Biological Significance of Degradation of Polyhydroxyalkanoates

Subhasree Ray and Vipin Chandra Kalia

Abstract Polyhydroxyalkanoates (PHAs) are biodegradable polymers produced by microbes under nutrient-deprived conditions. These polymers act as food reserves of the organism to survive under adverse environmental conditions. These molecules show high similarity with petroleum-based plastics. Hence, these are promoted as biodegradative alternatives to plastics. PHAs are degraded by the depolymerase enzyme primarily to generate energy for microbial growth. Extracellular degradation occurs on the PHA released from the lysed cell. Various factors are known to influence the degradative process such as humidity, temperature, monomeric composition, etc. The PHA degradation accompanies a decline in the polymer molecular weight and an increase in its crystallinity. Microbes involved in the PHA degradation contribute toward maintenance of ecosystem through the carbon cycle. The by-products of PHA degradation process can be subjected to different biological applications, especially in the energy and medical fields.

Keywords Intracellular depolymerase • PHA degradation • Hydroxy acids • Monomers

1 Introduction

Polyhydroxyalkanoates (PHAs) represent an interesting alternative to petroleum-derived plastics (Kalia et al. 2003; Reddy et al. 2003; Kumar et al. 2013, 2015a, 2016). Bacterial system can accumulate PHAs—high-molecular-weight biopolymers as storage compounds of carbon and reducing equivalents (Seebach and Fritz 1999). PHAs are also known to be osmotic neutral reservoirs of carbon and energy source. These inclusion bodies may contribute to more than 90% of the cell mass. These molecules can be biochemically synthesized from renewable resources (e.g.,

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biowaste, crude glycerol, etc.) and degrade completely in natural conditions (Jendrossek 2009; Kumar et al. 2009, 2015a, b, c, 2016; Patel et al. 2015a, b, 2016; Kalia et al. 2016). More than 90 bacterial genera are known to incorporate around 150 different hydroxy acid (HA) monomers to the PHA polymers. These polymers have been categorized as short-chain length (scl, C_3–C_5), medium-chain length (mcl, C_6–C_{14}), and long-chain length (lcl, $>C_{14}$) PHAs depending on their HA monomers. The PHA biosynthesis is controlled by *phaCAB* operon which governs a three-step conversion process of acetyl-CoA > acetoacetyl-CoA > 3-hydroxybutyryl-CoA. The latter is used by a broad substrate-specific key enzyme—PHA synthase—to produce PHAs (Singh et al. 2009, 2013, 2015; Kumar et al. 2014; Ray and Kalia 2016). All the proteins involved in PHA synthesis such as synthase, phasins, regulatory proteins, and those responsible for its degradation depolymerase are found to be integrated into a dense phospholipid layer, a structure known as "carbonosomes" (Jendrossek 2009; García-Hidalgo et al. 2013).

PHAs are quite attractive and have diverse biotechnological applications. PHAs act as a raw material for a variety of bioactive molecules such as vitamins, pheromones, fatty acids, probiotics, 3-hydroxyalkanoates, etc. However, their most important characteristic is their biodegradable nature. Most studies focus on PHA synthesis; however, it is equally interesting to learn how they are metabolized. Like all biological materials, it is quite easy to envisage the potential factors, which may be instrumental in their degradation, and the most relevant are the enzymes and the physicochemical composition of the substrate (Figs. 1 and 2).

2 Biodegradation of PHAs

The PHA biodegradation involves depolymerizing enzymes that convert them to their monomeric constituents, which undergo mineralization. The biological depolymerization process involves both extracellular and intracellular depolymerase enzymes. Intracellular degradation of PHAs involves hydrolysis of the stored material for carbon and energy generation. Extracellular degradation occurs on the polymer released from the cell by lysis. This degradation process depends upon the physicochemical composition of the polymer: stereoregularity, composition, crystallinity, and accessibility (Jendrossek 2001; Jendrossek and Handrick 2002). Under anaerobic conditions, the end products generally include CO_2, H_2O, CH_4/H_2S, and microbial biomass. In contrast, under aerobic conditions primary end products include CO_2, H_2O, and microbial biomass.

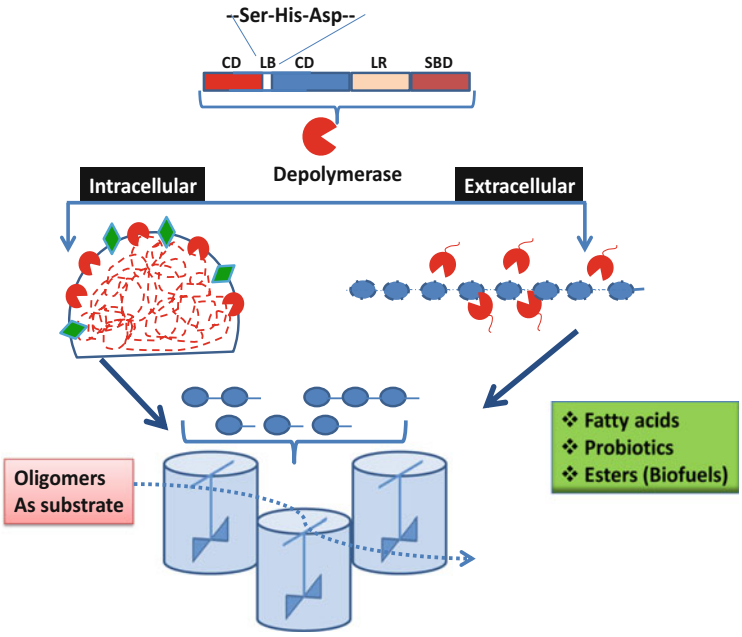

Fig. 1 PHA depolymerase activities and potential applications

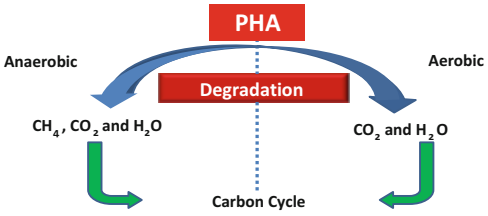

Fig. 2 General mechanism of biodegradation

3 PHA Depolymerase

The PHA-depolymerizing enzymes (*phaZ*) have gained significant attention in the last few decades (Abe et al. 2005). Depolymerases acting on scl-PHAs do not act on mcl-PHAs. Both bacteria and fungi possess these depolymerases, with higher (R) stereospecificity. Depending upon the substrate specificity, extracellular PHA depolymerases are classified into (1) scl-PHA depolymerase and (2) mcl-PHA depolymerase (Jendrossek 1998). *Bacillus megaterium* produces intracellular n-PHB depolymerase, capable of exhibiting extracellular PHB depolymerase activity as well. This unique property makes it more feasible for biodegradation (Cai et al. 2009). Standard methods for evaluating biodegradability have been established (Kalia et al. 2000).

PHA degradation competency is found to be dependent upon the following variables: (1) the environment (water, soil, etc.); (2) temperature, (3) shape, and texture of PHA; (4) the presence of inhibitors, dyes, etc.; (5) mobility; (6) tacticity; (7) crystalline; (8) molecular weight; and (9) type of functional groups present in its structure (Gu 2000; Artham and Doble 2008).

4 Bacterial PHA Depolymerases

Aerobic and anaerobic bacteria are known to degrade PHA. These bacteria can metabolize P(3HB) and its copolymer P(3HB-co-3 HV). PHA-degrading bacteria show specificity toward the monomeric composition of the polymer. In general, they can degrade either scl-PHA or mcl-PHA and some bacteria can metabolize both types (Takeda et al. 2000; Wang et al. 2009).

Intracellular PHA depolymerases (*i-PhaZ*) are found to be present in *Alcaligenes faecalis*, *Comamonas acidovorans*, *C. testosteroni*, *Pseudomonas fluorescens*, *P. lemoignei*, *Rhodospirillum rubrum*, *Ralstonia eutropha* H16, *R. pickettii*, etc. The *i-PhaZ* are specific for native PHA (n-PHA) and are usually located as transmembrane proteins on the PHA granules (Handrick et al. 2004; Uchino et al. 2007; Gebauer and Jendrossek 2006). Based on the physical state and location of the polymer, the PHA degradation process could either occur in amorphous state or denatured state. Apparently, the n-PHA gets metabolized by *i-PhaZ* releasing HA monomers, whereas the d-PHA being crystalline gets converted into HA monomers or oligomers by respective extracellular depolymerases (*e-PhaZ*) recorded in *Alcaligenes*, *Acidovorax*, and *Paucimonas* spp. (Braaz et al. 2003; Sugiyama et al. 2004; Gebauer and Jendrossek 2006; Hiraishi et al. 2010). Several PHA-degrading bacteria, such as *Stenotrophomonas* and *Pseudomonas* (among Gram-negative bacteria) and *Streptomyces* spp. and *Rhodococcus equi* (among Gram-positive bacteria), predominantly act on mcl-PHA. The genus *Rhodococcus* has gained importance due to its ability to degrade several hydrophobic substances which include scl-PHA, petroleum hydrocarbon, benzene ring, and PCB (Kobayashi et al. 2004; Kim et al. 2007; Ihssen et al. 2009) (Table 1).

Basically PHA depolymerase enzyme consists of three functional domains (amino acids): (a) a catalytic domain (320–420), (b) a linker region (50–100), and (c) a substrate-binding domain (40–60). The catalytic domain constitutes of a lipase box since they contain lipase-like catalytic triad that includes specific amino acid residues, i.e., serine (S), aspartic acid (D), and histidine (H), along with a pentapeptide signature sequence Gly-Xaa1-Ser-Xaa2-Gly, which is present in serine hydrolase (Jendrossek and Handrick 2002). Serine hydrolase side chain behaves as a nucleophile, which contains oxygen that attacks the ester bond. Here, the imidazole ring of the histidine residue enhances the rate of acidity and reactivity of the oxygen atom, while the carboxylic group of aspartate stabilizes the imidazole ring. Here, the hydroxyl group of the serine hydrolase side chain plays an important role in depolymerization (Jendrossek and Pfeiffer 2014)

Table 1 Biological applications of PHA deplolymerases

Origin	Bio-products	Applications	References
Pseudomonas sp.	R-hydroxyalkanoic acids	Helps in establishing PHA producers in soil and rhizosphere, and improves metabolism	Eugino et al. (2010)
Pseudomonas fluroscens		Drug delivery, protein microarray, protein purification, antibody immobilization in clinical diagnostics	Ihssen et al. (2009)
Pseudomonas putida CA-3	Monomers (3-hydroxydecanoic acids; R10)	Anti-proliferative activity	O'Connor et al. (2013)
P. fluoroscens GK13; *P. putida* KT2442	3-Hydroxyalkanoic acids	Potential to inhibit *Staphylococcus aureus* growth (Antibacterial property)	Martinez et al. (2014)
	PHACOS	Bactericidal to Gram-positive and Gram-negative bacteria	
Escherichia coli		Immobilized cell factories for biocatalysis and bio-transformation, Chaperone protein levels.	Wang et al. (2009)

(Table 2). PhaZ7 of *P. lemoignei* has considerable similarity to *B. subtilis* lipase—LipA. It has an additional domain. This lid-like domain reveals the presence of many hydrophobic amino acid residues, which includes Tyr105 (Tseng et al. 2006; Shah et al. 2007; Kim et al. 2007; Hermawan and Jendrossek 2010; Eugino et al. 2007, 2010; O'Conner et al. 2013) (Table 2). $PhaZ1_{Rru}$ has a similar sequence with the extracellular *PhaZ* of *Acidovorax* sp. having type II catalytic domain (lipase box) at N-terminus. In *R. rubrum*, it seems that it has an additional nonsoluble PHB depolymerase, comparatively less active than $PhaZ1_{Rru}$ (Kobayashi et al. 2003; Abe et al. 2005) (Table 2).

5 Fungal PHA Depolymerases

The process to degrade PHA is not limited to bacteria; many fungi and yeast play a major role in degrading P-3(PHB) and its copolymer P(3HB-co-3 HV) (Kim and Rhee 2003). Fungi are known to be potential candidates for PHA degradation process due to their higher rate of surface growth and high depolymerase activity (Table 2). Fungal *e-PhaZ* is specific for denatured PHA (d-PHA) and is found to be secreted by *Ascomycetes*, *Basidiomycetes*, *Deuteromycetes*, *Mastigomycetes*, *Myxomycetes*, *Zygomycetes*, etc. (Kim et al. 2000; Sang et al. 2002). *Penicillium* spp., *Aspergillus* spp., and *Variovorax* spp. are known to degrade PHB and its copolymer P(3HB-co-3 HV) by extracellular PHB depolymerase (Kim et al. 2000; Han and Kim 2002; Jendrossek and Handrick 2002; Nadhman et al. 2012; Seo et al. 2012) (Table 2). PHB depolymerase of *P. funiculosum* is a trimer having a M.Wt. of

Table 2 Biochemical characteristics of PHA depolymerases

Wild type strains	Depolymerase gene and source	MWt (KDa); aa	Active site	Type	pH; °C	References
Bacteria						
Bacillus thuringiensis ATCC35646	*phaZ; Escherichia coli* JM109	33; 300	$GWS_{102}MG$	IC-MCL	8;–	Tseng et al. (2006)
Bacillus sp. AF3		37			7;37	Shah et al. (2007)
Ralstonia eutropha H16	*phaZd1; phaZd2* *E. coli* JM109 S17-1	39.2;362	$GMS_{190}AG$	IC	8.5;–	(Jendrossek and Pfeiffer 2014)
		38.4;365	$GMS_{193}AG$	IC	8.5;–	
R. eutropha	*PhaZ5;PhaZ7* *Pseudomonas lemoignei*			IC, EC	8.5;–	(Jendrossek and Pfeiffer 2014)
R. eutropha H16	*PhaZa1 E. coli*			IC-MCL	7;–	Uchino et al. (2008)
R. eutropha H16	$PhaZ_{Reu}$ *E.coli*	78; 1400		IC-MCL	8;–	Sugiyama et al. (2004)
Ralstonia picketti T1				EC-SCL	8;–	
Wautersia eutropha H16	$PhaZd_{weu}$ HH16DZD1	39	$S^{190}D^{266}H^{330}$	IC-MCL	8.5–9;–20–30	Abe et al. (2005)
	E. coli (PE3ReZd1)					
Pseudomonas putida KT2442	*E. coli* DH5å M15; S17-1; PpAZ1; PpAZ2; Ppaz3	31	$S^{102}H^{248}A^{221}$	IC-MCL	8.8;–	De Eugenio et al. (2007)
Acidovorax sp.SA1				EC-SCL		Kobayashi et al. (2003)
Paucimonas lemoignei	*phaZ5 B. subtilis* WB800 Plasmid Pwb980	42.2		EC-SCL	8;–	Braaz et al. (2003)
Streptomyces ascomycinius	$PhaZ_{sa}$*(fkbU)* *E. coli; Rhodococcus sp.* T104	48.4	Ser $_{131}$-Asp_{209}-His_{269}	EC	6;45	Hidalgo et al. (2013)

Rhodobacter spheroides (ATCC17023)	*PhaZ$_{Rsh}$* (*ZP_00006106*) *E. coli* BLR(DE3)-PlYsS	46	$C_{178}A_{351}H_{384}$	IC-MCL	8;–	Kobayashi et al. (2004)
Bacterial strain HSJCM10698	*Type 2 dPHB* *E. coli* JM109 PUC19	46	D-x-D	EC	8;–	Takeda et al. (2000)
Thermus thermophilus HB8		42	S-D-(E)-H	EC	8;–	Uchino et al. (2007)
Rhodospirillum rubrum S1	ApdA	17.5		IC-MCL	1–12;–	Handrick et al. (2004)
	PhaZ1 R. rubrum SmRif	37	$S_{42}Asp_{138}His_{178}$	IC- SCL	8;–	Abe et al. (2005)
Fungi						
Penicillium simplicissimum LAR14	*dPHB*	36		EC	5;–	(Han and Kim 2002)
Bdellovibrio bacteriovorus HD100	*PhaZ$_{BD}$*	30	S-H-D	EC-MCL	10;4–45	Martinez et al. (2012)
Paucimonas lemoignei	*PhaZ7*			EC-SCL	9;–	Hermawan and Jendrossek (2010)
Variovorax sp. DSH1		26.5		EC-MCL		Seo et al. (2012)
Aspergillus sp.NA-25		57		EC	7;45	Nadhman et al. (2012)

mcl Medium chain length, *scl* Short chain length, *IC* Intra cellular, *EC* Extra cellular

33 kDa. It contains a catalytic triad which has residues Ser39, Asp121, and His155, respectively.

5.1 Biological Significance of PHA Depolymerases

The by-products generated by the action of PHA depolymerase have found important biological significances as biofuels, fuel additives, and probiotics and in the pharmaceutical industry. (Magdouli et al. 2015).

5.2 Biofuels

The major contributions of these HAs are toward biofuels, where they can be used directly to enhance the fuel efficiency. Besides, the esters of HAs (HAMEs) and (3HBME) with a combustion heat of 20–30 kJ/g were reported to be as good as biodiesel in terms of their combustion energy (Gao et al. 2011; Magdouli et al. 2015). Combustion heats were reduced when these biofuels were added to n-butanol and n-propanol (Chen 2011).

6 Medical Applications

6.1 Biocontrol Agents

The usage of antibiotics in animal husbandry has been increasing at an alarming rate. However, it is difficult to envisage the complete elimination of antibiotic from the system. Bacterial infections could be controlled by using short-chain fatty acids (SCFAs), as bacteriostatic agents. These bioactive molecules act by reducing the expression of virulence genes. Thus SCFAs can be exploited as biocontrol agents in animal husbandry. PHA degraded within the gastrointestinal tract has the potential to act as biocontrol agents. scl-PHAs and mcl-PHAs have been proposed to be the bioactive molecules for controlling bacterial infections (Defoirdt et al. 2009).

6.2 Antibacterials

The degradative product of PHAs can be used as building blocks of various chiral compounds and polyesters. 3-(HAs) can be used for the production of macrolides like elaiophylidene, pyrenophorin, grahamimycin A1, and colletodiol. The

bioconversion of 3HA to dioxanone amulets is used in the synthesis of different hydroxycarboxylic acids such as 2-alkylated 3HB and β-lactones. During adiposis treatment, 3HB is employed as an orally administered drug (Chen and Wu 2005). Pure monomeric units, 3-hydroxybutyric acid, can be used as a precursor of carbapenem and related antibiotics (Shivakumar et al. 2011). The HAs are employed for preparing biodegradable, implantable rods, which can be used to deliver antibiotics (sulperazone and duocid) in chronic osteomyelitis therapy (Chen and Wu 2005). *Pseudomonas fluorescens* GK13 harbors gene for PhaZGK13 depolymerase to depolymerize PHAs to monomers (HAs). The hydrolyzed products of polymer have greater potential to reduce bacterial infection, specifically those caused by *Staphylococcus aureus* (Martinez et al. 2014).

6.3 Drug Carriers

Dendrimers, a novel class of polymers, have been synthesized using 3HB monomers. These dendrimers possess biodegradability, monodispersity, and surface functional moieties, which enable them as promising drug carriers (Chen and Wu 2005). Monomers such as 3HB and 4HB prove effective in preparing novel β- and γ-peptides, which have improved resistance against peptidases and thus live longer in mammalian serum, making it suitable for cargo-drug delivery. 3HB suppresses glycolysis during hemorrhagic shock. Monomers are also used in the synthesis of fragrance (S-citronellol) and sex hormones. Also they possess antibacterial, antiproliferative, and hemolytic properties (Philip et al. 2007). The PHAs may be used to encapsulate cells.

6.4 Medical Devices

The HAs can be helpful in making various medical devices due to their biocompatible nature as they possess diverse specifications, mainly degradation rate and mechanical properties. The devices are sutured fastener (reattach tissue to the bone); meniscus repair device (repair of meniscus lesions); rivets and tacks (reattachment of soft tissues); staples and screws (fixation of soft tissues); surgical mesh, repair patch, and adhesion barriers (general surgery); cardiovascular patch (vascular patch grafting); orthopedic pins (bone and soft tissue fixations); stents; articular cartilage repair; etc. (Williams and Martin 2005; Valappil et al. 2006).

6.5 Anti-Osteoporosis Effect

A high concentration of 3HB induces ketoacidosis in humans (Tokiwa and Calabia 2007). 3HB oligomers are found to be a good energy substrate for injured patients as they undergo rapid diffusion in peripheral tissues. Also, it plays a major role in preventing brain damage by enhancing cardiac efficiency. It can regenerate mitochondrial energy in the heart. It has been shown that 3HB has potential to cure Parkinson's and Alzheimer's diseases by reducing the death rate of the human neuronal cells (Kashiwaya et al. 2000). (R)-3HB has a positive impact on osteoblasts growth and anti-osteoporosis activity. It enhances calcium deposition and serum alkaline phosphatase activity. It reduces the serum osteocalcin level and also prevents ovariectomization (bone mineral density reduction) (Zhao et al. 2007; Ren et al. 2010; Chen 2011).

6.6 Memory Enhancer

A unique property of the HA monomers and their oligomers is their ability to stimulate the Ca^{2+} channels and act as memory enhancers (Cheng et al. 2006; Xiao et al. 2007; Zou et al. 2009; Chen 2011; Magdouli et al. 2015).

6.7 Energy Factories

Depolymerase degrades PHA to produce oligomers and monomers. They are further utilized as a part of carbon assimilation machinery. So this hydrolytic enzyme provides the degradative by-product as C source to the predator bacterium in case of the intracellular degradation system. Very likely this enzyme serves as an energy reservoir factory for cells (Martinez et al. 2012).

6.8 Bio-Indicator

HAs are used for sensing the pollution and can be utilized as bio-indicators (Foster et al. 2001).

7 Conclusion

In summary, PHA depolymerase-producing organisms contribute toward ecosystem maintenance (through carbon cycle) and waste utilization and also provide building blocks for a plethora of industrially important substances. Thus, gaining the knowledge about the mechanism of PHA depolymerase from diverse microorganisms may enable us to exploit it for efficient bioconversion of PHAs.

8 Opinion

PHA depolymerase degrades PHA and its copolymers producing oligomers and monomers as their bio-product. (R)-3HB is a known chiral active compound. But the major concern with this polymer is their stinky smell during their production process. Gram-negative bacteria produce PHB along with lipopolysaccharides from their cell wall as a secretory material. Due to endotoxin, PHAs are not favorable for various applications such as medical and other industrial applications. So there is time to look forward for production of (R)-3HB through PHA depolymerization process, since chemical hydrolysis causes breakage of chemical bonds and lowers its quality. Employing depolymerase enzymes to degrade PHA industrial wastes will allow its recycling, leading to the production of value-added by-products—oligomers and monomers. These monomers have the potential to be helpful in PHA polymerization process.

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