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REVIEW ARTICLE

Microbial Cometabolism and Polyhydroxyalkanoate Co-polymers

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Abstract Polyhydroxyalkanoate (PHAs) are natural, biodegradable biopolymers, which can be produced from renewable materials. PHAs have potential to replace petroleum derived plastics. Quite a few bacteria can produce PHA under nutritional stress. They generally produce homopolymers of butyrate i.e., polyhydroxybutyrate (PHB), as a storage material. The biochemical characteristics of PHB such as brittleness, low strength, low elasticity, etc. make these unsuitable for commercial applications. Co-polymers of PHA, have high commercial value as they overcome the limitations of PHBs. Co-polymers can be produced by supplementing the feed with volatile fatty acids or through hydrolysates of different biowastes. In this review, we have listed the potential bacterial candidates and the substrates, which can be co-metabolized to produce PHA co-polymers.

Keywords *Bacillus* · Biowastes · Co-metabolism · Co-polymers · Polyhydroxyalkanoate · Gram-positive · Gram-negative

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Introduction

Biopolymers like polyhydroxyalkanoates (PHAs) have gained importance as these can be produced from natural and renewable substrates. Another important characteristic is their biodegradable nature. Their physical and chemical characteristics are very similar to synthetic plastics derived from petroleum products [1, 2]. The basic advantage of this biodegradable plastic is their non-polluting nature and potential to save fossil fuels. Diverse bacteria produce PHAs under nutritionally imbalanced conditions. The PHA biosynthetic pathway operates at high Carbon (C) concentrations and limitations of other nutrients (N, P, K, O, Mg, etc.) in the environment [3]. Here, instead of operating the tri-carboxylic acid cycle for generating energy, the metabolic pathway shifts towards PHA biosynthesis to produce granules, which act as C storage material [4]. Under normal physiological conditions, especially when the C:N ratio is low, i.e. N is present in sufficient quantities, NAD(P)H/NAD(P) ratio decreases and acetyl-CoA goes into the TCA cycle, releasing CoA for the next round of utilization. Accumulation of CoA inhibits the activity of β-ketothiolase, which blocks the PHA synthesis route. β-ketothiolase is the first enzyme of the PHA biosynthetic pathway. On the other hand, PHA production progresses when cell growth is reduced under N-limiting conditions. Here, NAD(P)H/NAD(P) ratio increases, which inhibits citrate synthase and isocitrate dehydrogenase activity resulting in the blockage of the TCA cycle. It leads to high acetyl-CoA concentration and lowers CoA, resulting in the activation of the enzyme—β-ketothiolase. The Phase I of PHA biosynthetic pathway become operative leading to the generation of acetoacetyl-CoA. It then gets transformed to 3-OH-butyryl-CoA, with the aid of NADPH–dependent acetoacetyl reductase in the Phase II. The whole process

terminates with the production of polyhydroxybutyrate (PHB), by polymerization of 3OH-butyrate monomers with the help of PHB synthase, i.e. Phase III [5, 6] (Fig. 1). The three enzymes of the PHB biosynthetic pathway are coded by genes: *phaA* (1179 nucleotides, nts), *phaB* (738 nts), and *phaC* (1767 nts), which are organized as CAB operon in *Ralstonia eutropha* (Fig. 2). The diversity of PHA synthases can be seen in organisms like: (1) *R. eutropha*, which has class I type, single subunit of PhaC (60–73 kDa), (2) *Pseudomonas oleovorans* having class II type—single PhaC subunit (60–65 kDa), (3) *Allochromaticum vinosum* and *Thiocapsa pfennigii* having class III, composed of two subunits PhaC (40 kDa) and PhaE (40 kDa), and (4) *Bacillus megaterium* representing class IV composed of subunits PhaC (40 kDa) and PhaR (22 kDa) (Fig. 3). Class I, II and IV type PHA synthases result in C3-C5 PHAs, whereas class III can result in more variable chain length PHAs (Fig. 3).

Polyhydroxyalkanoate (PHAs)

Bacteria have the potential to gather C in the form of PHAs to the extent of 90 % of the total dry cell mass (DCM). The composition of the PHAs depends upon the C chain length, which varies from: (1) C_3–C_5 i.e., short chain length PHA e.g., in *R. eutropha*, and (2) C_6–C_{14} i.e., medium chain length PHA e.g., in *Pseudomonas oleovorans* [3]. The nature of the biopolymers depends upon the growth medium, type and quantity of C source, bacterium, supplements, etc. Most bacteria produce homopolymers as PHB, however, a few have the potential to produce co-polymers, but need specific co-substrate to be present in the medium [1]. *R. eutropha* and *Chromobacterium violaceum* grown in the presence of valeric acid (VA) as supplemented material results in PHA co-polymers. The commercial value of

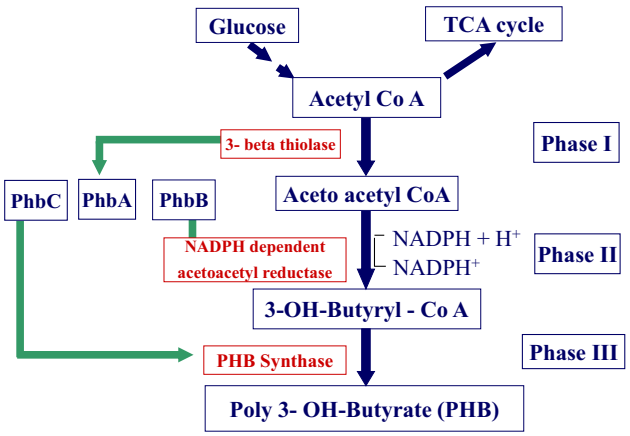

Fig. 1 Polyhydroxyalkanoate biosynthetic pathway. PHA is a synthesized by the action of enzymes: *PhbA* (*β-keto thiolase*), *PhbB* (*acetoacetyl-CoA reductase*) and *PhbC* (*PHA plymerase*). *TCA* tricarboxylic acid cycle

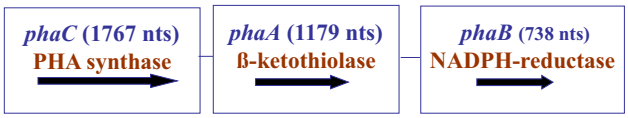

Fig. 2 *phaCAB* operon organization in *Ralstonia eutropha*

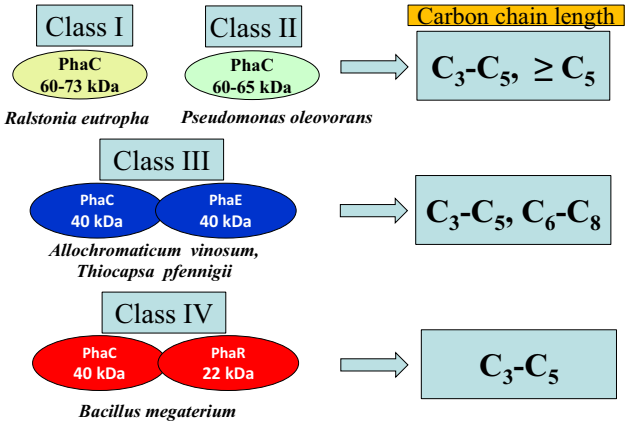

Fig. 3 Diversity of Polyhydroxyalkanoate (PHA) synthases

PHBs is lower as compared to co-polymers because of the following reasons: (1) brittle nature, (2) low strength, (3) high cost of production, (4) low elasticity, (5) low mechanical resistance, etc. [1]. PHA copolymers have characteristics, which can be compared to petroleum plastics. Here, the improvement in PHA strength is because of high molecular weight and variation in monomeric compositions. These changes can be achieved through variation in: (1) co-substrate, (2) feeding, (3) physiological conditions, (4) genetic modifications, (5) heterologous gene expressions (6) metabolic pathway modification [1, 7, 8]. 3HV monomers when incorporate into a PHA polymer chain, increase material characteristics of PHA co-polymer, such as: (1) melting point, (2) crystallinity, (3) stiffness, and (4) toughness. Co-polymers are thermoplastics, which have a melting temperature of 140 °C, which is close to that of polylactic acids [9].

PHA Co-polymers by Co-metabolism of Diverse Substrates

Gram-Negative Bacteria

Most PHA producers generally belong to gram-negative group of bacteria (Table 1) [10–35]. *Ralstonia* species are among the most widely studied PHA producers. They have an ability to produce homopolymers and co-polymers. *R. eutropha* could utilize mixtures of: (1) gluconoate + octanoate, and (2) glycerol + casein hydrolysate (CH) to produce PHB homopolymers, where PHA yield varied from 40 to 50 % of DCM [13, 18]. Different strains of

Table 1 Polyhydroxyalkanoate co-polymer production by co-metabolism of diverse substrates by gram-negative microorganism

Organism	Substrate	Homo-polymers		Co-polymer			References
		PHB		Type	Ratio mol (%)	Yield (% DCM)	
		mol (%)	Yield (% DCM)				
Alcaligenes eutrophus	Glucose + $(NH_4)_2SO_4$	100	78	–	–	–	[16]
Ralstonia eutropha PHB-4	Gluconate + octanoate	100	40.89	–	–	–	[18]
R. eutropha	Glycerol + caesin hydrolysate (CH)	100	50	–	–	–	[13]
Cupriavidus necator H16	Palm kernel oil + propionic acid (PA)	–	–	P(3HB-3HV-3HHx)	93:0:7	55.5	[25]
	Palm kernel oil + valeric acid (VA)	–	–	P(3HB-3HV-3HHx)	89:6:5	52.3	
C. necator DSM545	Glucose + VA	100	64.5	–	–	–	[33]
	FAME + VA	–	–	P(3HB-3HV)	4.3	63.4	
C. necator DSM7237	Glycerol + sunflower meal + levulinic acid	27 g/L	72.9	P(3HB-3HV)	22.5	66.4	[35]
C. necator	Crude glycerol + rapeseed meal	–	–	P(3HB-3HV)	2.8−8:55.6	NA	[32]
Cupriavidus sp. USMAA1020	γ-butyrolactone	–	–	P(3HB-4HB)	NA	52.4	[23]
Pseudomonas pseudoflava	Glucose + xylose	100	22	–	–	–	[10]
P. putida KTOY06	Dodecanoate + gluconate	–	–	P(3HD-3HDD-3HO- 3HHx)	NA	84.3	[19]
P. putida KT2440	Glucose + nonanoic acid	100	75	–	NA	NA	[21]
Burkholderia + *Acidobacteria*	Acetic acid (AA) + PA	100	NA	P(3HB-3HV)	0–74	NA	[34]
Aeromonas hydrophila CQ4	Dodecanoate + gluconoate	–	–	P(3HB-3HHx)		44.67	[20]
A. hydrophila 4AK4	Dodecanoate + PA	–	–	P(3HB-3HV-3HHx)	NA	37.2	[22]
	Lauric acid + 1,4-butanediol	–	–	P(3HB-4HB—3HHx)	NA	23.6	[28]
Azotobacter sp.	Glucose (5 % w/v) + FP (Fish peptone)	100	85	–	–	–	[11]
	Glucose (3 % w/v) + FP + NH_4Cl	100	74	–	–	–	
	Glucose (3 % w/v) + FP	100	79	–	–	–	
A. vinelandii UWD		–	–	P(3HB-3HV)	4.3	58.3	[12]
Comamonas acidovorans	Glucose + 1,4-butanediol	100	53	P(4HB)	0–96	40	[15]
Haloferax mediterranei	Rice bran + corn starch (1:8)	100	55.6	–	–	–	[17]
	Wheat bran + Corn Starch (1:2)	100	40.2	–	–	–	
Methylobacterium rhodesianum	Glycerol + CH + Casamino acids	100	65	–	–	–	[13]
E. coli JM109	Glucose	–	–	P(3HHx-3HO)	NA	54.0	[27]
Azohydromonas australica	Sucrose + Nitrogen	100	77.0	–	–	–	[31]
Mixed culture	AA + PA + Lactic acid	–	–	P(3HB-3HV)	31	NA	[14]
Mixed culture	Fermented molasses (VFAs)	100	65	P(3HB-3HV)	13	30	[30]
Mixed culture	AA + PA	100	78	P(3HB-3HV)	15–20	NA	[26]
Mixed culture (Waste activated sludge)	AA + Glucose	100	30	P(3HB-3HV)	3.1	NA	[27]
	AA + Bovine serum albumin (BSA)	100	29.1	P(3HB-3HV)	2.7	NA	
	AA + Glucose + BSA	100	30.8	P(3HB-3HV)	3.7	NA	

Table 1 continued

Organism	Substrate	Homo-polymers		Co-polymer			References
		PHB		Type	Ratio mol (%)	Yield (% DCM)	
		mol (%)	Yield (% DCM)				
Mixed culture (Activated sludge)	VFAs (AA + PA + VA + butyrate)	100	31–47	P(3HB-3HV)	53–69	48	[24]

a Not applicable *NA* Not available

PHA Polyhydroxyalkanoae

PHB Polyhydroxybutyrate

3HB 3-Hydroxybutyric acid

3HV 3-Hydroxyvaleric acid

4HB 4-Hydroxybutyric acid

3HO 3-Hydroxyoctanoate

3HHx 3-Hydroxyhexenoate

6HHx 6Hydroxyhexanoate

3HD 3-Hydroxydecanoate

3HDD 3Hydroxydodecanoate

Cupriavidus necator could produce P(3HB-3HV-3HHx) from vegetable oils, and glycerol supplemented with VA or levulinic acid, where 3HV and 3HHx components varied from 6 to 7 mol%. *C. necator* DSM545 produced homopolymers of PHB from glucose and VA, but co-polymers from FAME + VA [25, 33]. Similarly, *Pseudomonas* spp. could metabolize mixtures of sugars to PHB homopolymers. However, switching over to substrates such as dodecanoate and gluconaote mixtures resulted in PHA co-polymers—P(HD-HDD-HO-HHx) with *P. putida* and P(3HB-3HHx) with *Aeromonas hyrophila* CQ4. A variation in feed to dodecanoate + PA allowed *A. hydrophila* GAK4 to produce P(3HB-3HV-3HHx) [10, 19–22]. Mixed cultures of *Burkholderia* and *Acidobacteria* and other bacteria proved instrumental in transforming acetate and PA combination to P(3HB-3HV) [34]. A few other organisms, which produce PHA copolymers through co-metabolism of substrates are *Comamonas* and *Escherichia coli*, whereas others like *Azotobacter, Haloferax, Methylobacterium* and *Azohydromonas* did not produce co-polymers inspite of being provided with mixed substrates as feed [11, 13, 15, 17, 27].

Gram-Positive Bacteria

Among gram-positive bacteria, *Streptomyces*, *Corynebacteria*, *Clostridium*, *Nocardia*, *Rhodococcus*, *Staphylococcus* are capable of producing PHA co-polymers [4]. *Bacillus* spp. are among those few gram-positive bacteria, which have been gaining importance as PHA producers because of their unique metabolic characterstics. These are perhaps the only bacteria in this category, which can produce homopolymers and co-polymers of PHA from sugars and complex biowastes (Table 2) [1, 2, 7, 36–49]. *Bacillus* species are generally regarded as safe (GRAS) organisms [3, 7]. *Bacillus megaterium* OU303A and *Bacillus* sp. 88D utilized glucose, glycerol and acetate to produce PHB homopolymers, whereas addition of PA (<2.5 ml/L) allowed them to convert these mixtures into P(3HB-3HV). Here, 3HV content varied from 2.5 to 6.3 mol% [42, 43]. *Bacillus* sp. INT005 utilized butyrate to produce PHB, however, glucose in combination with different fatty acids (1 % v/v) resulted in PHA co-polymers with HV content varying from 1.5 to 29 mol% and total PHA yield ranging from 13 to 64.5 % DCM [38].

Bacillus licheniformis, *B. cereus, B. subtilis* and other *Bacillus* spp. could not produce PHA co-polymers from glucose or glycerol. However, use of defined mixed cultures of *B. cereus* and *B. thuringiensis* produced interesting results: (1) on pea-shell slurry (PSS) + glucose—only PHB 18.8 % of DCM was recorded, whereas (2) PSS + glucose + PA resulted in P(3HB-3HV::87:13), with a yield of 16.9 % of DCM. Addition of VA to PSS + glucose was also quite effective in producing co-polymer having 7–10 mol% of 3HV. In contrast, *B. cereus* EGU44 was also reported to show results which are quite similar to those recorded with defined mixed cultures of *Bacillus* [47]. *Bacillus thuringiensis* EGU45 was able to metabolize effluent from hydrogen production stage and yielded co-polymers of PHA with a 3HV content of 5–39 mol% [48].

Bacillus thuringiensis EGU45 could metabolize CG to PHA co-polymers. The composition of these co-polymers varied with the amount of PA or VA used as a

Table 2 Polyhydroxyalkanoate Co-polymer production by co-metabolism of diverse substrates by *Bacillus* sp

Organism	Substrate	Homo-polymers		Co-polymer			References
		PHB		Type	Ratio mol (%)	Yield (% DCM)	
		mol (%)	Yield (% DCM)				
Bacillus megaterium OU303A	Glucose (2 % w/v)	100	62	–	–	–	[42]
	Glucose (2 % w/v) + PA (<2.5 mL/L)	–	–	P(3HB-3HV)	97.5:2.5	58.6	
	Glycerol (2 % w/v)	–	–	P(3HB-3HV)	95:5	52	
	Glycerol (2 % w/v) + PA (<2.5 mL/L)	–	–	P(3HB-3HV)	86:14	57	
	Acetate (2 % w/v)	100	49	–	–	–	
	Acetate (2 % w/v) + PA (<2.5 mL/L)	–	–	P(3HB-3HV)	96.5:3.5	59	
B. megaterium DSM90	Glycerol	100	62.4	–	–	–	[46]
B. cereus ATCC14579	Caprolactone + octanoate	–	–	3HHx	NA	2–4	[36]
				P(3HHx-3HO)			
B. cereus UW85	γ- caprolactone	–	–	P(3HB-3HV-6HHx)	NA	NA	[37]
Bacillus sp.	Butyrate	100	NA	–	–	–	[38]
INT005	Glucose (0.1 % w/v) + Butyrate (1 % v/v)	–	–	P(3HB-3HHx)	98.5:1.5	32.9	
				P(3HB-4HB-3HHx)			
	Glucose (0.1 % w/v) + valerate (1 % v/v)	–	–	P(3HB-3HV)	51.5:48.5	18.8	
	Glucose (0.1 % w/v) + hexanoate (1 % v/v)	–	–	P(3HB-3HHx)	97.7:2.3	13.0	
	Glucose (0.1 % w/v) + octanoate (1 % v/v)	–	–	P(3HB-3HHx)	97.1:2.9	64.5	
	Glucose (0.1 % w/v) + decanoate (1 % v/v)	–	–	P(3HB-3HHx)	97.1:2.9	23.5	
	Glucose (0.1 % w/v) + γ-caprolactone (1 % v/v)	–	–	P(3HB-6HHx-3HHx)	97.3:2.7	23.2	
Bacillus sp. 88D	Glucose (2 % w/v)	–	–	P(3HB-3HV)	96:4	64.6	[43]
	Glucose (2 % w/v) + PA (<2.5 mL/L)	–	–	P(3HB-3HV)	87:13	59.8	
	Glycerol (2 % w/v)	–	–	P(3HB-3HV)	85:15	60.5	
	Glycerol (2 % w/v) + PA (<2.5 mL/L)	–	–	P(3HB-3HV)	96:4	60	
	Acetate (2 %w/v)	100:0	48	–	–	–	
	Acetate (2 %w/v) + PA (<2.5 mL/L)	–	–	P(3HB-3HV)	93.7:6.3	42	
Bacillus (Defined mixed strains:	Pea-shell slurry (PSS) + glucose	100	18.8	P(3HB-3HV)	87:13	16.9	[47]
B. cereus strains EGU3, EGU43 + EGU44 + EGU520 + *B. thuringiensis* EGU45	PSS + glucose + PA	–	–	P(3HB-3HV)	89:11	21.6	

Table 2 continued

Organism	Substrate	Homo-polymers		Co-polymer			References
		PHB		Type	Ratio mol (%)	Yield (% DCM)	
		mol (%)	Yield (% DCM)				
	PSS + glucose + VA	–	–	P(3HB-3HV)	90:10, 93:7	16–23	
B. cereus EGU44	PSS + glucose	100	30.0				[47]
	PSS + glucose + PA (0.5–2 % v/v)	–	–	P(3HB-3HV)	89:11, 84:16, 85:15	16 -22	
	PSS + glucose + VA (0.5–2 % v/v)	–	–	P(3HB-3HV)	83:17, 90:10	16 -24	
B. thuringiensis EGU45	Effluent from H_2-stage + glucose (1 % w/v) +						[49]
	1. M9 + GM2 media: 1X + 0.25X	NA	NA	P(3HB-3HV)	61:39	10	
	2. M9 + GM2 media: 1X + 0.5X	NA	NA	P(3HB-3HV)	62:38	7.6	
	3. M9 + GM2 media: 1X + 1X	NA	NA	P(3HB-3HV)	77:23	18	
	4. M9 + GM2 media: 1X + 2X	NA	NA	P(3HB-3HV)	95:5	21	
B. thuringiensis EGU45	Crude glycerol (CG) + Peptone (PE) + Yeast extract (YE) +						[48]
	1. PA (0.5 % v/v)	–	–	P(3HB-3HV)	89:11	53.9	
	2. PA (1.0 % v/v)	–	–	P(3HB-3HV)	94.7:5.3	37.3	
	3. PA (2.0 % v/v)	–	–	P(3HB-3HV)	98.2:1.8	44.2	
	4. VA (0.5 % v/v)	–	–	P(3HB-3HV)	95.7:4.3	37.8	
	5. VA (1.0 % v/v)	–	–	P(3HB-3HV)	98.2:1.8	48.5	
	6. VA (2.0 % v/v)	–	–	P(3HB-3HV)	99:1.0	56.3	
	CG + nutrient broth +						
	1. PA (0.5 % v/v)	–	–	P(3HB-3HV)	86.6:13.4	55	
	2. PA (1.0 % v/v)	–	–	P(3HB-3HV)	95.7:4.3	29	
	3. PA (2.0 % v/v)	–	–	P(3HB-3HV)	98.3:1.7	36	
	4. VA (0.5 % v/v)	–	–	P(3HB-3HV)	96.3:3.7	29.7	
	5. VA (1.0 % v/v)	–	–	P(3HB-3HV)	98.7:1.3	53.1	
	6. VA (2.0 % v/v)	–	–	P(3HB-3HV)	98.9:1.1	52.2	
	PSS	100	5.8	–	–	–	[2]
	PSS + glucose (1 % w/v)	100	7.7	–	–	–	
	Apple pomace (AP)	–	–	P(3HB-3HV)	64.3:35.7	3.8	
	AP + glucose (1 % w/v)	–	–	P(3HB-3HV)	75.9:24.1	7.5	

Table 2 continued

Organism	Substrate	Homo-polymers		Co-polymer			References
		PHB		Type	Ratio mol (%)	Yield (% DCM)	
		mol (%)	Yield (% DCM)				
	Onion peels (OP)	–	–	P(3HB-3HV)	80:20	8.4	
OP + glucose (1 % w/v)	–	–	P(3HB-3HV)	97.5:2.5	11.7		
Potato peels (PP)	–	–	P(3HB-3HV)	33:67	2.6		
PP + glucose (1 % w/v)	–	–	P(3HB-3HV)	90.9:9.1	38.2		
PS:AP:2:1 + glucose (1 % w/v)	–	–	P(3HB-3HV)	78.8:21.2	16.4		
PS:OP:1:2 + glucose (1 % w/v)	–	–	P(3HB-3HV)	63.4:36.6	20.5		
PS:PP:2:1 + glucose (1 % w/v)	–	–	P(3HB-3HV)	77:23	27.1		
Bacillus sp.	Glycerol	–	–	NA	NA	25–52	[39]
Bacillus sp.	*Madhuca* sp. Flowers (Sugars + malic acid)	–	–	P(3HB-3HV)	90:10	51	[41]
B. licheniformis PHA007	Glycerol	100	68.8	–	–	–	[45]
B. licheniformis DSM394	Glycerol	100	17	–	–	–	[45]
B. subtilis DSM10	Glycerol	100	18.9	–	–	–	[45]
B. cereus PHA037	Glucose	100	60.7	–	–	–	
B. thuringiensis R1	Glycerol	100	64.1	–	–	–	[40]
B. sphaericus NII0838	Glycerol	100	31.0	–	–	–	[44]

a Not applicable

NA Not available

PHA Polyhydroxyalkanoate

PHB Polyhydroxybutyrate

3HB 3-Hydroxybutyric acid

3HV 3-Hydroxyvaleric acid

4HB 4-Hydroxybutyric acid

3HO 3-Hydroxyoctanoate

3HHx 3-Hydroxyhexenoate

6HHx 6-Hydroxyhexanoate

3HD 3-Hydroxydecanoate

3HDD 3Hydroxydodecanoate

supplement. With PA in Peptone + Yeast extract (PE + YE) medium, PHA co-polymer had 3HV content in the range of 1.8–11 mol%. However, with VA in PE + YE medium, 3HV content varied from 1 to 4.3 mol%. On the other hand, CG + Nutrient broth (NB) supplemented with (1) PA resulted in 1.7–13.4 mol% of 3HV, and (2) VA resulted in 1.1–3.7 mol% of 3HV [49]. A very interesting result was recorded in an effort to provide supplemental fatty acids by hydrolysing different biowastes as mixtures in a wide range of ratios. Hydrolysates of PS was found to produce only AA, whereas apple poamace (AP) hydrolysates had only isovaleric acid. Hydrolysates of potato peels (PP) and onion peels (OP) produced mixtures of AA, butyric acid, and PA. This initial information was found to prove helpful in producing PHA co-polymers by co-metabolizing these biowatses by *B. thuringiensis* EGU45. PS alone was able to produce only homopolymers i.e., PHB, however, mixtures: (1) PS + AP, (2) PS + OP, and (3) PS + PP resulted in P(3HB-3HV), where HV content varied i.e., 21.2, 36.6, and 23.4 mol%, respectively. It implied that by co-metabolism, it is possible to divert PHA biosynthetic pathway from producing only homopolymers to different co-polymers [2].

Opinion

In order to produce co-polymers of PHA, it seems that in addition to bacterial genetic potential, we also need to choose a right combination of substrates and supplements. Thus co-metabolism is an important approach for producing PHA co-polymers of desired compositions. Among the PHA producers, *Bacillus* spp. are perhaps the most persistent. They have the ability to produce homopolymers and co-polymers as well from the cometabolizing substrates. It implies how *Bacillus* can engineer its metabolic pathway to produce PHA co-polymer. This property enables it to be a strong competitor as an industrial PHA producer in future.

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Compliance with Ethical Standards

Conflict of interest Authors declare no conflict of interests.

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