
Approaches for the Synthesis of Tailor-Made Polyhydroxyalkanoates

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Abstract

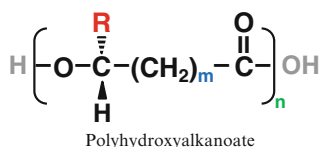
Polyhydroxyalkanoates (PHAs) are biodegradable and biocompatible thermoplastics. These have been proposed for a wide range of biotechnological applications, especially in the field of the medicine and chemistry. PHAs are produced by more than 300 bacterial species, the most efficient being *Cupriavidus necator* (formerly *Ralstonia eutropha*), *Alcaligenes latus*, and recombinant strains of *Escherichia coli*. PHAs are produced by fermentation using different culture systems, from batch culture to exponentially fed-batch cultures, and it is known that culture conditions, such as pH, aeration, and nutritional conditions, influence the chemical characteristic PHAs synthesized by microorganisms; because of that, it has been proposed that by manipulating the microbial metabolism and culture conditions, it is possible to design biopolymers with specific chemical properties. This paper describes four cases of PHAs production: the copolymers of poly-3-hydroxybutyrate-co-poly-3-hydroxyvalerate [P(3HB-co-3HV)] and poly-3-hydroxybutyrate-co-poly-3-hydroxyhexanoate [P(3HB-co-3HHx)], the medium-chain-length PHAs, the P3HB of ultrahigh molecular mass, and finally, the production of other short-chain-length PHAs, with a special emphasis on the species that have been reported for their production as well as the molecular and fermentation strategies evaluated in order to modify the chemical composition of PHAs.

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2.1 Introduction

Commercial interest in bioplastics has increased due to the possibility of replacing synthetic materials, which have disadvantages from the environmental perspective. In this regard, polyhydroxyalkanoates (PHAs) are a suitable option to substitute the plastics derived from petroleum.



PHA	R	m	Name of the monomer
SCL	H	1	3-hydroxypropionate (3HP)
	CH ₃	1	3-hydroxybutyrate (3HB)
	H	2	4-hydroxybutyrate (4HB)
	C ₂ H ₅	1	3-hydroxyvalerate (3HV)
	H	3	5-hydroxyvalerate (5HV)
MCL	C ₃ H ₇	1	3-hydroxyhexanoate (3HHx)
	C ₅ H ₁₁	1	3-hydroxyoctanoate (3HO)
	C ₇ H ₁₅	1	3-hydroxydecanoate (3HD)
	C ₉ H ₁₉	1	3-hydroxydodecanoate (3HDD)
	C ₉ H ₁₈	1	3-hydroxydodecenoate (3HDDe)
	C ₁₁ H ₂₃	1	3-hydroxytetradecanoate (3HTD)

$n = 1 - 230,000$ monomers.

Fig. 2.1 Chemical structure of polyhydroxyalkanoates. *R* alkyl group, *m* length of carbon chains, *n* number of monomers

PHAs are polyesters composed of 3-hydroxy fatty acid monomers (Fig. 2.1) (Chen 2010; Peña et al. 2014a). The main advantages of these biopolymers are their biodegradability and biocompatibility, making them suitable for a wide range of applications, from the traditional plastic industry to their use as materials in the biomedical field, with emphasis on chemical composition and purity of the product (Peña et al. 2014a; Leong et al. 2014). PHAs are synthesized by many microorganisms as energy reserve material. In general, it has been well documented that these polymers are produced under nutrient limitation, mainly nitrogen, phosphorus, or oxygen (Anderson and Dawes 1990; Peña et al. 2011, 2014a; Ienczak et al. 2013). There is a wide variability in PHA composition that includes homopolymers, heteropolymers, and up to 150 different types of monomers (Steinbüchel and Lütke-Eversloh 2003). The thermomechanical properties of PHAs and therefore their specific applications will depend on their chemical structure, specifically monomer composition (type, ratio, and distribution) and, in the case of homopolymers, their mean molecular mass (MMM). In this line, several attempts, that include genetic manipulation of microorganisms as well as changes on the culture conditions in which the cells are grown, have been evaluated in order to obtain materials of specific characteristics (Reddy

et al. 2003; Peña et al. 2014a; Leong et al. 2014). The subjects covered by this chapter include properties of PHAs and their applications, bacterial sources and PHAs biosynthesis, as well as the influence of culture conditions (i.e., medium composition, temperature, pH, etc.), which determine the composition of PHAs, including specific examples regarding the production of PHAs with different chemical compositions.

2.2 Chemical Structure, Physicochemical Properties, and Applications of PHAs

Polyhydroxyalkanoates (PHAs) are polyesters produced and accumulated by several bacteria as a carbon and energy reservoir. These polymers protect organisms against starvation and may enable them to survive under adverse conditions. The PHA accumulation occurs mainly under conditions of excess of carbon and limitation of other nutrients (Anderson and Dawes 1990; Peña et al. 2011, 2014a). These polymers are water-insoluble and they are stored in the cytoplasm as granules (Legat et al. 2010). The monomeric composition of PHAs depends primarily on the microbe and the type of the carbon used for growth. Based on their monomeric chemical

structure, three PHA groups can be defined: the short-chain-length PHAs (SCL-PHAs), with monomers from 3 to 5 carbon atoms, the medium-chain-length PHAs (MCL-PHAs) composed of units from 6 to 15 carbon atoms, and, finally, the long-chain-length PHAs (LCL-PHAs) with monomers of more than 15 carbon atoms (Peña et al. 2014a; Leong et al. 2014; Fig. 2.1). On the other hand, PHAs could also be classified as homopolymers, such as P3HB, or copolymers that could be found as SCL copolymers, MCL copolymers, and SCL-MCL-PHA copolymers. The thermoelastic properties of the PHAs will be influenced by the type, ratio, and distribution of the monomer units (Leong et al. 2014; Table 2.1); homopolymers of SCL-PHAs such as the poly-3-hydroxybutyrate P3HB are brittle and stiff materials, while copolymers of MCL-PHAs have improved elastomeric properties (Reddy et al. 2003). It must be emphasized that thermoprocessability, biodegradability, and biocompatibility of PHAs make them of great interest for biomedical applications such as the emerging field of tissue engineering (Hazer et al. 2012; Peña et al. 2014a; Leong et al. 2014).

2.3 Bacterial Sources of Polyhydroxyalkanoates

PHAs are produced by several bacterial and archaea species (Olivera et al. 2001; Chanprateep 2010; Peña et al. 2014a). It is noteworthy that species able to produce and accumulate these biopolymers could be found in diverse environments, from marine sediments with genera such as *Vibrio*, *Beneckea*, and *Paracoccus* (López-Cortés et al. 2010) to soil environments, where species such as *Azotobacter vinelandii*, *Bacillus* spp., *Cupriavidus necator*, and *Pseudomonas* spp. are natural producers of PHAs, or even species which can be found on extreme hypersaline environments, in which the *haloarchaeal* genera *Haloferax*, *Halococcus*, *Halobacterium*, *Halorubrum*, and *Haloarcula* are an interesting group for PHA production (Legat et al. 2010; Poli et al. 2011; Kumar et al. 2013). Until now only two species have been successfully used for PHA production at a commercial scale: *C. necator* and *Azohydromonas lata* (Chen 2009;

Ienczak et al. 2013); however, some of the genera and species listed above could bring advantages for the tailor-made production of these biopolymers, such as *Haloferax mediterranei*, which produces the poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)] copolymer in high-cell-density cultures, reaching PHA concentrations of up to 77.8 g L⁻¹ (Huang et al. 2006). Furthermore, production of PHAs using this archaea has additional advantages. For example, some archaea have the ability to grow on hypersaline conditions, using inexpensive carbon sources, and the feasibility to lyse cells using distilled water, which could be of great impact for PHA recovery costs (Hezayen et al. 2000; Huang et al. 2006; Poli et al. 2011). In contrast, *Pseudomonas* species are able to produce a wide range of MCL-PHAs from cheap and renewable substrates, such as plant oils. In addition, various *Bacillus* spp. are able to accumulate homopolymer P3HB and copolymer P(3HB-co-3HV) using different carbon sources as glycerol, carbohydrates, and biowaste (pea shells) and also produce MCL-PHAs, when various carbon sources are co-fed or when this bacterium was employed as host for overexpression of the biosynthetic operon *phaCAB* from *P. aeruginosa* and *C. necator* (Singh et al. 2009; Kumar et al. 2009, 2013, 2015). Another interesting case is *E. coli*, a nonnatural PHA producer; however, recombinant strains of this bacterium harboring PHA biosynthetic genes from *C. necator*, *A. lata*, *A. vinelandii*, or *Pseudomonas oleovorans* are important alternatives for the production of a wide range of PHAs, which *E. coli* can synthesize using a wide range of substrates. *E. coli* can grow in high-cell-density cultures and does not have PHA depolymerases, unlike natural PHA producers (Lee 1996; Olivera et al. 2001; Reddy et al. 2003; Chen 2009; Centeno-Lefja et al. 2014; Leong et al. 2014). In addition to the production of PHA by bacteria and archaea in pure cultures, the use of microbial mixed cultures for the production of these polymers is an attractive alternative. The mixed culture of several microbial species in one single process allows the use of very low-cost complex substrates, or mixtures of substrates, such as those present in waste materials (derived from agro-industry or other waste sources), with no sterilization requirements and with the possibility of a continuous process (Kleerebezem and Loosdrecht

Table 2.1 Mechanical properties and applications of PHAs

PHA	Tensile strength (MPa)	Elongation at break (%)	Young's modulus (GPa)	Crystallinity (%)	Applications	References
P3HB (1400 kDa)	161	45	2.8	71	P3HB with Mw. 1143 for scaffolds for nerve cells	Domínguez-Díaz et al. (2015); Chan et al. (2014)
P3HB (230 kDa)	43	5	–	46	Construction of cast film for the growth of human embryonic cells (HEK293) without any cytotoxic effect	Domínguez-Díaz et al. (2015)
P(3HB-4HB) (62–38 mol%)	–	48	0.65	–	Biomaterials for human dermal fibroblasts and orthopedic support. Furthermore, P(3HB-4HB) is a potential temporary substrate that can be used in transplantation to replace damaged bone or skin	Chanprateep et al. (2010)
P(3HB/3HB-co-3HHx) (100/70–30 mol%)	2.0	10	0.19	53.7	Construction of matrices as cell growth supporting materials for applications in skin engineering and in nerve regeneration	Li et al. (2008)
P(3HB/3HB-co-3HHx) (100/60–40 mol%)	3.5	17	0.32	50.8	–	–
P(3HO-co-3HHx) (88–12 mol %)	9.0	380	0.008	–	Scaffolds for tissue engineering of cardiac valves; suitable for engineering both soft and hard tissues	Jana et al. (2014)
P(3HB-co-3HV) (80–20 mol %)	32	50	1.2	–		

2007). In this type of cultures, it is very difficult to determine the species composition; however, some studies have started to identify some of the PHA-producing species present. Particularly interesting are those works reporting *Alcaligenes*, *Azoarcus*, *Amaricoccus*, *Comamonas*, *Achromobacter*, *Pseudomonas*, *Kluyvera*, *Acinetobacter*, *Paracoccus*, *Xanthobacter*, *Curto-bacterium*, *Flavobacterium*, and *Thauera* (Dionisi et al. 2005, 2006, 2007; Serafim et al. 2006; Lemos et al. 2008) as the dominant genera present in mixed cultures promoting high PHA accumulation.

2.4 PHA Biosynthesis Pathways

PHAs biosynthesis and its regulation has been well documented (Anderson and Dawes 1990; Slater et al. 1992; Steinbuchel and Schlegel, 1991; Peña et al. 2011, 2014a). The synthesis of

P3HB, the simplest SCL-PHA, involves three enzymatic reactions; the first reaction involved the condensation of two molecules of acetyl-CoA, mainly from the tricarboxylic acid (TCA) cycle, into acetoacetyl-CoA by the β -ketothiolase (encoded by *phaA*). Then, acetoacetyl-CoA gets reduced to 3-hydroxybutyryl-CoA (3HB-CoA) with the help of the enzyme acetoacetyl-CoA reductase (encoded by *phaB*). Finally, the PHA synthase (encoded by *phaC*) polymerizes the 3-hydroxybutyryl-CoA monomers to P3HB, with the subsequent liberation of CoA (Stubbe et al. 2005; Peña et al. 2011, 2014a). However, biosynthesis of PHAs with different monomeric compositions involved biosynthetic pathways of hydroxyacyl-CoA thioester precursors (Fig. 2.2). In the case of the biosynthesis of SCL-copolymers such as P(3HB-co-3HV), two pathways are involved, leading to C4 monomer (3-hydroxybutyryl-CoA) or to C5 monomer

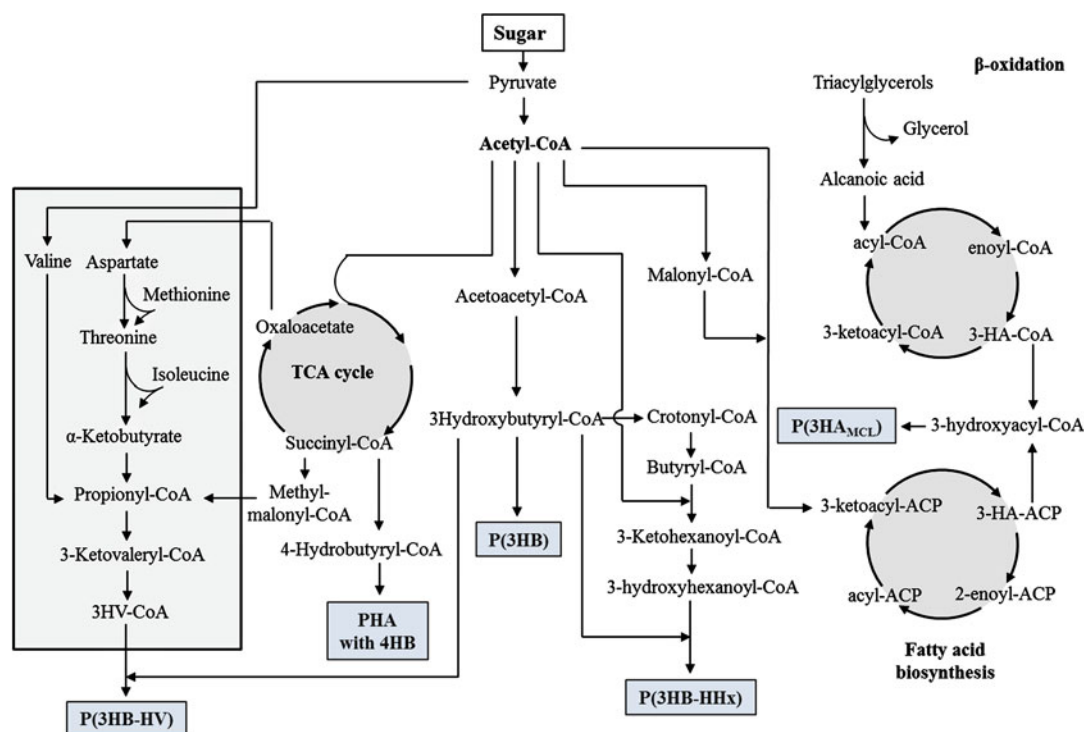


Fig. 2.2 Pathways involved in the biosynthesis of polyhydroxyalkanoates. Amino acid metabolic pathways, the tricarboxylic acids cycle, butyrate metabolism, fatty acid biosynthesis, and β -oxidation pathways (from left to right)

are shown. Abbreviations: ACP acyl-carrier protein, 3HB 3-hydroxybutyric acid, 3HA 3-hydroxyalkanoic acid, HV hydroxyvaleric acid, 4HB 4-hydroxybutyric acid, HHx hydroxyhexanoic acid, MCL medium chain length

(3-hydroxyvaleryl-CoA) (Steinbüchel and Schlegel 1991). As it has been previously described, the synthesis of the monomer of 3-hydroxybutyryl-CoA involves the condensation of two molecules of acetyl-CoA and its further reduction to 3-hydroxybutyryl-CoA which will be available for its incorporation into the copolymer by the PHA synthase (*phaC*). On the other hand, formation of the 3-hydroxyvaleryl-CoA involves the condensation of acetyl-CoA and propionyl-CoA into 3-ketovaleryl-CoA, a reaction catalyzed by the β -ketothiolase (*phaA*). Afterward 3-ketovaleryl-CoA is reduced by the acetoacetyl-CoA reductase (*phaB*) into the monomer 3-hydroxyvaleryl-CoA which could be incorporated into the growing polymer chain by the PHA synthase or polymerase (*phaC*). In this case, the propionyl-CoA, precursor of the 3-ketovaleryl-CoA, could be the result of the amino acid metabolism, from threonine, which could be converted in α -ketobutyrate and then reduced to propionyl-CoA with the help of the enzyme pyruvate dehydrogenase (Slater et al. 1998; Fig. 2.2), or it could be synthesized through β -oxidation during the growth of bacteria on fatty acids, amino acids, and other substrates that can first be converted into fatty acids (Steinbüchel and Schlegel 1991).

For the biosynthesis of the MCL-PHAs, which are composed of C₆ to C₁₅, two pathways are involved: one of these is the biosynthesis and degradation of fatty acids (β -oxidation pathway), wherein a wide variety of substrates are available for the polymer production (Lageveen et al. 1988; Timm and Steinbüchel 1990). From the fatty acid metabolism, precursors such as enoyl-CoA, hydroxyacyl-CoA, and ketoacyl-CoA could be used as substrates for the PHA polymerase for their further conversion into MCL-PHAs (Kraak et al. 1997; Lageveen et al. 1988; Fig. 2.2). Fatty acid biosynthesis is built by adding two carbons through intermediates linked to acyl-carrier protein (ACP), whereas in the β -oxidation pathway, two carbons are reduced from the fatty acyl substrates, the whole process liberates a molecule of acetyl-CoA in each cycle and their intermediates are linked to CoA (Fig. 2.2). Although, both fatty acid metabolic pathways are present in all

organisms, carbon sources can vary and affect MCL-PHA production. The capability of incorporating different hydroxyacyl-CoA units will be dependent on the PHA synthase (*phaC*). There are two types of these enzymes: the Type I which is harbored by organisms such as *C. necator* and synthesizes SCL-PHAs and the Type II which is present mainly in *Pseudomonas* and is able to polymerize MCL-PHAs.

2.5 Effect of the Culture Conditions on the PHAs Synthesized by Native and Recombinant Bacteria

It is known that the culture conditions affect the chemical characteristics of PHAs synthesized by microorganisms, and those chemical properties have an important effect on the mechanical properties and therefore the final applications of PHAs. In this section, some cases regarding the manipulation of the chemical composition of PHAs by the manipulation of strains and the culture conditions will be discussed (Tables 2.2 and 2.3).

Case 1: Production of the Heteropolymers P(3HB-co-3HV) and P(3HB-co-3HHx)

The copolymers poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)] and poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) [P(3HB-co-3HHx)] are conformed by monomers of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) or 3-hydroxyhexanoate (3HHx), respectively. Both copolymers are interesting candidates as alternative materials for replacement of petrochemical plastics. In the case of P(3HB-co-3HV), this was manufactured and commercialized by ICI (Biopol), Zeneca BioProducts, Biomer Inc. (Biomer), and Tianan Biologic (Enmat) (Braunegg et al. 1998; Chanprateep 2010).

Due to the presence of 3HV or 3HHx residues, polymer crystallinity is reduced, and these residues contribute to an increase of flexibility, elasticity, and melting temperature as compared with the homopolymer P3HB (Feng et al. 2002; Zhuang et al. 2014). In this line, when the molar ratio of 3HV is of only 20 mol %, the copolymer

Table 2.2 Production of polyhydroxyalkanoates under different culture conditions

PHA	Organism	Production scale	Culture conditions	Biomass (g L ⁻¹)	PHA content (%)	References
P3HP	Recombinant <i>E. coli</i>	Fed-batch fermentation 2 L	Crude glycerol Pure glycerol For both: 300 mM, 37°C and 400 rpm for 92 h	5.2 12.0	5.2 11.8	Andreeßen et al. (2010)
P3HB, P3HHx, P3HO, P3HD	Recombinant <i>E. coli</i>	Shake flasks	Decanoate Glucose and decanoate Cultures kept at 37 °C and 200 rpm	2.5–3.0 4.3–5.1	1.8–9.3 5.9–36.4	Li et al. (2011)
P4HB	Recombinant <i>E. coli</i>	Bioreactor 1 L	Glycerol (20 g L ⁻¹) with K-4HB (4 g L ⁻¹), 32 °C, pH 7, 800 rpm, 30 % DO ₂ , 1 L air/min, for 48 h Glucose (20 g L ⁻¹) with K-4HB (4 g L ⁻¹), propionic acid, and NZ-amines (1 g L ⁻¹), 32 °C, pH 7, 800 rpm, 30 % DO ₂ , 1 L air/min. for 48 h	N.S. 6.5–6.7	2.0–61.0 63.0–65.0	Kämpf et al. (2014)
P(3HB-co-3HHx) (90–10 mol %)	<i>E. coli</i> LS5218 (pBBJPC)	Shake flasks	M9 medium supplied with glucose (20 g L ⁻¹), 37 °C, 200 rpm, for 48 h	7.8	14.1	Wang et al. (2015)
P3HP	<i>E. coli</i> Q1911 (harboring both pHP302 and pHP513)	Baffled shake flasks 500 mL	100 mL of minimal medium with glycerol (20 g L ⁻¹) and glucose (3 g L ⁻¹)	4.9	10.2	Wang et al. (2014)
P(3HB-3HHx-3HO-3HD-3HDD-3HTD) (21.2, 6.1, 45.8, 11.0, 9.2, 6.8 mol %)	<i>E. coli</i> LS5218	Shake flasks batch 300 mL	50 mL of medium supplemented with glucose (30 g L ⁻¹), 30 °C, 250 rpm, for 72 h	6.5	12.1	Zhuang et al. (2014)
P(3HB-co-3HV) (85–15 mol %)	<i>E. coli</i> XL10	Fed-batch (first stage, glucose; second stage, propionate)	Continuous feeding of glucose (20 g L ⁻¹) and propionic acid (2 g L ⁻¹)	39.8	60.5	Liu et al. (2009)
P(3HB-co-3HHx) (60–40 mol %)	<i>C. necator</i> Re2133/pCB81	Shake flasks 250 mL	Butyrate (0.5 %)	0.6	65	Jeon et al. (2014)

N.S. not specified

has an excellent strength and flexibility (Luzier 1992). Besides, in some cases, these copolymers have better biocompatibility compared to either P3HB or polylactic acid, which makes them promising materials for medical fields, for example, in cardiovascular problems, wound-healing process, orthopedic issues, drug delivery, and

tissue engineering (Yang et al. 2002; Chen and Wu 2005; Table 2.1).

The ability to produce these copolymers is directly attributed to the specificity of polymerase synthase, which has been characterized only at preliminary level in *Bacillus* sp. (Lee et al. 2008). The P(3HB-co-3HV) is synthesized by several

Table 2.3 Culture conditions for the P3HB production with different molecular masses using recombinant *E. coli* strains and *Azotobacter* species

Organism	Mean molecular mass	Production scale	Culture conditions	Biomass (g L ⁻¹)	PHB content (%)	References
	Mw or Mn* (kDa)					
<i>E. coli</i> XL-1 Blue (pSYL105)	20,000*	Bioreactor 2.6 L	LB medium with glucose 20 g L ⁻¹ , pH 6, 37 °C	7.4	48	Kusaka et al. (1997)
<i>E. coli</i> JM109 (pGEM-phaC _{Ref} AB)	1800*	Shake flasks 500 mL with 100 mL medium	LB medium with glucose 20 g L ⁻¹ , 37 °C, 14 h culture time	3.2	33	Agus et al. (2006)
<i>E. coli</i> JM109 (pGEM-phaC _{Da} AB)	4000*			5.8	51	
<i>E. coli</i> JM109 (pGEM-phaC _{Ac} AB)	380*			2.7	24	
<i>E. coli</i> JM109 (pGEM-phaRCBspAB)	170–48*	Shake flasks	LB medium with glucose 20 g L ⁻¹ , 37 °C at 14 and 60 h of culture time	7.9–9.2	54–61	Agus et al. (2010)
<i>E. coli</i> JM109 (pGEM-phaRCBspAB)	1800*	Shake flasks	LB medium with glucose 20 g L ⁻¹ , 25 °C	5	24	Agus et al. (2010)
<i>E. coli</i> DH5α (pGETS109-pha) with order gene phaABC, phaACB, phaBAC, phaBCA, phaCAB, and phaCBA	2000–6200	Shake flasks	LB medium with glucose 20 g L ⁻¹ , 30 °C, 130 rpm at 72 h	8.0–11.2	31–57	Hiroe et al. (2012)
<i>A. vinelandii</i> UWD	4100	Shake flasks	5 % (w/v) beet molasses at 24 h	N.S.	N.S.	Chen and Page, (1994)
<i>A. vinelandii</i> OPN (ptsIIA ^{Nir-})	3670	Shake flasks	Low aeration conditions (200 mL PY medium)	N.S.	62	Peña et al. (2014b)
<i>A. chroococcum</i> 7B	2215	Shake flasks	Microaerophilic conditions	2.87	61.3	Myshkina et al. (2008)

N.S. not specified

bacteria such as *C. necator*, some species of *Bacillus*, *Azotobacter*, recombinant strains of *E. coli*, and *Haloferax mediterranei*. This last is a natural P(3HB-co-3HV) producer (Don et al. 2006). The composition of P(3HB-co-3HV) produced by bacterial sources could be manipulated by the kind of carbon sources employed. Several studies have shown that the supply of propionyl-CoA in cells is the key factor for the production of the 3HV fraction during the synthesis of P(3HB-co-3HV) (Aldor et al. 2002). Therefore, most attempts aimed to produce

P(3HB-co-3HV) copolymer or increasing the 3HV fraction are based on the strategies to improve propionate utilization. On the other hand, the P(3HB-co-3HHx) is generally produced from plant oil and fatty acids by several wild-type and recombinant bacteria. Previous reports have shown that it is possible to produce P(3HB-co-3HHx), containing greater than 20 % content of 3HHx monomer, using plant oils as carbon source (Kahara et al. 2004; Budde et al. 2011; Riedel et al. 2012). More recently, Jeon et al. (2014) demonstrated that engineered

C. necator can produce P(3HB-co-3HHx), when this strain was grown on mixed acids or on butyrate as carbon source. This strain produced a polymer containing up to 40 wt % of 3HHx monomer. It is important to point that this was the first report for the production of P(3HB-co-3HHx) copolymer in *C. necator* using butyrate. In this section the more recent attempts to improve composition and production of the copolymers P(3HB-co-3HV) and P(3HB-co-3HHx) will be discussed.

Choi and Lee (1999) described a strategy for production of copolymer P(3HB-co-3HV) at high concentration using a recombinant strain of *E. coli* with different feeding solutions containing propionic acid and glucose. In that study, a maximal copolymer concentration of 141.9 g L⁻¹ with a P(3HB-co-3HV) up to 62.1 wt % and a 3HV component of 15.3 mol % was reached. It has been reported that the copolymer composition can be manipulated by adding propionate in the feed (Fidler and Dennis 1992; Slater et al. 1992, 1998; Yim et al. 1996; Choi and Lee 1999). However, industrial production of propionate is more expensive than glucose (Poirier et al. 1995; Aldor et al. 2002) making difficult the scale-up process for P(3HB-co-3HV) production. In addition, propionate being toxic must be fed at relatively low concentrations (Steinbüchel and Lütke-Eversloh 2003). An alternative strategy has been to design genetically modified strains in which it is possible to induce the expression of a critical gene in the polymer-producing pathway (Aldor and Keasling 2001). Some examples of genetic modifications that increased P(3HB-co-3HV) synthesis have been reported in different bacteria. For example, Yang et al. (2012), by introducing the genes of propionyl-CoA transferase (*pct*), β -ketothiolase (*bktB*), acetoacetyl-CoA reductase (*phaB*) and PHA synthase (*phaC*) from *C. necator* into *E. coli* strain YH090, were able to produce P(3HB-co-3HV) with an ultra-high 3HV monomer composition reaching over 80 wt %. More recently, Yang et al. (2014) reported the *E. coli* strain (XL-1) harboring *E. coli* *poxB* L253F V380A gene along *C. necator* *prpE* (propionyl-CoA synthase) and *phaCAB* genes, which was able to produce propionyl-CoA

via citramalate pathway. When this strain was cultured in a defined medium having 20 g L⁻¹ of glucose as carbon source, P(3HB-co-3HV) was produced up to polymer content of 61.7 % based on dry weight. Furthermore, the 3HV monomer fraction in P(3HB-co-3HV) increased up to 5.5 mol % by additional deletion of the genes responsible for the metabolism of propionyl-CoA (*prpC* and *scpC*). Another interesting case is that of *Salmonella enterica* serovar *Typhimurium* that by expression of the *E. coli* (2R)-methylmalonyl-CoA mutase (YliK) and (2R)-methylmalonyl-CoA decarboxylase (YgfG) was able to biosynthesize P(3HB-co-3HV) from a single carbon source through the generation of propionyl-CoA from succinyl-CoA (Aldor et al. 2002).

It is important to point out that the production cost of these polymers can be significantly reduced by using activated sludge instead of pure substrates. This step enables easy operation, since this does not require sterile conditions and uses renewable substrates as carbon sources (Bosco and Chiampo 2010). Others have focused on the use of dairy waste (Pandian et al. 2010), sewage water (Hu et al. 1997; Wong et al. 2000), waste from food processing industry (Wong et al. 2000), as well as agricultural feed stocks (Solaiman et al. 2006). More recently, Narayanan et al. (2014) reported that the culture of *B. mycoides* DFC1 in rice husk hydrolyzate in combination with gluten hydrolyzate resulted in maximum synthesis of P(3HB-co-3HV) in the presence of valeric acid as co-substrate at different induction intervals and concentrations.

On the other hand, focus has been on the production of P(3HB-co-3HHx), which take advantage of the microbial fatty acid degradation pathways (Khanna and Srivastava 2005; Budde et al. 2011). A recent report of P(3HB-co-3HHx) production by *C. necator* has showed that the engineered *C. necator* accumulated P(3HB-co-3HHx) from fructose via the inverted β -oxidation pathway (Insomphun et al. 2015). Since the metabolic flux from acetyl-CoA to 3HB-CoA was too high in the natural PHA producer *C. necator*, a cellular content of 48 wt % P(3HB-co-3HHx) composed of 22 mol % 3HHx was obtained. In this context, Wang et al. (2015) with the purpose

to produce P(3HB-co-3HHx) from glucose as carbon source designed an *E. coli* recombinant strain, where they combined the BktB-dependent condensation pathway with the inverted β -oxidation cycle pathway, by cloning five exogenous genes (*bktB*, *phaB1*, *phaJ*, *ter*, and *phaC*). The resultant recombinant strain was able to produce a copolymer with a 3HHx fraction of 10 mol %. On the other hand, the biosynthesis of P(3HB-co-3HHx) from sugars involves an artificial pathway that allowed to build up the C6-monomer from three acetyl-CoA molecules, which is a challenge from metabolic engineering point of view. Based on this strategy, the recombinant *E. coli* strain PHB4 designed by Fukui et al. (2002) was able to accumulate P(3HB-co-3HHx) up to 48 wt % of dry weight from fructose, although the (3HHx) monomer composition in the copolymer was lower than 1.5 mol %.

Case 2: Production of Medium-Chain-Length PHAs (MCL-PHAs)

The MCL-PHAs may be used in diverse applications due to their better physical and mechanical properties as compared with the SCL-PHAs (Table 2.1). The MCL-PHAs are characterized due to their low degree of crystallinity, low melting point, and glass-transition temperatures combined with their improved flexibility, elasticity, and sticky properties that are required for applications in certain biomedical areas (Abe et al. 2012; Chen et al. 2014; Table 2.1). *Pseudomonas* spp. are able to produce MCL-PHAs, and their composition is directly related to the carbon source used as growth substrate. This is because the former monomers, as it was previously described (Fig. 2.2), are derived from intermediates of fatty acid biosynthesis or β -oxidation pathways; therefore, the nature of the PHA monomers produced by *Pseudomonas* species will depend on the metabolism of the specific carbon sources. When the carbon sources are carbohydrates, *P. aeruginosa* accumulates C10 (3-hydroxydecanoate; 3HD) from the biosynthetic fatty acid pathway as the predominant monomer. However, when the carbon source used are fatty acids, the precursors for the PHA synthesis are produced by β -oxidation pathway,

and the predominant monomers are C8 (3-hydroxyoctanoate; 3HO), C10, and C12 (3-hydroxydodecanoate; 3HDD) (Madison and Huisman 1999; Nitschke et al. 2011). In addition, *Pseudomonas* spp. produce MCL-PHAs due to their PHA synthases (type II), which are able to polymerize hydroxy acids of short and medium chain length (3HA_{SCL} and 3HA_{MCL}), covalently linked within the same polyester molecules (Steinbüchel and Lütke-Eversloh 2003; Chen et al. 2014). MCL-PHAs such as poly-3-hydroxydodecanoate (P3HDD) and poly-3-hydroxyoctanoate (P3HO) are of commercial interest, because these polymers exhibit a considerable interval of thermomechanical properties and elastomeric behavior.

Simon-Colin et al. (2008) showed that *P. gueszennei* was able to produce MCL-PHA copolymers with a great diversity in their structures and properties. The carbon sources include saturated and unsaturated monomers, from C4 to C14, but preferably C8 and C10 monomers. Furthermore, this strain was able to use a broad range of carbon sources as carbohydrates or fatty acids. For example, when the strain was grown in glucose, cells accumulated 3-hydroxybutyrate (3HB: 1.3 mol %), 3-hydroxyhexanoate (3HHx: 0.9 mol %), 3-hydroxyoctanoate (3HO: 22 mol %), 3-hydroxydecanoate (3HD: 62.8 mol %), 3-hydroxydodecanoate (3HDD: 6.2 mol %), 3-hydroxydodecenoate (3HDDE: 5.6 mol %), and 3-hydroxytetradecanoate (3HTD: 1.2 mol %). In contrast, when the bacterium was cultivated using oleic acid as carbon source, the MCL-PHAs included 3HTD (13.8 mol %) with less fraction mol of monomer 3HD (35.6 mol %). In another study, Simon-Colin et al. (2012) using sodium octanoate as sole carbon source observed that *P. gueszennei* synthesized MCL-PHAs mainly composed of 3HO accounting for up to 94 mol % and lower amounts of 3HHx and 3HD. Recently, in cultures of the *P. fulva* strain TY16 grown on petrochemical wastes as carbon source, the production of MCL-PHAs was reported (Ni et al. 2010). Interestingly, when this strain was grown in glucose, toluene, benzene, ethylbenzene, gluconic acid, and acetic acid, it was able to synthesize MCL-PHA copolyesters, containing saturated

and unsaturated units of 3HDD, 3HHx, 3HO, and 3HD. On the other hand, copolyesters – MCL-PHAs synthesized by *P. fulva* strain TY16 from octanoic and decanoic acids – were composed of repeating units of 3HHx, 3HO, and 3HD with a mean molecular mass (MMM) between 42 and 43 kDa (Ni et al. 2010).

Another interesting case was reported using the strain of *P. putida* KT2440, grown with nonanoic acid and glucose as carbon sources at a 1:1–1.5 (w/w) ratio for the PHA production. Under such conditions, this strain accumulated a biopolyester with the following composition: 3-hydroxynonanoate (66 mol %), 3-hydroxyheptanoate (32 mol %), and 3HV (1 mol %). The terpolymer produced exhibited a MMM of 11 kDa, with a polydispersion index of 1.8 (Sun et al. 2009). Chan et al. (2014) evaluated the PHA production employing the strain of *P. mosselii* TO7 through utilization of plant oils such as soybean and palm kernel oil as carbon sources. These authors demonstrated that this strain accumulated up to 50 % (cell dry weight) of poly-3-hydroxyoctanoate (P3HO) achieving a productivity of 2.05 g_{PHA} L⁻¹ h⁻¹.

Hori et al. (2011) evaluated the effect of temperature (within a range from 15 to 30 °C), in the biosynthesis of the PHAs produced by *P. aeruginosa* IFO3924. The results indicated that the MCL-PHA composition was closely dependent on the temperature and the culture time in which the biopolymer accumulation is carried out. At the beginning of the culture, 3HD and 3-hydroxydodecenoate (C12:1) units were found in the PHA samples at all temperatures evaluated (15, 20, and 25 °C). In contrast, the 3HO was detected only at 30 °C. On the other hand, when the maximum cellular content of PHA was achieved, 3HO and 3HD were the major monomer units present at all the temperatures tested. Haba et al. (2007) studied the effect of temperature (in the range of 18–42 °C) on PHA composition in cultures of *P. aeruginosa* 47 T2. In this study, *P. aeruginosa* was grown in mineral medium supplemented with urea as nitrogen source and 2 % of waste cooking oil. The results obtained indicated that the most abundant mono-

mer was 3HD, except for PHAs produced at 42 °C in which 3HO was the monomer present in greater proportion (43.2 %). At elevated temperatures, long chain monomers such as 3HD, 3HDD, and C14:1 decreased, whereas at 37 °C the content of unsaturated monomers (C12:1, C14:2, C14:1) increased. Later on a significant decrease was observed at 42 °C (Haba et al. 2007). Another interesting example is the production of MCL-PHAs by cultures of *P. mediterranea* using reagent-grade or partially refined glycerol (Pappalardo et al. 2014). The gas chromatography analysis indicated that the biopolymer structure was composed by six monomers: 3HHx, 3HO, 3HD, 3HDD, *cis* 3-hydroxydodec-5-enoate (C12:1Δ⁵), and *cis* 3-hydroxydodec-6-enoate (C12:1Δ⁶).

Besides *Pseudomonas*, recombinant strains of *E. coli* are also an alternative for production of MCL-PHAs. For example, Li et al. (2011) reported an *E. coli* recombinant strain harboring *phaA* and *phaB* genes from *C. necator* and the *phaC2* gene from *P. stutzeri* (pCJY02). When this strain was cultivated in medium with decanoate, the bacterium accumulated SCL-MCL-PHAs with a monomer composition of 3HB, 3HHx, 3HO, and 3HD in mol ratios of 43.2:12.8:10.3:33.6. However, when this strain was grown on decanoate and glucose, the recombinant strain synthesized the same biopolymer, but the mol ratios were 3HB (83.4), 3HHx (4.0), 3HO (5.6), and 3HD (7.0). These results indicated that it is possible to modulate the monomer content and type of the PHA accumulated by adding different carbon sources and manipulating metabolic pathways of the host. In the same line, Zhuang et al. (2014) designed in its *E. coli* host metabolic pathways to synthesize MCL-PHAs directly from glucose. Engineering the reversed fatty acid β-oxidation cycle, Zhuang et al. (2014) employed this route to generate the key intermediates for the production of MCL-PHAs in *E. coli*. By using a PHA synthase with broad substrate specificity and using glucose as carbon source, recombinant *E. coli* was able to produce MCL-PHA copolymers with monomer composition ranging from 4 to 14 carbons. The PHA compositions in mol %

were 3HB:21.2, 3HHx:6.1, 3HO:45.8, 3HD:11.0, 3HDD:9.2, and 3HTD:6.8 (Table 2.2).

Case 3: Production of Poly-3-hydroxybutyrate of High Molecular Mass (HMM-P3HB)

The third case of microbial PHAs is the poly-3-hydroxybutyrate (P3HB), the common homopolymer of SCL; it is composed by monomers of 3-hydroxybutyrate (3HB), which are linked by ester bonds between the hydroxyl group and the carbonyl groups of the two adjacent monomers (Fig. 2.1). This polymer has similar thermomechanical properties to those found in conventional petrochemical plastics (Chanprateep et al. 2010), and these properties of P3HB are highly dependent on the mean molecular mass (MMM) of the polymer (Peña et al. 2014a; Domínguez-Díaz et al. 2015). Previous reviews have pointed out two interesting bacterial sources for the production of P3HB of high molecular mass (HMM-P3HB): one of them belongs to the genus *Azotobacter*, which is able to accumulate P3HB that exhibits a high molecular mass (>1000 kDa), and the other are recombinant strains of *E. coli* (Peña et al. 2014a; Leong et al. 2014). Some of the most relevant cases are discussed here.

Several authors have studied the culture parameters that could affect the MMM of the P3HB synthesized by *Azotobacter*, finding that the composition of the culture medium, the oxygen availability, and temperature are some of the factors that could have an important effect on the MMM. For example, Chen and Page (1994) observed that the UWD strain of *A. vinelandii* accumulated P3HB with a high MMM (4100 kDa), when it was cultivated using beet molasses of 5 % (w/v) in contrast with cultures without this substrate. These authors suggested that the nitrogen compounds of the beet molasses such as organic acids and salts stimulate the synthesis of P3HB of very high molecular mass (Chen and Page 1994). More recently, Peña et al. (2014b), in shaken flask cultivations of mutant strain OPN, reported a polymer with an MMM of 3670 ± 270 kDa in cultures conducted under low aeration conditions (conventional shaken flasks) as compared with cultures under high aeration. They proposed that by manipulating the aeration

conditions of the culture and therefore the oxygen availability, it is possible to modify the MMM of the P3HB. Similar results were reported by Myshkina et al. (2008); these authors evaluated the P3HB production by *A. chroococcum* 7B, under microaerophilic conditions. Under low aeration condition, this strain was able to synthesize P3HB with a MMM of 2215 kDa and an increase on aeration negatively affected the MMM of P3HB. They also found that the optimal temperature for P3HB production with a high molecular mass was 30 °C; in contrast, at low (20 °C) or high (37 °C) temperatures, the MMM decreased.

On the other hand, an interesting example for production of P3HB with a high molecular mass is that reported for *E. coli* recombinant strains harboring the *C. necator* biosynthesis *phbCAB* genes (Kusaka et al. 1997). These authors reported, for first time, the production of a polymer with ultrahigh molecular mass (20,000 kDa) during the stationary phase of growth by culturing *E. coli* XL-1 Blue (pSYL105), in a bioreactor of 2.6 L, under controlled pH conditions at 6.0 with LB medium supplemented with glucose (20 g L⁻¹). Interestingly, when this *E. coli* strain was grown at pH within a range of 7.0–8.0, the molecular mass of P3HB decreased to values below 5000 kDa after 12 h of culture.

Another interesting case was reported by Agus et al. (2006), who demonstrated that MMM of the P3HB accumulated by recombinant strains of *E. coli* depends on the specific PHA synthase (type and organism of origin) employed. Their results indicated that P3HB with high number-average molecular mass (Mn: 1500–4000 kDa) were synthesized by PHA synthases from *C. necator* (type I), *Delftia acidovorans* (type I), and *Allochromatium vinosum* (type III). P3HB with the lowest Mn (170–790 kDa) were accumulated by PHA synthases from *Aeromonas caviae* (type I), *Pseudomonas* sp. (type III), and *Bacillus* sp. (type IV). On the contrary, these authors found out that the highest MMM were obtained using the PHA synthase from *D. acidovorans* (4000 kDa). They also observed that for the strain harboring the PHA synthase from *D. acidovorans*, an acid pH (4.8) favored the P3HB production with high Mn (2100 kDa) as compared with the

biopolymer produced under basic conditions (pH 7.4–7.8), where the Mn was 1500 kDa, and these results were similar to those observed by Kusaka et al. (1997). When they investigated the effect of temperature on the Mn of P3HB using *E. coli* recombinant strains with PHA synthase *D. acidovorans*, they found that at 37 °C the biopolymer exhibited a higher Mn (4300 kDa) than that accumulated in the condition of 30 °C (580 kDa). In contrast, when Agus et al. (2006) used a recombinant *E. coli* strain with PHA synthase from *Bacillus* sp., they found a different behavior to that observed with the strain containing PHA synthase from *D. acidovorans*. They found that the Mn of P3HB produced by the strain with PHA synthase from *Bacillus* sp. in the condition of 37 °C was lower (440 kDa) than the Mn of the P3HB synthesized at 25 °C (nearly 1900 kDa).

On the other hand, through rearrangement of gene order of *phbCAB* operon biosynthesis (*phaABC*, *phaACB*, *phaBAC*, *phaBCA*, *phaCAB*, and *phaCBA*) in recombinant *E. coli* DH5 α (pGES109-*pha*), Hiroe et al. (2012) found that it was possible to produce P3HB with different MMM in the range between 2000 and 6200 kDa. The results indicate that the MMM of P3HB accumulated by the six strains was higher during the exponential growth phase (12 h of cultivation) as compared with the biopolymer produced at the stationary phase (72 h). They also found an inverse correlation between MMM and P3HB synthase activity, in contrast to the accumulation percentage (quantified as dry weight), which increased as the synthase activity increased (Hiroe et al. 2012; Table 2.3).

Case 4: Production of Homopolymers of Short Chain Length: Poly-4-hydroxybutyrate (P4HB) and Poly-3-hydroxypropionate (P3HP)

Another example of SCL-homopolymers is the poly-4-hydroxybutyrate (P4HB; Fig. 2.1), which is one of the most promising PHA for biomedical applications, because of its unique properties, which include biodegradability, biocompatibility, nontoxicity, and superior mechanical properties. It must be emphasized that synthesis of P4HB requires precursor like 4-hydroxybutyric acid, 1,4-butanediol, or γ -butyrolactone (Valappil et al.

2007). Recently, Kämpf et al. (2014) investigated the production of P4HB using recombinant *E. coli* JM109 that harbors a 4-hydroxybutyric acid CoA transferase gene (*orfZ*) from *Clostridium kluyveri*, using glycerol and propionic acid. They found that biopolymer accumulation in the cells was of 80 % (dry weight) achieving 3.7 g L⁻¹ (Table 2.2). On the other hand, Le Meur et al. (2013) reported that recombinant *E. coli* JM109 was able to produce P4HB using xylose as carbon source and sodium-4-hydroxybutyrate (Na-4HB) as biopolymer precursor. The highest P4HB concentration achieved was 4.33 g L⁻¹ with a yield ($Y_{P4HB/Na-P4HB}$) of 92 % g g⁻¹. Also, Le Meur et al. (2014) using fed-batch high-density bacterial mass using glycerol as the sole carbon source along with precursor 4HB for biopolymer synthesis achieved a concentration of 15 g L⁻¹ of P4HB.

The last example is the poly-3-hydroxypropionate (P3HP), which combines the properties of P3HB and poly-2-hydroxypropionate (known as polylactic acid). Andreeßen et al. (2010) reported the conversion of glycerol to P3HP in an *E. coli* recombinant strain, harboring genes encoding for glycerol dehydratase (*dhaB1*) of *Clostridium butyricum*, the propionaldehyde dehydrogenase (*pduP*) of *Salmonella enterica*, and the PHA polymerase (*phaC1*) of *C. necator*. After 92 h of incubation at 37 °C with 300 mM of pure glycerol, 1.42 g L⁻¹ of P3HP were achieved with a yield of 17.5 mmol P3HP mol glycerol⁻¹ consumed, with the drawback production of ethanol (8.04 g L⁻¹), succinate (48.92 g L⁻¹), and acetate (0.26 g L⁻¹) as by-products. Another case was reported by Wang et al. (2014). They built a recombinant strain of *E. coli* (with *panM*, *panD*, *pp0596*, *ydfG*, *prpE*, and *phaC1*) for the P3HP production. This strain was able to produce 0.5 g L⁻¹ of biopolymer when it was cultivated in shaken flasks, using glycerol and glucose as carbon sources and without any addition of precursors. In cultures of the same strain in stirred bioreactors in fed-batch aerobic cultures, they obtained up to 10.1 g L⁻¹ of P3HP (Wang et al. 2013). In the same line, Gao et al. (2014) designed a recombinant stable *E. coli* strain harboring seven exogenous genes of P3HP synthesis pathway. This strain in aerobic fed-batch cultures

was able to produce 25.7 g L⁻¹ of biopolymer from glycerol.

2.6 Perspectives

PHAs are biomaterials of great importance not only due to their biodegradability and thermomechanical capabilities similar to those of the plastics derived from the petrochemical industry but also due to their biocompatibility, which is a characteristic required in medical and biomedical fields. In addition, the success of application of these biopolymers will depend on their chemical nature, mainly the monomer composition and mean molecular mass, and other properties which influence the mechanical properties, biodegradability, and biocompatibility of PHAs. Current advances in fermentation, purification technology, as well as the design of mutant strains by recombinant DNA technology would allow the tailor-made production of new PHAs. These tailor-made PHAs can be used as materials for biomedical uses, such as tissue engineering. From the economic viewpoint, the efforts are now focusing on the design of new strains, which can use complex substrates of very low cost, such as those present in waste materials, and having the versatility to produce PHAs with a wide chemical variety and molecular mass.

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