures, faster crystallization rates from the melt, a decrease in the glass-transition temperature and a large increase in the enthalpy of fusion; Kim et al. 1996a).

Methylated, methoxylated, ethoxylated and acetoxylated PHAs. Different species of *Pseudomonas* (*P. oleovorans*, *P. putida* and *P. citronellolis*) are able to accumulate UnPHAs containing branched monomers when cultured under appropriate conditions (Fritzsche et al. 1990b; Hazer et al. 1994; Scholz et al. 1994; Kim et al. 2003; Choi and Yoon 1994). *P. oleovorans* synthesized methyl-branched PHAs when grown in media containing 9-methyldecanoate, 8-methylnonanoate, 7-methylnonanoate or 7-methyloctanoate as the sole carbon source, or in combination with linear C_8–C_9 *n*-alkanoates. However, when 7-methylnonanoate, 5-methyloctanoate or 6-methyloctanoate or some dimethyl derivatives of alkenes (e.g. 2,6-dimethylhept-5-enoate) were used as the energy source, no PHA accumulation was observed, but copolyesters containing 5-methyloctanoic acid or 6-methyloctanoic

acid were obtained by cometabolism of these precursor together with octanoic acid (Fritzsche et al. 1990b).

The copolymer poly[(*R*)-3-hydroxy-7-methyl-6-octenoate-*co*-(*R*)-3-hydroxy-5-methylhexanoate] was obtained when *P. citronellolis* was grown in a medium containing citronellol as the sole carbon source (Choi and Yoon 1994).

Steinbüchel and co-workers also revealed the synthesis of methylated scl-PHAs when *C. necator*, *B. cepacia* and *Rhodococcus ruber* were cultured in a mineral salt medium containing tiglic acid (*C. necator*, *B. cepacia)* or (*R*)-3-hydroxypivalic acid (*R. ruber*), respectively (Füchtenbusch et al. 1996, 1998).

Recently, Dai et al. (2008) reported obtaining PHAs containing methylated monomers [(3-hydroxy-2-methylbutyrate and (*R*)-3-hydroxy-2-methylvalerate) in *Defluviicoccus vanus* related glycogen-accumulating organisms grown under anaerobic conditions. These authors reported that an increase in the relative proportion of methylated monomers in the copolymer lowered its crystallinity to a considerable extent.

PHAs having either methoxy or ethoxy groups were synthesized by *P. oleovorans* grown on pure 11-methoxyundecanoic acid or 11-ethoxyundecanoic acid. The PHA synthesized from 11-methoxyundecanoic acid consisted of (*R*)-3-hydroxy-7-methoxyheptanoate (main constituent) and (*R*)-3-hydroxy-9-methoxynonanoate (a minor monomer). However, the PHA produced from 11-ethoxyundecanoic acid contained (*R*)-3-hydroxy-5-ethoxypentanoate (at a slightly higher proportion) and (*R*)-3-hydroxy-7-ethoxyheptanoate. In both cases the monomers are generated through β-oxidation of each precursor (Kim et al. 2003). Furthermore, when this bacterium was fed with 2-octanone and octane, or mixtures of *n*-octylacetate and octane, two PHAs (polymer A, M_w 22,850, T_m 48ºC; and polymer B, M_w 29,930, T_m 50ºC) containing as monomers (*R*)-3-hydroxyhexanoate, (*R*)-3-hydroxyoctanoate, (*R*)-3-hydroxy-7-oxooctanoate and (*R*)-3-hydroxy-5-oxooctanoate (in polymer A) and (*R*)-3-hydroxyhexanoate, (*R*)-3-hydroxyoctanoate, 8-acetoxy-(*R*)-3-hydroxyoctanoate, 6-acetoxy-(*R*)-3-hydroxyhexanoate and 4-acetoxy-(*R*)-3-hydroxybutyrate (in polymer B) were accumulated (Jung et al. 2000).

The hydrophilicity conferred by the presence of alkoxy groups in the polymeric pendant chains results in an enhancement of the solubility of these polymers (Kim et al. 2003). Thus, the inherent hydrophobicity of mcl-PHAs could be strongly modified by the introduction of hydrophilic moieties into the side chain, increasing their applicability as biocompatible materials for a wide range of biomedical devices.

Epoxy-containing PHAs. Although most UnPHAs containing epoxide groups in the monomer side chains are obtained by chemical synthesis (reaction of *m*-chloroperbenzoic acid with unsaturated polyesters, see Sect. 3.2.1), different epoxidized bacterial polyesters have been obtained by direct fermentation. When *P. oleovorans* was grown in media containing 10-epoxyundecanoic acid and sodium octanoate, a PHA containing terminal epoxy groups in the side chains was obtained (Bear et al. 1997). Furthermore, a different bacterium, *P. stutzeri*, also accumulated an epoxidized PHA (containing as a monomer 3,6-epoxy-7-nonene-1,9 dioic acid) when cultivated on a mineral medium containing soybean oil as the sole carbon source (He et al. 1998). Additional studies revealed that when *Pseudomonas cichorii* was cultured in the presence of C_7–C_{12} 1-alkenes as the sole carbon sources, PHAs

containing repeating units with terminal epoxide groups were synthesized. The molecular weights of these polymers (which contained unsaturated, epoxidized and saturated units) were in the range 150,000–200,000 and differential scanning calorimetry thermograms showed that regardless of the monomer size and degree of epoxidation, the glass-transition temperature (T_g) was around −40ºC (Imamura et al. 2001). These authors also suggested that the first step of the biosynthetic pathway leading to the production of epoxidized monomers was catalysed by an enzyme that transforms 1-alkene into 1,2-epoxyalkane.

mcl-UnPHAs containing epoxy groups (as well as others containing unsaturations or halogens atoms, see above) are of special interest because these reactive groups can be modified chemically, thus leading to new polymers with different mechanical properties and, probably, new characteristics (Hazer and Steinbüchel 2007). Unsaturated and unsaturated epoxidized mcl-PHAs are used in cross-linking reaction with UV light, generating reticular structures which improve the elastomeric properties of the original polymers (Park et al. 1998a, b; Kim et al. 2001b; Ashby et al. 2000; Bassas et al. 2008).

PHAs containing carbonyl groups. When *P. oleovorans* was grown, under nitrogen-starvation conditions, in a two-liquid-phase fed-batch culture in the presence of 2-octanone and octane it accumulated UnPHAs containing (*R*)-3-hydroxyoctanoate, (*R*)-3-hydroxyhexanoate and some monomers bearing carbonyl groups [(*R*)-3-hydroxy-7-oxooctanoate and (*R*)-3-hydroxy-5-oxohexanoate]. The M_w of this polymer was 228,500 and its melting temperature was 48.5°C (Jung et al. 2000).

PHAs containing cyano groups. Although natural polyesters containing monomers bearing a cyano group (nitriles) are not common, some bacteria are able to synthesize these unusual polyesters through cometabolism. Lenz et al. (1992) reported that *P. oleovorans*, when cultured in chemically defined media supplied with 11-cyanoundecanoic acid and nonanoic acid as carbon sources, accumulated a PHA that contained monomers with cyano groups (9-cyano-3-hydroxynonanoate and 7-cyano-3-hydroxyheptanoate).

PHAs containing monomers bearing cycloalkyl groups. Polymers containing cycloalkyl monomers were isolated when *P. oleovorans* was grown in a mineral salt medium supplied with 5-cyclohexylvaleric or 4-cyclohexylbutyric acid and mixtures thereof with nonanoic acid (Andujar et al. 1997; Kim et al. 2001a). The PHA synthesized was a viscous and adhesive material with M_w of 52,000 and that showed two thermal temperatures (T_{g1} −16.3ºC and T_{g2} −32.6ºC), suggesting that it was a mixture of two polyesters with different cyclohexyl proportions.

PHAS containing alkyl ester derivatives as monomers. Cultures of *P. oleovorans* grown on media containing mixtures of methyl heptanoate, ethyl heptanoate or propyl heptanoate and heptanoic acid accumulated polymers containing as monomers certain (*R*)-3-hydroxy derivatives of these alkyl esters. The presence of monomers with pendant ester groups was maximum in the PHAs whose units were obtained from methyl heptanoate, whereas fermentations performed with ethyl heptanoate resulted in the formation of unsubstituted PHAs (Scholz et al. 1994).

PHAs containing (R)-3-hydroxyalkylthioalkanoic acids as monomers. A recombinant strain of *C. necator* (which expresses the PHA synthase of *P. mendocina*) is able

to synthesize poly[(3)-hydroxy-*S*-propyl-ω-thioalkanoate]s in cultures performed under PHA-production conditions and fed with either propylthiooctanoic acid or propylthiohexanoic acid and gluconic acid (Ewering et al. 2002). When propylthiooctanoic acid was supplied, a terpolyester of poly[(*R*)-3-hydroxypropylthiobutyrate-*co*-(*R*)-3-hydroxypropylthiohexanoate-*co*-(*R*)-3-hydroxypropylthiooctanoate] was obtained. However, when the PHA precursor was propylthiohexanoic acid, this bacterium accumulated a copolymer consisting of (*R*)-3-hydroxypropylthiobutyrate and (*R*)-3-hydroxypropylthiohexanoate {poly[(*R*)-3-hydroxypropylthiobutyrate-*co*-(*R*)-3-hydroxypropylthiohexanoate]}. However, when these alkylthioalkanoic acids were supplied to cultures of *P. oleovorans*, *P. aeruginosa* or *P. mendocina*, no PHAs containing thioalkanoates were synthesized, even when cosubstrates were added to the medium. Interestingly, *P. putida* KT2440 synthesizes a PHA with sulphur-containing monomers when grown in a medium containing propylthioundecanoic acid and equimolar amounts of nonanoic acid, and it is the only species of *Pseudomonas* able to synthesize these UnPHAs (Ewering et al. 2002).

PHAs containing thioether groups are light-yellow, translucent and very glutinous polymers that exhibit a typical thiol smell (Ewering et al. 2002). They might be used in medicine and in pharmacy owing to their antimicrobial properties and applications (skin substitute). Moreover, the presence of thioether groups in the side chains facilitates the production of new PHAs through chemical modifications of the sulphur atom (Ewering et al. 2002).

PHAs containing aromatic groups. The production of PHAs containing arylalkyl substituents was reported in *P. oleovorans*, *P. putida* and *P. cichorii* when these bacteria were cultured in media containing several ω-benzoylalkanoic acids (4-benzoylbutyrate, 5-benzoylvalerate, 6-benzoylbenzoylhexanoate, 7-benzoylheptanoate and 8-benzoyloctanoate) or ω-phenylalkanoic acids (5-phenylvaleric acid, 6-phenylhexanoic acid, 7-phenylheptanoic acid, 8-phenyloctanoic acid, 9-phenylnonanoic acid, 10-phenyldecanoic acid and 11-phenylundecanoic acid; Fritzsche et al. 1990a; Hazer et al. 1996; García et al. 1999; Honma et al. 2004).

Incubations of *P. cichorii* in media containing different ω-benzoylalkanoic acids revealed that PHAs containing a benzoyl side chain in the monomers were achieved with all the precursors indicated above, the polymer content being minimal (the lowest 3%) with 4-benzoylbutyric acid. The polymers synthesized from 4-benzoylbutyrate, 5-benzoylvalerate, 6-benzoylbenzoylhexanoate and 7-benzoylheptanoate were always homopolymers containing repeating units of the (*R*)-3-hydroxy-ω-benzoylalkanoic acid, whereas when the precursor added was 8-benzoyloctanoate, a copolymer mainly formed by (*R*)-3-hydroxy-6-benzoylhexanoate-*co*-3-hydroxy-8-benzoyloctanoate was synthesized (Honma et al. 2004). Studies addressing some of these polymers revealed that those containing benzoyl residues show different thermal properties from the usual alkanoates [i.e. the T_g of (*R*)-3-hydroxy-5-benzoylvalerate homopolymer is much higher than for PHB] and that their solubility in organic solvents also differs from that of common oxopolyesters (Honma et al. 2004)

When *P. oleovorans* was cultured in a medium containing 5-phenylvaleric acid as the sole carbon source, a homopolymer of poly[(*R*)-3-hydroxy-5-phenylvaletare] was accumulated (Fritzsche et al. 1990a); however, when *P. putida* was grown in

the same conditions it did not synthesize PHAs (Hazer et al. 1996; García et al. 1999). Surprisingly, in the cultures in which the ω-phenylalkanoic acids used had an alkyl chain with an odd number of carbon atoms (7-phenylheptanoic acid and 9-phenylnonanoic acid), the only PHA accumulated by *P. putida* was poly[(*R*)-3-hydroxy-5-phenylvalerate] (García et al. 1999). Furthermore, when ω-phenylalkanoic acids with an even number of carbon atoms in the alkyl chain (6-phenylhexanoic acid, 8-phenyloctanoic acid or 10-phenyldecanoic acid) were fed, different polymers were synthesized. When the carbon source was 6-phenylhexanoic acid, a homopolymer, poly[(*R*)-3-hydroxy-6-phenylhexanoate], was synthesized, whereas when 8-phenyloctanoic or 10-phenyldecanoic was supplied to the cultures, two copolymers, poly[(*R*)-3-hydroxy-6-phenylhexanoate-*co*-(*R*)-3-hydroxy-8-phenyloctanate] and poly[(*R*)-3-hydroxy-6-phenylhexanoate-*co*-(*R*)-3-hydroxy-8-phenyloctanate-*co*-(*R*)-3-hydroxy-10-phenyldecanoate], were obtained (García et al. 1999). Different PHAs containing mixtures of aliphatic and aromatic monomers were obtained when *P. putida* cultures were cofed with ω-phenylalkanoates and *n*-alkanoic acids (Olivera et al. 2001a). These copolymers were amorphous and had relatively low glass-transition temperatures (Abraham et al. 2001; Olivera et al. 2001a). The accumulation of poly[(*R*)-3-hydroxy-*n*-alkanoates-*co*-(*R*)-3-hydroxy-ω-phenylalkanoates] in pseudomonads has also been reported by other authors (Ward and O'Connor 2005).

Polymers containing nitrophenyl groups were isolated when *P. oleovorans* was grown in media containing 5-(2′,4′-dinitrophenyl)valeric acid and nonanoic acid (Arostegui et al. 1999). Thermal analysis indicated that this PHA constituted a mixture of two different polymers: one containing the nitrophenyl rings and the other tailored only with monomers derived from nonanoic acid. The nitrophenyl-containing polyester is an amorphous polymer having a T_g of 28°C (Arostegui et al. 1999).

When 5-*p*-tolylvaleric acid was used as the sole carbon source by a culture of *P. oleovorans*, production of poly[(*R*)-3-hydroxy-5-*p*-tolylvalerate] was achieved (Curley et al. 1996). This crystalline polymer had a glass-transition temperature of 18°C and a melting transition of 95°C. When *P. oleovorans* was cofed with 5-phenylvaleric acid and 5-*p*-tolylvaleric acid, a non-crystallizable copolymer containing as monomers (*R*)-3-hydroxy-5-*p*-tolylvalerate and phenylvalerate was obtained (Curley et al. 1996). A structurally related PHA was obtained when 9-*p*-tolylnonanoic acid was used as the monomer precursor (Hazer et al. 1996).

UnPHAs bearing phenoxy groups on their side chains were synthesized by *P. oleovorans* when it was cultivated in the presence of *ω*-phenoxyalkanoates (Ritter and von Spee 1994; Kim et al. 1996b). Ritter and von Spee (1994) reported the isolation of a PHA containing (*R*)-3-hydroxy-9-phenoxynonanoate and (*R*)-3-hydroxy-5-phenoxypentanoate units when *P. oleovorans* was grown with 11-phenoxyundecanoate, and Kim et al. (1996b) showed that this bacterium was also able to accumulate aromatic PHAs when grown in media containing 6-phenoxyhexanoate, 8-phenoxyoctanoate and 11-phenoxyundecanoate. In such cases the main repeating units were (*R*)-3-hydroxy-4-phenoxybutyrate (when the carbon source was either 8-phenoxyoctanoic acid or 6-phenoxyhexanoic acid) and (*R*)-3-hydroxy-5-phenoxypentanoate when the precursor was 11-phenoxyundecanoic acid. Moreover, a *P. putida* strain was also able to accumulate PHAs built from monomers bearing phenoxy groups

when cultured in media supplemented with 11-phenoxyundecanoic acid or with mixtures of 11-phenoxyundecanoic acid and octanoic acid (Song and Yoon 1996). The copolyester synthesized from 11-phenoxyundecanoic acid (showing a crystalline melting transition at 70°C and a glass transition at 14°C) contained as monomers (*R*)-3-hydroxy-5-phenoxyvalerate (75%) and (*R*)-3-hydroxy-7-phenoxyheptanoate (approximately 25%). When the culture was cofed with 11-phenoxyundecanoic acid and octanoic acid, a copolyester containing (*R*)-3-hydroxy-5-phenoxyvalerate, (*R*)-3-hydroxy-7-phenoxyoctanoate, (*R*)-3-hydroxy-9-phenoxynonanoate together with (*R*)-3-hydroxyoctanoate, (*R*)-3-hydroxyhexanoate and (*R*)-3-hydroxydecanoate as monomers was isolated. The molar ratio of aromatic monomers to aliphatic monomers in this PHA was proportional to the molar ratio of 11-phenoxyundecanoic acid to octanoic acid in the medium. An increment in the quantity of octanoic acid supplied in the feed shifted the major monomer unit in the polymer from (*R*)-3-hydroxy-5-phenoxyvalerate to (*R*)-3-hydroxy-7-phenoxyheptanoate (Song and Yoon 1996).

Furthermore, PHAs containing monomers with fluorinated phenoxy side groups were produced by *P. putida* when 11-(2-fluorophenoxy)undecanoic acid, 11-(3-fluorophenoxy)undecanoic acid, 11-(4-fluorophenoxy)undecanoic acid or 11-(2,4-difluorophenoxy)undecanoic acid was used as the sole carbon source (Takagi et al. 2004). When 11-(3-fluorophenoxy)undecanoic acid was the precursor of the monomers, a copolymer containing (*R*)-3-hydroxy-7-(3-fluorophenoxy)heptanoate (10%) and (*R*)-3-hydroxy-5-(3-fluorophenoxy)valerate (90%) was isolated. When the precursor was 11-(4-fluorophenoxy)undecanoic acid, the monomers present in the copolymer were (*R*)-3-hydroxy-7-(4-fluorophenoxy)heptanoate and (*R*)-3-hydroxy-5-(4-fluorophenoxy)valerate. Additionally, a homopolymer of poly[3-hydroxy-5-(2,4-difluorophenoxy)valerate] (with water-shedding properties) was obtained when cells were grown with 11-(2,4-difluorophenoxy)undecanoic acid. All these fluorophenoxy PHAs showed high crystallinity and a high melting point (around 100°C; Takagi et al. 2004).

The introduction of monomers containing thiophenoxy pendant groups into PHAs was performed by *P. putida* when this bacterium was cultured in a medium containing 11-thiophenoxyundecanoic acid as the sole carbon source (Takagi et al. 1999). This polymer contained (*R*)-3-hydroxy-5-thiophenoxyvalerate as the main constituent, as well as (*R*)-3-hydroxy-7-thiophenoxyheptanoate. This biopolyester is an amorphous elastic polymer with a glass-transition temperature of 4°C.

PHAs bearing methylphenoxy groups have been synthesized by *P. oleovorans* and *P. putida* growing in media containing 6-*p*-methylphenoxyhexanoic acid, 8-*p*-methylphenoxyoctanoic acid, 8-*m*-methylphenoxyoctanoic acid or 11-*p*-methylphenoxyundecanoic acid (Kim et al. 1999, 2000). Both microbes produced crystalline polymers (glass-transition temperature of 14°C and a melting temperature of 97°C) consisting of (*R*)-3-hydroxy-4-*p*-methylphenoxybutyrate and (*R*)-3-hydroxy-6-*p*-methylphenoxyhexanoate when grown with 8-*p*-methylphenoxyoctanoic acid, and a small amount of PHA was obtained when these bacteria were cultured with 8-*m*-methylphenoxyoctanoic acid. The cofeeding of these cultures with 8-*p*-methylphenoxyoctanoic acid and nonanoic acid/5-phenylvaleric acid produced random copolymers with compositions that depended of the precursor supplied to the medium (Kim et al. 1999).

The incorporation of *p*-cyanophenoxy and *p*-nitrophenoxy groups into PHAs synthesized by *P. putida* and *P. oleovorans* has also been reported (Kim et al. 1995a). However, the extent of the incorporation of these functional groups into PHA was significantly lower than the incorporations reported for other aromatic groups, and always required that octanoic acid be cofed to the culture.

3.1.2 PHAs with Elongated Backbones (Class 2)

PHAs containing as monomers hydroxyalkanoic acids different from (*R*)-3-hydroxyalkanoates have also been reported (Saito and Doi 1994; Schmack et al. 1998; Choi et al. 1999; Amirul et al. 2008). These polymers, which belong to both scl-PHAs and mcl-PHAs, have as a structural peculiarity an elongated backbone (Fig. 2). Among these UnPHAs, those copolymers containing as repeating units (*R*)-3-hydroxybutyric acid and 4-hydroxybutyric acid {poly[(*R*)-3-hydroxybutyrate-*co*-4-hydroxybutyrate], P(3HB–4HB)} are the paradigmatic examples.

Only a small group of wild-type bacteria able to produce P(3HB–4HB) have been reported to date. *C. necator* (Doi et al. 1989, 1990; Kunioka et al. 1989; Kim et al. 2005b), *Cupriavidus* spp. USMAA1020 (Amirul et al. 2008), *A. latus* (Hiramitsu et al. 1993; Kang et al. 1995), *C. acidovorans* (Saito and Doi 1994; Lee et al. 2004), *Comamonas testosteroni* (Renner et al. 1996), *Rhodococcus* sp. (Haywood et al. 1991) and *H. pseudoflava* (Yoon and Choi 1999; Choi et al. 1999) synthesize P(3HB–4HB) when cultured under appropriate conditions. Recently, the application of genetic engineering technology to producers of PHAs has allowed recombinant strains able to accumulate P(3HB–4HB) or poly(4-hydroxybutyrate) [P(4HB)] homopolymers to be obtained (Hein et al. 1997; Valentin and Dennis 1997).

The microbial biosynthesis of P(3HB–4HB) copolymers was achieved using different nutritional strategies. Most of them are based on the carbon sources used to feed the cultures (butyric acid and different sugars, or a single carbon source structurally related to butyric acid such as 4-hydroxybutyric acid, γ-butyrolactone or 1,4 butanediol; Saito and Doi 1994; Kunioka et al. 1989).

In other cases, *E. coli* strains have been engineered for the production of P(3HB–4HB) an P(4HB). An *E. coli* strain, coexpressing the 4-hydroxybutyric acid-CoA transferase from *C. kluyveri* and the PHA synthase from *C. necator* was able to accumulate P(4HB) when cultured in a medium containing 4-hydroxybutyric acid and glucose as carbon sources. When this strain was cultured in the same medium without glucose, a copolyester of P(3HB–4HB) was synthesized (Hein et al. 1997). Similarly, a strain of *E. coli* expressing the succinate degradation pathway from *C. kluyveri*, along with the *pha* genes from *C. necator*, is able to produce P(3HB–4HB) directly from glucose (Valentin and Dennis 1997). Moreover, different mutants of *C. necator* and *P. putida* handicapped in PHA biosynthesis, but harbouring the PHA synthase genes from *Thiocapsa pfennigii*, accumulated PHAs containing 4-hydroxyvalerate as the monomer when cultured in media containing laevulinic acid (Schmack et al. 1998; Gorenflo et al. 2001).

Additionally, UnPHAs containing 4-hydroxyvalerate (Valentin and Steinbüchel 1995), 4-hydroxyhexanoate (Valentin et al. 1994), 4-hydroxyheptanoate and

4-hydroxyoctanoate (Valentin et al. 1996), 5-hydroxyvalerate (Doi et al. 1987; Haywood et al. 1991), 5-hydroxyhexanoate (Doi et al. 1987; Valentin et al. 1996) or 6-hydroxyhexanoate (Labuzek and Radecka 2001; Tajima et al. 2003) have been isolated, suggesting that the position of the oxidized carbon in the monomers is not a crucial factor either for scl- or mcl-PHA synthase activities.

Analysis of the characteristics of the copolymers containing 4-hydroxybutyrate monomers revealed that they range from being highly crystalline to elastic, depending of the monomer content (Saito and Doi 1994). Furthermore, the homopolymer P(4HB) has good tensile strength and is quite similar to ultrahigh molecular weight PE. However, P(4HB) has as an advantage over PE its elongation at break, which is about 1,000% (Saito et al. 1996). Similar properties have been reported for copolymers containing 4-hydroxyvalerate as a monomer (Schmack et al. 1998). Moreover, Saito and Doi (1994) reported that the tensile strength of P(3HB–4HB) increases as a function of the 4-hydroxybutyrate fraction, showing that P(3HB–4HB) copolymers with high 4-hydroxybutyrate fractions are very strong thermoplastics elastomers. When the biodegradability of these copolymers was studied, it was observed that the erosion rate of P(3HB–4HB) films caused by depolymerases and lipases was strongly related to their monomer composition (Saito and Doi 1994; Saito et al. 1996).

3.1.3 PHAs Containing Thioester Linkages (Class 3)

A new family of bacterial biopolymers (thioester-containing PHAs) was reported by Lütke-Eversloh et al. (2001a, b). These authors showed that *C. necator* was able to synthesize and polymerize (*R*)-3-mercaptopropionic acid, thus generating a novel type of PHA that contains sulphur in the polymer backbone. Whereas in other PHAs the monomers are covalently linked by oxoester bonds, in these bioplastics they are linked by thioester bonds. For this reason, these polymers are referred to as polythioesters (Lütke-Eversloh et al. 2001a, b).

Different copolymers containing (*R*)-3-hydroxybutyrate, (*R*)-3-hydroxyvalerate, (*R*)-3-mercaptopropionate, (*R*)-3-mercaptobutyrate and (*R*)-3-mercaptovalerate were obtained when *C. necator* was cultured under the conditions required to synthesize PHAs (permissive conditions) in gluconate-containing minimal medium supplemented with the sulphur precursor substrates [(*R*)-3-mercaptopropionic acid, 3,3′-thiodipropionic acid or 3,3′-dithiodipropionic acid for the production of a copolymer containing (*R*)-3-mercaptopropionate units; (*R*)-3-mercaptobutyric acid for the synthesis of a copolymer containing as monomer (*R*)-3-mercaptobutyrate; and (*R*)-3-mercaptovaleric acid to produce a copolymer containing units of (*R*)-3-mercaptovalerate] (Lütke-Eversloh et al. 2001a, b; Lütke-Eversloh and Steinbüchel 2003; Kim et al. 2005a).

A different approach for the production of polythioesters involved the use of recombinant strains of *E. coli* expressing the genetic information required for a non-natural genetically engineered pathway (BPEC), which includes the genes *buk*, *ptb* and *phaEC*, encoding a butyrate kinase (*buk*) and a phosphotransbutyrylase

(*ptb*) from *Clostridium acetobutylicum*, and a type III PHA synthetase from *Allochromatium vinosum* or *Thiococcus pfennigi* (*phaEC*) (Lütke-Eversloh et al. 2002a; Lütke-Eversloh and Steinbüchel 2004; Thakor et al. 2005). With use of these transgenic *E. coli* strains, homopolymers of poly[(*R*)-3-mercaptopropionate], poly[(*R*)-3-mercaptobutyrate] and poly[(*R*)-3-mercaptovalerate] were obtained when the recombinant bacteria were cultured in a medium containing (*R*)-3-mercaptopropionic acid, (*R*)-3-mercaptobutyric acid or (*R*)-3-mercaptovaleric acid as precursors (Lütke-Eversloh et al. 2002a).

Furthermore, it has also been reported that the coexpression in this system of the PhaP1 phasin from *C. necator* strikingly increased the amount of poly[(*R*)-3-mercaptopropionate] accumulated by the recombinant *E. coli* strain when this bacterium was cultured in a medium containing (*R*)-3-mercaptopropionic acid as a precursor (Tessmer et al. 2007).

Taking into account that polythioesters differ from usual PHAs only in the substitution of oxygen atoms by sulphur atoms in the linkages of the polymer backbone leads to the assumption that the physicochemical properties that distinguish both kinds of polyesters should be a consequence of this structural difference. Analysis of the thermal properties of polymers containing sulphur atoms in the backbone linkages revealed that they exhibit marked differences from those of the corresponding oxygen-containing homologues. All the known polythioester homopolymers exhibited a lower melting temperature than the corresponding polyester homologues (Kawada et al. 2003). Furthermore, the copolymer poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxymercaptobutyrate], containing 40% or less of (*R*)-3-hydroxybutyrate, shows lower crystallinity and higher melting temperatures than the poly[(*R*)-3-hydroxybutyrate] homopolymer (Lütke-Eversloh et al. 2002a). Moreover, the thermal stability of poly[(*R*)-3-mercaptopropionate] is significantly enhanced in comparison with that of poly[(*R*)-3-hydroxypropionate]. Interestingly, the thermal stabilities of poly[(*R*)-3-mercaptobutyrate] and poly[(*R*)-3-mercaptovalerate] homopolymers were similar to those of poly[(*R*)-3-hydroxybutyrate] (Kawada et al. 2003). Analysis of the molecular weights of these polymers revealed that they ranged between 150,000 and 176,000 (Lutke-Eversloh et al. 2002b; Kawada et al. 2003; Tanaka et al. 2004; Impallomeni et al. 2007).

One surprising characteristic of polythioesters is that, in contrast to those polymers containing oxoester bonds, the thioester linkages are not susceptible to PHA depolymerases (Elbanna et al. 2003, 2004), thus constituting the only known kind of non-biodegradable biopolymers (Kim et al. 2005a).

3.1.4 Functional Analyses of the Different Proteins Involved in the Synthesis and Accumulation of UnPHAs

As indicated already, several approaches based on fermentation advances, biochemical studies or genetic engineering manipulations have allowed the production of many different PHAs (Lenz et al. 1992; García et al. 1999; Ahn et al. 2000; Lee et al. 2001; Lee and Choi 2001; Park et al. 2001; van Wegen et al. 2001; Ren et al. 2005b).

All these studies revealed that the synthesis of PHB and the copolymers tailored from (*R*)-3-hydroxypropionate, (*R*)-3-hydroxybutyrate, (*R*)-3-hydroxyvalerate or/and (*R*)-3-hydroxyhexanoate, their close structural analogues [(*R*)-3-mercaptolkanoates] or their derivatives (4-hydroxybutyrate, 4-hydroxyvalerate) requires the enzymes encoded at the *phaCBAPZ* cluster, and that the biosynthesis, accumulation and mobilization of mcl-PHAs and mcl-UnPHAs requires the proteins encoded by the genes included in the *phaC1ZC2DFI* locus (Fig. 6). In sum, the enzymes required to fully synthesize the monomers to be polymerized into well-known PHAs are also the same as those involved in the production of UnPHAs. However, in some special cases [e.g. synthesis of (*R*)-3-mercaptoalkanoates] a completely different biosynthetic pathway is required (Lütke-Eversloh et al. 2002a; Lütke-Eversloh and Steinbüchel 2004; Thakor et al. 2005).

Substrate Specificity of the Two Polymerases (PhaC1 and PhaC2) Involved in the Synthesis of mcl-UnPHAs

Although the two polymerases (PhaC1 and PhaC2) involved in the synthesis of aliphatic mcl-PHA were believed to have quite similar substrate specificity (Qi et al. 1997), recent studies performed with usual and unusual monomers have shown that some interesting differences do exist (Sandoval et al. 2007; Arias et al. 2008). The expression of either the gene *phaC1* or the gene *phaC2* in a *P. putida* mutant lacking the whole *pha* locus (*P. putida* U Δ*pha*) led to the production of two recombinant strains (*P. putida* U Δ*pha* pMC-*phaC1* and *P. putida* U Δ*pha* pMC-*phaC2*) that were able to synthesize several aliphatic and aromatic PHAs when cultured in media containing C_6–C_{10} *n*-alkanoic acids or C_5–C_9 *n*-phenylalkanoic acids. When *P. putida* U Δ*pha* pMC-*phaC1* was studied, the highest accumulation of polymers was observed when the precursors used were decanoic acid (for aliphatic PHAs) or 6-phenylhexanoic acid (for aromatic PHAs). However, when the recombinant strain studied was *P. putida* U Δ*pha* pMC-*pha*C2, it was observed that although it synthesized similar aliphatic PHAs (the highest accumulation was observed when hexanoic acid was the precursor), it only accumulated aromatic PHAs when the monomer was (*R*)-3-hydroxy-5-phenylvaleryl-CoA (Arias et al. 2008). These observations, which cannot be attributed to differences in the uptake (transport) of monomer precursors (in both cases the same strain – *P. putida* U Δ*pha* – was used), revealed that PhaC1 and PhaC2 recognize different substrates and also do so with different affinities. Variations in the substrate specificity of PhaC1 and PhaC2 were also observed when recombinant *E. coli* strains were used as PHA producers (Chen et al. 2004).

The elucidation of the molecular bases responsible for the different substrate specificities observed in the PHA synthases belonging to class II (Rehm 2003) has been approached by different authors (Wahab et al. 2006; Arias et al. 2008). Some structural studies have revealed the participation of a serine residue (Ser297) in the catalytic process, and the formation of two tetrahedral intermediates, stabilized by the formation of an oxyanion hole during PHA biosynthesis, has been

proposed (Wahab et al. 2006). Additionally, genetic engineering approaches have shown that the introduction of mutations in the genes encoding other polymerases (PHA synthase from *Aeromonas caviae*) leads to the production in this bacterium of different copolymers with quite variable composition, suggesting that this strategy could be useful not only for analysing the amino acid residues involved in the catalytic process (Tsuge et al. 2007), but also for expanding the number and characteristics of the PHAs accumulated by genetically manipulated strains. Despite all these advances, further experimental work is needed to clarify the nature of the intermediates as well as to establish the molecular mechanism responsible for the PHA polymerization reaction.

Depolymerases

All the genes, *phaZ* included, in the *pha* loci encode PHA depolymerases (commonly referred to as PhaZ) which are structurally related to esterases (all them contain a conventional lipase box; Ohura et al. 1999; Jendrossek and Handrick 2002; de Eugenio et al. 2007). These enzymes catalyse the release of (*R*)-3-hydroxy-acyl/aryl-CoA derivatives from intracellular polymers. The topological localization of PhaZ in the granule surface (Foster et al. 1994; Prieto et al. 1999; de Eugenio et al. 2007; Jendrossek 2007) and the inability of certain bacteria to mobilize scl-PHAs or mcl-PHAs when the different *phaZ* genes (involved in catabolism of either scl-PHAs or mcl-PHAs) are mutated support its physiological role (García et al. 1999; Sandoval et al. 2005, Sandoval et al. 2007; de Eugenio et al. 2007). Interestingly, these enzymes are able to hydrolyse most of the PHAs accumulated by bacteria (even those constituted by unusual monomers), revealing that the substrate specificity of PhaZ enzymes, as occurred with other esterases, is very broad (Sandoval et al. 2005). Some special PHAs (polythioester homopolymers) are not cleaved by depolymerases; hence, they remain almost unaltered. However, copolymers containing both oxoester and thioester linkages can be partially hydrolysed by extracellular [(*R*)-3-hydroxybutyrate] depolymerases, showing that PhaZ are specific for oxoester (but not for thioester) linkages (Elbanna et al. 2003, 2004).

The cleavage of certain UnPHAs by PhaZ often releases uncommon monomers that cannot be assimilated through the usual metabolic pathways. In such cases, when the catabolic route required for the degradation of an uncommon monomer does not exist, or has been mutated, the (*R*)-3-hydroxy derivative units released from the polymers are secreted into the culture broth (Olivera et al. 2001b). Similarly, when *phaZ* was overexpressed in a PHA-producer strain, the accumulation of granules of PHAs was never observed, since the PHAs are immediately hydrolysed. In such cases, the excess monomers generated (those not needed to support bacterial growth) are rapidly secreted (Sandoval et al. 2005). Thus, the transformation of a PHA-overproducer mutant of *P. putida* (*P. putida* U Δ*fadBA*; Olivera et al. 2001a, b) with a replicative plasmid containing the gene *phaZ* handicapped the accumulation of both aliphatic and aromatic PHAs in this bacterium (Fig. 5). Moreover, when this strain was cultured in media containing ω-phenylalkanoates, it efficiently converted (about 80%)

these compounds into (*R*)-3-hydroxy-ω-phenylalkanoates, which, since they could not be catabolized, were released into the culture broth (Sandoval et al. 2005).

The use of genetically engineered strains derived from different PHA producers overexpressing the *phaZ* gene seems to be a useful strategy to obtain different (*R*)-3-hydroxyalkanoates (even pure enantiomers) that, owing to their important chemical, biochemical and pharmaceutical uses, are of great biotechnological interest and have broad industrial applications (Jiang et al. 2004; Sandoval et al. 2005; Ren et al. 2005c; Yuan et al. 2008).

Role Played by PhaDFI Proteins

Very recently, a genetically engineered strain of *P. putida* lacking the *pha* cluster (*P. putida* Δ*pha*) and, therefore, unable to accumulate PHAs was used to analyse the role played by the Pha proteins in the synthesis of aromatic and aliphatic PHAs (Sandoval et al. 2007). For such experiments the *pha* genes (*phaC1ZC2DFI*) were expressed, in an isolated fashion or in tandem, in that mutant. The authors reported that transformation of this strain with a plasmid containing the whole *pha* locus restored the ability of the bacterium to synthesize all the PHAs accumulated by the wild type, that the expression of the gene *phaC1* alone (in the absence of *phaZC2DFI*) sufficed to ensure the synthesis of both aliphatic PHAs and aromatic PHAs, although in this recombinant strain, the number of granules was higher than in the wild type (Fig. 5), and that the coexpression in *trans* of the genes encoding PhaC1 and the phasin PhaF (one of the most abundant granule-attached proteins; Timm and Steinbüchel 1992; Prieto et al. 1999; Klinke et al. 2000; Sandoval et al. 2007) restores the original phenotype (only a few large granules). These data unequivocally showed that Pha proteins are the only enzymes required for the biosynthesis of both aliphatic and aromatic mcl-PHAs and that, at least in this species, these proteins cannot be replaced by other bacterial enzymes. They also showed that PhaF is involved in the coalescence of the granules of PHAs/aromatic PHAs, and that the functions played by all the Pha proteins (PhaC1ZC2DFI; mcl-PHA synthesis and granule architecture) can be performed by two single enzymes (PhaC1 and PhaF). In conclusion, *phaC1* and *phaF* would represent the minimum genetic information required for the synthesis of normal PHA granules in this bacterium (Sandoval et al. 2007).

Surprisingly, the deletion of the *phaDFI* genes in *P. putida* U decreased the synthesis of aliphatic PHAs and prevented the synthesis of aromatic polyesters (aromatic PHAs), suggesting that the rigidity of the phenyl groups present in the aromatic monomers requires some of these proteins (PhaD, PhaF or/and PhaI) to be properly organized in granules. A similar effect (a strong reduction in aliphatic PHAs synthesis and the absence of aromatic PHAs) was observed when *phaF* was deleted in *P. putida* U.

Furthermore, deletion of the *phaD* gene in *P. putida* U strongly reduced the synthesis of aliphatic and aromatic PHAs; this effect was fully restored when *phaF* was overexpressed in this mutant. Additional studies revealed that the overexpression of *phaF* in the wild type (*P. putida* U) or in the PHA-overproducer mutant

(*P. putida* U Δ*fadBA*) contributes to dramatically increasing granule size, increasing the intracellular amount of the polyesters (PHAs and aromatic PHAs) accumulated and increasing the cellular volume, pointing to the involvement of this protein in important cell cycle events. All these data confirm that PhaF has an important function in the synthesis of PHAs (particularly aromatic PHAs) as well as in the control of both the size and the number of PHA granules, and that its overexpression in different strains leads to the isolation of overproducer bacteria that could be used for the production of novel (or modified) polyesters (Sandoval et al. 2007).

Although for the time being the exact role of PhaD and phasins, at least at a molecular level, has not been clarified, it seems fairly evident that PhaF plays an important role in the organization of usual PHA and UnPHA granules, and that PhaD acts as a transcriptional activator of *phaF* (and probably of *phaI*; Fig. 6). However, further genetic experiments are needed to establish whether phasin PhaI serves only as a structural element involved in the organization of the nascent polymer chains, or whether this protein has a dual function (granule stabilization and *phaF* regulator), as proposed by Sandoval et al. (2007).

In sum, although many different polymers can be tailored by natural PHA-producer strains (or by the recombinant organisms constructed with genetic engineering, see above) the enzymes involved in the synthesis, accumulation and mobilization of all the scl- and mcl-PHAs produced by micro-organisms (usual PHAs or UnPHAs) require the proteins encoded by the genes belonging to the *phaCBAPZ* (in the case of scl-PHAs) and *phaC1ZC2DFI* (if they are mcl-PHAs) clusters.

3.2 UnPHAs Obtained by Chemical or Physical Modifications of Naturally Occurring One

The production of new polymers which improve (or sidestep) the limitations found in the bacterial polymers has been an important challenge for many scientists in recent decades. Chemical modification of well-known PHAs, one of the most important strategies followed, has allowed the production of different polyesters with novel or modified physiochemical properties (Arkin and Hazer 2002).

Although the chemical and physical modification of PHAs involves quite different reactions, mainly three different approaches involving (1) PHA functionalization, (2) the grafting/blocking copolymerization of PHAs and (3) the blending of the PHAs with other polymers, have been followed.

3.2.1 Functionalization of Microbial PHAs

Halogenation of PHAs

The production of chlorinated PHAs has mainly been achieved by passing chlorine gas through their solutions (Arkin and Hazer 2002). Usually, two different chemical

reactions have been carried out: (1) an addition reaction when unsaturated PHAs are the polymers to be modified or (2) a substitution reaction if the polymers to be functionalized are saturated hydrocarbons. In both cases the hydrolysis of the PHAs during the chlorination process is unavoidable. Once chlorinated, PHA can be converted into their quaternary ammonium salts, sodium sulphate salts and phenyl derivatives. Furthermore, Friedel–Crafts reactions between benzene and chlorinated moieties can yield cross-linked polymers (Arkin and Hazer 2002).

The determination of the melting transitions and glass-transition temperatures revealed that their values increased as a function of the chlorine content in the polymer (Arkin et al. 2000).

Very recently, production of other halogenated polymers (brominated PHAs) has been reported (Erduranli et al. 2008).

Epoxidation of Unsaturated PHAs

The introduction of epoxy groups in the structure of PHAs was performed by reacting the unsaturated side chains of these polymers {i.e. poly[(*R*)-3-hydroxyoctanoate-*co*-(*R*)-3-hydroxyundecenoate]} with *m*-chloroperbenzoic acid (Park et al. 1998a; Bear et al. 1997; Ashby et al. 2000). Epoxidized polyesters do not undergo substantial changes as regards either molecular weight or molecular weight distribution. However, a decrease in the melting transition temperature and in the enthalpy of melting was observed as the number of epoxide groups increased. Conversely, the glass-transition temperature increased in the epoxidized polymer (Park et al. 1998b).

Hydroxylation of Unsaturated PHAs

PHAs containing pendant diol groups were synthesized from unsaturated polymers {poly[(*R*)-3-hydroxyoctanoate-*co*-(*R*)-3-hydroxyundecenoate]} by treatment with $KMnO_4$ in cold alkaline solution (pH 8–9) at 20°C (Lee et al. 2000b). The chemical reaction did not cause PHA hydrolysis (or at least not to a high extent) since no severe reduction in molecular weight was observed. The hydroxylated polymers had a considerably enhanced hydrophilicity since they showed increased solubility in polar solvents such as an acetone/water mixture, methanol and dimethyl sulphoxide (Lee et al. 2000b).

Although $KMnO_4$ treatment allowed the hydroxylation of about half of the pendant vinyl groups present in the PHA, the reaction performed with 9-borobicyclononane (a hydroboration–oxidation reaction) hydroxylated most of the vinyl groups (almost 100%) present in poly[(*R*)-3-undecenoate] (Eroğlu et al. 2005).

Carboxylation of Unsaturated PHAs

Conversion of the olefinic carbons of PHAs into carboxyl groups has been achieved through different chemical reactions (Lee and Park 2000; Bear et al. 2001;

Kurth et al. 2002; Stigers and Tew 2003). One of them involves the oxidation of unsaturated PHAs {i.e. poly[(*R*)-3-hydroxyoctanoate-*co*-(*R*)-3-hydroxyundecenoate]} with $KMnO_4$ in the presence of $NaHCO_3$ (Lee and Park 2000). Although it allowed the transformation of 50% of the olefins into carboxylic functions, this method involves a decrease in the molecular weight of the polymer.

The second method of carboxylation also used $KMnO_4$ as an oxidation agent but, in this case, 18-crown-6-ether was employed as the phase transfer agent and $KMnO_4$ was employed as the dissociation agent. A reasonable retention of the molecular weight was observed when the pH of the reaction was carefully controlled (Bear et al. 2001; Kurth et al. 2002)

The third method implies the use of osmium tetroxide and Oxone, and a mixture of $2KHSO_5$, K_2SO_4 and $KHSO_4$ (Stigers and Tew 2003). With this reaction, a good yield of carboxylation is obtained; although a slight degree of backbone degradation was observed.

The transformation of olefins into polyesters containing carboxyl groups in their side chains increased the solubility of the polymers in polar solvents, showing that their hydrophilicity had been enhanced.

The production of PHAs containing carboxy-ended pendant groups has been performed through reactions which involved the conversion of the double bonds to thioethers via the free-radical addition of 11-mercaptoundecanoic acid (Hany et al. 2004) or (*R*)-3-mercaptopropionic acid. In these cases, the molecular weights of the modified polymers (despite the addition of mercapto acids to the double bonds) remained almost constant (Erduranli et al. 2008).

Glycopolymers

PHAs containing unsaturated or brominated side chains have been used as substrates for the production of glycoconjugates (derivatives of per-*O*-acetyl-1-thio-β-maltose-PHAs) via anti-Markovnikov additions. As expected, maltosyl-PHAs showed an enhanced hydrophilicity and hence better solubility and bioavailability (Constantin et al. 1999).

3.2.2 Cross-Linking of PHAs

Cross-linking is the process of the production of three-dimensional network structures from a linear polymer using chemical or physical methods. The cross-linking of polymer pendant groups has been achieved using different strategies, such as peroxide cross-linking, sulphur vulcanization and radiation-induced cross-linking. The main objective of chemical cross-linking is to improve the elastic response in bacterial elastomers without altering their biodegradability. In general, the cross-linked materials exhibited a decrease in tensile modulus, together with very low tensile strength and tear resistance. Moreover, because cross-linked PHAs are usually less sensitive to attack by PHA depolymerases, these polyesters could be used as photosensitive materials (e.g. microlithography; Kim et al. 2001b).

Peroxide Cross-Linking

Peroxide cross-linking has been successfully used to produce gels from PHAs. Peroxides are able to cross-link both saturated {e.g. poly[(*R*)-3-hydroxyoctanoate], PHO} and unsaturated {poly[(*R*)-3-hydroxyoctanoate-*co*-(*R*)-3-hydroxyundecylenate]} polymers (Gagnon et al. 1994a). *P. oleovorans* unsaturated PHAs were efficiently cross-linked (more than 90%) via a free-radical mechanism either thermally or under UV irradiation in the presence of benzoyl peroxide, benzophenone and/or ethylene glycol dimethacrylate (Hazer et al. 2001). The high degree of cross-linking achieved was owing to the presence of olefin groups in the polymer, which improved peroxide efficiency and reduced the probability of chain excision. To maintain the integrity of the polymer and to promote cross-linking versus scission chain reactions, multifunctional coagents are usually employed during peroxide cross-linking.

Sulphur Vulcanization

The cross-linking of some PHA copolymers {poly[(*R*)-3-hydroxyoctanoate-*co*-(*R*)-3-hydroxyundecenoate]} was achieved by adding a mixture of elemental sulphur or dipentamethylene thiuram hexasulphide (as a vulcanizing agent), zinc dibutyl dithiocarbamate (as an accelerator) and zinc oxide or stearic acid (as an accelerator activator; Gagnon et al. 1994b). An increase in the number of branches led to increased product elasticity. Unlike the case of peroxide cross-linking, the integrity of the material was preserved. However, sulphur vulcanization has as a disadvantage the fact that this procedure can only be used to cross-link unsaturated polymers.

Sulphur-Free and Peroxide-Free Cross-Linking

Largely unsaturated PHAs have been cross-linked by a very slow chemical procedure (it usually takes days), which implies the conversion of double bonds into epoxy groups and exposure to air (Ashby et al. 2000). Other epoxidized bacterial copolyesters, poly[(*R*)-3-hydroxyoctanoate-*co*-(*R*)-3-hydroxy-10,11-epoxyundecanoate], were cross-linked with succinic anhydride in the presence of 2-ethyl-4-methylimidazole or with hexamethylene diamine without a catalyst at 90°C (Lee et al. 1999a; Lee and Park 1999).

The cross-linking of *P. putida* unsaturated mcl-PHAs was performed by adding TiO_2 pigment powder. Later, pigmented PHA film was cured under either auto-oxidative or photo-oxidative conditions (van der Walle et al. 1999).

Radiation-Induced Cross-Linking

Although the chemical methods reported so far are effective for achieving polymer cross-linking, they have as an important disadvantage the fact that during the

reactions undesirable substances may be incorporated into the system. In contrast, the irradiation of polymeric materials avoids the addition (or generation) of contaminants to the matrix, thus allowing the production of very pure three-dimensional network structures with improved tensile strength. The cross-linking of poly[(*R*)-3-hydroxyoctanoate-*co*-(*R*)-3-hydroxyundecenoate] films by gamma irradiation has been reported by several authors (Ashby et al. 1998; Dufresne et al. 2001). Furthermore, chemical cross-linking can also be achieved by electron-beam irradiation of unsaturated PHAs. Even after cross-linking, the material was still biodegradable (De Koning et al. 1994).

3.2.3 Graft Copolymers of PHAs

Graft copolymers are polymers produced when molecular fragments belonging to others polymers have been inserted into their side chains (Hazer and Steinbüchel 2007). The condensation reaction between the carboxy-terminal groups of certain PHAs {poly[(*R*)-3-hydroxybutyrate]; poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyvalerate] or PHO} and the amine function of chitosan resulted in grafting of PHA polymers onto chitosan (Fig. 2). These grafted polymers formed opaque, viscous solutions in water and, once dried, afforded strong elastic films (Yalpani et al. 1991; Arslan et al. 2007).

Similar graft copolymers were also obtained by esterification of the carboxy-terminal groups of PHAs with cellulose hydroxyl functions. These copolyesters showed a slightly increased melting transition when compared with a physical mixture of PHB and cellulose (Yalpani et al. 1991).

UV irradiation and gamma irradiation are techniques widely used for the production of graft copolymers with improved physicochemical properties (Eroğlu et al. 1998; Jiang and Hu 2001; Kim et al. 2005c). UV irradiation of homogeneous chloroform solutions of PHO and monoacrylate–poly(ethylene glycol) (PEG), containing benzoyl peroxide as an initiator, resulted in monoacrylate–PEG–PHO-grafted copolymers. The surfaces and the bulk of the grafted copolymers became more hydrophilic as the content of monoacrylate–PEG in the copolymer increased (Kim et al. 2005c).

Furthermore, in an attempt to increase the strength of PHB, the homopolymer was immersed in isoprene solution (using heptane as a solvent) and irradiated with UV radiation. As a result, grafted copolymers showing much better ductility and strength than pure PHB were produced (Jiang and Hu 2001).

Gamma irradiation of mixtures containing either poly[(*R*)-3-hydroxynonanoate] and methyl methacrylate (Eroğlu et al. 1998) or acrylic acid and poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyvalerate] (Grondahl et al. 2005) also yielded graft copolymers with interesting properties.

In other cases, PHAs containing double bonds in the lateral pendant groups were grafted thermally using a polyazoester synthesized by the reaction of PEG and 4,4′-azobis(4-cyanopentanoyl chloride) (Hazer et al. 1999). A similar graft copolymer has also been obtained by UV irradiation of a homogeneous solution of

poly[(*R*)-3-hydroxyundecanoate] and monoacrylate–PEG (Chung et al. 2003). The presence of PEG in the graft copolymer conferred hydrophilicity to these compounds, enhancing their bioavailability.

Many other interesting chemical reactions involving the participation of different polymers, reagents, initiators and physicochemical conditions have also contributed to the production of many interesting graft copolymers (Hazer 1994, 1996; Ilter et al. 2001; Kim et al. 2002).

3.2.4 Block Copolymers of PHAs

Block copolymers are chemically synthesized polymers in which molecular fragments belonging to others polymers have been inserted into the backbones of a particular one (Hazer and Steinbüchel 2007). The aim is to obtain new PHAs with improved mechanical properties with respect to natural PHAs.

The synthesis of these compounds involves the participation of PHB derivatives (low molecular weight telechelic hydroxylated PHBs – PHB-diol) and other polyesters or polyurethanes (Reeve et al. 1993; Hirt et al. 1996b; Andrade et al. 2002a, b).

Atactic PHB segments containing primary hydroxy ends (PHB-diol) are usually produced by ring-opening polymerization of β-butyrolactone (Arslan et al. 1999), whereas PHBs with a structure similar to that of natural isotactic PHB were produced through the regioselective ring-opening polymerization of (*S*)-β-butyrolactone (Jedlinski et al. 1998). These compounds showed higher solubility than natural PHBs, but lacked crystallinity.

Block copolymers containing PHB and diethylene glycol were obtained by means of transesterification reactions using low molecular weight telechelic hydroxylated PHBs, diethylene glycol and *p*-toluene sulphonic acid as a catalyst (Hirt et al. 1996a).

A multiblock polymer containing PHB and PEG as block segments was produced by copolymerization of a low molecular weight PHB-diol (generated by alcoholysis of a high molecular weight bacterial PHB) and PEG in the presence of hexamethylene diisocyanate as a coupling agent (Li et al. 2005b; Zhao and Cheng 2004). A triblock copolymer containing the same partner components (PHB–PEG–PHB) was also synthesized by ring-opening polymerization of (*R*,*S*)-β-butyrolactone using PEG-based macroinitiators. The copolymer obtained was an amorphous solid that showed improved solubility with respect to pure PHB (Kumagai and Doi 1993; Shuai et al. 2000).

A PHB–monomethoxy PEG diblock copolymer was obtained through a transesterification reaction using PHB, PEG methyl ether and bis(2-ethylhexanoate) tin catalyst. The copolymers synthesized were amphiphilic and in water solutions formed colloidal suspensions which turned into PHB crystalline lamellae (Ravenelle and Marchessault 2002).

A copolyester containing PHB and PHO blocks was synthesized through polycondensation of PHB-diol and telechelic hydroxylated PHO (PHO-diol) in the presence of terephthaloyl chloride. The incorporation of the soft PHO block

together with the hard PHB block in a block copolymer, improved the mechanical properties of pure PHB (Andrade et al. 2002b, 2003).

Interestingly, PHA–poly(ethylene oxide) diblock copolymers were synthesized by *P. oleovorans* when cultured in fermentation media supplied with poly(ethylene oxide) (Ashby et al. 2002).

An amphiphilic triblock copolymer consisting of poly(ethylene oxide) and PHB as the hydrophilic and the hydrophobic blocks, respectively, was synthesized by coupling two chains of methoxy–poly(ethylene oxide)–monocarboxylic acid with a PHB-diol in the presence of 1,3-*N*,*N*′-dicyclohexylcarbodiimide (Li et al. 2003, 2005a). This compound shows better thermal stability than its respective precursors, undergoes thermal degradation in two separate steps, and has a strong tendency to form micelles in aqueous solution (Li et al. 2003, 2005a).

Hirt et al. (1996b) reported the synthesis and characterization of block copolyesterurethanes containing telechelic hydroxy-terminated poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyvalerate)] as the *hard segment* (crystallizable chain fragment) and poly(ε-caprolactone) or poly[(adipic acid)-*alt*-(1,4-butanediol; diethylene glycol; ethylene glycol)] as the *soft segment* (biodegradable fraction). A similar semicrystalline diblock copolymer {poly[(*R*)-3-hydroxyoctanoate]-*block*-poly(ε-caprolactone)}, in which PHO constituted the elastomeric soft segment and poly(ε-caprolactone) formed a more crystalline hard segment, was described by Timbart et al. (2004).

3.2.5 Blending of PHAs with Other Polymers

Polymer blends are physical mixtures of homopolymers or copolymers with different structures and properties (Verhoogt et al. 1994; Avella et al. 2000). Usually, blended polymers are formed by integration of a natural polymer (PHB or a derivative) and one or more synthetic ones, which improves or modifies some of the properties of the natural polymer. The techniques commonly employed to produce blended polymers are those based on either melt blending (direct mixing of component polymers in a molten state) or solution blending. The hydrophilicity, miscibility, crystallization behaviour, thermal characteristics, mechanical properties, polymer morphology and biodegradation rates of PHAs containing blends are largely influenced by the nature of the different components (Verhoogt et al. 1994; Ha and Cho 2002). In sum, blending procedures have allowed the production of many different PHA-containing mixtures with special characteristics and broad applications (Ha and Cho 2002). PHA blends are included in two different groups depending on the biodegradability of the mixtures: totally biodegradable blends and non-totally biodegradable blends.

Totally Biodegradable Blends

Totally biodegradable blends are polymer mixtures containing PHAs and another (or other) biodegradable polymer(s) as components. The blending of PHAs with certain biodegradable polymers usually improves the biocompatibility and the

biodegradability of the pure PHA (Verhoogt et al. 1994), thus increasing the medical applications. Different fully biodegradable PHA-containing blends have been studied. These include mixtures of PHB with cellulose derivatives (cellulose–acetate–butyrate and cellulose–acetate–propionate) (Scandola et al. 1992; Ceccorulli et al. 1993; Pizzoli et al. 1994), starch (Willet and Shogren 2002; Godbole et al. 2003), starch–acetate (Zhang et al. 1997a), PLA (Blumm and Owen 1995), poly(D,L-lactide) (Zhang et al. 1997b), poly(ε-caprolactone) (Kumagai and Doi 1992a, b; Lisuardi et al. 1992), poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyvalerate] (Kumagai and Doi 1992a; Owen et al. 1992; Organ and Barham 1993; Organ 1994; Barham and Organ 1994; Satoh et al. 1994; Yoshie et al. 2004), poly(6-hydroxyhexanoate) (Abe et al. 1994a), PHO (Horowitz and Sanders 1994; Dufresne and Vincendon 2000), poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyhexanoate] (Zhao et al. 2003), poly(β-propiolactone) (Kumagai and Doi 1992a), synthetic PHB (Pearce et al. 1992, 1994; Abe et al. 1994a, b, 1995; Pearce and Marchessault 1994), PHB-diol (Saad 2002), synthetic poly[(*R*)-3-hydroxypropionate] (Cao et al. 1998), poly(ε-caprolactone-*co*-lactide) (Koyama and Doi 1996), poly(vinyl alcohol) (Yoshie et al. 1995; Azuma et al. 1992), and poly(ethylene oxide) (Avella and Martuscelli 1988; Avella et al. 1991, 1993; Kumagai and Doi 1992b). The blending of PHO with PLA or PEG has also been reported (Renard et al. 2004).

Non-Totally Biodegradable Blends

This group of PHAs includes mixtures of polymers formed from a biodegradable polyester (usually PHB) and one or more non-biodegradable polymers (often chemically synthesized polymers). The presence of a non-biodegradable polymer in the blend usually restricts the access of hydrolytic enzymes (i.e. PHA depolymerases) to the biodegradable partner chain, thus preventing weight loss and increasing the stability of the polymeric mixture (Verhoogt et al. 1994). Examples of these polymers include blends of PHB with poly(vinyl acetate) (Greco and Martuscelli 1989; Kumagai and Doi 1992a, b), poly(epichlorohydrin) (Dubini et al. 1993; Sadocco et al. 1993), poly(vinylidene fluoride) (Liu et al. 2005), poly(methyl methacrylate) (Lotti et al. 1993), ethylene–propylene rubber (Greco and Martuscelli 1989; Abbate et al. 1991), ethylene–vinyl acetate (Abbate et al. 1991), poly(1,4-butylene adipate) (Kumagai and Doi 1992a), poly(vinylphenol) (Iriondo et al. 1995; Xing et al. 1997) or poly(cyclohexyl methacrylate) (Lotti et al. 1993).

For further information about blended polymers, their properties and applications, see the reviews by Ha and Cho (2002) and Yu et al. (2006).

4 Biotechnological Applications

Although high fermentation costs have hindered the use of PHAs as commodity materials, the development of a huge variety of PHAs, which differ in both monomer composition and physicochemical properties, has expanded the potential applications

of these polymers. Usual PHAs and UnPHAs are being used to obtain new products with high added value that can be applied in medicine (for making materials such as sutures, stents, implants, cardiovascular patches, slings, nerve guides, articular cartilage substitutes, tendon repair devices, wound dressing materials, bone-marrow scaffolds, carriers for bone morphogenetic proteins, bone fracture fixation systems, orthopaedic pins, and cell-growth-supporting materials; Sevastianov et al. 2003; Zheng et al. 2003; Sudesh 2004; Chen and Wu 2005; Valappil et al. 2006; Hazer and Steinbüchel 2007; Li et al. 2008), in pharmacy (as activators of the coagulation systems and the complementary reaction, as vehicles for drugs with antitumoral activity, as radiopotentiators, as chemopreventive, analgesic, antihelmintic, and nitric oxide synthase inhibitors, etc.; Olivera et al. 2001a; Sevastianov et al. 2003); in industry (as alternative sources to natural rubber; Mooibroek and Cornish 2000; van der Walle et al. 2001; Luengo et al. 2001; Sudesh 2004; Prieto et al. 2007; Bhatt et al. 2008; Li et al. 2008) as well as in other emerging fields of research (the tailoring of bioparticles for use in nanotechnology, the manufacture of biotags for protein purification, the design of new insecticides, production of composites for use in tissue engineering applications, etc.; Moldes et al. 2004; Misra et al. 2006; Rehm 2007; Li et al. 2008; Yao et al. 2008). The properties discovered in novel PHAs, such as polythioesters (high thermal stability and putative antibacterial activity) could broaden the medical applications of these compounds (Lütke-Eversloh and Steinbüchel 2004). Furthermore, taking into account that PHAs can be synthesized from many different carbon sources (crop plants, bagasse hydrolysates, paper mill waste water, whey, municipal waste water, sugar cane molasses, animal fats and vegetable oils, etc.; Ashby and Foglia 1998; Coats et al. 2007; Bengtsson et al. 2008; Koller et al. 2008; Mengmeng et al. 2008; van Beilen and Poirier 2008; Yu and Stahl 2008), PHA-producer organisms could play important ecological roles since they can be used to eliminate contaminants (Sudesh et al. 2007) or to recycle different industrial residues, even those that are toxic or dangerous for many species (Goff et al. 2007; Nikodinovic et al. 2008).

5 Concluding Remarks and Future Outlook

In view of the huge variety of PHAs reported in this review, it is evident that these polyesters not only represent a hopeful alternative to replace traditional plastics, but also, owing to their special properties and physicochemical characteristics, they offer a plethora of potential biotechnological applications (including ecological, medical, pharmaceutical and industrial uses). Additionally, the description of new PHA-producer strains isolated from unexplored (or until now scarcely studied) habitats (Ayub et al. 2009; Boiandin et al. 2008; De Lorenzo 2008; Kalyuzhnaya et al. 2008; Simon-Colin et al. 2008a, b; Singh and Mallick 2008; Yan et al. 2008), the design of novel methods for the study and detection of these polyesters even in mixed microbial populations (Berlanga et al. 2006; Monteil-Rivera et al. 2007; Russell et al. 2007; Dias et al. 2008; Foster et al. 2008; Grubelnik et al. 2008; Serafim et al. 2008; Werker et al. 2008) as well as recent advances in genetic,

metagenomic and metabolic engineering (Olivera et al. 2001a, b; Cowan et al. 2005; Solaiman and Ashby 2005; Kung et al. 2007; Sandoval et al. 2007; Tsuge et al. 2007; Velázquez et al. 2007; Dias et al. 2008; Kalyuzhnaya et al. 2008; Ruth et al. 2008) together with new approaches based on chemical synthesis and blending processes (Hazer and Steinbüchel 2007) will undoubtedly expand the number of PHAs that will become available in the near future. Furthermore, the fact that these polymers can be synthesized from different bacteria (Gram positive, Gram negative, aerobic or anaerobic), yeast and plants (Mittendorf et al. 1998; Terentiev et al. 2004; Zhang et al. 2006; Suriyamongkol et al. 2007; Ciesielski et al. 2008) when cultured under different nutrient and environmental conditions could allow advantage to be taken of many raw materials and their use in the elimination of industrial wastes (or subproducts) in different countries, thus facilitating the economic and industrial development of many areas of the planet.

In sum, although PHAs were discovered in 1925 (Lemoigne 1926), today, almost a century later, these compounds are one of the most promising biomaterials, as may be corroborated by reading the different chapters in this volume.

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Metabolic Engineering of Plants for the Synthesis of Polyhydroxyalkanaotes

Yves Poirier and Stevens M. Brumbley

Contents

Abstract Synthesis of polyhydroxyalkanoates (PHAs) in crop plants is viewed as an attractive approach for the production of this family of biodegradable plastics in large quantities and at low costs. Synthesis of PHAs containing various monomers has so far been demonstrated in the cytosol, plastids, and peroxisomes of plants. Several biochemical pathways have been modified to achieve this, including the isoprenoid pathway, the fatty acid biosynthetic pathway, and the fatty acid β-oxidation pathway. PHA synthesis has been demonstrated in a number of plants, including monocots and dicots, and up to 40% PHA per gram dry weight has been demonstrated in *Arabidopsis thaliana*. Despite some successes, production of

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PHAs in crop plants remains a challenging project. PHA synthesis at a high level in vegetative tissues, such as leaves, is associated with chlorosis and reduced growth in some plants. The challenges for the future are to succeed in the synthesis of PHA copolymer with a narrow range of monomer composition, at levels that do not compromise plant productivity, and in creating methods for efficient and economical extraction of polymer from plants. These goals will undoubtedly require a deeper understanding of plant biochemical pathways as well as advances in biorefinery.

1 Introduction

Despite the interesting properties of polyhydroxyalkanoates (PHAs) as biodegradable thermoplastics and elastomers, use of these bacterial polyesters as substitutes for petroleum-derived plastics remains a challenge, in part owing to the costs related to bacterial fermentation, making the synthesis of most PHAs substantially more expensive than that of commodity polymers, such as polyethylene. It is in the context of producing PHAs on a larger scale and at lower cost than is possible by bacterial fermentation that production of these polymers in plants, and particularly agricultural crops, is viewed as a promising approach (Brumbley et al. 2008; Poirier 1999; Poirier and van Beilen 2008; Suriyamongkol et al. 2007). Synthesis of PHAs in crops also fits into a larger concept of using plants as vectors for the renewable and sustainable synthesis of carbon building blocks that are currently almost exclusively provided by the petrochemical industry.

Synthesis of PHA in plants was first demonstrated in 1992 by the accumulation of poly[(*R*)-3-hydroxybutyrate] (PHB) in the cytoplasm of cells of *Arabidopsis thaliana* (Poirier et al. 1992b). Since then, a range of different PHAs have been synthesized, including various copolymers such as poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyvalerate] [P(HB–HV)] and medium-chain-length (MCL) PHAs, in a variety of plants, including corn, sugarcane, and switchgrass (Table 1; Poirier and van Beilen 2008). This has been achieved through the modification of various pathways localized in different subcellular compartments, such as the fatty acid and amino acid biosynthetic pathways in the plastid or the fatty acid degradation pathway in the peroxisome. Although the initial driving force behind synthesis of PHA in plants has been for the biotechnological production of biodegradable polymers, PHA synthesis in plants has also been used as a useful tool to study some fundamental aspects of plant metabolism (Poirier 2002).

2 Polyhydroxybutyrate

2.1 Synthesis of Polyhydroxybutyrate in the Cytoplasm

PHB is the most widespread and thoroughly characterized PHA found in bacteria. Despite its relative poor physical properties as a thermoplastic, PHB was initially targeted for production in plants. This is because the first bacterial PHA biosynthetic

Table 1 Summary of polyhydroxyalkanoates (PHA) produced in transgenic plants

PHA type	Specie	Tissue	Organelle	PHA (% dwt)	Reference
PHB	*Arabidopsis thaliana*	Shoot	Cytoplasm	0.1	Poirier et al. (1992b)
PHB	*A. thaliana*	Shoot	Plastid	40	Bohmert et al. (2000), Nawrath et al. (1994)
PHB	Oilseed rape	Shoot	Cytoplasm	0.1	Poirier and Gruys (2001)
PHB	Oilseed rape	Seed	Plastid	8	Houmiel et al. (1999)
PHB	Tobacco	Shoot	Cytoplasm	0.01	Nakashita et al. (1999)
PHB	Tobacco	Shoot	Plastid	<1.7	Arai et al. (2001), Bohmert et al. (2002), Lössl et al. (2003, 2005)
PHB	Corn	Shoot	Plastid	6	Poirier and Gruys (2001)
PHB	Corn	Cell suspension	Peroxisome	2	Hahn et al. (1999)
PHB	Alfalfa	Shoot	Plastid	0.2	Saruul et al. (2002)
PHB	Cotton	Fiber cells	Cytoplasm	0.3	John and Keller (1996)
PHB	Potato	Shoot	Plastid	0.01	Bohmert et al. (2002)
PHB	Sugar beet	Hairy root	Plastid	5.5	Menzel et al. (2003)
PHB	Sugarcane	Leaves	Plastid	1.9	Petrasovits et al. (2007)
PHB	Sugarcane	Stalks	Plastid	0.003	Petrasovits et al. (2007)
PHB	Switchgrass	Leaves	Plastid	3.7	Somleva et al. (2008)
PHB	Flax	Stem	Plastid	0.005	Wrobel et al. (2004)
PHB	Sugarcane	Leaves	Peroxisome	>1.0	Tilbrook et al. (2008)
P(HB–HV)	*A. thaliana*	Shoot	Plastid	1.6	Slater et al. (1999)
P(HB–HV)	*A. thaliana*	Whole plants	Cytoplasm	0.6	Slater et al. (1999)
P(HB–HV)	Oilseed rape	Seed	Plastid	2.3	Slater et al. (1999)
MCL-PHA[a]	*A. thaliana*	Whole plants	Peroxisome	0.6	Mittendorf et al. (1998)
MCL-PHA[a]	*A. thaliana*	Seed	Peroxisome	0.1	Moire et al. (2004), Poirier et al. (1999)
MCL-PHA[b]	Potato	Cell line	Cytoplasm	1	Romano et al. (2003)
MCL-PHA[c]	Potato	Shoot	Plastid	0.03	Romano et al. (2005)
SCL–MCL-PHA[d]	*A. thaliana*	Whole plants	Peroxisome	0.04	Arai et al. (2002), Matsumoto et al. (2005, 2006)
SCL–MCL-PHA[d]	Sugarcane	Leaves	Peroxisome	Not determined	Anderson et al. (2008)

dwt dry weight, *PHB* poly[(*R*)-3-hydroxybutyrate], *P(HB–HV)* poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyvalerate], *MCL* medium chain length, *SCL* short chain length

[a]PHA contained 3-hydroxyacid monomers from 6 to 16 carbons

[b]PHA contained only 3-hydroxyoctanoic acid

[c]PHA contained 3-hydroxyacid monomers from 6 to 12 carbons

[d]PHA contained 3-hydroxyacid monomers from four to eight carbons

genes cloned were for PHB synthesis from the bacterium *Ralstonia eutropha* (Schubert et al. 1988; Slater et al. 1988) and because this is one of the simplest pathways for production of a PHA. The cytoplasm was targeted as the first site for PHB synthesis because, in addition to containing acetyl-CoA, the building block for PHB, it also had the advantage that the bacterial enzymes could be directly expressed in this compartment without any modification of the proteins. Furthermore, an endogenous plant (*R*)-3-ketothiolase is present in the cytoplasm as part of the mevalonate pathway. Thus, creation of the PHB biosynthetic pathway in the cytoplasm was theoretically simpler, requiring only the expression of two additional enzymes (Fig. 1). The *R. eutropha phaB* and *phbC* genes, encoding, respectively, the acetoacetyl-CoA reductase and PHA synthase, were coexpressed in *A. thaliana* under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter, allowing a relatively high expression of the enzymes in a broad range of tissues (Poirier et al. 1992b). The highest amount of PHB measured in the shoots of these plants was approximately 0.1% dry weight (dwt). Detailed analysis of the PHB purified from *A. thaliana* confirmed that polymer was isotactic PHB and that the thermal properties of plant PHB were similar to those of bacterial PHB (Poirier et al. 1995). Furthermore, PHB accumulated in the form of granules that had a size and appearance very similar to those of bacterial PHB granules (Poirier et al. 1992b).

Plants expressing a high level of acetoacetyl-CoA reductase in the cytoplasm have shown a strong reduction in growth, with the most affected plants being approximately 5 times smaller by fresh weight (fwt) compared with wild-type plants (Poirier et al. 1992a). There was an overall good correlation between the extent of the growth reduction and the level of reductase enzyme activity. Although no abnormal phenotype was observed in plants expressing only the PHB synthase (and not producing PHB), combination of the acetoacetyl-CoA reductase with the PHB synthase led to a further reduction in growth compared with plants expressing only the reductase (Poirier et al. 1992a). Although the reasons for the dwarf

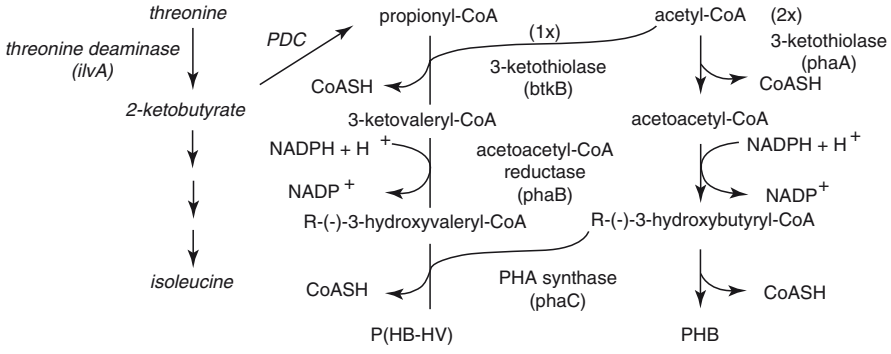

Fig. 1 Pathways of poly[(*R*)-3-hydroxybutyrate] and poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyvalerate] synthesis in plants. The pathways common to bacteria and transgenic plants are shown in *plain letters*, and the pathway specific to transgenic plants is shown in *italics*. *PDC* refers to the plant endogenous pyruvate dehydrogenase complex

phenotype have not been unambiguously determined, it is likely that the diversion of cytoplasmic acetyl-CoA and acetoacetyl-CoA away from the endogenous isoprenoid and flavonoid pathways might lead to a depletion of essential metabolites, such as sterols, which may affect growth. This is further supported by work showing that even a slight decrease in the expression of the *Arabidopsis* ATP-citrate lyase, which synthesizes cytosolic acetyl-CoA, resulted in severe stunting (Fatland et al. 2005).

Synthesis of PHB in the cytoplasm of rape leaf cells gave results very similar to *Arabidopsis* (Poirier 2002). Interestingly, overexpression of the bacterial (*R*)-3-ketothiolase in plants expressing the reductase and PHB synthase did not lead to a significant increase in PHB accumulation, indicating that (*R*)-3-ketothiolase activity was probably not limiting PHB synthesis in the cytoplasm, but rather that other factors, such as the low flux of acetyl-CoA, may be important.

Production of cytosolic PHB has been reported in leaves of *Nicotiana tabacum* through the coexpression of the *phaB* gene from *R. eutropha* and the PHA synthase from *Aeromonas caviae* (Nakashita et al. 1999). Although the bacterial genes were expressed under the strong promoter CaMV35S, expression of both proteins was relatively low and the amount of PHB detected in leaves was only 10 μg g^{-1} fwt. Inhibition of the mevalonate pathway at the level of the 3-hydroxy-3-methylglutaryl-CoA reductase led to a twofold increase in PHB level in tobacco cell lines, indicating a link between PHB synthesis and availability of acetyl-CoA (Suzuki et al. 2002). Similar low levels of PHB were obtained in potato expressing the phb enzymes in the cytosol (Poirier 2002). Transient expression of the three phb biosynthetic proteins has recently been demonstrated in in vitro bombarded slices of oil palm mesocarp tissues, although evidence for polymer production is still lacking (Omidvar et al. 2008). The oil palm mesocarp is the site for storage oil synthesis and oil palm is the most productive oil crop in the world. Although these first experiments on oil palm were done with cytosolic constructs, taking full advantage of the oil palm carbon flux through lipids for PHA synthesis will require directing the PHA pathway to the plastid.

Production of PHB in sugarcane cytosol has been tested using simultaneous biolistic transformation with the *phaA*, *phaB*, and *phaC* genes from *R. eutropha*. Out of 95 lines, only two were found to have any PHB and then it was at trace levels. The presence of all three enzymes was confirmed by western blot analysis (Petrasovits et al. 2007).

PHB synthesis has also been demonstrated in the cytoplasm of cells of cotton fiber cells (John 1997; John and Keller 1996). In this approach, PHA is not produced as a source of polyester to be extracted and used in the plastic industries, but rather as an intracellular agent that modifies the heat-exchange properties of the fiber. The *phaA*, *phaB*, and *phaC* genes from *R. eutropha* were expressed in transgenic cotton under the control of a fiber-specific promoter (John 1997; John and Keller 1996). PHB accumulated in the cytoplasm to 0.3% dwt of the mature fiber, a level similar to that for PHB production in *A. thaliana* cell cytoplasm, yet high enough to change the heat retention dynamic of the fiber, but no deleterious effect on fiber development was reported.

2.2 Synthesis of Polyhydroxybutyrate in the Plastid

The relatively limited supply of acetyl-CoA in the cytosol is thought to be responsible for the low accumulation of PHB as well as for the deleterious effects of transgene expression on plant growth observed in many plants. In this context, the plastid was viewed as a much better site for PHB synthesis, since this organelle has a larger flux of carbon through acetyl-CoA required for fatty acid biosynthesis. This is particularly true for the leucoplast of developing seeds of oil-accumulating plants, such as *Arabidopsis* and oilseed rape, or the mesocarp of oil palm fruits.

The phaA, phaB, and phaC proteins from *R. eutropha* were modified for plastid targeting by addition of the transit peptide of the small subunit of the ribulose bisphosphate carboxylase from pea (Nawrath et al. 1994). The modified bacterial genes were first expressed individually in *A. thaliana* under the control of the constitutive CaMV35S promoter, and later the transgenes were combined through crossings. Transgenic plants expressing only the plastid-targeted reductase and PHA synthase did not produce detectable PHB, providing further evidence that plastids do not have an endogenous 3-ketothiolase activity that could support PHB synthesis (Nawrath et al. 1994). However, plants expressing all three bacterial enzymes were shown to accumulate PHB inclusions exclusively in the plastids, with some organelles having a substantial portion of their volume filled with inclusions. The size and general appearance of these inclusions were similar to those of bacterial PHA inclusions (Nawrath et al. 1994). Interestingly, the quantity of PHB in these plants was found to gradually increase over time, with fully expanded presenescing leaves typically accumulating 10 times more PHB than young expanding leaves of the same plant. The maximal amount of PHB detected in presenescing leaves was 10 mg g^{-1} fwt, representing approximately 14% dwt. In contrast to PHB synthesis in the cytoplasm, expression of the PHB biosynthetic enzymes in the plastid was not accompanied by a large reduction in growth of these plants. However, leaf chlorosis was observed in plants accumulating more than 3–5% dwt. These results indicated that although the plastid can accommodate a higher production of PHB with minimal impact on plant growth compared with the cytoplasm, there was nevertheless a limit above which alteration of some of the chloroplast functions could be detected (Nawrath et al. 1994).

In contrast to the individual expression of the *R. eutropha phb* genes in plants followed by stacking through crossing, an alternative strategy was devised where all three plastid-modified *phb* genes were cloned into a single binary vector. By this approach, a number of lines were identified which accumulated PHB between 3 and 40% dwt (Bohmert et al. 2000). Whereas in a line accumulating 3% dwt most of the plastids contained some PHB inclusions, all plastids of mesophyll cells were packed with inclusion in the line containing PHB to 40% dwt. Interestingly, these transgenic plants showed a negative correlation between PHB accumulation and plant growth. Whereas plants containing 3% dwt PHB showed only a relatively small reduction in growth, plants accumulating between 30 and 40% dwt PHB were dwarf and produced no seeds (Bohmert et al. 2000). As previously observed by

Nawrath and colleagues, all plants producing above 3% dwt PHB showed some chlorosis (Bohmert et al. 2000; Nawrath et al. 1994). Together, these experiments demonstrate that although it is possible to further increase PHB production in plastids using new vectors, the approach of synthesizing PHB in the chloroplasts of shoots has its constraints.

Since the production of PHA in the plastid typically requires the expression of several enzymes, strategies devised to simplify the number of individual genes that must be expressed could have advantages. In this respect, a novel fusion protein composed of the 3-ketothiolase and acetoacetyl-CoA reductase from *R. eutropha* was created (Kourtz et al. 2005). This was a challenging project since the native thiolase and reductase enzymes act as homotetramers in bacteria. Nevertheless, one fusion protein exhibited thiolase and reductase activities in crude extracts of recombinant *Escherichia coli* that were only threefold and ninefold less than those of the individually expressed thiolase and reductase enzymes, respectively. Expression in *A. thaliana* of the plastid-targeted fusion enzyme, along with the plastid-targeted PHA synthase, resulted in plants accumulating roughly half the amount of PHB synthesized in plants expressing the individual enzymes (Kourtz et al. 2005).

To avoid the problems of stunted growth and chlorotic lesions associated with high PHB synthesis in transgenic plants expressing the gene constructs expressed under a strong constitutive promoter, a strategy was explored whereby the *phb* biosynthetic genes were expressed under the control of a chemically inducible promoter (Kourtz et al. 2007). In this approach, expression of the *phb* biosynthetic genes was delayed until a later stage of growth, once plant growth is well established. Expression in *A. thaliana* of the three plastid-modified *R. eutropha phb* genes under a promoter activated by a commercial ecdysone analog showed that whereas PHB synthesis could be effectively regulated by the chemical inducer, PHB accumulation was highest in the young leaves that were exposed to the inducer at an earlier stage of growth, and that high PHB accumulation remained associated with chlorosis (Kourtz et al. 2007).

The work described above was performed in the model plant *A. thaliana*, but numerous efforts have been made to bring the technology of PHA synthesis to crop plants. Among the first to attempt this were scientists at Monsanto, who have demonstrated the production of PHB in the plastids of corn leaves and stalk, as well as in the leucoplast of developing seeds of *Brassica napus*. In these experiments, the same *R. eutropha* genes modified for PHB production in the plastids of *A. thaliana* were used. Levels of PHB accumulation in corn leaves up to 5.7% dwt were reported (Poirier and Gruys 2001). Similar to the results obtained in *A. thaliana*, there was a progressive accumulation of PHB in corn shoots with time, with older leaves having more polymer than younger leaves. Furthermore, like *A. thaliana*, there was a correlation between leaf chlorosis and higher amount of PHB (Poirier and Gruys 2001). Perhaps one of the most striking observations made from the experiments in corn was the fact that whereas the leaf mesophyll cells showed few PHB granules, the bundle sheath cells associated with the vascular tissue were packed with granules. This unequal distribution of PHB was not due to the promoter used, since a similar pattern was seen for plants transformed with either the

CaMV35S or the chlorophyll A/B binding protein promoters, the latter promoter being known to be a strong promoter in mesophyll cells. Interestingly, a similar observation was made by the same group for *A. thaliana* plants transformed with the *phb* genes driven by the CaMV35S promoter, i.e., significantly more granules were found in cells surrounding the vascular tissue and epidermal cells compared with mesophyll cells (Poirier and Gruys 2001). These results suggest that the availability of plastidial acetyl-CoA for PHB synthesis may be quite different in various cell types, perhaps owing to metabolic channeling.

For the creation of the PHB biosynthetic pathway in developing seeds of *B. napus*, the three modified bacterial genes *phaA*, *phaB*, and *phaC* were put under the control of the fatty acid hydroxylase promoter from *Lesquerella fendeleri*, enabling strong expression for the developing seed (Houmiel et al. 1999). A PHB level up to 7.7% fwt of mature seeds was reported. Analysis of seeds by transmission electron microscopy revealed that PHB accumulated exclusively within the leucoplast, and that apparently every visible plastid contained the polymer. Seeds accumulating nearly 8% dwt PHB appeared normal and germinated at the same rates as nontransformed seeds (Houmiel et al. 1999). These results demonstrate that at least in the range 3–8% dwt PHB, the seed leucoplast appears a better production system than the leaf chloroplast. It is unknown at this point what the upper limit of PHB accumulation in seeds is and at what level PHB synthesis will start affecting the accumulation of lipids or proteins in the seed, two key factors that would impact on the viability of this approach in the biotechnological production of PHA in oilseed crops.

The first report of PHB production in a high biomass crop (more than 100 tons of biomass per hectare per annum) was in a *Saccharum* sp. (sugarcane). Each gene, *phaA*, *phaB*, and *phaC* from *R. eutropha,* was driven by the maize polyubiquitin promoter (ubi1) and *nos* terminator sequences and was cloned on a separate transformation vector. The three vectors were biolistically transformed into sugarcane callus simultaneously along with a construct containing the selectable marker gene *npt*II (Petrasovits et al. 2007). In sugarcane, the polymer accumulated in the leaves of chloroplast-targeted lines at levels up to 2.2% dwt and 0.01% in stems (Petrasovits et al. 2007). Purnell et al. (2007) conducted a replicated glasshouse trial using a random block design with six independent PHB-producing transgenic sugarcane lines and found that stalk height and weight and sugar levels were not affected by PHB accumulation. The ubi1 promoter is constitutively expressed and the PHB accumulation patterns reflected this. The highest PHB concentrations were found in the tips of the oldest leaf, with a gradient running from the leaf tip to base and a gradient running from leaves at the base of the plant to those at the top (Purnell et al. 2007).

In addition to the high-biomass crop sugarcane, PHB production has also been successful in the energy crop *Panicum virgatum* (switchgrass). To engineer switchgrass to produce PHB, a modified biosynthetic pathway was used where *phaA* and *phaB* were from *R. eutropha* and *phaC* was from a hybrid of *Pseudomonas oleovorans/Zoogloea ramigera* (Somleva et al. 2008). All three genes were combined together into a single binary vector each driven by its own promoter and transformed

into switchgrass via *Agrobacterium*. Two different promoters were tested, the constitutive rice ubiquitin 2 (rubi2) and the light-activated maize chlorophyll A/B binding protein promoter cab-m5. Similar to maize (Poirier and Gruys 2001) and sugarcane (Petrasovits et al. 2007), also plants that use the C_4 photosynthetic pathway, the highest accumulation of PHB granules appears to be in the chloroplast bundle sheath cells. However, unlike maize or sugarcane, some PHB accumulation was detectable in the chloroplasts of mesophyll cells of switchgrass. As with sugarcane, the highest PHB levels were found to accumulate in the older, mature leaves; however, the PHB levels (3.72% leaf dwt) were twice the PHB level of sugarcane (Petrasovits et al. 2007; Purnell et al. 2007). Whether this is because of all three genes being on same construct, the lower copy number because of *Agrobacterium*, the cab-5 promoter, the hybrid synthase, or a combination of some or all of these has yet to be determined in sugarcane.

Five additional crop plants have also been investigated for PHA production through expression of the PHB pathway in the plastid. Transformation of alfalfa, tobacco, potato, and flax with the three *R. eutropha* phb genes modified for plastid targeting were shown to give transgenic plants producing PHB in their leaves to a maximum level of 0.18, 0.32, 0.009, and 0.005% dwt (Bohmert et al. 2002; Saruul et al. 2002; Wrobel et al. 2004). Although the reasons behind the low level of PHB accumulation in these plants compared with *Arabidopsis*, corn, or sugarcane have not been fully elucidated, it has been demonstrated that constitutive expression of the bacterial 3-ketothiolase leads to a large decrease in the recovery of transgenic plants following transformation (Bohmert et al. 2002). The use of a construct where the bacterial 3-ketothiolase is expressed under the control of an inducible promoter led to an increased recovery of transgenic tobacco and potato producing PHB, although the amount of PHB produced remained relatively low at below 0.3% dwt (Bohmert et al. 2002). Transformation of in vitro cultured hairy roots of sugar beet with the same three *R. eutropha* genes modified for plastid targeting led to a significantly higher amount of PHB, with a maximum of 5.5% dwt (Menzel et al. 2003). In conclusion, although accumulation of PHB in the plastid appears to be problematic for several plants, the success encountered with *Arabidopsis*, rape, corn, sugarcane, switchgrass, and roots of sugar beet indicates that there is no fundamental barrier to relatively high production of PHA in the plastids of a number of crops encompassing either monocots or dicots. Like the synthesis of PHB in cotton fiber cytosol, accumulation of a low amount of PHB in the plastids of flax and cotton fiber was reported (John 1997; Wrobel-Kwiatkowska et al. 2007). For flax, PHB accumulation appeared to substantially modify the properties of the fibers, although it is unclear if these modifications are exclusively due to PHB accumulation or could also be due to secondary modification to plant metabolism affecting cell wall or lignin synthesis (Wrobel-Kwiatkowska et al. 2007).

As an alternative strategy to the transformation of the nuclear genome, transformation of the plastid genome with the *phb* gene has been examined. In theory, plastid transformation could lead to a higher level of polymer production because of the much larger copy number of transplastome compared with the nuclear genome. However, transformation of tobacco plastome with the *R. eutropha* polycistronic

operon containing the *phbA*, *phbB*, and *phbC* genes under the control of a bacterial promoter or of the plastid ribsomal RNA promoter (*Prrn*) has yielded plants synthesizing only a low amount of PHB (less than 0.1% PHB per gram dwt; Arai et al. 2001, 2004; Nakashita et al. 2001). Expression of the *R. eutropha* polycistronic operon under the control of the plant *psbA* promoter and the *psbA* 5′ untranslated region improved PHB accumulation up to 1.7 % dwt (Lössl et al. 2003). In these transgenic plants, a higher level of PHB was limited to the early stage of heterotrophic in vitro culture and the level decreased through autotrophic growth despite constant transcript levels. PHB amounts were also found to be highly variable in different tissues of the same plant. Furthermore, production of PHB in transplastomic tobacco was associated with growth retardation and male sterility (Lössl et al. 2003). Use of a transformation system where the plastidial polycistronic phb operon was under the control of an ethanol-inducible T7 RNA polymerase could solve the problem of growth retardation and sterility, but without further improvement in the yield of PHB (Lössl et al. 2005). Interestingly, transplastomic expression of only the 3-ketothiolase was associated with male sterility, again linking overexpression of the 3-ketothiolase with disruption of plant cell growth (Ruiz and Daniell 2005). Although further work is required to understand the factors limiting the stable production of PHB in transplastomic tobacco, it must be stressed that accumulation of PHA in tobacco and potato, either in the cytoplasm or in the plastid, has consistently been low compared with that in *Arabidopsis* or rape. In this context, it would be very interesting to know if the application of the transplastome approach to *Arabidopsis* and rape would give a similar or a higher amount of PHB compared with nuclear transformation.

PHB synthesized in plants is not thought to be degraded, since significant hydrolysis of PHA requires the presence of specialized bacterial enzymes, the PHA depolymerases (Jendrossek and Handrick 2002). PHA in plants is thus viewed as a final and largely unrecyclable carbon sink. This raises several interesting questions about how transgenic plants accumulating PHA can cope with a new carbon sink. For example, how does PHB synthesis in the plastids affect carbon flow to other compounds synthesized in the organelle, such as starch and fatty acids? How does the plant adjust, at the metabolic and genetic levels, to accommodate the synthesis of this new sink? Why are plants producing high amounts of PHB affected in their growth? Clearly, the tools of genomics, proteomics, and metabolic profiling could provide interesting answers to these questions and give general insights into plant biochemistry that would go well beyond PHA synthesis in plants.

In a first small-scale study of metabolite profiling, over 60 metabolites were measured in transgenic *A. thaliana* lines producing a high amount of PHB (Bohmert et al. 2000). Surprisingly, no changes in fatty acids were observed. There was, however, a correlation between an increase in PHB and a decrease in levels of isocitrate and fumarate, indicating a reduction in tricarboxylic acid cycle activity, leading perhaps to a reduction in pools of acetyl-CoA that may result in growth retardation. There was also a positive correlation between PHB accumulation and the levels of several sugars, such as mannitol, glucose, fructose, and

sucrose. Together, these data indicate that a high amount of accumulation of PHB in chloroplasts has negative and complex effects on plant metabolism that go beyond the chloroplast. At the gene expression level, no correlation could be found between the level of expression of the three *phb* genes and PHB accumulation, leaving unresolved the question of what limits PHB synthesis in the plastids. Preliminary data from metabolomic analysis, comparing leaves from nontransformed sugarcane with leaves from transgenic sugarcane lines producing PHB, found that the vast majority of the variation was a tissue culture effect and was not from the insertion of the PHB metabolic pathway and the selectable marker genes (Purnell et al. 2007).

2.3 Synthesis of Polyhydroxybutyrate in Mitochondria

Acetyl-CoA is found not only in the cytoplasm and plastids, but also in the mitochondria and peroxisomes, being primarily implicated in these organelles in the tricarboxylic acid and β-oxidation cycles, respectively. Mitochondria are important organelles involved in ATP synthesis, photorespiration, and programmed cell death. The N-terminal mitochondrial presequence from F1-ATPase β-subunit (ATPase-β) of *Nicotiana plumbaginifolia* was shown to be effective in targeting green fluorescent protein to the mitochondria (Petrasovits et al. 2007; Fig. 4). Although this signal was used to target *phaA*, *phaB*, and *phaC* from *R. eutropha* to sugarcane mitochondria, no PHB polymer accumulation was observed in transgenic sugarcane (Petrasovits et al. 2007).

2.4 Synthesis of Polyhydroxybutyrate in the Peroxisome

Synthesis of PHB in peroxisomes was described in transgenic black Mexican sweet corn suspension cell cultures (Hahn et al. 1999). In these experiments, the *phaA*, *phaB*, and *phaC* genes from *R. eutropha* were modified to add a peroxisomal targeting signal at the C-terminal end of each protein. Biolistic transformation of maize suspension culture with a mixture of all three genes lead to the isolation of transformants expressing all three enzyme activities and accumulating PHB up to 2% dwt (Hahn et al. 1999). As no transgenic plants have been obtained from these transformed cells, it is difficult at this point to evaluate the potential effects of PHB synthesis in peroxisomes on growth and metabolism.

In addition, PHB was produced in sugarcane expressing the *phbA*, *phbB*, and *phbC* proteins from *A. eutrophus* in the peroxisomes. Plants accumuated PHB at levels of more than 1% dwt of leaves collected from 2-month-old plantlets (Tilbrook et al. 2008). Further analysis of these plants will indicate if there are negative effects on PHB production in plant peroxisomes.

3 Poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyvalerate]

Because PHB homopolymer has relatively poor physical properties, extensive efforts have been invested in the synthesis of PHA copolymers that have better properties. Incorporation of (*R*)-3-hydroxyalkanoate monomers longer than four carbons into a polymer composed mainly of (*R*)-3-hydroxybutyrate leads to a decrease in the crystallinity and melting point compared with PHB homopolymer, and an improvement in plastic properties (De Koning 1995). The copolymer P(HB–HV), the first such copolymer that was extensively studied, is less stiff and is tougher than PHB, as well as being easier to process, making it a good target for commercial application (De Koning 1995). *R. eutropha* produces a random copolymer composed of (*R*)-3-hydroxybutyrate and (*R*)-3-hydroxyvalerate, P(HB–HV),when propionic acid or valeric acid is added to the growth medium containing glucose (Steinbuchel and Schlegel 1991). The biochemical pathway of P(HB–HV) synthesis from propionic acid is shown in Fig. 1. In *R. eutropha*, condensation of propionyl-CoA with acetyl-CoA is mediated by a distinct 3-ketothiolase, named btkB, which has a higher specificity for propionyl-CoA than the 3-ketothiolase encoded by the *phaA* gene (Slater et al. 1998). Reduction of 3-ketovaleryl-CoA to (*R*)-3-hydroxyvaleryl-CoA and subsequent polymerization to form P(HB–HV) are catalyzed by the same enzymes involved in PHB synthesis, namely, the acetoacetyl-CoA reductase and PHA synthase.

3.1 Synthesis of Poly[(R)-3-hydroxybutyrate-co-(R)-3-hydroxyvalerate] in the Cytosol

As described in a previous section, expression of the *R. eutropha* acetoacetyl-CoA reductase and PHB synthase in the cytosol of *A. thaliana* plants leads to the accumulation of only 0.1% of the PHB homopolymer (Poirier et al. 1992b). However, expression of the same reductase along with the PHA synthase from *A. caviae* leads to the accumulation of a similar amount of a PHA copolymer containing mostly (*R*)-3-hydroxybutyrate, but with 0.2–0.8 mol% (*R*)-3-hydroxyvalerate (Matsumoto et al. 2005). The PHA synthase of *A. caviae* has been previously shown to have unique substrate specificity, being capable of producing a PHA copolymer composed of monomers ranging from four to six carbons (Fukui and Doi 1997). Although several potential pathways could provide the propionyl-CoA or 3-hydroxyvaleryl-CoA thought to be required for the synthesis of P(HB–HV), including amino acid synthesis or degradation, as well as β-oxidation of odd-chain fatty acids, it is not known which of these pathways provides the substrate for copolymer synthesis in the cytosol. Interestingly, use of an in vitro mutated *A. caviae* PHA synthase having higher catalytic activity led to an approximate fivefold increase in PHA accumulation in the cytoplasm, indicating that the improvement of enzymatic properties though mutagenesis is a valuable approach to increase the amount of PHA produced in plants (Matsumoto et al. 2005).

3.2 *Synthesis of Poly[(R)-3-hydroxybutyrate-co-(R)-3-hydroxyvalerate] in the Plastid*

Because of the improved properties of P(HB–HV) copolymers over PHB, bacterial production of P(HB–HV), also known under the trade name Biopol™, was central to the marketing and commercial production of PHA by Monsanto in the mid-1990s. It was therefore natural that after the demonstration of high-level PHB synthesis in the plastids, efforts would be focused on the synthesis of PHA copolymers, such as P(HB–HV).

Since synthesis of P(HB–HV) in bacteria relies on the production of propionyl-CoA, it was necessary to create an endogenous pool of propionyl-CoA in plants that could be used by the PHA pathway. Furthermore, since the plastid was shown to be the best subcellular compartment for the synthesis of PHB from acetyl-CoA, it was also chosen as the site for P(HB–HV) synthesis from acetyl-CoA and propionyl-CoA. Although several metabolic pathways exist in prokaryotes and eukaryotes that can generate propionyl-CoA, the simplest strategy adopted was the conversion of 2-ketobutyrate to propionyl-CoA by the pyruvate dehydrogenase complex (PDC), an enzyme naturally located in the plastid (Slater et al. 1999). Although the PDC normally decarboxylates pyruvate to give acetyl-CoA, the same enzyme can also decarboxylate 2-ketobutyrate, albeit at low efficiency, to give propionyl-CoA. Since 2-ketobutyrate is also found in the plastid as an intermediate in the synthesis of isoleucine from threonine, both the substrate and the enzyme complex required for the generation of propionyl-CoA are present in this organelle. However, since the PDC would have to compete for the 2-ketobutyrate with the acetolactate synthase, an enzyme involved in isoleucine biosynthesis, the quantity of 2-ketobutyrate present in the plastid was enhanced through the expression of the *E. coli ilvA* gene, which encodes a threonine deaminase (Slater et al. 1999).

The genes encoding the *E. coli ilvA*, the *R. eutropha phaB* and *phaC*, as well as the *bktB* gene from *R. eutropha* encoding a 3-thiolase having high affinity for both acetyl-CoA and propionyl-CoA, were all modified by adding a plastid leader sequence to the enzymes. All genes were expressed under the control of the CaMV35S promoter. Constitutive expression of the ilvA protein along with bktB, phaB, and phaC proteins in the plastids of *A. thaliana* led to the synthesis of P(HB–HV) in the range 0.1–1.6% dwt, and with the fraction of (*R*)-3-hydroxyvalerate units being between 2 and 17 mol% (Slater et al. 1999).

Expression of the P(HB–HV) pathway in the leucoplast of *B. napus* seeds has also been achieved by putting the bacterial genes under the control of the seed-specific promoter from the *Lesquerella* hydroxylase gene. In these experiments, an isoleucine-insensitive mutant of the *ilvA* gene was coexpressed along with the *bktB*, *phaA*, and *phaC* genes, and all four genes were inserted in a single multigene vector. P(HB–HV) synthesis in the range 0.7–2.3% dwt was reported, with a (*R*)-3-hydroxyvalerate content of 2.3–6.4 mol% (Slater et al. 1999). Interestingly, there was an inverse relationship between the amount of PHA and the proportion of the (*R*)-3-hydroxyvalerate monomer, indicating a "bottleneck" in providing 3-hydroxyvaleryl-CoA to the PHA synthase. This bottleneck is thought to be caused by the inefficiency of the PDC in converting 2-ketobutyrate to propionyl-CoA.

4 Medium-Chain-Length Polyhydroxyalkanaoate

MCL-PHAs are typically described as elastomers, although their actual physical properties are very diverse, ranging from soft plastic to glue and rubber, and are primarily dependent on the monomer composition (De Koning 1995). Monomers present in MCL-PHAs typically include (*R*)-3-hydroxyacids from six to 16 carbons in length, and these monomers may also contain a wide spectrum of functional groups, including unsaturated bonds and halogenated groups (Steinbüchel and Valentin 1995).

There are two main routes for the synthesis of MCL-PHA in bacteria (Fig. 2; Steinbuchel and Fuchtenbusch 1998; Sudesh et al. 2000). The first is the synthesis of PHA using intermediates of fatty acid β-oxidation. This pathway is found in several bacteria, such as *P. oleovorans* and *Pseudomonas fragii*, which can synthesize MCL-PHA from alkanoic acids or fatty acids. Alkanoic acids or fatty acids present in the media are transported into the cell, where they are first converted to CoA esters before being directed to the β-oxidation pathway, where a number of 3-hydroxyacyl-CoA intermediates can be generated. Since the PHA synthase accepts only the *R* isomer of 3-hydroxyacyl-CoA and the bacterial β-oxidation of saturated fatty acids generates only the *S* isomer of 3-hydroxyacyl-CoA, bacteria must have enzymes capable of generating 3-hydroxyacyl-CoA. One potential enzyme is a 3-hydroxyacyl-CoA epimerase, mediating the reversible conversion of the *S* and *R* isomers of 3-hydroxyacyl-CoA, although no protein or gene encoding such activity has yet been unambiguously identified. In contrast, monofunctional enoyl-CoA hydratase II enzymes, converting enoyl-CoA directly to 3-hydroxyacyl-CoA, have

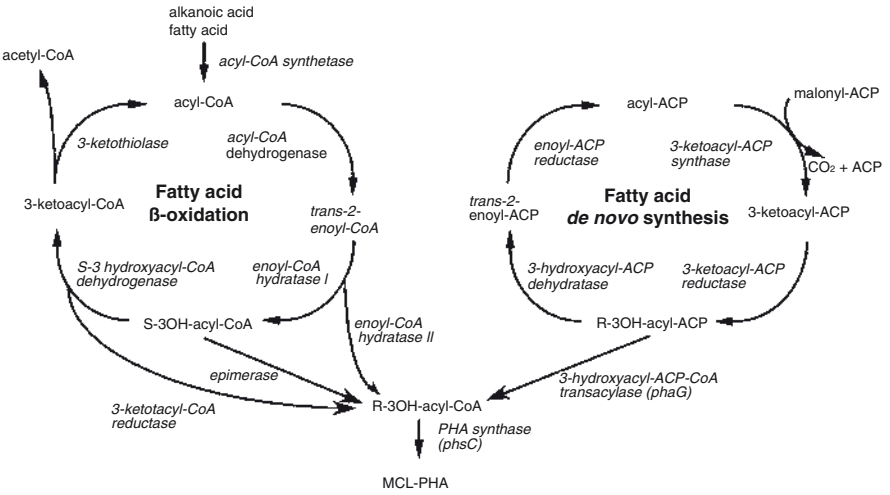

Fig. 2 Pathways for medium-chain-length polyhydroxyalkanaoate synthesis. Synthesis of medium-chain-length polyhydroxyalkanaoate in bacteria can be accomplished through the use of intermediates either of the fatty acid β-oxidation cycle (*left*) or of the de novo fatty acid biosynthetic pathway (*right*)

been identified in several bacteria, including *A. caviae* (Fukui et al. 1998; Reiser et al. 2000; Tsuge et al. 2000). Finally, it is speculated that a 3-ketoacyl-CoA reductase that could specifically generate 3-hydroxyacyl-CoA may exist in bacteria, although such an enzyme has not yet been unambiguously identified. It has, however, been shown that the enzyme 3-ketoacyl-acyl carrier protein (ACP) reductase, participating normally in the fatty acid biosynthetic pathway, may also act on the 3-ketoacyl-CoA to generate 3-hydroxyacyl-CoA, and thus contribute to MCL-PHA synthesis (Taguchi et al. 1999).

The second route for MCL-PHA in bacteria is through the use of intermediates of fatty acid biosynthesis (Fig. 2). This pathway is also found in numerous pseudomonads. In contrast to *P. oleovorans* and *P. fragii*, which can only synthesize MCL-PHA from related alkanoic acids present in the growth medium, *Pseudomonas aeruginosa* and *Pseudomonas putida* can synthesize a similar type of MCL-PHA when grown on unrelated substrates, such as glucose (Huijberts et al. 1992; Steinbuchel and Lutke-Eversloh 2003). In these bacteria, MCL-PHA is formed from the 3-hydroxyacyl-ACP intermediates of the de novo fatty acid biosynthetic pathway. PhaG is a key enzyme in this pathway, having a 3-hydroxyacyl-CoA-ACP transferase activity responsible for converting the 3-hydroxyacyl-ACP intermediate of the fatty acid biosynthetic pathway to 3-hydroxyacyl-CoA, the substrate for the PHA synthase (Rehm et al. 1998).

The first approach used to synthesize MCL-PHA in plants was to divert the 3-hydroxyacyl-CoA intermediates of the β-oxidation of endogenous fatty acids. Since β-oxidation in plants occurs in the peroxisomes, PHA biosynthetic proteins needed to be targeted to this organelle. The phaC1 synthase from *P. aeruginosa* was thus modified at the carboxy end by the addition of a peroxisomal targeting signal. The modified *phaC1* was expressed under the control of the CaMV35S promoter and transformed into *A. thaliana* (Mittendorf et al. 1998). Appropriate targeting of the PHA synthase in plant peroxisomes was demonstrated by immunolocalization. Transmission electron microscopy also showed the presence of typical PHA inclusions within the peroxisomes. The monomer composition of the MCL-PHA produced in plants reflected well the broad substrate specificity of the PHA synthase of *P. aeruginosa*. Thus, peroxisomal PHA was composed of over 14 different monomers, including saturated and unsaturated monomers ranging from six to 16 carbons (Mittendorf et al. 1998). The majority of (*R*)-3-hydroxyacids found in plant MCL-PHA could be clearly linked to the corresponding 3-hydroxyacyl-CoA generated by the β-oxidation of saturated and unsaturated fatty acids. The production of peroxisomal MCL-PHA was relatively low, with a maximal level of 0.4% dwt in 7-day-old germinating seedlings. In leaves, the PHA level decreased to approximately 0.02 % dwt. Interestingly, a two- to threefold increase in PHA was observed during leaf senescence. These data support the link between β-oxidation and PHA synthesis, since this pathway, in association with the glyoxylate cycle, are most active during germination and senescence, where they are involved in the conversion of fatty acids to carbohydrates. In contrast to PHB synthesis in the cytoplasm and plastid, no negative effects of peroxisomal MCL-PHA accumulation on plant growth or seed germination were observed, although this could be linked to the relatively low amount of PHA synthesized in the peroxsiome (Mittendorf et al. 1998).

Similar to the PHA synthase from *R. eutropha*, PHA synthase of *P. aeruginosa* is thought to accept only the *R* isomer of 3-hydroxyacyl-CoAs. As with bacteria, the wide range of monomers found in plant MCL-PHAs suggest that plants also have enzymes capable of converting the β-oxidation intermediates (*S*)-3-hydroxyacyl-CoA to the *R* isomer. Such enzymes could be either the 3-hydroxyacyl-CoA epimerase present on the plant multifunctional protein or an enoyl-CoA hydratase II activity that is specific for the generation of 3-hydroxyacyl-CoA from *trans*-2-enoyl-CoA. The latter enzyme has recently been identified in *A. thaliana* and several other plants, and has been shown to be involved in the degradation of unsaturated fatty acids (Goepfert et al. 2006). A third route for the synthesis of a narrow range of 3-hydroxyacyl-CoA is the hydration of *cis*-2-enoyl-CoA by the enoyl-CoA hydratase I activity of the multifunctional protein. The substrate *cis*-2-enoyl-CoA is derived from the β-oxidation of unsaturated fatty acids having a *cis* double bond at an even position, such as found in linoleic and linolenic acid (Poirier 2002).

Growth of transgenic plants in liquid media supplemented with detergents containing various fatty acids was used to study how to influence the quantity and monomer composition of PHA produced from β-oxidation. Addition of external fatty acids to plants resulted in both an increased accumulation of MCL-PHA and a shift in the monomer composition that reflected the intermediates generated by the β-oxidation of the external fatty acids (Mittendorf et al. 1999). For example, addition to the media of the detergent polyoxyethylene sorbitan esterified to lauric acid (Tween 20) resulted in a eight- to tenfold increase in the amount of PHA synthesized in 14-day-old plants compared with plants growing in the same media without detergent. The monomer composition of the MCL-PHA synthesized media containing Tween 20 showed a large increase in the proportion of saturated even-chain monomers with 12 carbons or fewer, and a corresponding decrease in the proportion of all unsaturated monomers. This shift in monomer composition is accounted for by the fact that β-oxidation of lauric acid, a 12-carbon saturated fatty acid, gives saturated 3-hydroxyacyl-CoA intermediates of 12 carbons and lower. Further experiments have shown that addition in the plant growth media of tridecanoic acid, tridecenoic acid (C13:1 □12), or 8-methylnonanoic acid resulted in the production of MCL-PHA containing mainly saturated odd-chain, unsaturated odd-chain or branched-chain (*R*)-3-hydroxyacid monomers, respectively (Mittendorf et al. 1999). These results demonstrated that the plant β-oxidation cycle was capable of generating a large spectrum of monomers that can be included in MCL-PHA even from fatty acids that are not present in significant quantities in plants. Furthermore, "feeding" experiments with these unusual fatty acids demonstrated that all (*R*)-3-hydroxyacids between six and 16 carbons that could be generated by the β-oxidation cycle (via the 3-hydroxyacyl-CoA intermediate) were found in the MCL-PHA. These results supported the concept that the monomer composition of PHA could be used as a tool to study the degradation pathway of fatty acids, including unsaturated fatty acids.

As an alternative to the addition of external fatty acids, modulation of the monomer composition of MCL-PHA synthesized in peroxisomes was also achieved by modifying the endogenous fatty acid biosynthetic pathway (Mittendorf et al. 1999). The first example of this approach was the expression of the peroxisomal PHA

synthase in a mutant of *A. thaliana* deficient in the synthesis of triunsaturated fatty acids. MCL-PHA produced from this mutant was almost completely deficient in all (*R*)-3-hydroxyacids derived from the degradation of triunsaturated fatty acids, including triunsaturated monomers (Mittendorf et al. 1999). Since numerous fatty acid desaturases have now been cloned and expressed in transgenic plants to control the number and position of unsaturated bonds in fatty acids, this approach could be extended to further modulate the proportion of a number of (*R*)-3-hydroxyacid monomers in PHAs.

The second approach used to influence the quantity and monomer composition of MCL-PHA was the coexpression of a medium-chain thioesterase in the plastid with a PHA synthase in the peroxisome. Studies on transgenic plants expressing a laurate acyl-ACP thioesterase in the plastid of either leaves or seeds of rape revealed the presence of a futile cycling of lauric acid, whereas a substantial portion of the unusual fatty acids were degraded through peroxisomal β-oxidation instead of accumulating in lipids (Eccleston et al. 1996; Eccleston and Ohlrogge 1998). These studies on lauric acid producing rapeseed indicated that expression of a thioesterase might be a way of increasing the carbon flux towards β-oxidation and peroxisomal PHA biosynthesis. This hypothesis was tested in *A. thaliana* by combining the constitutive expression of the peroxisomal PHA synthase with the caproyl-ACP thioesterase from *Cuphea lanceolata* in the plastid (Mittendorf et al. 1999). Expression of both enzymes led to a seven- to eightfold increase in the amount of MCL-PHA synthesized in plant shoots as compared with transgenics expressing only the PHA synthase. Furthermore, the composition of the MCL-PHA in the thioesterase/PHA synthase double transgenic plant was shifted towards saturated (*R*)-3-hydroxyacid monomers containing ten carbons or fewer. This shift is in agreement with an increase in the flux of decanoic acid towards β-oxidation triggered by the expression of the caproyl-ACP thioesterase (Mittendorf et al. 1999). Interestingly, constitutive expression of the related lauroyl-ACP thioesterase in *A. thaliana* was shown not to lead to an increase in the genes or enzymes involved in β-oxidation (Hooks et al. 1999).

The relation between fatty acid futile cycling and peroxisomal PHA synthesis was further extended to the developing seeds (Poirier et al. 1999). Synthesis of MCL-PHA has been demonstrated in seeds of *A. thaliana* by expressing the peroxisomal PHA synthase gene under the control of the seed-specific napin promoter. In such transgenic plants, MCL-PHAs accumulated to 0.006% dwt in mature seeds and the monomer composition was relatively similar to the PHA synthesized in germinating seedlings. Expression of both the PHA synthase and caproyl-ACP thioesterase in the leucoplasts of developing seeds resulted in a nearly 20-fold increase in seed PHA, reaching 0.1% dwt in mature seeds. Furthermore, as found with the expression of these two enzymes in whole plants, coexpression in seeds resulted in a large increase in the proportion of (*R*)-3-hydroxyacid monomers containing PHAs with ten carbons or fewer. These data clearly indicate that even though expression of the caproyl-ACP thioesterase in seeds leads to the accumulation of medium-chain fatty acids in triacylglycerides, there is still a significant proportion of these fatty acids that are channeled towards β-oxidation. This flux towards the β-oxidation cycle is thought to be quite significant, considering that there is only a fourfold difference between

the maximal amount of PHA synthesized in germinating seedlings (0.4% dwt), where β-oxidation is thought to be maximal, and the maximal amount of PHA synthesized in the developing seeds expressing the thioesterase (0.1% dwt), where metabolism should be mainly devoted to the synthesis of fatty acid synthesis instead of degradation.

Synthesis of MCL-PHA in the peroxisomes of developing seeds has also demonstrated the presence of an increased cycling of fatty acids towards β-oxidation in plants deficient in the enzyme diacylglycerol acyltransferase (DAGAT; Poirier et al. 1999). The *tag1* mutant of *A. thaliana* was shown to be deficient in DAGAT activity in developing seeds, resulting in a decreased accumulation of triacylglycerides and a corresponding increase in diacylglycerides and free fatty acids in mature seeds (Katavic et al. 1995). It was hypothesized that the imbalance created between that capacity of the plastid to synthesize fatty acids and the capacity of the lipid biosynthetic machinery of the endoplasmic reticulum to include these fatty acids into triacylglycerides might have two basic consequences: either fatty acid biosynthesis would be reduced (feedback inhibited) to match it with triacylglyceride biosynthesis, or excess fatty acids that cannot be included in triacylglycerides would be channeled towards β-oxidation. Expression of the peroxisomal PHA synthase in the *tag1* mutant resulted in a tenfold increase in the amount of MCL-PHA accumulating in mature seeds compared with expression of the transgene in wild-type plants (Poirier et al. 1999). Although these results do not address whether fatty acid biosynthesis is decreased in the *tag1* mutant, they nevertheless clearly indicate that a decrease in triacylglyceride biosynthesis results in an increase in the flux of fatty acids towards β-oxidation. Thus, carbon flux to the β-oxidation cycle can be modulated to a great extent and appears to play an important role in lipid homeostasis in plants, even in tissues that are primarily devoted to lipid biosynthesis, such as the developing seeds.

Analysis of futile cycling of fatty acids in developing seeds has been extended to transgenic plants accumulating the unusual fatty acids ricinoleic acid and vernolic acid (Moire et al. 2004). *A. thaliana* expressing either the *Ricinus communis* oleate 12-hydroxylase or the *Crepis palaestina* linoleate 12-epoxygenase under the control of the napin promoter was shown to accumulate approximately twofold more MCL-PHA in developing seeds compared with controls. Although relatively small compared with the increase in PHA observed in transgenic plants expressing the *C. lanceolata* capryl-ACP thioesterase, the twofold increase in MCL-PHA was quite significant considering that the steady level of hydroxy or epoxy fatty acids accumulated in transgenic seeds represented only 6.3 or 3.1 mol%, respectively. Thus clearly, a larger proportion of unusual fatty acids were being degraded via peroxisomal β-oxidation in developing seeds compared with the common fatty acids. Interestingly, microarray analysis of nearly 200 genes involved in fatty acid biosynthesis and degradation, including the genes encoding enzymes of the β-oxidation cycle, revealed no changes in gene expression in transgenic developing seeds expressing *C. lanceolata* caproyl-ACP thioesterase, *R. communis* oleate 12-hydroxylase, or *C. palaestina* linoleate 12-epoxygenase (Moire et al. 2004). These results indicated that analysis of peroxisomal PHA is a better indicator of the flux of fatty acid through β-oxidation than the expression profile of genes involved in lipid metabolism. Peroxisomal PHA as a metabolic marker has been further used to identify and characterize the contribution

of various peroxisomal enzymes, such as the enoyl-CoA isomerase, dienoyl-CoA isomerase, and enoyl-CoA hydratase II, to the degradation of unsaturated fatty acids in plants (Goepfert et al. 2005, 2006, 2008). Peroxisomal PHA has also been used in other eukaryotes, such as *Saccharomyces cerevisiae*, to study fundamental aspects of fatty acid degradation in peroxisomes (Bogdawa et al. 2005; de Oliveira et al. 2004; Maeda et al. 2006; Marchesini and Poirier 2003; Robert et al. 2005).

In the bacterial pathway of MCL-PHA synthesis from intermediates of fatty acid biosynthesis, the enzyme phaG plays a key role, catalyzing the conversion of 3-hydroxyacyl-ACP to 3-hydroxyacyl-CoA, the latter being the substrate for the PHA synthase (Rehm et al. 1998). The identification and cloning of the *P. putida phaG* gene opened up the possibility of synthesizing PHA copolymers in the plastids of plants from intermediates of fatty acid biosynthesis. Unfortunately, constitutive expression in the plastid of *A. thaliana* of only the phaG enzyme led to a marked deleterious effect on plant growth, the plants being dwarfed with crinkly leaves and the seed set being strongly reduced (V. Mittendorf, unpublished results). The reason for this phenotype is not known, but is thought to be perhaps due to interference of the transacylase with fatty acid biosynthesis. If this is the case, it would be interesting to know why this does not occur in bacteria expressing phaG. Coexpression in the plastid of the *P. aeruginosa* PHA synthase along with phaG did not conclusively lead to PHA accumulation in *Arabidopsis* (V. Mittendorf, unpublished results). Analogous experiments in potato led to similar conclusions, although evidence for the synthesis of a very small amount of MCL-PHAs was provided (Romano et al. 2005). Thus, despite the obvious advantages of the plastid as a location for the production of PHB and P(HB–HV), the synthesis in this organelle of PHA copolymer using fatty acid biosynthetic intermediates appears problematic at present.

The synthesis of MCL-PHA in potato cell lines has been demonstrated through expression of the PHA synthase from *P. oleovorans* in the cytoplasm (Romano et al. 2003). PHA could be detected only after feeding the cell lines with (*R*)-3-hydroxyoctanoic acid, with the PHA containing only the eight-carbon monomer. These results indicate that although no endogenous 3-hydroxyacyl-CoA could be detected in the cytoplasm, an acyl-CoA synthetase activity was present that was capable of converting (*R*)-3-hydroxyoctanoic acid (that originally comes from the external media) to the corresponding 3-hydroxyacyl-CoA. The amount of PHA detected reached up to 1% dwt (Romano et al. 2003).

5 Short-Chain-Length to Medium-Chain-Length Polyhydroxyalkanaote Copolymers

PHA copolymers have classically been defined as SCL copolymers when they contain monomers from three to five carbons and as MCL copolymers when they contain monomers from six to 16 carbons. However, a novel class of PHA copolymers has emerged with the discovery of PHA synthases capable of incorporating monomers with four to six or more carbons, such as the PHA synthase from *A. caviae* or

Pseudomonas sp61-3 (Fukui and Doi 1997). These "hydrid" SCL–MCL-PHAs containing a large fraction of (*R*)-3-hydroxybutyrate and a smaller fraction of (*R*)-3-hydroxyacids with six or more carbons have very good polymer properties, better than P(HB–HV), and have been named Nodax™, after its inventor, Isao Noda from Procter and Gamble (Noda et al. 2005). The synthesis of a such a "hybrid" PHA copolymer has been reported in *A. thaliana* expressing a PHA synthase from either *A. caviae* or *Pseudomonas* sp61-3 modified at the C-terminal end for targeting to the peroxisome (Arai et al. 2002; Matsumoto et al. 2005, 2006). Expression of these PHA synthases under the control of the CaMV35S promoter led to the accumulation of a PHA containing even-chain and odd-chain monomers ranging from four to six carbons for the *A. caviae* synthase, and from four to eight carbons for the *Pseudomonas* sp61-3 synthase. The maximal amounts of PHA accumulated in leaves were 0.02–0.04% dwt. Growth of transgenic plants expressing the *A. caviae* synthase in media containing Tween 20 increased the total amount of PHA synthesized without affecting appreciably the monomer composition (Arai et al. 2002). The incorporation of over 20 mol% of (*R*)-3-hydroxyvalerate into these PHAs raises the interesting question of the source of the odd-chain monomer. Although odd-chain monomers have been detected in MCL-PHAs synthesized from the expression of the *P. aeruginosa* PHA synthase in the peroxisome, the amount of odd-chain monomers was very low (less than 1 mol%). It is possible that an α-oxidation pathway could generate odd-chain intermediates from even-chain fatty acids and that this pathway is more active towards shorter-chain intermediates (i.e., six-carbon fatty acids). Although a gene involved in α-oxidation has been identified, the corresponding protein has not been linked to the peroxisome (Hamberg et al. 1999). Thus, despite evidence of a complete α-oxidation pathway in plants, the link between this pathway and the peroxisome needs to be established. PHA thus offers potentially a unique handle to study α-oxidation in plants.

PHA production in peroxisomes has also been reported in sugarcane. A six-enzyme strategy involving a broad substrate specificity PHA synthase was applied for production of PHA sugarcane peroxisomes. Gas chromatography–mass spectrometry analysis revealed accumulation of MCL-PHA copolymers in several lines, and SCL–MCL-PHA copolymer in one line. Although the content of SCL C_4 monomers was influenced by the expressed transgenes, MCL monomer content was apparently determined by a combination of the PHA synthase and the chain length of the fatty acids undergoing β-oxidation. Gel permeation chromatography showed that the SCL–MCL-PHA copolymer had a moderate weight-average molecular weight of 112,000, with a polydispersity index of 1.8 (Anderson et al. 2008)

6 Concluding Remarks and Future Perspectives

A spectrum of PHAs has now been successfully synthesized in plants using various metabolic pathways. These range from the stiff and brittle PHB to the more flexible P(HB–HV) plastic and MCL-PHA elastomers and glues. Experiments have shown

that in some cases very high amounts of polymer can be produced, however at a considerable metabolic cost. The challenge for the future is to achieve accumulation of adequate amounts of PHA (10% dwt or more) without affecting yield. For some agricultural production strategies, it will also be necessary to succeed in harvesting PHA without affecting the recovery of other plant products, such as oils, protein, sugar, or starch. This is an important contrast to the production of PHA by bacterial fermentation, where the system is designed to produce mainly PHA with little residual waste. A large-scale agricultural production of PHA may only be viable through the recovery of not only PHA, but also of all other valuable components of the crop. For example, in the case of an oil crop such as *B. napus*, one must be able to recover PHA and the oil, as well as still being able to use the delipidized protein-rich meal for animal feed. In the case of a carbohydrate-producing crop such as sugar beet or sugarcane, both sucrose and PHA would have to be recovered. There are alternative strategies. Crop plants could be grown only for biomass and PHA production. An example would be the synthesis of PHA in switchgrass, miscanthus, or sugarcanes, where the residual biomass remaining after PHA extraction could be used for production of sugars for fermentation and/or energy production. Alternatively, PHAs could be produced in parts of the plants that are not required for their current produce production, for instance, the leaves of sugarcane or sugar beets, leaving the respective stalks and tubers for sucrose production. We know thus far that PHB can be produced in the seed of rape to 8% dwt and 2–4% in the leaves of switchgrass and sugarcane without obvious deleterious effects on plant growth and germination. Thus, the goal of producing an adequate level of PHA in crops without yield penalties appears realistic, although more remains to be done.

The success of using transgenic plants as a source of novel material will not only depend on the production levels achieved, but also on whether the polymers can be extracted efficiently, economically, and ecologically from crops. Although a number of strategies have been described in the literature for the extraction of PHA, some relying on solvents and others not, further work is required to validate these extraction processes in the context of large-scale production in crop plants (Poirier 2001).

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Biosynthesis of Medium-Chain-Length Poly[(*R*)-3-hydroxyalkanoates]

Manfred Zinn

Contents

Abstract This chapter is focused on the production of medium-chain-length (mcl) poly[(*R*)-3-hydroxyalkanoate]s (PHAs) in pseudomonads. mcl-PHAs are, like all PHAs, isotactic polyesters and serve in bacteria as storage material for carbon and energy. In general, they are accumulated intracellularly in discrete granules under particular growth conditions that favor the biosynthesis, e.g., nitrogen or phosphorus limitation and carbon in excess. The nutrition, the culture conditions, and the type of bacterium have a strong influence on the mcl-PHA amount and the monomeric unit composition. The material properties of mcl-PHAs are very versatile and are mainly determined by the side chain of the polymer. The controlled supply of fatty acids results in structurally related mcl-PHAs and thus enables tailoring of material properties that range from fluidoplasts to elastomers to thermoplasts. Finally, the biosynthesis of mcl-PHA in high cell density cultures is discussed.

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1 Introduction

The class of polyhydroxyalkanoates (PHAs) is very versatile, with the largest group being the medium-chain-length (mcl) PHAs which consist of enantiomerically pure (*R*)-hydroxy fatty acids of between C_6 and C_{14} units. Since the discovery in 1983 of the Witholt group (de Smet et al. 1983) at the University of Groningen in the Netherlands, more monomers have been described in the literature (see also Guérin et al. 2009 and review by Kim et al. 2007).

The properties of mcl-PHAs are suitable for diverse applications, e.g., in engineering, agriculture, food applications, chemistry, pharmacy, and medicine, because of their inherent biodegradability and biocompatibility (Chen and Wu 2005a). Sophisticated bioprocesses and appropriate selection of the carbon substrate allow the production of tailor-made mcl-PHA. The chemical modification of functionalized PHA, including chlorination, cross-linking, epoxidation, hydroxylation, and carboxylation reactions, allows further tailoring of the material properties and was recently reviewed in detail by Hazer and Steinbüchel (2007). The largest potential of mcl-PHAs has to be seen in hydrophobic coatings of surfaces. Of particular interest are applications that need to be temporarily protected from adverse influences, e.g., microbial biofouling (Devassine et al. 2002; Zinn et al. 2000). New processing of mcl-PHAs includes the formation of composites with organic, inorganic, or metallic (nano-) particles (Il Yun et al. 2008; Yang et al. 2007) or fibers (Misra et al. 2006) to improve material properties such as tensile strength. A novel approach represents the application of mcl-PHA monomers. Recently, these (*R*)-3-hydroxy fatty acid monomers could be produced in a special bioprocess and represent an interesting group of building blocks for chemical synthesis of pharmaceutical agents (Ren et al. 2005b, 2007; Ruth et al. 2007; Wang et al. 2007; see also Guérin et al. 2009). A very interesting option is the chemical degradation of polyesters to alkyl esters for combustion (biodiesel; personal communication G. Chen and ISBP08, Auckland, New Zealand, 2008). To date, mcl-PHAs have not been commercialized and in many cases research groups produce their own polymers or acquire them from research laboratories.

2 mcl-PHAs: Their Chemical Structure and Biosynthesis in Prokaryotes

2.1 Chemical and Physical Properties

More than 150 different hydroxyalkanoic acids are known as PHA constituents (Chen and Wu 2005a; Rehm 2007); the largest percentage consists of mcl-PHA, but only few of them have been produced in large quantities and have been well characterized (Kessler and Witholt 1998; Witholt and Kessler 1999). Consequently, little is known about the chemical and mechanical properties of most of these polyesters.

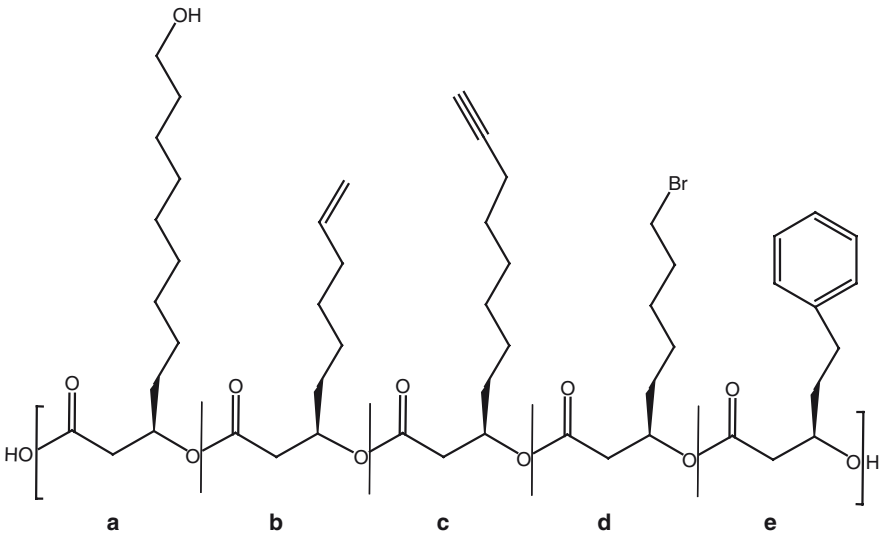

Fig. 1 Examples of functional groups found in medium-chain-length (mcl) polyhydroxyalkanoates (PHAs). **a** 3,12-Dihydroxydodecanoic acid (Lenz et al. 1992), **b** 3-hydroxy-8-nonenoic acid (Ren et al. 2005b), **c** 3-hydroxy-10-undecenoic acid (Kim et al. 1998), **d** 8-bromo-3-hydroxyoctanoic acid (Lenz et al. 1992), **e** 3-hydroxy-5-phenylvalerate (Fritzsche et al. 1990b)

To date, PHA monomers with straight, branched, saturated, unsaturated, and aromatic side chains have been identified (Steinbüchel and Valentin 1995) (see Fig. 1). Of special interest are functionalized groups in the side chains that allow further chemical modification, e.g., halogens, hydroxy, carboxy, epoxy, phenoxy, cyanophenoxy, nitrophenoxy, thiophenoxy, and methyl ester groups (Hazer and Steinbüchel 2007). The side chain length of the monomer and its functional group considerably influence material properties such as melting and glass-transition temperatures and crystallinity, and therefore determine their final application (Hazer and Steinbüchel 2007; Zinn et al. 2001). The weight-average molecular weights (M_w) range from about 60,000 to 360,000 and their polydispersities are in the range 1.6–2.4, which are significantly smaller than those observed for short-chain-length (scl) PHAs.

The crystallinity of poly[(*R*)-3-hydroxyoctanoate-*co*-(*R*)-3-hydroxyhexanoate] (PHO) , the most common type of mcl-PHA found in pseudomonads, is between 20 and 40%, resulting in low melting endotherms, which is typical for elastomers. It has been found by vibrational spectroscopy in combination with normal vibrational analysis that PHO has crystallization properties that are dependent on the chemical environment (Tao et al. 1995). As a consequence, a detailed crystal structure is still missing. Liu and Chen (2007) reported that the elongation of the side chain had a significant influence on the characteristics of mcl-PHA. Thus, the crystallinity was estimated to be enhanced on the basis of the melting endotherms and the mechanical properties such as tensile strength, stress at break, elongation at break, and Young's modulus (see Table 1). A similar effect had been described previously for the formation

Table 1 Properties of medium-chain length (*mcl*) polyhydroxyalkanoates (*PHA*) with different monomeric unit composition

mcl-PHA	$M_w \times 10^4$	M_w/M_n	T_g (°C)	T_m (°C)	*T* (MPa)	*S* (MPa)	*E* (%)	Young's modulus (MPa)
PHO[a]	28.6	2.40	−33.1	58.1	ND	ND	ND	ND
PHOU[a] (50 mol% HO)	29.0	1.90	−44.6	39.6	ND	ND	ND	ND
PHUE[a]	29.0	2.40	−49.3	NE	ND	ND	ND	ND
PHDD[b] (15 mol% HDD)	10.0	1.25	−44	53.0	8.7	5	188.7	3.6
PHDD[b] (39 mol% HDD)	15.7	1.45	−43	65.0	11.3	8.9	125	11.5
PHTD[b] (31 mol% HTD)	8.3	1.82	−40	58.1	7.57	7.11	275.1	31.73
PHTD[b] (49 mol% HTD)	9.5	1.43	−40	66.8	3.15	2.29	107.7	34

PHO poly(3-hydroxyocatanoate), *HO* 3-hydroxyocatanoate, *PHOU* poly(3-hydroxyoctanoate-*co*-3-hydroxy-10-undecenoate), *PHUE* poly(3-hydroxy-10-undecenoate), *PHDD* poly(3-hydroxydodecanoate), *HDD* 3-hydroxydodecanoate, *PHTD* poly(3-hydroxytetradecanoate), *HTD* 3-hydroxytetradecanoate, *ND* not determined, *NE* nonexistent

[a]Data taken from Hartmann et al. (2004); for the detailed composition, see also Table 2

[b]Data taken from Liu and Chen (2007)

of comb polymers. The elongated side chains induced side-chain crystallization by the formation of polyethylene-like crystal units (Hany et al. 2004).

2.2 Representative mcl-PHA Production Strains

Pseudomonads belonging to ribosomal RNA homology group I are able to accumulate PHA from fatty acids. Usually these substrates are poorly water miscible or/and toxic to bacteria at rather low concentration (Sun et al. 2007a); hence, their supply has to be well controlled in fermentations. Two basic types of carbon substrates lead to mcl-PHA formation: related and unrelated substrates. Related carbon substrates are substrates (typically fatty acids) that result in a mcl-PHA with very similar (*R*)-3-hydroxy acids but with the same or reduced carbon chain lengths (Huijberts et al. 1992b; Timm and Steinbüchel 1990). For example, nonanoic acid results in a mcl-PHA consisting of about 60 mol% (*R*)-3-hydroxynonanoate and about 40 mol% (*R*)-3-hydroxyheptanoate (Durner et al. 2001).

Most of the mcl-PHA production strains – with the exception of *Pseudomonas putida* GPo1 – accumulate alkanoic mcl-PHA also from unrelated carbon substrates through the fatty acid *de novo* synthesis pathway. Consequently, glucose may result in polymers containing (*R*)-3-hydroxy fatty acids with even carbon numbers, e.g., (*R*)-3-hydroxydecanoate, (*R*)-3-hydroxyoctanoate, (*R*)-3-hydroxyhexanoate,

and traces of (*R*)-3-hydroxbutyrate. Depending on the production strain and the growth conditions, the molar composition may vary significantly.

The production of mcl-PHA was extensively investigated with *Pseudomonas* species (Diard et al. 2002; Diniz et al. 2004; Eggink et al. 1992; Hartmann et al. 2006; Hoffmann and Rehm 2004; Hori et al. 1994; Huijberts and Eggink 1996; Kim et al. 2000, 2007). To understand the physiological functions of the strains better, the most important strains are described in more detail.

The first strain that was found to produce mcl-PHA was *Pseudomonas oleovorans* GPo1 (ATCC 29347; de Smet et al. 1983), which was later reclassified as *P. putida* GPo1 (van Beilen et al. 2001). A reclassification was considered to be necessary because this study revealed that the 16S ribosomal RNA sequence of *P. oleovorans* was identical to the one of *P. putida* ATCC 17633 which had been deposited earlier by Stanier (van Beilen et al. 2001). The strain GPo1 was originally isolated from oil-based cooling fluids in metal manufacturing (Lee and Chandler 1941) and is able to synthesize an impressive number of different mcl-PHAs under nitrogen, phosphorus, and other noncarbon limitations (Witholt et al. 1990) up to a maximal content of above 60% (w/w) (Jung et al. 2001; Kim 2002). In general, hardly any PHA is accumulated under carbon-limited growth conditions and from nonrelated carbon substrates. Interestingly, one exception has been identified: nonanoic acid supplied to continuous cultures ($D = 0.2\ h^{-1}$) as a single and growth-limiting carbon substrate can lead to significant amounts (16% w/w) of mcl-PHAs (Durner et al. 2001). *P. putida* GPo1 is capable of growing on *n*-alkane, *n*-alkanol, and alkanoic acids and producing structurally related mcl-PHAs (Brandl et al. 1988, de Smet et al. 1983; Fritzsche et al. 1990a; Gross et al. 1989; Lageveen et al. 1988; Lenz et al. 1992), side chains containing carbon–carbon double or triple bonds (Kim et al. 1998; Lageveen et al. 1988), or side chains with acetoxy, ketone, or aromatic groups (Curley et al. 1996; Fritzsche et al. 1990b; Jung et al. 2000; Kim et al. 1999; Fig. 1). Side chains with chlorinated (Doi and Abe 1990), brominated (Kim et al. 1992), fluorinated (Abe et al. 1990; Hori et al. 1994), as well as cyanoalkyl (Lenz et al. 1992) or epoxy (Bear et al. 1997) groups have also been reported. However, no carboxylated mcl-PHAs could be produced. Furthermore, with this strain it is possible to produce tailor-made mcl-PHA composed of defined proportions of different monomers in dual-nutrient-limited chemostat culture (Durner 1998; Durner et al. 2000, 2001; Hartmann et al. 2004; Zinn 1998).

P. putida U was originally isolated from a mud in a creek in Urbana, Illinois, USA, by the use of a *m*-cresol medium in 1963 (Dagley et al. 1964). The particularity of this strain is its capability to biosynthesize PHAs containing (*R*)-3-hydroxy-*n*-phenylalkanoic acids with acid residues larger than five carbon atoms (Garcia et al. 2000; Garcia et al. 1999). In contrast to GPo1 this strain accumulates mcl-PHA from unrelated carbon sources such as glucose and gluconate and generally does not need any nutrient limitation to accumulate mcl-PHA.

P. putida KT2440 is a derivative isolate of the toluene-degrading bacterium *P. putida* mt-2 that was isolated at the Research Institute for Microbial Diseases, Osaka University, Japan (Teruko 2002). The loss of the TOL plasmid pWW0 which is required for the oxidative catabolism of toluene or xylene resulted in the new

strain name. The genome of *P. putida* KT2440 has been completely sequenced and was published in 2002 (Nelson et al. 2002), which then allowed the design of a genome-wide oligonuceotide-based DNA microarray (Yuste et al. 2006). The strain *P. putida* KT2442 was first mentioned in 1981 (Bagdasarian et al. 1981; Franklin et al. 1981) and has a spontaneous rifampicin resistance. Hervas et al. (2008) compared the strains and found an almost identical expression profile for KT2440 and KT2442 when grown on a minimal medium with succinate as the carbon source.

P. putida CA-3 was isolated from a mixed culture in a styrene-containing bioreactor. The NCIMB Laboratories in Aberdeen, UK, further characterized the strain and found that it can utilize glucose and styrene as the sole carbon and energy source but is not able to degrade ethyl benzene. For mcl-PHA accumulation, a nutrient limitation enhances the production yield significantly, but the strain can accumulate mcl-PHA from glucose alone owing to the availability of the 3-hydroxyacyl-acyl carrier protein-CoA transacylase (PhaG) (O'Leary et al. 2005). The amino acid sequence derived was more than 99% identical to that of a transacylase from *P. putida* KT2440 (O'Leary et al. 2005).

Pseudomonas fluorescens BM07 was isolated from activated sludge in a municipal wastewater treatment plant in Chinju, Korea, in 2001 (Lee et al. 2001). This strain was able to produce up to 25.2% (w/w) mcl-PHA that contained 30.8 mol% 3-hydroxy-*cis*-5-dodecenoate and 4.5 mol% 3-hydroxy-*cis*-7-tetradecenoate from nonrelated carbon sources such as fructose and succinate. Interestingly, stearic acid was taken up by this strain and stored inside the cell as the free acid. *P. fluorescens*, especially strain GK13, is better known for its capability of degrading extracellular poly[(*R*)-3-hydroxyoctanoate] (Schirmer and Jendrossek 1994; Schirmer et al. 1993, 1995).

Pseudomonas citronellolis was found to produce mcl-PHA on many different substrates (Choi and Yoon 1994). Similar to *P. fluorescens*, this strain also produced unsaturated monomers from unrelated C_2–C_4 acids up to 9 mol% 3-hydroxy-*cis*-5-dodecenoate and up to 2.6 mol% 3-hydroxy-*cis*-7-tetradecenoate, with the largest content being (*R*)-3-hydroxydecanoate. A novel copolyester, poly(3-hydroxy-7-methyl-6-octenoate-*co*-3-hydroxy-5-methylhexanoate), was synthesized when *P. citronellolis* was grown on citronellol (Choi and Yoon 1994).

Pseudomonas aeruginosa was found to accumulate a carbon-storage compound as described by MacKelvie et al. (1968). Only in 1990 Timm and Steinbüchel found that this strain actually accumulates mcl-PHA from unrelated carbon substrates as demonstrated with gluconate (Timm and Steinbüchel 1990). The genetical organization of the mcl-PHA operon was identified by the same authors and was found to be similar to that of the other mcl-PHA producers (Timm and Steinbüchel 1992). The strain *P. aeruginosa* 42A2 produces PHA with unsaturated side chains and significant amounts of rhamnolipids when cultured on unsaturated fatty acids (Bassas et al. 2006; Haba et al. 2007). Different feeding strategies have been developed to reduce the rhamnolipid production (Dhariwal et al. 2008). In contrast to *P. putida* strains, *P. aeruginosa* can grow on intact triacylglycerols.

Another strain that is able to grow on triacylglycerols and oils is *Pseudomonas resinovorans* (coconut oil, soybean oil, tallow) (Ashby et al. 2001; Cromwick et al. 1996). These substrates may significantly reduce the production costs.

However, these oils represent new challenges in the production because of low water solubility as a feedstock and in downstream processing and purification of mcl-PHA because of similar solubility in organic solvents.

2.3 *Biochemistry of Biosynthesis*

Three different pathways could be identified for the formation of the mcl-PHA precursor 3-hydroxyacyl-CoA thioester (see Fig. 2; Huijberts et al. 1995; Rehm et al. 1998). β-Oxidation is the main pathway for mcl-PHA biosynthesis when bacteria

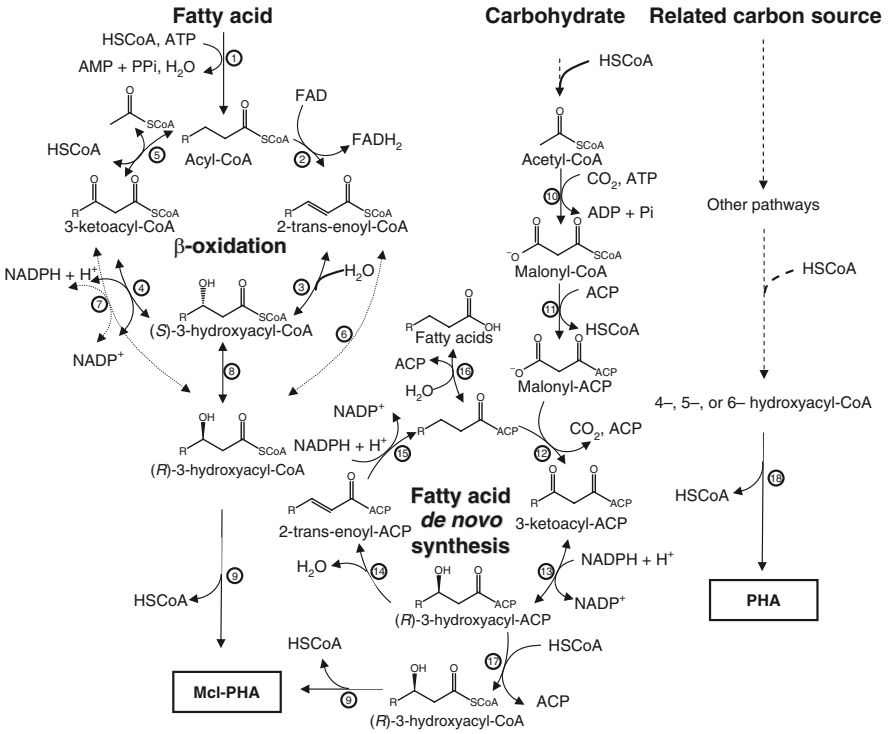

Fig. 2 Metabolic routes for mcl-PHA biosynthesis. *Pseudomonas putida* GPo1 synthesizes PHA through β-oxidation and *P. putida* KT2440 synthesizes PHA through fatty acid *de novo* synthesis. Special PHA consisting of 4-hydroxyalkanoate, 5- hydroxyalkanoate, or 6-hydroxyalkanoate can be produced by various bacteria when suitable precursors are supplied. *1* acyl-CoA synthetase, *2* acyl-CoA dehydrogenase, *3* enoyl-CoA hydratase, *4* NAD-dependent (*S*)-3-hydroxyacyl-CoA dehydrogenase, *5* 3-ketoacyl-CoA thiolase, *6* (*R*)-specific enoyl-CoA hydratase, *7* NADPH-dependent 3-ketoacyl-CoA reducatase, *8* 3-hydroxyacyl-CoA epimerase, *9* mcl-PHA polymerase, *10* acetyl-CoA carboxylase, *11* malonyl-CoA-acyl carrier protein (ACP) transacylase, *12* 3-keto-ACP synthase, *13* 3-keto-ACP reductase, *14* 3-hydroxyacyl-ACP dehydratase, *15* enoyl-ACP reductase, *16* acyl-ACP thiolase, *17* (*R*)-3-hydroxyacyl-ACP-CoA transacylase, *18* mcl-PHA polymerase

are grown on fatty acids (Kessler and Witholt 2001). Typically substrate-related production is the case, which means that the monomeric unit composition of the mcl-PHA is similar to that of the substrate but may be shortened by two carbon units. Thus, fatty acids with even carbon numbers result only in even-numbered (*R*)-3-hydroxyalkanoates, whereas odd-numbered fatty acids result only in odd-numbered (*R*)-3-hydroxyalkanoates. Phenylalkanoic fatty acids are integrated in analogy (Huijberts et al. 1995).

A second pathway results in 3-hydroxyacyl-CoA thiosters from *de novo* fatty acid synthesis, e.g., when carbohydrates or glycerol are supplied as the carbon substrate. As previously mentioned, this type of biosynthesis is considered to be non-substrate-related. In fact the monomers found in *P. putida* KT2442 grown on glucose included unsaturated monomers, e.g., (*R*)-3-hydroxydodecenoic and (*R*)-3-hydroxytetradecenoic acid (Huijberts et al. 1992a). It is worth mentioning that 4-pentenoic acid and 2-bromooctanoic acid inhibit the enzyme(s) linking the two pathways, fatty acid synthesis and β-oxidation (Lee et al. 2001).

As a third production pathway, acyl chain elongation has been reported for PHA production during growth of *P. putida* KT2442 on hexanoic acid (Huijberts et al. 1995). This is a rather unconventional process and may take place only under special growth conditions.

As previously mentioned, mcl-PHA-accumulating bacteria have better survivability when carbon starvation takes place (Ruiz et al. 2001). Interestingly, indications could be found that *P. putida* GPo1 simultaneously accumulates and degrades mcl-PHA (Ren et al. 2009; Zinn 1998; see also the model displayed in Fig. 3) as was previously found by Doi et al. (1990) in the case of *Cupriavidus necator*. This simultaneous process of synthesis and degradation of polyhydroxybutyrate (PHB) was confirmed by ^{14}C-glucose pulse experiments (Uchino and Saito 2008). The process might depend on intracellular concentrations of PHB-related metabolites or cofactors such as NADH, NAD, and CoA (Senior et al. 1972). In addition, phasins may also contribute to regulation of PHA synthesis and decomposition (Prieto et al. 1999a; York et al. 2001). PHB accumulation is only observed under nutrient-limited growth conditions because under optimal growth conditions PHB polymerase is inhibited by free CoA (de Eugenio et al. 2007, 2008) and because formation of the PHB precursor acetoacetyl-CoA is reversible, with the equilibrium on the side of acetyl-CoA (O'Leary et al. 2005). In *P. putida* KT2442, PhaZ is also expressed during PHA synthesis; however, when the cells were exposed to non-nitrogen limiting conditions, *phaZ* expression was promoted and the PhaZ concentration was found to be 4 times higher (Jendrossek and Handrick 2002). Limitation of vital nutrients might direct a regulatory system for transcription of *pha*Z, as suggested for *P. putida* CA-3 (Timm and Steinbüchel 1992). Transcriptional regulation is under investigation: For example, a regulatory gene *phaR* of *Pseudomonas lemoignei* has been identified with a putative helix–turn–helix motif which might indicate a DNA-binding protein and thus might be involved in the regulation of PhaZ expression (Hoffmann and Rehm 2004). In *P. aeruginosa*, two promoters are located upstream of *phaC1* and one

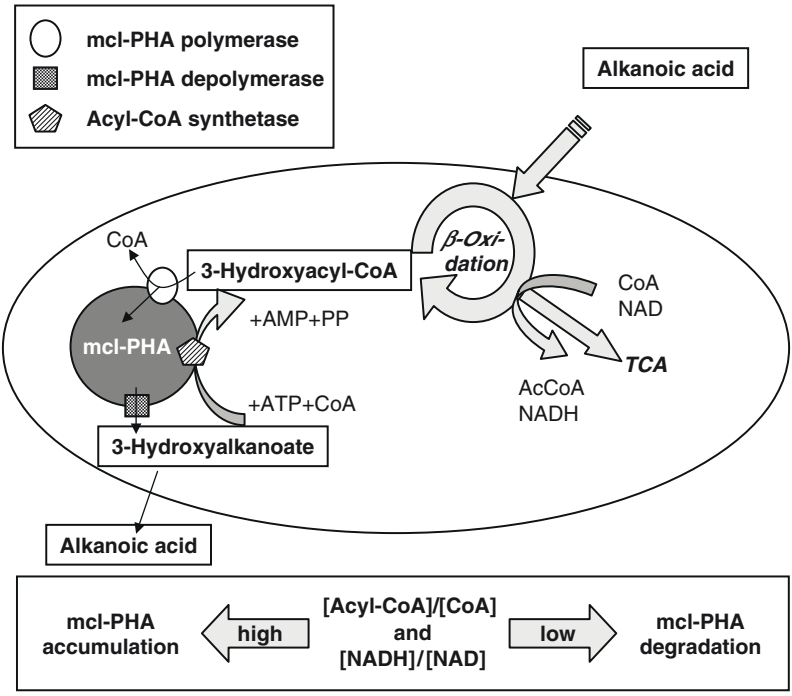

Fig. 3 Model for regulation of mcl-PHA at the enzymatic level in *P. putida* GPo1. Alkanoic acids of mcl (C_6–C_{14}) are taken up by the cell and enter the β-oxidation cycle. The mcl-PHA synthase preferentially polymerizes only (*R*)-3-hydroxyacyl-CoA of mcl. It is proposed that during accumulation degradation also takes place (Ren et al. 2009; Zinn 1998). Depending on the energetic status of the cell, the 3-hydroxyalkanoate can be excreted or recycled to (*R*)-3-hydroxyacyl-CoA by the acyl-CoA synthetase. Consequently, (*R*)-3-hydroxyacyl-CoA can be polymerized to mcl-PHA again or channeled back to the β-oxidation cycle and subsequently to the tricarboxylic acid cycle for energy gain

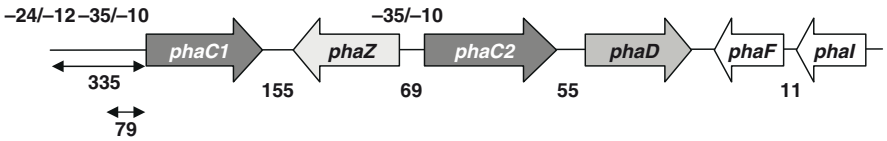

Fig. 4 The *pha* operon of *P. putida* GPo1 comprises two PHA polymerase genes (*phaC1*, 1,677 bp and *phaC2*, 1,730 bp), one depolymerase (*phaZ*, 849 bp), a regulatory gene (*phaD*, 615 bp), and two phasins with structural function (*phaF*, 767 bp and *phaI*, 419 bp). The mode of operation of promoters (–24/–12 bp, –35/–10 bp) is not clear yet (Huisman et al. 1991; Prieto et al. 1999c). *Numbers* indicate the number of base pairs

promoter is located upstream of *phaC2* (Kessler and Witholt 2001). Cotranscription of *phaC1*, *phaZ* and *phaC2*, or *phaC1* and *phaZ* was reported, but not confirmed (see Fig. 4; Kessler and Witholt 2001; Qi et al. 2000). The exact mechanism of

how transcription is activated and how PHA accumulation is triggered when growth becomes nutrient-limited has not yet been described. Even though PHA can be produced in larger amounts, not many details are known about the underlying mechanisms, including the detailed regulation of mcl-PHA accumulation and degradation.

2.4 Genetic Engineering

There are many publications about genetical engineering of the mcl-PHA-producing strains (Aldor and Keasling 2003; Kessler et al. 2001; Kim et al. 2006; Madison and Huisman 1999; Niamsiri et al. 2004; Steinbüchel and Lütke-Eversloh 2003). Here only a small and incomplete selection of publications is given that demonstrates the influence on production or material composition.

The gene for PHA polymerase C1 of *P. putida* GPo1 was successfully expressed in both *P. putida* GPp104 (PHA negative mutant of strain KT2442) and *Escherichia coli* JMU193 (Ren et al. 2005a). By the use of an inducible PalkB promoter, polymerase levels could be modulated over a wide range. The maximal polymerase levels in the *E. coli* host were about 3–4 times lower in comparison with the *P. putida* host. The PHA content per cell dry weight reached maximally 8 and 30% for recombinant *E. coli* and *P. putida*, respectively. In addition, the time course of polymerase expression in complex medium batch culture differed significantly in *P. putida* and *E. coli*. These results suggest that for optimal functioning of the PHA polymerase a molecular environment is required that is available in *P. putida* but not in *E. coli*.

As described above, *P. putida* KT2440 and KT2442 are able to accumulated mcl-PHA from nonrelated carbon substrates, unlike *P. putida* GPo1. The reason for this difference is the expression of the enzyme 3-hydroxyacyl-acyl carrier protein-CoA transferase (PhaG) in KT2440 and KT2442 (Rehm et al. 1998) as mentioned previously. Rehm et al. (1998) produced a chemical mutant of KT2440 by nitrosoguanidine mutagenesis and it was found that a mutant where PhaG was not produced any more showed a significant increase of PHA accumulation of up to 85% (w/w). Unfortunately, the strain was lost by the original laboratory. It might be interesting to see whether a *phaG* knockout mutant has the same increased accumulation as has been published.

P. putida KTOY06 was genetically engineered by the deletion of the genes of 3-ketoacyl-coenzyme A (CoA) thiolase (*fadA*) and 3-hydroxyacyl-CoA dehydrogenase (*fadB*). The β-oxidation pathway was weakened and therefore the carbon source tetradecanoic acid was converted to a mcl-PHA containing 31–49 mol% (*R*)-3-hydroxytetradecanoic acid as the main component, whereas the (*R*)-3-hydroxyhexanoic acid content remained almost constant at 3 mol%. The mechanical properties were influenced by the content of (*R*)-3-hydroxydodecanoic or (*R*)-3-hydroxytetradecanoic acid, respectively, as can be derived from Table 1.

3 General Production Processes

3.1 Basic Concepts of Nutrition

In 1840 the German chemist Justus von Liebig formulated the "law of the minimum" (von Liebig 1840), which strongly influenced the theoretical understanding of nutrition in biology. The law states that one nutrient always limits the amount of biomass that can be produced in a biological system. This comprehension ruled the experimental design by microbiologists for many years (van Niel and Hahn-Hagerdal 1999). Consequently, synthetic media for the cultivation of microorganisms are usually designed such that one specific nutrient limits the amount of biomass that can be formed (Egli and Zinn 2003). As a consequence, the biomass that can theoretically be produced in such a system is a function of the initial concentration and the biomass yield coefficient for this particular nutrient (Gottschal 1993).

A complementary approach focuses on the kinetic aspect of biomass growth. The studies by Blackman (1905) and Monod (1949) opened the way for the description of growth using kinetic models. In these models it is assumed that the concentration of a substrate and the affinity of the cell towards this substrate determine the rate of increase of biomass.

With the invention of the chemostat (Monod 1950; Novick and Szilard 1950) and better control systems, it was possible to investigate the stoichiometry (Herbert 1961) and kinetics of cell growth (Senn et al. 1994) in a better way. Quantitative studies revealed that the specific growth rate and the nature of the limiting nutrient have decisive influences on the cell composition (Goldman and Dennett 2000; Heldal et al. 1996), whereas studies of the kinetics focused on the affinity of cellular uptake systems towards nutrients and the observed specific growth rate (Button 1985; Button 1993; Kovarova-Kovar and Egli 1998).

Most mcl-PHA-producing bacteria start to accumulate PHA in a larger amount when their cell growth is impaired by the limitation of an essential nutrient (e.g., N, P, Mg, K, O, or S) in the presence of excess carbon (Lageveen et al. 1988). Typically, batch cultures become kinetically limited with the exhaustion of the essential nutrient. The term "nutrient starvation" is in this case the best description because the cell number is not increasing any more, but the specific biomass increases owing to PHA accumulation. In contrast, the cells in continuous cultivation are always in an exponential growth phase and therefore one speaks of "nutrient limitation", which reflects the stoichiometric perception (Zinn et al. 2004).

3.2 Batch and Fed-Batch Systems

In many cases, fed-batch cultures are carried out as two-phase fermentations. In the first phase, cells are cultured to a maximum cell density that can be reached by a minimal medium. In the second phase, only the carbon substrate for PHA accumulation

is supplied and the culture becomes starved for a nutrient (e.g., phosphorus). Since the accumulation is a linear process, this carbon feed can be done at a linear rate to cover cell energy maintenance and PHA accumulation. There are many deviations from this basic design and especially for high cell density cultivations (HCDCs) the first phase is also separated into two steps, namely, a batch and a fed-batch phase without significant PHA accumulation (Elbahloul and Steinbüchel 2009).

In the case of *P. putida* KT2440, a so-called one-step fed batch can be carried out where the cells are supplied with a highly concentrated medium to limit volume increase (Sun et al. 2009). This is possible because this strain does not require a nutrient limitation as mentioned previously. A significant problem with this strategy is the oxygen supply (Kellerhals et al. 2000). Other approaches follow more sophisticated control systems to reduce the fermentation problems and have been nicely reviewed recently (Sun et al. 2007b).

3.3 Chemostat

Continuous fermentation has been optimized for the efficient production of mcl-PHA (Hartmann et al. 2006; Huijberts and Eggink 1996; Prieto et al. 1999b). It has been shown that the PHA content decreased with a higher specific growth rate (Durner et al. 2000). Thus, a compromise between PHA content and productivity is required in a single-stage continuous process (Sun et al. 2007c). A clear advantage of chemostat cultivations is the tight control of the cell physiological functions by selected nutrient limitations (Egli 1999). Interestingly, it was found that nonanoic acid triggered mcl-PHA accumulation in *P. putida* GPo1 even under carbon-limited growth conditions (Durner et al. 2001). An explanation for this observation has not been given so far.

3.4 High Cell Density Cultivations

Economically feasible production of biomaterials with microorganisms requires high space–time yields (g $L^{-1} h^{-1}$). When the product is biomass-associated like PHA, high cell density (fed-batch) cultivation (HCDC) is most commonly used to reach maximal productivities (Lee et al. 1999; Riesenberg and Guthke 1999). Product concentrations well above 100 g L^{-1} and productivities of up to 4.9 g $L^{-1} h^{-1}$ can be reached with bacteria producing scl-PHA {e.g., poly[(*R*)-3-hydroxybutyrate] in fed-batch culture; Kim et al. 1996; Ryu et al. 1996; Wang and Lee 1997a, b}. However, a general problem of fed-batch processes is the change of growth conditions over time, especially when nonexponential feed profiles are applied. The biomass increase in fed-batch culture leads to an increasingly restricted supply of nutrients on a per cell basis, which in turn lowers the specific growth rate and, for example, negatively affects PHA synthesis (Suzuki et al. 1986a, b, 1988). The changing growth conditions also explain why

the composition of the PHA monomer cannot be controlled properly in batch or fed-batch cultures (Hartmann et al. 2006; Majid et al. 1999). However, recently it was shown that *P. putida* KT2440 can produce poly[(*R*)-3-hydroxyoctanoate-*co*-(*R*)-3-hydroxy-10-undecenoate] at almost constant composition on condition that a sophisticated feeding strategy was applied resulting in a constant growth rate (Sun et al. 2007b).

As mentioned above for high cell density fed-batch cultures, the two-step fermentation strategy has often been applied, where the cell division is separated from the PHA accumulation phase. With use of octanoic acid as a substrate, PHA contents of up to 75% (w/w) at a cell concentration of 55 $g L^{-1}$ and a volumetric productivity of 0.63 $g L^{-1} h^{-1}$ were obtained with *P. putida* GPo1 under nitrogen limitation (Kim et al. 2002). With *Pseudomonas* IPT 046, cell concentrations of up to 50 $g L^{-1}$ with a PHA content of 63% (w/w) and a volumetric productivity of 0.8 $g L^{-1} h^{-1}$ were reached under phosphate limitation using glucose and fructose as a mixed carbon source (Diniz et al. 2004). When oleic acid was used as a substrate for the cultivation of *P. putida* KT2442, a cell concentration of 141 $g L^{-1}$ with a PHA content of 51% (w/w) and a volumetric productivity of 1.91 $g L^{-1} h^{-1}$ were obtained under phosphate limitation (Lee et al. 2000).

In contrast to fed-batch cultures, steady-state continuous (chemostat) cultures are characterized by time-independent substrate availability and constant specific growth rate (Egli 1991; Pirt 1975). The specific cell productivity for mcl-PHA in high cell density chemostat culture is in the same range as found for regular fed-batch culture; nevertheless, the technique has not yet been applied on an industrial scale (Lee 1996a, b). In a case study by Mathys et al. (1999), a continuous alkane oxidation process (chemostat culture) with *P. putida* GPo1 was estimated to be as cost-efficient as a fed-batch process. However, the maximal volumetric mcl-PHA productivities reported for chemostat cultures are not satisfactory because they do not exceed 0.7 $g L^{-1} h^{-1}$, neither for *P. putida* GPo1 nor for *P. putida* KT2440 (Huijberts and Eggink 1996; Preusting et al. 1991).

A significant improvement could be achieved by using a two-stage chemostat process (Jung et al. 2001). Hereby the first stage consists of a regular chemostat, whereas the second one is fed with the fermentation broth of stage 1 and is additionally supplied with the mcl-PHA substrate. Jung et al. (2001) established a two-stage process with *P. putida* GPo1 and *n*-octane as a single carbon source in both bioreactors. Cell densities of 18 $g L^{-1}$ with a PHA content of 63% (w/w) and an overall volumetric productivity of 1.06 $g L^{-1} h^{-1}$ were obtained.

3.4.1 Use of Elevated Pressure to Enhance Oxygen Transfer in Bioprocesses

A challenge of PHA synthesis in high cell density cultures is the sufficient supply of oxygen (Sun et al. 2007c). Owing to the low solubility of molecular oxygen, adequate oxygenation of aerobic HCDC processes is still a challenging task in engineering (Knoll et al. 2005). The oxygen transfer rate (OTR) is a function of reactor design, stirrer speed, and aeration rate (combined in the term $k_L a$), and the

driving force, i.e., the gradient between dissolved O_2 and the O_2 concentration in the gas phase ($c^*_{o_2} - c_o$):

$$\mathrm{OTR} = k_L a(c^*_{O_2} - c_{O_2}). \quad (1)$$

Above a certain limit, the OTR can only be enhanced by raising the oxygen partial pressure in the reactor gas phase (Kellerhals et al. 1999; Sumino et al. 1992). Mostly the problem is solved by adding pure oxygen to the air supply (Castan et al. 2002), although costs are significantly increased and special safety precautions may be required (Schmid et al. 1999). Cultivation under increased atmospheric pressure has already been proposed as a valid alternative at an early stage of industrial biotechnology (May et al. 1934). The maximal OTR can be improved by a factor of 2.3 in normal, air-ventilated bioreactors simply by restricting off-gas flow, which results in an overpressure of 1.7 bar (Yang and Wang 1992). With specially equipped bioreactors, overpressures of up to 9 bar (1-MPa total pressure) are possible, resulting in a corresponding further improvement of the OTR (Knoll et al. 2005; Schmid et al. 2001). High-pressure reactors are still rarely used in industry in spite of the fact that the cost-efficiency of oxygen transfer can be improved by a factor of at least 1.4, even without considering costs for the purchase of pure oxygen (Knoll et al. 2005). The additional costs needed for pressurization of bioreactors up to 40 m^3 were estimated not to contribute significantly to the total investment costs for a new fermentation unit (Maier 2002). The Swiss Federal Laboratories for Materials Testing and Research (Empa) is currently investigating the influence of elevated atmospheric pressure on the biosynthesis of mcl-PHAs in *P. putida* strains. The bioreactor can be run in the fed-batch and chemostat mode up to an overpressure of up to 6 bar. The effect of pressure will be examined with fermentations of *P. putida* KT2440 and analyzed by gene arrays.

4 Production Processes to Tailor mcl-PHAs

4.1 Multiple Nutrient Limited Growth

It was shown a few years ago that PHA-accumulating bacteria are able to grow in chemostat cultures limited simultaneously by carbon and nitrogen substrates (Durner et al. 2001), a growth regime which is principally not possible in batch culture. It was concluded that dual (C, N) limited growth offers a new approach to tailor the PHA composition during biosynthesis because all carbon substrates are consumed to completion (Zinn et al. 2001, 2004).

The dual (C, N) limited growth ($D = 0.1\ h^{-1}$, C/N = 16 mol mol^{-1}) was used to produce functionalized mcl-PHA in *P. putida* GPo1 (Zinn and Hany 2005; Zinn et al. 2004). A given amount of 10-undecenoic acid in the carbon feed resulted in the formation of an identical proportion of olefinic (terminally unsaturated) PHA monomers in the polymer (Table 2). Furthermore, recent experiments revealed

Table 2 PHAs produced in continuous cultures grown on different carbon mixtures ($D = 0.1\ h^{-1}$; C/N = 15 $g\,g^{-1}$ in the medium feed) (data taken from Hartmann et al. 2006)

Experimental conditions	A	B	C	D	E	F
C-source feed (mol%)						
Octanoate	100	90	75	50	25	0
10-Undecenoate	0	10	25	50	75	100
Biomass and PHA production						
CDW (g L^{-1})	1.35	1.27	1.34	1.28	1.36	1.31
PHA content during steady state (%CDW)	37	27	29	37	27	30
PHA composition (mol%)						
Σ 3-hydroxyalkanoates	100	89.8	73.0	46.7	23.3	0
3-Hydroxyoctanoate	86.2	76.1	61.1	39.6	19.3	0
3-Hydroxyhexanoate	13.8	13.7	11.9	7.1	4.0	0
Σ 3-hydroxyalkenoates	0	10.2	27	53.3	76.7	100
3-Hydroxy-10-undecenoate	0	1.8	4.6	11.0	13.7	15.6
3-Hydroxy-8-nonenoate	0	8.4	18.8	36.8	52.6	69.5
3-Hydroxy-6-heptenoate	0	0	3.6	5.5	10.4	14.9

CDW cell dry weight

that the integration of an aromatic monomer [(*R*)-3-hydroxy-5-phenylvalerate] increased the glass-transition temperature from −38 to −6°C while maintaining functionality (olefinic monomers) at 10 mol% for all copolymer compositions (see Fig. 5; Hartmann et al. 2004). It is worth mentioning that storage of functionalized polymer needs to be considered carefully because loss of functionality could be observed in the case of terminal double bonds in the side chains (Schmid et al. 2001).

5 Conclusions

mcl-PHAs are very versatile in their composition and material properties. Owing to their elongated side chains, they are less crystalline than scl-PHAs and are suitable for all kinds of applications where elastomeric properties are required. Particular growth conditions allow control of the monomeric unit composition and thus enable tailoring of the functionality that may be used for chemical modification after biosynthesis – a property that is unique among polyesters. Owing to the high production costs of mcl-PHAs, bulk applications, e.g., in the packaging sector, have not been realized yet. Ongoing studies revealed clearly that waste streams could significantly reduce the production costs. Different sources of waste, such as vegetable oils (Song et al. 2008), animal fats, dairy whey, molasses, and meat-and-bone meal, have been considered to have a significant potential (Solaiman et al. 2006). Further, genetical engineering may help to introduce new pathways or optimize productivity in bacterial strains. To date, the largest field of applications of mcl-PHA has to be seen in high-tech applications, in particular in the

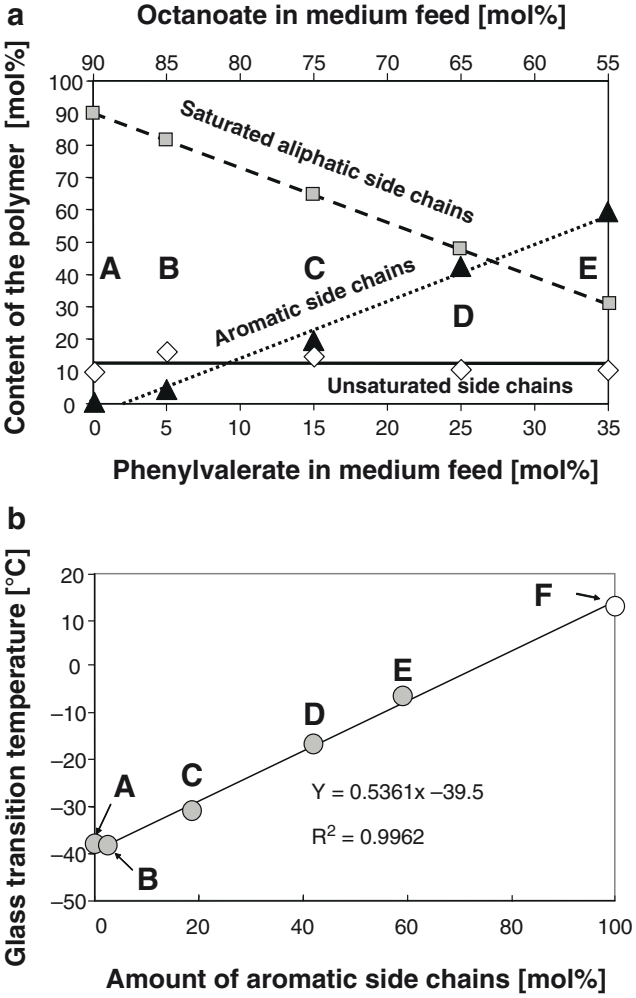

Fig. 5 Tailor-made mcl-PHA in chemostat cultivation ($D = 0.1\ h^{-1}$; C/N = 15 $g\,g^{-1}$ in the medium feed) of *P. putida* GPo1 with 10-undecenoate (fixed substrate feed at 10 mol% of total carbon feed), and variable amounts of 5-phenylvalerate and octanoate. **a** Composition of mcl-PHA as a function of the carbon feed composition, **b** glass-transition temperature of polymers with various contents of saturated and aromatic monomers. *Letters* indicate identical polymers in **a** and **b**. *F* indicates the glass-transition temperature of the homopolymer poly(3-hydroxyphenylvalerate) described by Fritzsche et al. (1990b). (Redrawn from Hartmann et al. 2004)

pharmaceutical and medical field, where quality, e.g., biocompatibility, is of great importance (Chen and Wu 2005b).

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Nodax™ Class PHA Copolymers: Their Properties and Applications

Isao Noda, S. Blake Lindsey, and Daniel Caraway

Contents

Abstract A family of promising polyhydroxyalkanoate (PHA) polyesters called Nodax™ class PHA copolymers, consisting of (*R*)-3-hydroxyalkanoate comonomer units with medium-size-chain side groups and (*R*)-3-hydroxybutyrate, are described. The bio-based biodegradable plastics made from renewable resources will be commercially available from Meredian. Because of the unique design of the molecular structure, the Nodax™ class PHA copolymers have a set of useful attributes, including polyolefin-like thermomechanical properties, polyester-like physicochemical properties, and interesting biological properties. Therefore,

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broad ranges of industrial and consumer product applications are anticipated. The structure and properties of the new PHA copolymers, as well as processing and conversion to various products are reviewed with some historical background of the development and future commercialization plans.

1 Introduction

Polyhydroxyalkanoate (PHA) is one of the promising bio-based biodegradable plastics made from renewable resources (Doi 1990). It is currently made by the bacterial fermentation of biomass, but a future possibility exists to produce PHA by using higher organisms (Poirier et al. 1995). Among a large number of known PHAs (Steinbüchel 1995), the so-called Nodax™ class PHA copolymers (Poliakoff and Noda 2004; Noda et al. 2005a, b), which will become available from Meredian (Bainbridge, GA, USA), are showing great potential to be a plastic resin of general utility across a broad array of applications.

The early development of this class of PHA copolymers was initiated by Procter & Gamble (Cincinnati, OH, USA) around the late 1980s. During the several decades of intensive research effort, Procter & Gamble accumulated a broad base of intellectual properties associated with this class of materials (Noda 1996, 1999). In 2007, Meredian took over the Nodax™ technology from Procter & Gamble for the full commercialization of this class of bioplastics. A large-scale commercial production of the PHA copolymers is scheduled to start in a few years. Given this new development, a comprehensive description of this class of material may be of interest to scientists and engineers in the field.

PHA copolymers having the Nodax™ class molecular structure exhibit a unique set of combined useful attributes, including polyolefin-like thermomechanical properties, polyester-like physicochemical properties, and interesting biological properties (Satkowski et al. 2001; Federle et al. 2002; Noda et al. 2004, 2005a, b). Therefore, broad ranges of industrial and consumer product applications are anticipated for this class of material. In this chapter, the basic structure and properties of the PHA copolymers, as well as their processing and potential conversion to various products, are reviewed with some historical background of the development and future commercialization plans.

2 Molecular Structure

The general molecular structure of the so-called Nodax™ class PHA copolymers is a random copolymer of predominantly (*R*)-3-hydroxybutyrate (3HB) and other (*R*)-3-hydroxyalkanoate (3HA) comonomer units, as shown schematically in Fig. 1. The secondary 3HA comonomer units must have side groups consisting of at least

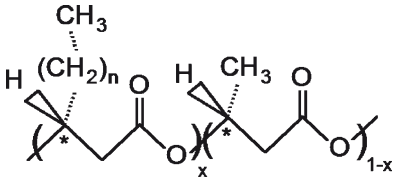

Fig. 1 Molecular structure of Nodax™ class polyhydroxyalkanoate (PHA) copolymer. The value of *x* is between 2 and 50%

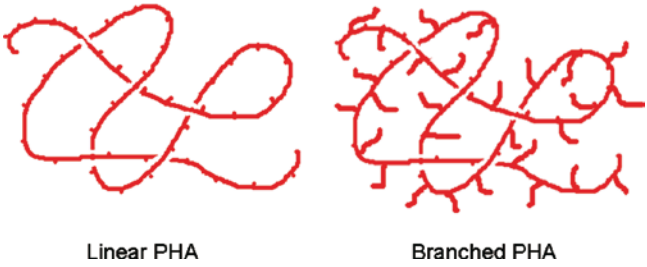

Fig. 2 Comparison between essentially linear PHA and moderately branched PHA copolymer

three carbon atoms. Examples of such 3HA units with medium-chain-length (mcl) side groups include (*R*)-3-hydroxyhexanoate (3HHx), (*R*)-3-hydroxyoctanoate (3HO), (*R*)-3-hydroxydecanoate (3HD), and (*R*)-3-hydroxyoctadecanoate (3HOd). Such PHA copolymers typically consist of at least 50 mol% 3HB and at least 2 mol% secondary mcl-3HA units.

The functional architecture of this class of PHA copolymers is substantially different from that of more familiar types of PHAs, such as poly[(*R*)-3-hydroxybutyrate] (PHB) homopolymer or poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxy valerate] (PHBV) copolymer. The size of the side groups in the conventional PHAs is limited only to short-side-chain types with no more than two carbon atoms. Although PHAs with only one or two carbon side groups may be viewed essentially as linear polymers, PHA copolymers with mcl side groups belong to a broad class of moderately branched polymers. The inclusion of a small amount of mcl-3HA units into the PHA polymer backbone leads to some profound changes in important physical properties of this class of copolymers.

The motivation behind the molecular design of Nodax™ class PHA copolymers closely follows that of the well-known industrial polyolefin linear low density polyethylene (LLDPE). LLDPE is a random copolymer of ethylene with a small amount of α-olefin units, such as 1-butene or 1-hexene, which will result in the formation of the polymer chain structure with mcl alkyl side group branches. In a similar manner, one can envision the possibility of creating a polymer structure of LLDPE with a PHA backbone having short alkyl side chains, as depicted in Fig. 2.

The effect of incorporating such short branches on the physical properties of highly crystallizable polymers such as polyethylene is well known. The branches act as a molecular defect, which disrupts the excessive regularity of the polymer chain and consequently lowers the melt temperature (T_m) and crystallinity. High-density polyethylene or PHB homopolymer without sizable side groups has high T_m and crystallinity. The material, therefore, is relatively hard and brittle and may be useful for making bottles and milk jugs. In contrast, copolymers with medium-size-chain side groups have much lower T_m and crystallinity. They become substantially more flexible and ductile and are suitable for making films and other soft articles.

3 Preparation Methods

3.1 Chemical Synthesis

It is noteworthy to point out that most of the Nodax™ class PHA copolymers studied in the early days were initially prepared by the classic chemical synthesis based on the ring-opening polymerization of chiral derivatives of lactone monomers (Schechtman and Kemper 1997; Federle 2002). The chemical synthesis program was pushed forward on the basis of the careful analysis of the biochemical synthetic pathway of existing PHA, which clearly indicated the possible existence of microorganisms capable of producing this type of copolymers. A large-scale production of PHA copolymers by the polymerization of rather expensive chiral monomers was certainly not a commercially very attractive option. On the other hand, a small-scale chemical synthesis in the laboratory allowed the researchers to examine the properties of a variety of new PHA copolymers, which had not yet been discovered in biological systems. The availability of a large number of novel PHA samples made by the chemical synthesis indeed greatly contributed to the early buildup of the broad intellectual properties related to this class of PHA copolymers (Noda 1996). The corresponding biosynthesis of the same PHA copolymers occurred much later, after the discovery of suitable microorganisms.

Figure 3 shows the basic scheme for the polymer synthesis. Details of the early chemical synthesis of mcl-3HA-containing PHA copolymers have been described elsewhere (Schechtman and Kemper 1997). It was recognized that sufficiently high molecular weights must be obtained to test the properties of these materials to their fullest potential. Particular care was taken to ensure random comonomer distribution along the chain and to ensure a high degree of isotacticity to closely approximate

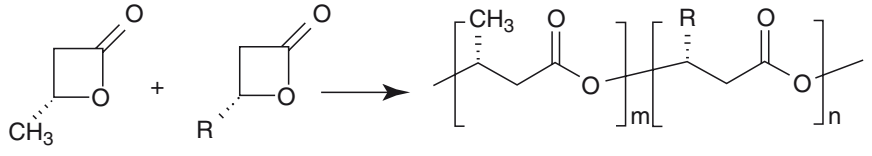

Fig. 3 Chemical synthesis of PHA copolymer by ring-opening polymerization (Noda et al. 2005b)

biosynthesized materials. Chiral 3-alkyl-β-propiolactone monomers were polymerized with zinc alkoxide initiator in dry toluene.

3.2 Biosynthesis

Many researchers in the past searched for the appropriate microorganisms capable of producing random copolymers of 3HB and mcl-3HA, now known as Nodax™ class PHA copolymers. Although some microbes were found to produce polymers containing various 3HA units, their specific origin as to where these 3HA came from was not certain (Brandl et al. 1989; Huisman et al. 1989; Timm and Steinbüchel 1990). Indeed, many of the early studies indicated that different 3HA fragments came from blends of PHB homopolymer and copolymers of mcl-3HA without 3HB units (Timm et al. 1990). Scientists from Kaneka in Japan (Shiotani and Kobayashi 1994) were the first to report the definitive discovery of microorganisms capable of producing copolymers of 3HB and 3HHx. By the mid-1990s, many other researchers started reporting the biosynthesis of various 3HA copolymers by using transgenic microorganisms (Abe et al. 1994; Caballero et al. 1995, Kato et al. 1996). Thus, the biosynthetic production of moderately branched PHA copolymers by a fermentation process has become possible (Lee et al. 2000; Chen et al. 2001).

The metabolic pathways utilized to produce poly[(*R*)-3-hydroxybutyrate-co-(*R*)-3-hydroxyhexanoate] (PHBHx) copolymer is shown in Fig. 4 (Noda et al. 2005a). Two units of acetyl-CoA forms the acetoacetyl-CoA with phaA thiolase, which is then converted to 3-hydroxybutyryl-CoA with phaB reductase. Parallel to these steps are the other metabolic pathways involving fatty acid biosynthesis (phaG) and

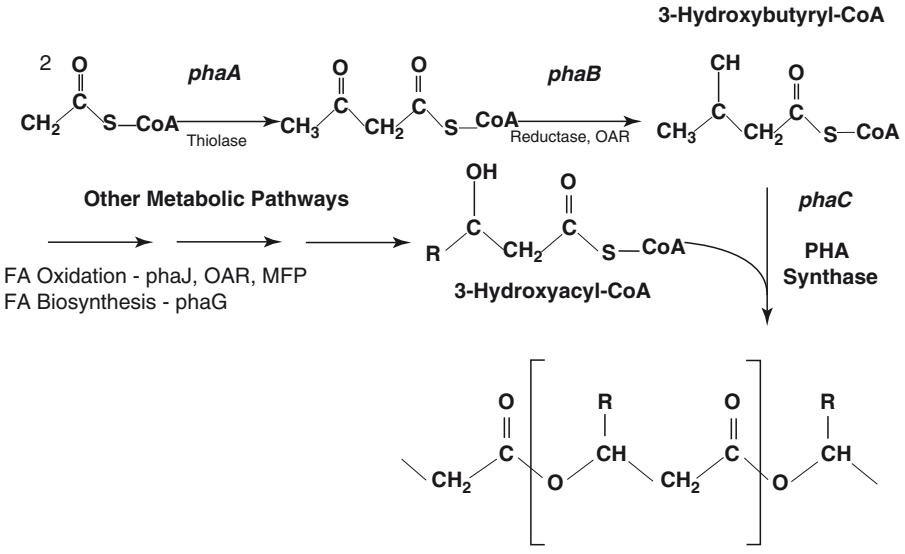

Fig. 4 Biosynthetic pathway of producing PHA copolymer (Noda et al. 2005b)

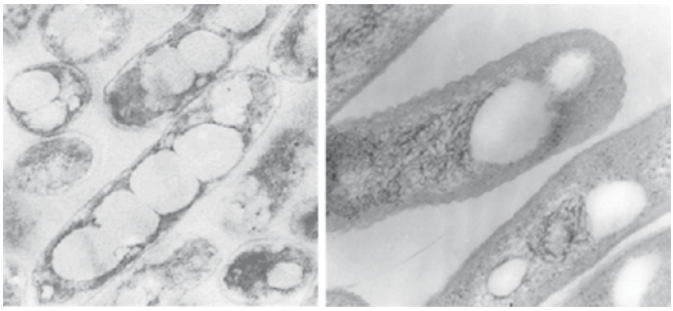

Fig. 5 Bacteria producing Nodax™ class PHA copolymers

fatty acid oxidation (phaJ, OAR, MFP), leading to the other 3-hydroxyacyl-CoA units. Finally, the copolymerization of 3HB-CoA and 3HA-CoA with phaC PHA synthase results in the production of this type of PHA copolymers. Figure 5 shows examples of microorganisms making different types of moderately branched PHA copolymers (Noda et al. 2005a).

4 Properties

4.1 Biological Properties

One of the very promising properties of biologically produced PHA copolymers is their rapid biodegradability (Noda et al. 2005a). Unlike any other type of biodegradable plastics, PHAs biodegrade under not only aerobic but also anaerobic conditions. Furthermore, PHA copolymers comprising mcl-3HA have a relatively low crystallinity compared with PHB homopolymer of PHBV copolymers. The lowered crystallinity in turn results in a very rapid degradation rate with microbial enzymes. Figure 6 show the degradation profiles of ^{14}C-labeled poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyoctanoate] (PHBO) samples under aerobic and anaerobic conditions. Details of the experimental protocols are reported elsewhere (Federle et al. 2002).

The result shows that this type of PHA copolymers can undergo a rapid biodegradation process, with or without oxygen. The PHA copolymer is shown to be readily mineralized by biodegradation to water and carbon dioxide, and in the case of the anaerobic condition also a small amount of methane. About 15–20% of the material is actually incorporated into the biomass.

It was found that the rates of both anaerobic and aerobic biodegradation of PHA copolymers are almost comparable to those of cellulose. This favorable biodegradability profile of PHA copolymers has a major practical implication. Articles made of this class

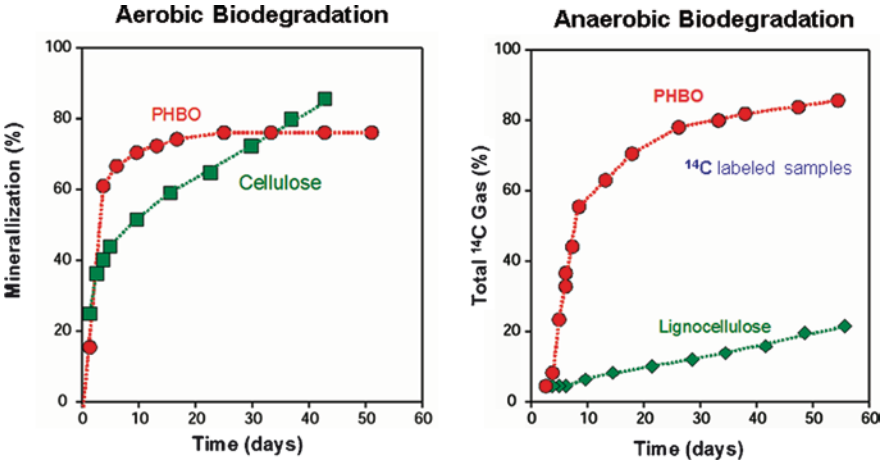

Fig. 6 Biodegradation of poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyoctanoate] (PHBO) copolymer (Noda et al. 2005b)

of polymers may be safely disposed of or flushed away in a common underwater environment, especially household septic tanks, where the access of free atmospheric oxygen is somewhat limited. Thin films and nonwoven fabrics made of PHA copolymers indeed undergo rapid biodegradation in a simulated septic tank at a rate comparable to tissue paper. A similar anaerobic biodegradation activity is expected in other environments, such as below the surface of rice paddies, rivers, and lakes.

It is also important to point out that the biodegradation of PHA copolymers will not take place unless the surrounding conditions support the biotic activities of microbes. The presence of moisture is essential for the degradation of even highly biodegradable materials, such as PHA and cellulose, as clearly demonstrated by the fact that books can be kept in a dry library for centuries. Likewise, commercial products made of PHA will not spontaneously biodegrade on the shelf, as long as they are kept in the ordinary low biotic environment. The same is true for a well-kept landfill with minimal water seepage, where disposed of articles are safely entombed. Thus, the biodegradation of products made of PHA copolymers may be designed and controlled according to the specific storage and use conditions.

4.2 Thermal Properties and Crystallinity

4.2.1 Melt Temperature

PHB homopolymer has a very high T_m, close to 180°C, which is close to its thermal decomposition temperature (Marchessault et al. 1990). The T_m of PHA may be controlled by the inclusion of 3HA comonomers along with 3HB units in a manner

analogous to LLDPE. The initial attempt to improve the properties of PHB homopolymer was to incorporate (*R*)-3-hydroxyvalerate (3HV) units into the polymer chain. Such a copolymer, PHBV, with very short ethyl side groups randomly distributed along the polymer chain, did indeed show some reduced T_m and crystallinity when a sufficient amount of 3HV units was incorporated. However, the efficacy of T_m and crystallinity lowering for a given amount of 3HV comonomer was surprisingly small.

It turned out that the molecular difference between 3HV and 3HB units with only one methylene group was too small to effectively disrupt the molecular regularity of the polymer chain. The ethyl side groups of 3HV comonomer units can be largely incorporated along with 3HB units without much structural disruption into the crystal lattice of PHB consisting of polymer chains with a relatively open helical conformation (Marchessault et al. 1990). Thus, the anticipated level of T_m and crystallinity lowering could not be achieved. It is difficult to bring down the T_m of PHBV much below 150°C by the moderate amount of incorporation of 3HV units. The relatively high T_m of PHBV imposes a definite practical limitation on the utility of PHBV copolymer as well as PHB homopolymer as general purpose commodity thermoplastics. The extent of thermal degradation during the melt processing of PHA becomes a major issue when the process temperature approaches the thermal degradation temperature of PHA. The thermomechanically induced degradation of PHA becomes noticeable at process temperature as low as 150°C (Satkowski et al. 2001).

Fortunately, it was later discovered that, unlike the ethyl side group of the 3HV unit, side chains having more than three carbons cannot be incorporated into the crystal lattice structure of PHB. Random incorporation of comonomer units, such as 3HHx, 3HO, and 3HD, which are rejected from the crystal structure, is an effective way of disrupting the excessive regularity of PHB homopolymer. Thus, it has become possible to dramatically reduce the crystallinity of PHA copolymers by adjusting the level of 3HA comonomer units having a medium chain of at least three carbons or more distributed along with the dominant 3HB comonomer units (Noda 1996; Satkowski et al. 2001).

Figure 7 shows the representative T_m data of PHA copolymers measured by differential scanning calorimetry as a function of the level of various 3HA comonomers, which are different from the dominant 3HB repeat units of the copolymer (Noda et al. 2005b). It should be noted that there are two very distinct groups of PHA copolymers showing different T_m-lowering trends. The T_m of PHBV copolymer does not change much from that of PHB homopolymer even at the level of 7 mol% incorporation of 3HV units. In contrast, the T_m of new PHA copolymers, consisting of mcl-3HA comonomer units, such as 3HHx, 3HO, and 3HD, is substantially lower than that of PHBV. Interestingly, the efficacy of T_m lowering for a given mole percentage incorporation of comonomer is essentially the same for all mcl-3HA comonomers. Thus, any mcl-3HA acts as the effective disruption of the regular structure of PHB, as long as the side group consists of at least three carbons.

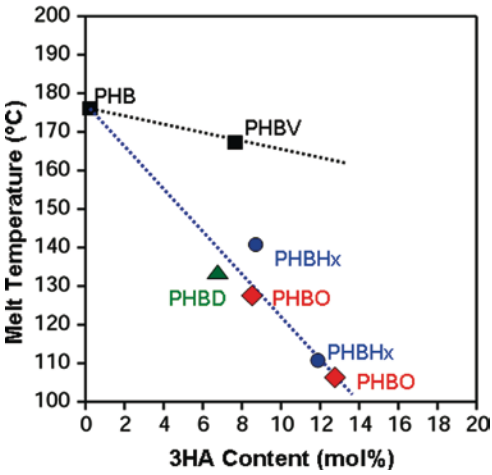

Fig. 7 Melt temperature of PHA copolymers (Noda et al. 2005b)

4.2.2 Crystallinity

Crystallinity of articles made of PHA copolymers is another property which can be adjusted by the incorporation of mcl-3HA units. Crystallinity influences the stiffness of the material. Because of the strict molecular stereoregularity of PHA created by the biosynthesis, the crystallinity of PHB homopolymer is known to become very high, often well in excess of 50%. Such high crystallinity results in the excessively hard and brittle material not well suited for many practical applications. The incorporation of mcl-3HA comonomer units, which cannot be incorporated into the PHB crystal lattice structure, should be an effective disruption to the excessive level of crystallinity.

Figure 8 shows the effect of 3HA incorporation into the PHB chain on the crystallinity of PHA copolymers measured by X-ray diffraction (Noda et al. 2005b). Again, two very distinct trends for the comonomer content dependence are observed. Although the level of crystallinity is not much affected by the incorporation of the 3HV unit, other 3HA units all systematically lower the crystallinity of PHA copolymers. As in the case of T_m lowering, the effect of mcl-3HA seems to be independent of the side group size, as long as it contains at least three carbons. Thus, both PHBHx and PHBO copolymers, with propyl and pentyl side groups respectively, show a similar crystallinity-lowering trend. Even poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyoctadecanoate] (PHBOd), a PHA copolymer with side groups much longer than 15 carbon atoms, shows a similar trend.

Although the T_m of PHA copolymers can be widely adjusted with the mcl-3HA comonomer composition, it is often set between 100 and 150°C to achieve the processing properties of typical commodity thermoplastics such as polyethylene. The crystallinity in this copolymer composition range is 20–40%, which produces very flexible low density polyethylene (LDPE) like materials.

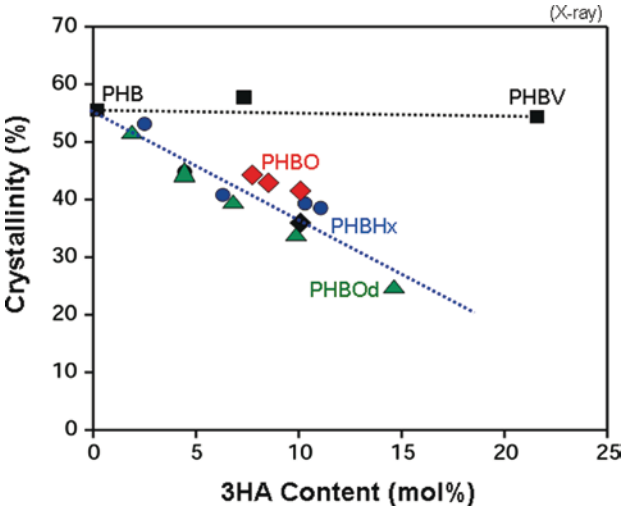

Fig. 8 Crystallinity of PHA copolymers (Noda et al. 2005b)

4.2.3 Glass-Transition Temperature

The glass-transition temperature T_g is another important thermal property of plastics with strong practical implications. It is well recognized that the T_g is closely associated with the segmental mobility of polymer chains, which in turn governs the toughness and other physical properties of the material. Unfortunately, the T_g of PHB homopolymer is somewhat high, such that the material becomes brittle upon cooling below 0°C. Even at room temperature, the high T_g and the limited polymer chain segmental mobility of PHB negatively affect the toughness and impact resistance of the material.

The incorporation of 3HA comonomer units into the PHB polymer chain reduces the T_g of copolymers (Marchessault et al. 1990). Figure 9 shows the T_g of various PHA copolymers with different lengths of side-chain groups (Noda et al. 2005b). There is a clear trend that the T_g of PHA copolymer is more effectively lowered by the incorporation of 3HA units with longer side chains. Thus, PHBHx, with a propyl side chain, has a lower T_g than PHBV, with an ethyl group, and PHBO, with a pentyl group, or poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxydecanoate] (PHBD), with a heptyl group, has an even lower T_g for a given level of 3HA incorporation. A higher level of 3HA incorporation also tends to lower the T_g of PHA copolymers.

4.3 Mechanical Properties

PHB homopolymer, and to some extent even PHBV copolymers, have traditionally suffered from the excessively stiff and brittle nature arising from the relatively high crystallinity of the material. The mechanical properties of PHB and PHBV are said

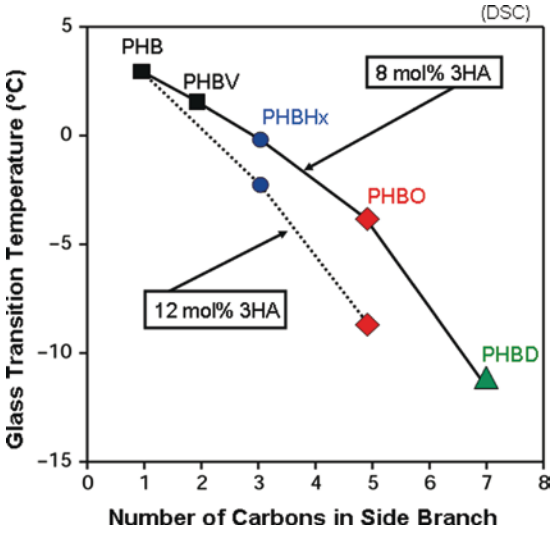

Fig. 9 Glass-transition temperature of PHA copolymers (Noda et al. 2005b)

to be similar to those of isotactic polypropylene (PP), but the lower ductility and impact strength prevent PHB and PHBV from effectively replacing commercial PP. In contrast, the properties of mcl-3HA containing PHA copolymers are closer to those of high-grade polyethylene (Satkowski et al. 2001). Because of the effective control of the excess crystallinity afforded by the incorporation of mcl-3HA units, the ductility and strength of the material are substantially increased. This improvement has made Nodax™ class copolymer an excellent candidate for the replacement for the existing general purpose commodity plastic resins.

Figure 10 shows typical tensile stress–strain curves of solution-cast PHA copolymer films after being aged for 7 days. The incorporation of 3HA units into the PHB polymer chain tends to decrease the stiffness and simultaneously increase the ultimate elongation of the material. These effects are more pronounced with mcl-3HA having longer side chains. The ability to produce remarkably tough and highly elastic material upon careful elongation under proper conditions is one of the interesting features of this class of PHA copolymers (Melik and Noda 2004). Such "hard elastic" films and fibers can be deformed over several times the size and are still able to snap back to the original dimension, as demonstrated in Fig. 11.

The stiffness of PHA copolymers can be controlled by the incorporation of mcl-3HA comonomer units (Noda et al. 2005b). Figure 12 shows Young's modulus of various PHA copolymers systematically decreases with the addition of 3HA comonomers. The effect of reduced crystallinity by incorporating more mcl-3HA is apparent. The incorporation of a higher level of mcl-3HA results in lower crystallinity, which in turn makes the material softer. The value of Young's modulus of PHA varied between that of very stiff polymers, such as poly(lactic acid) (PLA) and PP, and much softer material, such as LDPE.

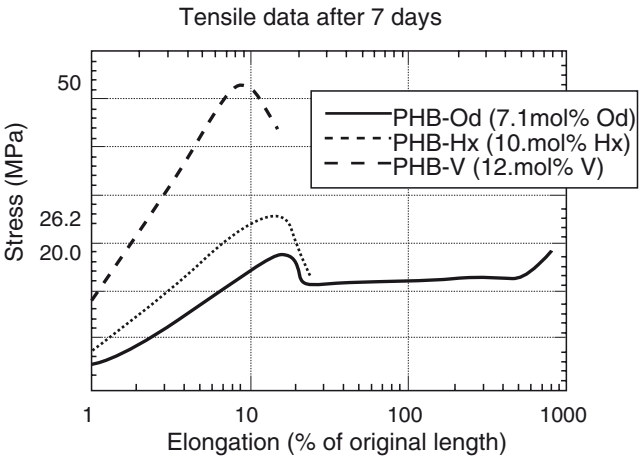

Fig. 10 Tensile stress–strain curves of PHA copolymers

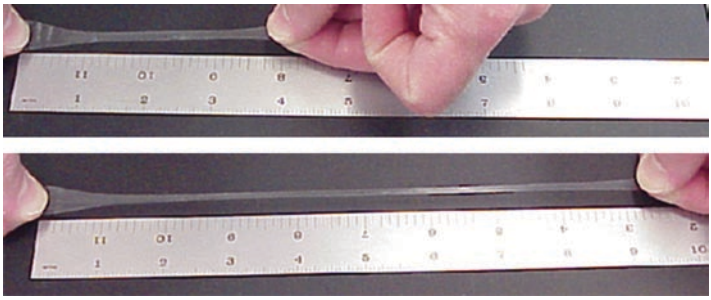

Fig. 11 Hard elastic films made of PHA copolymer

The stiffness of semicrystalline PHA is controlled not only by the overall crystallinity but also by the mechanical properties of the amorphous region of the material. This is shown by the effect of the length of the comonomer side chains, which influences the stiffness. Figure 13 shows Young's modulus of three different PHA copolymer samples with an identical level of mcl-3HA comonomers measured at room temperature (Noda et al. 2005b). Figure 8 indicates the expected crystallinity of these samples containing 10 mol% 3HA should be about 40%. However, the stiffness of the three samples is remarkably different, and PHA copolymer with longer side chain groups tends to be much softer.

The difference in the overall stiffness of semicrystalline PHA copolymers arises from the difference in the ductility of the amorphous region of the samples. The molecular segmental mobility of the amorphous portion of PHA is strongly influenced

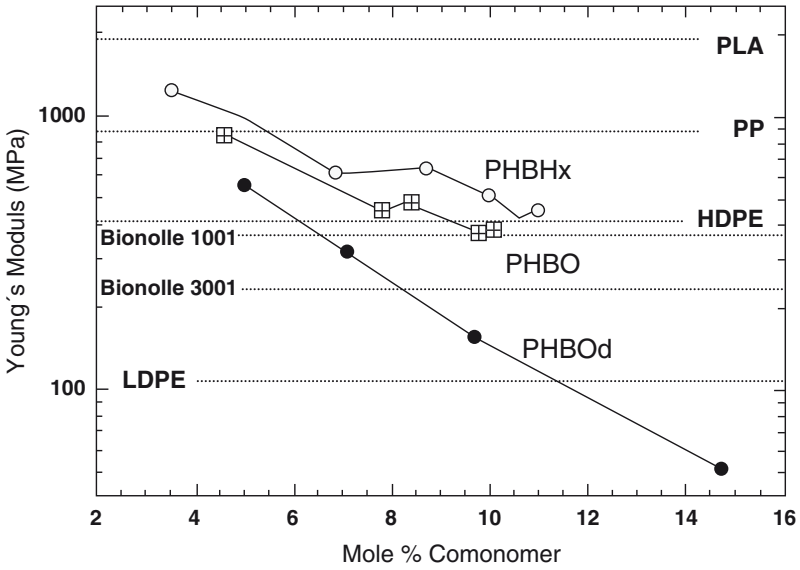

Fig. 12 Young's modulus of PHA copolymers (Satkowski et al. 2001)

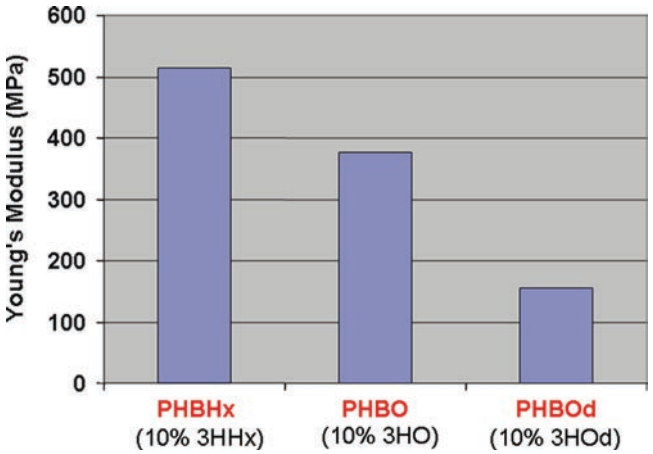

Fig. 13 Young's modulus of PHA copolymers (Noda et al. 2005b)

by the difference between the T_g and the end-use temperature. Thus, a PHA copolymer with a longer side chain group, which has lower T_g, should exhibit less overall stiffness, even if the crystallinity might be similar. A similar trend for the influence of the length of the side chain on the flexural modulus is shown in Fig. 14. Again, the increase in the side chain length resulted in much more flexible material.

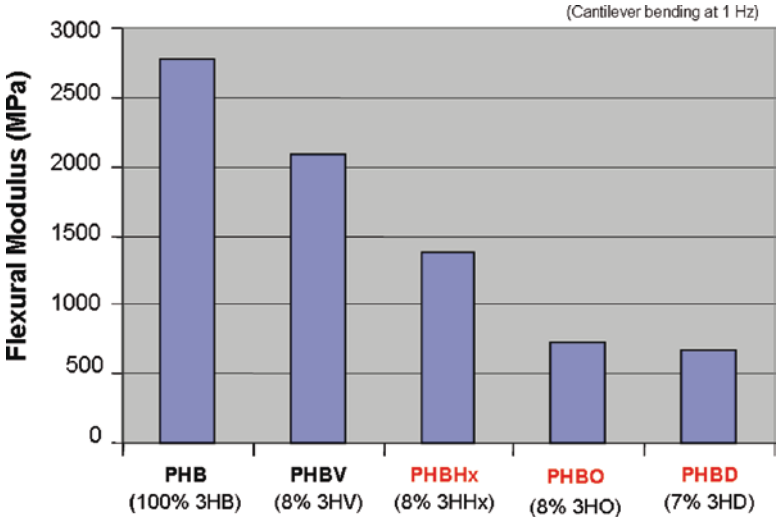

Fig. 14 Flexural modulus of PHA copolymers (Noda et al. 2005b)

4.4 Other Useful Properties

Many important physical properties, such as thermal and mechanical properties, of mcl-3HA containing semicrystalline PHA copolymers are relatively similar to those of conventional polyolefins. In contrast, its chemical properties are strongly affected by the presence of polar functional groups found in the molecular structure of polyester. For example, the surface energy of a PHA film is much higher than that of polyethylene, resulting in the superior wetting and ink printability useful for applications in flexible coatings and laminated papers. In addition to surface properties, PHA generally shows good compatibility and dispersibility for additives, such as pigments and fillers, as well as other biodegradable plastics, especially PLA (Noda et al. 2005a).

The effect of the presence of medium-sized branches on the physical properties also shows up in the solubility. Unlike the highly crystalline PHB and PHBV, mcl-3HA-containing PHA copolymers are readily soluble in so-called *green* solvents, such as ethyl acetate and acetone. It is possible to extract the polymer from biomass without using halogen-containing solvents such as chloroform (Noda and Schechtman 1999). This favorable solubility profile provides extra flexibility and economy in the extraction and purification of PHA copolymers.

A superior barrier property is another one of the interesting features of PHA copolymers. Table 1 shows the relative values of the water vapor and oxygen gas transmission rate for films made of various plastics. Even though PHA films can be very flexible, if the crystallinity is kept low, they exhibit excellent an barrier property for oxygen, carbon dioxide, and odors. Although PHA films are not as good as polyolefins, they also act as a reasonable barrier for moisture vapor. The optical transparency of thin films is also excellent for a flexible material.

Table 1 Relative gas permeability of polymer films

Polymer	Moisture	Oxygen
Saran™	1	10
PHA	90	40
PET	50	60
PP	10	2,300
PE	20	7,000
Natural rubber	1,000	24,000
Cellulose acetate	3,000	1,000

PHA polyhydroxyalkanoate, *PET* poly(ethylene terephthalate), *PP* polypropylene, *PE* polyethylene

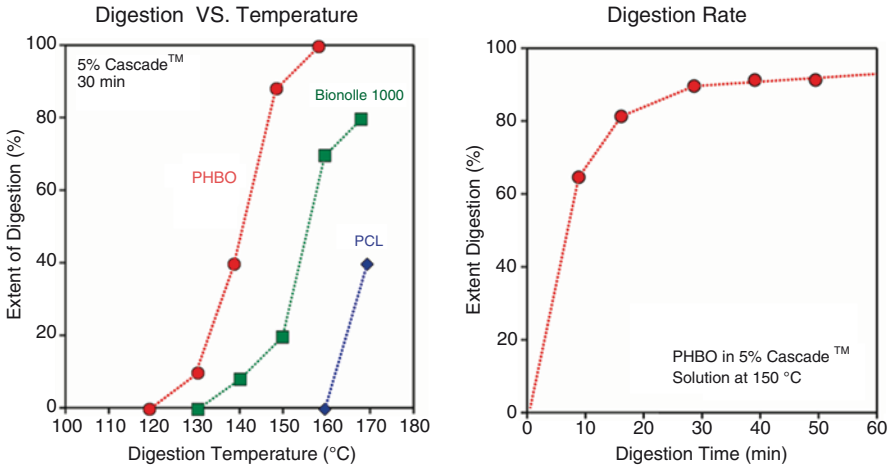

Fig. 15 Chemical digestion of PHA copolymer

PHA has much higher stability against hydrolysis compared with PLA. PHA does not decompose under normal temperature and humidity, and the level of hydrolytic degradation during the processing is low for a polyester resin. Interestingly, however, it degrades rapidly in alkaline solution at a high temperature (Noda 2005). Figure 15 shows the chemical digestion rate of PHA and other polyesters. That means PHA can be readily digested chemically during a process such as a de-inking step in paper recycling.

5 Processing and Conversion to Products

PHA copolymers can be converted into various forms and products, such as films, sheets, fibers, nonwovens, molded articles, pulps, powders, coating materials, laminates, and composites, using conventional processing steps such as extrusion and

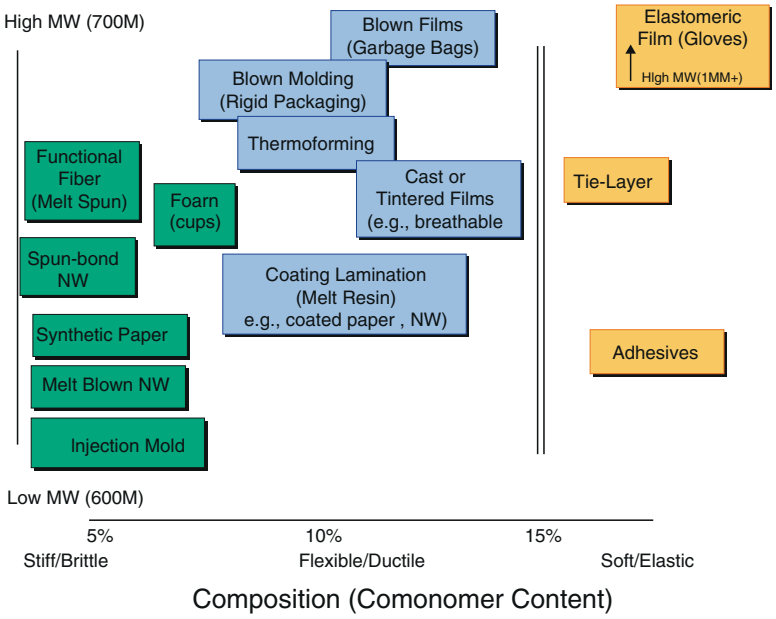

Fig. 16 Product design space of PHA copolymers (Noda et al. 2005a)

thermoforming. This broad processability of PHA copolymers arises from the melting point lowering resulting from the incorporation of mcl-3HA and superior compatibility of the material. Figure 16 shows a rough guideline for the design space of the type of copolymers which can be used for different applications (Noda et al. 2005a). Factors important to the conversion processes include the 3HA composition of copolymers, average molecular weights and molecular weight distributions, and the presence of processing aids such as nucleating agents and plasticizers. Figure 17 shows some examples of prototypes made from PHA copolymers.

6 Production and Commercialization

Meredian is committed to moving forward with large-scale production of PHA and will begin its operations in 2009 utilizing a pilot facility to validate production and process design prior to construction of its first full-scale PHA production facility in 2010. Meredian will focus its planned annual output of over 600 million pounds on specific market applications where the greatest value is provided to the marketplace. Meredian has an objective to build production facilities that are strategically located to support key customers while minimizing transportation costs.

Fig. 17 Prototypes of PHA products (Noda et al. 2005a; Poliakoff and Noda 2004)

7 Concluding Remarks

A family of promising PHA polyesters called Nodax™ class PHA copolymers, consisting of 3HA comonomer units with medium-size-chain side groups and 3HB, which will become commercially available from Meredian were described. Because of the unique design of the molecular structure, the Nodax™ class PHA copolymers have a set of useful attributes, including polyolefin-like thermomechanical properties, polyester-like physicochemical properties, and interesting biological properties, suggesting broad ranges of industrial and consumer product applications.

Advances in PHA technology will enable Meredian to be well positioned to serve the growing demand for materials produced from renewable resources. Leading companies around the world are focused on sustainable initiatives and the utilization of products produced exclusively from renewable starting materials that do not compete with the food supply and that also support multiple end-of-life options. Commercial success will be dependent upon the effectiveness of the current supply chain to efficiently transition to these new materials which will be required to meet ever-increasing demands for increased functionality. The properties of PHA within the portfolio of those described in this chapter have the unique capability to meet this challenge and exceed expectations.

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Manufacturing of PHA as Fibers

Tadahisa Iwata and Toshihisa Tanaka

Contents

Abstract Poly[(*R*)-3-hydroxybutyrate] and its copolymers have not been recognized as practical because of its stiffness and brittleness. Recently, we succeeded in obtaining strong fibers by two kinds of new drawing techniques from microbial polyesters produced by both wild-type and recombinant bacteria. The improvement of the mechanical properties of fibers is due not only to the orientation of the molecular chains but is also due to the generation of a planar zigzag conformation. The highly ordered and inner structures of strong fibers with tensile strength of over 1.0 GPa were analyzed by microbeam X-ray diffraction and X-ray microtomography

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with synchrotron radiation, respectively. The enzymatic degradation of strong fibers was investigated by using an extracellular polyhydroxybutyrate depolymerase. Furthermore, nanofibers were prepared by an electrospining technique from dilute solution and subcutaneous implantation of electrospun nanofibers was performed to investigate their bioabsorption behavior and tissue response. In this chapter, we present the processing, mechanical properties, molecular and highly ordered structure, enzymatic degradation, and bioabsorption of strong fibers and nanofibers produced from microbial polyesters.

Abbreviations

HFIP	1,1,1,3,3,3-Hexafluoro-2-propanol
P(3HB)	Poly[(*R*)-3-hydroxybutyrate]
P(3HB-*co*-4HB)	Poly[(*R*)-3-hydroxybutyrate-*co*-4-hydroxybutyrate]
P(3HB-*co*-3HH)	Poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyhexanoate]
P(3HB-*co*-3HV)	Poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyvalerate]
P(4HB)	Poly[(*R*)-4-hydroxybutyrate]poly(4-hydroxybutyrate)
PHA	Polyhydroxyalkanoate
SAXS	Small-angle X-ray scattering
UHMW-P(3HB)	Ultra-high-molecular-weight poly[(*R*)-3-hydroxybutyrate]
WAXD	Wide-angle X-ray diffraction

1 Introduction

Poly[(*R*)-3-hydroxybutyrate], P(3HB), accumulated in various bacteria, is extensively studied as a biodegradable and biocompatible thermoplastic with a melting point of 180°C (Alper et al. 1963; Doi 1990; Anderson and Dawes 1990). However, it is well known that the mechanical properties of P(3HB) materials markedly deteriorate by a process of secondary crystallization, since the glass-transition temperature (T_g) is about 4°C (Holmes 1988; De Koning and Lemstra 1993; Scandola et al. 1989). Accordingly, P(3HB) is considered as a polymer that is difficult to use in industrial applications because of its stiffness and brittleness.

Some research groups have attempted to improve the mechanical properties of P(3HB) films (De Koning and Lemstra 1994; Barham and Keller 1986; Kusaka et al. 1998, 1999; Aoyagi et al. 2003; Iwata et al. 2003) and fibers (Gordeyev and Nekrasov 1999; Schmack et al. 2000; Yamane et al. 2001; Furuhashi et al. 2004; Iwata et al. 2004). In the case of fibers, three groups have succeeded in obtaining melt-spun fibers with tensile strength of 190–420 MPa from P(3HB) produced by wild-type bacteria. However, the tensile strength of the fibers is not sufficient for industrial and medical applications such as fishing lines and sutures. Recently, we have succeeded in producing strong and flexible fibers with tensile strength of 1.3 GPa and elongation at break of 35% from ultra-high-molecular-weight P(3HB) [UHMW-P(3HB)] produced by recombinant *Escherichia coli* (Iwata et al. 2004). The strong fibers were processed

from amorphous fibers quenched in ice water near T_g by a method combining cold drawing into ice water and a two-step-drawing (a second-drawing) procedure at room temperature (Fig. 1a). More recently, we developed a new drawing technique (one-step drawing after isothermal crystallization) to obtain strong fibers from normal molecular weight (commercial grade molecular weight) P(3HB) (Tanaka et al. 2007c) and poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyvalerate], P(3HB-*co*-3HV) (Tanaka et al. 2006; Fig. 1b). Mechanical properties of PHA fibers together with common plastic fibers are summarized in Table 1.

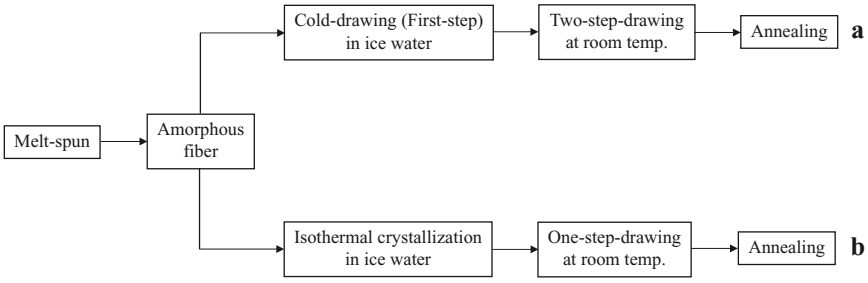


Fig. 1 Preparation methods for poly[(*R*)-3-hydroxybutyrate], P(3HB), and its copolymer fibers. (Reprinted with permission from Iwata 2005. Copyright 2005, Wiley-VCH Verlag GmbH & Co.)

Table 1 Mechanical properties of fibers processed from biodegradable polymers

Sample	Tensile strength (MPa)	Elongation at break (%)	Young's modulus (GPa)	References
P(3HB)	190	54	5.6	Gordeyev and Nekrasov (1999)
	330	37	7.7	Schmack et al. (2000)
	310	60	3.8	Yamane et al. (2001)
	416	24	5.2	Furuhashi et al. (2004)
	740	26	10.7	Tanaka et al. (2007c)
UHMW-P(3HB)	500	53	5.1	Tanaka et al. (2007c)
	1,320	35	18.1	Iwata et al. (2004)
P(3HB-*co*-3HV)	183	7	9.0	Ohura et al. (1999)
	210	30	1.8	Yamamoto et al. (1997)
	1,065	40	8.0	Tanaka et al. (2006)
P(4HB)	545	60	0.7	Martin and Williams (2003)
P(3HB-*co*-3HH)	46	200	–	Bond (2003)
	220	50	1.5	Jikihara et al. (2006)
	500	50	10.0	Tanaka et al. (2007a)
Poly(lactic acid)	570	35	6.0	
Poly(glycolic acid)	890	30	8.0	

P(3HB) poly[(*R*)-3-hydroxybutyrate], *UHMW-P(3HB)* ultra-high-molecular-weight poly[(*R*)-3-hydroxybutyrate], *P(3HB-co-3HV)* poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyvalerate], *P(4HB)* poly[(*R*)-4-hydroxybutyrate], poly(4-hydroxybutyrate) *P(3HB-co-3HH)* poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyhexanoate]

In these strong fibers, there are two kinds of molecular and crystal structures: an α-structure with a 2/1 helix molecular conformation and a β-structure with a planar zigzag one (Okamura and Marchessault 1967; Yokouchi et al. 1973; Orts et al. 1990). The generation mechanism and the distribution of both structures are revealed by microbeam X-ray diffraction, and 3D analysis of fibers was performed to observe the inner structure of fibers without pretreatment or damage in a synchrotron radiation facility (SPring-8), Japan. In this chapter, we firstly review the processing methods, mechanical properties, and molecular and highly ordered structure of strong monofilaments.

Nanofiber of biodegradable plastics such as P(3HB) and its copolymers is an attractive material for medical applications. We succeeded in obtaining well-organized nanofibers by an eletrospinning technique from dilute polymer solution. The fiber morphology and the crystalline structure of nanofibers are also reported in this chapter. In the last section, enzymatic and in vivo degradation of both monofilaments and nanofibers are presented.

2 High Tensile Strength Fibers

2.1 Cold-Drawn and Two-Step-Drawn Fibers Produced from UHMW-P(3HB)

2.1.1 Processing and Mechanical Properties

UHMW-P(3HB) was produced by *E. coli* XL1-Blue harboring a stable plasmid pSYL105 containing *Ralstonia eutropha* H16 PHA biosynthesis genes at pH 6.5 in Luria–Bertani and glucose medium at 37°C. The molecular weight of the P(3HB) sample used in this study was 3.72×10^6 (absolute M_w measured by multiangle laser light scattering) and 5.3×10^6 (relative M_w measured by gel permeation chromatography with polystyrene standard) with a polydispersity of 1.7 (Kusaka et al. 1998).

The amorphous fibers were obtained by quenching the melt-spun fibers of UHMW-P(3HB) in ice water. The cold drawing of amorphous P(3HB) fiber was carried out easily and reproducibly at a temperature below, but near to, the T_g of 4°C in ice water with two sets of rolls (Iwata et al. 2004). Both amorphous and cold-drawn fibers were transparent, indicating that only chain orientation and slide occurred without crystallization during the cold drawing. The cold-drawn amorphous fibers were kept at room temperature for several minutes to generate the crystal nucleus, and then two-step drawing was applied by a stretching machine at room temperature. The cold-drawn fibers were easily drawn at very low stress by more than 1,000%, but elastic recovery occurred on release from the stretching machine. Accordingly, the annealing procedure is required for fixing the extended polymer chains.

Table 2 Mechanical properties of cold-drawn; two-step-drawn and annealed fibers of UHMW-P(3HB) ($M_w = 5.3 \times 10^6$; Iwata et al. 2004)

Draw ratio			Tensile strength (MPa)	Elongation at break (%)	Young's modulus (GPa)
First[a]	Second[b]	Total			
–	–	As-spun	38	6	1.9
6	–	6	121	96	3.2
6	3	18	497	72	2.4
6	5	30	625	69	4.5
6	10	60	1,320	35	18.1

[a]Draw ratio of cold drawing in ice water by two sets of rolls
[b]Draw ratio of two-step drawing at room temperature by a stretching machine

The mechanical properties of cold-drawn, two-step-drawn, and annealed fibers of UHMW-P(3HB) are summarized in Table 2 (Iwata et al. 2004). The tensile strength and the elongation at break of as-spun fibers were only 38 MPa and 6%, respectively. After cold drawing six times in ice water, the tensile strength increased to 121 MPa. Interestingly, the elongation at break was also increased by cold drawing, indicating that the molecular chains align with the drawing direction and molecular entanglements were decreased by cold drawing. The tensile strength of two-step-drawn and annealed fibers linearly increased in the ratio of two-step drawing. When the total draw ratio reached 60 times (cold drawn six times and two-step drawn ten times), the tensile strength increased to 1,320 MPa (Fig. 2a, b). This value is higher than the values for polyethylene, polypropylene, poly(ethylene terephthalate), and poly(vinyl alcohol) of industrial grade and poly(glycolic acid) used as a suture. Thus, it was revealed that P(3HB) homopolymer is a very attractive material from the view point of mechanical properties.

2.1.2 Structure Analysis

The X-ray diffraction of a bundle of ten pieces of P(3HB) fibers (beam size 300 μm) as shown in Fig. 2c includes reflections simultaneously from both the α-structure (2/1 helix conformation; Okamura and Marchessault 1967; Yokouchi et al. 1973) and the β-structure (planar zigzag conformation; Orts et al. 1990) of P(3HB) that are drawn in Fig. 3. It is well known that P(3HB) crystallizes as an orthorhombic crystal system with unit cell parameters of $a = 0.576$ nm, $b = 1.320$ nm, and c(fiber axis) $= 0.598$ nm and space group $P2_12_12_1$ (α-structure; Yokouchi et al. 1973), and that the β-structure introduces from the orientation of free chains in amorphous regions between α-structure lamellar crystals; that is, the development of the β-structure is not really a transformation from the α-structure (Orts et al. 1990; Iwata et al. 2003, 2005; Iwata 2005). More recently, Iwata et al. (2008) reported a 3D crystal structure of the β-structure that has lattice parameters of $a = 0.528$ nm, $b = 0.920$ nm, and c(fiber axis) $= 0.469$ nm as an orthorhombic crystal system.

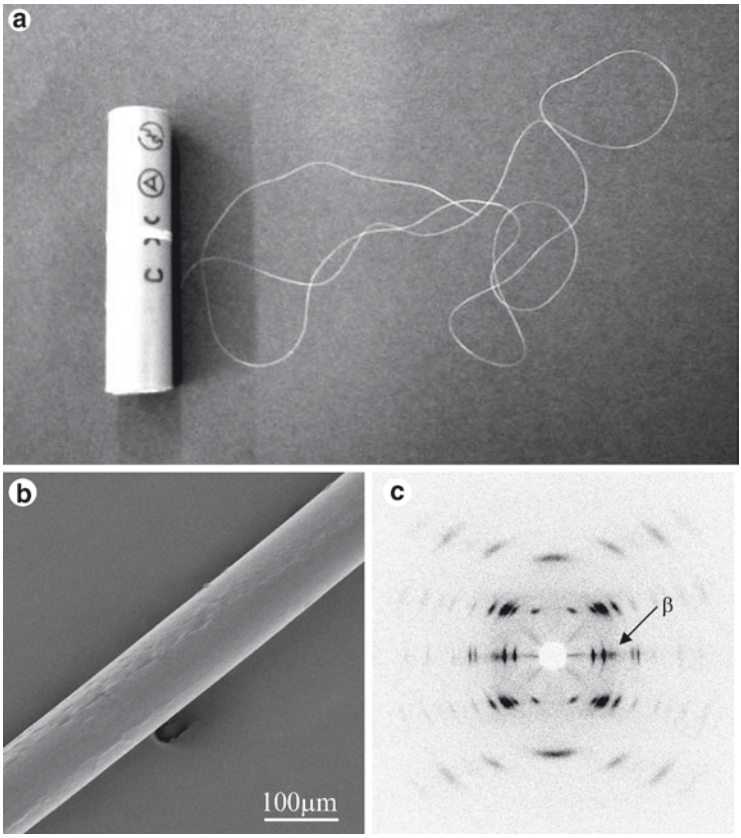
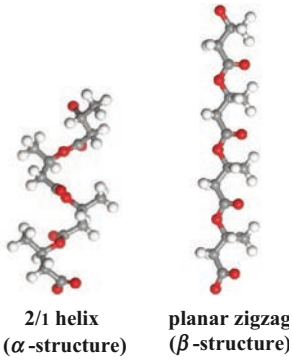


Fig. 2 **a** Ultra-high-molecular-weight P(3HB), UHMW-P(3HB), fiber processed by cold drawing (six times) in ice water and two-step drawing (ten times) at room temperature; and subsequently annealed at 50°C. **b** Scanning electron micrograph and **c** X-ray fiber diagram of UHMW-P(3HB) fiber. (Reprinted with permission from Iwata 2005. Copyright 2005, Wiley-VCH Verlag GmbH & Co.)

Fig. 3 Two types of molecular conformations of P(3HB): 2/1 helix (α-structure) and planar zigzag (β-structure). (Reprinted with permission from Ishii et al. 2007. Copyright 2007, Elsevier B.V.)



2.2 *One-Step-Drawn Fibers Produced from Commercial PHA*

2.2.1 Processing and Mechanical Properties

Cold-drawing and two-step-drawing methods are quite useful to obtain high-tensile-strength fibers. However, these techniques can also be used for high molecular weight P(3HB) produced by a recombinant *E. coli.* Accordingly, we developed a new drawing technique to process high-tensile-strength fibers from normal molecular weight PHA produced by the wild-type microorganism. Fibers of high-tensile-strength P(3HB) and its copolymer fibers were obtained from amorphous fibers by stretching the fibers after isothermal crystallization near the T_g as the new drawing method (Tanaka et al. 2006). Amorphous fibers of polymers were prepared by immediate quenching in an ice water bath near T_g of polymer produced by melt-spinning. The amorphous fibers before isothermal crystallization were transparent. Isothermal crystallization of the amorphous fiber was carried out in ice water for a certain period to prevent rapid crystallization and to allow the growth of small crystal nuclei. One-step drawing after isothermal crystallization was performed with a stretching machine at room temperature, followed by annealing in an oven to fix the extended polymer chains and increase the crystallinity of the fibers. These fibers were only slightly opaque even after the isothermal crystallization process for over 24 h. However, upon stretching, these fibers turned white.

The maximum draw ratio of amorphous commercial-P(3HB) fibers without crystallization (0 h) was about ten times in the initial length of the sample. However, the maximum draw ratio of the amorphous commercial-P(3HB) fibers decreased with increasing crystallization time. This value was constant at about four times with a crystallization time of over 40 h. Figure 4 shows the changes in the tensile strength of four times one-step-drawn commercial-P(3HB) fibers against the isothermal crystallization time (Tanaka et al. 2007c). The tensile strength of the fibers crystallized over a period of 0–24 h remained constant. However, the tensile strength of the fibers increased drastically when the crystallization time was prolonged

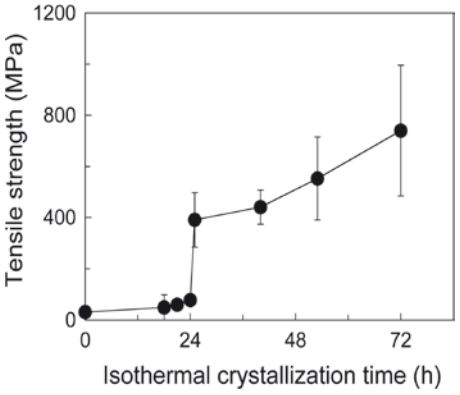


Fig. 4 Tensile strength of one-step-drawn commercial-P(3HB) fibers against isothermal crystallization time. (Reprinted with permission from Tanaka et al. 2007c. Copyright 2007, Elsevier Ltd.)

to more than 24 h. The tensile strength of the fibers crystallized for 72 h increased to 740 MPa. This is the highest tensile strength value reported to date for commercial-P(3HB) fibers (Gordeyev and Nekrasov 1999; Schmack et al. 2000; Yamane et al. 2001; Furuhashi et al. 2004) as shown in Table 1. The isothermal crystallization time of over 24 h is thought to be necessary to generate sufficient small crystal nuclei throughout the fiber. The mechanical properties of one-step-drawn fibers of commercial P(3HB) and UHMW-P(3HB) at each isothermal crystallization time are summarized in Table 3 (Tanaka et al. 2007c). The mechanical properties of one-step-drawn commercial-P(3HB) fibers were higher than in the case of UHMW-P(3HB) fibers. These results show that an increase in molecular weight has no effect on increasing the tensile strength of the fibers produced following the method reported here.

As an example of P(3HB) copolymers, high-strength commercial-P(3HB-*co*-3HV) and poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyhexanoate] [P(3HB-*co*-3HH)] fibers were produced by same method of stretching after isothermal crystallization (Tanaka et al. 2006, 2007a). The maximum draw ratio of P(3HB-*co*-3HV) fibers with isothermal crystallization time of 24 h was about ten times in the initial length of the sample. The mechanical properties of one-step-drawn P(3HB-*co*-3HV) fibers without and after isothermal crystallization are summarized in Table 4. The

Table 3 Mechanical properties of one-step-drawn P(3HB) fibers after isothermal crystallization near the glass-transition temperature (Tanaka et al. 2007c)

Sample	Draw ratio	Isothermal crystallization time (h)	Tensile strength (MPa)	Elongation at break (%)	Young's modulus (GPa)
Commercial P(3HB)	As-spun	0	20	8	0.9
	4	0	30	7	2.2
	4	25	390	44	5.2
	4	40	440	33	7.2
	4	72	740	26	10.7
UHMW-P(3HB)	As-spun	0	30	5	1.7
	4	0	40	4	2.4
	4	24	330	34	5.4
	4	44	500	53	5.1
	4	68	320	41	5.1

Table 4 Mechanical properties of one-step-drawn P(3HB-*co*-3HV) fibers after isothermal crystallization near the glass-transition temperature (Tanaka et al. 2006)

Draw ratio	Isothermal crystallization time (h)	Tensile strength (MPa)	Elongation at break (%)	Young's modulus (GPa)
As-spun	0	28	13	1.1
10	0	90	76	2.0
Nondrawn	24	27	15	1.2
5	24	710	50	6.8
10	24	1,065	40	8.0

tensile strength of as-spun fibers was about 30 MPa independent of isothermal crystallization. The tensile strength of ten times one-step-drawn fiber without isothermal crystallization was 90 MPa. However, the tensile strength of five and ten times one-step-drawn fibers after isothermal crystallization increased to 710 and 1,065 MPa, respectively. The mechanical properties of one-step-drawn P(3HB-*co*-3HV) fibers after isothermal crystallization are much better than those reported previously for this copolymer (Ohura et al. 1999; Yamamoto et al. 1997). Mechanical properties of fibers processed from other PHA, such as poly(4-hydroxybutyrate), P(4HB) (Martin and Williams 2003), and P(3HB-*co*-3HH) (Bond 2003; Jikihara et al. 2006; Tanaka et al. 2007a), reported previously are listed in Table 1. Our new drawing method by stretching after isothermal crystallization near T_g is an attractive procedure to obtain strong fibers from low molecular weight polyesters produced by wild-type bacteria.

2.2.2 Structure Analysis

To analyze the highly ordered structure of one-step-drawn commercial-P(3HB) and P(3HB-*co*-3HV) fibers, 2D wide-angle X-ray diffraction (WAXD) and small-angle X-ray scattering (SAXS) were performed (Tanaka et al. 2006, 2007c).

Figure 5 shows the scanning electron micrograph and WAXD pattern of four times one-step-drawn commercial-P(3HB) fibers after isothermal crystallization (Tanaka et al. 2007c). The surfaces of the fibers after isothermal crystallization had many fine voids that were evenly distributed throughout the fiber (Fig. 5a). The WAXD patterns of four times one-step-drawn P(3HB) fibers stretched at a crystallization time of over 24 h showed sharp reflections of both the α-structure and the β-structure (Fig. 5b).

Scanning electron micrographs and WAXD patterns of commercial-P(3HB-*co*-3HV) fibers after isothermal crystallization are shown in Fig. 6 (Tanaka et al. 2006).

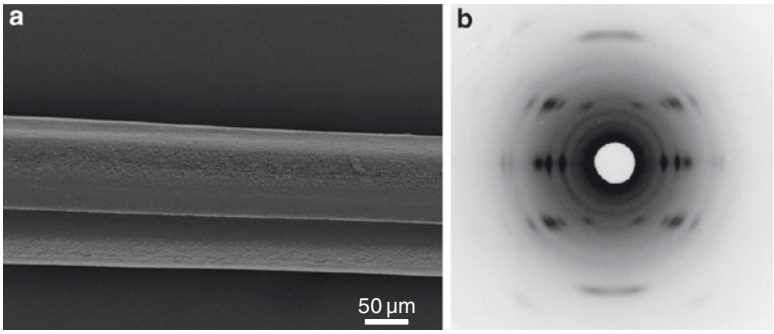


Fig. 5 **a** Scanning electron micrograph and **b** Wide angle X-ray diffraction (WAXD) pattern of four times one-step-drawn commercial-P(3HB) fibers after isothermal crystallization. (Reprinted with permission from Tanaka et al. 2007c. Copyright 2007, Elsevier Ltd.)

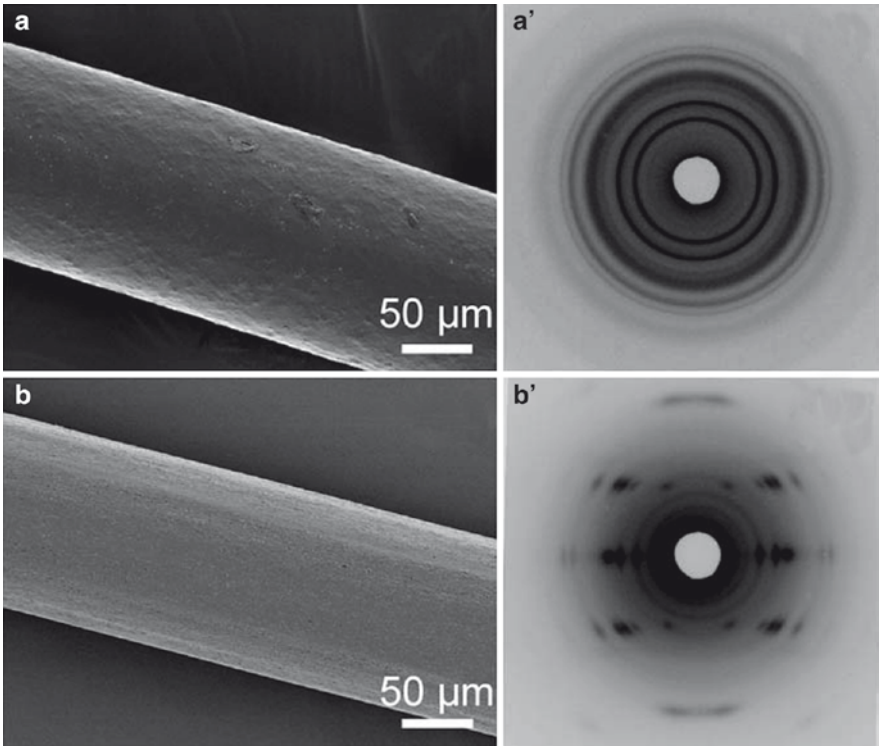


Fig. 6 Scanning electron micrograph and WAXD pattern of commercial-poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyvalerate], P(3HB-*co*-3HV), fibers after isothermal crystallization: **a**, **a′** non-drawn; **b**, **b′** ten times one-step-drawn. (Reprinted with permission from Tanaka et al. 2006. Copyright 2006, American Chemical Society)

The surface of the nondrawn P(3HB-*co*-3HV) fiber after isothermal crystallization was smooth throughout the fiber (Fig. 6a). However, the surface of the ten times one-step-drawn P(3HB-*co*-3HV) fiber after isothermal crystallization had many fine voids that were evenly distributed throughout the fiber (Fig. 6b), unlike the fibril-like lines generated parallel to the drawing direction of the ten times of the ten times one-step-drawn fibers without isothermal crystallization.

The WAXD pattern of a nondrawn fiber after isothermal crystallization showed only a ring pattern indicative of the unoriented α-structure crystal (Fig. 6a′) as observed with the as-spun fiber without isothermal crystallization. However, the WAXD patterns of ten times one-step-drawn fibers after isothermal crystallization showed the sharp reflections of both the α-structure and the β-structure along the equator (Fig. 6b′). The orientation of the α-structure crystals and the intensity of the β-structure reflection increased with increasing draw ratio for one-step-drawn fibers after isothermal crystallization. The orientation and crystallinity of fibers after isothermal crystallization were higher compared with those of fibers without isothermal crystallization. Many small crystal nuclei grow by slow crystallization

during the isothermal crystallization near the T_g. Drawing after isothermal crystallization leads to an increase in the orientation and crystallization of the molecular chains in the constrained amorphous region between these small crystal nuclei.

3 Microbeam X-Ray Diffraction Study

3.1 Two Kinds of Fiber Structures

Microbeam X-ray diffraction with beam sizes in the micrometer range is a useful and powerful method to investigate the in situ transition of the crystalline region and the local structure for monofilaments. To reveal the detailed fiber structure and the distribution of the two types of molecular conformations (α- and β-structure crystals) in monofilaments, microbeam X-ray diffraction was performed using synchrotron radiation at SPring-8, Japan. The beam size was focused to 0.5 μm with a Fresnel zone plate (Suzuki et al. 2001) and the monofilament was linearly scanned perpendicularly to the fiber axis with a step of 2 μm.

Figure 7a shows the microbeam X-ray diffraction patterns for a cold-drawn and two-step-drawn UHMW-P(3HB) monofilament obtained from three marked points in the microscope image (Iwata et al. 2004; Iwata 2005). In the microbeam X-ray fiber diagram of no. 1 (edge part), all the reflections were indexed with only the α-structure crystal. However, in the diagrams of nos. 2 and 3 (center part), the other reflection indexed by the β-structure was observed, together with the α-structure

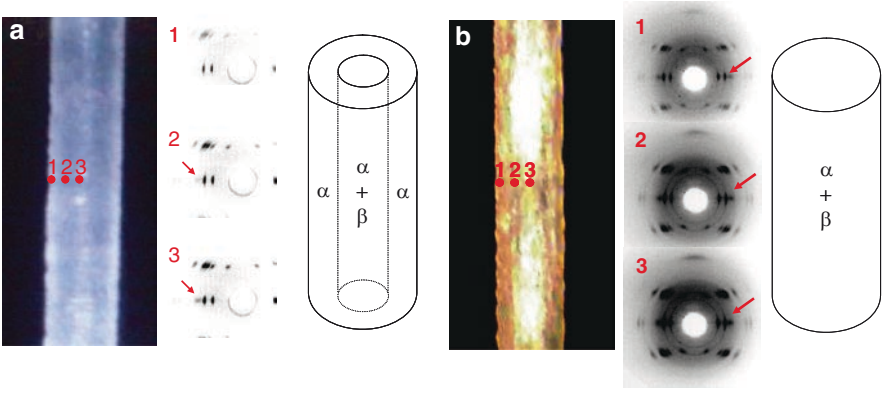


Fig. 7 Microbeam X-ray fiber pattern of drawn monofilaments, recorded from the three marked points in the microscope image, and schematic display, revealed by microbeam X-ray diffraction: **a** cold-drawn and two-step-drawn UHMW-P(3HB) monofilaments with core–sheath structure; **b** one-step-drawn P(3HB-*co*-3HV) monofilaments after isothermal crystallization with a uniform structure. The *arrows* indicate a reflection derived from the β-structure. (Reprinted with permission from Iwata 2005. Copyright 2005, Wiley-VCH Verlag GmbH & Co)

crystal. These results indicate that the strong two-step-drawn UHMW-P(3HB) fiber has a core–sheath structure with the α-stucture in the sheath region and with both α- and β-structures in the core region.

On the other hand, Fig. 7b shows the microbeam X-ray diffraction patterns for a one-step-drawn P(3HB-*co*-3HV) monofilament after isothermal crystallization obtained from three marked points in the microscope (Tanaka et al. 2006). All the X-ray diffraction patterns of one-step-drawn P(3HB-*co*-3HV) fibers have reflections indexed by both the α-structure and the β-structure. This result indicates that the strong one-step-drawn fiber does not have the core–sheath structure observed in cold-drawn and two-step-drawn UHMW-P(3HB) fibers as shown in Fig. 7a. In other words, one-step-drawn P(3HB-*co*-3HV) fibers after isothermal crystallization have a unique structure throughout consistent with both highly oriented α- and β-structure crystals. This structure supports the result that one-step-drawn fibers after isothermal crystallization have high tensile strength in spite of their low draw ratio.

3.2 Generation Mechanism of Planar Zigzag Conformation (β-Structure)

The presence of the β-structure crystals with a planar zigzag conformation is an important factor to generate high-strength P(3HB) materials. The existence of β-structure crystals is only observed by WAXD (Orts et al. 1990) and NMR measurements (Nishiyama et al. 2006), and it is difficult to prove by other analytical means, such as differential scanning calorimetry measurements. Until now the only known mechanism for generating the highly-ordered β-structure was derived from the observations of the cold-drawn and two-step-drawn UHMW-P(3HB) fibers (Iwata et al. 2004, 2005) and films (Aoyagi et al. 2003; Iwata et al. 2003). This mechanism proposed that the α-structure with lamellar crystals is produced by cold drawing, and then the β-structure with a planar zigzag conformation developed during the stretching in the second dimension of the constrained amorphous chains between α-structure crystals.

In the case of cold-drawn and two-step drawn UHMW-P(3HB) fibers, we found a new core–sheath structure. The core region consists of both α- and β-structures, whereas the sheath region consists of only the α-structure. The β-structure is generated from amorphous chains between lamellar crystals by the direct penetration of the stretching force in the core region. However, it was found that in the sheath region molecular chains are not strongly elongated by the rotation of lamellar crystals. As a result, the β-structure did not appear in the sheath region (Iwata et al. 2004, 2006).

However, in the case of one-step-drawn P(3HB-*co*-3HV) fibers after isothermal crystallization, the mechanism for generating α- and β-structure crystals is likely to be different from that in the case of cold-drawn and two-step-drawn fibers (Tanaka et al. 2006). The mechanism for the development of β-structure crystals for one-step-drawn P(3HB-*co*-3HV) fibers after isothermal crystallization is proposed as follows: (1) many small crystal nuclei grow in the amorphous region by slow crystallization during isothermal crystallization near the T_g, (2) the β-structure is developed first by the stretching of molecular chains in the constrained amorphous region

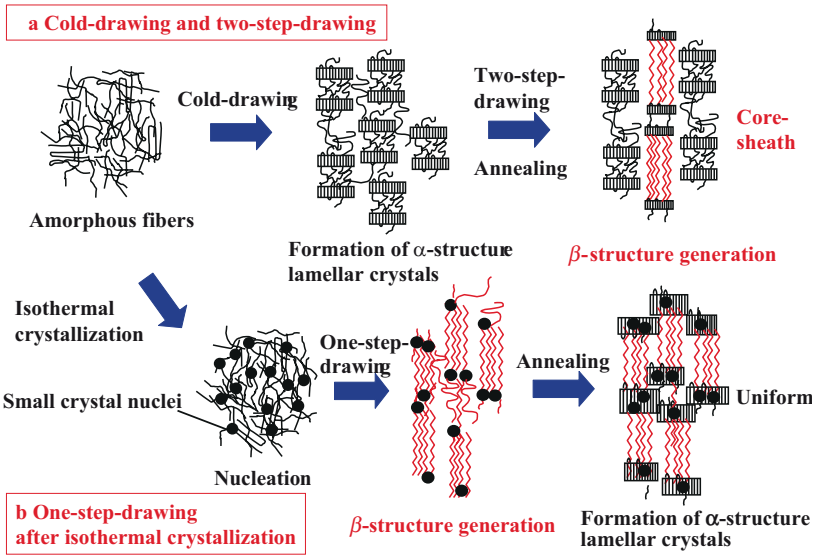


Fig. 8 The mechanism for generating the planar zigzag conformation (β-structure) in high-strength P(3HB) fibers by different drawing methods: **a** cold drawing and two-step-drawing; **b** one-step drawing after isothermal crystallization. (Reprinted with permission from Tanaka et al. 2006. Copyrigt 2006, American Chemical Society)

between small crystal nuclei, which act as cross-linking points, and then (3) the α-structure lamellar crystals with various thicknesses are generated from the small crystal nuclei during annealing. The generation mechanisms of a new core–sheath structure by the cold-drawing and the two-step-drawing procedure and a uniform structure by the one-step-drawing procedure are demonstrated in Fig. 8.

4 X-Ray Microtomography Study

The SAXS patterns of cold-drawn and two-step-drawn UHMW-P(3HB) fiber and one-step-drawn P(3HB-*co*-3HV) fiber are shown in Fig. 9. The SAXS pattern of a cold-drawn and two-step-drawn UHMW-P(3HB) fiber (Fig. 9a) yields clear two-spot reflections along the meridian, suggesting that there are lamellar crystals of systematic long period along the fiber axis. On the other hand, it is difficult to detect any spot reflection along the meridian in the SAXS patterns of one-step-drawn P(3HB-*co*-3HV) fibers after isothermal crystallization (Fig. 9b). Streak scattering can be clearly seen along the equator perpendicular to the drawing direction. In the past, this streak scattering along the equator was noted in highly oriented fibers and was considered to be due to voids in the fibers or the interface of the fiber. However, until now, there has been no direct evidence for a relation between the streak scattering and the presence of voids. Therefore, we made an attempt to analyze the inner structures of both UHMW-P(3HB) and P(3HB-*co*-3HV) fibers by using the X-ray microtomography method at the SPring-8 synchrotron radiation facility (Tanaka

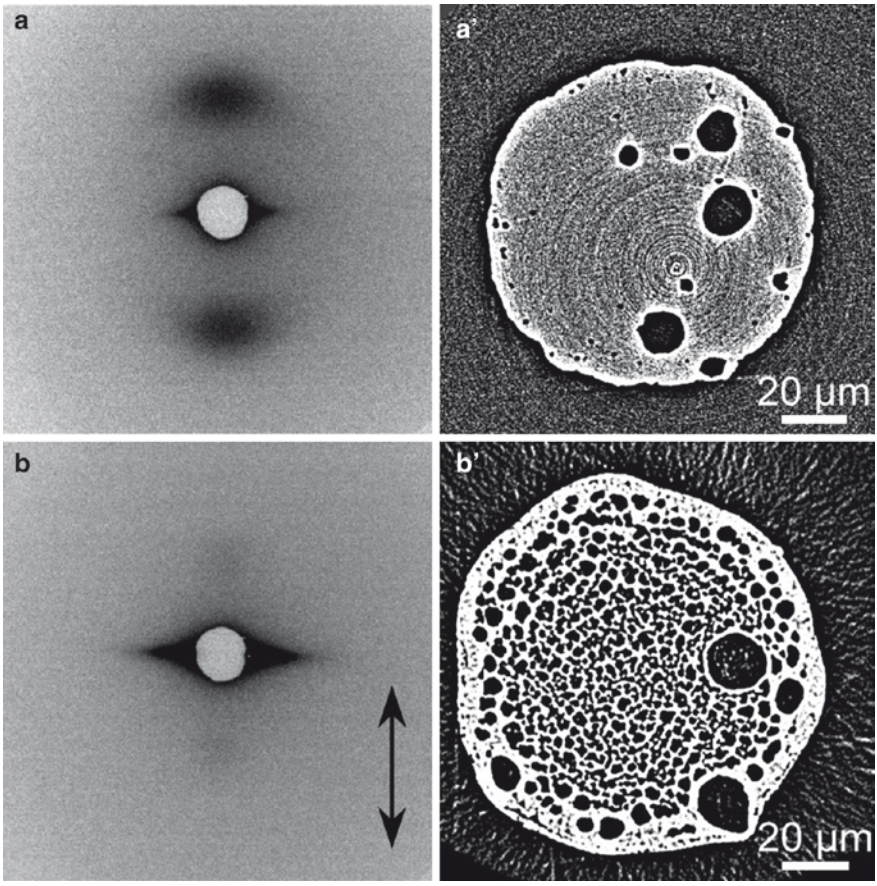


Fig. 9 **a**, **b** Small angle X-ray scattering patterns and **a′**, **b′** reconstructed images of cross sections perpendicular to the drawing direction of fibers: **a**, **a′** cold-drawn and two-step-drawn UHMW-P(3HB) and **b**, **b′** one-step-drawn P(3HB-*co*-3HV) after isothermal crystallization. The *arrows* indicate the drawing direction. (Reprinted with permission from Tanaka et al. 2007b. Copyright 2007, Elsevier Ltd.)

et al. 2007b). X-ray microtomography measurements using synchrotron radiation on polymer fibers with voids are considered to be a very interesting method for the evaluation and analysis of the inner structure of polymer materials without causing damage and the necessity of pretreatment.

A small piece of fiber was placed on a rod perpendicular to the fiber axis to rotate the fibers and to expose them to the X-ray beam. The images obtained were restructured to form cross-sectional images, and stereoscopic models were constructed by superimposing these images. The cross-sectional images perpendicular and parallel to the drawing direction of the UHMW-P(3HB) are almost uniform throughout the fiber as shown in Fig. 9a′. A few large holes seem to be generated by the entry of air or water during fiber preparation such as by melt-spinning.

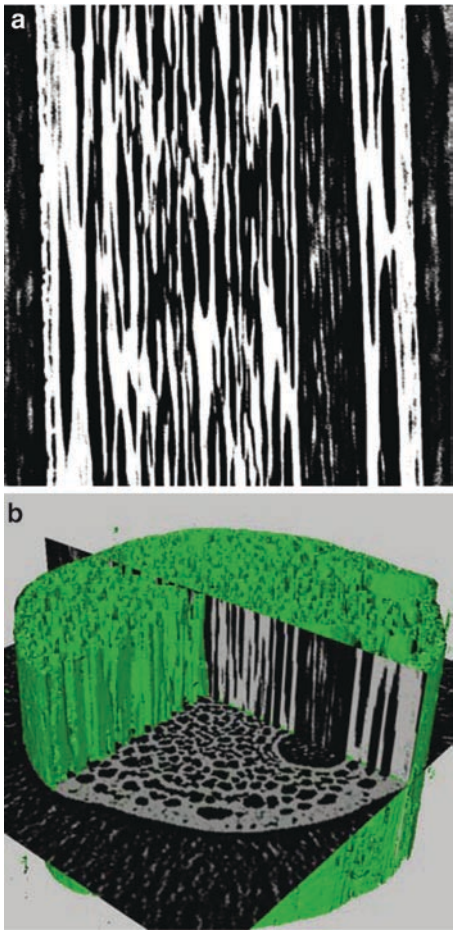


Fig. 10 Reconstructed images of ten times one-step-drawn P(3HB-*co*-3HV) fiber after isothermal crystallization: **a** cross sections parallel to the drawing direction and **b** stereoscopic models. (Reprinted with permission from Tanaka et al. 2007b. Copyright 2007, Elsevier Ltd.)

However, the one-step-drawn P(3HB-*co*-3HV) fiber has many fine voids of cohesive elliptic shape in the drawing direction (Figs. 9b′, 10). The results indicate that the streak scattering in the SAXS pattern shown in Fig. 9b is due to the presence of many fine voids throughout the fiber. The size and distribution of the voids in the one-step-drawn P(3HB-*co*-3HV) fibers after isothermal crystallization were analyzed by image analysis software. Most of the void diameters in cross sections perpendicular to the drawing direction were 1.0–1.6 μm and the average void diameter was 2.3 ± 1.5 μm.

Cross-sectional area versus load stress is an important parameter for evaluating the tensile strength of materials. To assess the mechanical properties of a uniaxial structure with many fine voids such as that of the one-step-drawn P(3HB-*co*-3HV) fiber after isothermal crystallization, one needs to know its true cross-sectional area. We therefore derived the cross-sectional areas of the oriented fiber from the cross-sectional images perpendicular to the drawing direction using image analysis

Table 5 Apparent and calculated cross-sectional areas and tensile strengths of ten times one-step-drawn P(3HB-*co*-3HV) fibers without and after isothermal crystallization near the glass-transition temperature (Tanaka et al. 2007b)

Isothermal crystallization time (h)	Apparent cross section		Tensile strength (MPa)[a]	Calculated cross section		Calculated tensile strength (MPa)[b]
	Area (μm^2)	Ratio (%)		Area (μm^2)	Ratio (%)	
0	1.21×10^4	100	90	1.21×10^4	99.9	90
24	1.29×10^4	100	1,065	0.68×10^4	52.7	2,020

[a]Obtained from the apparent cross-sectional areas in Table 4
[b]Recalculated from measured cross-sectional areas (1,065/0.527 = 2,020 MPa)

software. The calculated tensile strengths derived from the recalculated cross sections for one-step-drawn P(3HB-*co*-3HV) fibers without and after isothermal crystallization are summarized in Table 5. The recalculated cross-sectional area determined by X-ray microtomography analysis was the same as the apparent cross-sectional area for the one-step-drawn P(3HB-*co*-3HV) fiber without isothermal crystallization and agreed with an experimental result (Tanaka et al. 2006), as shown in Table 4. However, the recalculated cross-sectional area of the one-step-drawn P(3HB-*co*-3HV) fiber after isothermal crystallization was 52.7% of the apparent cross-sectional area. Therefore, the tensile strength determined by recalculation of the cross-sectional area for the latter fiber is considered to be 2,020 MPa. This calculated value indicates that the physical properties of P(3HB-*co*-3HV) fibers are highly suitable for their use as biodegradable materials.

The generation of many fine voids in one-step-drawn P(3HB-*co*-3HV) fibers after isothermal crystallization probably results from partial contraction of the polymer chains (in that way decreasing the free volume) and the entry of water during isothermal crystallization before drawing, or from thermal shrinkage of the polymer chains and outflow of water during annealing after drawing. The morphology of the 3D structure (Fig. 10) with many fine cohesive elongated voids in one-step-drawn fibers supports the latter explanation. In other words, fine voids develop by the partial shrinkage of polymer chains during the crystallization process at T_g with no significant change in the diameter of fibers. Therefore, a mechanism for generating many fine voids in one-step-drawn P(3HB-*co*-3HV) fibers after isothermal crystallization is proposed as follows: the large voids are formed by the entry of air or water when in the melt-spinning process; on the other hand, the fine voids are generated by the decrease of the free volumes of polymer chains during isothermal crystallization.

5 Electrospun Nanofibers

In response to the growing demand in the field of tissue engineering, PHA and its composites are thought to have good potential as emerging materials for medical devices such as sutures, bone plates, surgical mesh, and cardiovascular patches.

Recently, with the aim of developing porous materials or nonwovens, the formation of nanofibers with diameter ranging from several tens to hundreds of nanometers has been extensively studied (Reneker and Chun 1996; Morota et al. 2004). Electrospinning has emerged as one of the methods offering simplicity and versatility in preparing such biomaterials (Zong et al. 2005). Electrospun biomaterials facilitate better cell attachment and perfusion owing to the very high surface area to volume ratio and high porosity. In addition, electrospinning may provide an alternative method to produce fibrous materials with improved mechanical properties compared with solid-walled equivalents (Kim and Reneker 1999).

Scanning electron microscopy images of commercial-P(3HB) nanofiber spun from 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) solution with a polymer concentration ranging from 0.5 to 2.5 wt% are shown in Fig. 11a (Ishii et al. 2007). Whereas the nanofiber spun from 2.5 wt% solution had an average diameter of 560 nm, nanofibers spun from 1 and 0.5 wt% solutions had average diameters of 350 and 280 nm, respectively. This result indicates that the diameter of nanofibers can be controlled by the concentration of the polymers.

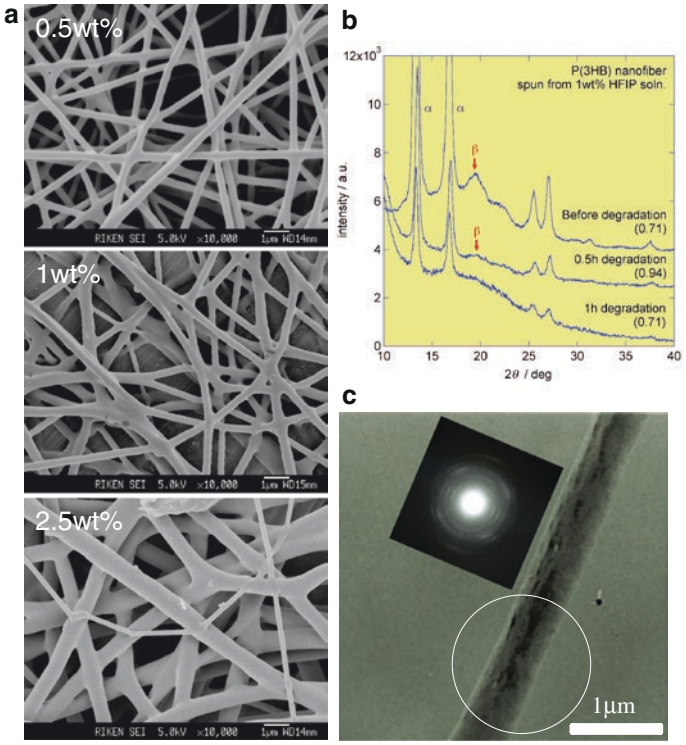


Fig. 11 **a** Scanning electron microscopy images, **b** WAXD profiles before and after partial enzymatic degradation, and **c** transmission electron microscopy image and electron diffraction pattern (*inset*) of P(3HB) nanofiber. (Reprinted with permission from Ishii et al. 2007. Copyright 2007, Elsevier B.V.)

Figure 11b shows the WAXD profiles of as-spun P(3HB) nanofiber mats spun from 1 wt% solution. In the profile before enzymatic degradation, diffraction peaks assigned to both an α-structure crystal with a 2/1 helix molecular conformation and a β-structure with a planar zigzag conformation are observed at $2\theta = 19.6°$.

A transmission electron microscopy image and the electron diffraction pattern of P(3HB) single nanofiber spun from 1 wt% HFIP solution are shown in Fig. 11c. Many arc-shaped reflections were observed in the diffraction pattern. A similar diffraction pattern has been observed for the drawn fibers and stretched films of P(3HB) in which molecular chains are highly oriented. Particularly, the appearance of (002) diffraction on the meridian suggests that the crystallographic *c*-axis of the α-structure P(3HB) crystal, i.e., the molecular chain axis, lies parallel to the fiber direction.

6 Enzymatic and In Vivo Degradation of Fibers

6.1 Enzymatic Degradation of Strong Fibers and Nanofibers

Enzymatic degradation of P(3HB) fibers with core–sheath structure consists of two kinds of molecular conformations, 2/1 helix (α-structure) and planar zigzag (β-structure), was performed in an aqueous solution containing extracellular PHB depolymerase from *Ralstonia pickettii* T1 at 37°C. Figure 12a and b shows the scanning electron micrographs of a P(3HB) fiber before and after partial enzymatic degradation (Iwata et al. 2006). It was revealed that this strong fiber was degraded by PHB depolymerase and the enzymatic erosion progressed from the fiber surface.

Figure 12c shows X-ray diffraction patterns of P(3HB) fibers before and after partial enzymatic degradation. Whereas the intensities of α-structure crystals remained unchanged before and after enzymatic degradation, the intensity of the β-structure decreased, in spite of the β-structure existing in the core region. This result indicates that the planar zigzag conformation (β-structure) is degraded faster than the 2/1 helix conformation (α-structure). Thus, the rate of enzymatic erosion can be controlled by the molecular conformation, in spite of the chemical structure being the same.

In the case of a cold-drawn and two-step-drawn P(3HB) monofilament with core–sheath structure, the β-structure was degraded faster than the α-structure, although the β-structure existed in the core region in fiber. Figure 12d shows a schematic display of the highly ordered structure of P(3HB) fiber with two kinds of molecular conformations. The sheath region consists of two domains that are lamellar crystals with 2/1 helix conformation (α-structure) and an amorphous region between these lamellar crystals. On the other hand, in the core region, the β-structure domains exist between lamellar crystals because of high orientation of amorphous chains. It is well known that the enzymatic degradation progresses from the amorphous region in the material surface. Accordingly, the enzymatic degradation of P(3HB) fibers firstly progressed from the amorphous region between α-structure lamellar crystals in the fiber surface (sheath region). Furthermore, the

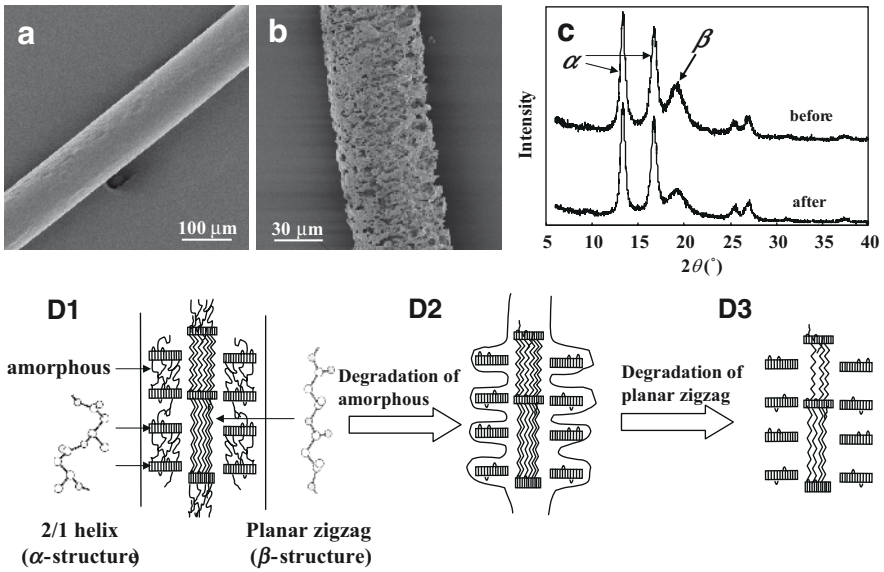


Fig. 12 Scanning electron micrographs of P(3HB) fiber before (**a**) and after (**b**) partial enzymatic degradation in an aqueous solution of PHB depolymerase purified from *Ralstonia pickettii* T1 at 37˚C. **c** Intensity profiles of equatorial lines in X-ray fiber diagrams before and after partial enzymatic degradation. α and β indicate the reflections derived from α- and β-structure crystals; respectively. **d** Enzymatic degradation behavior of P(3HB) fibers with two kinds of molecular conformations: α- and β-structure. (Reprinted with permission from Iwata et al. 2006. Copyright 2006, American Chemical Society)

enzyme molecules can penetrate the inside of the fiber by degrading the amorphous regions. Then, molecular chains of β-structure seem to be easily attacked by enzyme molecules, rather than those of α-structure, because the steric hindrance against the ester bond in the planar zigzag conformation is less than in the helix conformation. As a result, the intensity induced from the β-structure decreased and the α-structure crystal remained unchanged after partial enzymatic degradation. In a longer enzymatic degradation test, it was confirmed that all the fibers were completely degraded by PHB depolymerase.

Enzymatic degradation of one-step-drawn fibers and nanofibers was also performed in an aqueous solution containing extracellular PHB depolymerases. One-step-drawn fibers are degraded from fiber surfaces having many fine elliptic voids along the drawing direction (Tanaka et al. 2006, 2007c). On the basis of the X-ray diffraction analysis, the β-structure (planar zigzag conformation) is degraded faster than the α-structure (2/1 helix conformation) as in the case of two-step-drawn fibers (Fig. 13).

Figure 14 shows the scanning electron microscopy image of partially enzymatically hydrolyzed P(3HB) nanofibers spun from 1 wt% HFIP solution (Ishii et al. 2007). In contrast to the smooth surface of nanofibers before enzymatic treatment

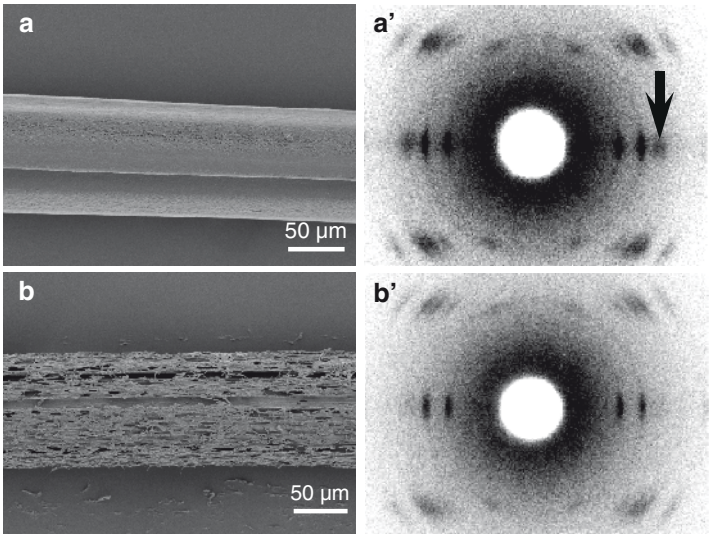


Fig. 13 Scanning electron micrographs (**a**, **b**) and WAXD patterns (**a′**, **b′**) of four times one-step-drawn commercial-P(3HB) fibers at an isothermal crystallization time of 40 h; follows by enzymatic degradation: degradation times of **a** 0 and **b** 1 h. The *arrow* indicates a reflection derived from the β-structure with planar zigzag conformation. (Reprinted with permission from Tanaka et al. 2007c. Copyright 2007, Elsevier Ltd.)

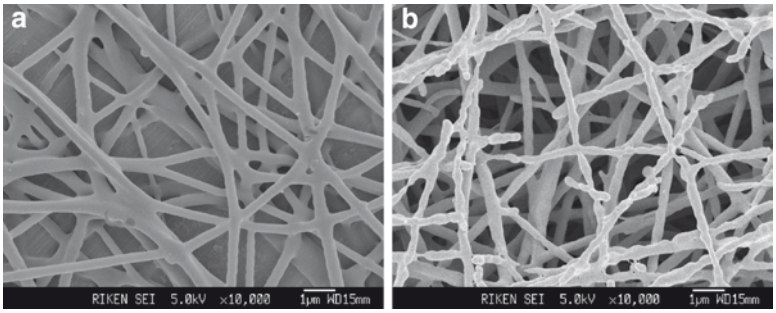


Fig. 14 Scanning electron micrographs of commercial-P(3HB) nanofibers before (**a**) and after (**b**) partial enzymatic treatment. (Reprinted with permission from Ishii et al. 2007. Copyright 2007, Elsevier B.V.)

as shown in Fig. 11, the enzymatically treated nanofibers had an irregular surface. This suggests that the degradation of P(3HB) nanofibers proceeded from the surface. The intensity of the β-structure decreased depending on the degradation time, whereas that of the α-structure remained unchanged during partial enzymatic degradation (Fig. 11b). This result further suggest that the β-structure degrades faster than the α-structure in spite of the fiber morphology and diameter.

More recently, we succeeded in revealing the crystal structure of PHB depolymerase from *Penicillium funiculosum* (Hisano et al. 2006). The trimer substrate of (*R*)-3-hydroxybutyrate with a planar zigzag conformation was perfectly bound in a crevice of the active site. This result suggests that the β-structure (planar zigzag conformation) is degraded faster than the α-structure (2/1 helix conformation), which was obtained from the enzymatic degradation of P(3HB) fibers with two kinds of molecular conformations. These results indicate that the rate of enzymatic degradation can be controlled by the molecular conformations of polymers.

6.2 In Vivo Degradation of Nanofibers

6.2.1 Morphological Changes

Four kinds of PHAs were electrospun to fabricate scaffolds with enhanced biocompatibility and bioabsorption. Subcutaneous implantation of the nanofibers in rat was performed to investigate their bioabsorption behavior and tissue response (Ying et al. 2008). The retrieved electrospun PHA scaffolds showed various changes in appearance after subcutaneous implantation (Fig. 15). After 4 weeks, both the electrospun P(3HB) and the P(3HB-*co*-5% 3HH) remained in their initial form. The electrospun P(3HB-*co*-7% 4HB) was fragmented into large pieces, whereas the electrospun P(3HB-*co*-97% 4HB) shrunk and became thinner. Even after 12 weeks, the electrospun P(3HB) showed no morphological change. However, significant changes were observed for the other three electrospun PHA

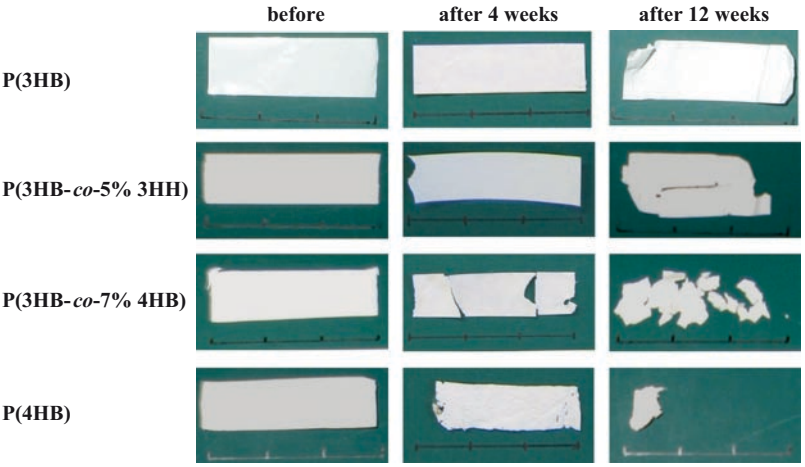


Fig. 15 Physical appearances of the electrospun polyhydroxyalkanoate (PHA) scaffolds before and after 4 and 12 weeks of subcutaneous implantation in rat. (Reprinted with permission from Ying et al. 2008. Copyright 2007, Elsevier Ltd.)

copolymers. The degree of degradation increased in the order P(3HB-*co*-5% 3HH), P(3HB-*co*-7% 4HB), and P(3HB-*co*-97% 4HB). The electrospun P(3HB-*co*-5% 3HH) displayed crevices on its surface, whereas the electrospun P(3HB-*co*-7% 4HB) was degraded into small fragments. Only a small piece of the electrospun P(3HB-*co*-97% 4HB) scaffold was retrieved, indicating enhanced bioabsorption of this 4HB-rich copolymer.

6.2.2 Histological Observation

The histological sections of the electrospun PHA scaffolds at different periods of subcutaneous implantation are shown in Fig. 16 (Ying et al. 2008). Histological observations indicate that all three electrospun copolymers elicited fairly mild tissue response relative to that of the electrospun P(3HB) throughout the course of the study. After 4 weeks of implantation, some parts of the electrospun P(3HB-*co*-97% 4HB) bordering the interface were degraded as evidenced by the small fragments that broke off from the main scaffold. More macrophages were found to be present along the interface connected to this copolymer in comparison with the electrospun P(3HB-*co*-7% 4HB) and P(3HB-*co*-5% 3HH). This phenomenon is desirable during wound healing because the presence of macrophages is necessary for the regeneration of

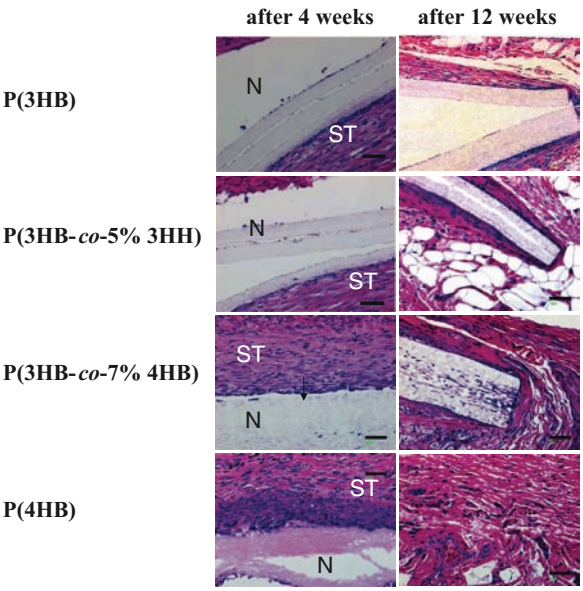


Fig. 16 Histological sections of the electrospun PHA scaffolds at different period of subcutaneous implantation. *Arrows* indicate the polymer surface. (Reprinted with permission from Ying et al. 2008. Copyright 2007, Elsevier Ltd.)

many cell types (Rappolee et al. 1988). The presence of thin connective tissue surrounding the electrospun P(3HB-*co*-97% 4HB) was also observed.

The most promising finding was the tissue response after 12 weeks of implantation for the electrospun P(3HB-*co*-97% 4HB). No fibrous encapsulation was observed around the degraded copolymer and there was also a substantial drop in the number of inflammatory cells. This observation is similar to the minimal inflammatory responses reported by Martin et al. (1999) in a study done on the biocompatibility of P(4HB) implanted subcutaneously in rats. In this study, the number of inflammatory cells surrounding the electrospun P(3HB-*co*-7% 4HB) and P(3HB-*co*-5% 3HH) did not appear to lessen. The muscle cells surrounding these two scaffolds appeared compact as a result of inflammatory reaction. After 12 weeks of implantation, the number of macrophages bordering the electrospun P(3HB) increased. Inflammation was obvious owing to the compacted muscle cells surrounding the scaffold. The difference in tissue response to the electrospun P(3HB-*co*-97% 4HB) and the electrospun scaffolds with a higher molar fraction of 3HB reflected their distinct physical properties. It has been reported that rigid polymers, such as P(3HB), elicit an acute inflammatory reaction because they exerts a continuous mechanical stimulus to the tissues surrounding the implants (Qu et al. 2006). Although the tissue response to the electrospun P(3HB-*co*-7% 4HB) and electrospun P(3HB-*co*-5% 3HH) was slightly more pronounced than that of the electrospun P(3HB-*co*-97% 4HB), the overall local tissue response to all three copolymers was found to be mild. The results have confirmed the biocompatibility of all three types of electrospun PHA copolymers.

7 Prospects

Plastics products have gained universal use, not only in food, clothing, and shelter, but also in the transportation, construction, medical, and leisure industries. Among plastics products, it is not an exaggeration to say that fibers play very important role in all fields. Unfortunately, until now it has been quite difficult to process highly functional fibers from biodegradable plastics. Especially, nobody has succeeded in obtaining high-performance fibers from microbial polyesters. Fibers have various properties depending on the diameter, the braid, the cord, the thread, etc. We proposed herein two kinds of new drawing techniques and some possibilities for the usage of microbial polyesters as fibers. The cold drawing from amorphous states is an attractive method for obtaining fibers with high tensile strength from highly crystalline polymers. To produce highly functional fibers, it is necessary to determine the mechanism of structure development. Time-resolved X-ray diffraction and simultaneous measurements by WAXD and SAXS using synchrotron radiation to understand the crystalline structure and the highly ordered structure are ideal methods for investigating dynamic structural changes such as crystallization, thermal behavior, and processing, in addition to microbeam X-ray diffraction and 3D microtomography.

We must establish a new research field in polymer science for producing nanocontrolled microbial polyester materials.

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

Philippe Guérin, Estelle Renard, and Valérie Langlois

Contents

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.

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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.

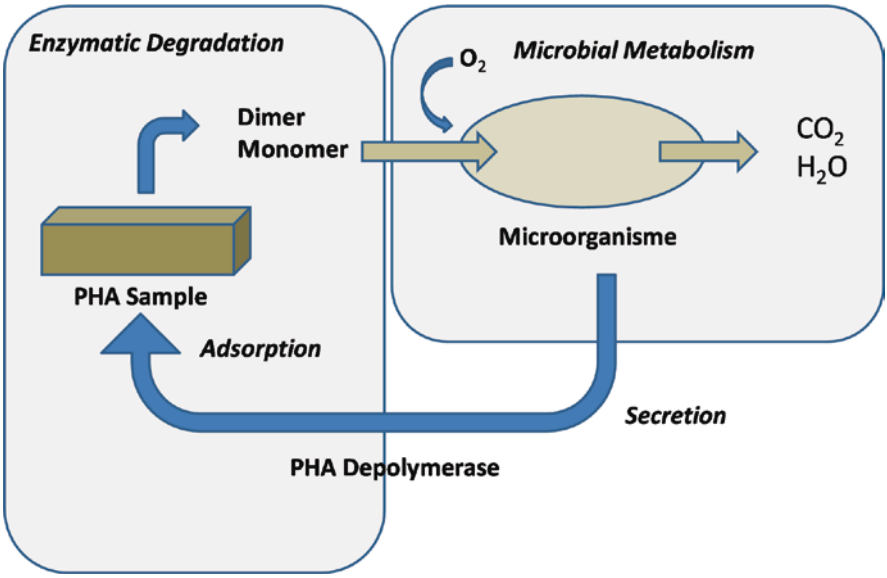

Fig. 1 Extracellular degradation

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

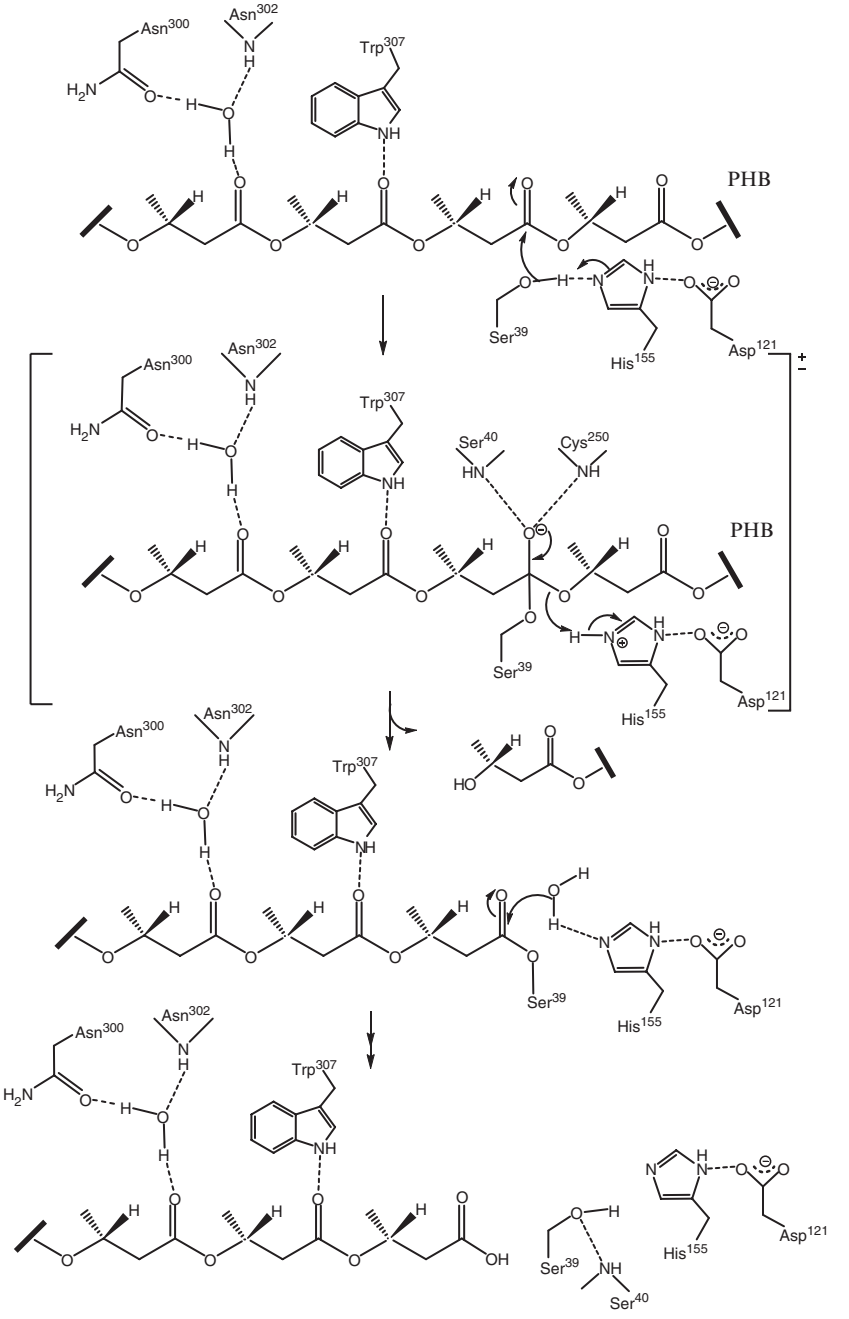

Fig. 2 A proposed mechanism for the action of the poly[(*R*)-3-hydroxybutyrate] (PHB) depolymerase from *Penicillium funiculosum* (Hisano et al. 2006)

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).

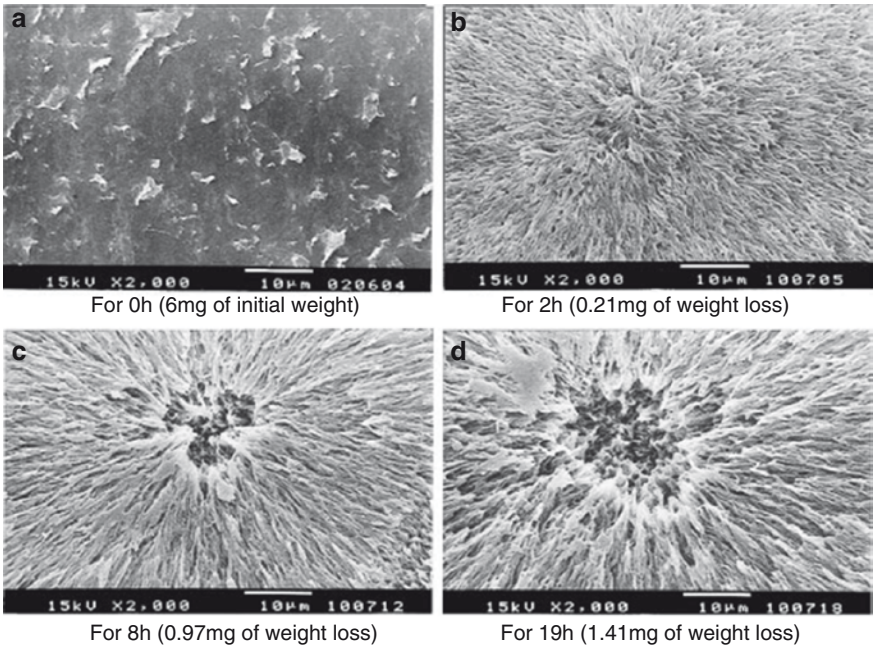

Fig. 3 Scanning electron micrographs of the PHB films crystallized at 120°C for 168 h from the melt before and after enzymatic erosion (Koyama and Doi 1997)

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,

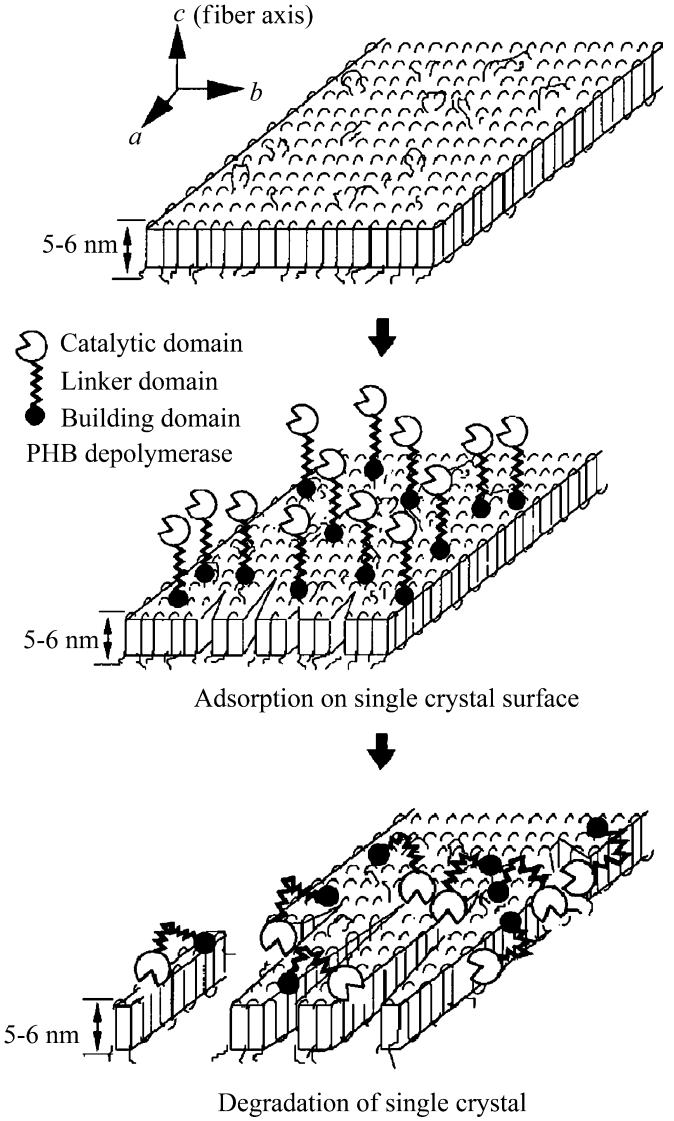

Fig. 4 Enzymatic hydrolysis of PHB single crystals by PHB depolymerase (Iwata 1999)

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].

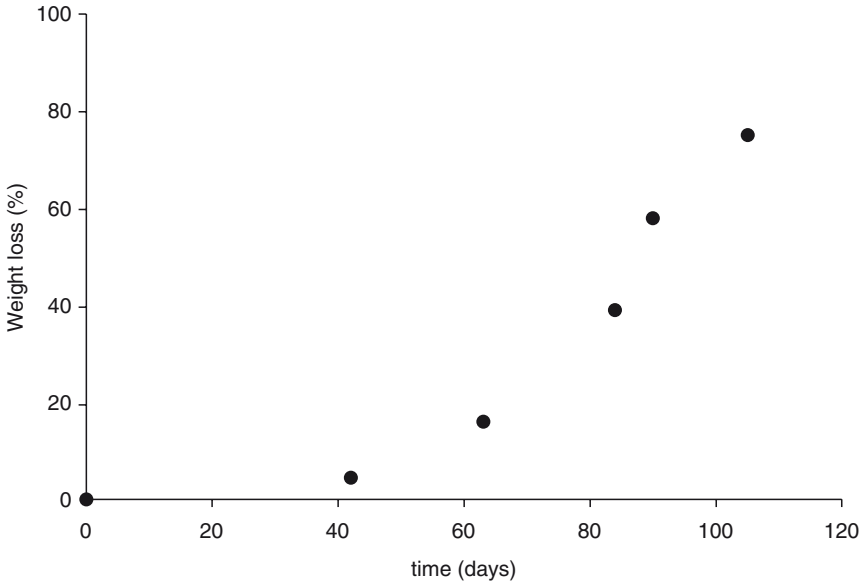

Fig. 5 Weight loss of oxidized poly[(*R*)-3-hydroxyoctanoate] [P(HO–COOH)] film during the hydrolytic degradation at pH 7.3 at 37°C (Timbart 2007)

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 pK_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

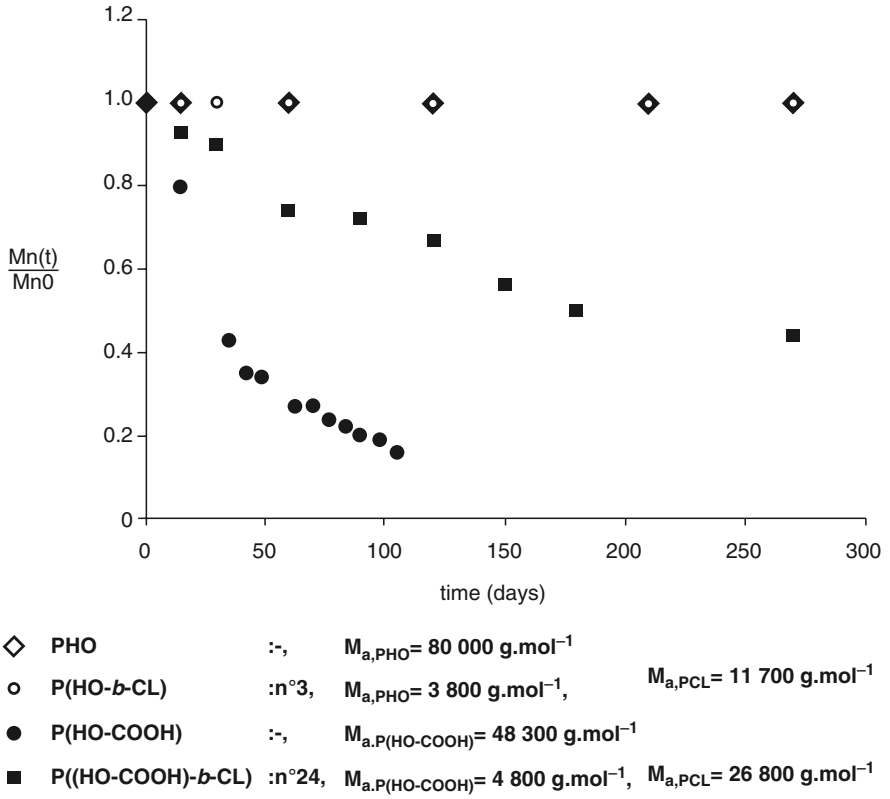

Fig. 6 Hydrolytic degradation of different copolymers at pH 7.3 at 37°C (Timbart 2007)

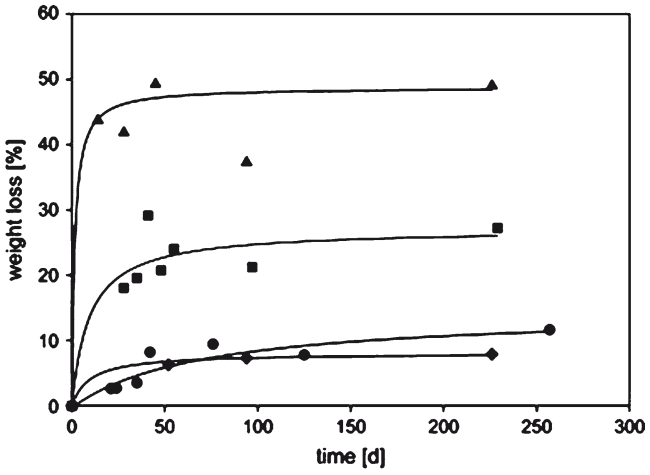

Fig. 7 Weight loss of different poly(HO_{75}–$COOH_{25}$) (PHOD)-*g*-poly(ethylene glycol) (PEG) films in phosphate buffer (pH 7.24, 37°C) with time: *circles* PHOD-*g*-PEG350 (grafting 52%), *squares* PHOD-*g*-PEG350 (grafting 28%), *diamonds* PHOD-*g*-PEG750 (grafting 29%), *triangles* PHOD-*g*-PEG2000 (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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Microbial Lactic Acid, Its Polymer Poly(lactic acid), and Their Industrial Applications

K. Jim Jem, Johan F. van der Pol, and Sicco de Vos

Contents

Abstract Lactic acid occurs widely in nature and is produced by many life forms from bacteria to human cells. Identified in 1780 as a component of sour milk, it has been used in food, drink, pharmaceutical, cosmetics, chemical and electronic industries in different forms, such as free acid (typically 80–92% in water), salts (e.g., calcium lactate), and esters (e.g., ethyl lactate). If one calculates the amount of its derivatives back to the equivalent amount of original lactic acid, the total global market volume in 2008 is estimated at around 260,000 tons of lactic acid (calculated at 100% concentration) for traditional applications [excluding poly(lactic acid)

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G.-Q. Chen (ed.), *Plastics from Bacteria: Natural Functions and Applications*, Microbiology Monographs, Vol. 14, DOI 10.1007/978-3-642-03287_5_13,

(PLA)]. PURAC has a market share of over 60%, and other producers share around 30–40% of the global commercial market for lactic acid and its derivates. Whereas lactic acid was historically produced through a chemical process, today more than 95% of the lactic acid produced is derived from biological resources (e.g., sucrose or glucose from starch) by microbial fermentation, which typically produces the L-(+) form of lactic acid. Only one producer still uses petroleum-based chemicals to synthesize racemic mixture of the D-(−) and L-(+) forms of lactic acid.

With the shortage and/or unstable supply of crude oil, its astonishing price variation, and the concerns of its environmental impact and the greenhouse gases released by petroleum-based plastic and chemical industries, bio-based plastics have gained significant attention. Among them, PLA is not only biodegradable, but is also regarded as most cost-competitive. NatureWorks, the largest producer of PLA, has expanded its annual production capacity from 70,000 to 140,000 tons in 2008–2009. Several other producers are setting up pilot plants in China and Europe. By 2020, the global annual production capacities of the lactic acid and PLA industries are very likely to exceed one million tons.

Compared with traditional petroleum-based plastics, PLA is still more expensive and usually has less desirable mechanical and physical properties, which limit its commercialization and applications. The recent commercialization of D-(−)-lactic acid and lactide has the potential to improve the mechanical and thermal characteristics of PLA resins and blends by the crystallization of stereocomplex PLA, which allows application in previously unattainable high-end markets.

The status and industrial (nonmedical) applications of microbial lactic acid and PLA are discussed in this chapter. The current issues and future work needed to develop the lactic acid and PLA industries are also reviewed. This chapter is meant to serve as an easy-to-read practical review for those interested in the general status and future of the lactic acid and PLA industries.

1 Lactic Acid and Its Derivatives

Lactic acid (2-hydroxypropionic acid) is perhaps the most widely occurring carboxylic acid in nature. The Swedish chemist Scheele first discovered it in 1780. It exists in two different forms (Fig. 1): the *dextrorotatory* form, called L(+)-lactic acid or (*S*)-lactic acid, and the *levorotatory* form, called D(−)-lactic acid or (*R*)-lactic acid. The plus and minus signs indicate the direction of the rotation of plane-polarized light produced by a chemical. These 2 stereoisomers (scientifically known as “enantiomers”) are produced by different enzymes [lactate dehydrogenases (LDH)] present in living organisms. In this chapter, they will be referred to as D-lactic acid (D-LA) and L-lactic acid (L-LA). Naturally formed lactic acid is usually in the L form, but D-LA may coexist with L-LA in some cases, especially if it is secreted by nonspecific microbes.

L-LA and D-LA are the mirror images of each other. In their pure forms, they have identical physical and chemical properties, except that they rotate plane-polarized light equally in opposite directions, and they may react differently with other asymmetric

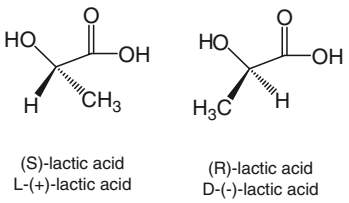

Fig. 1 Two stereoisomers of lactic acid. The *dotted lines* from the asymmetric center carbon atom project behind the plane of the paper

(chiral) reagents such as most enzymes in biological systems. An equal-molecular mixture (1:1) of D-LA and L-LA is "racemic" since it does not rotate polarized light any more. For many industrial applications, there is no difference between the performance of D-LA, L-LA, or any combination of them. For many biological or biochemical applications such as in the food, pharmaceutical, and agrochemical industries, as well as for certain polymer applications, there are very important differences between these two forms.

D-LA may cause metabolic problems. The daily intake of D-LA should be restricted to less than 100 mg kg^{-1} of body weight for adults (FAO/WHO 1967, 1968). Since babies are short of the enzyme to metabolize D-LA effectively, D-LA is not allowed to be added to food for babies or small children (FAO/WHO 1974; Nordic Working Group on Food Toxicity and Risk Assessment 2002). However, small amount of D-LA should not be considered to be very harmful either, based on the above daily intake limit of around 5–8 g of D-LA for regular adults.

Lactic acid and its derivatives have very wide applications in many different markets, such as food, pharmaceuticals, personal care, chemicals, electronics, and polymers. For the food industry, lactic acid may be added to confectionery, beverages, juices, wines, and dairy drinks (e.g., acidified milk drink or yogurt) as a taste or flavor enhancer, acidifier, and/or preservative. It may also replace phosphoric acid in beer production as an acidifier. Sodium and potassium lactate can inhibit bacterial growth (e.g., *Salmonella*, *Listeria*, and *Clostridium*) on raw poultry, meat, and steak to extend the shelf life, or on sausages and processed meat products to replace or reduce the usage of other preservatives. Calcium lactate is widely used to fortify the calcium content in soy milk and other nondairy beverages. Other lactate salts such as aluminum, copper, ferrous iron, magnesium, manganese, and zinc lactates are used in sports drinks or nutrition enhancers for mineral fortification. Sodium stearoyl lactylate is used as a bread conditioner.

Sodium lactate is used in pharmaceutical-grade injection solutions and kidney dialysis solutions. Mixtures of sodium lactate, lactic acid, and/or other organic acids may be used in cosmetics as a skin moisturizing or whitening agent. Detergent and personal care industries use lactic acid and its salts in acidic cleaners, toothpastes, hand-washes, shampoos, soaps, etc. for its cleaning, descaling, bacteriostatic, or biocidal effects, and because it is natural, safe, mild, and less corrosive than most other alternatives.

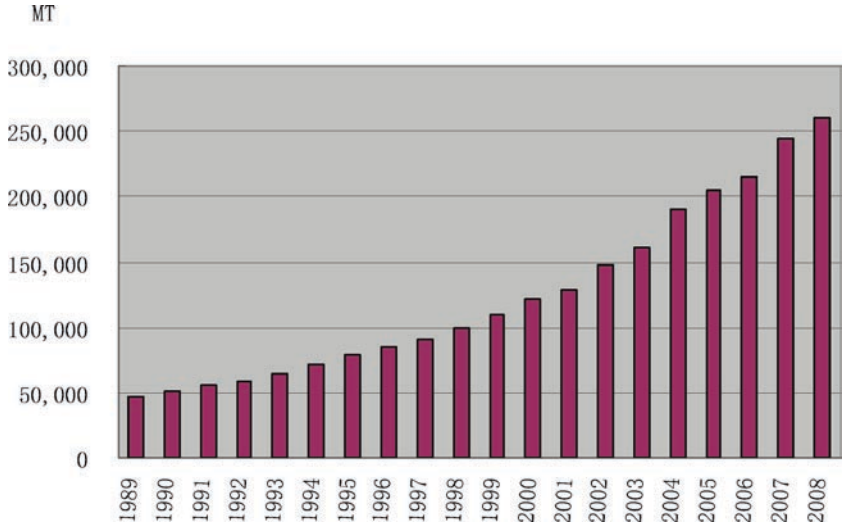

Fig. 2 The global commercial market volume of lactic acid and its derivatives, excluding poly(lactic acid) (PLA). Volumes are shown in the equivalent amount of 100% lactic acid

Lactic acid is also used in chemical (nickel) electroless metal plating. Lactic esters (e.g., methyl, ethyl, propyl, butyl, 2-ethylhexyl lactates) are used in the chemical and electronic industries as solvents for LCD processing, read/writable compact disk and IC production, and as cleaning solvents for wafers (edge bead removal) and printed circuit boards, because they are mild, safe, and biodegradable, with good solvency, appropriate viscosity, matching surface tension, low evaporation rate, high flash points, fully water miscible, etc. Lactic acid and its salts are also used in the leather deliming process, in the dying processes as a mild pH regulator, and in the textile industry. Its polymer, poly(lactic acid) (PLA), copolymers, and their applications will be discussed later.

The growth of the global commercial market volume of lactic acid and its derivatives (i.e., what is offered to the public, excluding captive usages such as lactic acid produced for internal PLA production within the same company or very closely related entities) is shown in Fig. 2; the average annual growth rate is 10%. The 2008 global traditional (non-PLA) market volume is estimated to be around 260,000 tons, calculated from the equivalent amount of 100% lactic acid (e.g., excluding the water and sodium portions of a sodium lactate solution).

2 Production of Lactic Acid by Fermentation

Lactic acid can be manufactured by chemical synthesis or microbial fermentation processes. The chemical synthesis process uses petroleum-based chemicals, which are subject to the potential supply problem of crude oil and its dramatic price variation

(between US $33 and 147 per barrel in 2004–2008). Another disadvantage of the chemical synthesis process is that it produces only the racemic (50:50) mixture of D-LA and L-LA, which is not desirable for the food, drink, and pharmaceutical industries due to the metabolic problems that D-LA may cause, and is not appropriate for the PLA industry, which typically requires lactic acid with high optical purity (e.g., over 98–99% of L-LA, with less than 1–2% of D-LA). For some industrial applications (e.g., metal plating and detergents), the optical purity of lactic acid does not matter; but these markets are relatively small. Only one commercial lactic acid producer known to the author today is still using the chemical synthesis process. Other commercial producers use microbes to ferment lactic acid from natural and annually renewable biological sources, mainly carbohydrates such as sucrose (from sugarcane or sugar beets), or glucose (hydrolyzed from starch).

This chapter focuses on the microbial fermentation process for lactic acid production. The first commercial operation was set up by Avery in the USA in 1881. Microbes contain enzyme(s) called LDH which can convert pyruvic acid to lactic acid. Depending on the particular microbe and the specificity of its LDH, the lactic acid fermentation process can produce rather pure D-LA or L-LA with high optical purity, or a mixture of them with low optical purity. Genetic engineering tools can be used to knockout the D-LDH gene(s) in the production strain to improve the optical purity of its L-LA fermentation process.

Many microbes can produce lactic acid, but a competitive commercial process requires a robust, fast-growing, low-pH, and high-yield strain with low-cost nutrient requirements. Typical *Lactobacillus* fermentation is anaerobic, requiring minimal energy for operation. When a fungus such as *Rhizopus oryzae* is used, the aerobic fermentation requires significant agitation and aeration with high energy cost and long fermentation time due to its slow growth and production rates.

For anaerobic fermentation, the most significant cost typically comes from medium components such as carbohydrate. Expensive components such as growth factors and vitamins should be avoided. During fermentation, two molecules of lactic acid are produced from one molecule of glucose through the "glycolysis" pathway. When lactic acid is formed, the pH of the broth is reduced and its acidity can inhibit the fermentation if it is not controlled. Typically, alkalis such as calcium carbonate, calcium hydroxide, or ammonium hydroxide are added to the fermentation broth to neutralize the lactic acid and to maintain the pH of fermentation at an optimal value for the particular production strain. However, calcium alkalis may precipitate lactic acid as calcium lactate after the fermentation broth has cooled down. Ammonium hydroxide does not cause lactic acid to precipitate.

After fermentation, lactic acid must be recovered from the broth and purified to meet its final specifications. Depending on the raw materials, microbes, and media used, the recovery steps and conditions may need to be adjusted. In principle, there is no difference in recovering and purifying D-LA compared with L-LA from the broth. Under extreme conditions (e.g., high temperature), D-LA and L-LA may be converted to each other through a process called racemization, and eventually reach equilibrium and become a racemic mixture. Therefore, processing at elevated temperature should be minimized to produce high optical purity lactic acid.

A typical downstream process includes (1) removal of production microbes (biomass) and solids (e.g., gypsum) from the broth, (2) recovery of crude lactic acid, and (3) purification of lactic acid. The biomass and solid waste can be separated from the liquid streams by various means, such as filtration, centrifugation, and decantation. If calcium alkali is used to control the fermentation pH, it produces calcium lactate precipitates which must be dissolved by acids such as phosphoric or sulfuric acid to extract lactic acid back into solution. After sulfuric acid has been added, calcium sulfate ($CaSO_4 \cdot 2H_2O$, known as gypsum) is formed and must be removed from the liquid stream as a major solid waste.

The recovered liquid stream contains crude lactic acid and impurities such as soluble proteins, residual sugars, salts, other acids, and complex color components which must be removed and purified mainly by chromatography, esterification, and/or distillation. Membrane filtration, crystallization, and/or evaporation may also be used to further purify the product stream, or to concentrate it to the desired degree. Typical commercial lactic acid products are sold at concentrations between 80 and 88%, or even 92–93%. At these concentrations some of the lactic acid molecules may be present as dimers and trimers at room temperature.

The purified lactic acid can be neutralized by various alkalis to produce lactic salt solutions such as sodium lactate and calcium lactate. Solid forms of lactic salts can be produced after evaporation, spray-drying, crystallization, and granulation. Esters can be produced by reacting lactic acid with alcohols (e.g., methanol, ethanol, propanol, butanol) and subsequent removal of unreacted free acid and alcohol from the product stream. Lactic acid can also be dehydrated to lactide and subsequently be polymerized to PLA through specific processes, which will be discussed later.

Since the lactic acid industry started around 120 years ago, many industrial producers have started up their lactic acid operations then stopped their business due to technical problems and/or market competition. The history of the lactic acid industry and the "retired" producers has been well reviewed (Benninga 1990). Current key lactic acid producers are listed in Table 1. Musashino Japan is the only one still using the chemical synthesis process to produce racemic lactic acid and its esters. Others use fermentation to produce optically pure lactic acid, mainly L-LA; but some Chinese companies ferment racemic mixture of L-LA and D-LA using nonspecific microbes.

Cargill owns the largest single lactic acid production plant (180,000 tons) in the world, but it has been mainly employed for their internal captive usage to produce PLA (Clydesdale and Whelan 2007). The largest lactic acid producer in the world, PURAC, supplies over 60% of the global market through its existing production facilities in the Netherlands, Spain, Brazil, and the USA. A new plant was put into operation in Thailand in late 2007 with 100,000 tons per year immediate production capacity and 200,000 tons per year design capacity (Fig. 3). With this new plant in Thailand, PURAC has freed other facilities for the commercial production of new high-end products such as lactides and D-LA.

The capacities of the production plants in Table 1 add up to over 470,000 tons, which is significantly higher than the actual global market demand, including the immediate needs for PLA. Despite the oversupply situation, new facilities have

Fig. 3 PURAC's new lactic acid plant in Thailand

Table 1 Key lactic acid producers, including those for captive poly(lactic acid) (PLA) usage

Key lactic acid producers in 2008–2009	Location of production plants
PURAC	Netherlands, Spain, USA, Brazil, Thailand
Cargill (for NatureWorks)	USA
ADM	USA
Galactic	Belgium
Musashino	Japan, China
JinDan	China
BBCA & Galactic	China
HaoKai	China
Sinolac (and Hyflux)	China
ShenDa	China
WuLiangYe	China
AnHua	China
KaiFeng	China
LeDa	China
BoFei (under construction)	China

been built and more building plans have been announced in China recently. Most of them are targeted to support future PLA projects even though the feasibility of the PLA industry in China has not been proven yet. These lactic acid projects may be dramatically ahead of the potential PLA projects. Besides, most Chinese local producers have not been able to produce lactic acid pure and economically enough to supply existing PLA pilot producers there.

3 Production of PLA

With the unstable supply and astonishing price volatility of crude oil, and the global warming situation, bio-based polymers have gained a lot of attention. Among them, PLA is the first one that has reached a commercial scale, is fully biodegradable under industrial composting conditions, and is generally regarded as the most promising and competitive bio-based plastic from annually renewable resources. Most other biodegradable plastics, such as Ecoflex™ (petroleum-based plastic from BASF), bio-based poly(hydroxybutyrate–hydroxyvalerate) (PHBV), and poly (butylene succinate) (PBS), currently petroleum-based, are typically 2–3 times more expensive than PLA and are produced on much smaller scales.

The polymerization processes and characteristics of PLA have been thoroughly reviewed (Garlotta 2001; Mehta et al. 2005; Henton et al. 2005) and will not be repeated in detail here. Polymerization of PLA has been conducted since 1932 by either a direct polycondensation (DPC) of lactic acid, or a ring-opening reaction of lactide, a cyclic dimer of lactic acid (Fig. 4). Both processes rely on highly purified polymer-grade lactic acid or lactide to produce PLA with good quality, high molecular weight, and a high yield. Crude lactic acid with impurities would strongly impact the production process, the yield, and the characteristics of PLA. Therefore, purification of lactic acid from the industrial fermentation process is of decisive importance. The optical purity (in addition to the chemical purity) of lactic acid or lactide also strongly affects the characteristics of PLA.

The DPC process dehydrates lactic acid into oligomers, which are then further polymerized to PLA with simultaneous dehydration. However, removal of water generated from the condensation of lactic acid is very difficult during the final stage of polymerization because the diffusion of moisture in the highly viscous polymeric

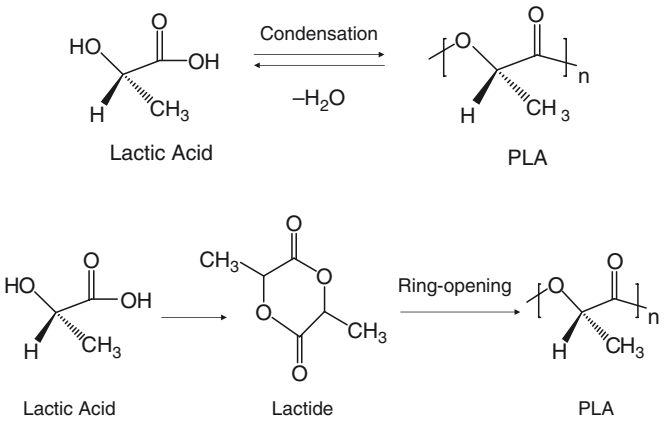

Fig. 4 PLA production processes: (1) direct polycondensation (DPC) of lactic acid and (2) ring-opening polymerization (ROP) of lactide

melt is very slow. The residual water trapped in the PLA melt may limit the achievable molecular weight and the characteristics of PLA. Consequently, it is rarely used, except by Tongji and Mitsui Chemical (Ajioka et al. 1995).

Most of the PLA production processes described utilize the more efficient conversion of lactide (the cyclic dimer of lactic acid) to PLA via the ring-opening polymerization (ROP) catalyzed by organometal catalysts (Henton et al. 2005). Lactic acid is dehydrated and polycondensed to its oligomers at high temperature and under a vacuum to remove moisture. Then lactide is obtained from catalytic depolymerization of these short PLA chains under reduced pressure. Residual lactic acid may be removed from lactide by various means, such as distillation or crystallization. The purified lactide is polymerized by a ring-opening reaction into PLA at temperatures above the melting point of lactide and below the degradation temperatures of PLA. The resulting PLA resin is solidified and/or crystallized into pellets. During the ROP of lactide, there is no moisture to be removed from the molten PLA resin.

The ROP process is sometimes referred to as a "two-step" process due to its clear intermediate lactide step, and the DPC process, without a lactide step, is referred to as "one-step." But both polymerization processes actually go through multiple processing steps to produce PLA from lactic acid. The typical ROP process used by NatureWorks is illustrated in Fig. 5 (Gruber 2004). Lactic acid is continuously converted into low molecular weight PLA prepolymers. The oligomers are then catalytically converted into lactide by *cyclization depolymerization* in another reactor. Residual lactic acid is removed from the molten lactide in the vacuum distillation column. Purified lactide in a polymer reactor goes through the ROP by an organic tin-catalyst without expensive solvents. Unreacted lactide is removed by vacuum

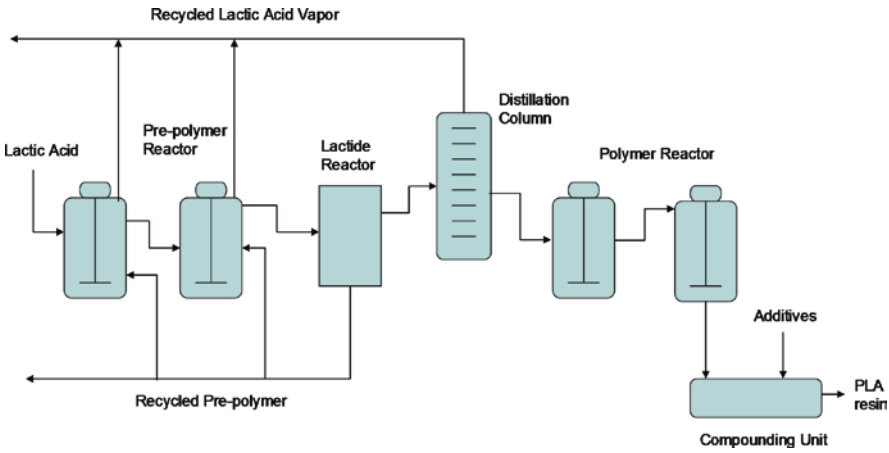

Fig. 5 Schematic illustration of integrated ROP process for PLA starting from lactic acid

for recycled. Purified PLA polymer is compounded with additives, and extruded to form resin pellets for crystallization and packaging.

Typically, polymer-grade L-LA with high chemical purity and optical purity (i.e., over 98–99% L-LA and less than 1–2% D-LA) is used for commercial PLA production. When L-LA is dehydrated at high temperature into L-lactide, some L-LA may be converted into D-LA. D-LA mixed with L-LA contributes to *meso*-lactide (the cyclic dimer of one D-LA and one L-LA) and heteropolymer PLA (with both D-LA and L-LA units). Heteropolymer PLA exhibits slower crystallization kinetics and lower melting points than homopolymer PLA (of pure L-LA units or pure D-LA units).

Currently, there is only one industrial-scale PLA production plant in the world, operated by NatureWorks in the USA, with a 70,000 tons capacity in 2002, expanded to 140,000 tons by mid-2009 (NatureWorks 2009). The second largest one is Hisun's 5,000 metric ton PLA plant in China. Other PLA facilities existing to date are smaller pilot plants for testing the process technology and feasibility. Multiple projects have been announced around the world. The current key PLA projects are listed in Table 2.

PURAC has been producing pharmaceutical-grade lactic acid based polymers for medical applications during the last 30 years. These medical applications (e.g., sutures, bone screws) are not the focus of this chapter, which covers only the large-scale industrial applications of PLA. In 2008, PURAC started a D-LA program and produced L-lactide and D-lactide for its PLA partners, such as Synbra. Synbra plans to introduce small volumes of expandable PLA (BioFoam™) to extend its range of expanded polystyrene (PS)-based foam products, before building a 50,000 ton per year expanded-PLA production facility (Schut 2008).

Table 2 Key PLA projects in 2008–2009

Key PLA projects in 2008–2009	Location of operation
NatureWorks	USA
Pyramid (Uhde and German Bioplastics)	Germany
Futerro (Galactic and Total)	Belgium
Sulzer	Switzerland
Synbra	Netherlands
Tate & Lyle (former Hycail)	Netherlands
Teijin	Japan
Toyobo	Japan
Hisun	China
BrightChina	China
SanJiang GuDe GuangShui	China
JiuDing	China
Zoxin	China
Tongji	China
PiaoAn	China
Confidential project	Asia

4 Markets and Applications for PLA

PLA is a thermoplastic polyester which can be spun to form fibers, stretched to form rigid films, extruded into sheets for thermoformed packaging, and injected into molds. It has been used mainly in food packaging (e.g., salad containers, cups, candy wraps, bottles), textiles (e.g., curtains, towels, apparels), nonwovens (e.g., wet-wipes, pillow stuffing), injection-molded parts (e.g., mugs, toys), etc. For flexible film applications such as shopping bags or mulch films, PLA needs to be blended to improve its flexibility and compostability. In other applications, PLA may require some modifications and improvements due to its unique performance profile. After it has been compounded to improve its impact strength and heat stability, it may be used for engineering plastic applications, such as the cases for computers, mobile phones, and radios. The typical applications of PLA are shown in Fig. 6.

The global PLA market volume information is not available to the public, but it must be smaller than the 70,000 metric ton capacity of NatureWorks's single production line up to early 2008, because other PLA pilot plants are still insignificant. On the basis of Japan's customs import data, the Japanese PLA market was about 6,000 tons per year in 2007–2008, with about 14% market growth and almost the same CIF price of about US \$2 kg^{-1}. If one assumes the Japanese market is around one fifth to one tenth of the global market, as commonly observed in other

Fig. 6 The application of PLA in different markets

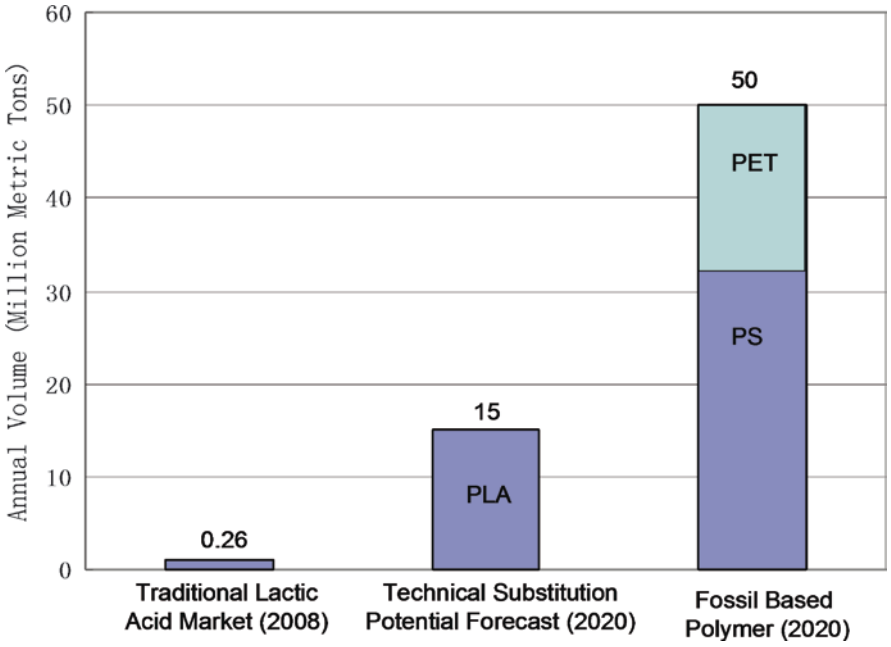

Fig. 7 Addressable PLA market size by replacing a fraction of the poly(ethylene terephthalate) and polystyrene market

markets, the global market of PLA in 2008 was perhaps around 40,000–60,000 tons. The second 70,000 tons per year line in NatureWorks's PLA production plant is scheduled to be operational in mid-2009 to support further market growth (Clydesdale 2008).

Since the characteristics of PLA are similar to those of poly(ethylene terephthalate) (PET) and PS, we may use the current 50 million tons global market volume of PET plus PS as a reference point to estimate the future PLA market volume. If PLA can replace a fraction of the PET/PS market, the global PLA market may easily grow to a multi-million-ton scale by 2020 (Fig. 7).

5 Characteristics and Modifications of PLA for Various Applications

As a rigid thermoplastic, PLA has basic properties comparable with those of PET and PS (Table 3), but rather different from those of polyethylene (PE) and polypropylene (PP). PLA has many unique beneficial characteristics (Table 4), such as superb transparency, glossy appearance, high rigidity (which allows downgauging of thermoforming parts), printing effects, and twist retention. These special characteristics make PLA a perfect fit for some market sectors, such as fibers, disposable

Table 3 Basic characteristics of PLA and traditional plastics

Polymer	Density (g mL^{-1})	T_g (°C)	T_m (°C)	Tensile strength (MPa)	Elongation (%)	Izod impact (J m^{-1}) (notched)
PLA	1.24	57–58	140–175	53	6	12.8
PS	1.05	100	–	45	5	21
PET	1.39	69	255	57	70	59
PP	0.9	−20	175	31	200	53

PS polystyrene, *PET* poly(ethylene terephthalate), *PP* polypropylene

Table 4 Beneficial characteristics of PLA for packaging and fiber applications

Packaging performance benefits

- Good clarity, gloss, and printability (similar to PET); good surface adhesion
- High stiffness (better than PET and PS) for downgauging of rigid packaging
- Lower processing temperatures with energy savings; sealable by heat, RF, and ultrasound
- Excellent resistance to fat, oil, and grease
- Good twist and dead-fold retention for candy wrap
- Good breathability and moisture vapor permeability for vegetables, fruits, and bread
- Natural resource-based; food contact safety and compliance; environmentally friendly

Fiber performance benefits

- Melt-processable natural-based fiber
- Staple fibers with good loft, bulk, resilience, insulation properties
- Filament-based fabrics with great hand, drape, and luster
- Great comfort and wicking performance
- Good durability in laundering

Table 5 Gas and moisture vapor permeability of PLA and other plastics

Polymer	Oxygen[a]	Moisture vapor[b] g.mil/100 sq.in./day	CO_2[a] cc.mil/100 sq.in./day/atm
PLA	38–42	18–22	183–200
PET	3–6	1–2.8	15–25
HDPE	130–185	0.3–0.4	400–700
PP	150–800	0.5–0.7	150–650
Nylon 6	2–3	16–23	10–12
EVOH	2–2.6	1.4–6.5	NA
HIPS	300–400	10	NA
PVC	4–30	1–5	4–50

HDPE high-density polyethylene, ethylene–vinyl alcohol copolymer, *HIPS* high-impact polystyrene, *PVC* poly(vinyl chloride), *NA* not available

[a]grams.mil per 100 square inch per day

[b]cc.mil per 100 square inch per day

cups, salad boxes, and cold food packaging. Not much modification is needed for these applications.

The special characteristics of PLA can make it a good fit for some applications but may also require modifications for some others. For example, the oxygen and moisture permeability of PLA is much higher than for most other plastics, such as PE, PP, and even PET (Table 5). Consequently, PLA can be used for the packaging of

fresh vegetable or fruits with better oxygen breathing effects. For packing fresh bread, PLA bags allow moisture from the warm bread to evaporate instead of condensing inside the bags. However, the high gas permeability of PLA can also cause problems for its application in bottles and may require some modifications (see below).

Some high-end applications such as packaging for electronics may require a significant modification of the characteristics of PLA to improve its mechanical strength, heat distortion temperature (HDT), durability, etc. Various ways of modifying PLA have been studied extensively but still require much more work; for example, compounding PLA with other polymers or fillers, branching or copolymerization of PLA resin, and crystallization of PLA parts. Some key approaches are summarized next.

5.1 Crystallization of PLA by Nucleating Agents

Depending on its optical purity, molecular weight, branching, the presence of additives, and the processing conditions, PLA can be amorphous, semicrystalline, or crystallized. These forms exhibit very different HDT and mechanical strength. Most thermoforming or injection-molding parts made from regular amorphous PLA (aPLA, such as Grade 2002 PLA from NatureWorks) have HDT at around 55–65°C. These aPLA parts lose their dimensional stability at temperatures above 55°C. Consequently, they can be used for only mid-to-room temperature applications and cannot be used for hot-fill bottles, coffee cups, and hot meal cutlery. The low HDT may also cause shipping problems, since the temperatures in containers during hot weather can be as high as 60–70°C. Typical aPLA parts are brittle and easily damaged upon impact. The poor mechanical properties of PLA (e.g., brittleness, crispiness, low impact strength, and poor tear strength) limit its high-end injection-molding applications, such as the casing of electronics.

These problems can be solved by increasing the degree of crystallinity of PLA parts. Crystallized PLA (cPLA) has much higher HDT and mechanical strength. PLA sheets and rigid films can by crystallized by biaxial orientation (BO). PLA filament, staple, and nonwoven fibers can be crystallized by high-speed spinning processes if they are performed correctly. Thermoforming and injection-molding PLA parts can be crystallized by adding nucleating agents such as superfine talc powder, DuPont's Biomax Thermal™ additive, or ethylene bis(strearamide) with appropriate annealing. However, the prolonged annealing time causes lower throughputs and higher production costs, which may limit the application and competitiveness. For example, cPLA spoons are rather expensive compared with PP spoons produced with a short process cycle time and cheaper resin.

Instead of adding nucleating agents, another cPLA approach uses the D form of PLA. Most commercial PLA available today are made of L-LA. Pure (optical purity 96–99%) D-LA was not commonly available on a large scale for the open market until PURAC started its D-LA production in 2008. The PLA homopolymers made

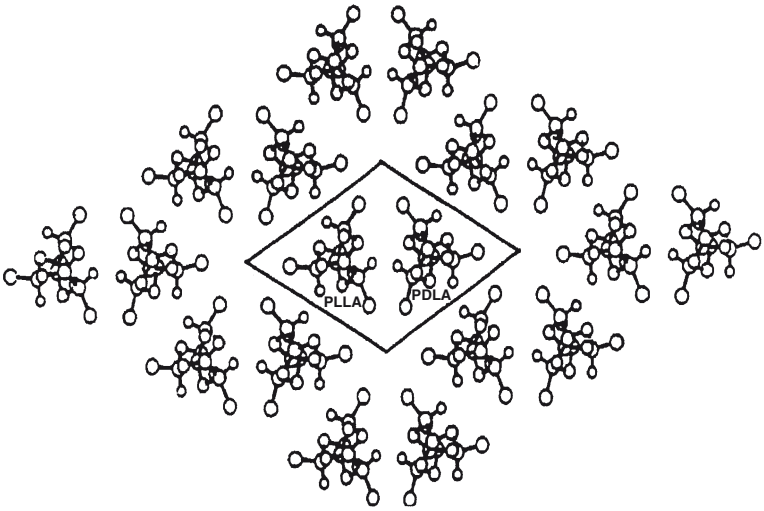

Fig. 8 Crystallization of stereocomplex PLA by blending poly(D-lactic acid) with poly(L-lactic acid) to form nucleating sites (Tsuji H. 2005)

from L-LA and D-LA are referred to as PLLA and PDLA, respectively. Blends of equal amounts of PDLA and PLLA can generate a stereocomplex crystalline form of PLA (scPLA), with a higher melting temperature (200–250°C compared with 180°C for PLLA homopolymer) and better heat resistance (with a HDT at more than 100°C compared with about 55°C for regular amorphous PLLA or amorphous PDLA). Even by adding only a small amount, say, 3–10%, of PDLA to regular PLLA, stereocomplex crystallites can form in the PLA melt, serving as a nucleating agent for PLA homocrystallization (Schmidt and Hillmyer 2000; Garlotta 2001; Anderson and Hillmyer 2006; de Vos 2008). With appropriate processing conditions, the HDT and mechanical strength of the final scPLA products can be increased much faster than in the talc crystallization process.

5.2 Compounding PLA with Other Polymers and/or Chemical Additives

Blending a polymer with other polymers, fillers, or fibers into multiphase compounds or composites is a proven method for developing its unique characteristics. Tremendous efforts have been invested in compounding PLA to raise its temperature tolerance and mechanical strength, and modifying its performance to expand its applications into high-end markets.

For durable high-end applications related to computers, electronics, and automobiles, other characteristics, such as flame resistance, good weatherability, and

Fig. 9 Japanese electronics products using the bio-based PLA/polycarbonate polymer blending

durability, are required. As a biodegradable plastic, PLA promptly degrades under industrial compositing conditions (about 58°C and 80–90% humidity) and requires significant compounding efforts to alter its biodegradability and to improve its durability. Consequently, PLA is commonly mixed with polycarbonate (PC), resulting in a phase-separated polymer blend, as widely done in Japan for electronics applications (Fig. 9). But the PLA/PC compounded products are not fully biodegradable any more. When the amount of PLA is over 50%, the compounded products are considered to be (partially) bio-based products and are widely accepted in Japan. A drawback of such compositions is that typical PC properties such as durability, impact, and scratch resistance are increasingly lost as the PLA content is increased.

PLA has rigidity similar to that of PET and PS, so it is not a natural fit for most flexible film applications such as shopping bags, garbage bags, and mulch films, which typically use PE and PP for their flexibility and stretchability. Plasticizers such as citrate esters can reduce the glass-transition temperature and the rigidity of PLA. For disposable film products such as agricultural mulch film and trash bags, the biodegradable characteristics of PLA preferably need to be maintained. Several "soft" biodegradable plastics such as PBS, PHB, PHBV, and EcoFlex™ (by BASF) can be used for blending with PLA to improve its flexibility.

PLA has also been blended with other nonbiodegradable polymers, such as poly(methyl methacrylate), acrylonitrile–butadiene–styrene, and Hytrel™ (from DuPont). Note that some polymers such as PE and PP are not compatible with PLA and cannot be used for compounding, unless special compatibilizers are used.

5.3 Compounding PLA with Nonplastic Materials

Nonplastic materials such as montmorillonite clay, glass fiber, and cellulose have been compounded with PLA to improve the characteristics of PLA products. Some low-end, large-volume applications such as mulch films may be extremely cost-sensitive and require compounding with low-cost materials to balance material costs and product value. Blending with low-cost filling materials such as starch, sawdust, biowaste, and stone powder has been widely tested, but it typically makes the characteristics of PLA parts worse. Before they are blended into PLA, fillers (especially starch and cellulose) must be thoroughly dried to avoid the degradation of PLA by moisture during processing. Reactive compatibilizers such as maleic anhydride can be used to improve the interfacial adhesion and performance of PLA/starch blends (Jang et al. 2007).

Disposable packaging materials with a relatively short usage time can benefit from better degradability or compostability. For example, the mulch film for covering wide areas of farmland demands the lowest possible costs and must be disposed of (or composted) easily after the harvest season. Bio-based fillers (e.g., starch) and catalysts enhancing degradation or disintegration of PLA may help dramatically to control the disposal of PLA mulch films on-site on the farmland after the growth season without the collection and transportation of waste film to industrial composting facilities.

5.4 Processing Technology To Improve PLA Performance

Sometimes, the improvement of PLA performance can be achieved simply by processing technology instead of compounding efforts. For example, coating, multilayering, annealing, and BO may yield desired barrier properties, transparency, and much better mechanical performance for PLA.

For example, PLA bottles work very well for fresh milk, juice, and mineral water in Western countries. But in China or less developed areas, mineral water bottles require a much longer shelf life (up to 1–2 years). With the extraordinarily high moisture vapor permeability of PLA (Table 5), the water level in PLA bottles can be significantly reduced after 3 months, due to the escape of moisture vapor through the PLA bottle wall. With similar high gas permeability, neat PLA and PET bottles both cannot be used for carbonated or alcoholic drinks such as sparkling water, coke, beer, and wine. Coating a gas barrier on the inner walls of PET bottles has successfully solved this problem in commercial beer packaging. However, the carbon vapor deposition technology used to coat PET bottles demands a processing temperature (over 85°C) which is higher than what neat PLA bottles can withstand; but scPLA may be able to withstand it. A different coating technology (PlasMax™ from the German company SIG) may work better for neat PLA bottles.

BO and high-speed fiber spinning processes offer alternative ways to crystallize PLA and improve its heat stability and mechanical strength dramatically.

When these processes are performed under appropriate conditions, entangled aPLA polymer chains can be aligned and annealed in an ordered manner to form cPLA. For example, the HDT of BO PLA films can be dramatically increased from 55 to 120°C or even higher, depending on the degree of crystallization achieved by the processing conditions. In this particular case, neat PLA is good enough for processing, with no (or very little) compounding needed.

5.5 Polymerization or Copolymerization To Modify PLA

Instead of polymerizing only lactic acid or lactide into PLA as being done today, it is possible to add other components to create the cross-linkage of PLA linear molecules, or to add other monomers (e.g., glycolide, eps-caprolactone, etc.) to form copolymers such as, poly(lactic acid -*co*- glycolic acid) with characteristics very different from those of PLA. The crystallization of the multiblock copolymer (PDLA-PLLA)n in a string of randomly linked PDLA and PLLA sequences of limited length is faster than the crystallization of a physical blend of high molecular weight homopolymers PLLA and PDLA (Fukushima et al. 2005).

6 Factors Helping the Growth of the PLA Industry

In the last two decades, PLA has attracted tremendous attention and interest. The major reasons are probably concerns about resource sustainability and environmental pollution. Currently, crude oil serves not only as an energy source for transportation etc., but also as raw material for chemical and plastic industries. Its role in energy supply may be replaced by solar, wind, and water power. But its role as a raw material can only be replaced by coal, natural gas, and biomaterials such as starch and cellulosic biowaste. Coal and natural gas will be depleted eventually just like crude oil. Therefore, future plastics must be produced from biomaterials. Current plastics produced from petroleum are typically not biodegradable and have caused more and more problems in terms of environmental pollution (e.g., solid waste pollution, greenhouse gases, global warming). Bio-based and biodegradable plastics such as PLA and PHBV are clearly potential solutions for these issues.

Consequently, Cargill took the leading role in establishing its PLA operation in 1994 with a 6,000 ton per year pilot plant. Since then, many major companies have joined the effort and entered the PLA industry, e.g., Dow, Mitsubishi, Mitsui, and Toyota. Some have built pilot plants (e.g., Hisun), whereas others have announced plans to do so, e.g., Galactic, COFCO, and Uhde. Many governments have also announced policies to support the development of PLA and other bio-based and/or biodegradable plastics. In the last 15 years, tremendous progress has been made to bring the production capacity of the PLA industry to over 100,000 tons per year. But this is still much smaller than the scale of traditional

plastics, at over one million tons. Besides the growing concerns and the potential further deterioration of the oil price and supply stability, global warming, and solid waste pollution, other key factors helping (or limiting) the development of the PLA industry are reviewed here.

6.1 Government Regulations and Public Awareness

Other than the oil price, which affects the cost difference between PLA and traditional plastics, the most important factor impacting the growth of the PLA industry may be government regulations. Compared with the cost and properties of mature traditional plastics, those of current commercial PLA products are still a disadvantage. To overcome the economic barrier for PLA, several governments, such as those of France, Korea, Italy, and Taiwan, have set up regulations to limit or penalize the usage of traditional plastics for some disposable applications such as trash bags, and to subsidize or provide tax advantages for biodegradable plastics. For example, the *Grüne Punkt* tax in Germany levied since 2007 has made PLA dramatically more cost-competitive since nonbiodegradable traditional plastics have a front-end tax up to ~1.27€ kg^{-1}. Public awareness, education, and stronger law enforcement can also boost the usage of PLA and other biodegradable plastics, especially for low-end, large-volume, cost-sensitive, disposable applications such as trash bags and disposable cups. Public concerns about environmental issues can force brand owners to use PLA disposable products for a green image.

6.2 Development of Compounding, Converting, and Process Equipment Technologies

With its unique properties, PLA typically requires the plastic converters to conduct test runs, optimize process conditions, and/or modify their process equipment. For example, the heating media of processing equipment may need to be replaced because of the lower operating temperature of PLA; the extruder configurations may need to be changed to avoid overheating and overshearing of melted PLA; the fiber spin speed may need to be increased to help the crystallization and properties of PLA fiber; a bone-dry dehumidifying dryer is needed to control the moisture contents of PLA resin, recycled scraps, and additives to less than 400-ppm moisture content to avoid the degradation of PLA during processing. With the current growing PLA markets, compounders and converters may justify the investment to optimize the PLA process to further expand its markets. A bigger market will further encourage efforts to develop or modify converting and processing equipment for PLA to produce better and more competitive PLA products.

6.3 Development of Polymerization Technology

Polymerizing lactic acid or lactide on laboratory scales into PLA is easy, but building a commercially feasible industrial PLA plant to produce competitive, high-quality PLA resin consistently is a different story. Proven industrial polymerization technology on pilot scales is needed for major polymer companies to build PLA production plants. Several companies are offering their PLA technology to the market, for instance, Udhe Inventa Fisher, Sulzer, and Hitachi. When their pilot plants are validated for large-scale operation, major polymer producers may start building full-scale PLA production plants. A second major PLA supplier will help comfort key polymer clients and allow them to commit to developing PLA application technology and replacing traditional plastics.

6.4 Reduction of Plant Cost and Entry Risk

To reduce the production cost of PLA, the scale of a fully integrated ROP PLA production plant should be over 50,000 tons per year. A small plant has a high unit production cost and low competitive edge; but a large plant requires a huge front-end capital investment and high entry risk. The minimal economical scale requirement mainly comes from the upstream lactic acid and lactide production, instead of the PLA polymerization part. If the lactide plant is decoupled from the polymerization plant, the size of the PLA polymerization plant can be much smaller than 50,000 tons per year, but can still be competitive. PURAC has just installed a 5,000 ton per year lactide pilot plant to supply PLA pilot plants. Once the concept has been proved, PURAC may build a 75,000 ton per year lactide production at its Thailand lactic acid production site to support multiple PLA production plants at the 20,000–30,000-ton scale with low entry risk but that are still cost-competitive. The PURAC Thailand plant uses non-GMO sugarcane or cassava as the carbon source to produce lactic acid and lactide. So, the resulting PLA will be classified as non-GMO products.

6.5 Infrastructure of Recycling or Composting PLA Waste

PLA waste can be handled in many currently existing end-of-life solutions such as incineration, mechanical recycling, and composting. However, PLA does not degrade effectively in atmospheric conditions or in most home composters. It requires industrial composting conditions (around 58°C and over 80% humidity) to degrade within several months. Appropriate garbage collection, separation, and composting systems must be installed for PLA parts to decompose and return to nature. PLA plants do not have enough critical quantity yet to establish their own recycling system as is being done for PET or other traditional plastics. This problem can be

resolved when more PLA products enter the market. PLA parts must be segregated from other plastics such as PET to avoid contaminating their recycling streams. Automatic identification (Near IR or Fourier Transformed IR) systems can be installed in the plastic recycling lines to segregate PLA from other plastics. Recently, the economic and environmental feasibility of chemically recycling PLA waste back to lactic acid is under testing.

7 Concluding Remarks and Future Perspectives

Compared with most other biodegradable plastics and/or bio-based plastics, PLA is by far the most important and promising one. The leading position of PLA is demonstrated by the current scale of the PLA industry, the number of pilot projects announced or under construction, the numerous products and applications of PLA in biodegradable and/or bio-based polymer markets, the number of polymer companies and converters involved with PLA, the news and attention created by PLA, the patents and publications related to PLA, etc.

PLA is both bio-based and biodegradable, with unique properties (Tables 3, 4). Other than that, the major reason for PLA's leading position is its competitive production cost and market price compared with most other biodegradable and/or bio-based plastics. The key cost difference of plastics usually comes from the costs of their monomers or raw materials, instead of the processing cost of producing these polymers from monomers. Lactic acid is perhaps the most cost-effective bio-based plastic monomer known to date due to its particularly advantageous position in the metabolic pathway. With corn starch and sulfuric acid prices at historic levels of around US \$0.3–0.4 kg^{-1} (2008 year-end price), a large quantity bulk order of 88–92% L-LA (with 8–12% water) was around US \$1.2–1.5 kg^{-1} (depending on the contract terms and specific purity requirements of different PLA producers) in 2008. Consequently, the price of PLA (for industrial, nonmedical applications) was typically around US \$1.8–2.5 kg^{-1} (2008 year-end bulk ex-works price, depending on the grade, volume, contract, etc.), relatively close to the price of traditional plastics (e.g., PET and PS) when the crude oil price was over US \$80–100 per barrel in mid 2008. In comparison, most other biodegradable polymers such as PHBV and PBS were sold at around US \$4–5 kg^{-1} in 2008.

Figure 10 shows the inherit advantage of lactic acid produced by the universal metabolic pathway shared by most living organisms from humans, to plants, to microbes. Through the anaerobic glycolysis pathway, two lactic acid molecules ($M_w = 90$) are produced from one glucose ($M_w = 180$) with a 100% theoretical yield (i.e., no loss at all). For the anaerobic fermentation of ethanol, the theoretical yield is around 51% with two CO_2($M_w = 44$) molecules lost per glucose. After ethanol has been dehydrated to ethylene and polymerized into bio-PE, the theoretical yield from glucose is only around 31% compared with 80% for PLA (Fig. 10). For PHBV-like biopolymer synthesized in microbial cells, the building block is typically acetyl-CoA, with around 51% theoretical yield after the loss of CO_2 (decarboxylation) from

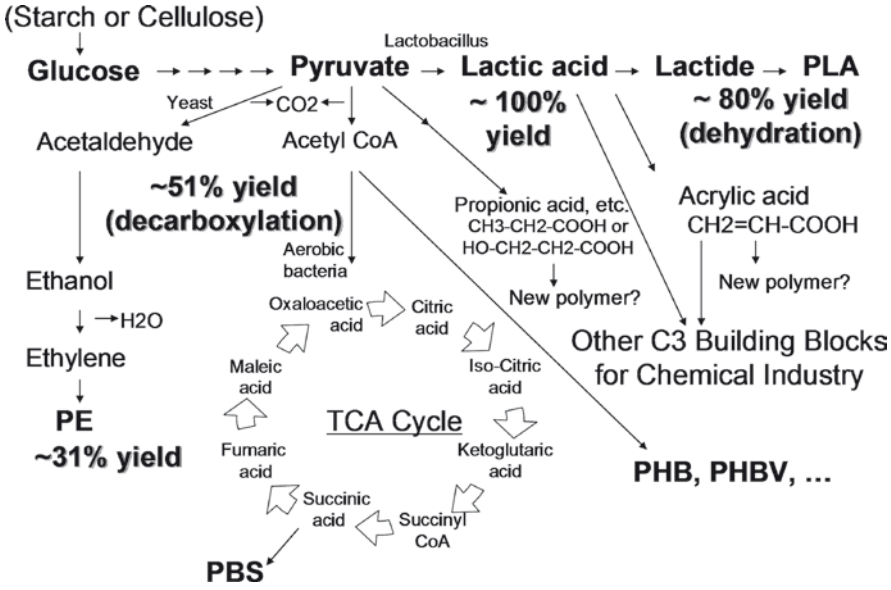

Fig. 10 Metabolic pathway of chemicals for bioplastic polymers and their theoretical yields

pyruvate. For PBS-like polymers, four-carbon molecules (e.g., butanediol and succinic acid) are typically produced by the tricarboxylic acid (TCA) cycle after the acetyl-CoA step with low yields. Advanced genetic engineering work is in progress trying to "fix" two CO_2 molecules into one glucose (with six carbons) to produce two four-carbon succinic acid molecules by reversing half of the TCA cycle. However, the energy for fixing CO_2 still has to be generated from the metabolization of glucose through acetyl-CoA into the TCA cycle with a theoretical yield of less than 51%. To date, succinic acid and butanediol from fermentation processes or bio resources still cannot compete with chemically synthesized products, and are much more expensive than lactic acid. Recently, several collaboration programs (i.e. between PURAC and BASF, DSM and Roquette, and Mitsubishi and PTT) have been announced to study or build their bio-based succinic acid projects.

In the future, lactic acid may be produced from biowaste (e.g., cellulose and lignocellulose) instead of starch from food crops to further reduce the cost and to increase the sustainability of PLA. A gypsum-free process is also under development to reduce solid waste to make PLA more environmentally friendly. Industrial-scale D-LA is being optimized to produce low-cost PDLA to improve PLA for high-end applications. Extensive efforts have been committed by the enzyme and fermentation industries and research institutes to these topics.

Many plastic companies have also been developing the polymerization, compounding, converting, and processing technologies for PLA. New biodegradable polymers or additives have been compounded with PLA to improve its characteristics for different applications and high-end markets. Multiple government regulations

have been implemented to restrict the usage of traditional plastics, and to promote biodegradable plastics, e.g., carbon credit system and tax benefits for biodegradable plastics. Infrastructures (e.g., garbage collection, composting, recycling systems) and promotion of public awareness are all improving for the development of PLA and other environmentally friendly plastics. With the depletion of fossil fuel reserves and the global awareness of environmental pollution, there is no doubt that low-cost, bio-based, and biodegradable plastics such as PLA will be very promising in the future plastic industry. In the next 10–20 years, we shall expect to see the PLA industry reach a multi-million-ton scale, similar to the traditional plastic industry today.

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Microbial Succinic Acid, Its Polymer Poly(butylene succinate), and Applications

Jun Xu and Bao-Hua Guo

Contents

Abstract Succinic acid is an important platform chemical derived from petrochemical or bio-based feedstocks and can be transformed into a wide range of chemicals and polymers. Increasing demand for biodegradable poly(butylene succinate) (PBS) will open up a new market for succinic acid. In this chapter, the synthesis of succinic acid is briefly reviewed. We focus on the polymerization, crystalline structure, thermal and mechanical properties, and biodegradability of PBS and its copolymers. PBS shows balanced mechanical properties similar to those of polyethylene and excellent performance during thermal processing. In addition, PBS and its copolymers can biodegrade in various environments, such as soil burial, river, sea, activated

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sludge, and compost. The properties and biodegradation rate can be easily tuned via copolymerization. Consequently, lowering the cost of the feedstock, molecular design, and further understanding of the processing–structure–performance of polyesters will be greatly beneficial to expand the market for succinic acid, PBS, and PBS copolymers.

1 Introduction

Traditionally, succinic acid is principally used in four application regions (Zeikus et al. 1999). The largest application region is as a surfactant, an additive as a detergent and foaming agent; the second is as an ion chelator, for preventing the corrosion and spot corrosion of metal in the electroplating industry; the third is as an acidulant, a pH regulator and flavoring agent in the food industry; the fourth is relative to pharmaceutical products, including the production of antibiotics, amino acids, and vitamins. The current worldwide use of succinic acid is around 20,000–30,000 tons per year and this is increasing by around 10% a year (Kidwell 2008).

Moreover, succinic acid is also a platform chemical used as a precursor of many commodity or specialty chemicals, including adipic acid, 1,4-butanediol, tetrahydrofuran, *N*-methyl pyrrolidinone, 2-pyrrolidinone, succinic acid esters, succinate salts, and γ-butyrolactone (Fig. 1) The derivatives of succinic acid are estimated have a market potential of 270,000 tons per year (Zeikus et al. 1999; Willke and Vorlop 2004).

At present, succinic acid can be made commercially by hydrogenation of fossil-derived maleic acid (anhydride). However, nonrenewability and the rising price of the fossil resources have limited the use of succinic acid for a wide range of applications. On the other hand, with development of genetic engineering and metabolic engineering of microbial strains, alternative cheap carbon resources, improvement of purification technology, and fermentative production of succinic acid from renewable resources can be more cost-effective than the fossil-based processes. An additional advantage of fermentation to produce succinic acid is fixation of the greenhouse gas CO_2 into the product.

Succinic acid and its derivative diamines and diols can be used as monomer units of a variety of plastics, such as polyesters, polyamides, and polyester amides (Bechthold et al. 2008). Among them, poly(butylene succinate) (PBS) and its copolymers are a family of biodegradable polyesters synthesized from succinic acid, butanediol, or other dicarboxylates and alkyldiols. Owing to their excellent thermal processability, balanced mechanical properties, and good biodegradability, they have attracted intense attention from both academia and industry. PBS products can find wide applications as supermarket bags, packaging film, mulch film, and other disposable articles. Owing to the steady growth of the market for biodegradable plastic, the demand is expected to increase rapidly. Consequently, the market for succinic acid will improve steadily and the ratio of bio-based succinic acid will improve steadily.

In this chapter, we will give a brief review of the production of succinic acid, PBS, and its copolymers. Because several reviews have been devoted to the fermentation

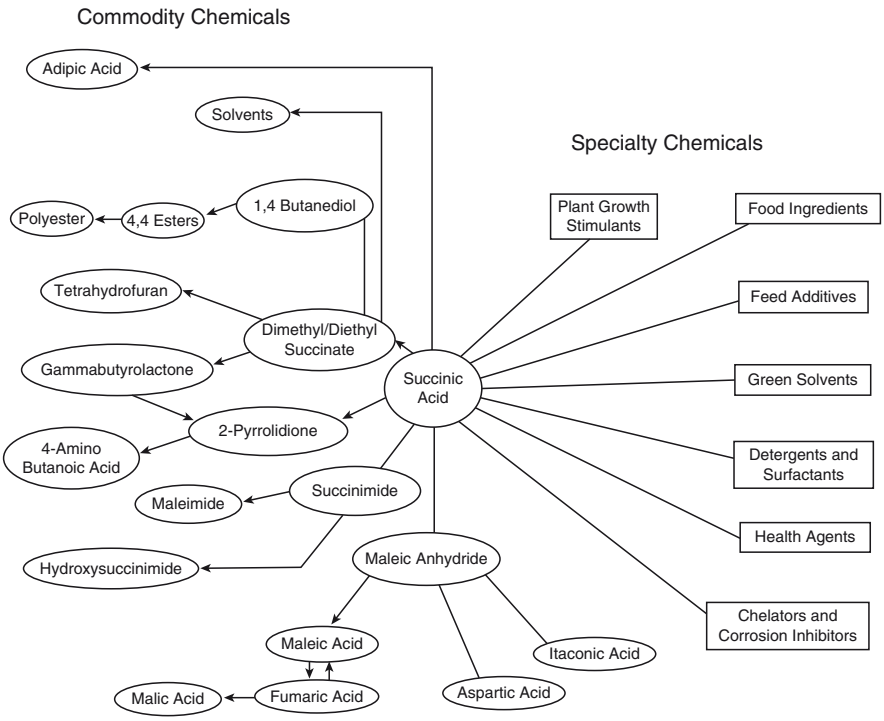


Fig.1 Various chemicals and products derived from succinic acid (Zeikus et al. 1999)

of succinic acid (Zeikus et al. 1999; Song and Lee 2006; McKinlay et al. 2007; Bechthold et al. 2008; Cukalovic and Stevens 2008), this chapter will give only a glimpse of the production of succinic and will focus on the synthesis, structures, and properties of PBS and its copolyesters.

2 Production of Succinic Acid

The methods of production of succinic acid can be divided into two categories: petrochemical and fermentation routes.

There are several industrial processes for succinic acid production: (1) oxidation of paraffins forms a mixture of different carboxylic acids, followed by separation of succinic acid; (2) catalytic hydrogenation of maleic acid or *trans*-maleic acid; (3) electrochemical synthesis from maleic anhydride in a bipolar membrane or nonmembrane cell; (4) production from acetylene, carbon monoxide, and water catalyzed by $[C_o(CO)_4]$ in an acid medium under a pressure of 2.94–49.03 MPa at 80–250°C. Among them, electrochemical synthesis is a generally applied process for succinic acid, which has the advantage of high yield, low cost, high purity of

the final product, and very low or no waste formation. Succinic acid produced by the electrochemical process can be used in food and pharmaceuticals.

Fermentation production of succinic acid from renewable resources has attracted intense interest in the past decades to lower the dependence on fossil resources and further reduce the price of the product. Succinic acid is one of the intermediates in the metabolic pathway of anaerobic and facultative microorganisms. Various microorganisms have been reported to produce succinic acid, such as typical gastrointestinal bacteria and rumen bacteria and some lactobacillus strains (Kaneuchi et al. 1988). Among them, *Actinobacillus succinogenes, Anaerobiospirillum succiniciproducens*, and *Mannheimia succiniciproducens* are the most promising strains to produce succinic acid at high yields. *A. succinogenes* variants can produce succinate up to 110 $g\,l^{-1}$ at a yield of 83–87 wt% if the pH is maintained with magnesium (Guettler et al. 1996a, b, 1999). A succinic acid productivity of 3.9 $g\,l^{-1}\,h^{-1}$ has been reported for *M. succiniciproducens* (Lee et al. 2002), which is the highest value that has been reported so far. *Enterococcus faecalis* RKY1 was also reported to produce succinic acid with a high productivity of up to 17.1 $g\,l^{-1}\,h^{-1}$ starting from 80 $g\,l^{-1}$ fumarate and glycerol in a continuous fermentation process (Ryu et al. 1999; Ryu and Wee 2001; Wee et al. 2002). The recombinant *Escherichia coli* AFP184 strain showed a production rate and yield substantially higher those of natural producers. Productivity up to 3 $g\,l^{-1}\,h^{-1}$ was obtained by dual-phase fermentations of sugar in high concentrations (Berglund et al. 2007). *E. coli* AFP111 produced succinic acid up to 99.2 $g\,l^{-1}$ with a productivity of 1.3 $g\,l^{-1}\,h^{-1}$ (Vemuri et al. 2002). Besides, corn starch, corn steep liquor (Agarwal et al. 2006), whey (Samuelov et al. 1999; Lee et al. 2003a; Wan et al. 2008), cane molasses (Agarwal et al. 2006; Liu et al. 2008), glycerol (Lee et al. 2001; Yazdani and Gonzalez 2007), and lignocelluloses (including wood hydrolysate – Kim et al. 2004, Lee et al. 2003b – and straw hydrolysate) have been tested to produce succinic acid by fermentation. In addition, a novel biorefining concept using cereals (e.g., wheat, corn) as a feedstock for microbial succinate production has been introduced (Du et al. 2007).

The downstream purification of succinic acid is also key for the fermentation process, which has a cost of about 60–70% of that of the total bioproduction process (Baniel and Eyal 1995). The separation and purification of succinic acid from the fermentation broth has been reviewed in the literature (Zeikus et al. 1999).

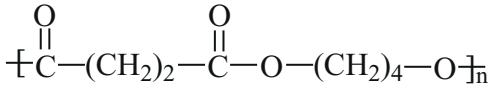
Compared with the chemical process, the fermentation process has the advantages of mild conditions, independence of the fossil feedstock, and fixation of CO_2. Theoretically, succinate fermentations consume 1 mol of CO_2 per mole of succinate produced: 1 mol of glucose and 2 mol of CO_2 are transformed into 2 mol of succinate. In practice, side products of the fermentation (acetate, formate, etc.) lead to a distortion of the balance. However, the fermentation process has the following disadvantages: large occupation space of the factory, longer fermentation time, a large demand for water and disposal of the wastewater, complicated separation and purification of the final product (Cukalovic and Stevens 2008). To make the fermentation process competitive, researchers are attempting to find more productive microbial strains that can resist high concentration of succinic acid and utilize cheap feedstocks and to develop novel separation and purification technology with low cost.

DSM and Roquette have worked together to build a demonstration plant in Lestrem, France, to produce several hundred tons of succinic acid per year from starch using an innovative enzyme-based fermentation technology (Kidwell 2008). The technology is expected to be applied on a commercial scale in 2011–2012. It is expected that the new biotechnology-based route could result in up to 40% reduction in energy requirements compared with the traditional chemical method, and could have a positive impact on reducing CO_2 emissions, as CO_2 is actually used in the fermentation process. In Japan, Mitsubishi has also attempted to industrialize fermentation production of succinic acid, which will be used as monomer units of the company's biodegradable plastics GS Pla.

3 Synthesis of PBS and Its Copolymers

3.1 Historical Outline and Recent Industrial Developments of PBS

PBS is a linear aliphatic polyester with excellent mechanical properties and biodegradability. Its molecular formula is as follows:



The pioneering work on the synthesis of PBS and other aliphatic polyesters was started by Carothers (1931). But because of poor condensation conditions at that time, the PBS obtained was of low molecular weight less, than 5,000, and was weak and brittle. In the past few decades, as an active response to environmental concerns about the disposal of nondegradable municipal and industrial plastic waste, aliphatic polyesters have attracted intense attention as degradable plastics. PBS with a molecular weight high enough for practical applications has been produced since the 1990s. In 1993, Showa High Polymer constructed a semicommercial plant with a capacity of 3,000 tones per year capable of manufacturing high molecular weight PBS under the trademark Bionolle, which was synthesized via melt condensation polymerization followed by a chain-extension method using diisocyanate as the chain-coupling agent to increase the molecular weight (Fujimaki 1998). The final product has a number-average molecular weight (M_n) ranging from 20,000 to 200,000 and a weight-average molecular weight (M_w) ranging from 40,000 to 1,000,000 (Takiyama et al. 1994a, b, 1995). Bionolle is suitable for making films, sheets, filaments, nonwoven fabrics, laminates, molded foam products, and injection-molded products and can be widely applied in agriculture, forestry, civil engineering, and other fields in which recovery and recycling of materials after use is problematic. But chain-extended PBS is thought to be not suitable for applications in the fields of medicine, personal care, cosmetics ,food-contact products, etc., which are

fields in which it could be most widely and significantly applied and where biosafety is a fundamental requirement.

Thereafter, researchers and companies attempted to manufacture high molecular weight PBS via direct melt polycondensation. PBS synthesized by this method has the same physical and mechanical properties as the product produced by the chain-extension method, but the former has better biosafety and biodegradability than the latter. Mitsubishi Chemicals built a 3,000 ton per year PBS production line and began the practical market introduction of PBS named GS Pla in April 2003. In 2006, Hexing Chemical, Anhui, China also established a 3,000 ton per year PBS manufacturing line for direct melt polycondensation, which was developed by Tsinghua University in Beijing, China. A facility capable of manufacturing PBS in a quantity of 10,000 tons per year is presently under construction by Hexing Chemical, and will run at the end of 2009. In October 2007, Xinfu Pharmactical, Hangzhou, China, built a PBS production line with one-step polymerization technology, which was supported by the Technical Institute of Physics and Chemistry, Chinese Academy of Sciences. The major manufacturers of PBS are listed in Table 1.

Table 1 Manufactures of poly(butylene succinate) (*PBS*) and its copolymers

Manufacturer	Product	Monomers	Production capacity (ton/year)
Hexing Chemical, China	PBS and its copolymers	Succinic acid, butanediol, branched alkanedicarboxylic acid	10,000
Xinfu Pharmaceutical, China	PBS, PBSA	Succinic acid, adipic acid, butanediol	3,000
Jinfa Tech, China	PBSA	Succinic acid, adipic acid, butanediol	300
BASF, Germany	Ecoflex	Adipic acid, terephthalic acid, butanediol	14,000
Eastmann, USA	East Bio	Adipic acid, terephthalic acid, butanediol	15,000
Showa, Japan	Bionolle	Succinic acid, adipic acid, butanediol	5,000
Mitsubishi Chemical, Japan	GS Pla	Succinic acid, lactic acid, butanediol	3,000
Mitsubishi Gas Chemicals, Japan	Iupec	Succinate, carbonate, butanediol	
Nippon Shokubai, Japan	Lunare	Succinic acid, adipic acid, ethylene glycol	
Ube, Japan	ETERNACOLL 3050	Decanedicarboxylic acid, ethylene glycol	
Ire Chemical, Korea	Enpol	Succinic acid, adipic acid, terephthalic acid, butanediol	
SK Chemicals, Korea	Skygreen	Succinic acid, adipic acid, butanediol, ethylene glycol	

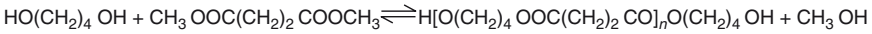
PBSA poly(butylene succinate-*co*-butylene adipate)

3.2 Synthesis of PBS

3.2.1 Transesterification Polymerization

Transesterification polymerization is a usual technology for synthesis of poly (ethylene terephthalate), the most common polyester nowadays, which is often used for spinning of synthetic fibers. So it is understandable that the pioneer synthetic method for PBS is transesterification polycondensation.

In the transesterification polymerization method, PBS is synthesized by melt polymerization starting from stoichiometric amounts of dimethyl succinate and 1,4-butanediol, or using an excess of 1,4-butanediol not above 10%, in the presence of a catalyst such as tetra-*n*-butyl-titanate or tetraisopropyl titanate, as shown in Scheme 1. The synthetic procedure can be separated into two stages: transesterification and polycondensation. Before reaction, the reactor is filled with nitrogen at room temperature to remove the air and avoid oxidation during the transesterification reaction, then the reaction system is heated to start transesterification under stirring at a temperature ranging from 150 to 190°C under the nitrogen atmosphere. After most of the methanol and water produced by transestericaton have been distilled off, the polycondensation is fulfilled under a vacuum at a higher temperature, e.g., 200°C, to remove the butanediol formed in the reaction and polymerize the oligomers to polymer. Via transesterification polymerization, PBS with M_n of 59,500 and M_w of 104,100 can be synthesized (Tserki et al. 2006a, b).



Scheme 1. Reaction formula showing polymerization of PBS from dimethyl succinate and 1,4-butanediol

3.2.2 Direct Polymerization of Succinic Acid and Butanediol to Synthesize PBS

Direct melt polymerization of succinic acid and butanediol to PBS. Direct polymerization of PBS is a polymerization process starting from dicarboxyl acid and alkyl diol without a chain-extension step. Direct melt polymerization of PBS can be conducted in two ways: one is where the polymerization is finished in the melt of succinic acid and butanediol, called "direct melt polymerization," the other is where the polymerization is finished in the solution of raw materials, namely, solution polymerization. Direct melt polymerization is simple and can produce PBS of high molecular weight, so it is considered the most promising process for PBS production owing to the economic considerations and the potential applications in food-contacting packages.

In direct melt polymerization, PBS is prepared using a two-step process. In the first step, esterification takes place at a temperature ranging from 150 to 200°C under atmospheric pressure or in a low vacuum. In the second step, polycondensation is followed under a high vacuum at a higher temperature, e.g., 220–240°C, for

deglycolization. To avoid oxidation, both esterification and polycondensation should be conducted in a nitrogen atmosphere.

$$\mathrm{HO(CH_2)_4OH + HOOC(CH_2)_2COOH \rightleftharpoons H[O(CH_2)_4OOC(CH_2)_2CO]_mOH + H_2O}$$

$$\mathrm{H[O(CH_2)_4OOC(CH_2)_2CO]_nO(CH_2)_4OH + H[O(CH_2)_4OOC(CH_2)_2CO]_mOH \rightleftharpoons}$$

$$\mathrm{\dagger O(CH_2)_4OOC(CH_2)_2CO\ddagger_n\dagger O(CH_2)_4OOC(CH_2)_2CO]_m OH + HO(CH_2)_4OH}$$

The keys to obtaining high molecular weight PBS are as follows:

1. During the esterification stage, the water formed must be removed from the system. The amount of water formed should reach up to 85–90%. Otherwise, the following polycondensation will continue for a longer period of time and one may not get PBS with sufficiently high molecular weight to remove water and by-products (such as tetrahydrofuran) in the esterification stage.
2. Esterification must be carried out at the proper temperature. The reaction rate of esterification improves with temperature, but more by-products will be formed. The major by-product is tetrahydrofuran, which forms owing to dehydration of butanediol. Tsinghua University and Hexing Chemical have developed a unique esterification technique to depress the formation of the by-product tetrahydrofuran. After adoption of the technique, the residual tetrahydrofuran content in the final PBS is less than 0.1%.
3. A sufficiently high vacuum is required to remove the butanediol and the small amount of water formed in the polycondensation stage. The Technical Institute of Physics and Chemistry, Chinese Academy of Sciences, has applied the cold trap technique in the flow line of a PBS preparation. It is reported that the cold trap can reach −120°C and so cause the small molecular material formed in the condensation stage to condense and can keep a stable high vacuum and improve the removal of small molecular compounds. Consequently, PBS with M_w higher than 2×10^5 can be prepared via this technology.
4. Catalysts with high reactivity and that are resistant to hydrolysis must be chosen to improve the molecular weight of PBS and to shorten the reaction time. At the same time, a thermal stabilizer an and end-capping reagent are incorporated to reduce degradation during polycondensation and the following thermal processing.

Many research groups have published results on catalyst activities and catalysis mechanisms. A wide variety of catalysts have been used in polycondensation of PBS, such as $Sc(CF_3SO)_3$, $Sc(NTf_2)_3$, titanium tetrabutoxide, titanium(IV) isopropoxide phosphate acid, and titanium and tin composite. It is generally accepted that compounds of antimony, tin, and titanium are the most active catalysts for polycondensation. Among these catalysts, titanium tetrabutoxide (or tetrabutyl titanate) and titanium(IV) isopropoxide are usually used (Takiyama et al. 1994a, b; Mochizuki et al. 1997); Yang et al. 2003.

Mochizuki et al. (1997) synthesized PBS with M_n up to 59,000 using tetra-*n*-butoxy titanate as a catalyst and polyphosphoric acid as a thermal stabilizer. Tetra-*n*-butoxy germanium is an effective catalyst for synthesis of high molecular weight

poly(ethylene succinate) (PES). Using titanium(IV) isopropoxide as a catalyst, Ahn et al. (2001) produced PBS with M_n of 77,000 and M_w of 141,000.

Solution polymerization of succinic acid and butanediol to PBS. Solution polymerization is a modified method of direct melt polymerization, which dissolves the raw materials in a solvent such as xylene or decahydronaphthalene. The esterification reaction and the condensation reaction in the solvent improve the removal of the small molecular material formed in the reaction process, so the reaction can be conducted at lower temperature, which can avoid oxidation of PBS, but at the cost of a longer reaction time.

Utilizing a new water trap that contains a 4-Å molecular sieve, Zhu et al. (2003) synthesized PBS with M_n of 24,800 after polycondensation for 70 h with $SnCl_2$ as a catalyst and dimethyl benzene as the solvent and water-removing agent. (Ishii et al. (2001) reported one-step synthesis of PBS by distannoxane-catalyzed polymerization of succinic acid and butanediol in a two-phase system of solvent (decalin) and molten polymer under azeotropic conditions at atmospheric pressure. When the reaction time was extended to 72 h in the presence of 0.001 mol% of 1-chloro-3-hydroxy-1,1,3,3-tetrabutyldistannoxane, PBS with M_n of 117,000 and M_w of 277,000 was obtained. Sun et al. examined the effect of different catalysts, such as $SnCl_2$, $Ti(OiPr)_4$, $Ti(OBu)_4$, $Sn(Oct)_2$, $Zn(Ac)_2$, and p- toluenesulfonate (*p*-TS), on the molecular weight of solution-polymerized PBS. Among these catalysts, $SnCl_2$ showed the most promising result: PBS with the highest M_w of 79,000 was observed after reaction for 12 h.

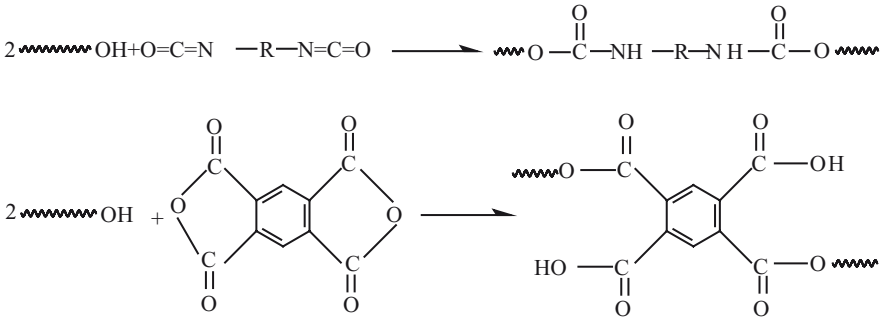
3.2.3 Condensation Polymerization Followed by Chain Extension

Chian extension is another method to produce high molecular weight PBS. Compared with the direct melt condensation polymerization, an additional chain-extension step was carried out to further improve the molecular weight of PBS. A chain extender with two functional groups can react with the terminal –OH or –COOH of PBS. Ideally, a chain extender molecule will couple two PBS chains.

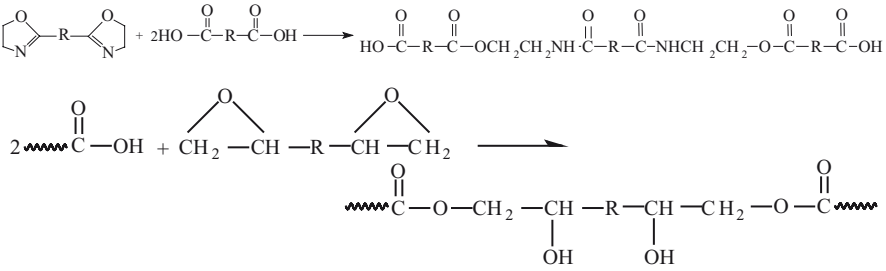
In this process, the polycondensation conditions are not so critical as those for the direct melt polycondensation. On the other hand, the disadvantage is that incorporation of the chain extender will reduce the biosafety and will affect the biodegradability of the PBS obtained. Consequently, chain-extended PBS cannot be applied as a food-contacting material.

A variety of chain extenders have been investigated for improvement of PBS molecular weight, such as isocyanate (Fujimaki 1998; Tserki et al. 2006a), oxazoline (Xu et al. 2007), anhydride, biscaprolactamate (Zhao et al. 2007), and epoxy compound. Diisocyanate and anhydride are suitable for chain extension of hydroxyl-terminated PBS, whereas oxazoline and expoxy are applicable for carboxyl-terminated PBS.

Showa Denko (Japan) produced high molecular weight PBS (Bionolle) through a polycondensation reaction of butanediol with the aliphatic dicarboxylic acid succinic acid followed by chain extension utilizing hexamethylene diisocyanate as the coupling agent. Chain extension of HO–PBS–OH with diisocyanate can produce PBS with a relatively high molecular weight. It was reported that the M_w of PBS can reach nearly 300,000 and the M_n can reach nearly 200,000 at the optimal conditions.



Bisoxazoline is a new type of chain extender, and can couple low molecular weight PBS during polycondensation, reaction extrusion, fiber spinning, etc. Chain extension of PBS with bisoxazoline will not result in the formation of small molecules and gelling, which is promising for industrial manufacturing. Xu et al. (2007) utilized bisoxazoline as a chain extender, which improved the M_n from 111,700 to 189,500.



3.2.4 Lipase-Catalyzed Synthesis of PBS

Lipase-catalyzed synthesis of PBS is a recently developed method, which is fulfilled at milder conditions without remnant metal salt. In 2006, Azim et al. (2006) reported *Candida antarctica* lipase B catalyzed synthesis of PBS from the monophasic reaction mixtures of diethyl succinate and 1,4-butanediol. The reaction temperature played an important role in determining the molecular weight of PBS. After polymerization for 24 h in diphenyl ether, PBS with M_n of 2,000, 4,000, 8,000, and 7,000 was produced at 60, 70, 80, and 90°C, respectively. The low molecular weight was due to precipitation after polymerization for 5–10 h, limiting the growth of the polyester chain length. Increasing the polymerization temperature from 80 to 95°C can result in the maintenance of a monophasic reaction mixture after 21 h, which led to production of PBS with M_w of 38,000 and polydispersity index of 1.39.

PBS synthesized by lipase catalyzation has a narrower polydispersity index than that synthesized by the methods described above and has no residual metal. However, at present the former has a lower molecular weight than the latter. In addition, the remaining lipase may cause a problem in the following thermal processing. The future trend will focus on further improvement of the molecular weight and utilize immobilized lipase as a catalyst.

Ring-opening polymerization of cyclic monomers, succinic anhydride and tetrahydrofuran, can also be applied for PHB synthesis. Catalyzed by aluminum triflate at 100°C for 48 h, PBS with M_n of 12,400 was obtained at a yield of 49% (Wang and Kunioka 2005).

3.3 Synthesis of PBS Copolymers and Branched PBS

To tailor-design the physical properties and biodegradation rate, PBS copolymers and branched PBS have been synthesized in the past few decades. In this review, we fill focus on the random PBS copolymers, which possess verified properties with change of the copolymer composition.

PBS random copolymers are obtained from succinic acid or succinate, 1,4-butanediol, and other dicarboxylic acids or alkylenediols, such as adipic acid, terephthalic acid, succinic acid with substituted side groups, sebacic acid, 1,3-propanediol, and other substituted glycols. Usually, copolymerization leads to a decreased degree of crystallinity, depressed heat distortion temperature, and improved elongation.

Despite their practical significance, however, there are difficulties in film blowing the aliphatic polyesters owing to their low melt strength and melt viscosity. To solve the problem associated with melt processing, lots of attempts have been made to increase the molecular weight of the polymer by incorporating a suitable chain-extending agent in the polymerization stage. As recognized, the rheological properties of a molten polymer are influenced not only by its molecular weight and molecular weight distribution, but also by the branch content of the polymer. In particular, rheological properties related to elongation are greatly affected by long-chain branches. Branching has been proved effective in enhancing processability in the processes involved with elongational flow, such as fiber spinning, film blowing, vacuum forming, and foaming. Branching increases the melt strength and confers a tension-hardening property on the polymer, which is of help for uniform extension in the polymer processing in which a high degree of orientation is required.

To obtain long branched PBS, a branching agent, trimethylol propane (Kim et al. 2001), glycerol, or other monomers with multifunctional groups are introduced. To avoid gel formation, the content of the branching agent must be very low, e.g., less than 0.5–1%.

4 Crystalline Structure and Properties of PBS and Its Copolymers

4.1 Crystalline Structure of PBS and Its Copolymers

PBS is a semicrystalline polyester. Its mechanical properties such as tensile strength, impact strength, and flexibility and its transparency and biodegradability depend on the crystal structures and the degree of crystallinity to some extent.

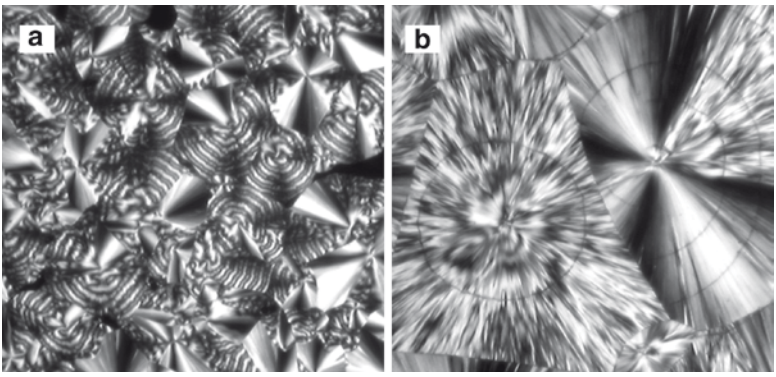


Fig. 2 Micrographs of poly(butylene succinate) (PBS) crystallized at **a** 70°C and **b** 90°C

The crystallization behavior of PBS is similar to that of polyethylene: the thickness of the lamellae depends upon the crystallization temperature, and thickening of the lamellae occurs by annealing.

When crystallized from quiescent melt, PBS forms spherulites as observed under the polarized optical microscope, as presented in Fig. 2. The size and the morphology of the spherulites are affected predominantly by the crystallization temperature. A banded spherulite consisting of circular bright and gray birefringent bands forms over a wide range of temperatures; with further increase of temperature, the regular bands disappear and a coarse-grained morphology prevails. Besides the normal spherulite morphology, some spherulites demonstrate a peculiar winglike region. The origin of the winglike region is still not clear and deserves further study.

When observed under the atomic force microscope, PBS film reveals surface modulations in the height image (Fig. 3), with the modulation period being the same as the band spacing observed under the polarized optical microscope. The atomic force microscope phase images in Fig. 4 reveal that the spherulite consists of radiating lamellar crystals, which splay and branch consecutively during growth, leading to filling of the whole space. This suggests that the cooperative twisting of the lamellar crystals leads to the formation of the banded spherulite.

The thickness of PBS lamellar crystals depend on the crystallization temperature (Gan et al. 2001). The thickness of the crystalline lamellar core increases steadily and that of the amorphous layer decreases with increase of the crystallization temperature. But the total degree of crystallinity varies little with temperature.

The unit cell parameters can be determined from the wide angle X-ray diffraction patterns of the single crystals, films, or fibers. PBS has two crystalline modifications: α and β form, depending on the crystallization conditions. The α form appears when PBS is crystallized from a quiescent melt, whereas the β form occurs under mechanical stress. Both of the modifications belong to the monoclinic system with the space group of $P2_1/n$. In both cases, the unit cell contains two molecular chains;

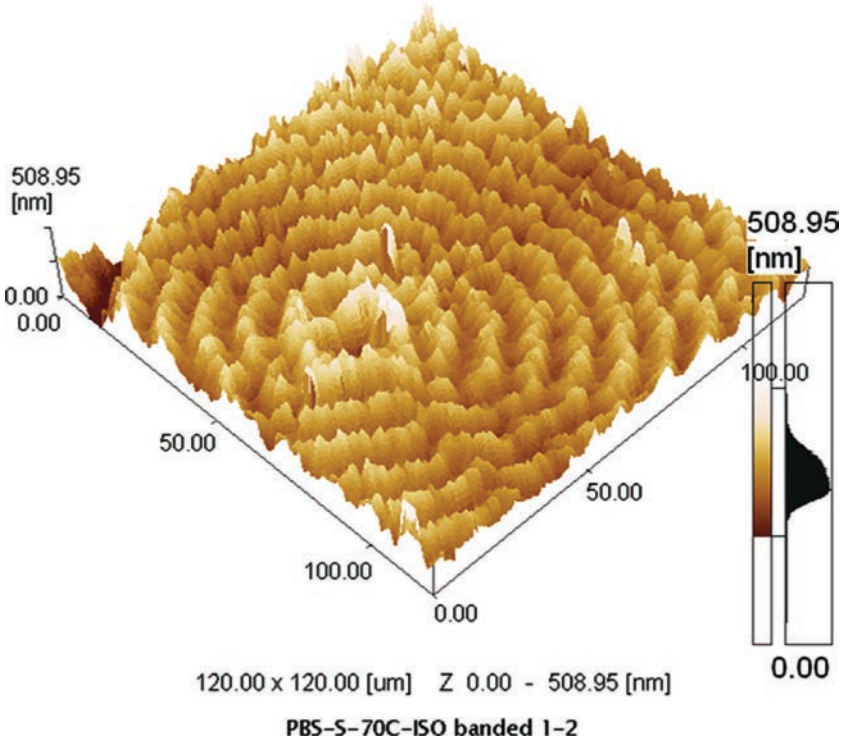


Fig. 3 Atomic force microscope (AFM) topograph of a PBS banded spherulite

the cell dimensions are $a = 0.523$ nm, $b = 0.912$ nm, c(fiber axis) = 1.090 nm, and $\beta = 123.9°$ for the α form (Ihn et al. 1995) and $a = 0.584$ nm, $b = 0.832$ nm, c(fiber axis) = 1.186 nm, and $\beta = 131.6°$ for the β form (Table 2). Figure 5 shows the powder diffractogram of α-form PBS, where the 2θ peak at 19.6°, 21.5°, 22.5°, and 28.8° corresponds to (020), (021), (110), and (111) deflection, respectively. The molecular conformations of the α and β forms are $T_7GT\ \bar{G}$ and T_{10}, respectively, where T, G, and $\bar{G}$ indicate *trans*, *gauche*, and *gauche* minus, respectively. The β form has a more extended chain conformation than the α form, as shown in Fig. 6. The solid-state transition between the α and β forms is reversible under the application and release of strain (Ichikawa et al. 1994).

The crystalline structure of PBS copolymers varies with the copolymer composition. For instance, poly(butylene succinate-*co*-butylene terephthalate) (PBST) demonstrates a PBS-type crystalline structure when the content of butylene terephthalate units is less than 30 mol% and shows a poly(butylene terephthalate)-type crystalline structure when the content of butylene terephthalate units is higher than 40 mol%, as demonstrated in Fig. 7. The other PBS copolymers reveal the same trend of crystalline structure dependence on the copolymer composition.

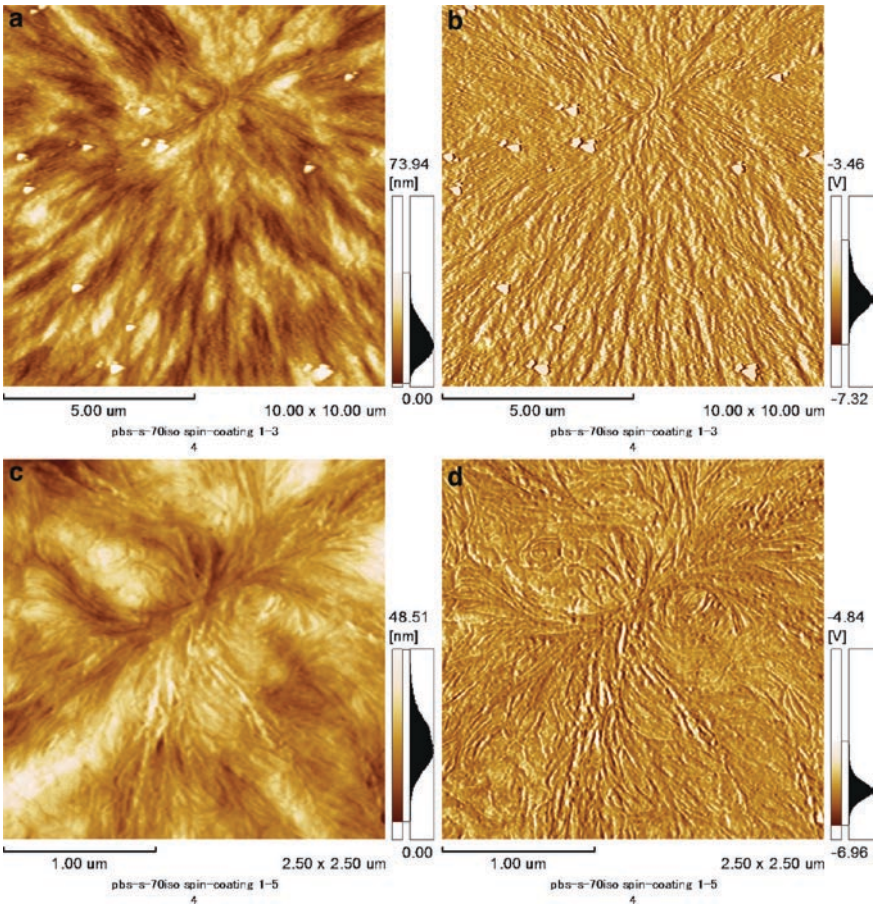


Fig. 4 AFM images of the lamellae in a PBS banded spherulite. The *left colum* and the *right column* shows the height and the phase images, respectively

Table 2 Unit cell parameters of the α and β forms of PBS (Ichikawa et al. 2000)

	α Form	β Form
Crystal system	Monoclinic	Monoclinic
Space group	$P2_1/n$	$P2_1/n$
Cell dimensions		
a (nm)	0.523 (2)	0.584 (5)
b (nm)	0.912 (3)	0.832 (11)
c(fiber axis) (nm)	1.090(5)	1.186 (7)
β (°)	123.9 (2)	131.6 (5)
Volume (nm^{-3})	0.4315 (30)	0.4320 (80)
Observed density (g cm^{-3})	1.28	–
Calculated density (g cm^{-3})	1.33	1.32
Number of chains running through the unit cell	2	2

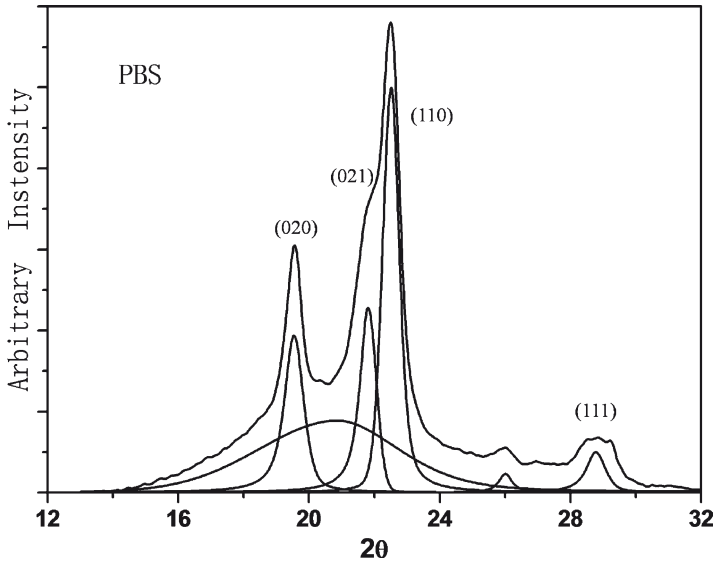


Fig. 5 Typical wide-angle X-ray diffraction (WAXD) pattern of melt-crystallized PBS film

4.2 Thermal Properties of PBS and Its Copolymers

The glass-transition temperature, melting point, heat distortion temperature, thermal degradation temperature, etc. are important parameters affecting the application and processing of semicrystalline polymer materials. These thermal parameters can be obtained via differential scanning calorimetry, dynamic mechanical analysis, thermogravimetric analysis, etc.

The semicrystalline PBS homopolymer has a melting peak around 112–116°C, which depends on the molecular weight and the thermal history of the sample preparation.

The melting temperature of PBS varies with the thermal history, e.g., the crystallization temperature (Papageorgiou and Bikiaris 2005; Yasuniwa et al. 2005). Figure 8 shows the differential scanning calorimetry melting curves of PBS isothermally crystallized at different temperatures. Cold crystallization appears in the PBS samples crystallized at low temperatures, for instance, 60–90°C. Some samples show multiple melting endotherms, among which the lower-temperature endotherm corresponds to the original lamellae formed during the previous isothermal crystallization, whereas the endotherm at the higher temperature arises from melting–recrystallization during the heating scan. The reorganization of the preformed metastable lamellae leads to lamellar thickening. PBS with low molecular weight reorganizes more easily and will show multiple melting peaks over a wider temperature range than that with higher molecular weight.

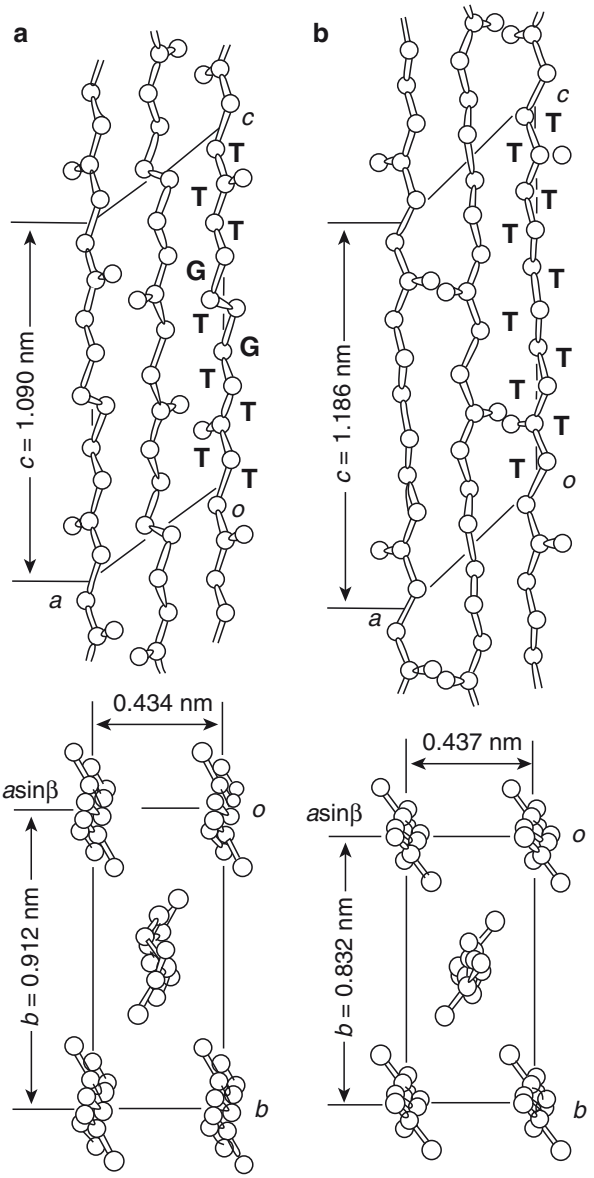


Fig. 6 Crystal structures of PBS of the **a** α form and **b** β form (Ichikawa et al. 2000)

The thermogravimetric analysis curve of PBS reveals that this polyester shows weight loss of 5, 50, and 90% at 325, 400, and 424°C, respectively, in air. It should be noted that this does not indicate that PBS polymer chains are thermally stable up to 325°C. On the contrary, PBS shows thermal degradation when extruded at 200°C, which is implied by the decrease of the shear viscosity with prolonged time.

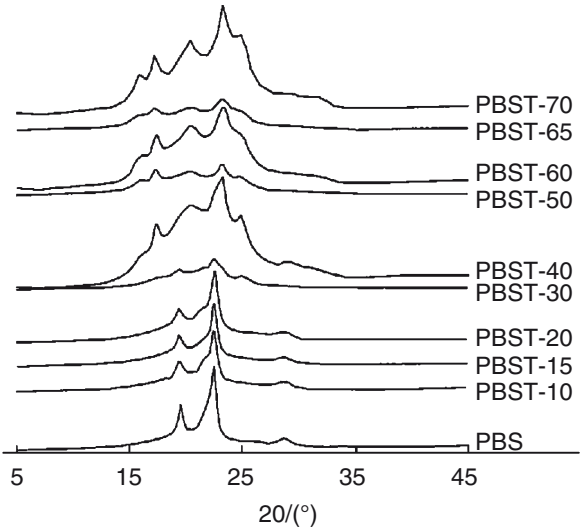


Fig. 7 WAXD patterns of PBS and poly(butylene succinate-*co*-butylene terephthalate) (PBST). The number after PBST indicates the percentage of the butylene terephthalate units

The thermal degradation temperatures of PBS are almost the same in nitrogen and air (Nikolic et al. 2003).

The thermal properties of PBS copolymer depend on the copolymer composition. The melting temperature and degree of crystallinity decrease with the increase of the comonomer content when it is less than 30 mol%, as presented in Figs. 9 and 10 and Tables 3 and 4. When the comonomer content is less than 15 mol%, the copolymers still have a melting temperature higher than 100°C, which is preferred for real applications.

The dependence of the glass-transition temperature on the copolymer composition varies with the chemical structure of the comonomer unit. Copolymerization with methyl succinic acid, dimethyl succinic acid, adipic acid, and propanediol results in a decreased glass-transition temperature , whereas copolymerization with benzyl succinic acid and terephthalic acid brings about an increased glass-transition temperature, as shown in Fig. 11.

4.3 Processing Properties of PBS

PBS is a typical thermoplastic and can be processed via various methods, such as extrusion, injection molding, film blowing, fiber spinning, and thermoforming. It has been reported that PBS can be processed into melt-blown, multifilament, monofilament, nonwoven, flat and split yarn, injection-molded products, film, paper laminate, sheet, and tape for applications in the textile and plastic industries.

The processability of thermoplastics is sensitive to the rheological properties. PBS is a shear-thinning melt, which demonstrates decreasing viscosity with the increase of shear rate. The non-Newton index of PBS homopolymer is around 0.7.

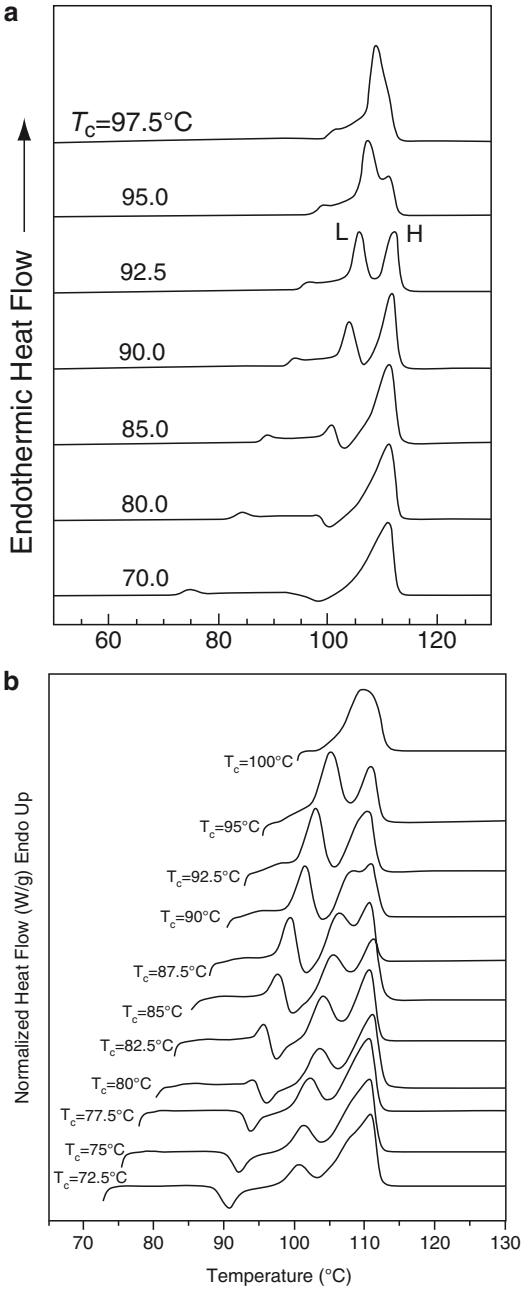


Fig. 8 Differential scanning calorimetry heating curves of PBS crystallized at different temperatures. **a** PBS with M_w about 2.5×10^5 (Yasuniwa et al. 2005), **b** PBS with M_w about 6,800 (Papageorgiou and Bikiaris 2005)

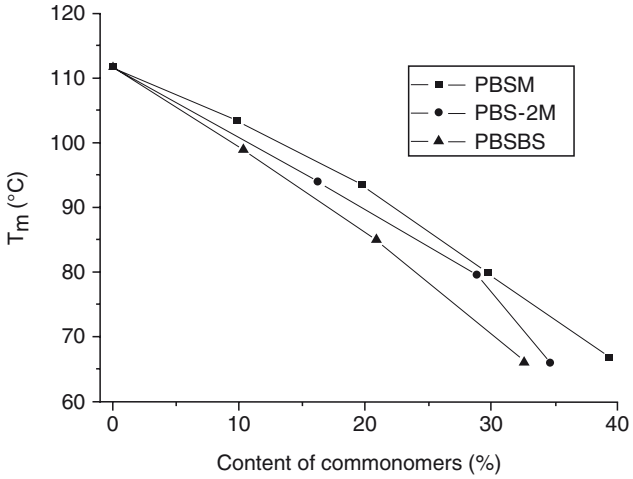


Fig. 9 Dependence of melting temperature of PBS copolymers on the chemical structure (Sun 2005)

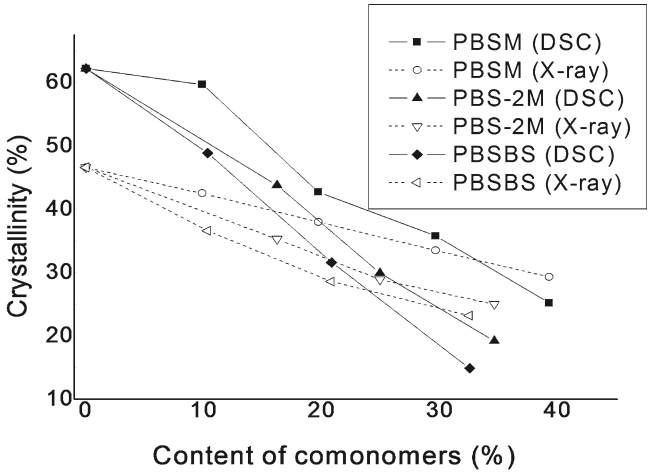


Fig. 10 Dependence of crystallinity of PBS copolymers on the chemical structure (Sun et al. 2005)

For film blowing, strain-hardening, namely, the tensile viscosity improves with the tension rate, is preferred for a stable process line. To obtain the goal, long branches were introduced into the PBS polymer chain.

4.4 Mechanical Properties of PBS

PBS has mechanical properties similar to those of polyolefins such as polyethylene. PBS possesses good tensile strength and impact strength, moderate rigidity, and

Table 3 Thermal properties of poly(butylene succinate-*co*-butylene terephthalate) (*PBST*) copolymers

Copolymer	$10^{-4}\bar{M}_n$	$10^{-4}\bar{M}_w$	Tensile strength (MPa)	Elongation (%)	T_m (K) (DSC)	T_g (K) (DMA)	ΔH_m (J g^{-1})	H_m/ (J g^{-1})	Crystallinity (%) DSC	Crystallinity (%) WAXS
PBS	3.09	5.86	35.17	82	385	255	67.4	110.3	61.1	39.66
PBST-10	3.08	5.04	36.17	294	379	256	79.1	110.3	71.7	48.77
PBST-15	2.16	3.98	36.01	>500	372	261	61.2	110.3	55.5	42.82
PBST-20	2.94	5.17	30.73	>500	363	264	57.0	110.3	51.7	41.57
PBST-30	2.94	5.70	23.11	>500	359	261	16.4	121.7	13.5	32.68
PBST-40	1.25	2.42	12.34	>500	405	257	9.7	145.5	6.7	28.01
PBST-50	1.32	2.53	15.42	>500	411	266	8.9	145.5	6.1	29.63
PBST-60	1.26	2.71	21.79	>500	427	280	17.4	145.5	12.0	30.99
PBST-65	1.72	4.04	23.33	>500	439	290	34.3	145.5	23.6	32.68
PBST-70	1.47	4.91	31.95	358	449	293	26.3	145.5	18.1	40.79
PBT					495	313[9]	50.1	145.5	34.4	

DSC differential scanning calorimetry, *DMA* dynamic mechanical analysis, *WAXS* wide-angle X-ray scattering, *PBT* poly(butylene terephthalate)

Table 4 Thermal properties of poly(butylene succinate-*co*-propylene succinate) (*PBSPS*) copolymers (Xu et al. 2008)

Polymer	T_m (°C)	ΔH_m (J g^{-1})	T_c (°C)	T_g (°C)	T_m^0 (°C)	$X_{C\text{-}DSC}$ (%)[a]	$X_{C\text{-}WAXD}$ (%)[b]	T_d (°C) (50 wt%)
PBS	113	69.8	76	−30.0	132	63.2	52.8	404
PBSPS-5	111	57.2	69	−31.3	124	51.8	49.9	385
PBSPS-10	105	56.0	60	−32.9	118	50.7	49.3	377
PBSPS-15	100	49.7	56	−33.5	114	45.0	47.7	368
PBSPS-20	96	43.4	52	−33.9	108	39.3	46.0	374
PBSPS-30	84	39.8	40	−34.8	103	36.0	43.9	371
PBSPS-40	76	37.3	39	−35.1	99	33.8	40.2	362
PBSPS-50	63	20.9	–[c]	−35.5	89	18.9	37.3	361
PPS	50	36.4	–[c]	36.0	–[c]	–[c]	44.6	378

WAXD wide-angle X-ray diffraction

[a] $X_{C\text{-}DSC}$ was calculated from the DSC method (110.5 J g^{-1}) for a 100% crystalline PBS.

[b] $X_{C\text{-}WAXD}$ was calculated from the X-ray method

[c] No crystallization peak was observed by DSC at the cooling rate of 10°C min^{-1}

hardness, and is a typical tough polymer. Table 5 shows the typical properties of PBS synthesized by various manufacturers. PBS is tougher than poly(lactic acid), but has lower rigidity and Young's modulus.

Copolymerization is a facile method to tune the mechanical properties of PBS. When the comonomer content is less than 30 mol%, the copolymers possess the PBS-type crystalline structure with low crystallinity, which leads to decreased tensile strength and enhanced elongation and impact strength (Tables 3 and 4). Figure 12 demonstrates the variation of the mechanical properties of PBST with the copolymer composition.

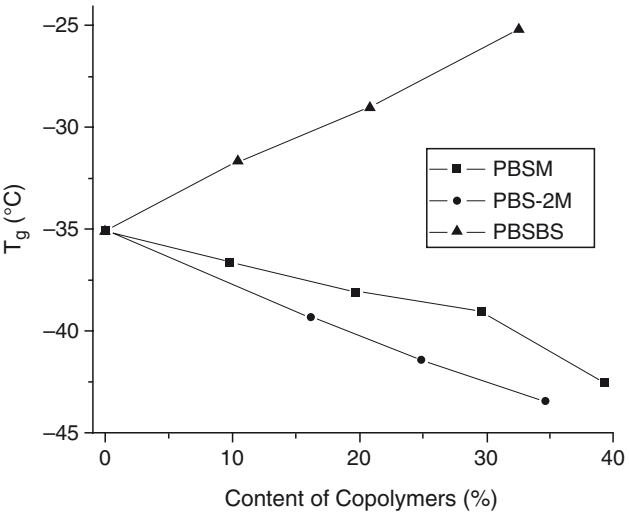


Fig. 11 Dependence of the glass-transition temperature of PBS copolymers on the chemical structure (Sun et al. 2005)

Table 5 Properties of PBS compared with poly(lactic acid) (*PLA*) and some common polyolefins (Ishioka et al. 2002)

Items	PLA (LACEA)	PBS (Bionolle) 1000	PBSA 3000	PP MA210	HDPE	LDPE F082
Glass-transition temperature (°C)	55	−32	−45	−5	−120	−120
Melting point (°C)	170–180	114	96	163	129	110
Heat-distortion temperature (°C)	55	97	69	110	82	49
Tensile strength (MPa)	66	34	19	33	28	10
Elongation at break (%)	4	560	807	415	700	300
Izod impact strength (J m^{-1})	29	300	>400	20	40	>400
Degree of crystallinity (%)		35–45	20–30	56	69	49

PP polypropylene, *HDPE* high-density polyethylene

4.5 Degradability of PBS and Its Copolymers

Compared with the conventional nondegradable plastics, environmentally degradable plastics, including PBS and its copolymers, are benign to the environment and can degrade into CO_2 and water through naturally occurring degrading enzymes and microorganisms after disposal. Consequently, these biodegradable plastics will find wider and wider applications as disposable film or articles. Degradation of PBS can be divided into hydrolytic degradable, enzymatic degradation, and biodegradation in environmental conditions, such as burial, activated sludge, and compost.

Table 6 Mechanical properties of PBS with different molecular weights (Sun et al. 2007)

M_n	Yield strength (MPa)	Maximum tensile strength (MPa)	Elongation (%)
79,000	18.8	37.6	355
57,000	18.1	35.7	221
40,600	18.6	35.0	167
32,500	18.2	34.0	25.2

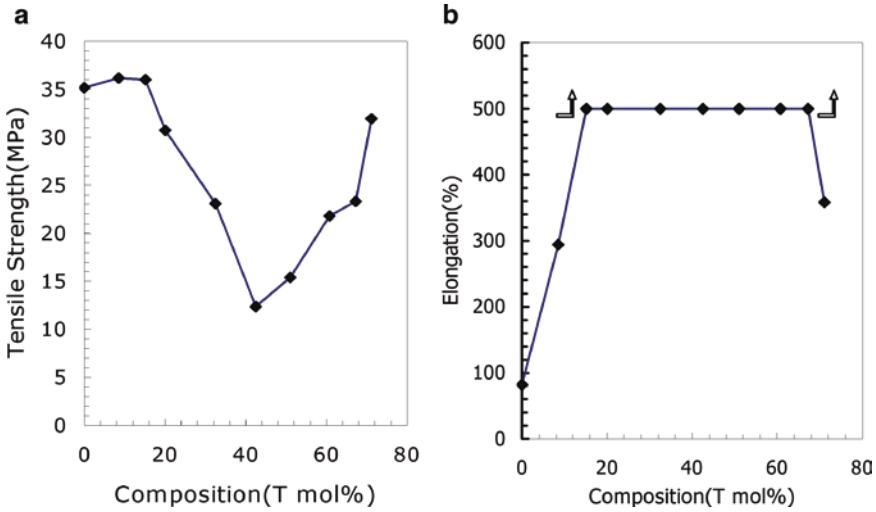


Fig. 12 Mechanical properties of PBST: **a** tensile strength, **b** elongation at break (Ding 2002)

The biodegradation rate of PBS and its copolymers is sensitive to the chemical structure (copolymer composition, molecular weight and its distribution), microscopic condensed state structures (lamellar thickness and degree of crystallinity), macroscopic shape of the articles, and the degradation conditions (microorganisms, temperature, pH, humidity, aerobic or anaerobic, etc.).

4.5.1 Nonenzymatic Hydrolytic Degradation

pH has a considerable effect on the rate of hydrolytic degradation. The higher the pH, the greater the hydrolytic degradation rate. At pH 13, PBS can lose 3–8% of its original weight after degradation for 9 days. At pH 10.6, the weight loss of PBS reaches up to 23% after hydrolytic degradation for 27 days. At pH 7.2, the weight loss is less than 10% after 9 weeks; but in the following period, the degradation accelerates greatly: PBS loses 75% of its weight after 15 weeks, as shown in Fig. 13 (Li et al. 2005). In the hydrolytic degradation, the molecular weight decreases steadily with time, which indicates that hydrolytic degradation proceeds via random chain scission, as revealed in Fig. 14.

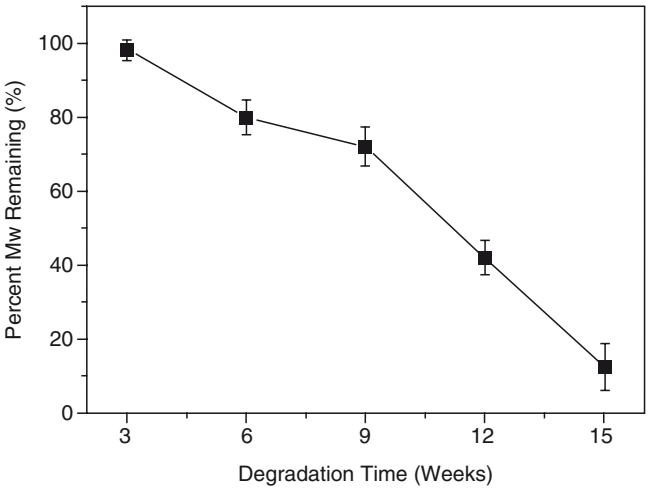


Fig. 14 Percentage of M_w remaining compared with day 0 values as a function of the degradation time in phosphate-buffered saline solution for PBS films. *Error bars* represent means ± SD for $n = 5$ (Li et al. 2005)

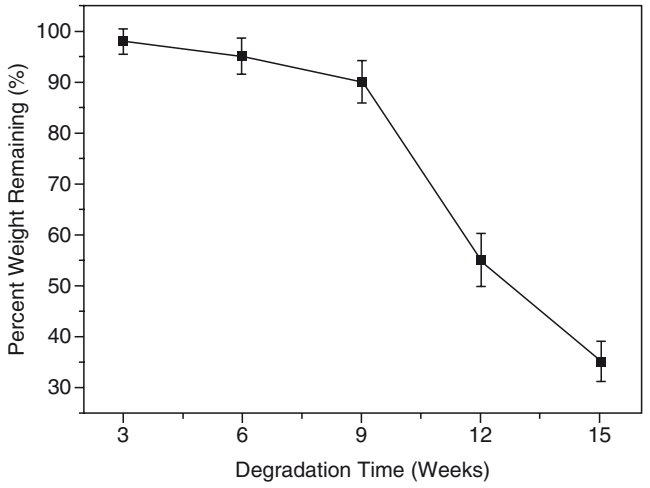


Fig. 13 Percentage of weight remaining compared with day 0 values as a function of degradation time for PBS films in phosphate buffered saline solution. *Error bars* represent means ± the standard deviation (SD) (Li et al. 2005)

Hydrolytic degradation of PBS is also affected by the thermal history, which will produce a different polymer morphology, which controls the diffusion of water into the polymer matrix: water penetrates at a higher rate in the amorphous region than in the crystalline region, which results in a higher hydrolysis rate in the amorphous region. Figure 15 shows the degradation profile of PBS samples crystallized at different temperatures. PBS isothermally crystallized at 60°C underwent the fastest hydrolytic

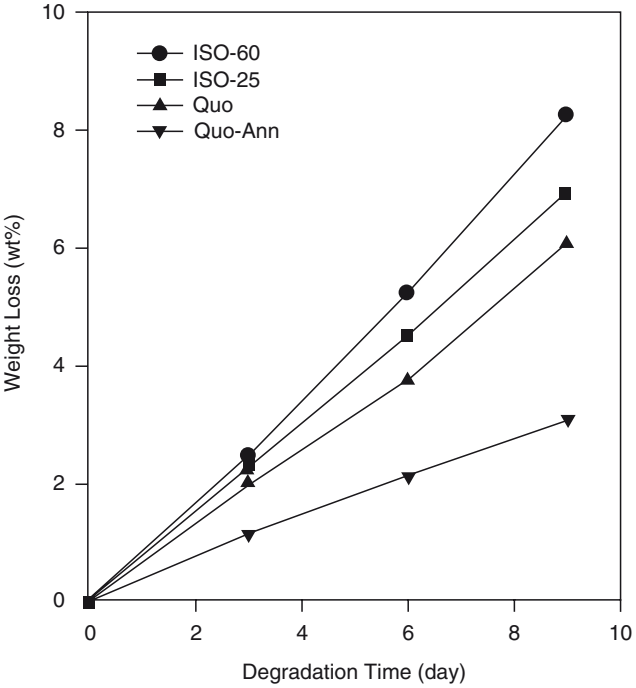


Fig. 15 The weight loss of PBS samples crystallized at different temperatures as a function of hydrolysis time in 1 N NaOH solution (Cho et al. 2001)

degradation, whereas that quenched and then annealed at 70°C demonstrated the lowest degradation rate.

The central part of the spherulites and spherulitic impinging lines is degraded first, followed by the other parts of the spherulites (Fig. 16). It was also found that a sample isothermally crystallized at 60°C, which had a spherulite composed of less densely packed fibrils, had a higher degradation rate compared with a melt-quenched sample, in spite of similar crystallinity. This result indicates that the internal structure of the spherulite also played an important role in hydrolytic degradation.

It is of particular interest that PBS has a higher hydrolytic degradation rate than poly(butylene succinate-*co*-butylene adipate) (PBSA) copolymers, as shown in Fig. 17 (Ahn et al. 2001). It is suggested that the presence of butylene adipate units may promote the hydrophobic nature of copolyesters, which would negatively influence the hydrolytic susceptibility by sterically hindering the access of nucleophiles.

Hydrolytic degradation is also a potential method for monomer recovery of PBS after its use. To overcome the very slow degradation rate of hydrolytic degradation in the solid state owing to the highly hydrolysis resistant crystalline residues (or extended chain crystallites) hydrolytic degradation in the melt has been developed without the aid of a catalyst (Tsuji et al. 2008). Hydrolytic degradation of PBS and PBSA was carried out over a temperature range of 180–300°C for periods of

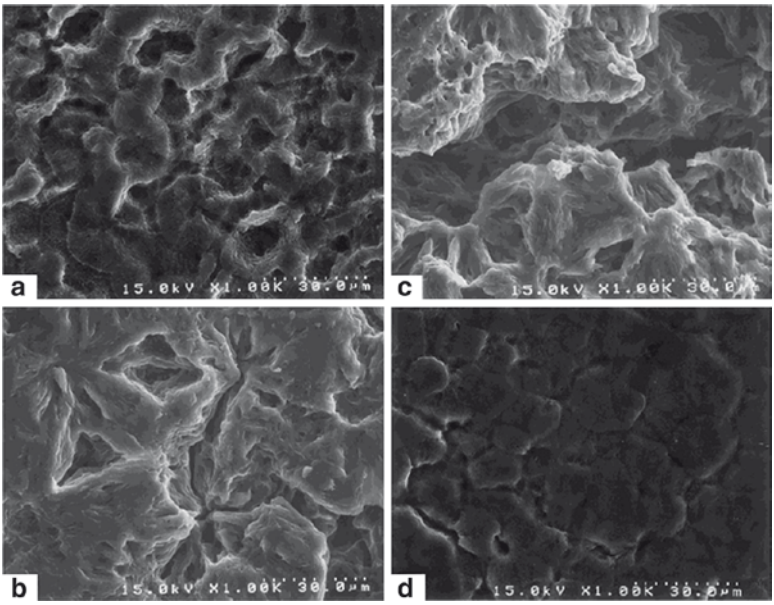


Fig. 16 Scanning electron microscopy photographs of the degraded surface of PBS samples crystallized at different temperatures after hydrolysis for 9 days: **a** melt quenched, **b** isothermally crystallized at 25°C, **c** isothermally crystallized at 60°C, and **d** melt quenched and annealed at 75°C(Cho et al. 2001)

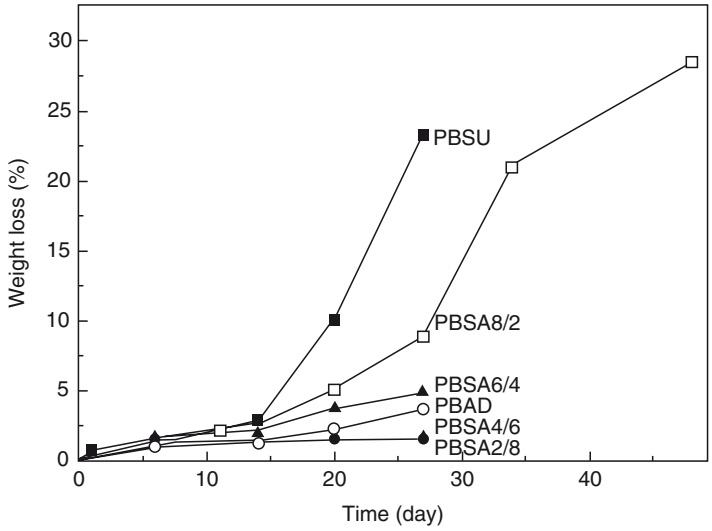


Fig. 17 Weight loss of poly(butylene succinate-*co*-butylene adipate) (PBSA) copolymers degraded at 30°C in ammonium chloride buffer solution (NaN_3 0.03%, pH 10.6, sample size: 20 mm × 20 mm × 0.4 mm; Ahn et al. 2001)

up to 30 min. Succinic acid and adipic acid were recovered at maximum yields of 65–80%, whereas butanediol was recovered at a maximum yield of only 30%, probably because of its decomposition.

4.5.2 Enzymatic Hydrolysis of PBS and Its Copolymers

The enzymatic degradation of aliphatic polyesters depends not only upon the chemical structure as regards the hydrophilic–hydrophobic balance within the main chain, but also upon the highly ordered structure as regards its specific solid-state morphology and degree of crystallinity (Pranamuda et al. 1995; Montaudo and Rizzarelli 2000; Mochizuki et al. 1997; Bikiaris et al. 2008). For instance, PBS fiber has a much lower degradation rate than PBS film: PBS fiber and film showed weight losses about 6 and 76% after being incubated with Lipase PS® at 50°C and pH 6.0 for 2 weeks (Taniguchi et al. 2002). The sharp contrast can be attributed to the fact that PBS fiber has a higher degree of crystallinity and order of crystal orientation than PBS film.

For poly(propylene alkanedicarboxylate)s, the chemical hydrolysis and enzymatic biodegradation are shown in Fig. 18. The enzymatic degradation rates of these aliphatic polyesters are about 40-fold higher than the rates of the nonenzymatic hydrolysis.

For aliphatic–aromatic PBS copolymers, the enzymatic biodegradation rate decreases with increasing aromatic comonomer content (Honda et al. 2003; Li et al. 2007), as indicated in Fig. 19.

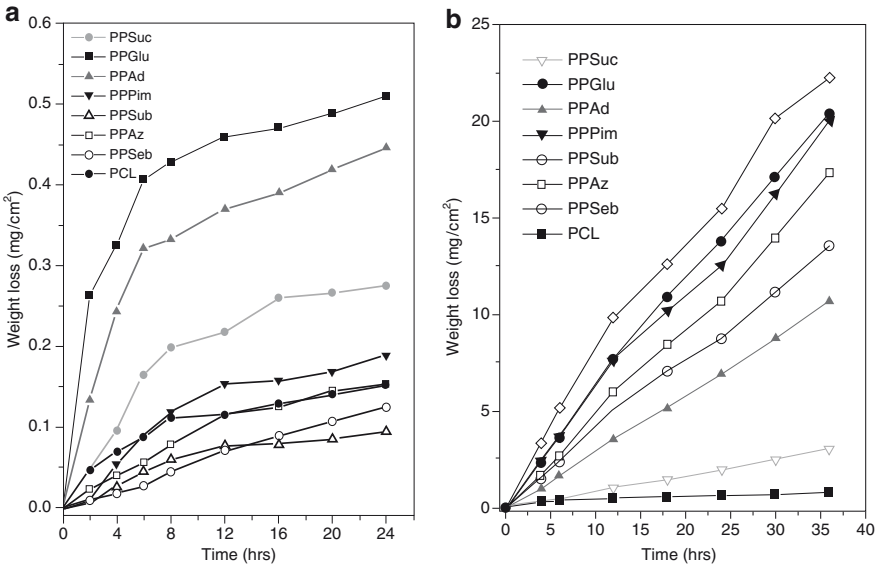


Fig. 18 Weight loss versus time for poly(propylene alkanedicarboxylate)s: **a** during chemical hydrolysis and **b** during enzymatic hydrolysis. Respective plots for poly(ε-caprolactone) are also presented for comparison (Bikiaris et al. 2008)

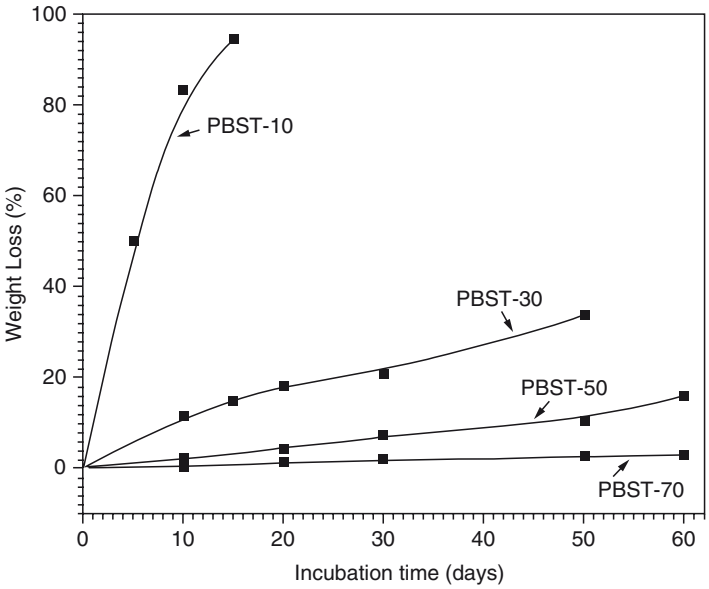


Fig. 19 The change in weight loss of PBST copolyester films degraded by the lipase from *Pseudomonas* (Lipase PS®) as a function of incubation time (Li et al. 2007)

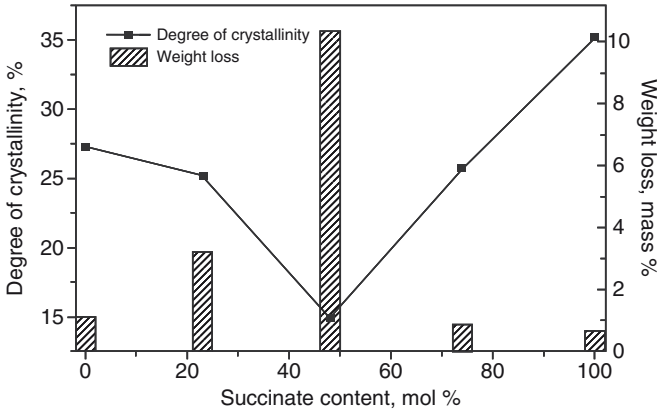


Fig. 20 Weight loss of PBSA degraded for 24 h in a buffer solution containing lipase from *Candida cylindracea*, and degree of crystallinity versus the polyester composition (Nikolic and Djonlagic 2001)

Besides the chemical structure, the degree of crystallinity is the major rate-determining factor for the rate of degradation of PBS copolymers. For aliphatic copolymers, such as poly(butylene succinate-*co*-ethylene succinate) and PBSA copolymers, those with the lowest degree of crystallinity demonstrate the highest degradation rate in lipase solution, as revealed in Fig. 20 (Mochizuki and Hirami

1997; Mochizuki et al. 1997; Nikolic and Djonlagic 2001). PBS and poly(butylene adipate) (PBA) homopolymer have almost the same enzymatic degradation rates, owing to the approximately similar degrees of crystallinity.

The enzymatic hydrolysis of PBS proceeded by a surface-etching mechanism to give 4-hydroxybutyl succinate as the main product with traces of succinic acid and 1,4-butanediol (Taniguchi et al. 2002; Lee et al. 2008). Enzymatic degradation proceeds only on the surface of the solid substrate, accompanied by both the surface erosion and the weight loss, because the enzymes cannot penetrate the polymer systems of the solid substrate. The enzymes degrade selectively amorphous or less ordered regions, which allow enzymes to diffuse into the substrate although the crystalline regions are also eventually degraded. In this process, the molecular weight and molecular weight distribution do not change during the enzymatic degradation because only the polymer on the surface of the substrate is degraded and the low molecular weight degradation products are removed from the substrate by solubilization in the surrounding aqueous medium.

Enzymatic degradation is not affected by the molecular weight of aliphatic polyesters and the degradation trends for low molecular weight (M_w 6,300) and high molecular weight (M_w 29,000) PBS were similar (Song and Sung 1995). The lack of any dependence of the enzymatic degradation on molecular weight would indicate that the lipases produced by microorganisms are endotype enzymes, which randomly split bonds in the polymer chains. Thus, the variations in the rate of enzymatic degradation are not attributable to molecular weight differences (Nikolic and Djonlagic 2001).

4.5.3 Environmental Biodegradation of PBS and Its Copolymers

PBS and its copolymers can be degraded in various environments, such as natural water, soil burial, activated sludge, and compost. The environmental biodegradation rate is sensitive to both the properties of the polyester and the environment, e.g., the microorganisms, nutrient, temperature, and humidity. For instance, Bionolle has different biodegradation rates in various environments, as presented in Table 7 (Fujimaki 1998). Table 8 shows biodegradation properties of several aliphatic polyesters in different waters (Kasuya et al. 1998). It has been demonstrated that

Table 7 Relationship between polymer structures and biodegradability of the aliphatic polyester Bionolle (Fujimaki 1998)

Biodegradability tests	PBS	PBSA	PES
In hot compost	Normal	Rapid	Normal
In moist soil	Normal	Rapid	Normal
In the sea	Slow	(Rapid)	Slow
In water with activated sludge	Slow	Slow	(Rapid)

PBS polybutylene succinate, *PBSA* polybutylene succinate adipate copolymer, *PES* poly(ethylene succinate)

Table 8 Weight-loss (*WL*) biodegradabilities and biological oxygen demand (*BOD*) biodegradabilities of aliphatic polyester films in different natural waters for 28 days at 25°C (Kasuya et al. 1998)

	Freshwater (river)		Freshwater (lake)		Seawater (bay)		Seawater (bay)	
Sample	WL biodeg.[a] (%)	BOD biodeg.[b] (%)	WL biodeg.[a] (%)	BOD biodeg.[b] (%)	WL biodeg.[a] (%)	BOD biodeg.[b] (%)	WL biodeg.[a] (%)	BOD biodeg.[b] (%)
Poly(3HB)	100±0	75±16	93±7	52±7	41±16	27±10	23±13	14±10
Poly(3HB-*co*-14% 3HV)	100±0	76±2	100±0	71±1	100±0	84±2	100±0	78±5
Poly(3HB-*co*-10% 4HB)	100±0	90±1	74±26	55±17	70±30	51±27	59±15	43±14
Poly(ε-caprolactone)	100±0	75±8	100±0	77±1	100±0	79±2	67±21	56±9
Poly(ethylene succinate)	100±0	83±2	100±0	77±2	2±1	1±1	5±2	3±2
Poly(ethylene adipate)	100±0	70±3	95±5	68±8	100±0	65±13	57±14	46±13
Poly(butylene succinate)	2±1	3±1	22±14	12±8	2±2	1±1	2±3	2±0
Poly(butylene adipate)	24±7	20±4	80±13	50±10	34±2	20±2	11±10	10±5

3HB 3-hydroxybutyrate, *3HV* 3-hydroxyvalerate, *4HB* 4-hydroxybutyrate

[a] Weight-loss biodegradability

[b] BOD biodegradability

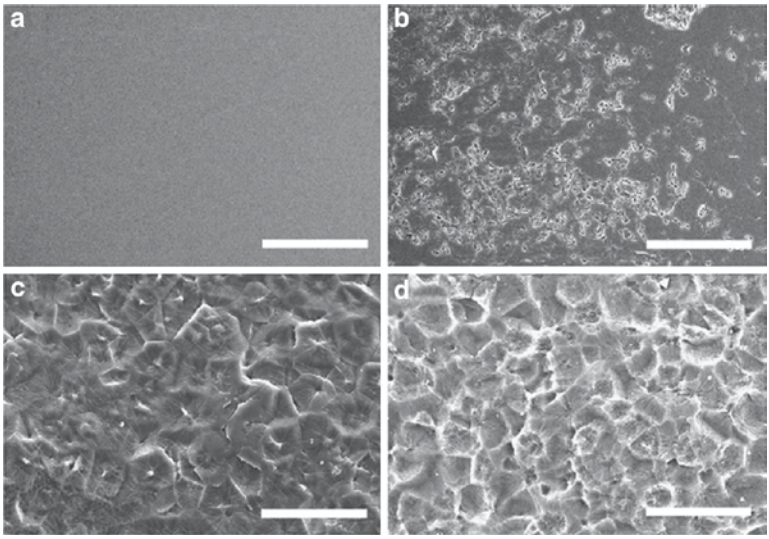


Fig. 21 Scanning electron micrographs of PBS film surfaces prior to (**a**) and following degradation by the NKCM1706 strain for 5 days (**b**), 10 days (**c**), and 20 days (**d**). The *white bars* indicate a length of 20 mm (Ishii et al. 2008)

PES degrades much faster in freshwater than in seawater. In contrast, PBS degrades slowly in both freshwater and seawater.

Biodegradation in soil. The degradability of biodegradable plastics depends on the degrading organisms existing in the environment. The percentages of degrading bacteria for PBS in the soil environment have been estimated to be 0.2–6.0% of the total colonies. Bacteria phylogenetically related to the genus *Roseateles* showed very high PBS-degrading activity (Suyama et al. 1998).

When degraded in soil burial, PBS film will lose weight at a rate of around 0.2–1 mg cm^{-2} (Zhang et al. 2001; Ishii et al. 2008) or 0.5% after biodegradation for 30 days. Figure 21 shows the surface morphology of PBS film before and after biodegradation in soil containing the fungal NKCM1706 strain. In addition, the M_w of the residual film remained constant during the degradation, indicating that the degradation began from the surface of the film and not the inside.

The biodegradability of PBS copolymers also depends on the copolymer composition. For the aliphatic copolyesters, the greatest biodegradability occurs at about 30–50 mol% comonomer content, as demonstrated in Figs. 22 and 23. This may be attributed to the lowest degree of crystallinity in the middle composition range. For aliphatic–aromatic PBST, the biodegradability in soil burial decreases with increased content of the aromatic comonomers (Zhang et al. 2001).

Biodegradation in activated sludge. Biodegradation of PBS and its copolymers is faster in activated sludge than in soil burial. For PBS film, a considerable change in the film morphology could be observed after it had been immersed in activated

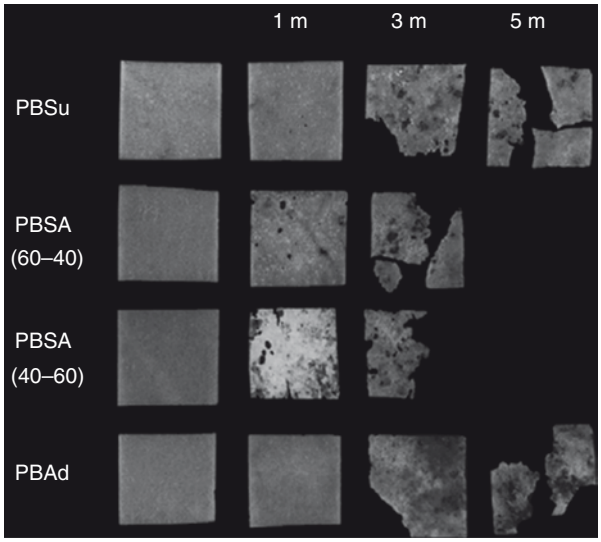


Fig. 22 Photographs of homopolyesters and copolyesters after soil burial degradation for 1, 3, and 5 months (Tserki et al. 2006b)

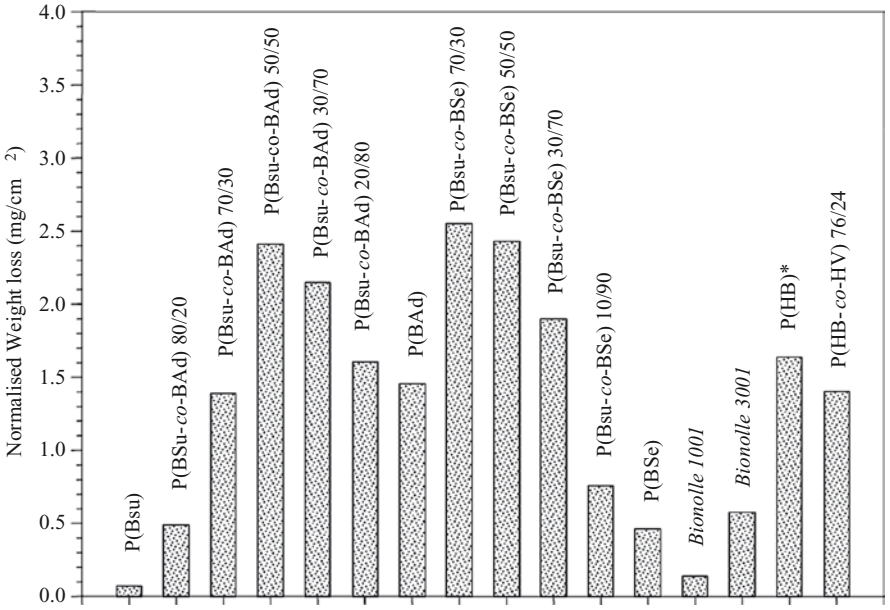


Fig. 23 Normalized weight losses for PBSA, poly(butylene succinate-*co*-butylene sebacate BSe), Bionolle, polyhydroxybutyrate and poly(hydroxybutyrate-*co*-hydroxyvalerate) 76/24 film samples in soil burial tests for 15 days (Rizzarelli et al. 2004)

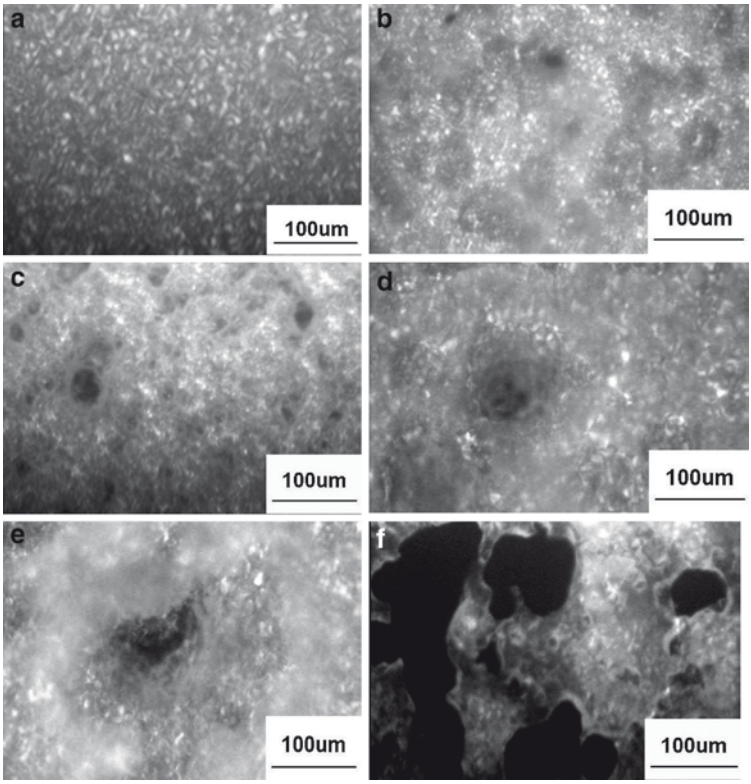


Fig. 24 Polarized optical micrographs of PBS degraded in activated sludge for different periods of time: before biodegradation (**a**); degraded for 1 week (**b**), 2 weeks (**c**), 4 weeks (**d**), 6 weeks (**e**), and 10 weeks (**f**) (Ding 2002)

sludge for 2 weeks. After 10 weeks of biodegradation, big holes were observed in the PBS film, as shown in Fig. 24.

The molecular weight has a considerable effect on biodegradation of PBS in activated sludge. After biodegradation for 12 weeks, PBS with an intrinsic viscosity of 1.05 dl g^{-1} has a weight loss of about 80%, which is much larger than the weight loss of PBS with an intrinsic viscosity of 1.79 dl g^{-1}, about 16% (Fig. 25).

The biodegradation rate depends on the chemical structure of the polyesters. For PBSA copolymers, the biodegradation rate in activated sludge improves with increase of the butylene adipate content from 0 to 20 mol%, as presented in Fig. 26. PBSA with 20 mol% of the butylene adipate fraction will disintegrate into pieces after biodegradation for 6 weeks. For poly(butylene succinate-*co*-butylene methylsuccinate) (PBSM), the biodegradability arises with increase of butylene methylsuccinate content from 0 to 40 mol%, as shown in Fig. 27. This phenomenon can be attributed to the decreased degree of crystallinity. For PBS copolymers with

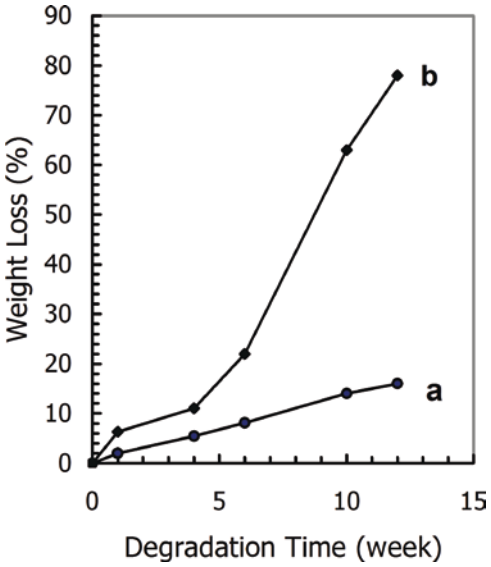


Fig. 25 Biodegradation of two PBSs with different M_w in activated sludge: **a** PBS with intrinsic viscosity of 1.793 dl g^{-1}; **b** PBS with intrinsic viscosity of 1.049 dl g^{-1} (Ding 2002)

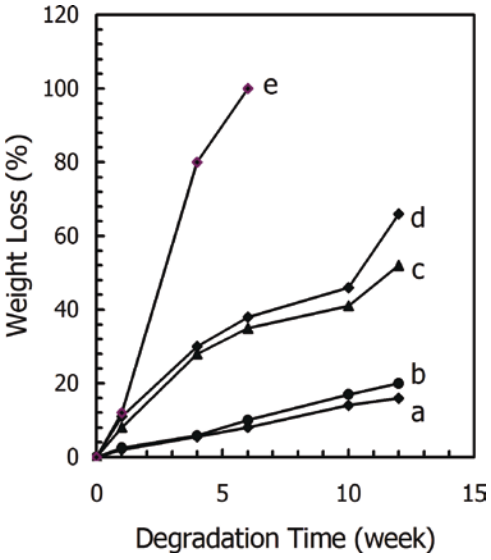


Fig. 26 Biodegradation of PBSA copolymers in activated sludge: **a** PBS, **b** "Poly(butylenes succinate-co-5 mol% butylene adipate) copolymer (PBSA-5), **c** Poly(butylenes succinate-co-10 mol% butylene adipate) copolymer (PBSA-10), **d** PBSA-15, **e** PBSA-20 (Ding 2002)

substituted succinic acid as the comonomer, at the same comonomer content of 10 and 20 mol%, the degradation rate follows the order

PBSM≈poly(butylenes succinate-co-butylene dimethylsuccinate) PBS2M> PBSA > poly(butylenes succinate-co-butylene benzylsuccinate) PBSBS > PBS.

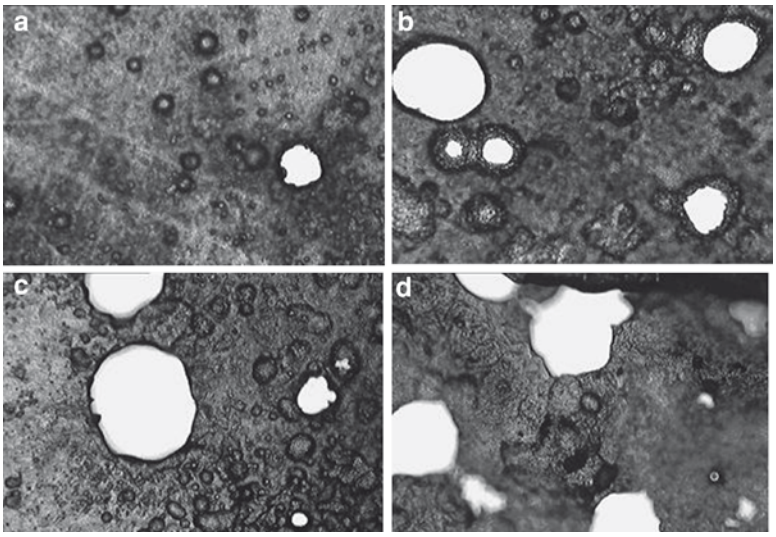


Fig. 27 Micrographs of poly(butylene succinate-*co*-butylene methylsuccinate) copolymers after biodegraded in activated sludge for 2 weeks: **a** poly(butylene succinate-*co*-10 mol% butylene methylsuccinate), **b** poly(butylene succinate-*co*-20 mol% butylene methylsuccinate), **c** poly(butylene succinate-*co*-30 mol% butylene methylsuccinate), **d** poly(butylene succinate-*co*-40 mol% butylene methylsuccinate) (Sun 2005)

Figures 28 and 29 reveal the film morphology of different PBS copolymers at the same comonomer content of 20 mol% after biodegradation in activated sludge for 2 and 3 weeks, respectively. PBSM and PBS2M show biodegradation holes, whereas PBSBS does not demonstrate clear biodegradation traces.

For PBST copolymer, with increase of butylene terephthalate comonomers, the biodegradability increases first, then decreases sharply. PBST-10 has a higher biodegradability than PBS homopolymer. The biodegradation profile with time is given in Fig. 30. The weight loss of PBS increases steadily with biodegradation, whereas that of poly(butylenes succinate-co-10 mol% butylene terephthalate) (PBST-10) and poly(butylenes succinate-co-15 mol% butylene terephthalate) (PBST-15) levels off after the initial 4–6 weeks. This fact indicates that the aromatic units are not so susceptible to biodegradation as the aliphatic units.

Biodegradation in compost. Biodegradation in compost is a standard protocol to judge whether a plastic is biodegradable or compostable. The biodegradation rate of PBS in compost is sensitive to the shape and size of the specimen (Zhao et al. 2005; Yang et al. 2005). PBS powder shows a degradation rate comparable to that of the film. PBS pellet degrades more slowly and shows a final weight loss of less than 15% after degradation in compost for 3 months, as demonstrated in Fig. 31. In contrast, PBSA powder has biodegradation rate similar to that of the film (Yang et al. 2005).

PBSA has a higher biodegradation rate than PBS. The weight loss of PBSA and PBS is around 90 and 55% after degradation in 60°C compost for 3 months

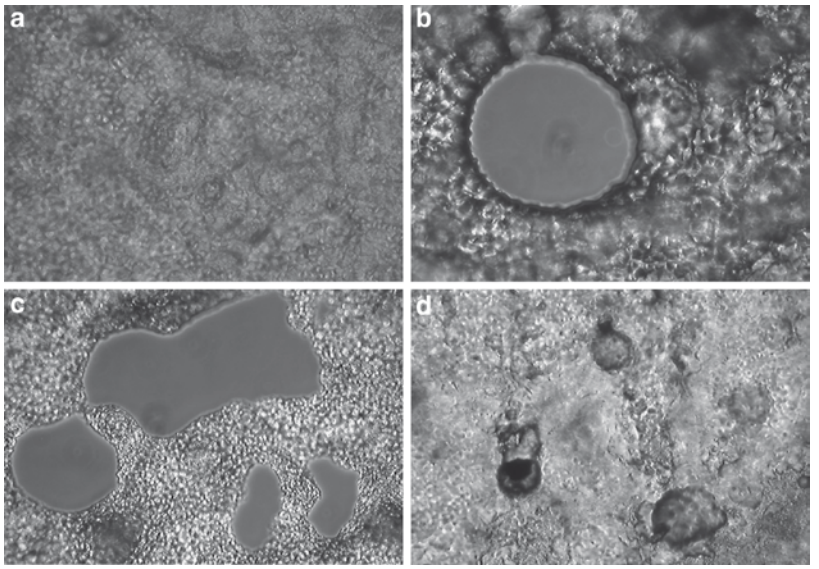


Fig. 28 Micrographs of different PBS copolymers after being biodegraded in activated sludge for 2 weeks: **a** poly(butylene succinate-*co*-20 mol% butylene adipate), **b** poly(butylene succinate-*co*-20 mol% butylene methylsuccinate), **c** poly(butylene succinate-*co*-20 mol% butylene dimethylsuccinate), **d** poly(butylene succinate-*co*-20 mol% butylene phenylsuccinate) (Sun 2005)

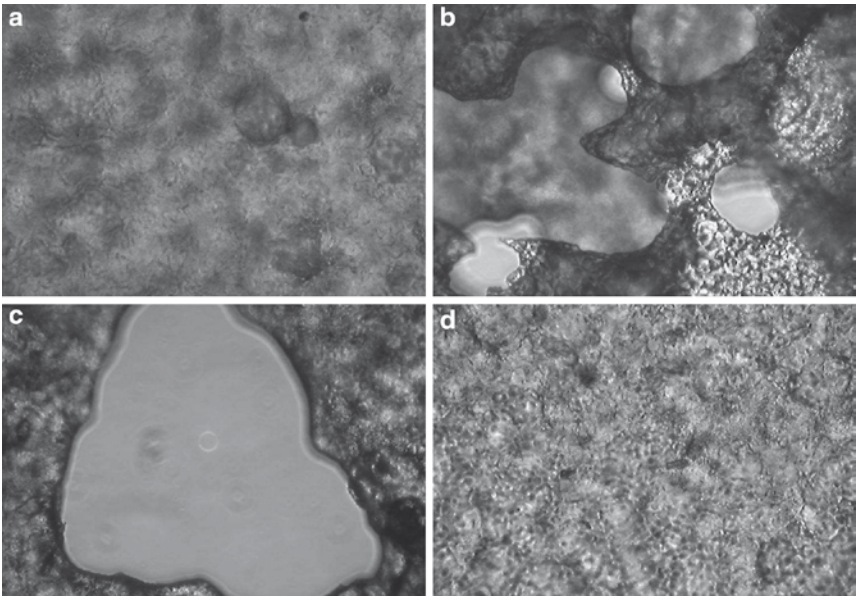


Fig. 29 Micrographs of different PBS copolymers after being biodegraded in activated sludge for 3 weeks: **a** poly(butylene succinate-*co*-20 mol% butylene adipate), **b** poly(butylene succinate-*co*-20 mol% butylene methylsuccinate), **c** poly(butylene succinate-*co*-20 mol% butylene dimethylsuccinate), **d** poly(butylene succinate-*co*-20 mol% butylene phenylsuccinate) (Sun 2005)

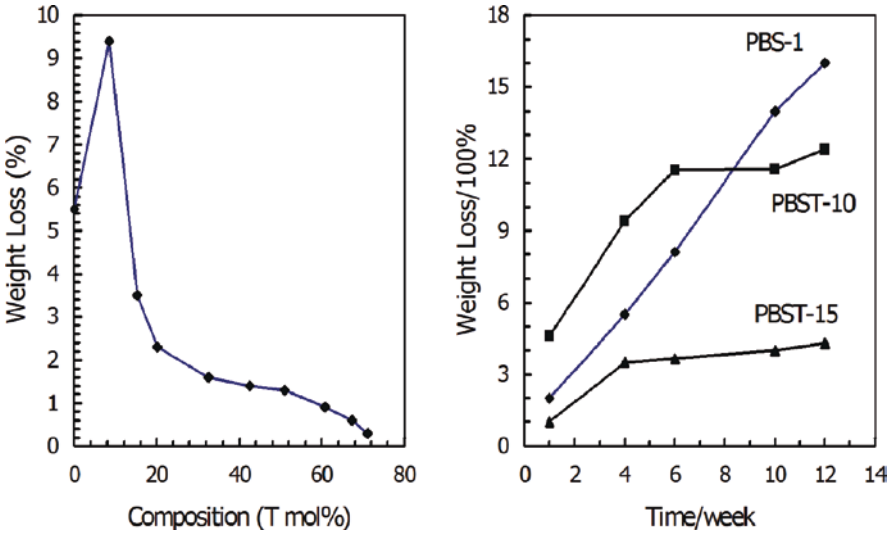


Fig. 30 Weight loss of various PBSTs after biodegradation in activated sludge: **a** dependence of the weight loss on copolymer composition after degradation for 4 weeks; **b** biodegradation profiles of PBS and two PBSTs with time (Ding 2002)

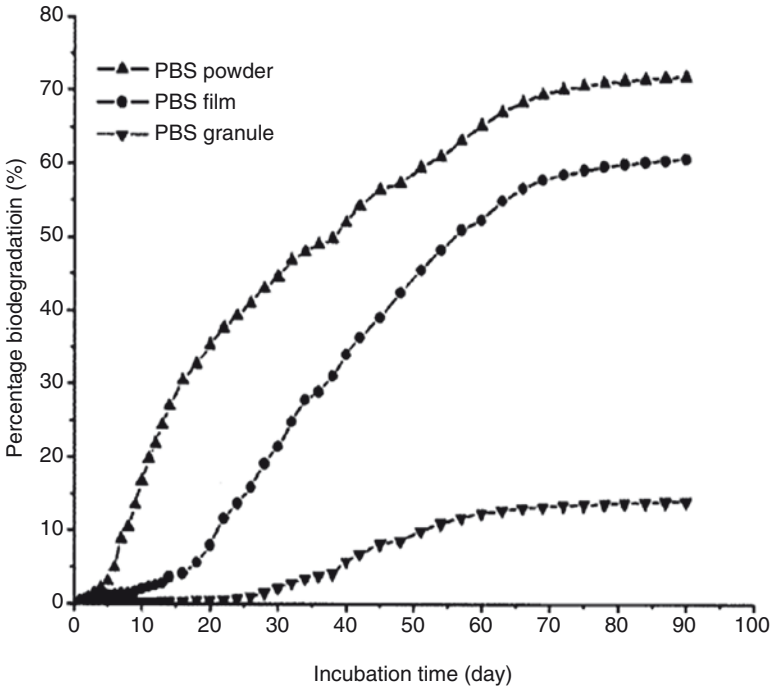


Fig. 31 Biodegradation of PBS samples with different shapes under controlled composting conditions at 60°C (Zhao et al. 2005)

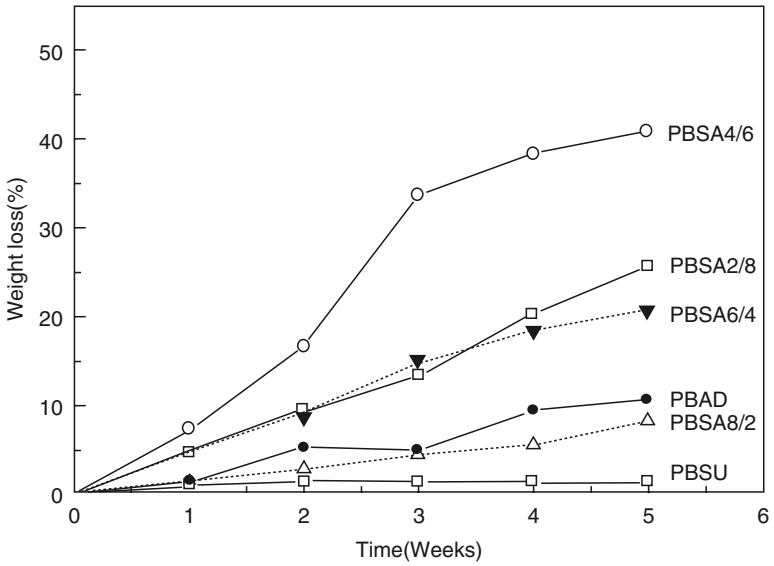


Fig. 32 Weight loss of the PBSA copolyesters degraded at 30°C, 90% humidity in the composting soil (sample size 20 mm×20 mm×0.4 mm) (Ahn et al. 2001)

(Zhao et al. 2005). The biodegradation rate of PBS in 30°C compost is also lower than that of PBA, which can be attributed to the lower molecular mobility of the former. PBSA with 60 mol% butylene adipate units showed the highest biodegradability (Fig. 32), owing to the lowest degree of crystallinity at the copolymer composition (Ahn et al. 2001).

Various microorganisms able to degrade PBS and its copolymers have been isolated, including bacteria and fungi, such as *Bacillus stearothermophilus* (Tomita et al. 2000), *Microbispora rosea, Excellospora japonica* and *Excellospora viridilutea* (Jarerat and Tokiwa 2001), *Penicillium chrysogenum* (Zhu et al. 2003), *Bacillus pumilus* and *Acidovorax delafieldii* (Hayase et al. 2004), and *Aspergillus versicolor* (Zhao et al. 2005).

5 Application of PBS

PBS decomposes naturally into water and CO_2. In this era of increasing environmental awareness, it is thus attracting attention as a promising eco-friendly alternative to common plastics. The scope of the application fields is growing. So far, PBS has found applications in agriculture, fishery, forestry, civil engineering, and other fields in which recovery and recycling of materials after use is problematic. For example, PBS is actually used as packaging materials,

Fig. 33 Disposable articles made by injection molding of PBS. From *left* to *right*: knife, golf nail, spoon, fork, inner liner of shoes

vegetation nets, mulching film, compost bags, etc. Figure 33 shows some articles produced from PBS.

6 Concluding Remarks and Future Perspectives

Owing to its biodegradability, wide processing window, and balanced mechanical properties, PBS and its copolymers have attracted much interest in the past two decades. Via copolymerization with different monomer units, the thermal properties, mechanical properties, and biodegradability can be precisely tailored.

The major competitive field for PBS-type polyesters will be blown film, which is a big market owing to requirements of packaging and mulch. PBS and its copolymers with high molecular weight or branched structure are suitable for the production of tubular film and biaxially oriented film.

The flexibility will limit the applications of pure PBS polyester; however, the disadvantage can be overcome by blending it with starch or polylactide. Blending with these rigid plastics will improve the modulus of the final products.

At the present stage, petroleum- or coal-based succinic acid is still cheaper than bio-based feedstock. With future development of fermentation technology and the finding of new microbial strains, bio-based succinic acid will become competitive.

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Microbial Ethanol, Its Polymer Polyethylene, and Applications

He Huang

Contents

Abstract Polyethylene (PE) is an important engineering material. It is produced through the ethylene polymerization process. Ethylene can be produced through steam cracking of ethane, steam cracking of naphtha or heavy oils, or ethanol dehydration. With the increase of the oil price, bioethylene, produced through ethanol dehydration, is a more important production route for ethylene. Green PE, which does not pollute the environment with CO_2 or nonuseable by-products, is made from bioethylene as a monomer. Microbial ethanol production techniques derived from different feedstocks, the chemistry and process of bioethylene production, and the status of green PE research are the main topics of this chapter. Different pretreatment techniques used during microbial ethanol production are described. The research

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status of cellulose ethanol is given in detail. On the basis of the ethanol dehydration chemistry principle, the progress of research and development on catalysts and the process for dehydration of ethanol to ethylene is also described.

1 Introduction

Ethylene, a basic building block for the chemical industry, can be produced through steam cracking of ethane, steam cracking of naphtha or heavy oils, or ethanol dehydration. The major use of ethylene is as a monomer for the production of polyethylene (PE), polystyrene, polyglycols, etc. The production route of ethylene from coal, natural gas, and biorenewable resources has been researched and developed in recent years owing to the high cost of oil. Green PE (also known as renewable PE) is made out of ethanol, which becomes ethylene after a dehydration process. The final product (PE) is identical to naphtha- or gas-based PE; therefore, the physical properties for conversion into plastics products are maintained as are its recycling properties. The world's first announced ethanol-based chemical plant, totally integrated from sugarcane to PE, was recently announced by Dow Chemical Company, in conjunction with Crystalsev, a large sugar and ethanol producer in Brazil. The plant is said to be projected to produce 770 million pounds per year of renewable linear low-density PE (LLDPE). Construction began in 2008, and it is slated to start production in 2011. Roughly 2 tons of ethanol is needed to make 1 ton of PE, as dehydration removes half of the weight as water, from the sugarcane-based ethanol, before conversion into ethylene (C_2).

As shown in Fig. 1, green PE based on biorenewable resources can be produced through biological and chemical processes, and is completely recyclable. One of the main environmental benefits of this project will be the sequestration of roughly 2 kg of CO_2 per kilogram of PE produced, which comes from the CO_2 absorbed by the sugarcane while growing, minus the CO_2 emitted in the production process. In this chapter, microbial ethanol produced from different feedstocks, the chemistry and process of bioethylene production through ethanol dehydration, and the status of green PE research will be introduced.

2 Microbial Ethanol

Microbial ethanol is derived from biorenewable feedstocks, including wood, corn stalks, and other plant waste, as a resource through microbial strains and other biological fermentation processes. Bioethanol production is a relatively mature process among biomass transformations. Many forms of biomass can be used as industrial raw materials for bioethanol manufacture. A large industry with mature technology has developed for the production of bioethanol. Ethanol has many advantages, such as a high heat value and compatibility as a fuel with current engine technology. It is an important replacement fuel. Currently, this is the major development direction of the world's fuel ethanol production.

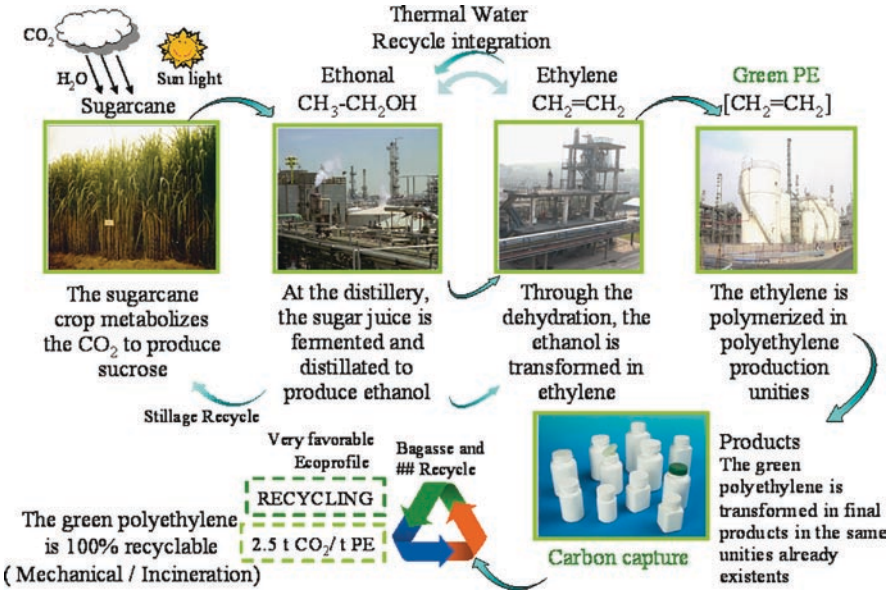

Fig. 1 Green polyethylene

2.1 Feedstock

Resources that contain sugars can be used as a feedstock for ethanol fermentation. There are many biomass resources in the world that can be used to produce ethanol by fermentation. According to the traditional approach, they can be divided into three categories – starch, sugar, and cellulose materials:

1. *Starch materials*. These are the most important raw materials which can be used for production of ethanol by microbial strains. Sweet potato, cassava, maize, potato, barley, rice, and sorghum all belong to starch materials.
2. *Sugar materials*. These materials mainly include sugarcanes and sugar beets.
3. *Cellulose materials*. These are the most promising raw materials for ethanol production. These materials include crop stalks, forest logging and wood processing residues, straws, paper mill and sugar-containing-cellulose wastes, and parts of solid waste.

The fuel ethanol industry is different from the traditional alcohol industry. It requires a large-scale production and high-purity products. This industry requires that its raw materials be accessible, and the process must be simple, reliable, and economical. Though the technology that employs sugar and starch materials to produce ethanol is already well developed, the raw materials mainly come from the human food chain, and eventually will cause a food security issue. Thus, it is difficult to meet the long-term energy demand. Therefore, the production of ethanol from sugar or starch materials is subject to a lot of restrictions.

Among the three main raw materials, cellulose is the world's most abundant biomass resources, though it has not been fully utilized. Global production of biomass is estimated to be about 200×10^9 tons each year, of which 90% is wood cellulose. About 8×10^9–20×10^9 tons of the original biomass still is not utilized each year. At present, the enzymatic hydrolysis of cellulose biomass is considered to be the most promising technology. In this area, some studies have been done, but industrial-scale production is still being hampered owing to the technical problems or the lack of biomass refining methods. However, cellulose ethanol can reduce greenhouse gas emissions by 70–90% and significantly improve the environmental situation, so it has the potential to impact the development direction of the fuel ethanol industry.

2.2 *Starch Ethanol*

Corn, wheat, and potato are the economic crops which are rich in starch. In addition to starch, the materials also contain protein, cellulose, and pectin substances. The feedstock with rich amylase is required in the alcohol fermentation industry. At present, the technology producing alcohol with starch feedstock is widely used in the USA and European countries. Figure 2 shows the general production process (Dong et al. 2007).

2.2.1 Pretreatment of Starch Material

Starch material is pretreated to increase the starch enzymatic hydrolysis efficiency. Typically, the pretreatment includes mechanical grinding or superfine pulverization. During the treatment process, the criterion to evaluate the pretreatment method is based on several parameters, including increasing the hydrolysis rate, reducing the

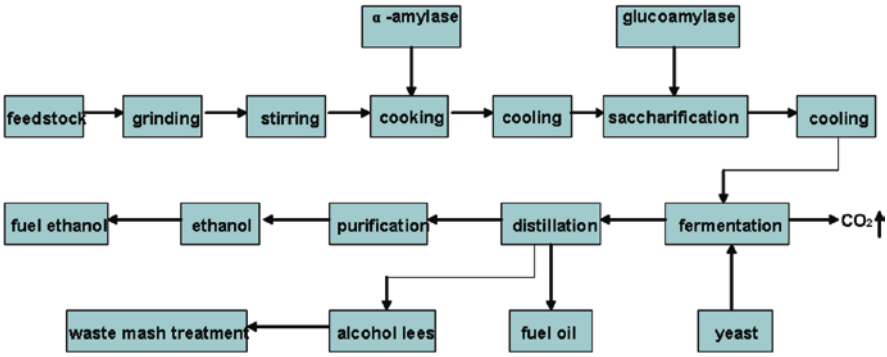

Fig. 2 The general process of ethanol production from starch feedstock

loss of carbohydrates, diminishing the excess production of by-products which inhibit the hydrolysis and fermentation, and improving the performance–price ratio of the technological process.

2.2.2 Hydrolysis of Starch

To fully exploit ethanologenic microorganisms such as yeasts or the bacterium *Zymomonas mobilis*, starch must be hydrolyzed into glucose units prior to alcohol fermentation. This can be achieved using two kinds of enzymes, namely, α-amylase and amyloglucosides. The process is called double-enzyme hydrolysis.

The feedstock is first ground. The plant cell tissue of the raw materials is destroyed during the grinding process, and this favors the dissociation of starch. Subsequently, water is added and the mixture becomes a syrup with suitable concentration. After it has been preheated, maxilase (α-amylase) is added into the powder syrup. Then, the syrup is heated to 95°C by steam injection, and transferred to a cooking pot for heating for 2 h at 95–105°C. After that, it is cooled to 60°C and glucoamylase is added. Finally, the cooking mash is transferred into a fermentor after being heated for 30 min. The purpose of cooking is to convert the starch from a particle state into a gelatinization state. Through saccharification the gelatinized starch is converted to sugar. Ethanologenic microorganisms convert the sugar to ethanol.

In recent years, the liquefaction process by adding enzyme has been widely applied to amylohydrolysis and achieved good results with the development of the enzyme industry. The saccharification process that uses double enzymatic fermentation is an important preprocessing step in the production of starch ethanol.

2.2.3 Fermentation

2.2.3.1 Strains

The characteristics of ideal ethanol fermentation microbes include rapid fermentation, strong ethanol tolerance, few by-products, and strong tolerance of osmotic pressure and temperature. The efficient processes for converting glucose into ethanol all use yeast, such as *Saccharomyces cerevisiae* and Kaersibo yeast (*Saccharomyces carlsbergensis*)

2.2.3.2 Fermentation Technology

The conventional ethanol fermentation process uses liquefied starch as a substrate. It involves two separate operations which have significant consequences, namely, the presaccharification of starch and ethanol fermentation. Since ethanol production is inhibited by end products, low cell concentration and the substrate, simultaneous saccharification and fermentation (SSF) has

been unitized. Some studies have attempted to carry out the steps of saccharification and fermentation in the same reaction pot (Oscar and Carlos 2007). In contrast, the SSF process combines two steps into one and correspondingly improves the rate of hydrolysis. In the cost-effective alternative process, the product inhibition on saccharification by glucose can be diminished since the glucose produced from oligosaccharides is consumed immediately by the cells and converted into ethanol. However, a serious problem confronted by bacterial fermentation is decreased ethanol yields owing to the consumption of microorganisms which can produce amylase.

2.3 Sugarcane Ethanol

Sugarcane contains sufficient sugar that it can be converted into ethanol directly. The process for sugarcane is as follows: Sugarcane materia → pressing → fermentation → ethanol.

Sugarcane is recognized as one of the best energy crops in the world. It has a high yield of biomass that can be used .Sugarcane stalk contains more than 13% sucrose, which can be used to produce ethanol directly through fermentation.

During the process of fuel ethanol production using sugarcane, the first step is to extract the sugarcane juice. After fermentation, distillation, and the dehydration processes, the juice can be transformed into high-purity ethanol (Oscar and Carlos 2007). Conventional sugar processing can be used to extract cane stalk juice, and the process for producing molasses ethanol can be directly applied to the production of cane juice ethanol without any changes in process engineering.

In the process for producing fuel ethanol using sugarcane as the feedstock it is not necessary to focus on the pretreatment of the feedstock, but it is necessary to improve the fermentation process. Sugarcane juice can easily cause the problem of yeast flocculation in the fermentation process because of increased proteins and colloids. So the breeding of deflocculating yeast is needed to improve the ability to exhaust impurities and prevent the tower from being blocked by colloid material. For the distillation operation, improved distillation equipment is required to enhance the ability of tower tray to prevent colloid-type substances from plugging the tower. Species of high sugar content sugarcane are needed to enhance the ethanol fermentation output and reduce the operating costs for the purification of downstream products in the sugarcane cultivation industry.

2.4 Cellulose Ethanol

The major components of cellulose feedstock include cellulose, hemicellulose, and lignin. The first step is the pretreatment of raw materials, removing the inhibitors of saccharification and fermentation. Second, cellulose is hydrolyzed into simple sugars by the action of acid or enzyme. Third, ethanol is formed by fermentation

from pentose and hexose. The final step is the purification of ethanol (through distillation or filtering), the gasification of residual solids, and treatment of wastewater.

Figure 3 shows the general process of ethanol production with cellulose feedstock.

2.4.1 Pretreatment of Cellulose Material

Pretreatment is required to make cellulose more accessible to the enzymes that convert the carbohydrate polymers into fermentable sugars. The goal is to break the lignin seal and disrupt the crystalline structure of cellulose. The ideal pretreatment process should be economic and include (1) high hemicellulose and cellulose conversion, (2) low sugar decomposition, and (3) reduced by-products (Sun and Cheng 2002).

Pretreatment methods are shown in Table 1 (Yang and Chlarles 2008; Lee et al. 2008).

Steam explosion is the most commonly used method for pretreatment of lignocellulosic materials. In the processing, chipped biomass is exposed to high-pressure saturated steam and then the pressure is swiftly reduced, which makes the materials undergo an explosive decompression. Steam explosion is typically initiated at a temperature of 160–260°C (corresponding to a pressure of 0.69–4.83 MPa) for several seconds to a few minutes before the material is exposed to atmospheric pressure. The process causes hemicellulose degradation and lignin transformation owing to the high temperature, thus increasing the potential for cellulose hydrolysis (Sun and Cheng 2002).

In the steam explosion process, the addition of H_2SO_4 (or SO_2) or CO_2 in the steam explosion can effectively improve enzymatic hydrolysis, decrease the production of inhibitory compounds, and lead to more complete removal of hemicellulose.

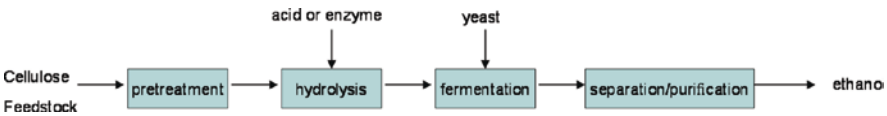

Fig. 3 The general process of ethanol production from cellulose feedstock

Table 1 Some pretreatment processes of lignocellulosic materials

Pretreatment method	Examples
Mechanical method	Ball milling, comminution, extraction
Explosion method	Steam explosion, CO_2 explosion, ammonia fiber explosion
Acid hydrolysis	Dilute acid (H_2SO_4, HCl), concentrated acid (H_2SO_4, HCl), acetic acid
Alkaline hydrolysis	NaOH, lime, ammonia
Organic solvent	Methanol, ethanol, butanol, benzene
Biological pretreatment	Lignin peroxidase, manganese-dependent peroxidases

The advantages of steam explosion pretreatment include the low energy requirement compared with mechanical comminution and no recycling or environmental costs.

Recently, our group (Yan et al. 2008) reported a novel pretreatment method for cellulose materials. A cycle spray flow-through reactor was designed and used to pretreat corn stover in dilute sulfuric acid medium. The dilute sulfuric acid cycle spray flow-through (DCF) process enhanced xylose sugar yields and cellulose digestibility and increased the removal of lignin. With the DCF system, xylose sugar yields of 90–93% could be achieved for corn stover pretreated with 2% (w/v) dilute sulfuric acid at 95°C during the optimal reaction time (90 min). The remaining solid residue exhibited enzymatic digestibility of 90–95% with cellulase loading of 60 FPU g^{-1} glucan that was due to the effective lignin removal (70–75%) in this process. Compared with the flow-through and compress hot water pretreatment process, the DCF method produces a higher sugar concentration and higher xylose monomer yield. The novel DCF process provides a feasible approach for lignocellulosic material pretreatment.

2.4.2 Hydrolysis and Saccharification of Cellulose

The methods for saccharification of cellulose include acid saccharification and enzymatic saccharification.

Enzymatic hydrolysis is a common method. Enzymatic hydrolysis of cellulose is carried out by cellulase enzymes which are highly specific. Cellulases are usually a mixture of several enzymes. At least three major groups of cellulases are involved in the hydrolysis process: endoglucanase, cellobiohydrolase, and β-glucosidase. The cost of enzymatic hydrolysis is low compared with acid or alkaline hydrolysis which have corrosion problems because enzyme hydrolysis is usually conducted under mild conditions (pH 4.8 and 45–50°C). Both bacteria and fungi can produce cellulases for the hydrolysis of lignocellulosic materials. These microorganisms can be aerobic or anaerobic, mesophilic or thermophilic. Most studies for commercial cellulase production have focused on fungi even though many cellulolytic bacteria, particularly the cellulolytic anaerobes and *Bacteroides cellulosolvens*, produce cellulases with high specific activity because the anaerobes have a very low growth rate.

2.4.3 Fermentation

The process of fermentation of cellulose materials differs from that of starch or sugar materials: (1) the hydrolysate of the lignocellulosic material is toxic to the fermentative microorganisms; (2) there is more xylose in the hydrolysate. So, the utilization of pentose is one of the most important decisive factors for the process economics (Zaldivar et al. 2001).

The methods for fermentation of cellulose ethanol include direct and indirect fermentation, mixed strain fermentation, SSF, simultaneous saccharification and cofermentation (SSCF), nonisothermal SSF, and immobilized cell fermentation.

The direct fermentation method is based on the direct fermentation of cellulose by cellulolytic bacteria, without acidic or alkali pretreatment. It has the features of simple equipment and low cost, but with low ethanol yield and production of organic acid and other by-products. The problems can be solved partly by mixed strain fermentation.

SSF and SSCF are preferred since both operations can be done in the same tank, resulting in lower costs (Oscar and Carlos 2007).

Cellulose hydrolysis and fermentation of sugar solution carried out in the same tank is referred to as SSF. Simultaneous saccharification of both cellulose (to glucose) and hemicellulose (to xylose and arabinose) and cofermentation of both glucose and xylose carried out by genetically engineered microbes that ferment xylose and glucose in the same broth is referred to as SSCF.

Since cellulose hydrolysis and fermentation of the sugar solution are carried out continuously in the same tank and glucose is continuously fermented, the feedback inhibition to cellulose due to the increase of glucose concentration is avoided. From a technological aspect, this method simplifies the equipment needed, saves production time, and improves the production efficiency. But there are some inhibitory factors, such as the inhibition of xylose and incoordination of the saccharification and fermentation times (Oscar and Carlos 2007).

2.4.4 Purification

The traditional method for alcohol purification is distillation. When the alcohol concentrations in the mixture and in the steam reach 95.57 and 97.6%, respectively, the mixture cannot be further purified, so a second distillation must be carried out. At present, the domestic secondary distillation methods include calcium oxide dehydration, azeotropic distillation, extraction distillation, absorption, molecular sieves, and vacuum dehydration. In addition, chemical film separation is a very promising alcohol purification technology, but it is still in the experimental stage.

3 Ethylene via Dehydration of Microbial Ethanol

3.1 Background

The shortage of global petroleum resources is driving up costs in the ethylene industry, whereas the demand for ethylene-derived product is increasing. Therefore, the competitive advantage of oil-cracked ethylene is diminished in today's market. Many countries hope that bioethylene from renewable biomass for ethylene will replace nonrenewable fossil resources. It is important to develop bioethylene in these countries with extremely scarce fossil resources. In fact, the future economic development strategy calls for replaceable energy development and a cyclic economy. The development of a mature bioethylene industry is an important part of

reducing petroleum dependence. In this section we focus on the chemical and technical processes involved in the dehydration of microbial ethanol to ethylene.

3.2 Chemistry

3.2.1 Catalysts for Microbial Ethanol Dehydration

An efficient catalyst is the key technology to the industrialization of dehydration of microbial ethanol to ethylene. Reported catalysts for ethanol dehydration include activated clay, phosphoric acid, sulfuric acid, activated alumina, transition metal oxide, transition metal composite oxide, heteropolyacid, and zeolites (Huang et al. 2008). Catalysts based on activated alumina are the most important kind of catalyst for ethanol dehydration, and are the catalysts dominantly used in current industrial production. The most representative of these catalysts is Syndol (with a main composition of Al_2O_3–MgO/SiO_2), developed by Holcon Science and Design Company in the 1980s. This catalyst was used to dehydrate a solution of about 50,000 tons per year of ethanol on the largest dehydration installation system at that time, carrying out the ethanol dehydration reaction at 335–450°C, with a LHSV (liquid hourly space velocity) of 0.7 h^{-1}. The conversion of ethanol and the selectivity of ethylene were about 97–99 and 94.5–98.9%, respectively, for this process. The lifetime of Syndol is about 8–12 months, after which it needs to be regenerated (Kochar et al. 1981). Although catalysts based on activated alumina have distinguished characteristics such as high stability, renewability, and high purity of the product, they still have some disadvantages, such as low activity, low LHSV of the feedstock, high reaction temperature, and high energy consumption. To overcome these disadvantages, many researchers have developed various binary and composite metal oxides based on activated alumina.

In the 1980s, researchers began to study zeolites for dehydration reactions of alcohols. These zeolites included A, Y, β, HZSM-5 SAPO-34, and mordenite type. These studies discovered that zeolite catalysts in ethanol dehydration are better than oxide catalysts owing to the lower reaction temperature. Zeolite catalysts have a higher operation LHSV, higher ethanol conversion rate, higher ethylene selectivity, and allow a lower concentration of ethanol raw materials than oxide catalysts. Among the zeolites investigated, HZSM-5 molecular sieve is the most popular for dehydration of ethanol to ethylene. This is the most promising catalyst for commercial use. Currently, research work is mainly concentrated on the modification of HZSM-5, such as by ion exchange, hydrothermal treatment, and controlling surface acidic active sites and surface acid strength distribution, to improve the hydrothermal stability, anticoke ability, enhancing the activity and selectivity of HZSM-5. Recent studies have been reported by Zhang et al. (2008) on the effect of the phosphorus content on the performance of phosphorus modified HZSM-5 catalysts in dehydration of ethanol to ethylene. They found that the main product was ethylene at 300–440 K and the anticoke ability of the catalysts modified by phosphorus of 3.4 w.t.% was improved, owing to the presence of weak acid sites after phosphorus modification. When the *P* loading is below 3.4%, ethylene and higher hydrocarbons were found at high temperature.

3.2.2 Mechanism for Microbial Ethanol Dehydration

Under different reaction conditions, the main products of the ethanol dehydration reaction are ethylene and ether. Although many researchers have made great efforts to reveal the mechanism for ethanol dehydration, disputes exist when the mechanisms are applied to a particular reaction process. Some researchers think that the dehydration of ethanol to ethylene is a parallel reaction process, whereas others think that it is a parallel continuous reaction process, namely, dehydration of ethanol to ether and further dehydration to ethylene.

Many of the trial results have indicated that the coexistence of an acid and alkaline center is conducive to dehydration of ethanol to ethylene. Hassan suggested the catalytic mechanism of ethanol dehydration catalyzed by solid acid or alkaline catalysts in 1982 (Abd El-Salaam and Hassan 1982). He claimed the acid and alkaline center of the catalyst cooperated in the process of ethanol dehydration. Although the reaction was mainly catalyzed by the acid center, the existence of a modest alkaline center could promote this reaction. Hassan considered ethanol was absorbed in the acid and alkaline center on the surface of the catalyst and then formed adsorption-state compounds, which could further dehydrate to ethylene and release the acid and alkaline center. The mechanism of dehydration of ethanol to ethylene catalyzed by activated alumina suggested by Cosimo et al. (1998) is shown Fig. 4.

Recently, Kondo et al. (2005) studied the mechanism of ethanol dehydration in acid solutions and on the surface of zeolites respectively. Acid-catalyzed ethanol dehydration on zeolites was shown to proceed via a covalent ethoxy group (C_2H_5O) as a stable intermediate, and this was directly observed by infrared spectroscopy (Fig. 5).

3.3 *Process Description*

Currently, dehydration of ethanol to ethylene uses gas-phase–solid-phase dehydration. Ethanol is pretreated and then the gas state is fed into the reactor. The dehydration of ethanol to ethylene is a very endothermic reaction. The column reactor was first used to improve the efficiency of heat transfer. Heat transfer was further improved through the development of the adiabatic reactor (Raymond et al. 1989). Very few studies have reported fluidized bed reactors for ethanol dehydration.

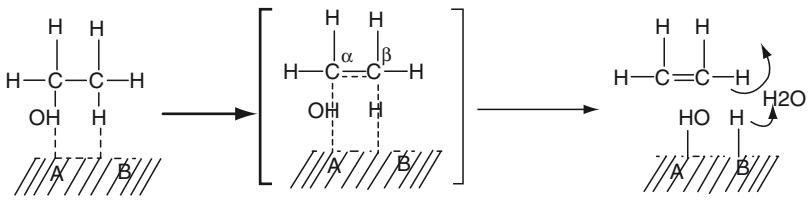

Fig. 4 Mechanism for dehydration of ethanol to ethylene on Al_2O_3: (**a**) Lewis acid center and (**b**) base center

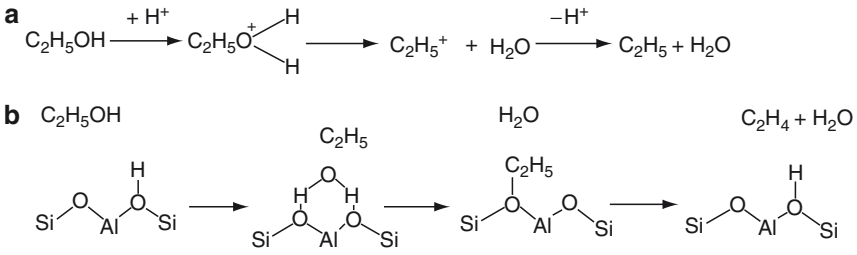

Fig. 5 Comparison of mechanisms of ethanol dehydration (**a**) in acidic solutions and (**b**) on zeolites

Table 2 Different technologies for of dehydration of ethanol to ethylene

Technology	Lummus technology	Halcon/SD technology	Petrobras technology	Sinopec technology
Development corporation	Lummus, USA	Halcon/SD, USA	Petrobras, Brazil	China Sinopec Group

Only Lummus reported a fluidized bed reactor for use in a trial ethanol dehydration study. The fluidized bed reactor operated with a temperature of about 399°C, a one-way ethanol conversion rate of greater than 99.5%, and an ethylene yield of more than 99% (before distillation; Tsao and Zasloff 1979). Dehydration of ethanol to ethylene is a relatively mature technology. Different processes have been developed by many companies according to their respective catalyst (as shown in Table 2).

Halcon/SD designed a polymer-grade ethylene production process as shown in Fig. 6. The production process includes two parts (reaction and separation). First, the ethanol is vaporized, and then is fed through the fixed-bed reactor filled with catalyst. The reaction takes place under adiabatic conditions. Ethanol is vaporized and preheated to 125°C, then is passed through the first stage of the catalyst bed in the reactor with four stages (Pearson 1983). When the ethanol contacts the catalyst, it is converted into ethylene. Because the reaction is performed in the adiabatic reactor, the reaction velocity decreases with the raw ethanol gas proceeding through the catalyst bed. Gas from the first stage of the catalyst bed is heated to the reaction temperature and is then sent to the reactor in the second stage of the catalyst bed; some conversion of ethanol into ethylene also occurs there. A similar conversion also happens in the third and the fourth stages. Gas leaving the reactor contains a small amount of crude ethanol that did not react and also ether, acetaldehyde, methane, carbon dioxide, etc.. After recovery of some heat by waste heat boilers, the gas is sent into the water quenching tower, and ethanol and water are removed (Barrocas et al. 1980). The rough ethylene is compressed to 1 MPa (gauge), then sent to the alkali washing tower to remove acid, and then cooled to 10°C. The rough ethylene is sent to a fixed-bed gas dryer, so that the water content meets the technical requirement. At this time, the ethylene purity is 98% (mole fraction; Kochar 1980). If there is a need for the production of polymer-grade ethylene, ethylene will be sent to the ethylene tower and the stripper tower for further refinement.

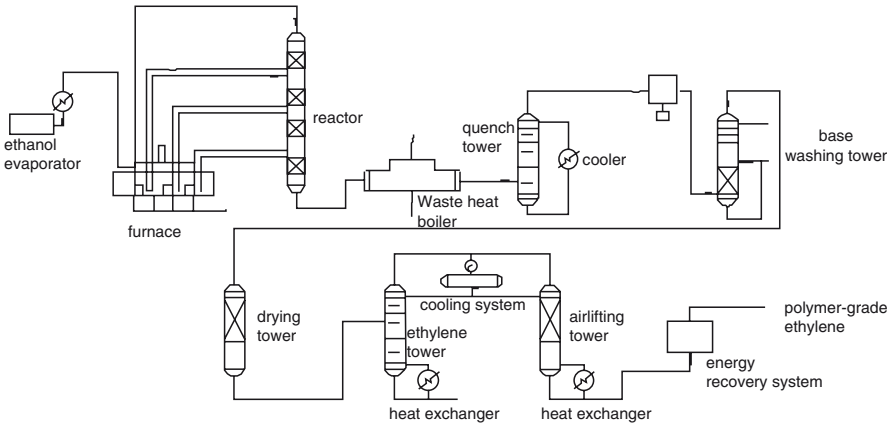

Fig. 6 Halcon/SD technical process plans

Coupling technology is a systematic approach to improving the energy efficiency of the system. When the purification of ethanol and catalytic dehydration are integrated into a system, the energy efficiency is improved. The ethanol vaporization requires low-temperature heat resources which are commonly associated with geothermal energy or the waste heat in a coal-fired plant.

With current technology, the coupling process is as follows. Ethanol fermentation liquid is purified by distillation, and is directly transferred to the catalytic dehydration process in a gaseous state (Uytterhoeven et al. 1988). The ethylene effluent from the reactor is heat-exchanged with ethanol solution to heat the ethanol to the temperature necessary for catalytic dehydration. Thus, some of the heat needed to preheat the ethanol for catalytic conversion to ethylene is recovered from the ethylene effluent. In this transformation, heat is conserved. After the ethylene effluent at low temperature has been further washed, dried, and refined, the remaining effluent is high-purity ethylene. The coupling technology can obviously improve the overall energy consumption efficiency.

4 Polyethylene and Applications

PE is the most plastic polymer. It is widely used in industry, agriculture, packaging, as well as daily life because of its low price and good performance. PE occupies a prominent position in the plastics industry (Huang and Rempel 1995). At present, industrial production of PE is dependent upon petroleum derivatives. The PE industry is facing new challenges with reduced global oil supply and increased oil demand. Environmental pollution caused by petrochemical industry practices is driving additional changes in this industry. Renewable biomass conversion to ethanol, dehydration into ethylene (Isao et al. 2005; Guangwen et al. 2007), and further

polymerization to PE provides an alternative raw material route to meet global needs. Dow Chemical Company and Brazil's Crystalsev, a major ethanol producer, in July 2007 formed a joint venture to produce PE from sugarcane. Japan's National Institute of Advanced Industrial Science and Technology is working together with industry and academia to build a production unit with an annual output of one million tons of bioethanol-based ethylene and propylene. China Petrochemical Corporation also plans to set up a process for production of PE from bioethanol. This shows that bio-based PE has a strong competitive edge in the market. Bio-based PE and petroleum PE are both made from high-grade ethylene; thus they have the same nature. Different reaction processes can be used to produce different products. These products include low-density PE (LDPE), LLDPE, medium-density PE (MDPE), high-density PE (HDPE), ultrahigh molecular weight PE, modified PE, and ethylene copolymers (Dewart and Everaert 1998; Safaa and Tarun 2008; Ana et al. 2008). The PE can be used for the production of film, hollow products, fibers, and daily-use products such as sundries by the commonly used blow molding, extrusion, injection molding, and other processing methods.

4.1 Bio-Based Polyethylene Used in Films

More than 60% of LDPE production is used for thin films produced by blow-molding. These films have good transparency and a certain degree of tensile strength. They are widely used in a variety of packaging materials for agricultural products, processed fish and meat produce, clothing, medicine, chemical fertilizers, and industrial products. Composite film produced by the extrusion process is used for packaging heavy objects. Since 1975, HDPE film products have been developed because of their excellent advantages such as high-intensity, low-temperature resistance, moisture-proof properties, printability and good mechanical property.

The largest use of LLDPE is also for thin films. Its strength and resilience are better than those of LDPE, and it also has better puncture resistance and rigidity. Although the transparency is poor, it is still slightly better than that of HDPE. A PE coating can be squeezed into several forms such as paper, foil, or plastic film, what can be made into polymer composites (Baann and Lindahl 2001).

4.2 Bio-Based Polyethylene Used in Pipe Plate

Pipes produced by squeezing HDPE and LDPE have many advantages, such as flexibility, corrosion resistance, easy installation, and structure stability. The pipes are widely used in many areas, such as water supply and drainage, gas, heat supply, wire- and cable-protecting tube, agricultural water-saving engineering, industrial sewage, and mineral transportation. PE pipes have become second largest plastic pipe category, behind PVC pipes.

4.3 Bio-Based Polyethylene Used in Fiber

PE fiber is named Yilun in China. Low-pressure PE is usually the raw material, and the spinning process transfers PE into the fiber. Yilun is mainly used as fishing nets, ropes, and so on. Ultra-high-strength PE fiber (strength of up to 3–4 GPa) has been developed. This kind of high-strength PE fiber can be used as a composite for bullet-proof vests and automotive and marine work.

4.4 Bio-Based Polyethylene Used in Hollow Products

Hollow containers, manufactured by the blow-molding process, is the main usage of HDPE. Large hollow and barrier containers are currently the main products produced by the hot spot process. High molecular weight and HDPE with a high melt viscosity and a small vertical stretch effect is needed to meet the requirement of the heavy weight of the large-scale hollow container's roughness in the processing of blowing. The use of high molecular weight and HDPE not only solves the problem of processing described above, but also endows the products with many advantages, such as great strength, excellent impact performance, high ESCR (environmental stress crack resistance) and high-intensity stack ability. For barrier containers, although HDPE exhibits lots of advantages, including good water barrier properties, its barrier performance for some gases, such as O_2 and CO_2, and polar hydrocarbon solvents is very poor. When HDPE is used in the storage of food, there is a risk of food spoilage and loss of taste, and storage of chemicals such as hydrocarbon solvents can result leakage of solvent. Also, HDPE used in the storage of toxic liquid could have contaminated the environment. Therefore, to compete with the markets for metals and glass materials, it is necessary to further improve HDPE's resistance to permeation performance for all kinds of materials; this is also one of the most active subjects studied regarding HDPE hollow containers.

5 Concluding Remarks and Future Perspectives

Bioethylene and green PE is one of the successful biorefinery processes. To compete with PE produced from oil resources, the green PE process must be improved and developed continuously. The efficient process improvement requires much knowledge and technology so that ethanol can be manufactured at low-cost from nonfood resources; improvements are especially needed in the areas of cellulose pretreatment technology, fundamental ethanol dehydration chemistry, process and equipment development, the performance enhancement of downstream products, and so on. The successful operation of green PE industrial equipment has opened up a new era for bio-based materials, and will accelerate the quick development of the biorefinery industry. The experience developed during this process will be very important for the utilization of biorenewable resources.

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Microbial 1,3-Propanediol, Its Copolymerization with Terephthalate, and Applications

Hongjuan Liu, Xianjin Ou, Sheng Zhou, and Dehua Liu

Contents

Abstract Poly(trimethylene terephthalate) (PTT) fibers, as a new type of polyester, are characterized by much better resilience and stress/recovery properties than poly(ethylene terephthalate) (PET) and poly(butylene terephthalate) (PBT). PPT chains are much more angularly structured than PET and PBT chains and such chains can be stretched by up to 15% with a reversible recovery (Ward et al. 1976). These properties make PTT highly suitable for uses in fiber, carpet, textile, film, and engineering thermoplastics applications. 1,3-Propanediol (PDO), as one of the polyester raw materials for PTT, has also attracted interest.

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In the 1990s three technical processes for the production of PDO were developed. The first process used acrolein, and was developed by Degussa-Hüls; this technology was sold to DuPont in 1997. The second was developed by Shell with ethylene oxide as the substrate. These two processes are all classic chemical processes. Recently, the biological process of PDO production with glycerol or glucose as raw materials in one or two steps has been considered as a competitor to the traditional petrochemical routes. It has advantages such as mild reaction conditions, good selectivity of product, environmental friendliness, and use of a renewable feedstock. With the development of biotechnology, especially gene manipulation technology, microbial PDO has prospects for the production of PTT.

1 Introduction to 1,3-Propanediol

1,3-Propanediol, well known as PDO, is an organic compound with the formula $HOCH_2CH_2CH_2OH$. Usually PDO is colorless, has a slightly sweet taste, and is a viscous liquid which is easily miscible with water and ethanol. More details on the physical properties of PDO are shown in Table 1.

PDO has been known by for more than 100 years, and has been applied widely in many industrial applications, such as composites, adhesives, laminates, coatings, moldings, aliphatic polyesters, and antifreeze. From the 1990s, more and more efforts were paid to the preparation of PDO on a large scale because of its attractive potential application in the synthesis of fibers of poly(trimethylene terephthalate) (PTT), which is thought of as another promising compound compared with its similar counterparts poly(ethylene terephthalate) (PET) from the 1950s and poly(butylene terephthalate) (PBT) from the 1970s. PTT was considered as one of six new chemicals in 1998 in the USA.

2 PDO Production by Chemical Methods

All the chemical processes for the preparation of PDO have a similar intermediate, 3-hydroxypropionaldehyde, which is chemically synthesized using different chemical catalysts with a variety of feedstocks, and then is reduced to PDO using hydrogen

Table 1 Properties of 1-3-propanediol (Daubert and Danner 1989)

Properties	Values	Properties	Value
Molecular mass	76.10 Da	Viscosity	52 cP (20°C)
Boiling point	214.4°C (101.3 kPa)	Density	1.0526 g/cm (20°C)
	103.0°C (1.33 kPa)	Flash point	ASTM D-92 79°C
Melting point	−26.7°C	Rate of evaporation	0.016 (nBuOAc =1)
Refractive index	1.4386 (25°C)	Surface tension coefficient	46.2 mN/m

under chemical catalysis. This step is usually easy compared with the preparation of 3-hydroxypropionaldehyde. Two processes which have industrial applications have been developed by Degussa using propylene and Shell using ethylene oxide, respectively. Some other methods have also been reported.

2.1 Degussa Process Using Propylene as a Feedstock

This process consists of a three-step reaction: propylene is oxidized to acrolein, acrolein is hydrated to 3-hydroxypropionaldehyde, which then is reduced to PDO (Arntz 1991).

1. Oxidation of propylene to acrolein

$$CH_2{=}CHCH_3 + O_2 \rightarrow CH_2{=}CHCHO$$

2. Selective hydration to 3-hydroxypropionaldehyde

$$CH_2{=}CHCHO + H_2O \rightarrow HOCH_2CH_2CHO$$

3. Catalytic hydrogenation to PDO

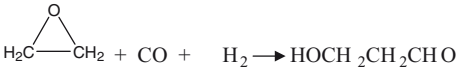
$$HOCH_2CH_2CH_2OH + H_2 \rightarrow HOCH_3CH_2CH_2OH$$

The key step in this process is the hydratation of acrolein, where various by-products are generated, and the quality of PDO is dependent on the hydroxylation of acrolein. So a high-performance catalyst which can selectively hydrate acrolein to form 3-hydroxypropionaldehyde is important for the industrial application of this process. Meanwhile, the instability and toxicity of acrolein are negative factors in this process (Fig. 1).

2.2 Preparation of PDO Using Ethylene Oxide as a Feedstock

This route consists of a two-step reaction. Firstly, ethylene oxide is carbonylated with carbon monoxide and hydrogen to form 3-hydroxypropionaldehyde. 3-Hydroxypropionaldehyde is purified and then reduced to PDO by hydrogen. The chemical catalysts used in the route are important for the selectivity and yield of the products, especially in the first reaction. The availability on a large scale with low cost and stability of ethylene oxide merits the use of this route, but the high cost of equipment and difficulty in preparing the catalysts hamper its industrial applications (Slaugh et al. 1995, 2001).

(1) Hydroformylation of ethylene oxide to 3-HPA



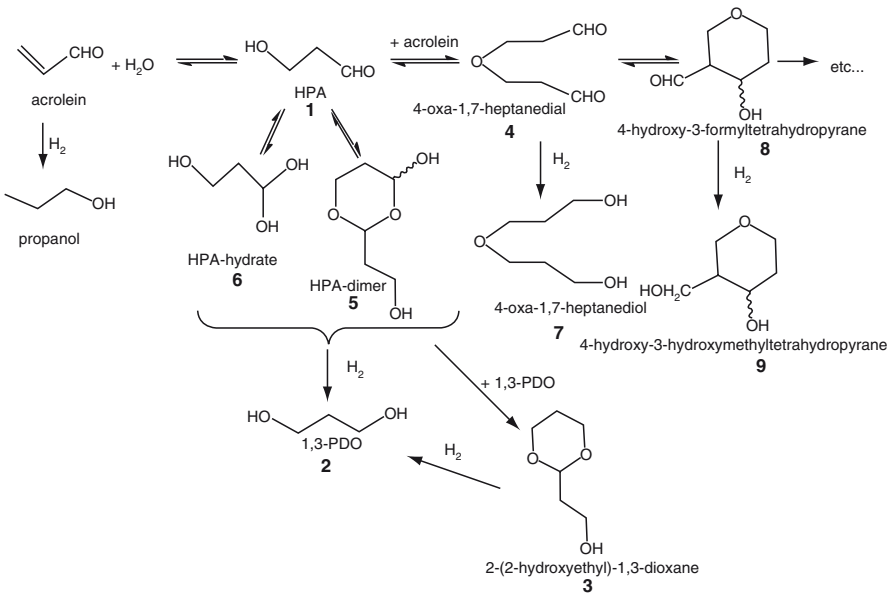


Fig. 1 Reactions involved in the process of the preparation of 1,3-propanediol (PDO) from propylene

(2) Catalytic hydrogenation to PDO

$$HOCH_2CH_2CHO + H_2 \rightarrow HOCH_2CH_2CH_2OH$$

2.3 Preparation of PDO via Selective Dehydroxylation of Glycerol

PDO can also be prepared by reductive dehydroxylation of the second carbon of glycerol, which includes three steps: acetalization, tosylation, and detosyloxylation. Firstly, glycerol is acetalized with benzaldehyde, a preferred hydroxyl group protection reagent, to protect the first and third hydroxyl groups of glycerol. Then the second conversion is the tosylation of acetalized glycerol at the first and third hydroxyl groups into 5-hydroxy-2-phenyl-1,3-dioxane, to transform the hydroxyl group into an easy-leaving state (a tosyloxyl group in this case). The final step of the conversion is a detosyloxylation reaction followed by a hydrolysis reaction to remove the tosylated hydroxyl group of glycerol and release the protecting reagent (Fig. 2) (Wang et al. 2003).

One problem with this process is the by-product generated in the first step, which will lower the yield of desired compounds and add to the difficulty of operations.

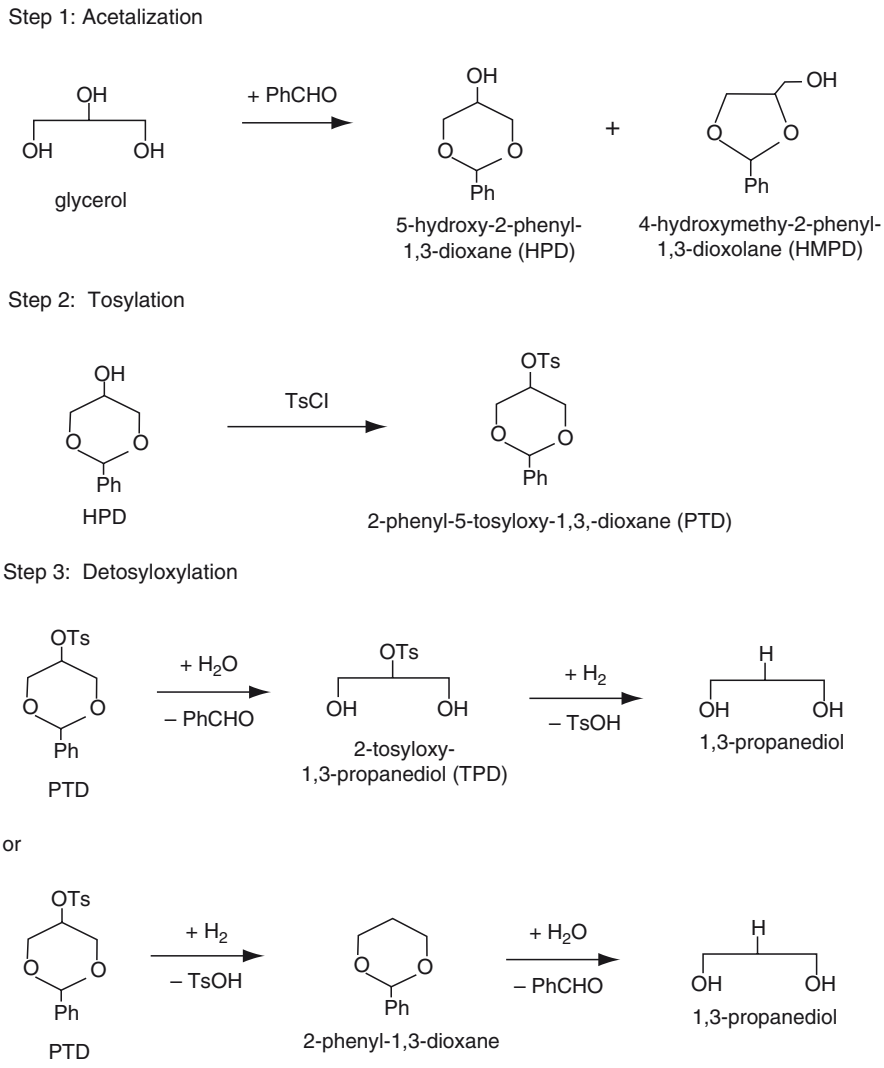


Fig. 2 Reactions involved in the selective dehydroxylation of glycerol to PDO

2.4 Other Processes Reported for the Preparation of PDO

Formaldehyde and acetaldehyde easily undergo aldol-condensation reactions to form 3-hydroxypropionaldehyde using KOH as a catalyst, and then aluminum isopropoxide catalyzes the reduction of 3-hydroxypropionaldehyde to PDO after removal of KOH via an ion-exchange resin. This route was firstly reported in a patent from India. The formaldehyde and acetaldehyde feedstocks used in this method

are cheap and are available on a large scale. But the yield and selectivity of the condensation reaction of formaldehyde and acetaldehyde need to be proved, and the cost of the catalyst is another factor that needs to be taken into account (Malshe 1999).

3 PDO Production by Microbial Fermentation

Today a bioprocess for the preparation of an industrial chemical is an attractive option compared with a traditional chemical process. The same is valid for preparation of PDO, and the biosynthesis process is attracting more and more attention because of the relatively low cost, mild reaction conditions, and environmental friendliness.

3.1 Microorganisms and the Metabolic Pathway

As early as the nineteenth century, it was found that microorganisms can generate PDO by metabolizing glycerol. Only a narrow range of microorganisms are able to produce PDO by fermenting glycerol, including *Klebsiella pneumoniae*, *Enterobacter agglomerans*, *Citrobacter freundii*, *Clostridium acetobutylicum*, *Clostridium butyricum, Clostridium pasteurianum*, *Lactobacillus brevis*, and *Lactobacillus buchneri* (Zeng et al. 1994; Barbirato et al. 1995; Schutz and Radler 1984; Boenigk et al. 1993; Biebl and Marten 1995; Forsberg 1987). These bacteria belong to different families and genera (Fig. 3).Among them, more attention has been paid to *K. pneumoniae, C. freundii*, and *C. butyricum* because of their higher PDO yield and productivity in PDO production. In addition, *Aspergillus niger* and *Aspergillus oryzae* have also been reported to be able to produce PDO from glycerol, but a very low production capacity was obtained (Yuan et al. 2006)

The production of PDO from glycerol is generally performed under anaerobic conditions in the absence of other exogenous reducing equivalent acceptors (Laffend et al. 1997). The PDO pathway is a dismutation process (Fig. 4). Through the oxidative pathway, glycerol is dehydrogenated by an NAD^+-linked glycerol dehydrogenase to dihydroxyacetone, which is then phosphorylated by PEP- and ATP-dependent dihydroxyacetone kinases. Through the parallel reductive pathway, glycerol is dehydrated by the coenzyme B_{12} dependent glycerol dehydratase to form 3-hydroxypropionaldehyde. 3-Hydroxypropionaldehyde is then reduced to the major fermentation product PDO by the $NADH_2$-linked PDO dehydrogenase, thereby regenerating NAD^+ (Fig. 3).

The PDO production is regulated by the *glp* regulon and the *dha* regulon (Zeng and Bieb 2002). The gene expression in the *dha* regulon can be induced by dihydroxyacetone (Forage and Lin 1982). The genes coding for the enzymes of glycerol metabolism, i.e., glycerol dehydratase (*dhaB*, *dhaC*, *dhaE*), PDO oxidoreductase (*dhaT*), glycerol dehydrogenase (*dhaD*), dihydroxyacetone kinase (*dhaK*), and a

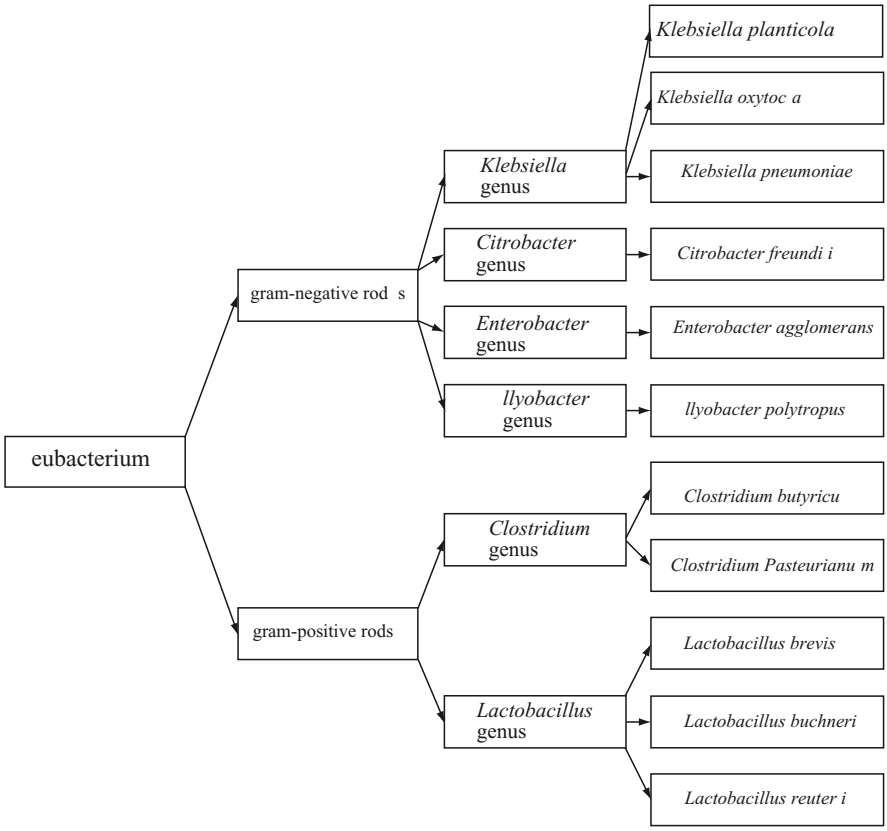


Fig. 3 The classification chart of wild-type bacteria reported in PDO production

putative regulatory gene (*dhaR*), have been cloned and sequenced (Fig. 5) (Sprenger et al. 1989; Tong et al. 1991; Tobimatsu et al. 1996; Daniel and Gottschalk 1992; Seyfried et al. 1996).

It has also been reported that, in the presence of oxygen, glycerol is metabolized through a completely different pathway regulated by the *glp* regulon. The glycerol is first converted to glycerol 3-phosphate under the catalysis of glycerol kinase, and then glycerol 3-phosphate is oxidized to dihydroxyacetone phosphate by a dehydrogenase. The *glp* regulon is constituted from six genes, including *glpFK* (membrane protein and glycerokinase), *glpTQ* (glycerophosphodiester phosphodiesterase), *glpABC* (anaerobic *sn*-glycerol 3-phosphate dehydrogenase, and *glpD* (*sn*-glycerol 3-phosphate dehydrogenase). The regulator gene *glpR* is near to *glpD*, which encodes an inhibitor of 33 kDa. The genes *glpE* and *glpG* are located between *glpD* and *glpR*; however, the function of the former two genes is not clear (Forage and Lin 1982; Koch et al. 1964; Ruch and Lin 1975).

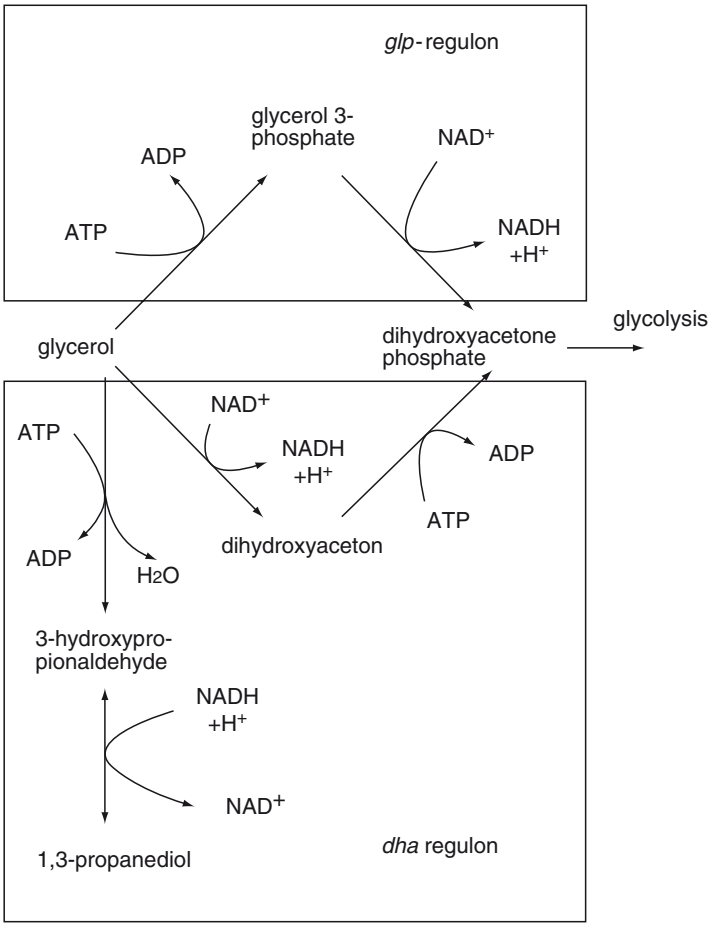


Fig. 4 Glycerol metabolic pathway in *Klebsiella pneumoniae*

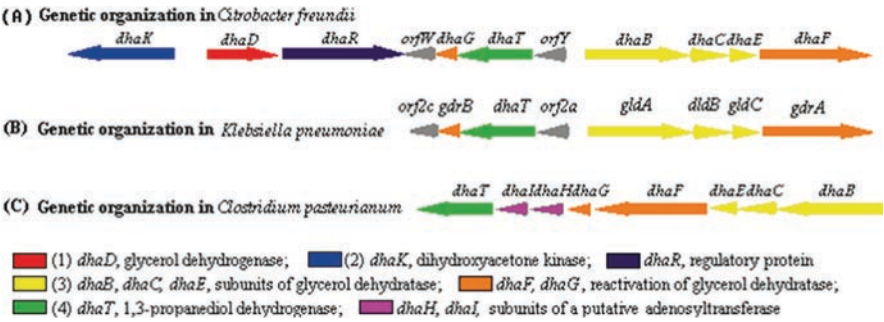


Fig. 5 Structure and function of genes in the *dha* regulon

In addition to these native PDO producers, recombinant organisms have also been constructed to enhance PDO production with the development of recombinant DNA technology. The main effective methods for genetic modification are decribed next.

3.1.1 Gene Overexpression of Key Enzymes

Glycerol dehydratase, as an example of a key enzyme, was demonstrated to be a rate-limiting enzyme (Abbad Andaloussi et al. 1996; Ahrens et al. 1998) in the PDO biosynthesis pathway, and is encoded by the three structural genes *gldA*, *gldB*, and *gldC* in the case of *K. pneumoniae* (Tobimatsu et al. 1996) and *dhaB*, *dhaC*, and *dhaE* in the case of *C. freundii* (Seyfried et al. 1996). Overexpression of the glycerol dehydratase gene is expected to enhance PDO production.

3.1.2 Knocking Out Genes Responsible for the Formation of Undesired By-Products

During the production of PDO, the synthesis of by-products such as acetic acid, lactic acid, and ethanol will decrease the yield of PDO. On the other hand, a high concentration of accumulated by-product, such as lactic acid, will also lead to difficulties in extracting PDO. So, blocking the synthesis of the by-products would be a good way to increase the PDO yield.

3.1.3 Strain Construction To Produce PDO from Glucose Directly

Lots of work has been done to construct a strain to utilize glucose because glucose as a substrate is much cheaper than glycerol in PDO production. However, there is no microbial wild-type strain has been found capable of converting glucose directly to PDO by now.

The conversion of glucose to PDO requires the combination of two natural pathways: glucose to glycerol and glycerol to PDO. The best natural pathways for the production of glycerol are found in yeast. *Saccharomyces cerevisiae* produces glycerol from the glycolytic intermediate dihydroxyacetone 3-phosphate using two enzymes: dihydroxyacetone 3-phosphate dehydrogenase and glycerol 3-phosphate phosphatase. The natural pathway for the production of PDO from glycerol requires two enzymes: glycerol dehydratase and PDO dehydrogenase. To construct a single organism to produce PDO from glucose, one could insert the glycerol pathway into a natural PDO producer, or the PDO pathway into a natural glycerol producer. Although both of these approaches seem simple and direct, there are problems involving natural regulation of the pathways. The third way is to build both pathways into a host that could do neither step (Fig. 6). DuPont has successfully constructed strain of *Escherichia coli* containing the genes from *S. cerevisiae* for glycerol production and the genes from *K. pneumoniae* for PDO

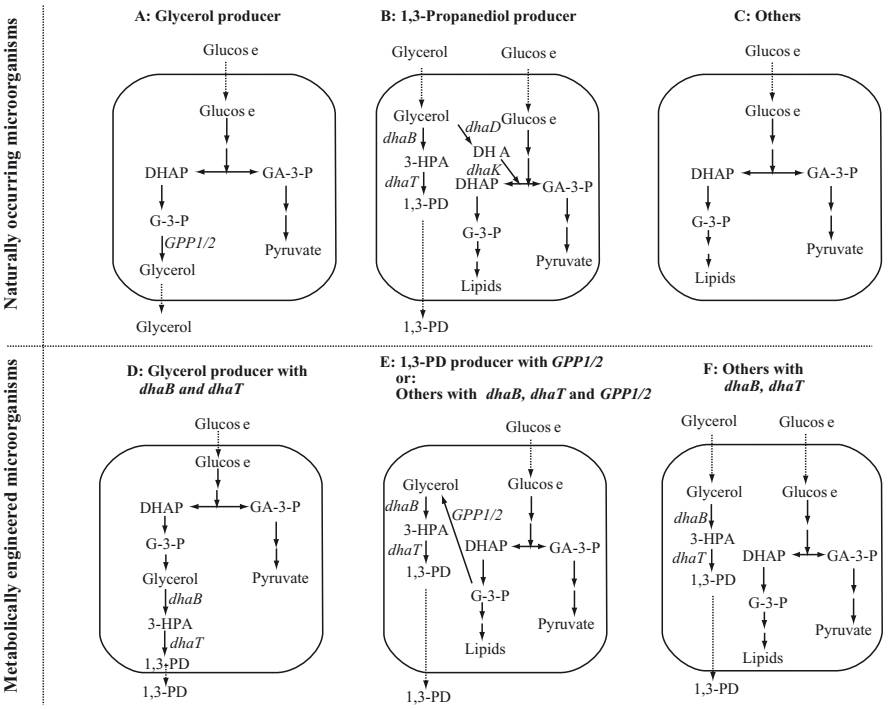


Fig. 6 Construction of recombinant microorganisms producing PDO from glucose (Biebl et al. 1999)

production. The final concentration of PDO produced by engineering *E. coli* reached 135 g/L (Nakamura and Whited 2003) using glucose as a feedstock. *E. coli* provides several advantages of other systems. *E. coli* is closely related to *K. pneumoniae* and *C. freundii*, but it does not naturally produce glycerol or PDO, and there is no natural regulation to overcome. *E. coli* is the most completely studied organism and its metabolism and physiological characteristics are well characterized. A large number of metabolic mutants have been constructed and analyzed. In addition, *E. coli* has been used in large-scale fermentations and production on an industrial level.

3.2 Fermentation Technology

PDO can be produced by microorganisms by fermentation and some by-products were obtained, such as lactic acid and 2,3-butanediol. The metabolite pathway of glycerol fermentation under anaerobic conditions and the fermentation product are described in Fig. 7.

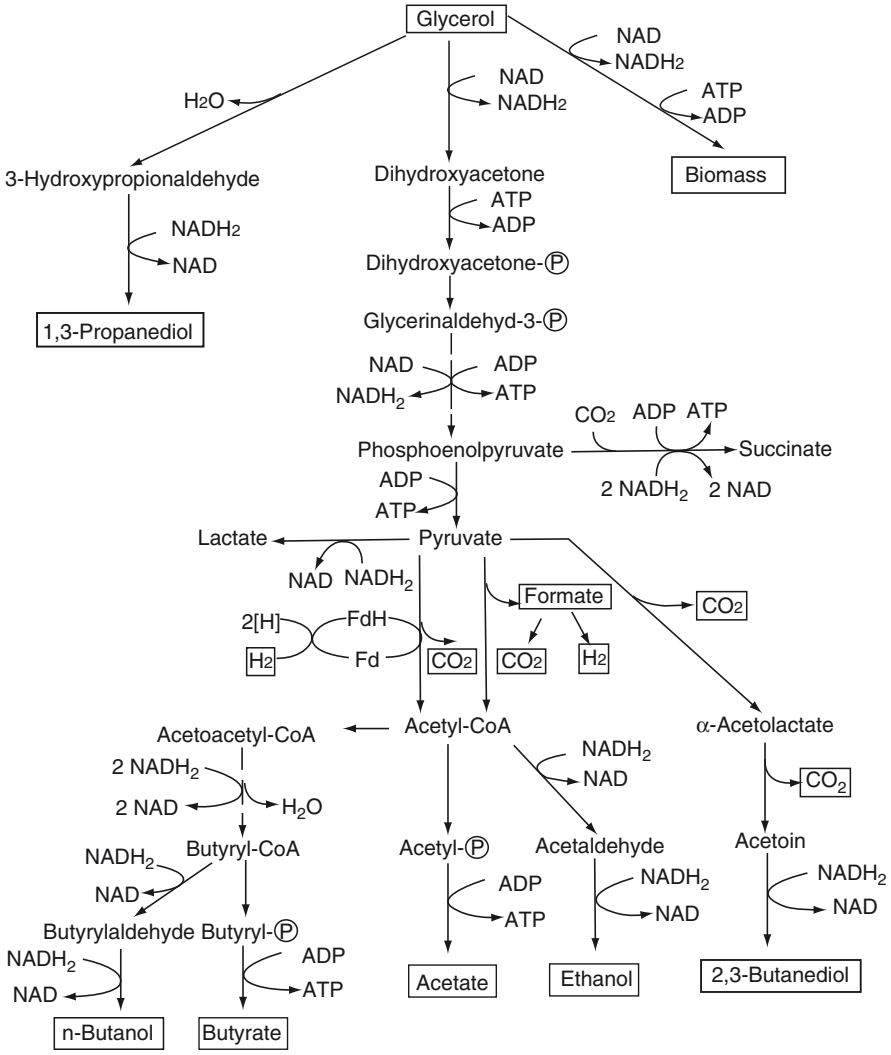


Fig. 7 Biochemical pathways of glycerol fermentation under anaerobic condition

Generally, the maximum concentration of PDO was obtained in batch and fed-batch cultures. Continuous culture has the advantage of relatively high productivities, but the PDO concentration was lower than that of batch fermentation. Günzel et al. (1991) studied the batch fermentation of PDO by *C. butyricum* DSM in a 2-L whisk and a 1.2-L airlift fermenter; the PDO concentration was 50–58 g/L. Cameron et al. (1998) studied the fed-batch fermentation in a 5-L fermenter with *K. pneumoniae* ATCC 25995; a PDO concentration and productivity of 73.3 g/L and 2.3–2.9 g/(Lh) were obtained. Menzel et al. (1997) studied the continuous fermentation of

glycerol by *K. pneumoniae* DSM 2026 in a 2-L fermenter; a higher productivity of 4.9–8.8 g/(Lh) was obtained, but the PDO concentration was just 35.2–48.5 g/L.

To enhance the PDO production and decrease the production cost, some new technology has been developed in the past few decades.

3.2.1 Micro-Aerobic Fermentation of PDO

The conventional PDO fermentation was performed under anaerobic conditions. However, recently, many investigations have focused on optimizing the fermentation conditions as well as exploring the metabolic mechanism in *K. pneumoniae* under micro-aerobic or mild aerobic conditions (Wang et al. 2001; Huang et al. 2002; Chen et al. 2003). In comparison with anaerobic cultivations, microbial production of PDO under micro-aerobic conditions has attracted much attention owing to low equipment investment and power consumption on an industrial scale. Under aerobic conditions, the conversion is mainly catalyzed by the *glp* system (Forage and Lin 1982). Cheng et al. (2004) compared different aeration strategies in the fed-batch fermentation of PDO by *Klebsiella oxytoca*; a PDO concentration of 69.6 g/L was obtained in a 5-L fermenter with an anaerobic–aerobic two-stage strategy. Liu et al. (2007) studied the PDO fermentation from glycerol by *K. pneumoniae* under micro-aerobic conditions and the final PDO concentration, molar yield, and volumetric productivity were 72 g/L, 57%, and 2.1 g/(L h), respectively. In the study of Zheng et al. (2008), the PDO concentration reached 74.07 g/L, and a high PDO yield and productivity of 0.62 mol/mol and 3.08 g/(L h), respectively, were obtained using *K. pneumoniae* under aerobic conditions.

3.2.2 PDO Production Using Glucose as an Auxiliary Substrate

Glucose, as a cheap carbon source, is often used as a hydrogen-donor substrate instead of the fraction of glycerol to provide both reducing equivalents for PDO formation and ATP for biomass. A higher yield of PDO to glycerol was obtained by using glucose as a cosubstrate. Abbad Andaloussi et al. (1995) studied the variation of carbon atoms and electronic flow of the PDO continuous fermentation of *C. butyricum* DSM 5431 by using glucose as the auxiliary substrate; the PDO yield increased from 57 to 92% compared with when glycerol was used as a substrate. In the study of Biebl and Marten (1995), a high concentration of glucose in the culture medium appeared to strongly inhibited the glycerol dehydrogenase and PDO dehydrogenase activities involved in the production of PDO (Malaoui and Marczak 2001).

3.2.3 PDO Production by Crude Glycerol

To enhance the yield of PDO and decrease the cost of production, strategies involving the utilization of raw materials by cells capable of resisting the impurities in the medium have been utilized (Papanikolaou et al. 2000; Barbirato et al. 1998) Raw,

unpurified glycerol, which does not need further purification, has been used in this type of fermentation. For example, a large quantity of low-cost by-product crude glycerol is produced in biodiesel production. Conversion of glycerol into higher value-added products such as PDO can decrease the production cost and it is an important process to integrate with biodiesel production. Tsinghua University studied the PDO fermentation by the genetically modified strain HR526 with the by-product glycerol of biodiesel; the PDO concentration reached 106 g/L. In 2008, the demonstration was finished in the pilot plant and a facility with capacity of 4,000 ton/year PDO is running at Hunan Rivers Bioengineering Company, China (Xu et al. 2009).

3.2.4 Using Glucose as the Substrate To Produce PDO

There has been growing interest in a more economical route that utilizes glucose as a lower-cost feedstock. The process was realized either by two-stage fermentation and mixed culture, or by recombinant microorganisms, because there is no natural microorganism that can directly convert glucose into PDO by now. Haynie and Wagner (1996) studied the mixed culture of yeast producing glycerol and bacteria producing PDO by using glucose as a substrate, but because of the different culture conditions and the formation of PDO as a result of glucose concentration inhibition, the mixed culture is not the best option. The two-stage fermentation technology has been studied to convert glucose into PDO. The first step is to use recombinant *E. coli* or yeast to convert glucose into glycerol; the second step is to convert the glycerol converted in the first step to PDO (Huang et al. 2002). Also, as mentioned already, DuPont developed a metabolically engineered organism that could produce PDO at a rate of 3.5 g/(L h), with concentration of 135 g/L and a yield of 0.62 mol PDO/mol glucose.

3.3 Separation and Extraction

PDO produced via fermentation contains residual organic impurities such as water, glucose, organic acids, salts, glycerol, and other compounds. Isolation and purification of PDO from a fermentation source therefore has significant and unmet challenges. Given the high boiling point and hydrophilicity of PDO, economic separation of PDO from those contaminants is difficult. The downstream processing of biologically produced PDO usually includes three main steps as shown in Fig. 8.

The first step is the separation of microbial cells, mostly by using membrane filtration or high-speed centrifugation, including pretreatment such as adjusting the pH with a base or adding a flocculant (e.g., chitose or synthetic cationic flocculants based on polyacryamide) into the broth. Flocculation precipitation on an industrial scale attracts attention owing to its simplicity if cheap and effective flocculants are available. Chitosan and polyacrylamide have been tested for this purpose. By combined use of chitosan and polyacrylamide at optimal concentrations of 150 and 70 ppm, respectively, the soluble

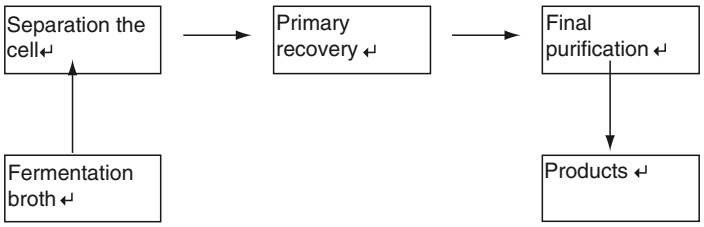


Fig. 8 General process for the PDO separation and purification from the fermentation broth

protein in the broth decreased to 0.06 g/L, and the recovery ratio of the supernatant liquor to the broth was greater than 99% (Hao et al. 2006). The second step is the removal of impurities and primary separation of PDO from the fermentative broth. The last step is final purification of PDO by vacuum distillation and/or preparative liquid chromatography. Many methods have been studied, for example, using evaporation for removal of water, ethanol, and acetic acid, electrodialysis for desalination, alcohol precipitation, and dilution crystallization for removal of proteins and salts, solvent extraction and reactive extraction, ion-exchange chromatography, adsorption with activated charcoal or a molecular sieve, and pervaporation with a zeolite membrane (Roturier et al. 2002; Wilkins and Lowe 2004; Adkesson et al. 2005; Cho et al. 2006; Malinowski 1999; Ames 2002; Sanz et al. 2001; Gong et al. 2004; Malinowski 2000; Hao et al. 2005, 2006) Although several methods have been developed for the separation and purification of PDO, disadvantages also exist. For example, the energy consumption of evaporation and distillation is high; the loss of PDO, leading to a low PDO yield during electrodialysis and the membrane pollution, can be very serious; and the energy consumed using chromatography is even higher than that consumed using simple evaporation and distillation because the PDO solution is diluted and the resin or adsorbent has low selectivity and capacity. Many extractants have been studied for liquid–liquid extraction of PDO but no effective extractant has been found for hydrophilic PDO. The process of reactive extraction is complicated and the trace amount of aldehyde in PDO is prohibitive for polymerization of PTT, which is difficult to control.

The methods and technologies studied so far have their limitations or drawbacks in terms of yield and energy consumption. For further development, classic separation techniques need to be improved or combined with other new technologies for PDO to be produced on a large scale.

4 PTT Production with PDO

PTT was first patented in the 1940s, but the high costs of production of high-quality PDO, the starting raw material for PTT, became a hindrance to industrialization of PTT. It was not until the 1990s that commercial production of PTT was possible,

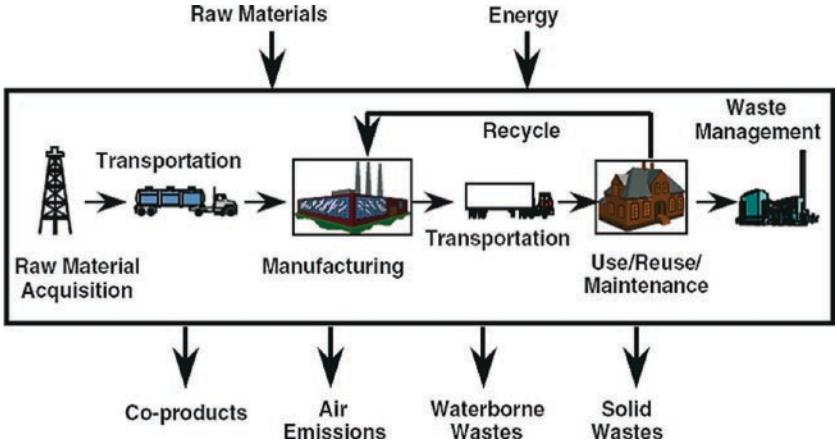


Fig. 9 Product life and environmental effects of poly(trimethylene terephthalate) (PTT) (Kurian 2005)

when Shell Chemicals developed a method of producing PDO at low cost. With the development of microbial PDO production, the PTT synthesized with microbial PDO has received much attention from researchers.

Sorona, a PTT polymer produced by DuPont with microbial PDO, can be easily modified to achieve desirable functional properties as well, i.e., excellent physical and chemical properties, dimensional stability, low moisture absorption, easy care, good weather resistance, easy processability, and recyclability. It has been shown to have advantages over other polymers, from the raw material to ultimate disposal when considering the effect on the environment (Fig. 9). For example, greenhouse gas emission in the manufacture of bio-PDO has been demonstrated to be about 40% less than for petrochemical PDO; the overall PTT polymerization process is more energy efficient than for PET; polymerization and downstream processing of Sorona uses less energy compared with PET owing to the lower temperatures required, both for processes involving remelt and for dyeing; and recycling of Sorona is made much easier by the absence of heavy metals in the product, compared with PET and nylon (Kurian 2005).

Therefore, PTT produced with microbial PDO will have promising applications in the near future in fibers for apparel and carpets, films, and engineering components, which will significantly enlarge the scale of PDO production.

4.1 Introduction to PTT

PTT is a semicrystalline polymer synthesized by the condensation of PDO with either terephthalic acid or dimethyl terephthalate. The unique, semicrystalline molecular structure of PTT features a pronounced “kink”, as shown in Fig. 10

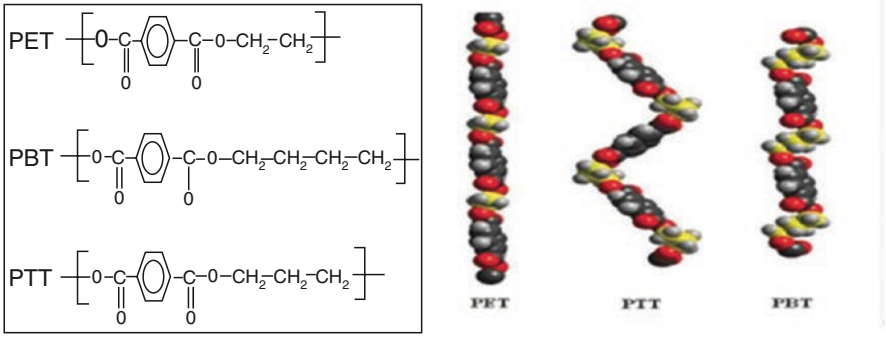


Fig. 10 Molecular formulas and semicrystalline structure differences of poly(ethylene terephthalate), PTT, and poly(butylene terephthalate)

(Hu et al. 2008), which gives serious beneficial properties to PTT compared with PET and PBT.

The molecular structure of a zigzag shape can translate tensile or compressive forces at a molecular level to bending and twisting of bonds rather than simply stretching, which is analogous to the tensile behavior of a coiled spring compared with a straight wire. So PTT shows better stretch-recovery characteristics than other traditional polymers. Also other properties of PTT are between those of PET and PBT, such as the crystallization rate and glass-transition temperature. Hence, engineering plastics is probably an important application field of PTT.

4.2 The Production of PTT

PTT is a semicrystalline polymer synthesized by the condensation of PDO with either terephthalic acid or dimethyl terephthalate, followed by polymerization. Studies of PTT had never gone beyond academic interest until recent years because one of its raw materials, PDO, was very expensive and available only in a small volume. PTT received less attention compared with PET and PBT. However, recent breakthroughs in PDO synthesis made PTT available in industrial quantities, thus offering new opportunities in carpet, textile, film, packing, and engineering thermoplastics markets.

The polymer production is similar to the PET synthesis and involves direct esterification and ester-interchange polymerization (Fig. 10). For PTT, Shell Chemicals developed a low cost method of producing high-quality PDO, the starting raw material for PTT. There are two routes to synthesize PTT, namely, the transesterification of dimethyl terephthalate with PDO and the esterification route with terephthalic acid and PDO. In the first stage of polymer synthesis, terephthalic acid or dimethyl terephthalate is mixed with PDO to produce oligomers having one to six repeat units with the help of a catalyst. In the second stage ,this oligomer is

polycondensed to a polymer with 60–100 repeat units. The catalyst used in the first step also accelerates the polycondesation reaction. Generally, this objective can be fulfilled by two methods. The first is the use of a lower process temperature, which reduces the processing time in the melt phase to a minimum and keeps oxygen out completely. The second way involves selecting a sufficient amount of a catalyst and adding stabilizers such as phosphorus compounds or sterically hindered phenols.

4.3 The Properties of PTT Made from PDO

Although PTT was first synthesized by Whinfield and Dickson in 1941, it was never commercialized because of the lack of an economical source of trimethylene glycol monomer. Recently, Shell Chemicals announced the development of a technology to make trimethylene glycol economically via the hydroformylation of ethylene oxide. With a cheap monomer source, it is now possible to commercialize PTT at a competitive price. PTT has an odd number of methylene units between the terephthalate moieties, in contrast to PET and PBT, which have even numbers of methylene units. PET molecules are more fully extended. Two carboxyl groups of each terephthaloyl group are situated in opposite directions. Also, all bonds are in the *trans* conformation, with successive phenylene groups at the same inclination along the chain. PTT differs from this conformation with bonds of the $OOO(CH_2)_3OOO$ unit having the sequence *trans–gauche–gauche–trans*, leading to the contraction of the repeating unit and, because of the opposite inclinations of successive phenylene groups along the chain, the molecule takes on an extended zigzag shape.

As a promising polymer, PTT's trimethylene units are organized in a highly contracted and helically coiled *gauche–gauche* conformation. PTT nanofibers exhibit high surface smoothness, length uniformity, and mechanical strength. PTT fiber opens up an avenue for novel optical fibers for wavelengths ranging from the visible to the near infrared, and they can be arbitrarily positioned, bent, intertwined, twisted, tensed, and assembled into different structures.

PTT's outstanding resilience, relatively low melt temperature, and ability to rapidly crystallize offer potential opportunities for its use in carpet, textile, film, packing, engineering thermoplastics, and other marketplaces, particularly those dominated by nylons, PET, and PBT. In addition, it is reported that the promising aromatic polyester PTT can be enzymatically hydrolyzed using diverse enzymes.

4.4 The Market and Applications for PTT

PTT was first synthesized in 1941, but owing to the high costs of high-quality PDO, one of the raw materials to produce PTT, it was not commercially available.

In 1998, PTT was finally introduced to the market by Shell Chemicals under the trade name Corterra, since an economical process for the production of PDO had been developed. PTT fibers are commercially produced today by DuPont and Shell. PTT has special characteristics as a fiber. It is particularly interesting in carpet fibers, where it has shown outstanding resiliency and chemical resistance. Also, this polymer shows potential in the field of engineering thermoplastic polymers and fabrics.

PTT was introduced as a commercial aromatic polyester polymer, joining others such as PET and PBT. This semicrystalline polymer is produced industrially by the condensation of PDO with either terephthalic acid or dimethyl terephthalate. The breakthroughs in PDO synthesis have made it available in industrial quantities, and this offers new opportunities for the carpet, textile, film, packing, and engineering thermoplastics markets.

The applications of PTT in the textile industry include filament yarns, staple fibers, and bulked continuous filament yarns for carpets. In blends with other synthetic fibers such as Lycra or natural fibers such as cotton, PTT enables a variety of end products to be produced that have a soft feel, good drape, and good stretch and recovery qualities (Carr et al. 1997; Yonenaga 2000; Dupeuble 2001; Kathiervelu 2002). One of the most recent applications of PTT is sewing thread which will endow clothing products with added value by appropriate extensibility, recovery, and dimensional stability. The extraordinary properties of PTT are very useful for sports and leisurewear as well as elastic interlinings and shirting fabrics. The wear performance of carpets made from PTT is equal to or better than that nylon, without the staining or cleaning problem. PTT has exhibited very good performance in apparel: softness, stretch, and brilliant lasting colors in both knit and woven fabrics, in hosiery and intimate apparel, linings, denim, swimwear, etc. Another application of PTT is upholstery because of its good stretch recovery, dye and print capability, stain resistance, and resiliency.

5 Outlook

Although polyester polymers have been known for a long time, the world polyester market is still growing. More and more new applications are under development. This has many causes. In contrast to many other polymers, recycling is easy. Polyesters contain only carbon, hydrogen, and oxygen. For these reasons it is likely that the use of PDO will grow at a high rate in the next few decades. The bio route is a very interesting future option. The realization of this route depends on the progress of process development to obtain fiber grade quality PDO and the carbohydrate to oil price ratio. If the whole cost of PDO production by the biological method were decreased to a level to compete with that of the chemical method, through development of the separation and fermentation technology, the PTT produced by microbial PDO would have good prospects.

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Microbial *cis*-3,5-Cyclohexadiene-1,2-diol, Its Polymer Poly(*p*-phenylene), and Applications

Guo-Qiang Chen

Contents

Abstract This chapter describes the production of cis-3,5-cyclohexadiene-1,2-diol (DHCD) from aromatic compounds, their polymerization into poly(p-phenyelene) (or PPP), and the properties and applications of the polymer. Large-scale synthesis of DHCD has been demonstrated, and DHCD is widely used in the pharmaceutical industry, as well as in chemical industries for polymer productions. Recent study including different types of dioxygenases, strain development by recombination, and genetical modification were done to develop the process technology for commercialization of this new polymer and chemical intermediates.

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1 Introduction

Research into new methods for the production of polymers is attractive mainly for two reasons. The first is the desire to improve the environmental impact of current polymer-producing processes by the use of more environmentally compatible reagents and milder conditions. The second is that the important polymer properties, such as chirality, temperature–structure profiles, electrical characteristics, optics, and biodegradability, are being investigated. These new methods will create new markets for polymers with entirely new properties and will also serve existing markets by the introduction of more environmentally acceptable processes. One of the most attractive methods is biotechnological, specifically biocatalytic, polymer synthesis, which is economical and environmentally compatible (Dordick 1992a, b; Ballard et al. 1994).

Novel monomers may be able to be synthesized from simple starting materials by biocatalysis, and subsequently polymerized into new materials with novel properties (Ballard et al. 1994). The common soil microorganism *Pseudomonas putida* is able to synthesize *cis*-3,5-cyclohexadiene-1,2-diol (DHCD) from benzene, catalyzed by the enzyme dioxygenase. Polymerization of DHCD produces polymers which are precursors for poly(*para*-phenylene) (PPP) synthesis (Ballard et al. 1983a, b, 1988). PPP and other polyaromatics are key materials with great potential in industries demanding high performance, such as electronics and aerospace. PPP can be considered as the ultimate polyaromatic since the product is composed entirely of phenylene rings. The intractability of this structure had precluded its synthesis in a way allowing useful applications (Ballard et al. 1994).

In this chapter, we will describe the biosynthetic approach to PPP from DHCD and the strain development to produce DHCD. Properties and applications of PPP, and the development of polyaromatics will also be considered.

2 Synthetic Approaches to PPP

Polyaromatics are polymers that formed by direct linkage between carbon atoms of aromatic rings. PPP is one of the most widely researched and used polyaromatics (Fig. 1). Owing to its novel properties, such as high tensile strength, high Young's modulus, and thermostability, PPP is considered to have high potential for applications in the electronic and aerospace industries.

PPP was first synthesized by Goldschmiedt (1886). He applied the Wurtz–Fittig reaction which coupled *para*-dibromobenzene using sodium, and the product was determined to be a tridecamer (13 subunits) by elemental analysis. In 1936, the same monomer was used and a PPP containing up to 16 benzene rings was obtained (Busch et al. 1936).

Owing to the inertness of benzene, it is not an easy task to synthesize PPP using traditional methods of fabrication. However, various syntheses of PPP have still been attempted since the 1950s.

The direct polymerization of benzene is definitely one route that can be followed. This technique was developed by Kovacic and others in the 1960s

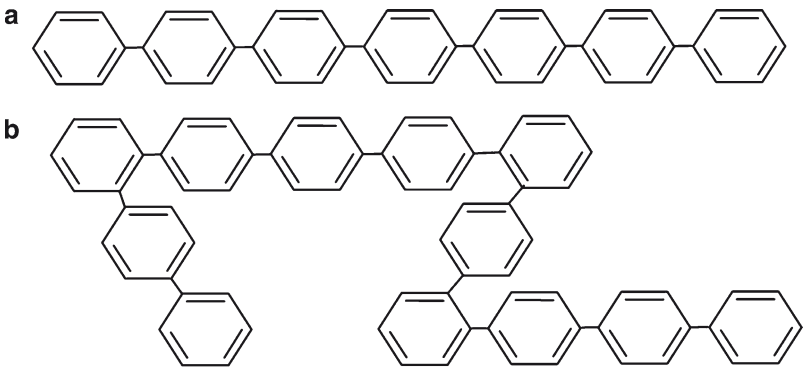
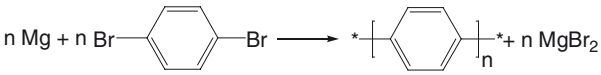


Fig. 1 Chemical structures of poly(*p*-phenylene) (PPP). **a** PPP ultimately composed of a 1,4 unit, **b** PPP containing a 1,2 unit

(Kovacic and Kyriakis 1962, 1963; Kovacic and Oziomek 1964; Speight et al. 1971). The process is known as oxidative cationic polymerization. It used $AlCl_3$ as a Lewis acid catalyst and $CuCl_2$ as an oxdidant. The polymer produced was a black material, containing a mixture of 1,2 (*ortho*) and 1,4 (*para*) units as well as chemical defects such as chlorination and intermolecular cross-links (Brown et al. 1986; Berresheim et al. 1999). The products should be more correctly defined as oligomers rather than polymers, as the chain lengths were between ten and 15 phenylene rings. And it was difficult to remove the $CuCl_2$. Nevertheless, this technique is still successful as a route to PPP (Kovacic and Jones 1987).

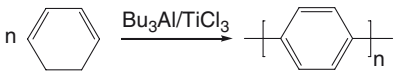
Kovacic's method was improved by Arnautov and Kobryanskii (2000) using an oxidative polycondensation route. The PPP obtained had a higher molecular weight (Sun et al. 2005).

A second synthesis option for PPP was developed by Yamamoto et al. (1978), in which *para*-dibromobenzene is polymerized using a nickel catalyst in the presence of magnesium (Scheme 1). This is an example of directly using Grignard chemistry to form a macromolecule. However, the result is still not ideal, since the growth of the chain length does not go beyond 10–12 phenylene rings.



(1)

An early attempt to use polymers of cyclohexadiene as a route to PPP was by Marvel and coworkers (Marvel and Hartzell 1959; Cassidy et al. 1965). This process involves the direct polymerization of cyclohexa-1,3-diene using a Ziegler catalyst (Scheme 2; Ballard et al. 1994).



(2)

Marvel et al. used 5,6-dibromocyclohexa-1,3-diene, which was converted into poly(5,6-dibromo-1,4-cyclohex-2-ene) and subsequently into PPP with elimination of

HBr (Berresheim et al. 1999). However, this method of synthesis has two main defects. First, the poly(cyclohexene) produced contains 1,4 and 1,2 units. Second, there are a number of bromo-substituted intermediates and thus the aromatization is incomplete.

Recently, Grubbs et al. improved this method by using trimethylsiloxy protecting groups and a nickel catalyst. The polyphenylene produced contained up to 96% of 1,4 units (Gin et al. 1991, 1992a, b, 1994a, b). Another improvement was made by Natori et al. (2006), in which soluble polyphenylene homopolymers with a controlled polymer chain structure were synthesized by the complete dehydrogenation of poly(1,3-cyclohexadiene) having 1,2 units and 1,4 units.

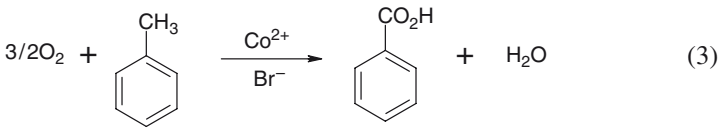
Although the route from cyclohexadiene to PPP is not mature enough, it offers an innovative approach which seeks to avoid the difficulties of direct fabrication of PPP, by creating a precursor polymer to be subsequently converted into PPP. Given this intent, one possible option is to use the appropriate derivative of cyclohexa-1, 3-diene that can be polymerized by a 1,4 addition reaction. Synthesis of such derivatives using organic chemistry has been studied but is not practical and cost-effective. However, the biological oxidation of benzene and its derivatives, which proceeds via a dihydroxylated cyclohexadiene intermediate, offers the potential for large-scale synthesis (Ballard et al. 1994).

3 Biocatalytic Production of *cis*-DHCD

Benzene *cis*-diol, namely, *cis*-DHCD, is a chiral chemical which is important for the pharmaceutical and chemical industries (Hack et al. 1994; Wahbi et al. 1996, 1997). It is one of the intermediates during biological aromatic oxidation in some microorganisms. Bacteria such as *P. putida* can be genetically modified to achieve the synthesis and highly efficient production of *cis*-DHCD.

3.1 Aromatic Oxidation in Microorganisms

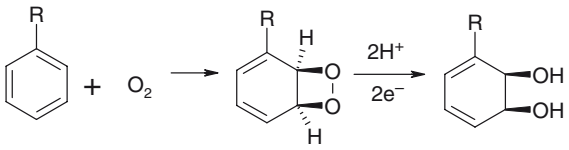
Aerobic microorganisms can utilize various aromatic compounds as carbon sources (Doelle 1975; Stanier and Ornston 1973). Oxygenase enzymes catalyze the first step of the degradation of aromatic substrates by functionalizing the benzenoid ring using O_2 as an oxdidant. The two major groups of oxygenases are monooxygenases and dioxygenases (Ballard et al. 1994).



(3)

The differences are unique between biological oxygenase reactions and classical organic chemical processes. As is shown in Scheme 3, the industrial process is used

to produce the aromatic dibasic acids from *ortho*-xylenes, *para*-xylenes, and *meta*-xylenes. A temperature between 150 and 200°C and oxygen under pressure are required. Owing to the stability of the aromatic ring, 98% of the product is benzoic acid. In contrast, oxidation by dioxygenase results in almost exclusive attack on the aromatic nucleus (Scheme 4), with the aromatic substrate dioxidized by addition of dioxygen. The product of this reaction has been shown to be *cis*-DHCD (Gibson 1990), which is without parallel in organic chemistry.



(4)

The *cis*-DHCDs are subsequently further degraded to central metabolic intermediates such as pyruvate and fumarate (Doelle 1975). This metabolism of aromatics via *cis*-DHCDs is a property of certain bacteria. Higher organisms and mammalian systems oxidize aromatics to *trans*-DHCDs with an entirely different reaction mechanism and do not provide options for synthesis of *cis*-DHCDs (Ballard et al. 1994).

3.2 Aromatic Dioxygenases

Since the 1960s, many aromatic dioxygenases have been discovered and identified from various bacterial species and they can catalyze reactions to obtain over 300 types of arene *cis*-diols (Gibson et al. 1970; Gibson and Parales 2000; Mason and Cammack 1992; Lipscomb et al. 2002; Bui et al. 2000; Reiner and Hegeman 1971; Subramanian et al. 1979, 1985). Dioxygenases can be grouped into benzene dioxygenase, toluene dioxygenase (TDO), and benzoate dioxygenase (BZDO) in terms of catalytic mechanism, enzyme structure, and reaction substrate (Bagneris et al. 2005; Costura and Alvarez 2000; Friemann et al. 2005; Kim et al. 2003; Shindo et al. 2005; Yildirim et al. 2005).

Most dioxygenases have in common that they have relatively low specificity and similar enzyme structures. Many of them can catalyze a broad range of aromatic substrates, such as benzene, toluene, naphthalene, and chlorobenzene (Quintana and Dalton 1999; Raschke et al. 2001). In general, dioxygenase enzymes contain an iron–sulfur center (Mason and Cammack 1992). For example, benzene dioxygenase enzyme from *P. putida* comprises three subunits: the first one an iron–sulfur-centered dioxygenase, the second an electron transfer protein also possessing an iron–sulfur center, and the third a flavoprotein (Axell and Geary 1973; Harpel and Lipscomb 1990; Subramanian et al. 1985; Geary et al. 1984; Ensley and Gibson 1983). Similarly, TDO is composed of three subunits: flavoprotein reductase, ferredoxin, and a terminal iron–sulfur dioxygenase (ISP_{TOL}; Butler and Mason 1997). The catalytic mechanisms are also similar: two electrons are passed from reduced NADH through subunits to dioxygen and the substrate (Fig. 2).

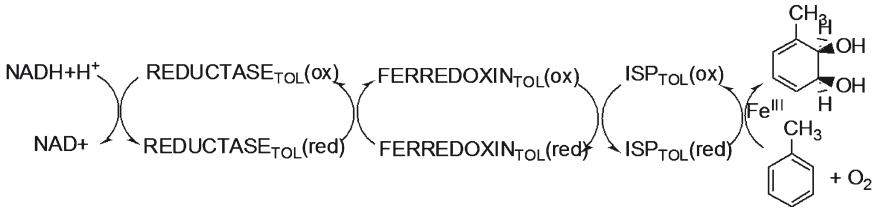
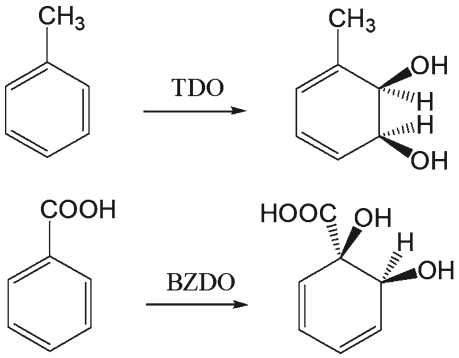


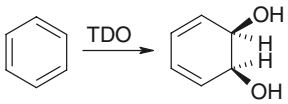
Fig. 2 Electron transportation in the toluene dioxygenase (TDO) system (Subramanian et al. 1979)

Fig. 3 Differences between dioxydation reactions catalyzed by TDO and benzoate dioxygenase (*BZDO*)



BZDO is one of the most special dioxygenases owing to its catalytic mechanism. Other dioxygenases convert aromatic rings to *cis*-diols by adding two hydroxyl groups to two or three sites (Bui et al. 2000). In contrast, BZDO, as well as its homogeneous enzyme toluate dioxygenase, catalyzes the reaction to produce *cis*-diols with hydroxyl groups at one or two sites (Yamaguchi et al. 1975; Lipscomb et al. 2002; Fig. 3).

BZDO enzyme also contains an iron–sulfur center. But it consists of two components: an oxygenase of an $(\alpha\beta)_3$ subunit structure, in which the α contains a Rieske [2Fe–2S] cluster and a mononuclear iron site, and a reductase with one FAD and one [2Fe–2S] cluster (Yamaguchi et al. 1975; Yamaguchi and Fujisawa 1978, 1980, 1982). In the process of dioxidation of benzoate by BZDO, the iron ion plays an important role (Fig. 4).



(5)

The application of bacterial strains containing these dioxygenases has used for transformation of environmental pollutant aromatic compounds to nonaromatic compounds (Cavalca et al. 2004; Parales and Haddock 2004). Moreover, the *cis*-diols produced by dioxygenases are attractive because of their inimitable chiral structures and their potentials in industrial synthesis for useful chemical products

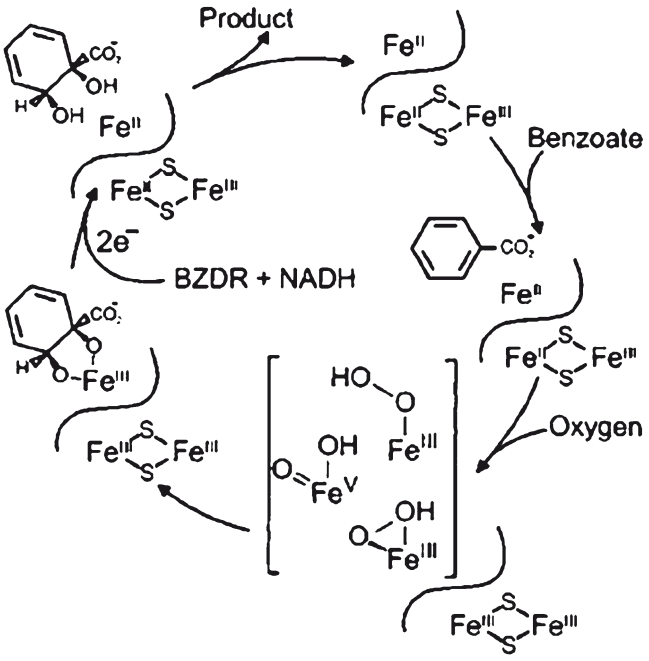


Fig. 4 Catalytic metabolism of BZDO. *BZDR* benzoate dioxygenase reductase. (Lipscomb et al. 2002)

(Boyd and Bugg 2006). The most widely researched and used is benzene *cis*-diol, or *cis*-DHCD, the reaction to produce it being catalyzed by benzene dioxygenase or TDO (Scheme 5). One of the applications of *cis*-DHCD is to synthesize the novel polymer PPP.

3.3 Synthesis of cis-DHCD

For synthesis of chiral *cis*-diols, biotransformations using bacterial strains containing dioxygenase have advantages over purely chemical reactions, including enantiospecificity, high yields, low economic cost, and environmental friendliness (Reddy et al. 1999). And there are two key issues that have to be addressed: one is the selection of an appropriate enzyme source and a bacterial strain; the other is the development of appropriate process technology (Ballard et al. 1994).

The development of appropriate process technology for using the biocatalyst and the subsequent product recovery is critical for achieving an economic and reliable process, particularly in cases such as biotransformation of benzene, where oxygen and toxic, volatile, poorly water soluble substrates are needed (Ballard et al. 1994).

Biocatalysts can be used for synthetic purposes in different forms, such as immobilized cells or enzymes, dissolved enzymes or intact cells (Lilly et al. 1990;

Lilly 1977). For the conversion of benzene to DHCD, the intact cell as a catalyst is preferred for several reasons. First, the reaction requires NADH, which can be supplied by the cell. Second, appropriate host strains have been supplied by strain development which are resistant to toxic organic substrates and can take up a range of aromatic compounds. Moreover, for the option of using dissolved enzymes as synthetic catalysts, it has been unsuccessful in isolating the three-protein enzyme complex (Yeh et al. 1977; Axcell and Geary 1975; Zamanian and Mason 1987; Mason and Cammack 1992).

Some problems are to be solved in the process of synthesis of DHCD by a growing culture during fermentation. One is the growth inhibition by aromatics and DHCDs at high concentration. Another is the recovery of DHCD downstream of the biotransformation, to dissolve the product in a nonaqueous organic solvent, as DHCD is an unstable compound in acidic aqueous media. This also requires keeping the pH above 7 to avoid degradation of DHCD to phenol, which clashes with the fact that dioxygenase activity is optimal at pH 7.

As a source of the enzyme, a microbial strain should be able to tolerate a significant concentration of benzene or other aromatic compounds. And the enzyme efficiency or a high rate of benzene oxidation is another key factor. Microorganisms used in the early studies for the metabolic pathway identification were characterized by high sensitivity to benzene (Gibson et al. 1968a, b), and are thus not suitable for cost-effective, large-scale operation. ICI isolated microorganisms from sites where levels of hydrocarbon contamination were significant. These bacteria showed rapid growth ability in benzene-saturated, aqueous solution. One of the strains, *P. putida* NCIMB 11767, was selected for development (Ballard et al. 1994). By application of chemical mutations to inactivate the DHCD dehydrogenase, a mutant strain was selected which had lost the ability to further metabolize DHCD as a carbon source. Further mutation was done to derive constitutive expression of the dioxygenase and to overcome the catabolic repression of dioxygenase synthesis by glucose. Finally, one of the strains, *P. putida* UV4, which showed a high level of DHCD accumulation from benzene after growth on glucose, was selected for process development. This catalyst was widely used for the production on a ton scale of DHCD and other substituted DHCDs from a wide range of aromatic substrates (Taylor 1982).

Recently, genetic modifications on microbial hosts have been preferred as an improvement in the cost-effective and large-scale fermentation. Natural strains containing dioxygenases are difficult to use for direct production of DHCDs owing to their long growth period, low benzene transformation efficiency, and difficulties in controlling growth conditions. New strains have been developed through genetic engineering, for example, a host strain harboring a plasmid containing dioxygenase gene and other related components to overexpress dioxygenase enzyme.

P. putida is an appropriate host strain for DHCD production. The strain mt-2 (ATCC 33015) was isolated from soils in the early 1960s by Hosokawa and others (Nozaki et al. 1963). It is able to grow on *meta*-toluate as the sole carbon source owing to its pWW0 plasmid. KT2440 is a mutant strain of mt-2, widely used as a host for *Pseudomonas* gene cloning and expression. The most important

character of KT2440 is the ability to transform and convert various aromatic compounds, utilizing benzoic acid as the sole carbon source and energy through the β-ketoadipate pathway.

P. putida KT2442 is a mutant strain of KT2440. It has the high environmental tolerance of KT2440. Since *Pseudomonas* species provide a good basis for genetic manipulation, which is necessary for strain development, KT2442 is considered to be a suitable host for DHCD synthesis. It is reported that *Pseudomonas stutzeri* 1317 (Chen et al. 2004) and *Aeromonas hydrophila* 4AK4 (Chen et al. 2001; Ouyang et al. 2005) are also able to utilize broad-range substrates and survive well in organic solvents (Jiménez et al. 2002; Chen et al. 2001, 2004). These three bacterial species can be used for highly efficient biotransformation of benzene to DHCD.

Escherichia coli JM109 harboring TDO genes has been used successfully for large-scale production of DHCD (Quintana and Dalton 1999; Qu et al. 2003), in which the TDO gene *tod* is constructed into plasmid pKST11. More recently, Ouyang et al. (2007a) constructed this *tod* gene into plasmid pSPM01, which was introduced into *P. putida* KT2442, *P. stutzeri* 1317, and *A. hydrophila* 4AK4. These three strains showed higher efficiency of DHCD production than *E. coli* JM109 (pKST11). Moreover, in contrast with the requirement of isopropyl β-D-thiogalactopyranoside induction for *E. coli* JM109 (pKST11), isopropyl β-D-thiogalactopyranoside was not needed to realize biotransformation by these three strains. To make a further improvement, Ouyang et al. (2007b) integrated the *vgb* gene, which encoded the *Vitreoscilla* hemoglobin protein that enhanced the oxygen microbial utilization rate under low dissolved oxygen concentration, into the *P. putida* KT2442 genome. The mutant strain *P. putida* KTOY02 (pSPM01) showed higher oxidation ability and higher production of *cis*-diols was achieved. *P. putida* KT2442 was also genetically modified to transform benzoic acid (benzoate) to benzoate *cis*-diol, also named 1,2-dihydroxycyclohexa-3,5-diene-1-carboxylic acid (Sun et al. 2008). These results indicate that *P. putida* KT2442 could be used as a cell factory to biotransform aromatic compounds.

There are three principal industrial modes of fermenting microorganisms: batch, fed-batch, and continuous fermentation, among which the continuous fermentation is the most demanding process, with nutrients continuously fed to the fermentor while an equal amount of spent growth medium is withdrawn to achieve a highly consistent product. The batch and fed-batch processes are simpler, the first one comprising inoculation of a sterile fermentor containing the growth medium with a live bacterial culture, and the latter one comprising continuous addition of a controlled amount of an essential growth element (Ballard et al. 1994). For a laboratory-scale fermentation for research, batch and fed-batch processes using shake-flasks and 3-6 L fermentors are mostly used for convenience.

Batch operation to synthesis of DHCD requires the supply of a biocatalyst, that is, a strain as the enzyme source, oxygen or air, and an aromatic reactant. Attention should be paid to the supply of the aromatic reactant since the aromatic reactant has damaging effects on microbial cells, and thus cannot be added to the reactor in too great an amount at one time. A detailed study of toluene hydroxylation kinetics (Woodley et al. 1991) showed that the aqueous toluene concentration should be

maintained between 0.05 and 0.20 g L^{-1} for optimization of biocatalytic activity. A concentration of toluene higher than 0.20 g L^{-1} would be toxic to the cells, whereas at concentration lower than 0.05 g L^{-1} poor use is made of the available dioxygenase activity. As the aromatic reactant is poorly water soluble and volatile, it may reach the bacteria via three routes: aqueous phase, vapor phase, or organic phase (Ballard et al. 1994). Bacteria may either catalyze the reaction with the aromatic reactant in the aqueous phase via mass transfer from the vapor or organic phase, or via direct contact with the nonaqueous phase, or both. However, vapor-phase addition has resulted in low reaction rates and product concentration (de Bont et al. 1986; van den Tweel et al. 1986). In contrast, organic-phase addition has been done successfully on the laboratory scale by dissolution of the reactant in a selected organic solvent (Harrop et al. 1992; Collins and Woodley 1993; van den Tweel et al. 1987; Yarmoff et al. 1988).

A two-phage cultivation system has been developed in the biotransformation of benzene (Quintana and Dalton 1999; Qu et al. 2003). To reduce benzene toxicity, a 3–10 times volume of water-insoluble organic solvent, such as tetradecane or liquid paraffin, was used for dissolution of benzene before the benzene was added into the culture broth. However, this large volume of organic solvent would reduce the effective working volume of the bioreactor, leading to increasing DHCD production cost and complicating the downstream extraction procedure. These disadvantages may hinder the commercial-scale production of *cis*-diols and the productivity of DHCD using the recombinant *E. coli* system was limited.

With use of *P.putida* KT2442 as the host, which has high resistance to benzene or its derivatives and high dioxygenase enzyme activity, the highest yield of benzene *cis*-diol ever reported, near 60 g L^{-1} on the 5-L fermentor scale, was achieved (Ouyang et al. 2007a).

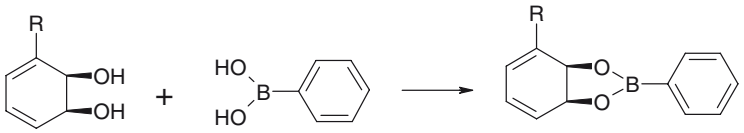
For a ton scale operation, a simpler approach has been successfully used to supply the reactant to cells via the aqueous phase. The reactant is added at a controlled rate so that that the supply is matched by the reactant dihydroxylation to DHCD. A control system is needed to maintain the correct aqueous-phase reactant concentration as the activity of the cells varies during the stages of the biotransformation (Ballard et al. 1994).

3.4 Recovery of cis-DHCD

The DHCD product is more water-soluble than the starting material aromatic compounds; thus, it is a problem to isolate the compound. Other water-soluble components in the cells, such as proteins and nucleic acids, may create difficulties in extracting the DHCD into an organic solvent. It is also important to keep the pH above 7 to avoid DHCD dehydrating into phenol (Ballard et al. 1994).

There are several methods for isolating the DHCD product in a short sequence in high yield and with high purity. An elegant method is the formation of an insoluble phenylboronate complex (Herbert et al. 1990; Scheme 6). This reaction occurs

when the phenylborate is added stoichiometrically to the DHCD. The precipitated product can be filtered and recovered. Subsequently, when the phenylboronate adduct is broken, the phenylborate can be recovered and reused, and the DHCD can be recrystallized.



(6)

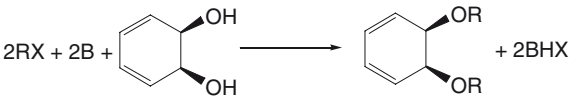
Another method of recovery of DHCD is to absorb it onto an insoluble and hydrophobic solid such as diatomaceous earth (Güuzel et al. 1990) or charcoal, by passing the aqueous solution down a column. The column binds DHCD and DHCD may be eluted by washing the column with a polar organic solvent such as methanol.

The most obvious way of isolating DHCD is to extract it from the aqueous medium into an organic solvent and then recrystallize it. This method needs careful selection of solvent and operation owing to the high water solubility of DHCD.

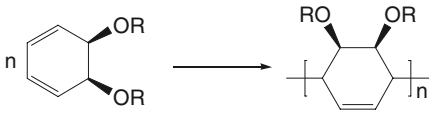
Some DHCD products have been found to be unstable as solids left at room temperature over time; exothermic decomposition results in the formation of phenol. DHCDs produced by ICI are formulated as solutions in ethyl acetate containing a small amount of basic triethylamine stabilizer, and are fully stable at room temperature (Ballard et al. 1994).

4 Polymerization: From *cis*-DHCD to PPP

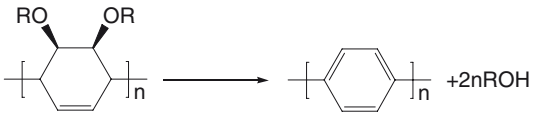
The process of synthesis of PPP from *cis*-DHCD is as follows: first, derivatization of *cis*-DHCD (Scheme 7); second, polymerization of *cis*-DHCD derivatives (Scheme 8); finally, the aromatization to obtain PPP (Scheme 9).



(7)



(8)



(9)

4.1 Derivatives of cis-DHCD

For synthesis of PPP from *cis*-DHCD, the first step is the derivatization of DHCD. The reason not to directly polymerize DHCD is that after its polymerization, which is in fact difficult, the subsequent aromatization step gives rise not to PPP but to a polymer of undefined structure containing nonaromatic and phenolic groups (Ballard et al. 1994).

Derivatization of DHCD can be carried out at and above pH 7.4 without the formation of phenol, by the reaction shown in Scheme 7, where RX can be an acid chloride, anhydride, or iodide, and B can be an organic tertiary base. The thermal properties of derivatives of DHCD have to be identified after the polymerization and aromatization processes. The aromatization process can be carried out smoothly at a temperature greater than 100°C. Acetic anhydride is often used to produce cis-3,5-cyclohexadien-1,2-diol diacetate (DHCD-DA). The dimethylcarbonate (DMC) derivative has also been used for the majority of polymerization work covered by a range of ICI patents (Ballard et al. 1983a, b, 1984; Chenshire 1984; Nevin and Shirley 1985).

4.2 Polymerization of cis-DHCD Derivatives

In early experiments, contamination by small amounts of impurities such as phenol made experiments attempting the polymerization of DHCD and its derivatives unsuccessful. When pure DHCD was used, the initiating radicals facilitated the formation of phenol, also inhibiting the polymerization. However, most of the acyl derivatives could be polymerized by using radical initiators either as the pure compound or dispersed in an organic solvent in which they were insoluble. The details of the polymerization, including conversion rate, molecular weight, temperature, and the relationships among these parameters, were described by Ballard et al. (1994).

Most effective polymerizations were achieved in the absence of solvent and would proceed almost to completion without difficulty. For example, with use of benzoyl peroxide as an initiator, the conversion rate of DHCD-DA would reach 90% in 40 h. Moreover, the variation in molecular weight, which was represented by the number-average degree of polymerization and the dispersity, showed that the bulk polymerization of these monomers was similar to that of acrylic esters (Ballard et al. 1994).

The relationship between polymerization rate and concentration of monomer and catalyst is shown in Eq. 1:

$$R_p = \frac{-d[M]}{dt} = K[M]_0^{3/2}[I]_0^{1/2} \tag{10}$$

where $[M]_0$ and $[I]_0$ are the initial monomer and initiator concentrations, respectively (Ballard et al. 1994). This is a general feature of the polymerization of

vinyl monomers such as styrene and methyl methacrylate. It was also found that the polymerization had a pressure coefficient and the rate of polymerization at 3,000 atm was 5–7 times greater than at atmospheric pressure. But the molecular weight was not significantly higher (Ballard et al. 1988, 1994).

The molecular weight of the polymer is sensitive to the concentration of the monomer, and would be reduced markedly by the presence of an aromatic solvent. Thus, the polymerization should be performed in the absence of solvent to obtain a high molecular weight. The reaction temperature is also a sensitive parameter. For example, the molecular weight increases by a factor of 5–10 times when the polymerization is carried out at 60°C as compared with 90°C. Through a consideration of the temperature effect on the polymerization rate, the energy of activation is calculated to be 16.9 kcal mol^{-1} using the Arrhenius equation (Ballard et al. 1994). The molecular weight of the polymer can be markedly increased by using deuterated analogs of DHCD. This effect of deuteration is well known in radical polymerization of vinyl monomers, and is due to the retardation of the bimolecular termination reaction and possibly to differences in the proton or deuteron abstraction from the monomer, leading to degradative chain transfer (Ballard et al. 1988).

Polymerization in organic diluents, in which the polymer is insoluble, has been achieved by using dispersing agents consisting of a poly(methyl methacrylate) backbone with a side chain derived from 12-hydroxystearic acid. Polymer powders from acetate, benzoate, and methylcarbonate derivatives of DHCD have been obtained. Particularly, the benzoate and methylcarbonate derivatives are polymerized at high rates and give polymers with molecular weights up to one million (Ballard et al. 1994).

4.3 Aromatization Process to PPP

The polymers of DHCD derivatives can be aromatized by heating the polymers as fibers or films in the solid state and in solution (Ballard et al. 1994). The process is done with reaction shown in Scheme 9, accompanied by elimination of two molecules of the acid for each phenylene group formed. In the case of DHCD-DA aromatization, the ROH is acetic acid.

The DMC derivative is another preferred one for aromatization for practical reasons, as the eliminated acid is methylcarbonic acid, which decomposes to methanol and carbon dioxide. This DHCD-DMC aromatization process is catalyzed by alkali metal salts and a tertiary organic nitrogen compound. Tertiary bases such as *n*-octylamine, the oligo bases, and other bases of low volatility are the preferred catalysts for the process at 240°C using 0.5 mol%. Moreover, the base can be removed by volatilization at 350°C after the conversion is complete. This is definitely an advantage over using metal salts (Ballard et al. 1994).

N-Methylpyrrolidone is not only a catalyst for the conversion to PPP, but is also a good solvent for the partially aromatized poly(DHCD-DMC) with up to 35 mol% of phenylene groups. Thus, it is a good base for the study of the initial stages of the

aromatization process. It has been demonstrated that the process is autocatalytic, that is, as the relative number of phenylene groups in the chain increases, the adjacent DHCD-DMC residues are more readily aromatized. And the aromatization does not produce chain scission (Ballard et al. 1994).

There is a fundamental difference between aromatization in the solid state and in solution. The conversion in solution up to 26% does not involve chain scission as the conformational changes occurring are accommodated. On the other hand, in the solid state, indirect evidence shows that chain fracture takes place, accompanied by crystallization of polyphenylene (Ballard et al. 1988).

Alternative methods of polymerization to obtain a polymer chain with no 1,2 phenylene units have been developed at the California Institute of Technology (Gin et al. 1992a). In this process, *cis*-5,6-bis(trimethylsiloxy)-1,3-cyclohexadiene (DHCD-TMS) is prepared by the reaction in Scheme 7. It is then polymerized by an organometallic catalyst, bis(allyltrifluroacetonickel(Ni^{2+})), to give an exclusively 1,4 polymer in 93% yield. Poly(DHCD-TMS) cannot be aromatized, so it is converted to poly(DHCD-DA) by removal of the trimethylsilane groups with a fluoride ion and methanol and is reacetylated using acetic anhydride. This is aromatized thermally, and the PPP is shown to be of longer chain length than polymers from radical-initiated polymerization (Ballard et al. 1994).

5 Properties and Applications of PPP

Some properties of PPP are related to the aromatization process (Ballard et al. 1994). The level of the crystallinity of PPP is affected by the aromatization temperature. When the process is done below the glass-transition temperature (T_g) of the precursor molecule, which is 185°C, a predominantly amorphous PPP powder or coating is produced. The subsequent annealing of this powder at temperatures below or above 290°C has quite distinct effects on the crystallinity. When the temperature is below 290°C, there is no recognizable increase in crystallinity. In contrast, once the temperature goes up above 290°C, a high level crystallinity polymer is produced. On the other hand, if the precursor molecule is aromatized above its T_g, small crystals would be formed during the aromatization process and impede the further reorganization of the macromolecules, making the maximum possible crystallinity unachievable. But these small crystals are absent from the polymer produced by aromatization below T_g. The distinct change of crystallinity at 290°C also shows that it is associated with the increase in chain mobility and thus this temperature is the T_g for amorphous PPP.

The T_g of the precursor molecule rises with the increase in aromatization degree, as more phenyl groups are formed and the flexible precursor becomes more rigid. The phenyl groups are formed initially in blocks and are not randomly distributed along the chain, so the T_g increases smoothly. After the aromatization degree reaches about 30%, the T_g increases more rapidly (Ballard et al. 1994).

Another property of PPP related to the aromatization process is the thermal stability. One of the optimal options of the process is to carry it out in an inert atmosphere, complete it by heating at 260°C, and finally at 320°C, to remove the amine catalyst and oligomers. The PPP coatings obtained in such a way can be used at temperatures near 400°C, and in the absence of oxygen, even at 500°C. In these conditions, no recognizable decomposition of PPP occurs. At temperatures above 400°C in air, only a small amount of weight is lost in the form of hydrogen and methane. Breakdown of phenyl groups only occurs at a significant rate at 600–800°C. In an inert atmosphere such as N_2, at a temperature of 900°C, only 7% of weight is lost with production of oligophenyls containing three to 11 phenyl units, and no benzene or diphenyl is produced. In contrast, other aromatic polymers and coatings can only withstand temperature of 350°C for short periods without significant breakdown (Ballard et al. 1994). This thermal stability of PPP is among the best in all polymer materials.

Other properties of PPP are also the among best of all artificial and natural polymer materials, such as (1) it is the most resistant to acid corrosion, it is only dissolved slowly by 98% sulfuric acid, much superior to other polymer materials; (2) it is the most resistant to radiation, without any changes under radiation of 8.95×10^8 rad from cobalt; (3) it is the hardest to burn; (4) it has the highest rigidity; and (5) its refractive index of 1.833 is higher than that any other organic polymer.

Oligophenyl absorbs in the infrared, at about 800 cm^{-1}. It has been shown that when the unit number of PPP increases, the absorption wavenumber decreases, and this decrease is in direct ratio to $1/\alpha^2$, in which α is the unit number. This is due to the decline of the vibrational energy of Π*** conjugation by polymerization. As a result, vibration would happen even from irradiation by a low-energy wave, and thus the absorption takes place. If the unit number was 20, the theoretical absorption wavenumber would be 2 cm^{-1}, and the frequency would be 60 Hz. This would meet the need for materials with absorption of long electromagnetic waves. Moreover, since the structure of PPP is symmetrical, its dielectric constant is very low, and it would not be activated by a general electric or magnetic field.

As described above, PPP has novel rigidity and thermal stability, and its density is only 1.228 $g\,ml^{-1}$, one sixth that of iron. This indicates that PPP can be suitably applied in special environments where materials of high rigidity, thermal stability, and corrosion resistance are needed, for example, aerospace.

PPP coatings are very good electrical insulators, with electrical resistivity of 10^{13}–$10^{16}\,\Omega$cm. However, the treatment with n- and p-type dopants such as sodium naphthalide, ferric chloride, and AsF_5, the electrical resistivity decreases markedly to that of a semiconductor. The electrically insulating coatings have been studied for use in the design of advanced liquid crystal displays for computers. By deposition PPP on glass plates coated with an electrically conducting layer of indium tin oxide (ITO), the plates encapsulate a solution of liquid-crystalline molecules. The supertwisted birefringent effect enhances the visualization of the display. It has also been shown that a light-emitting diode (LED) can be constructed with PPP (Grem et al. 1992). A glass plate is coated with a layer of ITO and aluminum with PPP sandwiched between them. The application of 12 V at a frequency of 60 Hz

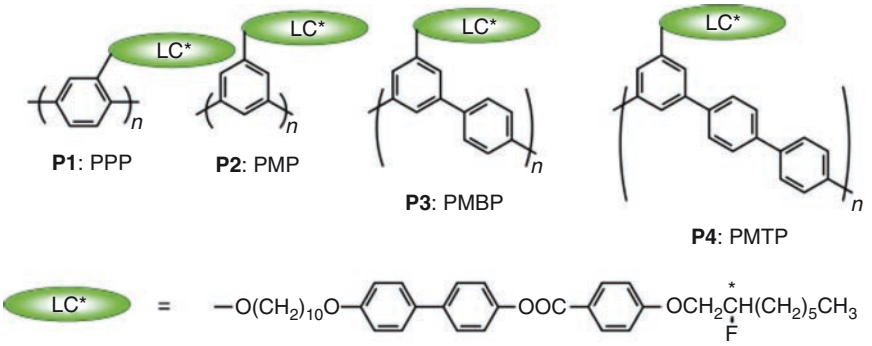


Fig. 5 Chemical structures of chiral liquid-crystalline conjugated polymers*PMP* poly (*meta*-phenylene), *PMBP* poly (*meta*-biphenylene), *PMTP* poly(*meta*-terphenylene) (Suda and Akagi 2008)

between the electrodes produced blue light. This type of LED differs from that of poly(phenylene vinylene) (Burroughes et al. 1990), which emits radiation of yellowish light. From these studies PPP may be suggested for development of computer screens. In addition, the blue emitted radiation is photochemically active and may be used for electroluminescent devices.

Recently, liquid-crystalline polyphenylene derivatives have been synthesized through substitution of a fluorine-containing chiral liquid-crystalline group into side chains, with an aim to develop ferroelectric liquid-crystalline conjugated polymers (Fig. 5; Suda and Akagi 2008). These are attracting interest because they can afford anisotropies in electrical and optical properties when they are macroscopically aligned. This study also elucidated that PPP can be used to prepare new types of polymer materials.

6 Summary and Future Developments

In this chapter we have described the production of DHCDs from aromatic compounds, their polymerization into PPP, and the properties and applications of the polymer. Large-scale synthesis of DHCD has been demonstrated, and DHCD is widely used in the pharmaceutical industry, as well as in chemical industries for polymer productions. A review of these points was given by Ballard and others in 1994. We have added recent study results obtained since then, such as research on different types of dioxygenases, strain development by recombination, and genetical modification. More research needs to be done, on one hand, to develop the process technology for commercialization of this new polymer and chemical intermediates. On the other hand, research on a series of DHCDs and their derivatives and new applications of the polymers would create many new opportunities for industry.

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