Chapter 18
Bacilli and Polyhydroxyalkanoates: An Intracellular Granule Having Promising Feature as a Resource for Production of Bioplastics

Priya Patel and Nasreen S. Munshi

Abstract Polyhydroxyalkanoate (PHA) is biodegradable biopolymer produced by microorganisms as lipid inclusion body under the stressful environmental conditions. They possess the properties analogous to petrochemically derived synthetic plastics and can serve as novel resource for production of bioplastics. Varieties of prokaryotes in diverse niches have been reported to accumulate PHA when there is excess carbon and/or limited nitrogen or phosphorous. *Bacillus* spp. are prominent source for industrial production of PHA as they are predominant in nature. Different *Bacillus* spp. are reported to utilize a wide range of substrates such as sucrose, glucose, fructose, starch and others for production of PHA. On the other hand, few *Bacilli* accumulate PHA while using inexpensive biowastes such as pea-shell slurry, fish solid waste, activated sludge, sugar industry wastewater and others as substrates. This allows sustainable management of waste along with generating a valuable by-product. They are known to synthesize PHA homopolymer as well as copolymers. The Food and Drug Administration (FDA) has considered *Bacillus* as Generally Regarded As Safe organisms (GRAS), allowing its application for large-scale bioplastic production. Further, the absence of immunogenic lipopolysaccharide layer in *Bacillus* spp. allows biomedical applications of produced PHA. The main emphasis of this article is to summarize the generalized, metabolic and genetic features of *Bacillus* spp. associated with PHA production and providing substantial information for exploiting capabilities of *Bacillus* spp. for industrial PHA production.

Keywords Polyhydroxyalkanoate · *Bacillus* spp. · Biodegradable · Biopolymer · Industrial PHA production · Bioplastic · Biowastes · PHA granule

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18.1 Introduction

Plastics and their products are basic necessity of individual's life. Low cost, light weight, water resistance, rust free and robustness are promising features of plastic, permitting its wide applications for societal benefits (Thompson et al. 2009). Concurrent to its enormous applications, plastic pollution has become threat for biosphere. Approximately more than 300 Mt. plastic is produced annually. Plastic manufacturing and utilization rate in India, China and Brazil are increasing exponentially (Koller 2017). These non-biodegradable petrochemically derived synthetic plastics accumulate as such in environment causing harm to wildlife, marine animals, humans and environment (Sathya et al. 2018).

Biodegradable polymers having properties similar to synthetic plastics are considered to be potential substitute of petrochemically derived plastics (Koller 2017). When discarded in surroundings, the polymers which get entirely converted into CO_2 and H_2O within fixed duration are designated as "biodegradable polymers" (Sathya et al. 2018). Nature-based and chemical-based polymers are the two main categories of biodegradable polymers. Further, as depicted in Fig. 18.1, animal-based, agro-based and microbe-based polymers are subcategorized under naturally derived polymers (Mulchandani and Katiyar 2020). Nature-based polymers are also called as "biodegradable biopolymers" as they are obtained from biological materials (Pittmann and Steinmetz 2017). Chemical-based polymers are initially extracted from biological sources in the monomeric form and then polymerized by using various chemicals. For example, polylactic acid (PLA) and polybutylene succinate

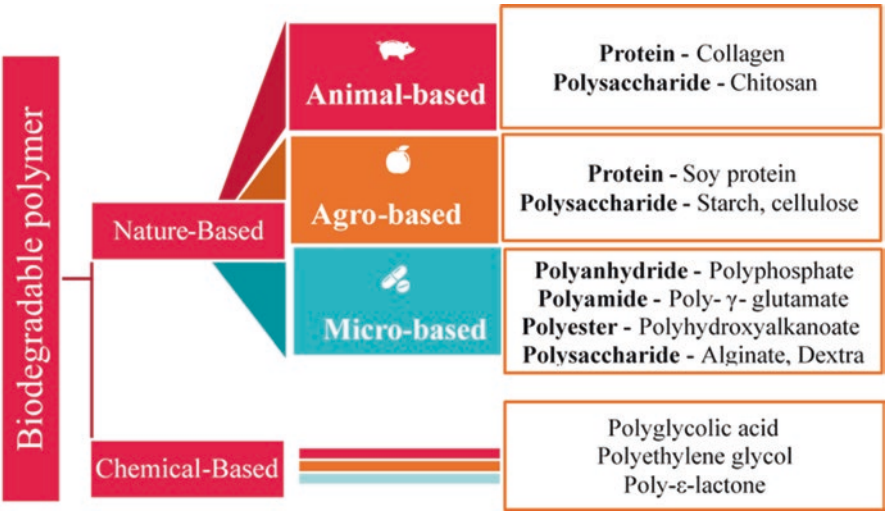

Fig. 18.1 Categories of biodegradable biopolymers: there are two main types of biodegradable polymers: nature-based and chemical-based. Further, nature-based polymer is divided into three subtypes, i.e. animal-based, agro-based and microbe-based. Examples are illustrated in different categories of polymers

(PBS) get polymerized in the presence of lactic acid and succinic acid, respectively. In contrast to this, nature-based biopolymers are advantageous as they do not require polymerization after extraction (Kourmentza et al. 2017). Amongst all the nature-based biopolymers, starch is widely used as packaging material due to its biodegradable nature other than being cost-effective and non-hazardous. But the major drawback is that it lacks thermoplastic properties (Sanyang et al. 2015). Chemical treatment of starch with plasticizer can impart thermoplastic properties to it (Coats et al. 2016). Microbe-based biopolymer – polyhydroxyalkanoate (PHA) – is an intracellular granule synthesized by bacteria in the presence of excess substrate and/or nutrient limiting condition. They possess features similar to polypropylene including thermoplasticity and is also biodegradable in nature (Pagliano et al. 2017; Hassan et al. 2016; Mokhtarani et al. 2012; Gamba et al. 2017). Also, biodegradability of PHA is higher than PLA and starch (Coats et al. 2016). Different bacterial strains have been reported to accumulate PHA by utilizing several carbon sources as well as agricultural and industrial waste as feedstock (Chua et al. 2003). The stated characteristics of PHA allow its wide acceptance as a source of bioplastics suitable for applications in packaging, medical, pharmaceutical, agriculture and food industries (Coats et al. 2016; Goudarztalejerdi et al. 2015).

Prokaryotes such as *Ralstonia eutropha*, *P. putida* CA-3, *P. putida* mt-2, *P. putida* F1, *Sphingobacterium* sp. ATM, *Bacillus odyssey* SUK3, *P. desmolyticum* NCIM 2112, etc. are capable of PHA production (Nikodinovic et al. 2008; Tamboli et al. 2010; Sato et al. 2008). Although many types of PHA producers are reported till date, *Bacillus* spp. are considered as valuable bioresource for industrial PHA production as they are predominant in nature (Mohapatra et al. 2017). They are capable of accumulating PHA homopolymer as well as copolymers (Singh et al. 2009). Moreover, they are also stated as "Generally Regarded As Safe organisms" (GRAS) by the Food and Drug Administration (FDA) (Singh et al. 2009; Mohapatra et al. 2017). Hence, *Bacillus* spp. are appropriate bio-resource for industrial PHA production. In this chapter, the generalized characteristics of PHA are described along with an account on how the abilities of *Bacillus* spp. can be exploited for industrial PHA production.

18.2 Biodegradable Biopolymer: Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHA) are water insoluble polyester granules situated inside bacterial cytoplasm (Colombo et al. 2017; Goudarztalejerdi et al. 2015; Kumar et al. 2004). They are synthesized by bacteria in the presence of excess carbon and/or limited phosphorus, sulphur, oxygen or nitrogen (Goudarztalejerdi et al. 2015; Sathya et al. 2018).

18.2.1 Monomeric PHA and Its Derivatives

PHA biopolymer comprises of hydroxyalkanoic acid monomers (Goudarztalejerdi et al. 2015; Sathya et al. 2018). The general structural formula of hydroxyalkanoic acid or PHA monomer is shown in Fig. 18.2, where R represents an alkyl group and n ranges from 1 to 3. A total number of carbon atom in PHA monomer ranges from 3 to 14 on the basis of type of R group incorporated (Pradhan et al. 2020). The PHA biopolymer consists of long chain of monomeric PHA ranging from ~100 to 30,000 units (Pradhan et al. 2020; Jacquel et al. 2008; Jiang et al. 2016; Basnett and Roy 2010). Table 18.1 denotes the type of PHA monomer and a number of C atoms present in it according to incorporated alkyl group.

PHAs are classified into four categories on the basis of biopolymer chain length and the type of monomeric unit incorporated in biopolymer chain. On the basis of chain length, they are classified as short-chain length PHA (*scl*-PHA) and medium-chain length PHA (*mcl*-PHA). The *scl*-PHA comprises of 3–5 carbon atoms, whereas *mcl*-PHA contains 6–14 carbon atoms (Kourmentza et al. 2017; Ciesielska and Kiewisz 2016). On the basis of monomeric unit, two types of PHAs are there, one is homopolymer PHA and the other is copolymer PHA. In homopolymer PHA, biopolymer chain is consisted of identical type of PHA monomer, whereas biopolymer chain of copolymer PHA contains different types of PHA monomers (Pradhan et al. 2020). Classification of PHA is defined in Fig. 18.3.

The *mcl*-PHAs have properties similar to elastomers and are semi-crystalline in nature. They are used for preparation of drug delivery matrix, surgical sutures and implants. On the other hand *scl*-PHAs have tensile strength similar to polypropylene, they are used for formulating food packaging material and disposable items (Kourmentza et al. 2017; Pradhan et al. 2020). The *scl*-PHAs are predominantly accumulated in bacteria as compared to *mcl*-PHAs (Pradhan et al. 2020). More than 150 different kinds of PHAs have been reported so far (Pittmann and Steinmetz 2017; Sathya et al. 2018; Pradhan et al. 2020; Kourmentza et al. 2017). Table 18.2 illustrates the types of PHAs produced by different species of bacteria (Singh et al. 2015).

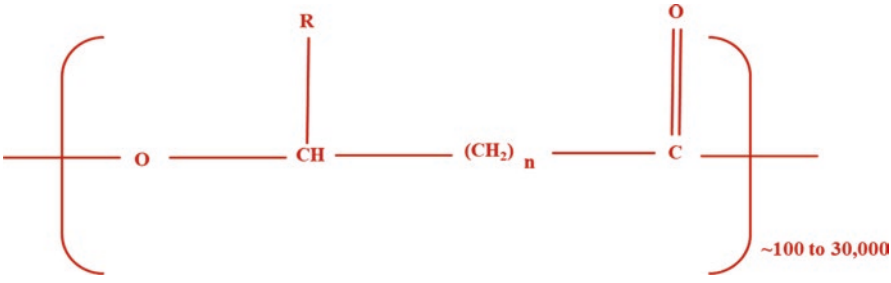

Fig. 18.2 General formula of monomeric PHA: the formula shown is of hydroxyalkanoic acid, the monomeric subunit of PHA biopolymer where R indicates an alkyl group and n ranges from 1 to 3. Long chain of hydroxyalkanoic acid in PHA biopolymer contains multiple monomers

Table 18.1 Type of alkyl group and number of C atoms present in monomeric PHA

Alkyl group (−R)		Total number of C atoms in PHA monomer	n	Type of PHA monomer
Type	Molecular formula			
Hydrogen	–H	C_3	1	Poly(3-hydroxypropionate)
		C_4	2	Poly(3-hydroxybutyrate)
		C_5	3	Poly(3-hydroxyvalerate)
Methyl	$–CH_2$	C_4	1	Poly(3-hydroxybutyrate)
		C_5	2	Poly(3-hydroxyvalerate)
		C_6	3	Poly(3-hydroxyhexanoate)
Ethyl	$–C_2H_6$	C_5	1	Poly(3-hydroxyvalerate)
		C_6	2	Poly(3-hydroxyhexanoate)
		C_7	3	Poly(3-hydroxyheptanoate)
Propyl	$–C_3H_8$	C_6	1	Poly(3-hydroxyhexanoate)
		C_7	2	Poly(3-hydroxyheptanoate)
		C_8	3	Poly(3-hydroxyoctanoate)
Butyl	$–C_4H_{10}$	C_7	1	Poly(3-hydroxyheptanoate)
		C_8	2	Poly(3-hydroxyoctanoate)
		C_9	3	Poly(3-hydroxynonanoate)
Pentyl	$–C_5H_{12}$	C_8	1	Poly(3-hydroxyoctanoate)
		C_9	2	Poly(3-hydroxynonanoate)
		C_{10}	3	Poly(3-hydroxydecanoate)
Hexyl	$–C_6H_{14}$	C_9	1	Poly(3-hydroxynonanoate)
		C_{10}	2	Poly(3-hydroxydecanoate)
		C_{11}	3	Poly(3-hydroxyundecanoate)
Heptyl	$–C_7H_{16}$	C_{10}	1	Poly(3-hydroxydecanoate)
		C_{11}	2	Poly(3-hydroxyundecanoate)
		C_{12}	3	Poly(3-hydroxydodecanoate)
Octyl	$–C_8H_{18}$	C_{11}	1	Poly(3-hydroxyundecanoate)
		C_{12}	2	Poly(3-hydroxydodecanoate)
		C_{13}	3	Poly(3-hydroxytridecanoate)
Nonyl	$–C_{9–}H_{20}$	C_{12}	1	Poly(3-hydroxydodecanoate)
		C_{13}	2	Poly(3-hydroxytridecanoate)
		C_{14}	3	Poly(3-hydroxytetradecanoate)
Decyl	$–C_{10–}H_{22}$	C_{13}	1	Poly(3-hydroxytridecanoate)
		C_{14}	2	Poly(3-hydroxytetradecanoate)
Undecyl	$–C_{11–}H_{24}$	C_{14}	1	Poly(3-hydroxytetradecanoate)

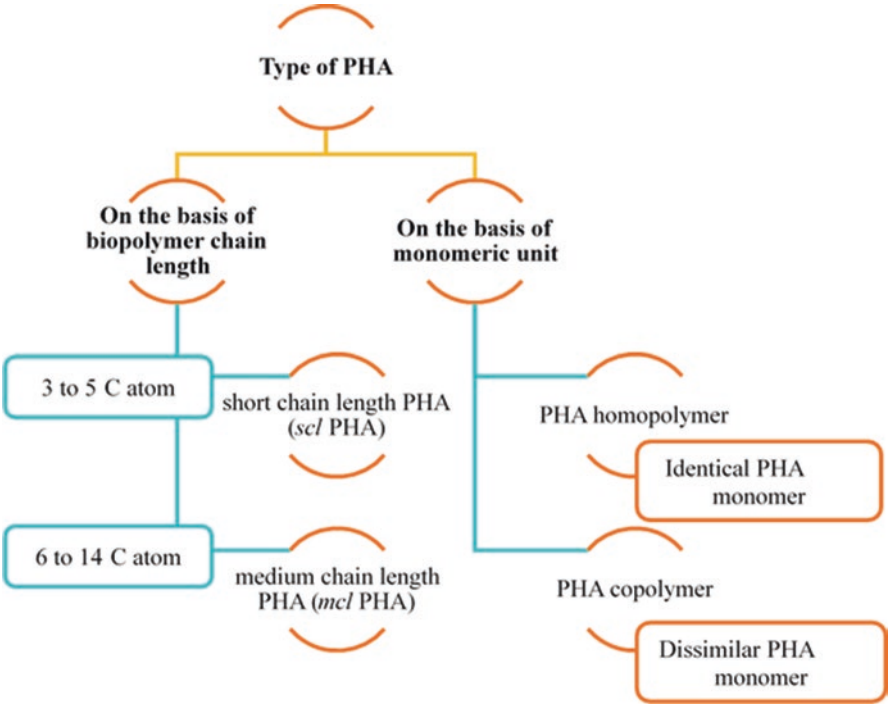

Fig. 18.3 Classification of PHA: there are four types of PHA classified on the basis of biopolymer chain length and on the basis of monomeric unit. Short-chain length PHA (*scl*-PHA) contains 3–5 carbon atoms, whereas medium chain length PHA (*mcl*-PHA) contains 6–14 carbon atoms. PHA biopolymer chain having identical monomeric unit is termed as PHA homopolymer while that with different monomeric units is termed as PHA copolymer

18.2.2 PHA as Carbon and Energy Reserves for Prokaryotes

PHAs are the inclusion bodies acting as intracellular carbon reservoir in bacterial strain (Sowinski et al. 2010). PHA granules constitute about 90% of the cell dry weight (CDW) (Bhuwal et al. 2013; Strong et al. 2016). *Ralstonia eutropha* was capable of accumulating 80% PHA of cell dry weight (Mokhtarani et al. 2012). When availability of carbon sinks in surrounding, PHA granules are used as carbon and energy source. Hence, they augment the survival of bacterial strain under stressful conditions existing in water and soil environments. UV irradiation, salinity, desiccation, osmotic shock, thermal stress and oxidative stress are the few stressful conditions occurring in water and soil environments where PHAs are used as carbon and energy source. Taxonomically diverse species of bacteria are inhabiting in soil niches. They have to deal with fluctuating conditions existing there. It has been reported that PHA-producing strain is protected during starvation as compared to mutant strain deficient in PHA production. But the co-relation

Table 18.2 Types of PHAs produced by bacteria

Name of PHA	Type of PHA		Name of PHA-producing bacteria	Substrate	Yield	References
	Chain length	Monomeric unit				
P(3HB)	NA	Homopolymer	*Enterococcus* sp. NAP11	Cardboard industry wastewater	79.27%	Bhuwal et al. (2013)
P(3HB)	NA	Homopolymer	*Brevundimonas* sp. NAC1	Cardboard industry wastewater	77.63%	Bhuwal et al. (2013)
Biopolymer having P(3HO), P(3HD), P(2HDDE), P(3HTD), P(3HHDE)	NA	Copolymer	*P. aeruginosa* strain SDS3(HQ 230975)	Crude oil	23.13%	Goudarztalejerdi et al. (2015)
Biopolymer having P(3HO), P(3HD), P(2HDDE), P(3HTD), P(3HHDE)	NA	Copolymer	*P. aeruginosa* strain XB7(KF 44738)	Crude oil	21.87%	Goudarztalejerdi et al. (2015)
Biopolymer having P(3HO), P(3HD), P(2HDDE), P(3HTD), P(3HHDE)	NA	Copolymer	*P. stutzeri* strain PS-SRU-ICU (JF 264901)	Crude oil	23.26%	Goudarztalejerdi et al. (2015)
Biopolymer having P(3HO), P(3HD), P(2HDDE), P(3HTD), P(3HHDE)	NA	Copolymer	*P. aeruginosa* strain H1 (JX 100389)	Crude oil	20%	Goudarztalejerdi et al. (2015)
Biopolymer having P(3HO), P(3HD), P(3HDDE), P(3HHDE).	*mcl*-PHA	Copolymer	*P. putida* F1, *P. putida* mt-2 and *P. putida* CA-3	BTEX mixture	0.25 ± 0.04 g/l	Nikodinovic et al. (2008)

NA not available, *P* poly, *(3HB)* 3-hydroxybutyrate, *(3HO)* 3-hydroxyoctanoate, *(3HD)* 3-hydroxydecanoate, *(2HDDE)* 2-hydroxydodecanoate, *(3HTD)* 3-hydroxytetradecanoate, *(3HHDE)* 3-hydroxydecahexanoate, *(3HHDE)* 3-hydroxydodecanoate

between PHA accumulation and survival strategy is strain specific depending on the suboptimal growth preceding to starvation (Sowinski et al. 2010). PHA-producing bacteria are reported in microbial mats pursuing an essential role in stress tolerance and biofilm formation (Campisano et al. 2008; Sowinski et al. 2010). Reducing equivalents produced during PHA degradation plays a vital role in energizing chemotaxis process in the surrounding environments having lower quantity of reducing power (Kadouri et al. 2003; Sowinski et al. 2010). Moreover, EPS production is observed in PHA accumulating strains (Aneja et al. 2004). NADH generated during PHA degradation gets accumulated into EPS which serves as energy reservoir, useful for bacteria under stressed conditions (Sowinski et al. 2010).

Apart from this, biodegradable nature of PHA permits its utilization as carbon and energy source by microbial communities existing in the environment. PHA gets depolymerized into oligomer by intracellular PHA depolymerase (i-PhaZ) and extracellular depolymerase (e-PhaZ) (Grage et al. 2009; Philip et al. 2007). Former acts on native PHA granules and later converts the partially degraded PHA granules into oligomers. These oligomers are transformed into monomers by hydrolases. The monomers generated can be utilized as source of carbon and energy by surrounding microbial community (Sowinski et al. 2010). Figure 18.4 displays the possible role of PHA in stressful environment.

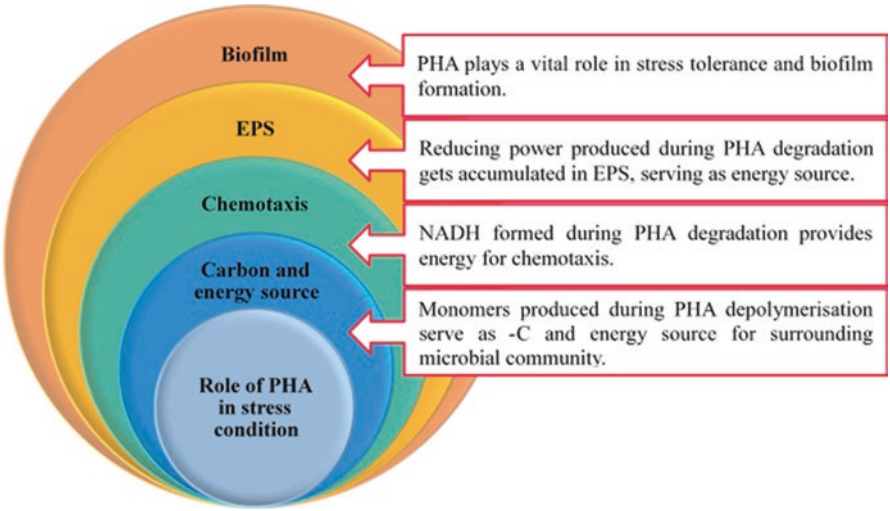

Fig. 18.4 Possible roles of PHA in stressful environment: monomeric PHA synthesized during biodegradation of polymeric PHA by depolymerase enzyme is used as carbon and energy source by diverse microbial communities existing in the surrounding environment. During its breakdown, reducing equivalent formed is used as energy source for chemotaxis. These reducing powers also get accumulated in EPS serving as energy source. Even PHA is essential in stress tolerance and for formation of microbial mats

18.2.3 Structure of PHA

Carbonosomes PHA granules are composed of proteins and phospholipid layer which is resistant to physical and chemical agents (Jendrossek 2009; Jendrossek and Handrick 2002). The innermost part of granule is made up of polyesters (Grage et al. 2009). PHA synthase (PhaC), PHA depolymerase (PhaZ), regulatory protein (PhaR) and phasins (PhaP) are four proteins present in PHA granule (Grage et al. 2009; Potter and Steinbuchel 2005). Structure of PHA granule is shown in Fig. 18.5.

PHA synthase plays a crucial role in converting hydroxyalkanoic acid into PHA polyester. They are classified into four classes – I, II, II and IV – as described in Fig. 18.5b. PHA synthase of class I and II is composed of 61–70 kDa PhaC subunit. Class III PHA synthase comprises of PhaC similar to that of class I PhaC and PhaE, both having 40 kDa molecular weight, whereas class IV PHA is composed of 40 kDa PhaC and 20 kDa PhaR. Class I, III and IV PhaC are reported to synthesize *scl*-PHA in contrast to class II PhaC which forms *mcl*-PHA (Grage et al. 2009; Potter and Steinbuchel 2005, 2006). As mentioned earlier, PhaZ are of two types, i-PhaZ present on the surface of PHA inclusion bodies and e-PhaZ secreted by

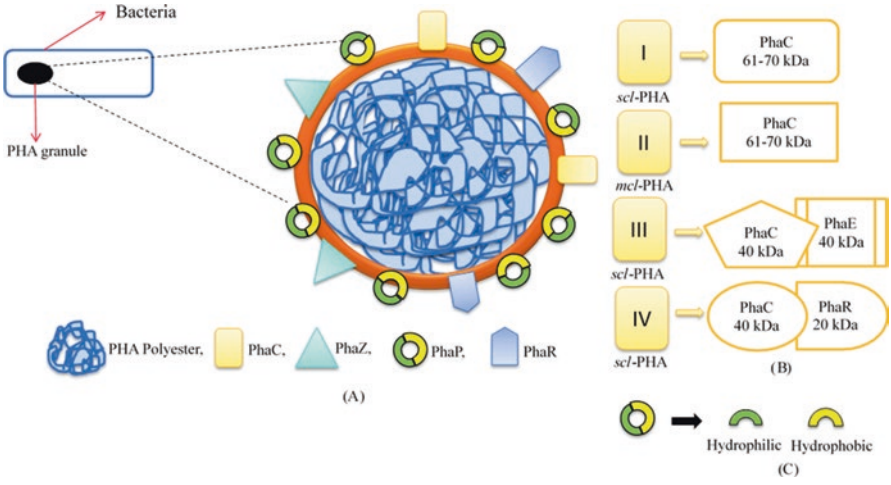

Fig. 18.5 Structure of PHA granule and its components: (**a**) intracellular PHA carbonosomes, (**b**) four classes of PhaC, (**c**) PhaP protein components. PHA carbonosomes are composed of hydroxyalkanoic acid polyester core surrounded by PhaC, PhaZ, PhaP and PhaR proteins. PhaZ are PHA depolymerase, important for degradation of PHA, while PhaR is a regulatory protein, essential for regulating the process of granule formation. PhaC is PHA synthase and PhaP is phasin proteins. There are four classes of PhaC which are essential for synthesizing biopolymer PHA. Class I and II of PhaC have single subunit of 61–70 kDa and are essential for synthesizing *scl*-PHA and *mcl*-PHA, respectively. Class III PhaC leads to formation of *scl*-PHA and is a combination of 40 kDa PhaC and 40 kDa PhaE. Class IV PhaC is made up of 40 kDa PhaC and 20 kDa PhaR, playing vital role in formation of *scl*-PHA. PhaP protein which forms a major part of the granule is amphibolic in nature having outer hydrophilic layer facing towards cytoplasm and inner hydrophobic layer facing granular inner side

many bacteria. Both are very essential for biodegradation of PHA granules (Grage et al. 2009; Sowinski et al. 2010). PhaR is the transcriptional regulatory protein required for regulation of PHA synthesis and PhaP production (Grage et al. 2009). Phasins are non-catalytic surface proteins having low molecular weight ranging between 11 and 25 kDa. They are produced in huge amount, comprising ~5% of cellular proteins (Grage et al. 2009). Their amphipathic layer as shown in Fig. 18.5c is composed of hydrophobic domain facing inside of granules and hydrophilic domain facing towards cytoplasm, thus, creating an interface between granule and cytoplasm (Grage et al. 2009; Mezzina and Pettinari 2016). They are essential for expression and activation of PhaC and PhaZ and, hence, are essential for PHA synthesis as well as degradation. They are also important for sorting of PHA granules and even affect size and number of granules. Few phasins act as chaperone proteins. Further, they belong to four families – PF09361, PF09602, PF09650 and PF05597 (Mezzina and Pettinari 2016).

Two models as presented in Fig. 18.6 have been reported till date for formation of PHA granules – micelle formation model and budding model (Potter and Steinbuchel 2006; Rehm 2006). According to the first model, initially PhaC proteins are randomly distributed in the cytoplasm, and as polymerization event initiates, it gets arranged in the form of micelle. Later on with increase in the biopolymer chain length, it gets distributed on the surface of PHA granules. After this, phasins and PhaR get accumulated on the granule surface. On the other hand, the budding model states that the PhaC are located between the phospholipid bilayer and carry out the polymerization of granule. The formed granule is then released into the cytoplasm. Then, the other proteins get attached to the outer surface of granule. Budding model is similar to the formation of eukaryotic neutral lipid. Micelle formation model is widely accepted as compared to the budding model (Potter and Steinbuchel 2006). *B. megaterium* seems to follow the budding model for PHA granule formation (Valappil et al. 2007a). It is believed that the PhaC possesses all the characteristics essential for formation of granule (Rehm 2006).

18.2.4 Comparative Aspects of Plastics and PHA

PHA have properties similar to petrochemically derived synthetic plastics like polyethylene and polypropylene (Numata et al. 2009; Bhuwal et al. 2013). Table 18.3 describes the general properties of PHA (Bugnicourt et al. 2014). Average molecular mass of PHA is 4.0×10^6 Da (Verlinden et al. 2007).

Further, the mentioned general property of PHA varies according to the type of biopolymer polymerized. Poly(3-hydroxybutyrate) denoted as P(3HB) is the most common type of PHA biopolymer accumulated by bacteria (Numata et al. 2009). It was the first biopolymer to be isolated from *B. megaterium* in the year 1920 by Maurice Lemoigne at the Pasteur Institute (Philip et al. 2007; Verlinden et al. 2007; Numata et al. 2009; Potter and Steinbuchel 2005). It has 162–181 °C melting temperature, −4 to 18 glass transition temperature, 19–44 MPa tensile strength, 1.2–4

Gpa Young's module, 0.8–4.5% elongation to break and 50–80% degree of crystallinity (Pradhan et al. 2020). Table 4 elucidates the properties of diverse types of PHA and polypropylene.

18.3 Bacilli and PHA

18.3.1 Diversity of PHA-Producing Bacillus *Species*

Members belonging to the genus *Bacillus* are able to accumulate varieties of PHA by utilizing ample of carbon sources. Table 18.5 displays the list of PHA-producing *Bacilli*. For PHA production, they were able to feed on simplest nutrient source such as glucose to complex hydrocarbons such as dyes and industrial effluents. Cost associated with PHA production decreases when any waste is used as substrates (Wen et al. 2010). *Bacillus* spp. were able to accumulate PHA while feeding on low-cost C source (Mohapatra et al. 2017).

18.3.2 Nutrients Essential for PHA Production by Bacilli

As described earlier, microorganisms accumulate PHA when there is excess substrate and/or growth limiting conditions. Feast and famine are the two terminologies describing the substrate availability. Former describes excess substrate and later indicates limited substrate availability. In famine conditions, the microorganisms will limit their cellular activity to minimum level essential for cell viability. They limit their activity by lowering the level of RNA transcription and/or enzyme activity. Further, when famine is followed by feast, initially the available substrate is utilized for PHA production instead of cellular growth. This happens because of the absence of essential enzymes required for cell growth. Thus, PHA accumulation is the physiological adaptation of microorganisms depending on substrate concentration (Albuquerque et al. 2010; Beun et al. 2002). In other words, such type of microorganisms does not require nutrient limiting conditions for PHA accumulation, and hence, they show growth-associated biopolymer production (Shi et al. 2007). Thus, type of microorganisms and microbial growth rate are the two internal factors affecting PHA accumulation. Moreover, the growth limiting conditions also arise by external factors such as limiting nutrients like N and P or in the presence of electron acceptors, viz. O_2, nitrate and phosphorous (Albuquerque et al. 2010; Shahid et al. 2013; Saharan et al. 2014). Growth-limiting nutrients will minimize the cellular growth as described earlier, and excess substrate is directed towards PHA production. Such microorganisms require limited nutrients and excess substrate for PHA production. In few organisms, it has been reported that C:N ratio also affects PHA

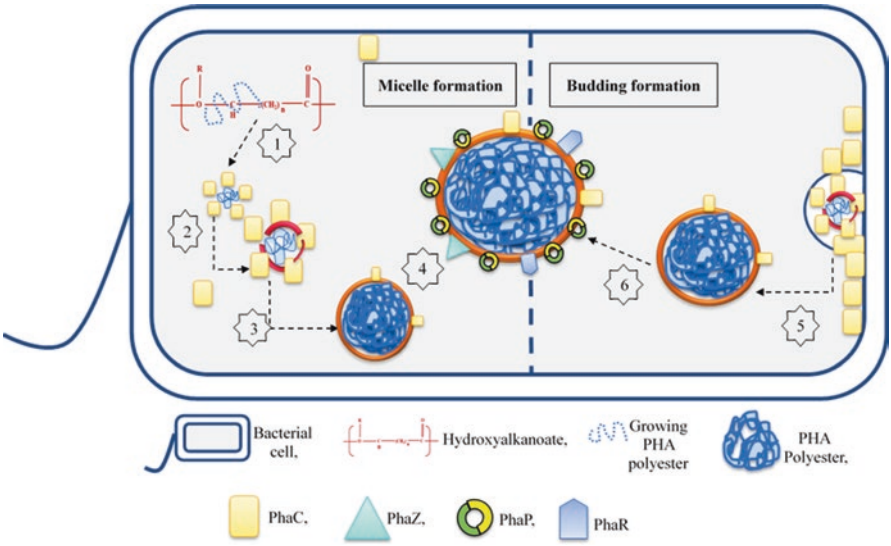

Fig. 18.6 Mechanism of PHA granule formation: two mechanisms for PHA granule formation are known – micelle formation (shown on left) and budding formation (shown on right). According to the most widely accepted micelle formation model, randomly distributed PhaC in cytoplasm initiates formation of granular body from hydroxyalkanoic acid (1) and (2). The granular body then enlarges into native granule acquiring PhaP, PhaZ and PhaR (3) and (4), while budding formation model depicts that the PhaC are located in the intracellular membrane and buds off into the granule (5) and (6)

accumulation (Shi et al. 2007; Wen et al. 2010; Saharan et al. 2014). Figure 18.7 indicates the pictorial outline of PHA production in varied nutrient conditions.

B. megaterium PNCM 1890 prefer urea as nitrogen source over sodium nitrate, ammonium chloride and ammonium sulphate, showing high PHB production. Researchers believe that uptake of low molecular weight, polar and uncharged urea for PHB formulation is higher as compared to remaining inorganic and ionic sources. The strain was capable to accumulate 3.91 g/L of PHB when C:N and C:P ratios were 14.3 g/g and 21.4 g/g, respectively, within 24 h (Danez et al. 2020). *B. megaterium* BA-019 produced 42% PHB of CDW in the presence of sugarcane molasses and urea as carbon and nitrogen source, respectively. The C:N ratio was 10 mol/mol, and incubation period was 24 h (Kulpreecha et al. 2009) (Table 18.4).

PHA production by *B. megaterium* DSM 509 was observed when grown in MM medium with different carbon sources such as glucose, glycerol, succinic acid, citric acid, acetic acid, pentanoic acid and octanoic acid. Then from this, medium cells were transferred into MM medium without N (MM-N), and PHA was extracted. Monomeric composition of PHA extracted from MM medium indicated *scl*-PHA, whereas that from MM-N indicated *mcl*-PHA. Authors suggested that the *scl*-PHA may get degraded when transferred from MM to MM-N in order to provide energy for synthesizing *mcl*-PHA (Shahid et al. 2013). *Bacillus flexus* is reported to

Table 18.3 General properties of PHA

Property	Unit	PHA
Glass transition temperature (T_g)	°C	2
Melting temperature (T_m)	°C	160–175
Degree of crystallinity (X_{cr})	%	40–60
Young's modulus (E)	GPa	1–2
Tensile strength (σ)	MPa	15–14
Elongation to break (ε)	%	1–15

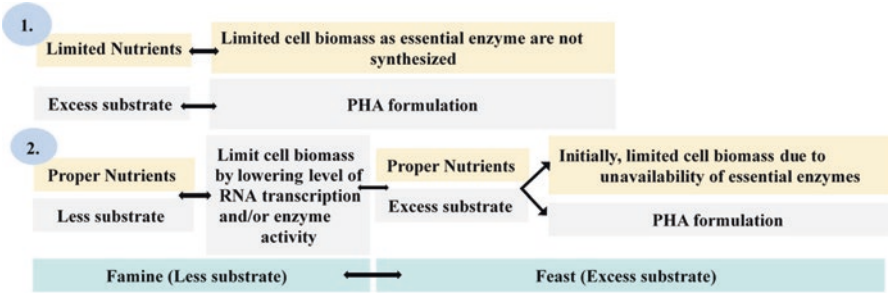

Fig. 18.7 Generalized mechanism for microbial PHA production under different nutrient conditions: as indicated in mechanism 1, limited nutrients hinder synthesizing of enzymes essential for cell biomass and hence limit growth. Excess substrate is directed towards PHA formulation, whereas as seen in mechanism 2, instead of nutrients, substrate is limited (famine) which again limits cell biomass development by lowering the level of RNA transcription and/or enzyme activity. When famine is followed by feast (excess substrate), initially cell biomass is limited as essential enzymes are unavailable, and excess substrate is directed for PHA formulation

produce high PHA in nitrogen-limiting conditions (Somashekara et al. 2009). In contrast to this, *Bacillus thuringiensis* EGU45 showed higher P(3HB-co-3 HV) (1.5–3.5 g/L) copolymer production in the presence of excess nitrogen or low C:N ratio and crude glycerol as carbon source (Kumar et al. 2015). *Bacillus mycoides* RLJ B-017 was unable to accumulate PHB at high oxygen transfer rate (OTR). It accumulated 69.4 ± 0.4% PHB of CDW in the presence of sucrose as carbon source and di-ammonium sulphate (Borah et al. 2002). The non-photosynthetic microorganisms, *Bacillus thuringiensis* EGU45 and *Bacillus cereus* EGU44 were capable of producing 11.3% PHB of CDW in the same medium where they were initially subjected for H_2 production (Patel et al. 2011) (Table 18.5).

Table 18.4 Properties of different types of PHA and polypropylene

Type of PHA and polypropylene	T_g (°C)	T_m (°C)	X_{cr} (%)	E (GPa)	σ (MPa)	ε (%)	References
Polypropylene	−14 to −6	160–169	50	1.1–2	28–40	20–75	Pradhan et al. (2020)
Polypropylene	−10	176	NA	1.7	38	400	Strong et al. (2016)
Polypropylene	−10	176	50–70	NA	38	400	Verlinden et al. (2007)
LDPE	−30	130	NA	0.2	10	620	Strong et al. (2016)
LDPE	−125 to −90	105–125	43	0.14–0.3	7–17	200–900	Pradhan et al. (2020)
HDPE	−125 to −90	130–137	79.8–81	0.7–1.4	20–40	100–1000	Pradhan et al. (2020)
P(3HB)	−4 to 18	162–181	50–80	1.2–4	19–44	50–80	Pradhan et al. (2020)
P(3HB)	−1	180	NA	3.5	40	5	Strong et al. (2016)
P(3HB)	2	177	60	NA	43	5	Verlinden et al. (2007)
P(3HO)	−35	60	30		10	300	Basnett and Roy (2010)
P(3HB-co-3 HV)	10 to −6	137–170	NA	0.7–2.9	Up to 690	30–38	Ciesielska and Kiewisz (2016)
P(3HB-co-3 HV)	−13 to 10	64–171	53–56	0.14 to 8.7	1.8 to 51	1–970	Pradhan et al. (2020)
P(3HB-co-3 HV)	−1	145	56	NA	20	50	Verlinden et al. (2007)
P(3HB-co-20%3HD)	−8	130	NA	NA	17	680	Ciesielska et al. (2016)
mcl-PHA	−48.46	43.95	NA	NA	NA	NA	Nikodinovic et al. (2008)
mcl-PHA	−40	80	NA	NA	20	300	Ciesielska and Kiewisz (2016)
mcl-PHA	~ − 40	~60	40	NA	Higher	Higher	Pradhan et al. (2020)
scl-PHA	179	4	NA	3.5	5	40	Ciesielska and Kiewisz (2016)
scl-PHA	~ − 180	~ − 0	70	NA	Lower	Lower	Pradhan et al. (2020)
P(3HB-co-20 mol% 3 HV)	−1	145	NA	0.8	20	50	Strong et al. (2016)
P(3HB-co-6 mol% 3 HV)	−8	133	NA	0.2	17	680	Strong et al. (2016)
P(3HB-co-mol16% 4B)	−7	150	45	NA	26	444	Verlinden et al. (2007)
P(3HB-co-mol10% Hx)	−10	176	34	NA	21	400	Verlinden et al. (2007)
P(3HB)	−11	161	NA	NA	NA	NA	Contreras et al. (2013)
P(3HB)	−16	136.8	NA	NA	NA	NA	Contreras et al. (2013)

NA not available, *LDPE* low-density polyethylene, *HDPE* high-density polyethylene, *P* poly, *(3HB)* 3-hydroxybutyrate, *(3HO)* 3-hydroxyoctanoate, *3(HV)* 3-hydroxyvalerate, *3(HD)* 3-hydroxydecanoate, *(4B)* butyrate, *(Hx)* hexanoate, T_g glass transition temperature, T_m, melting temperature, X_{cr} degree of crystallinity, E Young's module, ε elongation to break, σ tensile strength

Table 18.5 Diversity of PHA-producing *Bacilli*

Type of *Bacilli*	Type of PHA	PHA yield	Nutrient source	Incubation period	Study level	References
B. megaterium	P(3HB)	186.8 mg/g	Glycerol reagent grade (GRG)	48 h	Flask level	Cardozo et al. (2016)
B. megaterium uyuni S29	P(3HB) with 161Tm	30%	Glucose	NA	Bioreactor level	Contreras et al. (2013)
	P(3HB) with 136.8 T_m*	70%	Glucose	NA	Bioreactor level	
Isolate AWW belonging to genus *Bacillus*	P(3HB)	41.66%	Glucose	48 h	Flask level	Getachew and Woldesenbet (2016)
	P(3HB)	54.16%	Fructose	48 h	Flask level	
	P(3HB)	48.83%	Sucrose	48 h	Flask level	
	P(3HB)	51.61%	Corn cob	48 h	Flask level	
	P(3HB)	38.55%	Teff straw	48 h	Flask level	
	P(3HB)	26.92%	Banana peel	48 h	Flask level	
	P(3HB)	63.41%	Peptone	48 h	Flask level	
	P(3HB)	51.25%	Ammonium nitrate	48 h	Flask level	
Isolate ASS belonging to genus *Bacillus*	P(3HB)	35.45%	Glucose	48 h	Flask level	
Isolate LAW belonging to genus *Bacillus*	P(3HB)	28.88%	Glucose	48 h	Flask level	
Isolate FPS belonging to genus *Bacillus*	P(3HB)	23.59%	Glucose	48 h	Flask level	
Isolate KAS belonging to genus *Bacillus*	P(3HB)	16.66%	Glucose	48 h	Flask level	
Isolate KIS belonging to genus *Bacillus*	P(3HB)	28.57%	Glucose	48 h	Flask level	
Isolate WW belonging to genus *Bacillus*	PHA	46.28%	Glucose	48 h	Flask level	Getachew and Berhanu (2016)
Isolate RS belonging to genus *Bacillus*	PHA	35.45%	Glucose	48 h	Flask level	
Isolate SS belonging to genus *Bacillus*	PHA	34.04%	Glucose	48 h	Flask level	
Bacillus sp. N-2	P(3HB)	20%	Glucose	5 days	Flask level	Hassan et al. (2016)

(continued)

Table 18.5 (continued)

Type of *Bacilli*	Type of PHA	PHA yield	Nutrient source	Incubation period	Study level	References
6 BC1 *Bacillus* co-culture	P(3HB)	150 mg/L	Glucose	48 h	Flask level	Kumar et al. (2014)
	P(3HB)	855 mg/L	Pea-shell slurry	48 h	Flask level	
	P(3HV)	20 mg/L	Pea-shell slurry	48 h	Flask level	
5 BC1 *Bacillus* co-culture	P(3HB)	230 mg/L	Glucose	48 h	Flask level	
	P(3HB)	1620 mg/L	Pea-shell slurry	48 h	Flask level	
	P(3 HV)	25 mg/L	Pea-shell slurry	48 h	Flask level	
5 BC2 *Bacillus* co-culture	P(3HB)	230 mg/L	Glucose	48 h	Flask level	
	P(3HB)	1595 mg/L	Pea-shell slurry	48 h	Flask level	
	P(3HV)	15 mg/L	Pea-shell slurry	48 h	Flask level	
4 BC1 *Bacillus* co-culture	P(3HB)	250 mg/L	Glucose	48 h	Flask level	
	P(3HB)	430 mg/L	Pea-shell slurry	48 h	Flask level	
	P(3HV)	30 mg/L	Pea-shell slurry	48 h	Flask level	
4 BC2 *Bacillus* co-culture	P(3HB)	205 mg/L	Glucose	48 h	Flask level	
	P(3HB)	960 mg/L	Pea-shell slurry	48 h	Flask level	
	P(3HV)	20 mg/L	Pea-shell slurry	48 h	Flask level	
4 BC3 *Bacillus* co-culture	P(3HB)	190 mg/L	Glucose	48 h	Flask level	
	P(3HB)	570 mg/L	Pea-shell slurry	48 h	Flask level	
	P(3HV)	30 mg/L	Pea-shell slurry	48 h	Flask level	
3 BC1 *Bacillus* co-culture	P(3HB)	185 mg/L	Glucose	48 h	Flask level	
	P(3HB)	875 mg/L	Pea-shell slurry	48 h	Flask level	
	P(3HV)	25 mg/L	Pea-shell slurry	48 h	Flask level	
2 BC1 *Bacillus* co-culture	P(3HB)	230 mg/L	Glucose	48 h	Flask level	
	P(3HB)	780 mg/L	Pea-shell slurry	48 h	Flask level	
	P(3HV)	40 mg/L	Pea-shell slurry	48 h	Flask level	
2 BC2 *Bacillus* co-culture	P(3HB)	220 mg/L	Glucose	48 h	Flask level	
	P(3HB)	835 mg/L	Pea-shell slurry	48 h	Flask level	
	P(3HV)	35 mg/L	Pea-shell slurry	48 h	Flask level	

Bacillus sp.	PHA	NA	Marine sample	NA	NA	Wecker et al. (2015)
Bacillus odysseyi SUK3	PHA	58%	Mixture of red HE8B, red M5B, remazol red, orange 3R, rubine, golden yellow HER and direct blue GLL	48 h	Flask level	Tamboli et al. (2010)
Bacillus megaterium BA-019	P(3HB)	42%	Molasses and sucrose	24 h	Fed-batch	Pagliano et al. (2017)
Bacillus cereus SPV	P(3HB)	61.07%	Sugarcane molasses	50 h	Shaken flask	Akaraonye et al. (2012)
	P(3HB)	51.37%	Sugarcane molasses	50 h	Fermenter level	
B. megaterium DSM 509	PHA	48%	Octanoic acid	24 h	Flask level	Shahid et al. (2013)
Bacillus megaterium	PHB	5.61 g/L	Glucose and ammonium sulphate	64 h	Bioreactor	Mohanrasu et al. (2020)
Bacillus megaterium SRKP-3	PHB	11.32 g/L	Dairy waste, Rice bran and Sa water	36 h	Feed-batch	Pandian et al. (2010)
Bacillus drentensis BP17	PHB	5.55 g/L	Pineapple peel solution	36 h	Flask level	Penkhrue et al. (2020)
Bacillus sp. *CFR 67*	PHA	524 mg/L	Wheat bran hydrolysate	72 h	Flask level	Sreekanth et al. (2013)
Bacillus tequilensis	P(3HB-co-3 HV)	59%	Synthetic acids	48 h	Flask level	Reddy et al. (2014)
		36%	Acidogenic fermented food waste (AFW)			

NA not available, T_m melting temperature, *P* Poly, *3(HB)* 3-hydroxybutyrate, *3(HV)* 3-Hydroxyvalerate

18.3.3 Metabolic Overview for PHA Production in Bacilli

The monomeric PHA/hydroxyalkanoic acid is synthesized by different metabolic pathways. The type of –R group incorporated in monomeric PHA depends on the type of substrate and the capability of microorganisms to metabolize the available substrate (Sudesh et al. 2000). The common intermediate in the pathway is hydroxyacyl-Co-A, as shown in Fig. 18.8a (Chen 2010). The type of acyl group present in it reveals the formation of a particular type of PHA. For example, 3-hydroxybutyryl-Co-A contains butyryl group which gets polymerized into 3-hydroxybutyric acid/polyhydroxybutyrate [3(PHB)] (Chen 2010; Pradhan et al. 2020). Polymerization event is catalysed by PHA synthase (PhaC) (Grage et al. 2009; Chen 2010; Pradhan et al. 2020). It is the most common enzyme involved in all PHA production pathways. The metabolic pathway of PHB, the most common kind of PHA, is well established. As shown in Fig. 18.8b, it involves two enzymes, viz. β-ketothiolase (PhaA) and acetoacetyl-CoA reductase (PhaB), along with PhaC. The microorganisms act upon available substrate and convert it into acetyl CoA. Further, PhaA transforms it into acetoacetyl-CoA which gets converted into 3-hydroxybutyryl-CoA by PhaB (Pradhan et al. 2020; Chen 2010; Beun et al. 2002). Like all other biochemical pathways, PHA synthesis also involves wide varieties of enzymes and a few of them are listed in Table 18.6.

Apart from this, *B. thuringiensis* strain YBT-1520 was reported to show PHB production from acetyl Co-A via two dissimilar pathways. One involved three traditional enzymes, viz. PhaA, PhaB and PhaC, while other involved enzymes designated as AtoB, FadB and Crt. The enzyme AtoB converts acetyl CoA into acetoacetyl-CoA. Then it gets transformed into Crotonoyl CoA via

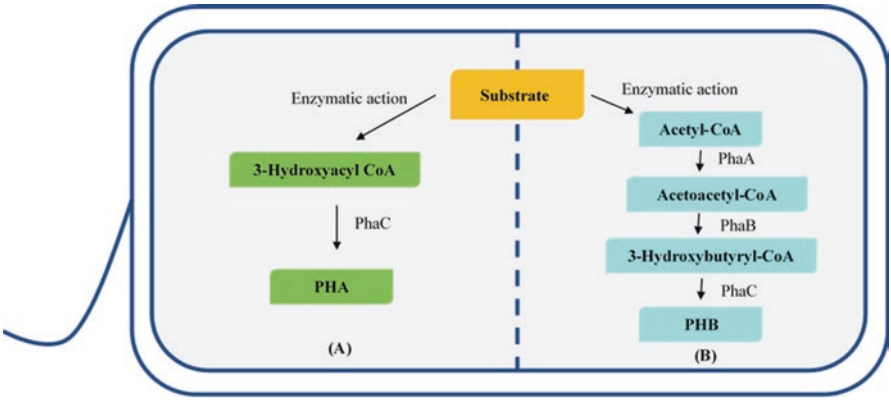

Fig. 18.8 Metabolic pathways for PHA production in bacterial cells. (**a**) PHA synthesis via 3-hydroxyacyl CoA intermediate and (**b**) PHB synthesis via acetyl-CoA: Formation of particular type of PHA depends on the type of acyl group. For example, butyryl is the acyl group leading to formation of PHB

Table 18.6 Enzymes involved in biosynthesis of PHA and gene encoding them

Enzyme	Gene	Type of PHA	Organism	References
β-ketothiolase	*pha*A	P(3HB-*co*-3HHx)-	*Aeromonas caviae*	Fukui and Doi (1997) and Fukui et al. (1998)
NADH-acetoacetyl-CoA dehydrogenase	*pha*B			
(*S*)-specific enoyl-CoA hydratase (crotonase)	$phaJ_{Ac}$			
PHA synthase	*pha*C			
β-ketothiolase	NA	P(3HB)	*Rhizobium* (*Cicer*) sp. strain CC 1192	Chohan and Copeland (1998)
NADPH-dependent acetoacetyl-CoA reductase	NA			
PHA synthase	NA			
3-ketoacyl-ACP synthase III (FabH)	*fab*H	PHA	*Aeromonas caviae*	Taguchi et al. (1999)
Malonyl-CoA-ACP transacylase (FabD)	*fab*D			
PHA synthase (PhaC*Ac*)	$phaC_{Ac}$			
3-ketothiolase	NA	Poly(3HB-co-4HB)	*Clostridium kluyveri*	Valentin and Dennis (1997)
Acetoacetyl-CoA reductase	NA			
PHA synthase	NA			
Succinic semialdehyde dehydrogenase	*suc*D			
4-hydroxybutyrate dehydrogenase	4*hb*D			
4-hydroxybutyrate-CoA: CoA transferase	*orf*Z			
β -ketothiolase	NA	Poly(3HB-co-3HV-co-4 HV	*Alcaligenes eutrophus*	Valentin and Steinbuchel (1995)
Acetoacetyl-CoA reductase	NA			
3-hydroxyacyl CoA dehydrogenase	NA			
Acyl CoA dehydrogenase	NA			
Acyl CoA synthase	NA			
Acyl CoA transferase	NA			
β-hydroxyacyl-CoA hydrolase	NA			
PHA synthase	NA			
PHA synthase	*pha*C	PHA	*Bacillus megaterium*	Mccool and cannon (2001)
Class IV PHA synthase	$phaRC_{Bm}$	P(3HB)	*Bacillus megaterium* NBRC15308T	Tomizawa et al. (2011)
Class IV PHA synthase	$phaRC_{By}$	P(3HB)	*B. cereus* YB-4	Tomizawa et al. (2011)
PHA synthase	*pha*C and *pha*R	PHA	*Bacillus* sp. INT005	Satoh et al. (2002)

NA not available

(S)-3-hydroxybutynyl Co-A by FadB. Lastly, Crt acts upon crotonoyl CoA and forms (R)-3-hydroxybutynyl CoA, the common intermediate of both pathways which is polymerized by PhaC (Gong et al. 2012). *B. cereus* has been reported to formulate PHA via acetyl CoA metabolism or via fatty acid oxidation. It contains enzymes such as PhaA, PhaB, PhaJ, PhaRC and FadB for PHA production. PhaJ converts crotonoyl CoA to R-3-hydroxybutyryl CoA. PhaRC functions like PhaC while the remaining enzymes function as described before (Tsugeet et al. 2015).

Generally, PHA biosynthesis competes with TCA cycle for assimilating acetyl-CoA. Under stable growth condition, acetyl-CoA gets oxidized via TCA cycle and NADH generated is utilized for ATP production. In nutrient-deprived state, cell growth is limited, and the NADH accumulates (Faccin et al. 2013). This NADH will inhibit citrate synthase indicating the availability of sufficient amount of ATP and precursor for biosynthesis. Thus, rate of TCA cycle declines (Kim and Gadd 2008). Further, acetyl CoA generated due to the presence of excess substrate is directed towards PHA biosynthesis (Faccin et al. 2013). Decline in TCA cycle is even related with the absence of oxygen. Due to unavailability of O_2, NADH does not get oxidized via electron transport chain (ETC) and gets accumulated there. This inhibits citrate synthase, and rate of TCA cycle reduces (Kim and Gadd 2008; Faccin et al. 2013). Though unavailability of O_2 causes failure of TCA cycle, PHA accumulation is also limited. The reason behind this is oxidation of enzymes essential for PHA production. The inhibitory effect of O_2 over PHA production is solely dependent on the individual organisms (Borah et al. 2002). For appropriate PHA production, optimum aeration is required. Availability of O_2 above and below optimum level reduces PHA accumulation (Faccin et al. 2013).

18.3.4 Molecular Evidences for PHA Production by Bacilli

Biosynthesis of PHA includes numerous classes of enzymes. Three genes termed as *pha*A, *pha*B and *pha*C encode three key enzymes β-ketothiolase, acetoacetyl-CoA reductase and PHA synthase of PHB production pathway. Other genes involved in PHA production are listed in Table 18.6.

B. thuringiensis R1 possesses *pha*P, *pha*Q, *pha*R, *pha*B and *pha*C genes, as depicted in Fig. 18.9. Gene designated as *pha*P encodes for phasins protein and *pha*Q encodes for transcriptional regulatory protein essential which regulates *pha*P activity. Apart from this, *pha*R encodes for unknown product essential for activity of *pha*C. Gene length of these genes along with their promoter regions and

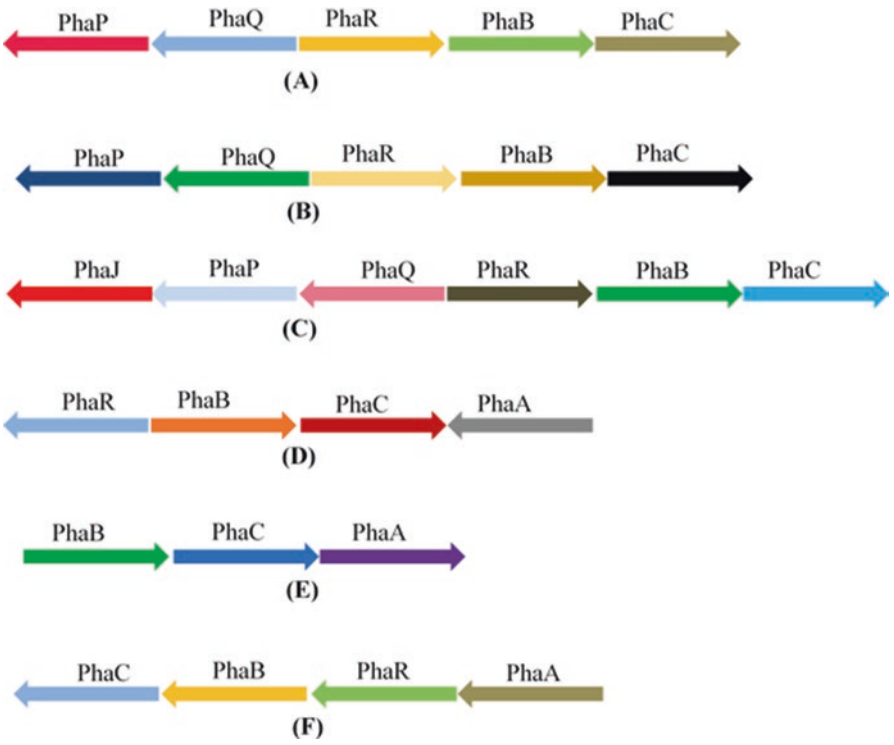

Fig. 18.9 Arrangement of PHA synthesis operon in different *Bacillus* spp. (**a**) *B. thuringiensis* R1, (**b**) *B. megaterium*, (**c**) *B. cereus* subgroups, (**d**) numerous *Bacillus* spp., (**e**) *B. thuringiensis* serovar chinensis CT-43 and *B. megaterium* QM B1551 and (**f**) *B. anthracis* CDC 684 and *B. megaterium* WSH-002

Table 18.7 Nucleotide sequence of R1 promoter and RBS of genes essential for PHA formulation in *Bacillus thuringiensis* (Desetty et al. 2008)

Gene	Size (bp)	Promoter sequence	RBS sequence
*pha*P	522	−10 (^{1099}CACATTTAA^{1091})	^{556}TTGGGG^{551}
*pha*Q	450	−35 (^{1119}AACTGA^{1114})	^{1053}GAGGTG^{1048}
*pha*R	528	−10 (^{1143}AAATAAAAT^{1130})	^{1171}CAGAAT^{1176}
*pha*B	741	−35 (^{1765}TTTCTA^{1770})	^{2682}AAGGAG^{2687}
*pha*C	1083	−10 (^{2606}ATATGTAAT^{2614})	^{2682}AAGGAG^{2687}

ribosomal binding sites (RBS) is listed in Table 18.7 (Desetty et al. 2008). *B. megaterium* have *phaP* (513 bp), *phaQ* (441 bp), *phaR* (609 bp), *phaB* (744 bp) and *phaC* (1089 bp) as shown in Fig. 18.9b (Valappil et al. 2007a). Few *B. cereus* subgroups as shown in Fig. 18.9c have *pha*R-*pha*B-*pha*C operon located in same direction and *pha*P-*pha*Q-*pha*J operon in its contradictory direction. Later operon is found

upstream of former one. The gene *pha*J was found to be involved in directing monomer supply via β-oxidation for PHA formation (Tsugeet et al. 2015).

Wide variations in orientation of genes crucial for PHA production were reported in numerous *Bacillus* spp. It was reported that operon *pha*RBCA as shown in Fig. 18.9d was found in *B. anthracis* A0248, *B. anthracis* Ames, *B. anthracis* "Ames Ancestor", *B. cereus* Q1, *B. cereus* 03BB102, *B. cereus* AH820, *B. cereus* B4264, *B. cereus* G9842, *B. cereus* AH187, *B. cereus* biovar anthracis str. CI, *B. cereus* ATCC 10987, *B. cereus* E33L, *B. megaterium* DSM 319, *B. thuringiensis* BMB171 and *B. thuringiensis* serovar konkukian, 97–27. Moreover, authors had reported operon *pha*BCA as illustrated in Fig. 18.9e, in *B. thuringiensis* serovar chinensis CT-43 and *B. megaterium* QM B1551. They had even shown that *B. anthracis* CDC 684 and *B. megaterium* WSH-002 have *pha*CBRA operon, as shown in Fig. 18.9f (Kumar et al. 2013).

18.3.5 Strategy for PHA Accumulation and Recovery

Microorganisms show higher PHA accumulation in favourable environmental conditions. As described earlier in nutrient-depleted environments, few microorganisms ensure higher PHA yield. Growth conditions favourable for microbes to accumulate PHA vary from organism to organism (Albuquerque et al. 2010; Shahid et al. 2013; Saharan et al. 2014; Beun et al. 2002). Also, yield of PHA obtained varies with the time at which PHA is extracted (Pandian et al. 2010; Kumar et al. 2015; Pagliano et al. 2017; Rathika et al. 2018). Moreover, inoculum size and pH also affect the yield of extracted PHA. Higher PHA yield extracted from *B. subtilis* RS1 was obtained with 10% inoculum size and at pH 7 (Rathika et al. 2018). *Bacillus* sp. BPPI-14 and *Bacillus* sp. BPPI-19 showed higher PHA yield at pH 7 and 37 °C using glucose as sole source of carbon (Mohammed et al. 2019). Hence, optimization of PHA accumulation media is required for enhancing PHA yield. Utilization of cheap nutrient source will reduce the cost of industrial PHA production (Verlinden et al. 2011; Mohapatra et al. 2017; Kourmentza et al. 2017).

Another factor which affects industrial level PHA production is the cost associated with PHA recovery. Effective PHA recovery scheme plays an important role in gaining higher PHA yield. As demonstrated in Fig. 18.10, approaches for PHA recovery can be divided into six steps, viz. cellular biomass harvesting, pretreatment, non-PHA cellular biomass disruption, PHA extraction, drying and purification (Kourmentza et al. 2017; Sathya et al. 2018; Jacquel et al. 2008). As PHA is intracellular product, concentration of cellular biomass is carried out. It is harvested using centrifugation or filtration (Kourmentza et al. 2017). The harvested biomass is subjected for pretreatment prior to cell lysis. The main aim of pretreatment is to weaken the microbial cell wall, which involves physical techniques such as lyophilization, ultrasonication and high temperature. The cell lysis of pretreated biomass is carried out using chemical, enzymatic or biological method. The agents used for cell lysis should not deteriorate PHA. Chemical method involves usage of sodium

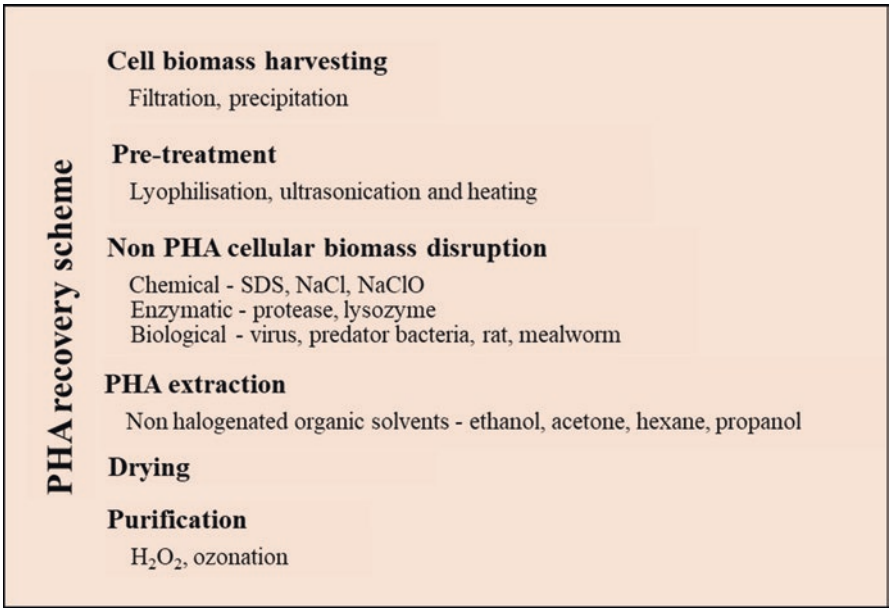

Fig. 18.10 PHA recovery scheme: it includes six steps, viz. cell biomass harvesting, pretreatment, non-PHA cellular biomass disruption, PHA extraction, drying and purification

chloride (NaCl), sodium hypochlorite (NaClO) or sodium dodecyl sulphate (SDS). Enzymatic method includes application of enzymes such as proteases and lysozyme (Kourmentza et al. 2017; Jacquel et al. 2008). For biological cell lysis, researchers have used virus and predatory bacteria. Some researchers have fed the rats and mealworms with biomass, and PHA was recovered from faeces (Kourmentza et al. 2017). Amongst all, chemical methods are widely accepted as they are eco-friendly and do not involve the use of halogenated solvents. Moreover, enzymatic methods are costly, and biological methods are time-consuming (Kourmentza et al. 2017; Jacquel et al. 2008). Traditionally, PHA was extracted using hazardous chlorinated halogenated solvents, such as chloroform, 1, 2-dichloroethane and methylene chloride (Jacquel et al. 2008). Nowadays, PHA extraction is carried out using non-halogenated organic solvents in which PHA is soluble. It includes solvents such as acetone, n-hexane, propanol and ethanol (Kourmentza et al. 2017; Jacquel et al. 2008). Extracted PHA is dried and obtained in the form of powder (Kourmentza et al. 2017; Jacquel et al. 2008). For purification of biopolymer, H_2O_2 or ozonation has been employed (Horowitz et al. 2001; Madkour et al. 2013). Application of cheap and environment-friendly PHA recovery technique is beneficial which needs to be optimized (Kourmentza et al. 2017).

18.3.6 Techniques Involved in Characterization of PHA

A wide range of sophisticated techniques are employed for characterization of extracted PHA powder. Fourier-transform infrared spectroscopy (FTIR), nuclear magnetic resonance (NMR) and gas chromatography-mass spectroscopy (GC-MS) are the most common techniques used for determining the functional group incorporated in PHA. Apart from this, X-ray diffraction (XRD), gel permeation chromatography (GPC), differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) were used for determining mechanical and thermal properties of PHA (Pradhan et al. 2020; Sathya et al. 2018; Mohapatra et al. 2017; Gumel et al. 2012; Godbole 2016; Johnston et al. 2018). Details of all these techniques are described in Table 18.8.

PHA extracted from *B. licheniformis* AS3-2 was characterized using FTIR (Shah 2012). PHA extracted from *Bacillus cereus* was characterized using scanning electron microscopy (SEM), FTIR, XRD, NMR, DSC and thermal gravimetric analysis (TGA) and confirmed as PHB (Babruwad et al. 2015). PHB-co-PHV extracted from *Bacillus megaterium* OUAT 016 was characterized using FTIR, NMR, XRD and TGA (Mohapatra et al. 2020).

18.3.7 Challenges for Bacilli to Produce PHA

Bacillus spp. are capable of producing PHA using cheap substrates including waste materials. Moreover, they are capable of tolerating high pH and high osmotic pressure. Despite of such facts, the major drawback associated with its application for industrial scale PHA production is its sporulating nature (Wu et al. 2001; Mohapatra et al. 2017). Spore formation may utilize energy generated via PHB degradation (Wu et al. 2001). Normally, it was supposed that PHB degradation diverts energy and carbon source for sporulation (Kominek and Halvorson 1965). *B. cereus* has been reported to accumulate PHB prior to sporulation which subsequently gets degraded during spore formation event (Navarro et al. 2006; Kominek and Halvorson 1965). PHB accumulated by *B. thuringiensis* using glucose is utilized for spore formation (Benoit et al. 1990). *Bacillus* sp. JMa5 showed spore formation in nutrient-limiting conditions with low PHB yield. Besides this, it showed growth-associated PHB production. Hence, authors conclude that low levels of nutrients induce sporulation which may limit PHB accumulation (Wu et al. 2001). Further, acidic pH and low level of potassium show decline in spore formation (Mohapatra et al. 2017). *Bacillus* spp. SPV was unable to sporulate in acidic pH and showed PHB accumulation (Valappil et al. 2007b). The antisporogenic agent α-picolinic acid prevents conversion of vegetative *Bacillus cereus* T cells to sporulating cells. TCA cycle enzymes essential for sporulation seem to be synthesized during shift from vegetative phase to sporulation phase. α-picolinic acid is inhibitory to aconitase synthesis and prevents TCA cycle essential for spore formation and not for

Table 18.8 Techniques used for characterization of PHA

Name	Role	Information obtained			References
FTIR	Used for identification of functional group of PHA	–R group	Wave frequency (cm^{-1})		Pradhan et al. (2020)
		–CH	2962–2853		
		–C=O	1742–1709		
		–C–O or –C–C	1300–1709		
		–OH	3460–3407		
NMR	Used to recognize functional group and polymeric content of PHA biopolymer	–R group	^{1}H NMR (ppm)	^{13}C NMR (ppm)	Pradhan et al. (2020)
		–CH	5.2–5.26	67.8–68.5	
		$–CH_2$	2.17–2.7	31.09–41.3	
		$–CH_3$	1.25–1.6	19.95–21.4	
		–C=O	NA	169.1–169.5	
GC-MS	Used for analysing monomeric composition of PHA	Methyl esters of PHA are subjected to GC-MS analysis and functional groups are identified on the basis of retention time			Lee and Choi (1997)
XRD	Useful for studying crystalline nature of PHA	% Xc = At − Aa/At × 100 At = area of crystalline peak Aa = area of amorphous peak			Pradhan et al. (2020)
GPC	Used for determining mw, Mn and polydispersity index	Polydispersity index indicates molecular mass distribution and was calculated by determining Mw/Mn ratio			Pradhan et al. (2020)
DSC	Used for understanding thermal properties such as T_m and X_c of PHA	% $X_c = \Delta H_m/\Delta H_f \times 100$ ΔH_m = measured enthalpy of polymer ΔH_f = enthalpy of 100% pure PHB (146 J/g)			Pradhan et al. (2020), Gunaratne and Shanks (2005), and Kulkarni et al. (2010)
TGA	Used for analysing thermal stability and degradation as well as resistance temperature of PHA	TGA graph indicates the reduction in weight of PHA with rise in temperature. PHA degradation takes place as the temperature increases. Degradation of PHA correlates with decrease in weight of PHA			Pradhan et al. (2020)

NA not available, *Mw* average molecular weight, *Mn* number average molecular weight, T_m, melting temperature, X_c crystallinity

biomass development. Thus, this antisporogenic agent prevents spore formation (Hanson et al. 1963). Such sort of manipulation of environmental conditions may be supportive in regulating the sporulating nature of *Bacillus* spp. and thus permitting its usage for industrial scale PHA synthesis. Moreover, study by Wang et al. (2016) shows that sporulation in *B. thuringiensis* is not associated with PHB degradation.

They even observed that many *Bacillus* spp. lack *pha*C and *pha*Z genes but still sporulate, indicating individuality of spore formation over PHB degradation. There arises a need of strategies for controlling sporulation for application of such strains in industrial PHA production.

Apart from this, the *Bacillus* spp. possess thick cell wall which makes PHA extraction difficult (Wu et al. 2001; Mohapatra et al. 2017). *B. flexus*, grown in inorganic rich medium contains less diaminopimelic acid and amino acids in cell wall. This allows easier cell lysis, and so higher PHA recovery was found (Divyashree and Shamala 2010). Similar techniques can be used for efficient recovery of PHA from *Bacillus* spp.

18.3.8 Approaches for Improving Properties of PHA for Industrial Application

Most of the biopolymers possess poor mechanical properties. Brittle and fragile nature of biopolymers limits their industrial scale application (Vieira et al. 2011). In order to improve their mechanical properties, they are either blended with plasticizer and/or cross-linking agent (Jantrawut et al. 2017) or blended with other polymers (Mohapatra et al. 2017; Narancic et al. 2018). Plasticizers have low molecular weight and are non-volatile compounds. They are widely used in polymer industry for enhancing the properties of polymers. They are known to reduce Tg of polymers and supplement their biodegradation (Vieira et al. 2011). Usage of eco-friendly nature-based biodegradable plasticizer is advantageous over conventional plasticizer such as phthalates (Vieira et al. 2011). Thermal and mechanical properties of PHBV films improve after blending with biodegradable plasticizers such as soybean oil (SO), epoxidized soybean oil (ESO), dibutyl phthalate (DBP) and triethyl citrate (TEC) (Vieira et al. 2011). Cross-linking agent forms intermolecular cross linkages with biopolymer, permitting suitable biopolymer film formation (Jantrawut et al. 2017). Further, application of some additives along with plasticizer enhances enzymatic degradation of PHB (Vieira et al. 2011).

Blending of P(3HB) extracted from *B. megaterium* Ti3 with polyethylene glycol enhances biocompatibility of P(3HB) film (Israni et al. 2020). The blends of PHB extracted from *B. cereus* strain VIT-SSR1 isolated from industrial effluents were prepared with chitosan. Biocompatibility of these blends was investigated on L929 mouse fibroblast cell line with MTT assay. They proved to be biocompatible and hence can be used for drug delivery (Evangeline and Sridharan 2019). PHBV extracted from *B. aryabhattai* PHB10 was blended with polyethylene glycol, and cytotoxicity was analysed on human keratinocytes (HaCat cells). Approximately 99% cells were viable, and hence this blend can be employed for skin graft application (Pillai et al. 2020).

18.3.9 Commercial Applications of PHA Obtained from Bacilli

Microbially originated biodegradable PHA have plenty of applications. They are used for manufacturing of packaging material and biomedical products (Mohapatra et al. 2017; Chen 2010; Sathya et al. 2018). Moreover, they are used as drug delivery carriers, as pharmaceutical products/drugs and as biofuels (Chen 2010). PHA are non-toxic in nature and hence are biocompatible, allowing its biomedical applications (Pradhan et al. 2020). They are even used for agricultural purposes (Pradhan et al. 2020; Sowinski et al. 2010).

Commercially, manufacturer entitled as PHB Industrial S.A., Brazil, have employed *Bacillus* spp. under Biocycle trademark. They are exploited for P(3HB) production from sugarcane (Ciesielska and Kiewisz 2016). PHB extracted from pigmented *Bacillus* sp. C1 (2013) (KF626477) was biocompatible in nature and hence can be used as drug delivery carrier (Pati et al. 2020). PHB of *B. thuringiensis* is non-toxic and is suitable for biomedical purpose (El-Abd et al. 2017). PHB-co-PHV extracted from *B. megaterium* OUAT 016 was found to be biocompatible and can be used as drug delivery carrier (Mohapatra et al. 2020). P(3HB-co-HV) extracted from *B. thermoamylovorans* was esterified using methanol and H_2SO_4. This, P(3HB-co-HV) methyl ester can be used as biofuel (Sangkharak et al. 2020). PHA levofloxacin nanoparticles were prepared using PHA extracted from *B. subtilis* NCDC0671, and its levofloxacin releasing efficacy was proved to be efficient. Hence, they can be used for delivering levofloxacin drug (Umesh et al. 2017).

18.3.10 PHA Depolymerase of Bacilli *and Biodegradation*

The most attractive feature of PHA is its biodegradability. It is composed of 100% natural biobased resources (Pradhan et al. 2020). In aerobic conditions, they get transformed into CO_2 and H_2O, whereas in anaerobic conditions, CH_4 is obtained additionally. It undergoes thermal degradation as well as enzymatic and non-enzymatic degradation (Pradhan et al. 2020; Nesic et al. 2020). It gets degraded when exposed to soil or compost and even in marine sediments (Nesic et al. 2020). It is prone to get degraded by microbial PHA depolymerase (i-PhaZ and e-PhaZ) or non-enzymatically inside animal tissues. It takes about few months to a year to get degraded in anaerobic conditions. Degradation rate boosts up in UV light. PHA polymers with high T_m take longer duration for degradation. Apart from this, PHA with low molecular weights gets degraded faster. Factors affecting rate of PHA degradation are temperature, pressure, moisture, surface area, pH and type of microorganism (Pradhan et al. 2020).

The role of i-PhaZ and e-PhaZ is described in earlier section, i.e. in functions of PHA and structure of PHA granule. *Bacillus megaterium* has i-PhaZ designated as PhaZ1 and degrades PHB into 3-hydroxybutyric acid monomers (Chen et al. 2009). *B. thuringiensis* possess P(3HB) depolymerase designated as PhaZ (Huang et al.

2012). *Bacillus* sp. strain NRRL B-14911, *B. megaterium*, *B. pseudofirmus* and *Bacillus* sp. strain SG-1 have e-PhaZ (Ma et al. 2011). *B. megaterium* N-18-25-9 contains e-PhaZ gene designated as $phaZ_{Bm}$ (Takaku et al. 2006). *B. thuringiensis* subsp. *israelensis* ATCC 35646 contains gene designated as *phaZ* have function similar to intracellular P(3HB) depolymerase (Tseng et al. 2006).

18.4 Future Prospects

Biodegradable biopolymers are recognized as potential substitute for conventional petrochemical-based plastics. *Bacillus* spp. are considered as promising agents for PHA production. A wide range of lab level investigations on *Bacillus* and PHA have been carried out till date. But their application in actual biopolymer industry needs attention. Moreover, varied spectrum of microbial PHA is known to exist, but most of the studies are constricted to P(3HB). Finding out additional forms of PHA having plastics like properties might be advantageous. Genetically engineered *Bacillus* strain ensuring higher PHA-producing capabilities can be the targeted aim of future research. Methyl esters of PHA have properties similar to biofuel so exploring the application of PHA is obtained using waste substrates, as biofuel will allow sustainable management of waste along with formation of valuable by-product. The PHA polymeric proteins such as PhaP, PhaZ and PhaC may be applicable as potential drug delivering tools.

18.5 Conclusions

Plastic pollution is one of the major concerns in the world. Biodegradable biopolymers being solutions to such issue have gained much attention amongst scientific community. Primarily, microbial PHA are significantly valuable as they are solely biobased and totally biodegradable. They are carbon-rich inclusion bodies synthesized by microorganisms in response to stress conditions. The polyester PHA granules are comprised of hydroxyalkanoic acid, PhaP, PhaC, PhaZ and PhaR. Micelle formation and budding are the two strategies known for PHA formulation in microorganisms. *Scl*-PHA have properties identical to polystyrene, whereas *mcl*-PHA are widely accepted in medical industry.

First and foremost concern for using PHA in bio-industry depends on the choice of microbial strain and cost-effective nutrient source. *Bacillus* spp. are known to serve as appropriate bacteria for industrial application. They are also recognized to produce PHA from varieties of inexpensive waste. They are known to produce PHA in the presence of excess substrate with either nutrient-deprived or non-nutrient-deprived conditions. Metabolic pathway of PHA production and operons encoding enzymes of pathway in *Bacilli* are well known. The media conditions known to enhance PHA accumulation in bacilli need to be optimized in order to increase the

yield. Accomplishing appropriate cost-effective eco-friendly PHA recovery strategy can enable us to meet the need of industries. Advances in modern science have led to development of many sophisticated techniques for characterization of PHA. Features such as sporulation and thick cell wall associated with *Bacillus* spp. hinder its applicability for industrial scale PHA manufacturing. Abundant research had been done to solve such issues. Moreover, blending of PHA extracted from *Bacilli* with plasticizer or cross-linking agent has increased its biocompatibility, making them suitable for biomedical applications. Blending also decreases the time required for PHA degradation. The enzymes i-PhaZ as well as e-PhaZ from *Bacilli* origin play a vital role in biodegradation of PHA. Thus, *Bacillus* spp. are promising resources for bioplastic industry.

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