Chapter 6 Polyhydroxyalkanoates (PHA): natural polymers produced by bacteria, an option for the replacement of plastics

Capítulo 6 Polihidroxialcanoatos (PHA): polímeros naturales producidos por bacterias, una opción para el remplazo de los plásticos

FONSECA-BARRERA, Itzel del Carmen†*, MENDOZA-GARCÍA, Patricia Guillermina, PEÑA-MONTES, Carolina and RAMÍREZ-HIGUERA, Abril

Tecnológico Nacional de México - I. T. Veracruz, Unidad de Investigación y Desarrollo de Alimentos, Av. Miguel Ángel de Quevedo No. 2779, Col. Formando hogar, 91987 Veracruz, Ver, México.

ID 1st Author: *Itzel del Carmen, Fonseca-Barrera* / **ORC ID:** 0000-0003-3562-9899, **CVU CONACYT ID:** 950657

ID 1st Co-author: *Patricia Guillermina, Mendoza-García* / **ORC ID:** 0000-00001-6838-0861, **CVU CONACYT ID:** 270773, **SNI CONACYT ID:** 77817

ID 2nd Co-author: *Carolina, Peña-Montes* / **ORC ID:** 0000-0002-4767-1210, **CVU CONACYT ID:** 277236

ID 3rd Co-author: *Abril, Ramírez-Higuera* / **ORC ID:** 0000-0002-1430-2689, **CVU CONACYT ID:** 242658

DOI: 10.35429/H.2022.6.1.64.81

I. Fonseca, P. Mendoza, C. Peña and A. Ramírez

* patricia.mg@veracruz.tecnm.mx

A. Marroquín, L. Castillo, S. Soto, L. Cruz. (Coord.) CIERMMI Women in Science TXIX Biological Sciences. Handbooks-©ECORFAN-México, Querétaro, 2022.

Abstract

Synthetic plastics have facilitated the transport of food and various products; however, their time to degrade has caused severe environmental problems due to their accumulation in seas and rivers. Polyhydroxyalkanoates (PHA) have been proposed as an alternative to synthetic plastics due to their biodegradable characteristics and similar properties to polypropylene and polystyrene. PHA are polymers produced by bacteria such as *Bacillus* spp., *Streptomyces* spp., *Staphylococcus* spp., *Cupriavidus necator*, *R. eutropha* and *Alcaligenes latus* that accumulate the polymer in intracellular lipid granules that serve as their energy source. This review aims to provide an overview of research in recent years on identifying PHA-producing strains, methods for their extraction, factors affecting their production, the study of their structure and film-forming characteristics, and their applications and future developments related to PHA.

Polyhydroxyalkanoates, Intracellular, Bioplastics, Environmental

Resumen

Los plásticos sintéticos han facilitado el transporte de alimentos y de diversos productos, sin embargo, el tiempo que requieren para degradarse ha ocasionado graves problemas ambientales debido a su acumulación en mares y ríos. Los polihidroxialcanoatos (PHA) han sido propuestos como una alternativa para la reducción al uso de sintéticos plásticos debido a sus características biodegradables y propiedades similares al polipropileno y al poliestireno. Los PHA son polímeros producidos por bacterias, por ejemplo, *Bacillus* spp., *Streptomyces* spp., *Staphylococcus* spp., *Cupriavidus necator*, *Rastonia eutropha* y *Alcaligenes latus* que acumulan al polímero en gránulos lipídicos intracelulares que sirven como su fuente de energía. El objetivo de esta recopilación es proporcionar un panorama sobre las investigaciones que se han realizado en los últimos años acerca de la identificación de cepas productoras de PHA, los métodos para su extracción, factores que afecten su producción, el estudio de su estructura y características para la formación de películas; así como las aplicaciones y los futuros avances relacionados con los PHA.

Polihidroxialcanoatos, Intracelular, Bioplásticos, Ambiental

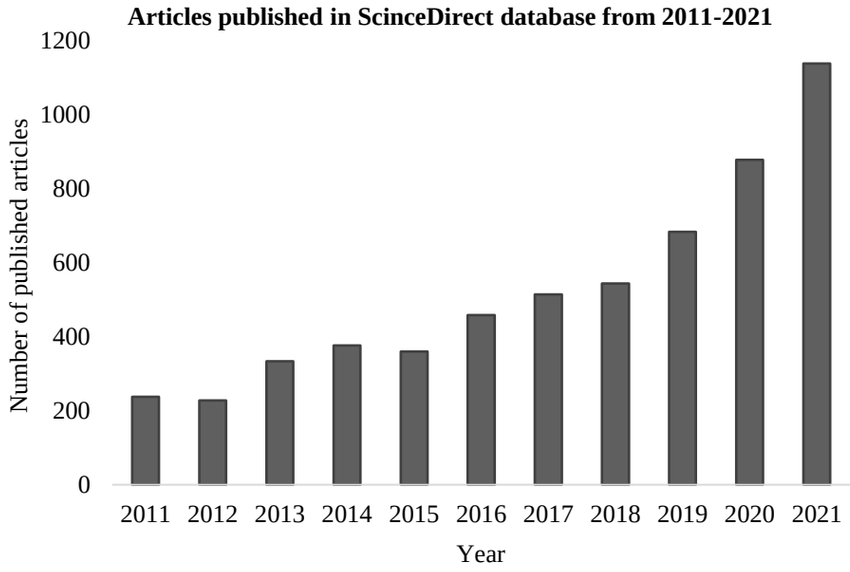
6.1 Introduction

The increase in the production of plastics by industry has caused their accumulation in the seas and soils, causing dramatic changes in the biosphere and affecting the survival of several species (Luengo *et al.*, 2003). The production of plastics has increased significantly, reaching 350 million tons annually. One of the main reasons for this is the advantages of these materials, such as durability, flexibility, and low production cost. However, it is estimated that more than 250 000 tons of plastic float in the sea, negatively affecting marine wildlife (Heidbreder *et al.*, 2019). In most cases, plastic waste reaches the ocean after being mismanaged on land. Plastic pollution has gained political attention, causing challenges for its reduction. Globally, it has been estimated that plastic pollution causes about $13 billion in financial damages annually (Nielsen *et al.*, 2019). It has been reported that the five countries originating the most significant amounts of plastic pollution are China, Indonesia, the Philippines, Vietnam, and Sri Lanka, which are responsible for more than half of the plastic leakage from land to sea. However, the leading cause of these leaks is the export of waste from other countries, so measures have been taken, such as the return of waste to the exporting countries (UNEP, 2014; Gregson and Crang, 2018; Nielsen *et al.*, 2019).

New alternatives have been sought to reduce the production and use of plastics worldwide. Polyhydroxyalkanoates (PHA) are polyesters stored intracellularly by different bacteria as a carbon source. They are made up of fatty acid chains with ester bonds between their hydroxyl group and the carbonyl group of the following monomer (Muhammadi *et al.*, 2015). Due to their characteristics, like conventional plastics, have been taken as an alternative to replace synthetic polymers. According to the ScienceDirect database, the number of publications on PHA production increased from 238 articles in 2011 to 1139 by 2021 (Graphic 6.1). Studies have focused on increasing the yield in the production of PHAs, their biomedical applications, the use of low-cost carbon sources, physicochemical modifications of the polymer to improve its characteristics and the search for new PHA-producing strains.

At the industrial level, countries such as the United States, China, Italy, Canada, and Germany produce polyhydroxyalkanoates such as polyhydroxybutyrate (PHB) and PHB-co-hydroxyvalerate (PHV) copolymers from bacteria such as *Rastonia eutropha*, *Cupriavidus necator*, *Aeromonas hydrophila* and *Pseudomonas putida*. TianAn, a Chinese PHA producing company, has reported PHA production of 10 thousand to 50 thousand tons per year. Nodax, a U.S. company, annually produces 91 thousand tons of different types of PHA. The thermoplastic nature of PHA makes it a candidate for a wide range of standard manufacturing techniques, including injection molding, extrusion, film-forming and blow molding. This compilation aims to provide an overview of the research carried out in recent years on identifying PHA-producing strains, methods for their extraction, factors affecting their production, the study of their structure and film-forming characteristics, as well as applications and future developments related to PHA.

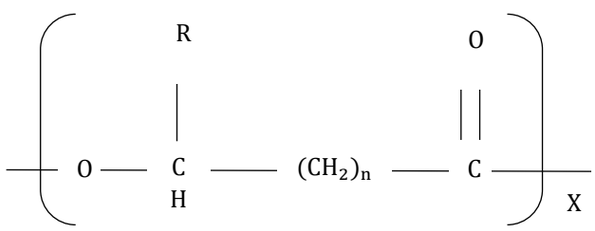
Graphic 6.1 Articles published in Sciencedirect on Polyhydroxyalkanoates



6.2 Chemical structure of PHA

PHAs are polyesters of 3,4,5 and 6 hydroxyalkanoic acids with a general structure shown in Figure 6.1. The radical group is regularly substituted by an unsaturated or saturated alkyl group ranging from C_1 to C_{13}. PHAs are linear polymers that form ester bonds between the carboxyl group of one monomer and the hydroxyl group of the next. More than 150 types of hydroxycarboxylic acids have been identified as PHA monomers. In all PHAs that have been characterized, the hydroxyl-substituted carbon atom has the R configuration due to the stereo-specificity of PHA biosynthetic enzymes. Because of this chirality at the center of the structure, the chemical synthesis of PHAs is complex. The monomer composition, macromolecular structure, and physicochemical properties of PHA vary depending on the microorganism and carbon source used for their growth (Berezina and Martelli, 2014; Muhammadi *et al.*, 2015; Prados and Maicas, 2016).

Figure 6.1 The general structure of PHAs



R group	No. of carbons	PHA polymer
Metyl	C_4	Poly (3-Hydroxybutyrate)
Etyl	C_5	Poly (3- hidroxivalerate)
Propyl	C_6	Poly (3-hydroxyhexanoate)
Butyl	C_7	Poly (3-hydroxyheptanoate)
Pentyl	C_8	Poly (3-hydroxyoctanoate)
Hexyl	C_9	Poly (3-hydroxynonanoate)
Heptyl	C_{10}	Poly (3-hydroxydecanoate)
Octyl	C_{11}	Poly (3-hydroxyundecanoate)
Nonyl	C_{12}	Poly (3-hydroxydodecanoate)

*Source: Muhammadi et al.,*2015

According to their chain size, PHA can be classified into short-chain (3-5 carbon atoms), medium-chain (6-14 carbon atoms) and long-chain (more than 14 carbon atoms) hydroxyalkanoic acids. It has been reported that strains such as *Alcaligenes eutrophus* can only synthesize short-chain PHA. On the other hand, *Pseudomonas oleovorans* are associated with the production of medium-chain PHA (Muhammadi *et al.,* 2015). The synthesis of both short-chain and medium-chain PHA has also been reported. For example, in the presence of propionic and valeric acid, *Alcaligenes eutrophus* produces copolymers of 3-hydroxybutyrate acid and 3-hydroxyvalerate acid (Anderson *et al.*, 1990). *Aeromonas caviae* is another bacterial strain that produces a copolymer of hydroxybutyrate and 3-hydroxyhexanoate. Boyadin *et al.* (2008) reported that *Photobacterium leiognathi* and *Vibrio harveyi* produced 2 to 3 PHA heteropolymers (hydroxybutyric, hydroxyvaleric and hydroxyhexanoic acid). As observed in these studies, the type of PHA depends on the producing bacteria; therefore, in the following section, different sources of isolation of PHA-producing strains and some of their yields will be mentioned.

6.3 PHA-producing microorganisms, isolation sources and nutritional factors that affect its production

Some bacteria accumulate substances, such as PHA, when external energy exceeds the cell need for growth. PHA is utilized when external energy input is insufficient to maintain cell growth, division, or viability. About 300 different bacteria, including Gram-positive and Gram-negative bacteria, have been reported to accumulate PHA. These bacteria accumulate PHA intracellularly in lipid granules in the presence of excess carbon sources and limited nitrogen source conditions. Once the microorganism depletes this carbon source, PHA is depolymerized and metabolized to act as a carbon and energy source (Muhammadi *et al.*, 2015; Prados and Maicas, 2016).

Several genera capable of synthesizing PHA have been identified, among which the *Bacillus* and *Pseudomonas* genera stand out. The source of isolation can be soil samples, marine sediments, dairy products, used oils or waste from different industries, for example. Ching *et al.* (2007) isolated bacteria from marine sediments, four of the twenty phenotypically different colonies were positive in the Nile red staining test. The bacteria were PHA producers. They identified by 16S rDNA sequencing that the four isolates belonged to the genus *Vibrio* with 98% identification. In the same year, Jamil *et al.* isolated a PHA-producing strain from sediment samples from the Karachi coast. The 16S rRNA gene analysis identified it as *Pseudomonas* sp. with 94 % homology. Sangkharak and Prasertsan (2012) isolated PHA-producing bacteria from different sources (pickle waste, nitrogen-rich soils, cow and chicken manure, and municipal garbage). In total, fifty strains were isolated and tested positive with Nile blue. Six different genera were identified, including *Bacillus* spp., *Proteus* spp., *Pseudomonas* spp., *Aeromonas* spp., *Alcaligenes sp*. and *Chromobacterium* spp. Nevertheless, the bacterium identified as *Bacillus licheniformis* JN 162418 was determined to have the highest PHA production (6.58 g /L).

In 2019, Alshehrei collected soil samples from Saudi Arabia to determine the presence of PHA-producing bacteria. Twenty strains tested positive for PHA production using Sudan black. It was determined that strain F15 belonged to the *Bacillus* genus and had the highest PHA production (4.3 g/L). Mohammed *et al.* in 2019, isolated two different PHA-producing *Bacillus* species from plastic waste landfills. Both bacteria tested positive using Sudan black B and Nile blue A. They reported that the highest PHA production for *Bacillus* BPPI-14 (49.46 ±2.79%) was obtained using glucose as a carbon source, a temperature of 37 °C and a pH of 7. It has also been possible to isolate PHA-producing strains from oil-contaminated soils. Al-Ardawy and Taj-Aldeen (2020) identified the presence of PHA granules in *Lactiplantibacillus plantarum* isolated from dairy products and *Pseudomonas* isolated from oil. They determined that *Lactiplantilactobacillus plantarum* had the highest PHA production (1.5 g /L) compared to *Pseudomonas* spp. 1 (0.95 g/L).

Isolation sources of PHA-producing bacteria can be varied, and their production conditions can also be a significant effect when considering their yield. Yüksekdag *et al.*, (2007) analyzed different carbon and nitrogen sources in PHB production from *Streptococcus thermophilus* Ba21S; their results showed that the percentage of PHB increased when using sucrose (35.56%) compared to the control (glucose) (12.47%) opposite case when changing the nitrogen source where it decreased from 21.15% (Protease peptone) to 3.69 % when using L-cysteine. Sharma *et al.*, (2012) studied PHA production in a *Pseudomonas putida* LS46 at high and low concentrations of ammonium sulfate (4 g/L and 1 g/L). They determined that the highest production occurred when using nitrogen at low concentrations in a 48h incubation period. Other examples of PHA-producing genus and their isolation source, as well as their PHA production and carbon source used for PHA synthesis, are shown in Table 6.1. Each bacterium synthesizes PHA differently depending on the carbon source and the enzymes involved in the process. In section 6.3, some of the most common routes for PHA synthesis will be described.

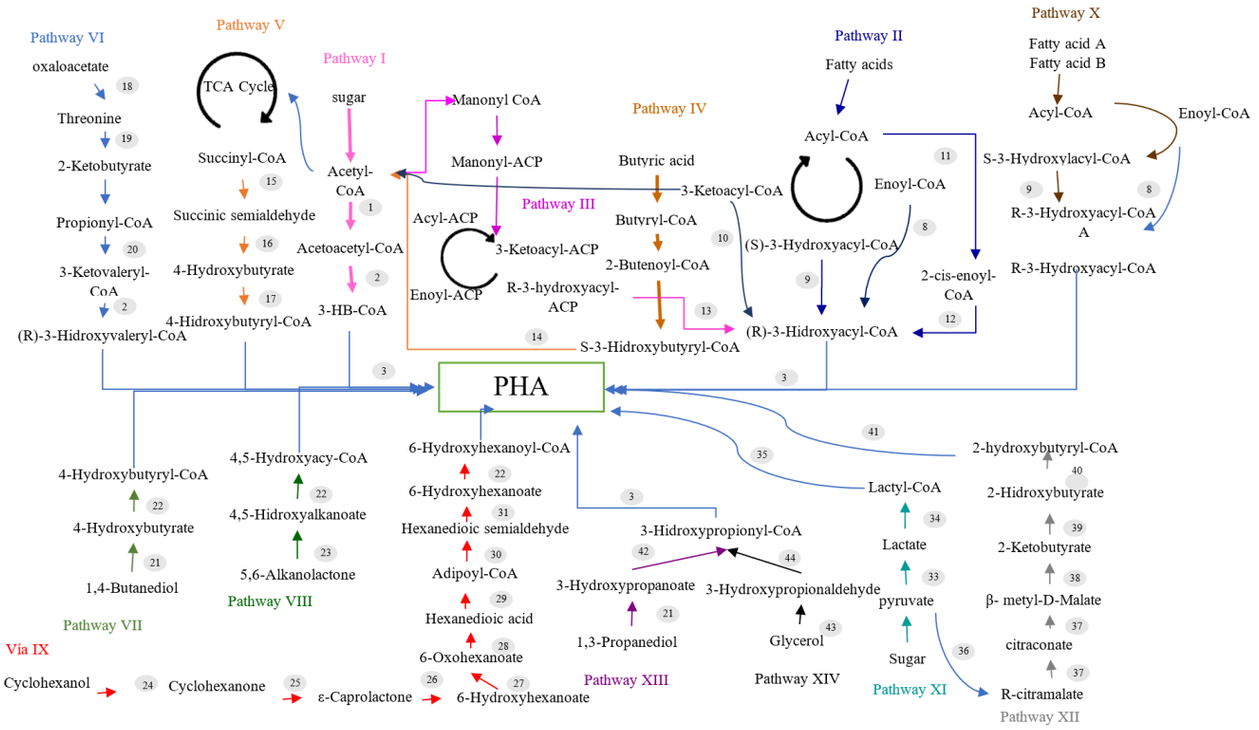
Table 6.1 PHA-producing bacteria isolated from different sources and their polymer production yield using different carbon sources

Bacteria	Insulation source	Carbon source	PHA (g/L)	Author
Vibrio MK4	Soil samples from Katch Island.	Sesame oil	4.22	Arun *et al.*, 2009
Lactiplantilactobacillus plantarum CW10 *L. casei* WWD3	Fermented milk Whey	Starch	3.5 3.3	Monilola and Makinde, 2020
Bacillus subtilis MSBN17	Sea sponge Callyspongia diffusa	waste pulp, tamarind powder and palm sugar	2.46	Sathiyanarayanan *et al.*, 2013
Bacillus spp.	Liquid wastewater samples from the "salwa" industry	Fructose	2.2	Ataei *et al.*, 2008
L. casei *L. plantarum* A *L. brevis* C3 *P. halophilus* B1 *S. thermophilus* E1	Different dairy products	Glucose	1.9 0.66 0.86 0.11 0.19	Aslim *et al.*, 1998
Pseudomonas chlororaphis PA23	Soybeans	Nonanoic acid	0.9601	Sharma *et al.*, 2017
Halomonas AT1214	Shrimp shell	Yeast extract	0.270	Simon-Colin *et al.*, 2008
Pseudomonas aeruginosa AU1292	Sediments from the Karachi coast	Yeast extract	0.376	Jamil *et al.*, 2007
Vibrio M20	Marine sediment	Glucose	0.176	Chien *et al.*, 2007

6.4 Metabolic pathways for the synthesis of PHA

A total of 14 pathways have been reported for PHA synthesis (Figure 6.2). Regularly, short-chain PHA is synthesized by pathway I, where two molecules of acetoacetyl-CoA are condensed to one molecule of acetoacetyl-CoA by the enzyme β-ketothiolase (PhaA). Acetoacetyl-CoA is converted to 3-hydroxybutyrate-CoA by the enzyme NADPH-acetoacetyl-CoA reductase (PhaB). Subsequently, the ester bond is catalyzed by PHA synthase (PhaC) action to form poly (3-hydroxybutyrate). In pathway II, the substrates originated from the β-oxidation of fatty acids. In this pathway, different hydroxyalkanoate monomers are generated by the action of (R)-enoyl-CoA hydratase (PhaJ), acyl-CoA oxidase and 3-ketoacyl-CoA reductase (Sharma *et al.*, 2021). The V-XIV pathways are engineered pathways leading to the production of unconventional PHA, for example, the use of a recombinant *E. coli* to produce poly-4-hydroxybutyrate using glucose as a carbon source. This strain contained genes encoding succinate degradation from *Clostridium kluyvery* and PHA synthase from *Ralstonia eutropha*. The native succinate semialdehyde dehydrogenase genes (*sad* and *gabD*) in *E. coli* were inactivated to enhance carbon flux to poly-4-hydroxybutyrate biosynthesis via pathway 5 (Zhou *et al.*, 2012).

Figure 6.2 Pathways for PHA synthesis



Enzymes for PHA synthesis: 1(β-Ketothiolase);2 (NADPH dependent acetoacetyl-CoA); 3(PHA synthase); 8 (R-Enoyl-CoA hydratase); 9 (Epimerase); 10(3-ketoacyl-CoA reductase);11(Acyl-CoA oxidase, putative); 12 (Enoyl-CoA hydratase, putative); 13 (3-Hydroxyacyl-ACP-CoA transferase); 14 (NADH-dependent acetoacetyl-CoA reductase);15 (Succinic semoaldehyde dehydrogenase); 16 (4-Hydroxybutyrate dehydrogenase); 17(4-Hydroxybutyrate-CoA transferase); 18 (Aspartokinase I, Homoserine kinase, Threonine synthase); 19 (Threonine deaminase); 20 (BktB(PhaA)); 21 (Alcohol dehydrogenase, Aldehyde dehydrogenase); 22 (Hydroxyacyl-CoA synthase, putative); 23 (Lactonase, putative); 24 (Cyclohexanol dehydrogenase); 25(Cyclohexanone monooxygenase); 26 (Caprolactone hydrolase); 27 (6-Hydroxyhexanoate dehydrogenase); 28 (6-Oxohexanoate dehydrogenase); 29 (Semialdehyde dehydrogenase, putative); 30 (6-Hydroxyhexanoate dehydrogenase, putative); 31 (Hydroxyacyl-CoA synthase, putative); 32 (3-Ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA dehydrogenase); 33 (Lactate dehydrogenase); 34(Propionate CoA-transferase); 35 (Type II PHA syhthase); 36(α-Isopropylmalate synthase); 37(3-Isopropylmalate dehydratase); 38 (3-Isopropylmalate dehydrogenase); 39 (2-Hydroxybutyrate dehydrogenase); 40 (Propionate CoA-transferase);41(Type II PHA syhthase); 42 (Propionyl-CoA synthase); 43 (Glycerol dehydratases); 44 (Propionaldehyde dehydrogenase)

Source: Modified from Meng et al., 2014

Each pathway involves several enzymes that play an essential role in PHA synthesis. However, PHA synthase is one of the enzymes in charge of defining the chain length size of PHA. The different PHA synthases are classified into four categories based on their structure, composition and chain length. Class I comprises a subunit that polymerizes short-chain PHA, and class II comprises a subunit that polymerizes medium-chain PHA. Class III and IV are heterodimer enzymes formed by two subunits, class III is constituted by a subunit of PhaC and another subunit of PhaE, and class IV comprises two subunits, one of PhaC and PhaR (Tripathi *et al.*, 2021). PhaC from *Rastonia eutropha* has been known to polymerize PHA monomers consisting of 3 to 5 carbons such as poly(3-hydroxypropionate), poly(3-hydroxybutyrate), poly(4-hydroxybutyrate), poly(3-hydroxyvalerate) and copolymers of hydroxypropionate and 4-hydroxybutyrate).

Once PHA is formed, it is stored in lipid granules inside the cell, where their number and size depend on the producing bacterium. About 8-13 granules ranging from 0.2 to 0.5 µm have been reported for *Alcaligenes eutrophus*. However, in *Pseudomonas oleovorans*, it is estimated to accumulate around one to two large granules (Muhammadi *et al.*, 2015). On the other hand, PhaC identified in the *Pseudomonas* genus can polymerize 6 to 14 carbon monomers (Meng *et al.*, 2014). Once PHA is formed, it is stored in lipid granules inside the cell, where their number and size depend on the producing bacterium. About 8 to13 granules ranging from 0.2 to 0.5 µm have been reported for *A. eutrophus*. However, in *P. oleovorans,* it is estimated to accumulate around one to two large granules (Muhammadi *et al.*, 2015).

The variety of PHA, synthesized by the different pathways, has led to this polymer having physicochemical properties that differ from one other giving them applications in different areas. Some examples of different PHA synthesized and their physicochemical properties will be mentioned in section 6.5.

6.5 Physicochemical properties of PHAs

PHAs are biodegradable thermoplastics that are insoluble in water but soluble in chlorinated hydrocarbons such as chloroform. They have poor resistance to acids and bases. The properties of PHAs differ depending on their chemical composition. In some cases, their properties can be similar to common polymers. The PHB has some characteristics similar to polypropylene (Table 6.2). PHBs found in pure form are brittle because it presents an elongation percentage lower than 15 %. However, they have Young's modulus above 1 GPa, indicating that it is a rigid material (Bugnicourt *et al.*, 2014). The use of a polyhydroxybutyrate/polyhydroxyvalerate (PHB-HV) blend reduces the percentage crystallinity and Young's modulus from 60 % (PHB) to 53 % and from 1700 MPa to 1200 MPa, respectively (El-Hadi *et al.*, 2002). Another author who studied the blend of these polymers was Arcos-Hernandez *et al.* (2013), who studied the properties of a 38%mol PHB -62%mol HV copolymer. Their data determined that this presented Young's modulus of 867 MPA and an elongation percentage of 5%. A glass transition temperature of -12.4 °C and a degradation temperature of 268.1 °C.

Corre *et al.* (2012) studied the properties of different commercial PHA (Enmat Y1000P, Mirel F1006, Mirel F3002 and P226). Their results showed that the commercial PHA Enmat Y1000P presented values of Young's modulus (2624 MPa) and maximum tensile stress (32.2 MPa) higher than P226 and Mirel F3002, in addition to Young's modulus increased from 2624 MPa at day 0 to 4329 MPa at day 74. This process could be due to a secondary crystallization presented by this type of material.

Wecker *et al.* (2015) analyzed PHA synthesized by an *Enterobacter* sp. FAK 1384 determined that the PHA was a medium-chain of different polymers (3-hydroxydecanoate, 3-hydroxyoctanoate, 3-hydroxydodecenoate, 3-hydroxydodecanoate and 3-hydroxyhexanoate and 3-hydroxytetradecanoate). They determined that it had a crystallinity index of 0.26, a melting point temperature of 47 °C and a glass transition temperature of -47 °C. In 2016, Ray *et al.* analyzed PHA by the strain *Pannonibacter phragmitetus* ERC8. They determined by the nuclear magnetic resonance that the synthesized PHA was a copolymer of 3HB and 3HA (medium-chain PHA). The polymer's melting temperature was 163.29 °C with a percentage crystallinity of 48.55 %. The thermogravimetric analysis determined that requiring 500 °C for its complete degradation.

Perez-Arauz *et al.* (2019) reported the production of a heteropolymer composed of 98.2% mol HB, 0.75% mol HV and 1% mol HA from the *Cupriavidus necator* strain. They determined that the melting temperature for this polymer was 159.4°C with a crystallinity percentage of 22.2 %. Regarding its mechanical properties, the maximum stress of 2.7 MPa and an elongation of 25.7 % were obtained. The films elaborated from this polymer also showed a water vapor permeability (2.59×10^{-13} kg m s^{-1} $Pa^{-1}m^{-2}$) lower than that reported for those elaborated from PHB (6.9^{-7} kg m s^{-1} $Pa^{-1}m^{-2}$)

Table 6.2 Maximum tensile stress, % elongation and degradation temperature of different PHB copolymers and traditional synthetic polymers

Samples	Tm	σ (MPa)	ε (%)
PHB	177	43	5
P(3HB-co-20%mol3HV)	145	20	50
P(3HB-co-16%mol4HB)	150	26	444
P(3HB-co-15%mol 3HHx)	115	23	760
P(HB-co-10%mol HV)	150	25	20
P(HB-co-20%mol HV)	135	20	100
P(HB-co-10%mol HHx)	127	21	400
P(HB-co-17%mol HHx)	120	20	850
Polypropylene	170	34	400
Polystrene	110	50	-
Polyethylene	130	-	500
HDPE	135	29	620
LDPE	130	10	7300
PET	262	56	-

Tm: melting temperature.
σ: Tensile strength.
ε: Elongation at break.
HDPE: high-density polyethylene.
LDPE:low-density polyethylene.
PET: poly(ethylene terephthalate).

Source: Modified of Muhammady et al., 2015

6.5 Methods for the identification of PHA-producing bacteria

A method traditionally used for the detention of PHA granules is the use of Sudan black dye B, which consists of growing colonies in agar culture media where the dye previously dissolved in 96% ethanol is added. Once the dye is added for 20 minutes, the dye is decanted, and 96% ethanol is added for 1 minute. The colonies that present a bluish-black will be taken as positive for the production of the polymer. It is a convenient method for arresting a large number of PHA-producing bacteria (Schlegel *et al.*, 1970; Godbole, 2016). Phanse *et al.*, 2011, used this method to screen bacteria isolated from different sources (including domestic and industrial wastewater, dairy waste and soil samples). Twenty-three isolates tested positive for PHA production, showing a blue-black staining of their colonies, of which twelve belonged to the genus *Bacillu*s, five to the genus *Pseudomonas*, and four to the genus *Azotobacter* and two isolates to *Staphylococcus*.

Another dye employed for the detection of PHA is the use of Nile blue, a basic water-soluble oxazine. Heat-fixed smears are stained with 1% Nile blue A at 55 °C for 10 minutes and then washed with water and 8% acetic acid for one minute to remove excess dye. The stained smears are examined at 460 nm because PHA granules show orange fluorescence (Ostle and Holt, 1982). In 2017, Mascarenhas and Aruna identified PHA-producing bacteria isolated from different sources (raw honey samples, sugar industry waste, used machine oil and petroleum-contaminated soil) using Sudan black B and Nile blue. Their results showed that 81 strains isolated from these sources were positive when screened with Sudan black B. However, only 34 positive strains fluoresced in ultraviolet light when secondary screening was performed with Nile blue. Evangeline and Sridharan (2019) identified one PHA-producing bacterium out of the five isolated soil samples collected from the Ranipet area using Sudan black B and Nile blue as the screen. The bacterium was identified as *Bacillus cereus* VIT-SSR1.

Nile red, a derivative of Nile blue, is a sensitive screening method that allows observation of PHA accumulation in viable bacteria and is proposed by Spikerman *et al.* (1999). This method uses a stock solution of 0.25 mg of Nile red dissolved in 1 mL of dimethyl sulfoxide. This solution is poured into the sterile culture medium with a final concentration of 0.5 μg of Nile red per milliliter. The agar plates are exposed to ultraviolet light at 312 nm; as with blue, samples positive for PHA accumulation will fluoresce.

6.6 Methods used for PHA extraction

Cell disruption and removal of the protein sheet surrounding the PHA granules are necessary to extract PHA granules. Several protocols have been described for obtaining PHA. Among the methods mentioned in the literature are solvent extraction, chemical digestion, and enzymatic and physical methods (Visakh, 2014; Muhammadi *et al.*, 2015).

Most of the extraction methods use solvents such as chloroform and methanol. Modification in permeability of cell walls and dissolution of PHA in the solvent are the mechanisms of PHA extraction. Separation of PHA from the solvent can be performed by evaporation or precipitation of the polymer with a non-solvent material (Visakh, 2014). Due to the degradation of endotoxins present in Gram-negative bacteria, extraction by this method has been used for medical applications (Mohammadi and Ghaffari-Moghaddam, 2014). Regularly, the solvent ratio used is 20 parts for one part of the polymer, making it a costly method for the industry. (Perez-Rivero *et al.*, 2019). Currently, strategies have been sought for the use of chloroform, such as dimethyl carbonate. Samori *et al.* (2015) used this solvent to extract PHA from mixed cultures (*Amaricoccus sp.*, *Azoarcus sp*. and *Thauera*). They obtained a purity of 98 % and a polymer molecular weight of 1.2 MDa.

Another method studied is digestion, where sodium hypochlorite dissolves cellular substances such as proteins, carbohydrates, lipids and nucleic acids. PHA can be separated from the solution by centrifugation. Despite being a less expensive method, it has been reported that it can reduce up to 50% of the molecular weight of PHA because it is a potent oxidant, so the extraction conditions must be adequately designed to control the decrease in molecular weight (Tripathi *et al.*, 2021). Ramsay *et al.* (1990) extracted PHB from *Alcaligenes eutrophus* using sodium hypochlorite and surfactants (SDS and Triton X-100). They obtained purity of 97 to 98 % with a molecular weight between 730000 Da and 790000 Da when using surfactants, higher than that obtained by using only sodium hypochlorite (87% purity and molecular weight of 680000 Da). Heinrich *et al.* (2012) extracted PHB from *Ralstonia eutropha* H16 strain using sodium hypochlorite 13 % (v/v). They determined that they obtained a purity of 95.66% with an extraction percentage of 91.32%. Using gas chromatography, they determined that the weight of the extracted polymer was in the range of 460000 and 830000 Da lower than that reported for PHA extracted by chloroform (1700000kDa). The reduction in the molecular weight of PHA has led to the use of sodium hypochlorite and chloroform for polymer extraction. Hahn *et al.* (1994) obtained PHB from *A. eutrophus* using 30% concentrated hypochlorite and chloroform in a 1:1 ratio. Their results showed that polymer recovery was higher using this combination (97%) than sodium hypochlorite alone (91%). They also reported that the molecular weight of PHA did not significantly reduce its molecular weight (1020000 Da) with the control (chloroform: 1272000 Da). In 2013, Hamieh *et al.* used a sodium hypochlorite solution to lyse the *Lactobacillus acidophilus* cell wall for 24 hours. Chloroform was added to dissolve the PHA, and the polymer was precipitated with an acetone-ethanol solution 1:1 (v/v) to remove the membrane lipids. Chloroform was evaporated at 70 °C to obtain polymer crystals (Jacquel *et al.*, 2008).

Homes and Lim (1989) developed the enzymatic digestion method as an alternative to solvent extraction. The procedure starts with heat treatment of the cell mass, followed by enzymatic hydrolysis, treatment with surfactants and final decolorization with hydrogen peroxide. Although enzymes lead to high recovery levels, their high cost is a disadvantage. Some enzymes have high activity in protein dissolution but little effect on PHA degradation. Yasotha *et al*., (2006) extracted a medium-chain PHA synthesized by *Pseudomonas putida* using the commercial protease enzyme Alcalase. Their results showed a purity of 92.6% with an extraction of 90%. Another author who used this enzyme to obtain PHA was Kathiraser *et al.* (2007), who obtained a medium-chain PHA synthesized by *Pseudomonas putida*. Their analysis determined a purity of 92.63 % lower than that reported using chloroform (96.6%). In 2012, Neves and Müller studied the use of the proteases Corolase® L10, Alcalase® 2.4L, Corolase® 7089 and Protemax® FC and glycosidases Celumax® BC, Rohament® CL and Rohalase® for the extraction of P(3HB) and P(3HB-co-3HV) synthesized by *Cupriavidus necator*. The Celumax® BC enzyme obtained the cell membrane's best lysis, obtaining a recovery of 93.2 % of P(3HB-co-3HV) with a purity of 94%. Like the previous ones, the enzymatic digestion method has been combined with other methods.

Extraction using high pressure has also been used for the industrial extraction of PHA. The instruments used in this method destroy the cell wall. For its use, the pressure, temperature, biomass concentration, size, shape and strength of the cell wall must be considered. Cultures in the logarithmic phase have a lower resistance to cell wall rupture, while in the stationary phase, the resistance increases due to the increase in wall thickness (Tripathi *et al.,* 2021). Ghatnekar *et al.* (2002) extracted PHA produced by *Methylobacterium* sp. V49 using a ceramic cell disruption valve (APV Gaulin). Using a pressure of 400 kg cm^{-2} and two homogenization cycles resulted in 95 % recovery and 80 % purity of PHA. Hwang *et al.* (2006) obtained PHA and synthesized *Haloferax mediterranei* using ultrasonication with an amplitude of 20 kHz and a power of 525 W.

6.7 Methods for structural characterization of PHA

One of the most common methods used for the structural characterization of PHA is Fourier transform infrared spectroscopy (FTIR). It is a technique used to obtain an absorption spectrum of a sample. This method has been used for the detection of PHA in intact bacteria. This method can identify the carbonyl group in the fatty acid chain depending on the absorption peak. According to Hong *et al.* (1999), using this technique has identified PHA of short chains (such as hydroxybutyrate), medium chains (such as hydroxyoctanoate and hydroxydecanoate) or the combination of both, showing characteristic absorbance bands at wavelengths of 1728 cm^{-1}, 1740 cm^{-1} and 1732 cm^{-1}. Table 6.3 shows the characteristic bands of short- and medium-chain PHAs synthesized by *Azotobacter vinelandii* UWD, *Pseudomonas mendocina* AS 1.2329 and *Pseudomonas pseudoalcaligenes* AS 1.2328.

Table 6.3 Comparison of results from Fourier-transform infrared (FTIR) spectra and gas chromatography (G.C.) for component analysis of polyhydroxyalkanoates (PHA)

FTIR analysis		GC analysis:
Wave-number (cm^{-1})	**Possible PHA components**	**PHA monomer composition (%)**
1728 1262	PHB	100HB
1739 1261 2925	HB and mclHA	92HB, 8HD
1739 1260 2924	HB and mclHA	98HB, 2HO
1744 1165 2926	mclHA	0.3HB, 58HO, 41HD
1744 1162 2926	mclHA	22HO,78 HD
1744 1665 2928	mclHA	0.6HB,19HO, 80HD
1744 1165 2928	mclHA	0.4HB,20HO, 80HD
1739 1257 2926	HB and mclHA	23HB, 39HO, 38HD
1739 1258 2926	HB and mclHA	14HB, 50HO, 36HD
1739 1257 2926	HB and mclHA	22HB, 40HO, 38HD
1744 1168 2929	mclPHA	0.3HB, 59HO, 41HD
HA: polyhydroxyalkanoate. HB: polyhydroxybutyrate. HO: polyhydroxyoctanoate. HD: polyhydroxydecanoate. mcl: medium chains		

Source Hong et al.,1999

In 2013, Sathiyanarayanan *et al.* identified PHA synthesized from *Bacillus subtilis* isolated from a marine sponge (*Callypongia diffusa*) by FTIR analysis, reporting absorption bands at 1728 cm^{-1} and 1283 cm^{-1} corresponding to the C=O and C-O, respectively. The bands at 1230 cm^{-1}, 1382 cm^{-1} and 1184 cm^{-1} represented the $-CH_2$, $-CH_3$ and C-O-C. Finally, they reported an absorption band at 3450 cm^{-1} corresponding to the hydroxyl group. Another study with similar results was by Evangeline and Sridharan (2019), who extracted PHA synthesized by *Bacillus cereus* VIT-SSR1 and identified it by FTIR using KBr and a wavelength of 4000 cm^{-1} at 400 cm^{-1}. Their results showed an absorption band at 1724.34 cm^{-1} corresponding to the carbonyl group, characteristic of short chains of PHB monomers. The absorption bands at 3419.79 cm^{-1} correspond to the hydroxyl group.

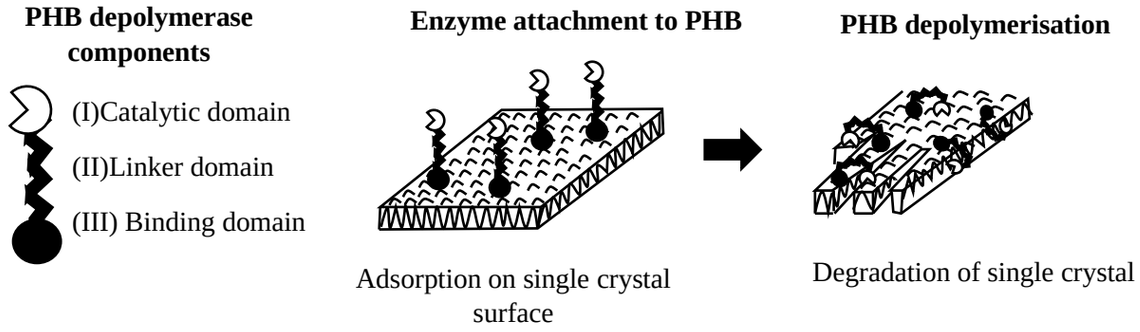
This method can be combined with gas chromatography coupled to mass (GCMS) which helps to quantify and determine the proportion in which each PHA is present in the structure. To perform this analysis, a methanolysis of PHA is necessary. In addition to the use, the temperatures to use the equipment and the temperature increase must be contemplated. Sathinarayanan *et al.* (2013) analyzed by GCMS the PHA synthesized by *Bacillus subtilis* MSBN17 and determined the presence of a retention peak at 2,548 min corresponding to a PHB. This value was close to that reported by Evangeline and Sridharan (2019) using an elite-5MS capillary column. They analyzed PHA produced by *Bacillus cereus* VIT-SSR1 peaks at times 3.69 (3-HB), 11.81, 17.26 and 21.40. However, this is non-pure, so it presented other retention peaks. Wecker *et al.* (2015) analyzed PHA synthesized by an *Enterobacter* sp. FAK 1384 was isolated from a marine source by GCMS. Their results determined a PHA consisting of 62% mol-3-hydroxydecanoate, 18% mol 3-hydroxyoctanoate, 12% mol of 3-hydroxydodecenoate, 7.6% mol of 3-hydroxydodecanoate and a small fraction of 0.3% mol 3-hydroxyhexanoate and 1.3% mol of 3-hydroxytetradecanoate. The composition of the medium-chain PHA induced a higher elasticity and elongation of the extracted polymer, having a crystallinity index of 0.26, indicating an amorphous polymer.

Another method used to identify PHA is nuclear magnetic resonance (NMR). The composition of the hydroxyalkanoate units can be identified by analyzing the nuclear magnetic field of the NMR spectra. This method allows for a differentiation between blends and hetero polymers of PHA and provides details about the topology and functional groups in the polymers. Another advantage is that the hydrolysis step of the polymer can be avoided (Godbole, 2016). Kathiraser *et al*., (2007) used this technique to compare the structural variation of PHA synthesized *by Pseudomonas putida* extracted by two different methods (solvent and enzymatic). Their results showed that there was no variation in the structure of PHA. The spectra showed a peak at 2.58, corresponding to CH_2. The peak at 5.1 ppm belonged to the CH group. The peaks at 5.30 ppm and 5.52 ppm determine the presence of a side chain where protons bind to two ethylene carbon sequences (-CH=CH-) associated with the unsaturated monomer. The signal at 1.5 ppm represents the first side chain of the monomers and at 1.3 is attributed to the methyl (CH_2) of the monomers. The bit at 0.89 ppm indicates the terminal methyl group (CH_3). In 2017, Sharma *et al.* used this technique to analyze PHA synthesized by *P. chlororaphis* PA23-63. The spectrum identified five peaks corresponding to protons on the methylene group, a hydroxyl group, a methyl group attached to a carbonyl group, a saturated methyl side-chain group, a methyl group and a terminal methyl group. FTIR identified the presence of different functional groups such as aliphatic C-H bonds, = C-H bonds, =CH bonds and =C-O bonds, and stretching of the =O bond and deformation of the =C-H bond. It was observed that the C-H methylene peaks were more prominent in the medium-chain PHA.

6.8 Biodegradability of PHAs

PHAs are biodegradable in different environments, making them an attractive option for replacing plastics. The general degradation process consists of the breakdown of the polymer into shorter chains by hydrolytic depolymerase, followed by the PHA trimers or dimers being processed by lipases and hydrolases. PHA depolymerizes are carboxylesterases that have a catalytic triad (serine-Histidine-aspartic acid) as their active site (Knoll *et al.,* 2009). Extracellular PHA depolymerase is the most studied protein that consists of three main domains: (III) a binding domain responsible for surface adsorption and breakdown of the polymer structure; (II) a linker domain that joins the binding domain to the catalytic domain; and (I) a catalytic domain that cleaves the PHA and any available dimer/trimer in two parts (Figure 6.3). Hydrolytic enzymes have excellent adhesion to amorphous surfaces due to their less ordered structure, making them more accessible to enzyme action (Meereboer *et al.*, 2020).

Figure 6.3 Single PHB crystal enzymatic degradation by PHB depolymerase



Source: Meereboer et al., 2020

The short and medium-chain PHA can be degraded by many bacterial genera such as *Bacillus*, *Clostridium*, *Comamonas*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Staphylococcus*, *Streptomyces*, among others. Different fungi have also been used for PHA degradation, such as *Acremonium*, *Aspergillus, Candida*, *Paecilomyces* and *Emericelopsia* (Kim *et al.*, 2013; Bugnicourt *et al.*, 2014; Meereboer *et al.*, 2020).

In 2016, Ong and Sudesh analyzed three different PHA (poly 3-hydroxybutyrate and combinations of PHB and Polyhydroxyhexanoate) and their effect on the soil microbial community. Films made from these PHAs were buried in the soil for eight weeks. The results concluded that poly 3-hydroxybutyrate-co-21%mol 3-hydroxyhexanoate showed the presence of holes three weeks after initiation of the test. Metagenomic analysis revealed that some of the significant Phyla found at the site included *Actinobacteria, Firmicutes*, and *Proteobacteria* that can degrade PHA. In the following year, Volova *et al.* (2017) studied the microbial degradation of polyhydroxyalkanoates with different chemical compositions. They determined that the surface community of bacteria differed between polymers. The polyhydroxybutyrate was degraded by bacteria of the genus *Mitsuaria, Chitinophaga* and *Acidovorax*, which could not degrade the other three types of pHA. *Roseateles depolymerans*, *Streptomyces gardneri* and *Cupriavidus*, were poly(3HB/4HB)-degrading species. *Roseomonas massiliae* and *Delftia acidovorans* degraded poly(3HB/3-hydroxyvalerate), and poly(3HB/3-hydroxyhexanoate) was degraded by species of *Pseudoxanthomonas* sp., *Pseudomonas fluorescens*, *Ensifer adhaerens*, and *Bacillus pumilus*. The microbial community formed on the polymer surface and the soil microbial community differed depending on the polymer composition.

6.9 Applications of the PHA

The properties of this compound and the mixture of different PHA have broadened the possible applications for its use. Due to their biocompatibility, biodegradability and low cytotoxicity to cells, PHA has been used for packaging, medical equipment (gloves) and fishing nets. It has been proposed to manufacture resorbable saturates, pharmaceuticals, and transplants for tissue engineering and pharmacology (Chee *et al.*, 2010).

Shishatskaya *et al.* (2004) used PHA synthesized by the bacterium *Ralstonia eutropha* to realize monofilament sutures. The preparation uses PHB (340000 Da) and a copolymer of PHB and polyhydroxyvalerate (PHV). Their results showed that the suture adequately held the muscle-fascial incision in animals and that the animals did not show any adverse reaction to the PHA fibers. Hufenus *et al.* (2011) elaborated on polylactic acid fibers and a mixture of PHB and 8mol% of 3-hydroxyvalerate (Tianan). They studied their biocompatibility in human dermal fibroblasts. Their results showed that there was no toxicity. The fibroblasts that grew were adhering to the fibers covering after a culture period of 1 week. Degradation tests showed a reduction of maximum stress up to 33% after four weeks of incubation. Another biomedical application given to PHA is in bone tissue engineering, Meischel *et al.* (2016) evaluated the response of bone to PHA composite implants in rat femurs. PHB constituted these implants with zirconia dioxide, Herafill® and Mgalloy WZ21. Their results showed that implants with 30% Herafill, PHB and zirconium dioxide possessed the highest bone accretion values and that the mechanical properties were similar to that of bone. In agriculture, this polymer has been used to reduce ghost fishing caused by nets made of synthetic polymers that, when discarded, cause a negative economic and ecological impact as they continue to catch and retain fish (Amelia *et al.*,2019).

Another application of PHA in agriculture is the controlled release of pesticides and insecticides, producing films for crop protection or seed encapsulation (Sharma *et al.*, 2021). PHA has also been used to make shopping bags, containers, and utensils such as rakes, feminine hygiene products, and cosmetic containers, among others (Chen, 2009).

6.10 Financing

This work has been funded by CONACYT [scholarship number: 815355, 2022]; Tecnológico Nacional de México [Project TecNM code: 14073.22-P].

6.11 Conclusions

The accumulation of plastics in the soil and the oceans is becoming more evident and alarming. It is enough to walk along the streets or beaches of some countries to observe this accumulation. Garbage on the streets has become so common that people walk through this waste without caring. This problem is caused due to poor waste management by governments. This does not exempt us as citizens from blame since the industry's increased plastic production is due to the growing consumer demand. Recyclable materials have been proposed to reduce the accumulation of plastics. However, people's lack of awareness causes the recycling process to be inefficient. It is easier to throw garbage on the streets where we walk than find a recycling point.

Using biodegradable polymers has been another option studied for years to replace plastic. While many are easy to degrade, their mechanical properties are inadequate to match or resemble the properties of currently used synthetic plastics. PHAs, as mentioned in this chapter, have the advantage of the show a wide variety of structures that can be used individually or in combination to improve their characteristics. PHB is the most reported biodegradable polymer for plastics in the literature because it is produced by a wide variety of bacteria and has properties similar to polypropylene. Another advantage of PHA is that it can be degraded by enzymes of the PHA-producing bacteria or bacteria living naturally in soils and seas. They can be degraded in less than two months, depending on environmental conditions. Since they are fatty acid monomers, their applications have been widely studied in medicine. However, applications have also been proposed in agriculture. The varied applications of PHA have led to their industrial production in different countries such as China, the United States and Canada. Despite more sources of isolation of producing bacteria, genetic modifications to increase polymer production and chemical modifications of the polymer to give them other applications are still being studied. PHAs have proven to be a viable candidate to replace the use of plastics, but industry and individuals have yet to determine if their value is less than that of plastics.

6.12 References

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