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REVIEW ARTICLE

Challenges and Opportunities for Customizing Polyhydroxyalkanoates

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Abstract Polyhydroxyalkanoates (PHAs) as an alternative to synthetic plastics have been gaining increasing attention. Being natural in their origin, PHAs are completely biodegradable and eco-friendly. However, consistent efforts to exploit this biopolymer over the last few decades have not been able to pull PHAs out of their nascent stage, inspite of being the favorite of the commercial world. The major limitations are: (1) the high production cost, which is due to the high cost of the feed and (2) poor thermal and mechanical properties of polyhydroxybutyrate (PHB), the most commonly produced PHAs. PHAs have the physicochemical properties which are quite comparable to petroleum based plastics, but PHB being homopolymers are quite brittle, less elastic and have thermal properties which are not suitable for processing them into sturdy products. These properties, including melting point (T_m), glass transition temperature (T_g), elastic modulus, tensile strength, elongation etc. can be improved by varying the monomeric composition and molecular weight. These enhanced characteristics can be achieved by modifications in the types of substrates, feeding strategies, culture conditions and/or genetic manipulations.

Keywords Bioplastic · Co-polymers · PHA synthases · Strategies · Thermo-mechanical properties

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Abbreviations

PHB	Polyhydroxybutyrate
PHA	Polyhydroxyalkanoate
HA	Hydroxyacids
3HB	3-Hydroxybutyrate
3HV	3-Hydroxyvalerate
4HB	4-Hydroxybutyrate
3H5PV	3-Hydroxy-5-phenylvaleric acid
3HHp	3-Hydroxyheptanoate
3HP	3-Hydroxypentanoate
3H2MB	3-Hydroxy-2-methylbutyrate
3H2MV	3-Hydroxy-2-methylvalerate
3HHx	3-Hydroxyhexanoate
3HHx=	3-Hydroxyhex-5-enoate
6HHx	6-Hydroxyhexanoate
3HHpe	3-Hydroxyheptenoate
3HO	3-Hydroxyoctanoate
3HN	3-Hydroxynonanoate
3HNe	3-Hydroxynonenoate
3HD	3-Hydroxydecanoate
3HDD	3-Hydroxydodecanoate
3HDDE	3-Hydroxydodecenoate
3HHD	3-Hydroxyhexadecanoate
3HHDE	3-Hydroxyhexadecenoate
3HTD	3-Hydroxytetradecanoate
3HTDE	3-Hydroxytetradecenoate
3HOD	3-Hydroxyoctadecanoate
3HUD	3-Hydroxyundecanoate
3HUDE	3-Hydroxyundecenoate
mcl	Medium chain length
M_n	Number average molecular weight
M_w	Weight average molecular weight
PDI	Polydispersity index
scl	Short chain length
T_g	Glass transition temperature

T_m Melting temperature

Introduction

Physical and thermoplastic properties of polyhydroxyalkanoates (PHAs) and their biodegradable nature make them the best alternative to synthetic plastics. PHAs with varying composition can be produced by microbes by fermenting an array of substrates [1, 2]. High cost of production and difficulties in processing the homopolymers of PHAs makes them less favourable for large scale usage [2–5]. Efforts to improve the monomer composition and their molecular weight (M_w) have resulted in polymers, which are more suitable for downstream processing. These aspects in PHAs are affected by the type of bacteria, substrate, feeding strategies and physiological conditions. All these components can be manipulated to attain polymer with desirable properties [6–8]. PHAs are divided into different classes based on the carbon (C) chain length: (1) short (scl-PHAs; 3–5Cs), (2) medium (mcl-PHAs; 6–14Cs), and (3) long (lcl-PHAs; 17–18Cs). These include some of the commonly produced PHAs e.g. PHB, P(3HB:3HV), P(3HB:3HHx), P(3HB:4HB:3HHx), P(3HB:3HV:3HHx), P(3HB:3HHx:3HO:3HD), etc. Although more than 150 monomers of hydroxyacids (HAs) have been reported to be produced, however, their incorporation leads to a limited variety of PHAs. The distinction among PHAs is made largely on the basis of the type of PHA synthases present in the bacteria and their corresponding specificities for CoA thioesters of HAs chain length [9–11]. This comprises the first critical step towards the process of tailoring the PHA. In addition, the other determinants are the interconnected pathways, which supply the raw material—CoA thioesters of HAs, for PHA synthases e.g. glucose and fatty acid (FA) metabolic pathways etc. [10–13]. This implies that the types of substrate and strategies to exploit these metabolic capabilities are important for manipulating the PHA composition as well as their M_w. Monomer composition and M_w affects the thermal properties, e.g. melting point (T_m), glass transition temperature (T_g) and mechanical properties e.g. crystallinity, elastic module, elongation and tensile strength. These properties are important for applications of these polymers in different fields. It has invariably been demonstrated that lower 3HB or higher non-3HB content in the polymer, enhances the quality of the polymer. The major challenges to make PHAs economically viable, is to make them easier to process and handle. Manipulating the genetic makeup of bacteria appears to be the most obvious choice for achieving these targets [4, 5, 14]. Interestingly, this can be also achieved by adopting strategies such as selection of bacteria, suitable substrates, appropriate feeding regime, and variations in physiological conditions [15]. The present article provides an insight into these approaches that can potentially provide us with the polymer of our choice.

Factors Affecting PHA Characteristics

Monomeric Composition and Molecular Weight

Among over 150 monomers, that have been reported so far, only a small number of homopolymers and copolymers of PHAs are known to be produced under normal physiological conditions (Table 1). In most cases, scl-PHA are produced, which include PHB, P(3HB:3HV), P(3HB:4HB), P(3HB:3HP) and P(3HB:3HV:4HB) [3, 4, 16, 17]. PHAs having mcl-HAs include P(3HB:3HB:3HHx), P(3HHx:3HO:3HD), and P(3HHx:3HO:3HD:3HDD). PHA polymers, which are less commonly observed, include other non-PHB homopolymers of 4HB, 3HV, 3H5PV, 3HHx, 3HHp, 3HO and 3HN. The M_w of PHA covers a wide range from 0.5×10^5 to 35×10^5 Da and their polydispersity index (PDI) varies between 1.1 and 6.0 [4]. PDI is the index for the distribution of molecules within a polymer sample, which is measured as ratio of the weight (M_w) versus number (M_n) average molecular weight. Thus, it determines the heterogeneity of a polymer mixture in relation to size of the molecules. The wider range of PDI makes it difficult to ascertain the homogeneity of the copolymer, especially those which have vastly different M_w and M_n values. This is due to the uncertain pattern of its thermal and mechanical attributes. The thermoplastic, crystallization, elastic behavior, mechanical resistance properties of PHAs depend largely upon its composition and M_w [18]. Thus, the homopolymer—PHB, which has low M_w of $5–10 \times 10^5$ Da, is highly crystalline and brittle in nature [19]. However, certain factors which govern these aspects and can be used to customize them towards more desirable ones.

Bacterial PHA Synthases

PHA diversity both in terms of its monomer composition and M_w is due to variations in the substrate specificity of the PHA synthases. The PHA synthases vary in their abilities to recognize specific HA monomers and their activities are regulated by the supply of HAs flux to PHA pathways. These together significantly affect the overall outcome of the PHA metabolism. This in turn is governed by the metabolic and physiological capabilities of bacteria. Moreover, the ratio of PHA synthase actively polymerizing

Table 1 Diversity of biochemical characteristics of bacterial polyhydroxyalkanoates

Substrate and conditions	PHA composition (mol%)					M_w:Da $\times 10^5$	PDI	Refs.
	3HB	3HV	4HB	3HHx	Others			
Azotobacter (γ-proteobacteria)								
Glucose/starch, shake flask (SF)	100	–	–	–	–	12–16	–	[69]
Molasses + food sugar/vinasses	100	–	–	–	–	5.9–8.9	–	
Fatty acids	100–78	0–22	–	–	–	8.2–16.5	–	[70]
Aeromonas (γ-proteobacteria)								
Lauric acid + VA, SF(VA@ 24 h)	66	21	–	13	–	12.7	2.3	[65]
Pseudomonas (γ-proteobacteria)								
Glucose, SF	–	–	–	3	3HO:13, 3HD:42, 3HTD, 3HDD:5, 3HDDE:37	3.4	3.7	[83]
Dodecanoic acid, SF	–			15	3(HO:HD:HDD::40:30:15	1.0	1.2	[27]
Tetradecanoic acid, SF	–	–	–	7.71	3HO:43.4, 3HD:26.1, 3HDD:16.5,3 HTD:6.2	–	–	
Nonanoic/undecanoic acid, SF	87	13	–	–	–	22–28	1.6–1.9	[83]
Oleic acid, SF	–	–	–	1	3(HO:HD:HDD:HTDE ::34:30:12::23	1.2	2.9	
Palm oil + palm oil cake, a. P/N limitation b. No nutrient limitation	a. 83–87 b. 25	a. 5–8 b. 71	–	–	3HHD:3HOD a. 4:4–5, b. 2:3	–	–	[29]
Cupriavidus (β-proteobacteria)								
Fructose, fed batch (FB)	100	–	–	–	–	6.5	1.8	[74]
1,4-Butanediol (1,4-B), SF	88	–	12	–	–			
Fructose + 1,4-B, FB, C/N:4–4200	36–95	–	5–64	–	–	1.0–2.6	1.1–2.5	
Oleic acid + 1-pentanol/VA +1,4-B/γ-butyrolactone, SF C/N:10–30	58–90	3–22	4–28	–	–	–	–	[64]
VA + 4HB-Na, SF (72 h)	10	40	50	–	–	11.0	1.1	[52]
Fructose + VA + 4HB, SF (72 h)	10–11	6–23	66–84	–	–	1.8–6.6	2.8–3.5	
I-pentanol + 1,4-B + oleic acid	64–73	8–32	4–19	–	–	3.4–8.2	2.6–3.4	[81]
I-pentanol + 1,4-B + palmitic acid	49–63	4–18	33	–	–	14.6–17	1.7–2.0	
Comamonas (β-proteobacteria)								
Mixed organic acids (POME), FB	79–92	8–21	–	–	–	3.4–10.8	1.1–2.6	[80]
Methylobacterium (α-proteobacteria)								
5-Hexenoic acid	65–100	–	–	0–27.4	3HHx:0–20.5	147–353	1.2–2.0	[39]
5-Hexenoic acid + methanol	28–94	–	–	3–24	3HP:0–3, 3HHx:2.6–44	207	1.4	
Bacillus (Firmicutes)								
Fatty acids, SF	100–59	0–48	–	–	–	8.4	3.9	[30, 39]
Glucose/hexa-/octa-/decanoate	97–98	–	–	1–2.9	–	3.1–5.2	1.9–2.9	[30]
4-Hydroxybutanoate, SF	96	–	1.8	1.7		5.1	1.9	
ε-Caprolactone	97	–		2.0	6HHx:0.7	3.7	2.8	

Table 1 continued

Substrate and conditions	PHA composition (mol%)					M_w:Da $\times 10^5$	PDI	Refs.
	3HB	3HV	4HB	3HHx	Others			
Sugars/pea-shells hydrolysate, SF	100	–	–	–	–	–	–	[43, 48]
Pea-shells hydrolysate + PR, SF	87	13	–	–	–	–	–	[17]
Haloferax (Archaea)								
Hydrolyzed whey permeate	94	6		–	–	0.01	1.5	[38]
Hydrolyzed whey permeate + VA + γ-butyrolactone at 34.25 h	73	21.8	5.1	–	–	0.01	1.5	
Enriched mixed culture								
AC	98.4	1.6	–	–	–	4.9	1.9	[61]
AC, pulse feeding (PF)	90–96	3–4	–	1	3H2 MB:0–4, 3H2MV:1	8.1	2.0	[7]
PR, PF	12	63	–	6	3H2 MB:6, 3H2MV:14	4.5	2.0	
BU, PF	83	7	–	2	3H2 MB:5, 3H2MV:2	9.0	1.7	
VA, PF	12	78	–	1	3H2 MB:5, 3H2MV:4	6.2	3.9	
IVA: isovaleric acid	91	7	–	–	3H2MV:2	6.1	2.7	[61]
Fermented molasses (FM), VFA, PF	85	15	–	–	–	6.5	2.3	[8]
Simulated FM, VFA, PF	80	20	–	–	–	3.9	2.7	
Alkaline fermentation liquid	74	24	–	–	3H2MV:2	8.5	2.7	[61]
Molasses, (LA + VFA), PF	56–70	13–43	–	1–23	3H2 MB:0–2, 3H2MV:0–1	3.5–4.3	1.8–2.1	[7]

AC acetic acid, *PR* propionic acid, *BU* butyric acid, *VA* valeric acid, *IVA* iso-valeric acid, *LA* lactic acid, *VFA* mixture of AC + PR + BU + VA, *4HB-Na* sodium salt of *4-hydroxybutyrate*, *POME Palm oil mill effluent*

the HAs to that of the enzymes supplying these CoA thioesters of HA monomers has a negative effect on PHA M_w [20]. The PHA synthases may be grouped under four categories. Class I and class II PHA synthases consist of single subunit i.e. PhaC, which has a preference for utilizing CoA thioesters of scl-HAs and mcl-HAs, respectively. Class III PHA synthases, composed of two subunits—PhaC and PhaE, prefer the CoA thioesters of scl-3HA with 3–5 C-chain length. Out of the two different kinds of subunits, the PhaC is similar to PhaC of class I and II while the second subunit PhaE has no similarity to other PHA synthases. Class IV PHA synthases are similar to the class III PHA synthases, however, instead of PhaE it has another subunit—PhaR. This has been widely shown to produce scl-PHAs [9–11]. The PHA synthases of different organisms fall under different classes suggesting that diversity of PHAs may be limited in wild type bacteria under normal culture and physiological conditions. This is limited to scl-PHA for class I and III PHA synthase bearing bacteria, e.g. *Ralstonia eutropha* and *Allochromatium vinosum*. *R. eutropha* which is the most widely used for PHA production is reported to produce PHB, P(3HB:3HV), P(3HB:4HB) and P(3HB:3HV:4HB) from a wide range of substrates like sugars, glycerol, FAs, dairy products, agricultural-industrial waste, etc. [21–23].

Pseudomonas with class II PHA synthase has the highest ability to produce mcl-PHA with 6–12C chain length monomers efficiently utilizing glucose, FAs, agricultural and oily wastes. PHAs produced by *Pseudomonas* are reported to have: 3HHx, 3HHp, 3HHpe, 3HO, 3HN, 3HNe, 3HD, 3HDD, 3HDDE, 3HHD, 3HHDE, 3HTD, 3HTDE, 3HOD, 3HUD, 3HUDE [24–28]. *Pseudomonas aeruginosa*, *P. oleovorans*, *P. resinovorans* and other *Pseudomonas* sp. have been reported to simultaneously produce 4–12C chain length 3HA units i.e. scl-mcl PHAs e.g. P(3HB:3HV:3HHD-3HOD) [29].

Bacillus, which has the class IV PHA synthase, has the capacity to produce P($3HA_{scl}$:$3HA_{mcl}$) which distinctly categorizes it for exploitation for various applications [9, 10, 30]. The versatility of this organism lies within its capacity to metabolize structurally unrelated C-sources like glycerol, glucose etc. [31]. *Bacillus* can produce scl-PHA but is also known to accumulate mcl-PHA i.e. 3HHx. PHAs varying from PHB, P(3HB:3HV), P(3HB:3HHx), P(3HB:4HB:3HHx) to P(3HB:6HHx:3HHx) depending upon the substrate [3, 4, 30, 32, 33]. *Bacillus* provides a robust option as potential PHA producer under more stringent conditions [3, 4]. In addition, certain unique features of *Bacillus* spp. makes it more competent over others, such as high PHA accumulation, lack of PHA depolymerase, lack of lipopolysaccharide endotoxin [3]. *Bacillus* spp. are capable of producing copolymers of PHA from glycerol and glucose, even under non-limiting N-conditions [4, 34, 35]. All these properties make *Bacillus* the most suitable contender as an industrial work-horse for PHA production.

Haloferax mediterranei an haloarchaea, having *phaEC* genes for encoding PHA synthase, although not widely studied, has been reported to produce P(3HB:3HV) utilizing starch, glucose, or other cheaper C sources such as industrial by-products [36–38]. Since *H. mediterranei* survives in extreme salinity, it circumvents the contamination problem and consequently greatly reduces the sterility requirement. Apart from these, other microorganisms, e.g. *Comamonas*, *Klebsiella*, *Methylobacterium*, *Microlunatus*, *Rhizobium*, *Rhodococcus*, *Sphingomonas* etc. have been shown to produce PHA [3, 39]. However, in these cases, most of the scl-PHAs have 3HB, 3HV and 4HB monomers and the yields are comparatively low. Compared to pure cultures, the use of mixed culture enriched under the Aerobic Dynamic Feeding or Feast and Famine conditions is more convenient. The advantage of using mixed cultures is the production of co-polymers with a broad range of PHA composition depending on the substrate used due to their metabolic capacities to utilize a wider range of substrates [8, 40]. Moreover, mixed cultures have better survival under stringent environmental conditions and variations occurring specially in biowaste feed, thus, providing an economical option by eliminating the need to sterilize the feed [17, 41–45].

Heterologous Expression of phaC

The differential expression of *phaC* gene in various host organisms has revealed the unique ability of the synthase [4, 19]. Subsequently, it has been emphasized that the PHA synthase governs the polymer quality both in terms of composition as well as size. The composition and M_w/M_n of PHA copolymer can be influenced e.g. PHA with mcl-HAs can be obtained with *R. eutropha* or *Bacillus* having *phaC* gene from *Pseudomonas* while with expression of *phaC* gene from *Bacillus* yielded scl-PHAs [4, 31]. *Escherichia coli* was used as a host for expressing *phaC* from various organisms including *Bacillus* spp. [4, 19]. It was realized that the metabolic background of the host also contributes towards the wide substrate selectivity of PHA synthase. This was evident when significant PHA production and changes in the M_n were observed by $phaRC_{YB4}$ (*B. cereus* YB4) in comparison to $phaRC_{Bm}$ (*Bacillus megaterium*) expressed in genetically modified *E. coli* [19]. With *E. coli* JM109 expressing $phaRC_{Bm}$, a PHA with 89×10^6 (M_n) was obtained in comparison to $phaRC_{YB4}$, yielding a PHA of only 20×10^4 (M_n). Thus, using different combinations of *phaR* and *phaC* subunit of *B. cereus* and *B. megaterium*, it was thus revealed that PHA synthase of *B. cereus* has the capacity to produce PHA at least three times

higher to that of *pha*RC_{Bm} [19]. Ability of *Bacillus* to endure large genome scale reduction and accept genes from distantly related organisms makes it a potential candidate in addition to *E. coli* [3, 13].

Rearrangements Within pha Operon

The genomic analyses done on the *pha* operon for naturally existing gene orders have revealed two contrasting forms i.e. *phaCAB* and *phaBCA* existing among PHA producers (*R. eutropha* and *Bacillus* spp.), respectively. In case of the mcl-PHA producer (*Pseudomonas* spp.) and another Gram negative bacterium (*Burkholderia* spp.), more than one type of gene arrangements have been reported. PHA operon has been well studied within *E. coli* to evaluate the spatial arrangement of the three genes on PHA quality and production [46]. The highest PHA yield of 6.38 g/l was obtained when *E. coli* strain was expressing operon *phaCBA*, however, M_w was quite low. There was enhancement in the M_w with the gene combinations of *phaACB* (38×10^5), *phaBAC* (41×10^5), *phaBCA* (50×10^5), and *phaABC* (62×10^5) [46]. Thus, it is anticipated that *Pseudomonas putida* (*phaABC*) has the inbuilt capacity to synthesize PHA of high M_w. Incidentally, *Bacillus thuringiensis* and other *B. cereus* group members posses *phaRBCA* naturally, that is reported to yield high M_w PHA within the metabolic milieu of *E. coli* [46]. Thus, such information may prove helpful in understanding and exploiting intrinsic abilities of these potential organisms to produce high M_w PHA.

Substrate and Feeding Regime

The composition of polymers is greatly affected by the type of substrate used and physiological conditions (Table 1). Substrates used for PHA production cover a wide range including sugars (glucose, fructose, sucrose, maltose, and lactose), starch, glycerol, FAs and its derivatives, methanol etc. [35, 45, 47]. Among biowastes—agricultural, industrial and dairy byproducts have been used as a cheap source of raw material [3, 4, 35, 45, 47–49]. While a combination of different substrates, especially the addition of precursor substrates may lead to different combinations of monomers, feeding regime also affects the PHA monomeric compositions [50, 51]. Most of the carbohydrate rich substrates, i.e. glycerol, agricultural wastes, dairy waste, etc. promotes the incorporation of 3HB, 3HV and 4HB with most of the PHA producers except *Pseudomonas*. Sugars, especially glucose, which is the simplest C-source, are the preferable substrates for PHB production [48]. Among pure substrates FAs are potential sources to obtain mcl-HA in polymer. Monomer composition of PHA obtained with *Pseudomonas* varies linearly with type of FAs used. Synthetic acids and their mixture are successfully employed for this purpose. In a recent report, glycerol was observed to produce PHB with higher M_w and improved mechanical strength than that observed on other sugars [4, 45]. Addition of precursor substrates further improves the copolymer composition towards non-3HB monomers. 3HV content increases with odd number C sources such as propionate and valerate [4, 17]. γ-Hydroxybutyric acid, 1,4-butanediol, γ-butyrolactone have been reported as potential precursor substrates for 4HB unit. In addition, gluconate is also reported to enhance 4HB and 3HV incorporation into the PHA produced by *Bacillus cereus* SPV in comparison to only 4HB when these were grown on fructose and sucrose [51]. A mixture of 1-pentanol (3HV precursor) and γ-butyrolactone or 1,4-butanediol (direct precursors of 4HB monomers) significantly produced terpolyester P(3HB:3HV:4HB) of different compositions [51, 52].

Wastes being diverse in their composition provide a wider range of C-sources for achieving diversity in PHA composition. P(3HB:3HV) production has been reported from biowaste as feed supplemented with precursors substrates, e.g. *Ralstonia* (on molasses, whey and other agricultural wastes), *Bacillus* (on pea-shells) [3, 4, 17, 49]. *H. mediterranei* on hydrolyzed whey as substrate produced P(3HB:6 % 3HV), which improved to P(3HB:21.8 % 3HV:5.1 % 4HB) while the yield increased to 87.5 % when sodium valerate and γ-butyrolactone was used to supplement the feed [38]. *Pseudomonas* can produce comparatively wider range of PHA on different wastes such as P(3HB:3HV) on whey [53], P(3HO:3HD:3HDD) on coprah oil [54], P(3HHx:3HO:3HD:3HDD:3HTD) on delignified rye grass hydrolysate [55] and P(3HHx:3HO:3HD:3HDD:3HDDE:3HTD:3HTDE) on soy molasses [26]. As also observed with FAs, oily waste can provide a wider range of monomers in PHA [56, 57]. By co-feeding soybean oil and γ-butyrolactone as C sources, P(3HB:4HB) can be synthesized by *R. eutropha* KCTC2662 having 4HB fractions of 6–10 mol% [22]. Tercopolymers P(3HB:3HB:3HHx) was reported from palm kernel oil mixed with 3HV precursors [58, 59].

A fermented acidogenic effluent is a potential alternative and low cost substrate for PHA with comparable yield and properties as obtained with synthetic acids. Volatile fatty acids (VFAs) present in the effluent are utilized as substrates by PHA producing bacteria and the composition of PHA depends upon the VFA profile. Acetate and butyrate promote higher content of 3HB in polymer. Interestingly, the non-PHB yield may be higher in content with acidogenic effluents in comparison to synthetic substrates. P(3HB:3HV) may be formed with HV content in the range 15–31 %, varying linearly with the amount of propionate and valerate [8]. The enriched cultures of glycogen

accumulating organisms were reported to utilize VFA from fermented sugarcane molasses to produce copolymers having 3HB (56–70 mol%), 3HV (13–43 mol%), 3HHx (1–23 mol%), 3H2 MB (0–2 mol%) and 3H2MV (0–2 mol%) which varied with VFA composition [7]. In addition to 3HV, presence of propionate in media also promoted 3H2MB and 3H2MV in the polymer as these could be formed through the combination of acetyl- and propionyl-CoA [60, 61]. Lactate is more likely to promote 3HB in a polymer by both pure and mixed cultures [7].

Adopting certain strategies while feeding with the chosen substrate may improve the PHA yield and composition. Different type of fermentation processes and bioreactors have been employed for PHA production, including submerged fermentation and solid state fermentation. Solid state fermentation may provide efficient and economical alternative to conventional submerged fermentation [47]. Continuous feeding ensures a supply of 3HV precursors throughout the process. Feeding substrate (acetic, propionic, and lactic acids) at the rate of 8.5 g COD/l/d periodically after every 2 h led to the synthesis of co-polymer P(3HB:3HV) and polymer (PHB) from activated sludge enriched in sequence bed reactor (SBR) [62]. With fermented molasses as source of VFAs being fed continuously, relative to pulse wise feeding resulted in enhancing the 3HV content in PHA from 31 to 39 %. The lower yield of 3HV in PHA may be due to the depletion of precursors at the end of each pulse [8, 47]. It resulted in the formation of only PHB. This transition of polymer type formed in culture leads to block or blend polymer formation [63]. Concentration and availability of substrate may also influence the composition. Increasing the lauric acid from 0.068 to 0.342 wt% increased 3HB content while it had a negative effect on non-3HB content [64]. Adding the precursor substrate after a certain period of cultivation on growth promoting conditions may avoid an inhibitory effect of 3HV (valeric acid, propionate etc.). Adding these precursors along with other C-sources such as glucose/fructose precursors also serve the same purpose [17, 52, 65].

Although biowaste and fermented effluent have the advantage of promoting higher monomer diversity, it has been observed that the PHA produced from such feed has lower M_w and is heterogenous in nature in comparison to that observed with pure or synthetic substrates. In comparison to fermented effluent, polymer with single synthetic acid has higher M_w up to 3.0×10^6 Da, which may be due to higher rates of polymerization [66]. M_w of the copolymers was observed to be $4.5–9 \times 10^5$ Da with acetate and propionate, 8.5×10^5 Da, for P(3HB:3HV) with molasses and $3.5–4.3 \times 10^5$ Da for copolymer with fermented molasses [7, 67]. The disruption of polymer chain by 3HV units causes the average M_w to decrease with increasing monomer units, e.g. it declined from 6.5×10^5 to 2.2×10^5 Da when 3HV content improved from 15 to 39 % [68]. Moreover, the PHA obtained here with synthetic polymer is more or less homogenous [7]. Interestingly, Bengtsson et al. [7], successfully obtained narrow range PDI with fermented waste also. In accordance to these results, negative effect of organic acids present in fermented waste was observed by others as well. The M_w of 4.1×10^3 and 5.9×10^2 kDa was obtained with *Azotobacter vinelandii* UWD and *A. chroococcum* 7B on 5 and 2 % w/v molasses which further decreased on increasing molasses or acetate concentration in the feed. In contrast, M_w in the range $1.2 \times 10^3–1.6 \times 10^3$ kDa was achieved on pure C-source (glucose, sucrose, starch or FAs) [69, 70].

Culture Conditions

Accumulation of PHA under stress conditions is well documented. Therefore, the culture conditions under which the PHA producing bacteria are cultivated e.g. nutrient level, pH, aeration rate, cultivation time etc. can alter the composition to a certain extent. Uncontrolled pH may lead to decreased PHA yield due to higher pH stress and higher maintenance requirement. P(3HB:3HV) synthesized by mixed bacterial culture had higher 3HV up to 48 mol% at pH 9.5 in comparison to pH 8.5 and P(3HB:3HV:4HB) synthesized by *Delftia acidovorans* had increased 3HV (29–46 mol%) and 4HB (39–50 mol%) at pH 8.5 in comparison to pH 5.0 [71, 72]. The pH can also have an indirect effect on PHA composition as the VFA profile produced under acidogenic fermentation can be manipulated by varying it [67]. In addition to this, nutrient level in media as compared to C affects the quality of PHA. The study of kinetics of P(3HB:4HB) production by *Cupriavidus necator* strain A-04 has shown that production of 4HB monomers was high under nitrogen (N) rich medium whereas, 3HB incorporation increases in N-limiting media [73]. Under N-limiting conditions 4HB precursors flux goes to 3HB-CoA, thus more 3HB is incorporated into polymer P(3HB:4HB) by *C. necator*. Thus, under different C/N level varying range of 4HB (0–94 mol %) was attained [74]. Similar results of increasing 3HV and 4HB content with an increasing C/N ratio was also observed. It attained a maximum value at C/N 10–15 with different combinations of precursor substrates (butyrolactone/1,4-butanediol with pentanol/valeric acid) [64]. *B. cereus* strain SPV could produce PHB under sulphur, phosphorous or N-limiting conditions, but the 3HV unit in PHA was incorporated in potassium limiting media [32]. *Thermus thermophilus* utilized whey as substrate to produce PHA up to the content of 35 % containing 3HV, 3HHp, 3HN and 3HUD on media containing 24 % (v/v) whey under nutrient limitation [75]. Since, N-rich

conditions induce higher growth of cells and incorporation of 4HB, it increases the PDI as compared to that observed for PHA produced under N-limitation. *C. necator* produced PHA with M_w of 5.2×10^2 kDa when cultivated under high C/N ratio, which improved to 8.2×10^2 kDa under low C/N ratio [74]. In contrast, M_w of PHA obtained with *Azohydromonas lata* declined with decreasing C/N and C/P ratio. In this case, the highest values of M_w—2.5×10^3 kDa was recorded at C/N ratio of 20 which declined 20 times at C/N ratio of 6. Similarly, M_w—2.0×10^3 kDa observed at C/P ratio of 8 also declined three times at C/P ratio of 0.8 [76]. In contrast to most studies, where N limitation seems to favour PHA production, recent work has revealed that *Bacillus* spp. can produce PHA and its co-polymer independent of N concentration, especially on glycerol as feed [35].

Another factor that significantly affects the M_w is the agitation rate. Increasing the agitation rate from 50 to 250 rpm resulted in a remarkable increase in 3HB from 5 to 54 mol% in P(3HB:3HV:4HB) while there was a decline in 3HV i.e. from 50 to 30 mol% and 4HB i.e. from 47 to 16 mol% [72]. Along with this, M_w is strongly affected by aeration conditions and improved from 14.8×10^2 to 16.7×10^2 kDa with increase in the aeration rate from 250 to 190 rpm [77]. Cultivation time of the reactor is also observed to alter the monomers. 3HB and 3HV content were predominant in polymer obtained with *C. necator* at initial time, but, 4HB increased with time attaining the maximum level at 60–90 h [52]. Transition in the composition of PHA cultivated under SBR showed higher 3HB and 3HV at initial time, followed by higher concentration of 3HHx in later cultivation hours as the substrate for scl-PHA [7]. Integration of PHA production with H_2 may yield an improved and efficient PHA. During the process, the shift in the reactor from anaerobic static bioreactor to shake conditions, a modified composition was observed [17, 33, 42, 44, 78].

Thermal Properties

Thermal properties of PHA polymer determine the ideal temperature conditions required for their processing and utilization. These affect the polymer morphology and their mechanical properties. T_m for a polymer determines the point of transition from a crystalline phase into a solid amorphous phase. PHA polymers are stable at temperature below 160 °C, thus preferable polymer should have a T_m below 160 °C. A considerable difference in melting temperature and degradation temperature is desirable to enable convenient polymer processing. T_g marks the transition of glassy and brittle state of polymer below T_g to soft and plastic polymer above T_g. Thus the polymers having T_g comparatively low e.g. below room temperature are soft and flexible in texture. The T_g and T_m for PHA were between −53 and 10 °C and undetectable to 179 °C, respectively (Table 2). Variations in T_g and T_m were observed to be affected by monomer composition of the PHA [6, 7].

The melting point is more significantly determined by the 3HB content and also by the presence on mcl-HAs. The homopolymer PHB has melting point in the range 174–179 °C which is higher than the other homopolymers—P(3HV) and P(4HB) i.e. 103 and 53 °C, respectively. As observed (Table 2), T_m for PHA progressively declines with decreasing 3HB content. P(3HB:3HV) with the 3HV content varying on or after 5–70 mol% has the T_m going down from 170 to 87 °C, respectively. A similar trend is observed in case of T_g for these polymers that declined from 2.25 to −13 °C, respectively [65, 74, 79]. T_g for pure PHB is 2.5–10 °C while it goes down to −16 °C in case of P(3HB:3HV) with 90 mol% 3HV content (Table 2). P(3HB:5 % 4HB) has the higher T_m and T_g i.e. 169 and −2 °C in comparison to P(3HB:38 % 4HB) i.e. 152 and −10 °C, respectively [74]. Interestingly, P(3HB:3HHx) with 3HHx equal to 10–12 mol% has quite lower values (T_m: 96–127 °C) in comparison to P(3HB:10–20 % 3HV) (T_m: 137–156 °C) and P(3HB:16 % 4HB) (T_m: 150 °C) having relatively similar content of 3HB. This may be due the presence of mcl-HA 3HHx [7, 8, 30, 65, 74, 80]. Tercopolymer P(3HB:3HV:4HB) with higher 3HB content e.g. monomer ratio 73:8:19 mol%, respectively, has the T_m equal to 131 °C and T_g equal to −10 °C while these values with monomer ratio 4:3:93 mol%, are 55 and −51.6 °C, respectively [52, 81]. Similarly, another tercopolymer—P(3HB:3HV:3HHx) had the T_m 101 °C and T_g −1.9 °C at monomer ratio of 75:13:12 declining to T_m 54 °C and T_g −5.14 °C at monomer ratio of 48:24:28, respectively [65]. It is interesting to note that in tercopolymer having 3HB:3HV:3HHx in the ratio 70:25:5 and 56:43:1, T_m is observed to be 129 and 155 °C, respectively, which is quite high in spite of having lower 3HB content in comparison to the polymer with a corresponding monomer ratio of 75:13:12 (T_m: 101 °C). This is due to the lower level of 3HHx in their makeup. However, T_g is not much affected by this scenario [7, 65]. T_m for P(11 % 3HB:23 % 3HV:66 % 4HB) is 91.8 °C, which is quite low in comparison to T_m for P(3HB:24 % 3HV) i.e. 138.0 °C although both have similar 3HV content [52]. Similarly, effect of mcl-HAs in composition can be marked in different cases such as lower T_m of P(3HB:3HV:3HHx) and P(3HB:3HHx:3HO) in contrast to P(3HB:3HV:3HHx) and P(3HB:3HHx:3HHx), even if these have similar or higher 3HB content (Table 2). Polymer with 17 mol% non-3HB containing 3H2 MB, 3H2MV and 3HHx has melting point equal to 137 °C which is more or less equivalent to T_m observed for

Table 2 Variability in thermal and mechanical properties of the polyhydroxyalkanoates due to monomeric composition

Polyhydroxyalkanoates		T_m (°C)	T_g (°C)	Elastic modulus (GPa)	E:B[a] (%)	Tensile strength (MPa)	References
Composition	Mol%						
Homo-polymers							
3HB	100	171–179	2.5–10	1.1–3.5	0.4–5.0	19–40	[52, 65, 74, 83]
4HB	100	53	−48	149	1000	104	[49]
Co-polymers							
3HB:3HV	95:5	170	2.2	–	–	–	[65]
	80–90:10–20	137–156	−1 to 1.7	0.8–1.2	50–100	20–32	[7, 8, 29, 80, 83]
	70–80:20–30	138–139	−6 to −0.1	1.37	30	70	[74, 80]
	50–60:40–50	113–138	−16 to −10	–	–	–	[74]
	30:70	87	−13	–	–	–	
3HB:4HB	95:5	169	−2	1.23	10.7	1.36	[74]
	84:16	150	–7	–	–	–	
	76:24	161	−5	0.79	22.2	0.87	
	62:38	152	−10	0.66	48.0	2.98	
3HB:3HHx	88–90:10–12	96–127	−1.2	0.5	113–400	9.4–21	[65]
3HB:4HV	90:10	159	–	–	242	24	[47]
Tercopolymer							
3HB:3HV:4HB	73:8:19	131	−10.0	0.10	316	12	[81]
	63:4:33	–	−14.0	0.10	937	9	
	49:18:33	–	−16.0	0.03	554	2	
	12:12:76	87.3	−21.1	0.14	9	4	[52]
	11:23–24:55–56	92–100	−15 to −17	0.4–0.6	3–5	9–10	
	10:40:50	88	−13.7	0.12	300	9	
	4:3:93	55	−51.6	0.13	430	14	
3HB:3HV:3HHx	75:13:12	101	−1.9	0.07–0.1	740–833	12.8–14.3	[7, 65]
	70:25:5	129	−7.2	–	–	–	
	67:20:13	58–68	−6 to −3.6	–	–	–	
	56:43:1	155	−5.5	–	–	–	
	48:24:28	54	−5.1	–	–	–	
3HB:3HHx:3HHx=	94:3:3	153–168	2.0	–	–	–	[39]
	89:6:5	145	−7	–	–	–	
	65:18:17	145–165	−11	0.19	7.4	3.86	
3HB:3HHx:3HO	94:3:3	126	−4	0.39	15	22	[56]
Other co-polymers							
3HB:3HA	98:2	150–167	1	0.95	16	26	[29, 56]
	94–96:4–6	133	−8	0.22	680	17	

Table 2 continued

Polyhydroxyalkanoates		T_m (°C)	T_g (°C)	Elastic modulus (GPa)	E:B[a] (%)	Tensile strength (MPa)	References
Composition	Mol%						
3HB:3HV:3HHx:3H2 MB:3H2MV	90:4:1:4:1	171	4.6	–	–	–	[7]
	84:7:2:5:2	137	2.8	–	–	–	
	74:24:2	101	2.68	–	–	–	[61]
	12:63–78:1–6:5–6:4–14	90–98	−12 to −14	–	–	–	[7]
	11:63:6:6:14	89	−14.0	–	–	–	
3HB:3HV:3H2MV	7:82:11	96	–	–	–	–	[61]
3HB:3HP:3HHx:3HHx=	52:0:27:21	139	−12	–	–	–	[39]
	51:3:17:29	148	−11	0.04	10.3	1.0	
	28:3:25:44	105	−16	–	–	–	
3HB:3HHx:3HO:3HD	89:6:4:1	111	−6	0.07–0.21	188–493	4–10	[56]
3HB:3HV:3HHD:3HOD	83–87:5–8:4:4–5	115–120	−13 to −14	0.2	701–723	18	[29]
3HHx:3HO:3HD:3HDD:3HDDE:3HTD:3HTDE	1–3:13–38:30–42:5–12:0–37:tr:0–23	37–40	−49 to −53	–	–	–	[83]
3HHx:3HO:3HD:3HDD	0–15:0–93:0–92:0–56	53–75	−37 to −44	0.19–0.35	188–346	5.8–8.7	[27]

[a] Elongation to break

P(3HB:3HV) with 15–18 mol% 3HV i.e. (124–166 °C) [7, 82]. Absence of 3HB and presence of mcl-PHA resulted in the lowest T_m as well as T_g of 37–40 and −49 to −53 °C in P(3HHx:3HO:3HD:3HDD:3HDDE) [79]. Albuquerque et al. [8] reported that the T_g (−1 to −16 °C) and T_m (137–147 °C) obtained with mixed cultures are lower than the pure cultures. The second minor value of T_g is an indicative of an heterogenous nature of the polymer as the mixture of two types of polymers, presence of block or blend polymer. Heterogenity in the polymers can also be depicted by T_m. In such cases, T_m may be higher than the expected range of copolymers, which may be due to the presence of homopolymer PHB in block or mixture. Dai et al. observed the polymer containing cumulative 45 and 80 mol% of 3HB, 3HV, 3H2MB and 3H2MV with glycogen accumulating organism mixed culture, having higher T_m equal to 156.4 and 154.9 °C, respectively, indicating the heterogenous nature [6]. Following the same trend melting point for polymer with 30, 44 and 53 mol% non-3HB content is 144.5, 155.0 and 153.7 °C, respectively [7]. Polymer detected as copolymer by GC obtained with glucose, octanoic and oleic acid have a higher T_m (177–179 °C) and T_g (3–4 °C) as expected for a copolymer having mcl-HAs. This observation suggests that the polymer was heterogenous having mixture of homopolymer PHB and copolymers, which are then separated using acetone fraction [83].

Mechanical Properties

Mechanical properties including crystallanity, elongation, tensile strength, etc. are usually assessed for PHA polymer to quantify its processability. Within the bacteria, the polymers are present in the form of spherical granules, generally 50–500 nm in diameter. Extracellular PHA is often crystalline having about 50–80 % of crystallinity [56, 84]. The crystallinity of PHA, which depends on M_w and monomer composition, in turn, affects its thermal, mechanical properties and biodegradability [85]. Homopolymer PHB which has low M_w (up to 1×10^3 kDa) has high crystallinity and brittleness [4]. There are two crystallization forms observed for PHB: α-form having lamellar crystalline nature and β-form having planar zigzag conformation. The polymers with higher M_w have β-form imparting it the better mechanical strength and thus, can be converted to films and fibers [18, 85, 86].

Depending upon the nature of PHA polymers ranging from non-crystalline to highly crystalline, crystallanity index may vary between 0 and 70 % increasing with 3HB monomers. Homopolymer PHB have higher crystallinity index i.e. 44 % followed by copolymers with varying monomer composition e.g. 40 % index for P(3HB:30 % 3HV) [87]. P(3HB:8 % 3HV) has the crystallanity index of 57.3 % which decreases to 44 % in polymer with

15–18 mol% of 3HV content [82]. Incorporation of long chain monomers decreases melting enthalpy and thus, the crystallinity. Increase in 3HV content up to 39 % provides the amorphous matrix to the polymer. Chanprateep et al. [74] were successful in attaining thermoplastic ranging from brittle to flexible plastic depending on variations in 4HB units. Presence of a large number of non-3HB monomers makes it more amorphous leading to no distinct T_m. The copolymer P(60 % 3HB:2 % 3H2 MB:13 % 3HV:1 % 3H2MV:23 % 3HHx), P(3HB:4.3–8.5 % 4HB:17.6–22.0 % 3HHx) are amorphous in nature [7, 88]. Nevertheless, the polymers P(3HB:3HV:3HHx) with 1.2–5.4 mol% of 3HV and 1.4–15.1 mol% 3HHx are not entirely amorphous [16]. Conclusively, higher content of mcl-HA, e.g. 3HHx promotes the amorphous nature of the polymer.

The elastic modulus indicates a measure of PHA's stiffness and ranges from flexible mcl-PHA (0.002 GPa) to brittle scl-PHA (3.5 GPa). Elongation at break is the capacity of any material to which it can be stretched without breaking. Since elongation has the values ranging from 0.4 % for P(3HB) to ~1000 % for P(4HB), PHA can have varied nature, i.e. hard and rigid having lowest value to soft elastic having higher values for this property. Tensile strength, the stress required to break a material while stretching or pulling it, for PHA lies in the range of 0.9–190 MPa (Table 3). The elastic modulus decreases with decreasing 3HB and increasing mcl-HAs while elongation to break has the opposite trend. PHB has the highest elastic modulus equal to in the range 1.1–3.5 GPa and lowest elongation to break value in the range 0.4–5.0 however, the tensile strength is in a considerable range of 19–40 MPa (Table 3). P(3HB:25 % 3HV) has the lower elastic modulus i.e. 1.37 MPa as compared to P(3HB:9 % 3HV) i.e. 1.62 MPa. Elastic modulus is comparatively lower for P(3HB:4HB) related to P(3HB:3HV). Elastic modulus decreases (1.23–0.1 GPa) and elongation to break increases (10–1080 %) as mole fraction of 4HB increases from 5 to 90 mol% and thus polymer becomes more flexible [49, 74]. In tercopolymer 4HB unit increased elasticity (% elongation) and 3HV increased the elastic modulus. Elongation for P(10 % 3HB:6 % 3HV:84 % 4HB) is 300 %, which is ten times higher than P(3HB:9 % 3HV) with the equivalent 3HV unit and the elastic modulus is 13 times higher i.e. 392 MPa for P(11 % 3HB:23 % 3HV:66 % 4HB) than P(3HB:64 % 4HB) having equivalent 4HB unit [52]. P(3HB:3HV:4HB) with a corresponding monomer ratio 73:8:9 has values for elastic modulus and elongation equal to 0.1 GPa and 316 %, which are better than those observed with a monomer ratio of 64:32:4 (0.14 GPa and 19 %, respectively) but inferior to those with a corresponding monomer ratio of 63:4:33 (0.1 GPa and 937 %, respectively) [81]. Similarly, effect of 3HHx was evident on the elastic modulus and elongation. Presence of 3HHx results in the lower elastic modulus up to 0.002 GPa and elongation to break to increase up to 740–833 % [65]. Somehow, tensile strength showed quite a variation and was observed to be higher with less monomer diversity and content e.g. higher value of 190 MPa obtained with P(3HB:9 % 3HV) [74]. High M_w PHA can have high mechanical strength. As reported by Iwata, ultra-high M_w PHB having M_w equal to 5.3×10^3 kDa can have a tensile strength up to 1320 MPa [18].

Conclusion

Homopolymers such as PHB, the most widely reported PHAs, have low strength (low M_w), are brittle (highly crystalline), and susceptible to degradation (high elastic modulus and high T_m). These properties limit their range of applications and processability. Copolymers of different HAs help to enhance the thermo-mechanical characteristics of PHAs. Strategies to produce copolymers include usage of carbohydrates and FAs as feed. Sugars, agricultural wastes, molasses, whey majorly promote scl-PHA, whereas

Table 3 Physical properties of biopolymers and synthetic polymers

Polymer	Melting temperature (°C)	Glass transition (°C)	Young's modulus (GPa)	Elongation to break (%)	Tensile strength (MPa)	References
Polyhydroxybutyrate	175–180	4	3.5–4	3–11	11–40	[29, 47, 49, 74]
Biopol products (<28.4 % 3HV)	102	−8 to −9	–	–	–	[74]
P(3HB-11.8 % 3HHx)	143	−7	0.16	50.1	10.5	[47, 49]
High density polyethylene	112–132	–	0.4–1.0	12–700	18–33	[47, 74]
Low density polyethylene	88–100	−36	0.05–0.2	126–600	10–78	[29, 47, 74]
Polypropylene	170–176	−10	0.6–1.7	400–900	27–38	[29, 47, 49, 74]
Polystyrene	110–240	100	3.0–3.1	3–4	50	[47, 49]
Polyvinylchloride	100–260	82	3.4	20–80	10–60	[47]
UV degradable bag	–	–	0.7	384	24	[74]
6,6-Nylon	265	–	2.8	60	83	[47]

mcl-PHAs can be obtained with long C-chain length FAs or oily wastes [89, 90]. In contrast to PHB, copolymer of PHAs having non-3HB contents like 3HV and 4HB can be achieved by addition of precursors: (1) 3HV by supplementing the feed with odd number C-sources like propionate, valerate, 1-pentanol etc., and (2) 4HB units from substrates like γ-hydroxybutyric acid, 1,4-butanediol, γ-butyrolactone. Feeding regime strategies like: (1) adding precursors after 24 h of cultivation, (2) using continuous periodic feeding instead of pulse feeding, and (3) varying the cultivation time, etc. also prove beneficial. Culture conditions, including pH, aeration rate (rpm), C/N ratio, etc. strongly influence the composition and M_w of the PHA, which consequently affect the PDI, the index for heterogeneity of the polymer. Lower T_m and T_g obtained in copolymer is beneficial to give elastic and flexible texture at normal temperature. These properties can be manipulated by introducing changes in the genes involved in PHA synthesis, especially the expression of *phaC* in different hosts. *R. eutropha,* with class I PHA synthase produces scl-PHA, while *Pseudomonas* class II PHA synthase produces mcl-PHAs. *Bacillus* has unique ability to produce scl- and mcl-PHAs. *Bacillus* spp. are among those few organisms which can produce homopolymers and copolymers from pure substrates and biowastes of diverse origins [91]. *Bacillus* like *E. coli*, can be subjected to a wide range of genetic manipulations in terms of genome reduction, horizontal gene transfer, heterologous expression, etc. *Bacillus* has a natural genetic ability where genes in the *pha* operon are best suited for high quality PHAs. *Bacillus* being a GRAS organism has been shown to also produce H_2, in addition to PHA from a wide range of biowastes and under even non-stressed conditions [92]. It will be perhaps not immature to state that *Bacillus* is likely to be the organism of choice for producing tailored PHAs, which can be commercialized.

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