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Review

Start a Research on Biopolymer Polyhydroxyalkanoate (PHA): A Review

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Abstract: With the impending fossil fuel crisis, the search for and development of alternative chemical/material substitutes is pivotal in reducing mankind's dependency on fossil resources. One of the potential substitute candidates is polyhydroxyalkanoate (PHA). PHA is a carbon-neutral and valuable polymer that could be produced from many renewable carbon sources by microorganisms, making it a sustainable and environmental-friendly material. At present, PHA is not cost competitive compared to fossil-derived products. Encouraging and intensifying research work on PHA is anticipated to enhance its economic viability in the future. The development of various biomolecular and chemical techniques for PHA analysis has led to the identification of many PHA-producing microbial strains, some of which are deposited in culture collections. Research work on PHA could be rapidly initiated with these ready-to-use techniques and microbial strains. This review aims to facilitate the start-up of PHA research by providing a summary of commercially available PHA-accumulating microbial cultures, PHA biosynthetic pathways, and methods for PHA detection, extraction and analysis.

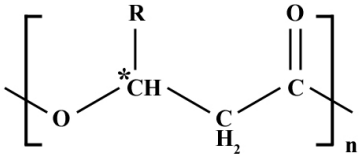
Keywords: polyhydroxyalkanoate; PHA; biopolymer; bacteria; archaea; pathway; PHA detection; PHA extraction; PHA characterization

1. Introduction

PHA is a family of naturally-occurring biopolyesters synthesized by various microorganisms. First discovered by Lemogine in 1926 [1], PHA has since attracted much commercial and research interests due to its biodegradability, biocompatibility, chemical-diversity, and its manufacture from renewable carbon resources [2]. A PHA molecule is typically made up of 600 to 35,000 (*R*)-hydroxy fatty acid monomer units [3]. Each monomer unit harbors a side chain *R* group which is usually a saturated alkyl group (Figure 1) but can also take the form of unsaturated alkyl groups, branched alkyl groups, and substituted alkyl groups although these forms are less common [4]. Depending on the total number of carbon atoms within a PHA monomer, PHA can be classified as either short-chain length PHA (scl-PHA; 3 to 5 carbon atoms), medium-chain length PHA (mcl-PHA; 6 to 14 carbon atoms), or long-chain length PHA (lcl-PHA; 15 or more carbon atoms) [3]. About 150 different PHA monomers have been identified and this number continues to increase with the introduction of new types of PHA through the chemical or physical modification of naturally-occurring PHA [5], or through the creation of genetically-modified organisms (GMOs) to produce PHA with specialized functional groups [6]. These features gave rise to diverse PHA properties which can be tailored for various applications ranging from biodegradable packaging materials to medical products. PHA is also considered as pharmaceutically-active compound and currently investigated as potential anti-HIV drugs, anti-cancer drugs, antibiotics, *etc.* [7,8]. The production of various types of PHA material, their properties and downstream applications was recently reviewed by Philip *et al.* [9], Olivera *et al.* [10], Chen [11], and Rai *et al.* [7].

The intense research and commercial interest in PHA is evident from the rapid increment in PHA-related publications. Web of Science citation report (Thomson Reuters, New York, NY, USA) revealed that in the last 20 years, PHA-related documents have increased by almost 10-fold while citations have increased by more than 500-fold with an average citation count of about 1100 citations per year. This has fuelled the growth of knowledge and development of techniques related to microbial PHA production. With this ready information, research work on PHA could be rapidly initiated either through using microbial strains previously deposited in culture collections or through isolating and characterizing novel PHA-producing microbes. Figure 2 is an illustration of the typical workflow processes for PHA research. This review aims to facilitate the start-up of PHA research by providing an overview of PHA-accumulating microbes currently available in culture collections, PHA biosynthetic pathways, techniques for microbial PHA detection and characterization, PHA polymer extraction, and polymer characterization.

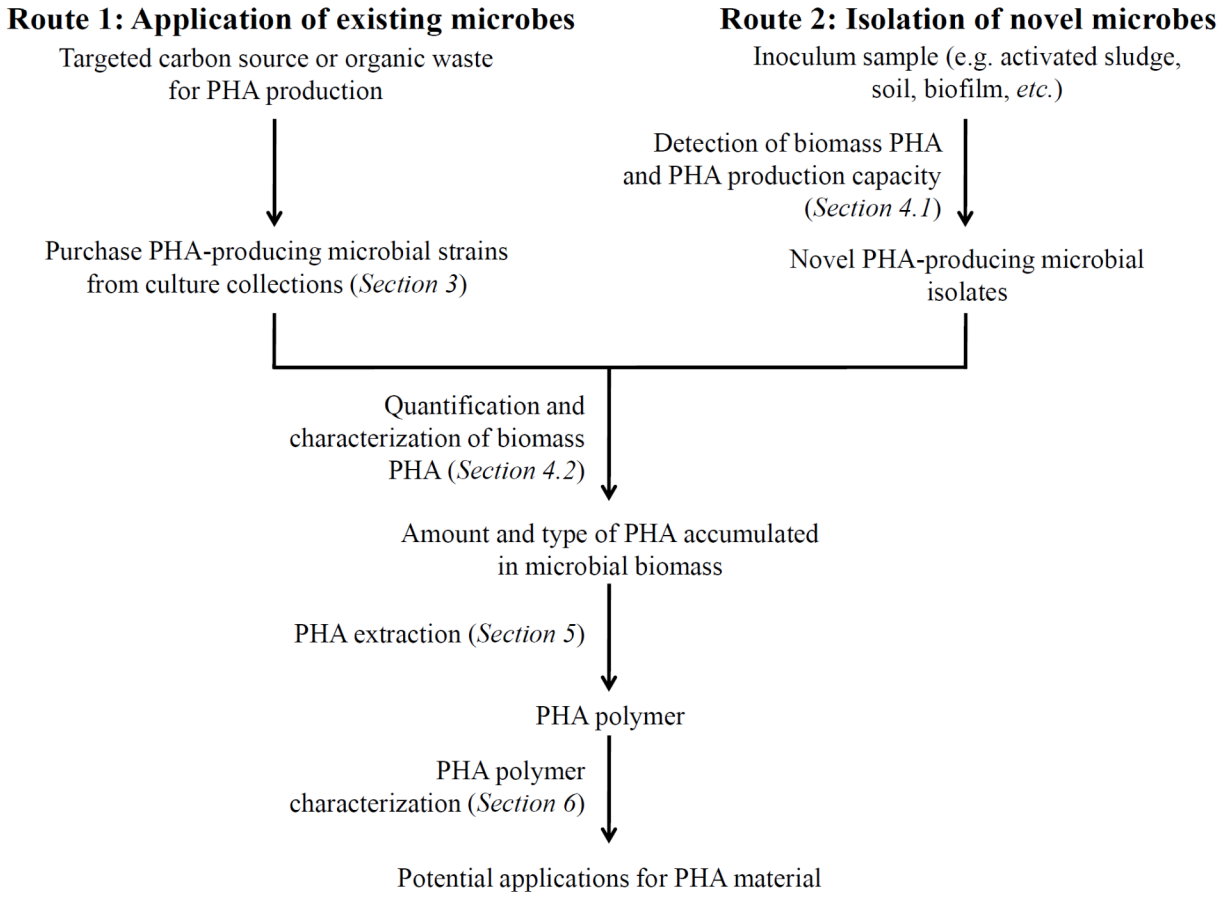
Figure 1. Polyhydroxyalkanoate (PHA) chemical structure. The nonmenclature and carbon number for PHA compounds is determined by the functional alkyl *R* group. Asterisk denotes chiral center of PHA-building block.



Poly(3-hydroxyalkanoate)

R group	Carbon no.	PHA polymer
methyl	C_4	Poly(3-hydroxybutyrate)
ethyl	C_5	Poly(3-hydroxyvalerate)
propyl	C_6	Poly(3-hydroxyhexanoate)
butyl	C_7	Poly(3-hydroxyheptanoate)
pentyl	C_8	Poly(3-hydroxyoctanoate)
hexyl	C_9	Poly(3-hydroxynonanoate)
heptyl	C_{10}	Poly(3-hydroxydecanoate)
octyl	C_{11}	Poly(3-hydroxyundecanoate)
nonyl	C_{12}	Poly(3-hydroxydodecanoate)
decyl	C_{13}	Poly(3-hydroxytridecanoate)
undecyl	C_{14}	Poly(3-hydroxytetradecanoate)
dodecyl	C_{15}	Poly(3-hydroxypentadecanoate)
tridecyl	C_{16}	Poly(3-hydroxyhexadecanoate)

Figure 2. Schematic workflow processes for PHA research.



2. PHA Biosynthetic Pathways

PHA plays a pivotal role in priming microorganisms for stress survival. PHA promotes the long-term survival of bacteria under nutrients-scarce conditions by acting as carbon and energy reserves for both non-sporulating and sporulating bacteria. Additionally, bacteria that harbor PHA showed enhanced stress tolerance against transient environmental assaults such as ultraviolet (UV) irradiation, heat and osmotic shock [12]. PHA biosynthetic pathways are intricately linked with the bacterium's central metabolic pathways including glycolysis, Krebs Cycle, *β*-oxidation, *de novo* fatty acids synthesis, amino acid catabolism, Calvin Cycle, and serine pathway [4,13–17]. Many common intermediates are also shared between PHA and these metabolic pathways, most notably being acetyl-CoA. In some PHA-producing microbes such as *Cupriavidus necator*, *Chromatium vinosum*, and *Pseudomonas aeruginosa*, the metabolic flux from acetyl-CoA to PHA is greatly-dependent on nutrient conditions [18]. Under nutrient-rich conditions, the production of high amounts of coenzyme A from Krebs Cycle blocks PHA synthesis by inhibiting 3-ketothiolase (PhaA) such that acetyl-CoA is channeled into the Krebs Cycle for energy production and cell growth [19] (Figure 3). Conversely, under unbalanced nutrient conditions (*i.e.*, when an essential nutrient such as nitrogen and phosphorus is limiting in the presence of excess carbon), coenzyme A levels are non-inhibitory allowing acetyl-CoA to be directed towards PHA synthetic pathways for PHA accumulation [19,20]. This metabolic regulation strategy in turn enables the PHA-accumulating microbes to maximize nutrient resources in their adaptation to environmental conditions.

To date, much insight has been gained on metabolic pathways for scl-PHA and mcl-PHA synthesis through studies using wild-type strains and heterologous expressions in recombinant strains [3]. In-depth reviews on these various PHA biosynthesis pathways and the enzymes involved have been provided by Chen [11], Lu *et al.* [4], Madison and Huisman [13], and Khosravi-Darani *et al.* [21]. Figure 3 shows the various routes of scl-PHA synthesis (pathways A to J) and mcl-PHA synthesis (pathways J to M) while Table 1 provides a summary of the enzymes involved. Although the biosynthesis of PHA from (*R*)-hydroxyalkyl-CoA ([*R*]-3-HA-CoA) precursors were most commonly reported, the diversity of PHA precursors is not restricted to (*R*)-3-HA-CoA alone [4]. Putative metabolic routes, such as pathways L and M (Figure 3), were recently proposed to expound for the metabolism of cyclohexanol to 6-hydroxyhexanoyl-CoA and 4,5-alkanolactone to 4,5-hydroxyacyl-CoA (4,5-HA-CoA) [11]. Nevertheless, the current knowledge on biosynthetic pathways is largely confined to (*R*)-3-HA-CoA precursors and falls short of accounting for the chemically-diverse PHA monomers and PHA monomers of lcl-PHA. There remains much about biosynthetic pathways waiting to be uncovered. Further studies to verify putative pathways as well as unraveling new biosynthetic pathways are anticipated to facilitate the creation of PHA materials that could be tailored for specific application needs.

Figure 3. PHA biosynthetic pathways. Dotted lines represent putative pathways. Numbers represent enzymes involved in the chemical reactions that are summarized in Table 1. (3-H2MB-CoA, 3-hydroxy-2-methylbutyryl-CoA; 3-H4MV-CoA, 3-hydroxy-4-methylvaleryl-CoA; 3-HV-CoA, 3-hydroxyvaleryl-CoA; 3-H2MV-CoA, 3-hydroxy-2-methylvaleryl-CoA; 4-HB-CoA, 4-hydroxybutyryl-CoA; 3-HB-CoA, 3-hydroxybutyryl-CoA; (*R*)-3-HA-CoA, (*R*)-3-hydroxyacyl-CoA; 4,5-HA-CoA, 4,5-hydroxyacyl-CoA).

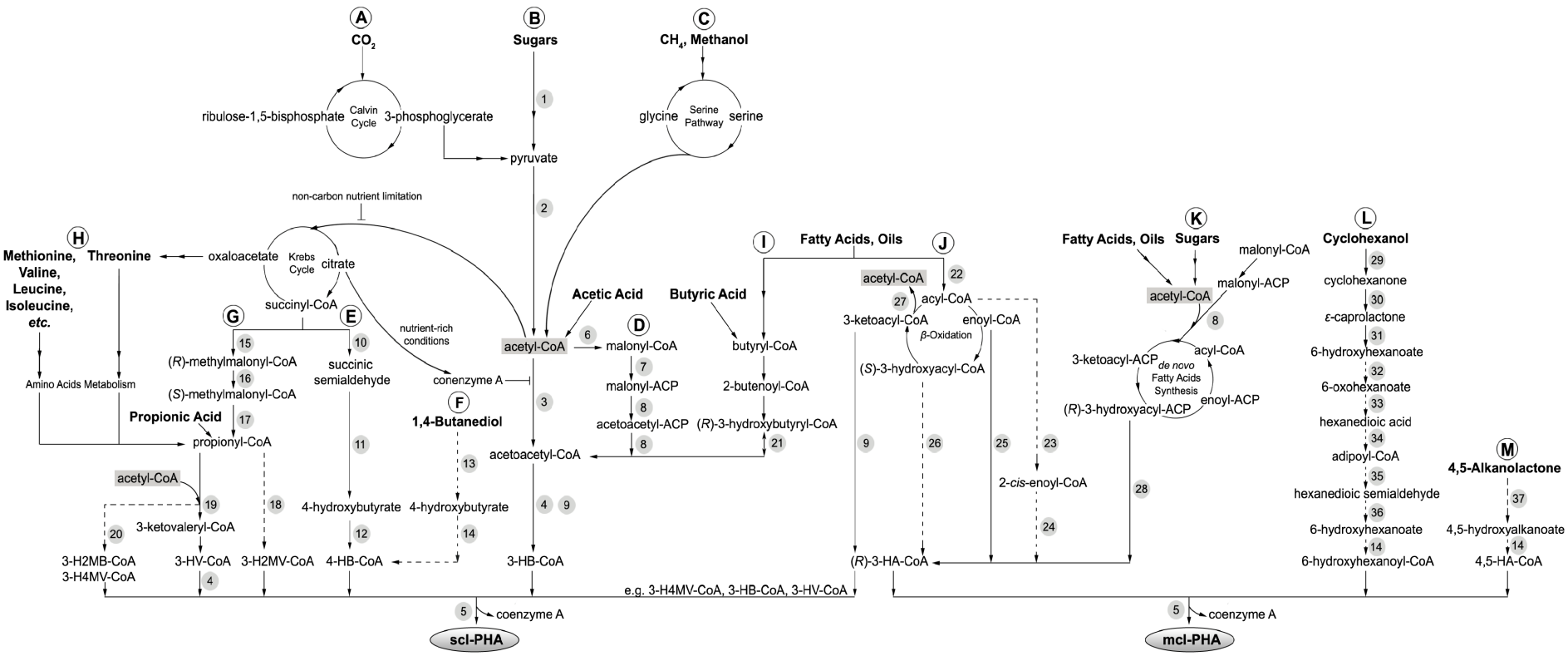


Table 1. Enzymes involved in PHA biosynthesis pathways.

No.	Enzyme	Abbreviation	Species	Reference
1	Glyceraldehyde-3-phosphate dehydrogenase	-	*Cupriavidus necator*	[22]
2	Pyruvate dehydrogenase complex	-	*Cupriavidus necator* and *Burkholderia cepacia*	[22]
3	3-Ketothiolase	PhaA	*Cupriavidus necator*	[23]
4	NADPH-dependent acetoacetyl-CoA reductase	PhaB	*Cupriavidus necator*	[23]
5	PHA synthase	PhaC	*Cupriavidus necator* and various	[12,23]
6	Acetyl-CoA carboxylase	ACC	*Escherichia coli* K-12 MG1655	[24]
7	Malonyl-CoA:ACP transacylase	FabD	*Escherichia coli* K-12 MG1655	[24]
8	3-Ketoacyl carrier protein synthase	FabH	*Escherichia coli* K-12 MG1655	[24,25]
9	NADPH-dependent 3-Ketoacyl reductase	FabG	*Pseudomonas aeruginosa*	[26]
10	Succinic semialdehyde dehydrogenase	SucD	*Clostridium kluyveri*	[27]
11	4-Hydroxybutyrate dehydrogenase	4HbD	*Clostridium kluyveri*	[27]
12	4-Hydroxybutyrate-CoA:CoA transferase	OrfZ	*Clostridium kluyveri*	[27]
13	Alcohol dehydrogenase, putative	-	*Aeromonas hydrophila* 4AK4	[28]
14	Hydroxyacyl-CoA synthase, putative	-	Mutants and recombinants of *Cupriavidus necator*	[29]
15	Methylmalonyl-CoA mutase	Sbm	*Escherichia coli* W3110	[30]
16	Methylmalonyl-CoA racemase	-	*Nocardia corallina*	[31]
17	Methylmalonyl-CoA decarboxylase	YgfG	*Escherichia coli* W3110	[30]
18	Ketothiolase, putative	-	-	[32]
19	3-Ketothiolase	BktB	*Cupriavidus necator*	[33]
20	Ketothiolase, putative	-	-	[32]
21	NADPH-dependent acetoacetyl-CoA reductase	-	*Rhizobium* (*Cicer*) sp. CC 1192	[34]
22	Acyl-CoA synthetase	FadD	*Pseudomonas putida* CA-3 and *Escherichia coli* MG1655	[35,36]
23	Acyl-CoA oxidase, putative	-	-	[37]
24	Enoyl-CoA hydratase I, putative	-	-	[37]
25	(*R*)-Enoyl-CoA hydratase	PhaJ	*Pseudomonas putida* KT2440	[38]
26	Epidermase	-	-	[37]
27	3-Ketoacyl-CoA thiolase	FadA	*Pseudomonas putida* KT2442	[39]
28	3-Hydroxyacyl-ACP:CoA transacylase	PhaG	*Pseudomonas mendocina*	[40]
29	Cyclohexanol dehydrogenase	ChnA	*Acinetobacter* sp. SE19 and *Brevibacterium epidermidis* HCU	[41]
30	Cyclohexanone monooxygenases	ChnB	*Acinetobacter* sp. SE19 and *Brevibacterium epidermidis* HCU	[41]

Table 1. ***Cont.***

No.	Enzyme	Abbreviation	Species	Reference
31	Caprolactone hydrolase	ChnC	*Acinetobacter* sp. SE19 and *Brevibacterium epidermidis* HCU	[41]
32	6-Hydroxyhexanoate dehydrogenase	ChnD	*Acinetobacter* sp. SE19 and *Brevibacterium epidermidis* HCU	[41]
33	6-Oxohexanoate dehydrogenase	ChnE	*Acinetobacter* sp. SE19 and *Brevibacterium epidermidis* HCU	[41]
34	Semialdehyde dehydrogenase, putative	-	-	[11]
35	6-hydroxyhexanoate dehydrogenase, putative	-	-	[11]
36	Hydroxyacyl-CoA synthase, putative	-	-	[11]
37	Lactonase, putative	-	Mutants and recombinants of *Cupriavidus necator*	[29]

3. PHA-Producing Microbial Strains from Culture Collections

The PHA bioaccumulation trait is widespread among the bacterial and archaeal domains with PHA-producing microbes occurring in more than 70 bacterial and archaeal genera [4,42]. Bioaccumulated PHA is stored in the form of intracellular lipid granules in these microbes [43]. Acting as biocatalysts, these PHA-producing microorganisms enable the coupling of a myriad of carbon catabolic pathways together with PHA anabolic pathways, thereby playing a key role in the diversification of PHA production from various carbon sources. These carbon sources include saccharides (e.g., fructose, maltose, lactose, xylose, arabinose, *etc.*), *n*-alkanes (e.g., hexane, octane, dodecane, *etc.*), *n*-alkanoic acids (e.g., acetic acid, propionoic acid, butyric acids, valeric acid, lauric acid, oleic acid, *etc.*), *n*-alcohols (e.g., methanol, ethanol, octanol, glycerol, *etc.*), and gases (e.g., methane and carbon dioxide) [1,44]. Wastestreams, which provide a free source of carbons, have also been identified for PHA production [45]. These include waste frying oil, vinegar waste, waste fats, food waste, agricultural waste, domestic wastewater, plant oil mill effluents, crude glycerol from biodiesel production, plastic waste, landfill gas, *etc.* The deposition of some PHA-producing microbial strains in culture collections has made these strains commercially available. Microbial strains from culture collections are generally well-documented in terms of their genetics and biochemistry underlying carbon assimilation and PHA accumulation. With this knowledge, it enables the appropriate microbes to be selected according to the targeted carbon source, facilitating the rapid start-up of PHA-related research and/or industrial production. Table 2 provides a summary of carbon substrate utilization and PHA production by deposited bacterial and archaeal strains.

Table 2. PHA-producing microbial strains available in culture collections.

Microorganism	Culture collection number [b]	Carbon source	PHA monomer or polymer [c]	PHA content (%CDM)	Average PHA productivity (g L^{-1} h^{-1})	Reference
Gram-negative bacteria						
Azohydromonas australica (formerly *Alcaligenes latus*)	ATCC 29713, DSM 1124, IAM 12664, LMG 3324	Malt waste	P3HB	70.1	0.445	[46]
Azohydromonas lata (formerly *Alcaligenes latus*)	ATCC 29714, DSM 1123, IAM 12665, LMG 3325	Sucrose	P3HB	50.0–88.0	0.050–4.940	[47–49]
		Fructose, glucose	P3HB	76.5–79.4	0.121–0.128	[50]
Azotobacter beijerinckii	DSM 1041, NCIB 11292	Glucose	P3HB	24.8	0.090	[51]
Burkholderia cepacia (formerly *Pseudomonas multivorans* and *Pseudomonas cepacia*)	ATCC 17759, DSM 50181, NCIB 9085	Xylose	P3HB	58.4	NG	[52]
		Glycerol	P3HB	31.3	0.103	[53]
		Fructose, glucose, sucrose	P3HB	50.4–59.0	NG	[50]
Burkholderia sp. USM	JCM 15050	Lauric acid, myristic acid, oleic acid, palmitic acid, stearic acid	P3HB	1.0–69.0	NG	[54]
Caulobacter vibrioides (formerly *Caulobacter crescentus*)	DSM 4727	Glucose	P3HB	18.3	0.008	[55]
Cupriavidus necator H16 (formerly *Hydrogenomonas eutropha* H16, *Alcaligenes eutrophus* H16, *Ralstonia eutropha* H16 and *Wautersia eutropha* H16)	ATCC 17699, DSM 428, KCTC 22496, NCIB 10442	Fructose, glucose	P3HB	67.0–70.5	0.052–0.067	[50]
		4-Hydroxyhexanoic acid	P3HB	76.3–78.5	NG	[56]
		Corn oil, oleic acid, olive oil, palm oil	P3HB	79.0–82.0	0.041–0.047	[57]
		Acetate, butyrate, lactic acid, propionic acid	3HB, 3HV	3.9–40.7	0.001–0.037	[58]
		CO_2	P3HB	88.9	0.230	[59]

Table 2. *Cont.*

Cupriavidus necator (formerly *Hydrogenomonas eutropha*, *Alcaligenes eutrophus* N9A, *Ralstonia eutropha* N9A and *Wautersia eutropha*)	DSM 518	4-Hydroxyhexanoic acid	P3HB	65.8–66.2	NG	[56]
Cupriavidus necator (formerly *Hydrogenomonas eutropha*, *Alcaligenes eutrophus* TF93, *Ralstonia eutropha* TF93 and *Wautersia eutropha*)	ATCC 17697, DSM 531	4-Hydroxyhexanoic acid	P3HB	67.2	NG	[56]
		CO_2	P3HB	60.0	0.600	[60]
Cupriavidus necator [a] (formerly *Hydrogenomonas eutropha*, *Alcaligenes eutrophus*, *Ralstonia eutropha* and *Wautersia eutropha*)	CECT 4623, KCTC 2649, NCIMB 11599	Glucose	P3HB	76.0	2.420	[61]
		Potato starch, saccharified waste	P3HB	46.0	1.470	[62]
Cupriavidus necator (formerly *Hydrogenomonas eutropha*, *Alcaligenes eutrophus*, *Ralstonia eutropha* and *Wautersia eutropha*)	DSM 545	Molasses	P3HB	31.0–44.0	0.080–0.120	[63]
		Glucose, propionic acid	P3HB3HV	80.0	0.820	[64]
		Waste glycerol	P3HB	14.8–36.1	0.330–4.200	[65]
Halomonas boliviensis LC1	ATCC BAA-759, DSM 15516	Hydrolyzed starch	P3HB	56.0	NG	[66]
Hydrogenophaga pseudoflava	ATCC 33668, DSM 1034	Lactose, sucrose	P3HB3HV	20.2–62.5	0.018–0.117	[67]
		Hydrolyzed whey and valerate	P3HB3HV	40.0	0.050	[68]
Methylobacterium extorquens	ATCC 55366	Methanol	P3HB	40.0–46.0	0.250–0.600	[69]
Methylobacterium extorquens	ATCC 8457, DSM 1340, NCIB 2879, NCTC 2879	Methanol	P3HB	35.0–62.3	0.183–0.980	[70,71]
Methylocystis sp. GB25 [a]	DSM 7674	Methane	P3HB	51.0	NG	[72]
Novosphingobium nitrogenifigens Y88	DSM 19370, ICMP 16470	Glucose	P3HB	81.0	0.014–0.021	[73]
Paracoccus denitrificans	ATCC 17741, DSM 413	*n*-Pentanol	P3HV	22.0–24.0	NG	[74]

Table 2. *Cont.*

Pseudomonas aeruginosa	NCIM 2948	Cane molasses, fructose, glucose, glycerol, sucrose	P3HB	12.4–62.0	0.012–0.110	[75]
Pseudomonas aeruginosa PAO1	ATCC 47085	Oil and wax products from polyethylene (PE) pyrolysis	mcl-PHA	25.0	NG	[76]
Pseudomonas frederiksbergensis GO23 [a]	NCIMB 41539	Terephthalic acid from polyethylene terephthalate (PET) pyrolysis	mcl-PHA	24.0	0.004	[77]
Pseudomonas marginalis	DSM 50276	1,3-Butanediol, octanoate	scl-mcl-PHA, mcl-PHA	11.9–31.4	NG	[78]
Pseudomonas mendocina	ATCC 25411, DSM 50017	1,3-Butanediol, octanoate	scl-mcl-PHA	13.5–19.3	NG	[78]
Pseudomonas oleovorans	ATCC 8062, DSM 1045	4-Hydroxyhexanoic acid	scl-mcl-PHA	18.6	NG	[56]
Pseudomonas putida CA-3 [a]	NCIMB 41162	Styrene	mcl-PHA	31.8	0.063	[79]
		Styrene from polystyrene (PS) pyrolysis	mcl-PHA	36.4	0.033	[80]
Pseudomonas putida GO16 [a]	NCIMB 41538	Terephthalic acid from polyethylene terephthalate (PET) pyrolysis	mcl-PHA	27.0	~0.005, 0.008 [d]	[77]
Pseudomonas putida GO19 [a]	NCIMB 41537	Terephthalic acid from polyethylene terephthalate (PET) pyrolysis	mcl-PHA	23.0	~0.005, 0.008 [d]	[77]
Pseudomonas putida GPo1 (formerly *Pseudomonas oleovorans*)	ATCC 29347	Alkenes, *n*-alkanes	mcl-PHA	2.0–28.0	NG	[81]
		n-Alkanoates	scl-mcl-PHA, mcl-PHA	5.0–60.0	NG	[82,83]

Table 2. *Cont.*

Pseudomonas putida KT2440	ATCC 47054	Nonanoic acid	mcl-PHA	26.8–75.4	0.250–1.110	[84]
		4-Hydroxyhexanoic acid	mcl-PHA	25.3–29.8	NG	[56]
		Glucose	mcl-PHA	32.1	0.006	[85]
Pseudomonas putida F1	ATCC 700007, DSM 6899	Benzene, ethylbenzene, toluene	mcl-PHA	1.0–22.0	NG	[86]
Pseudomonas putida mt-2	NCIMB 10432	Toluene, *p*-xylene	mcl-PHA	22.0–26.0	NG	[86]
		Acetic acid, citric acid, glucose, glycerol, octanoic acid, pentanoic acid, succinic acid	mcl-PHA	4.0–77.0	NG	[87]
Thermus thermophilus HB8	ATCC 27634, DSM 579	Whey	scl-mcl-PHA	35.6	0.024	[88]
Gram-positive bacteria						
Bacillus megaterium	DSM 90	Citric acid, glucose, glycerol, succinic acid	P3HB	9.0–50.0	NG	[87]
Bacillus megaterium	CCM 1464, DSM 509, IFO 12109, NBRC 12109	Citric acid, glucose, glycerol, succinic acid, octanoic acid	P3HB, scl-mcl-PHA, mcl-PHA	3.0–48.0	NG	[87]
Various *Bacillus* spp. type strains	Refer to [89]	Acetate, *n*-alkanoate, 3-hydroxybutyrate, propionate, sucrose, valerate	3HB, 3HV, 3HHx	2.2–47.6	NG	[89]
Corynebacterium glutamicum	ATCC 15990, DSM 20137, NCIB 10337	Acetic acid, citric acid, glucose, glycerol, succinic acid	P3HB, mcl-PHA	4.0–32.0	NG	[87]
Corynebacterium hydrocarboxydans	ATCC 21767	Acetate, glucose	3HB, 3HV	8.0–21.0	NG	[90]
Microlunatus phosphovorus	DSM 10555, JCM 9379	Glucose	3HB, 3HV	20.0–30.0	NG	[91]
Nocardia lucida	NCIMB 10980	Acetate, succinate	3HB, 3HV	7.0–20.0	NG	[90]

Table 2. *Cont.*

Rhodococcus sp. [a]	NCIMB 40126	Acetate, 2-alkenoate, 1,4-butanediol, 5-chlorovalerate, fructose, glucose, hexanoate, 4-hydroxybuytrate, lactate, molasses, succinate, valerate	P3HB3HV	4.0–53.0	NG	[90]
Various *Streptomyces* spp. type culture	Refer to [89]	Glucose	P3HB	1.2–82.0	NG	[89]
Archaea						
Haloferax mediterranei	ATCC 33500, CCM 3361, DSM 1411	Vinasse	P3HB3HV	50.0–73.0	0.050–0.210	[92]
		Hydrolyzed whey	P3HB3HV	72.8	0.090	[93]
		Glycerol and crude glycerol from biodiesel production	P3HB3HV	75.0–76.0	0.120	[94]
Various archaeal strains	Refer to [95]	Fructose, glucose, glycerol	P3HB, P3HB3HV	0.8–22.9	<0.001–0.021	[95]

[a] Refers to patent strain; [b] ATCC, American Type Culture Collection (Manassas, VA, USA); CCM, Czech Collection of Microorganisms (Masaryk University, Brno, Czech Republic); CECT, Colección Española de Cultivos Tipo (Universidad de València, Edificio de Investigación, Burjassot, Spain); DSM, German Collection of Microorganisms and Cell Cultures GmbH (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany); IAM, Institute of Molecular and Cellular Biosciences (The University of Tokyo, Japan [collection transferred to JCM]); ICMP, International Collection of Microorganisms from Plants (Plant Diseases Division, DSIR, Auckland, New Zealand [also known as PDDCC]); IFO, Institute for Fermentation, Osaka (Yodogawa-ku, Osaka, Japan [collection transferred to NBRC]); JCM, Japan Collection of Microorganisms (RIKEN BioResource Center, Tsukuba, Ibaraki, Japan); KCTC, Korean Culture Center of Microorganisms (Department of Food Engineering, Yonsei University, Seoul, Republic of Korea); LMG, Laboratorium voor Microbiologie, Universiteit Gent (Gent, Belgium); NBRC, NITE Biological Resource Center (Department of Biotechnology, National Institute of Technology and Evaluation, Kisarazu, Chiba, Japan); NCIB, National Collection of Industrial Bacteria (Torry Research Station, Aberdeen, Scotland, UK [incorporated with NCIMB]); NCIM, National Collection of Industrial Microorganisms (National Chemical Laboratory, India); NCIMB, National Collections of Industrial Food and Marine Bacteria (Aberdeen, Scotland, UK); NCTC, National Collection of Type Cultures (Public Health England, UK); [c] PHA is indicated as monomers unless nuclear magnetic resonance (NMR) verification was performed; [d] Refers to the maximal PHA productivity rate reported; CDM: cell dry mass; NG: not given.

3.1. Gram-Negative Bacteria

To date, most PHA-producing bacteria were found to be Gram-negative bacteria [4]. Scl-PHA is usually synthesized in high amounts by bacterial species from *Azohydromonas*, *Burkholderia*, and *Cupriavidus*. *A. lata* (ATCC 29714) is reportedly capable of producing between 50% and 88% cell dry mass (%CDM) of poly(3-hydroxybutyrate) (P3HB) from various sugars including glucose, fructose and sucrose [47–50] while *Burkholderia* sp. USM (JCM 15050) could synthesize up to 69 %CDM of P3HB from fatty acids [54]. *C. necator* is a hydrogen-oxidizing (Knallgas) bacterium which could produce PHA by fixing CO_2 through Calvin Cycle [21]. The well-characterized *C. necator* H16 (ATCC 17699) could shift between heterotrophic and autotrophic mode for growth and PHA production. Emerging evidence also suggested that both heterotrophic and autotrophic PHA biosynthesis can occur concurrently in this bacterium [16]. The unique physiology of *C. necator* H16 (ATCC 17699) meant that it was able to utilize chemically-diverse carbon substrates such as CO_2, sugars (*i.e.*, glucose and fructose), *n*-alkanoic acids (*i.e.*, 4-hydroxyhexanoic acid), vegetable oils (*i.e.*, olive oil, corn oil, and palm oil) for P3HB accumulation in the range of 67 to 88.9 %CDM [50,56,57,59] (Table 2). Owing to the high scl-PHA production capacity, wild-type and mutant bacterial species from the aforementioned genera are widely employed in industrial PHA production. Recent updates on the application of Gram-negative bacteria in industrial-scale PHA production could be found in review articles by Chen [96] and Chanprateep [97].

Scl-PHA was also produced by Gram-negative methylotrophs. *Methylobacterium extorquens* (ATCC 55366) and *Paracoccus denitrificans* (ATCC 17741) produced up to 46 %CDM P3HB from methanol and up to 24 %CDM poly(3-hydroxyvalerate) (P3HV) from *n*-pentanol, respectively [69,74]. Due to the cheaper cost of methanol compared to pure sugar substrates, the use of methylotrophs for industrial scl-PHA production could reduce PHA cost. However, further studies would be required to enhance the PHA content and PHA productivity of methylotrophs before they can be considered as attractive inoculum alternatives for industrial scl-PHA production [3].

The production of mcl-PHA is often reported in *Pseudomonas* sp., and usually occurs at PHA contents between about 1 and 30 %CDM (Table 2). Higher mcl-PHA contents have also been observed in *P. putida* mt-2 (NCIMB 10432), which could produce up to 77 %CDM mcl-PHA from octanoic acid [87]; and *P. putida* KT2440 (ATCC 47054), a mutant of *P. putida* mt-2 lacking the TOL plasmid, which could produce up to 75.4 %CDM using nonanoic acid [84]. Aside from mcl-PHA, some Pseudomonad species have also been reported to synthesize scl-mcl-PHA copolyesters. These species include *P. marginalis* (DSM 50276), *P. mendocina* (ATCC 25411), *P. putida* GPo1 (ATCC 29347), and *P. oleovorans* (ATCC 8062) when *n*-alkanoates and 1,3-butanediol were provided as carbon sources [56,78,82]. For most of these Pseudomonad species, 3-hydroxybutyrate (3HB) is typically incorporated as a minor constituent in scl-mcl-PHA (between less than 1 mol% and 7.8 mol%) [78,82]. An exception is *P. oleovorans* (ATCC 8062) where its cultivation on 4-hydroxyhexanoic acid resulted in a copolymer predominated by 3HB (92.4 mol%) [56]. Pseudomonads are also well-known for their bioremediation properties including the biodegradation recalcitrant and/or toxic aromatic carbon substrates [98], and have been successfully applied in the treatment of contaminated effluents, exhaust gas and soils [99–101]. Recent studies demonstrated that aromatic-degraders *P. putida* F1 (DSM 6899), *P. putida* mt-2 (NCIMB 10432), and *P. putida* CA-3 (NCIMB 41162) could bioconvert

toxic pollutants benzene, toluene, ethylbenzene, xylene (BTEX) and styrene to mcl-PHA [86]; *P. putida* CA-3 (NCIMB 41162) and other Pseudomonads including *P. aeruginosa* PAO1 (ATCC 47085), *P. frederiksbergensis* GO23 (NCIMB 41539), *P. putida* GO16 (NCIMB 41538) and *P. putida* GO19 (NCIMB 41537) could utilize crude pyrolysis products from various plastics (*i.e.*, polystyrene [PS], polyethylene [PE] and polyethylene terephthalate [PET]) for mcl-PHA production [76,77,80], which offers the potential benefit to off-set waste treatment cost through PHA recovery.

PHA accumulation has been observed in Gram-negative extremophilic bacteria as well. These bacteria accumulate PHA under unique cultivation conditions with either high salinity or elevated temperatures. The halophilic *Halomonas boliviensis* LC1 (DSM 15516) could grow and produce 56 %CDM of scl-PHA P3HB from starch hydrolysate under moderately saline conditions (0.77 M NaCl) [66] while the thermophilic *Thermus thermophilus* HB8 (ATCC 27634) synthesized up to 35.6 %CDM of scl-mcl-PHA copolymer from whey at a high cultivation temperature of 70 °C [88]. Compared to other Gram-negative bacteria, extremophiles are advantageous in terms of their lower sterility demand as well as their potential for direct application with waste effluents originally high in salt concentrations or temperatures, eliminating the need and cost involved for pre-treatment of waste effluents.

The main concern with Gram-negative bacteria however, is the presence of lipopolysaccharide (LPS) endotoxins in the bacteria's outer cell membrane, which may co-purify with crude PHA polymer during the extraction process [7]. LPS endotoxin is a pyrogen which can elicit a strong inflammatory response [102], rendering the PHA polymer unsuitable for biomedical applications. Removal of LPS endotoxin can be achieved through the treatment of PHA polymer with oxidizing agents (*i.e.*, sodium hypochlorite and NaOH, ozone, hydrogen peroxide, and benzoyl peroxide), with repeated solvent extractions, or with solvent extraction followed by purification with activated charcoal [7,103,104]. These methods however, increase the overall cost of PHA production and lead to changes in PHA polymer properties (*i.e.*, reduction in molecular mass and polydispersity).

3.2. Gram-Positive Bacteria

PHA production in Gram-positive bacteria has been reported in genera *Bacillus*, *Caryophanon*, *Clostridium*, *Corynebacterium*, *Micrococcus*, *Microlunatus*, *Microcystis*, *Nocardia*, *Rhodococcus*, *Staphylococcus*, *Streptomyces* [4]. Compared to Gram-negative bacteria, Gram-positive bacteria were mostly found to produce scl-PHA [89] and at lower PHA contents between about 2 and 50 %CDM (Table 2), which is why Gram-positive bacteria have yet to be adopted for commercial PHA production. A high scl-PHA content of 82 %CDM has been previously reported for *Streptomyces* sp. (ATCC 1238) growing on glucose, but the value may be an overestimation by crotonic acid assay [89,105]. Some microbial strains are able to synthesize mcl-PHA or scl-mcl-PHA copolymers if suitable carbon substrates and conditions are provided. A study by Shahid *et al.* [87] demonstrated that *B. megaterium* (DSM 509) formed exclusively P3HB from glycerol and succinic acid in a mineral medium supplemented with nitrogen, but started to synthesize scl-mcl-PHA upon sub-culturing to the same medium and in the absence of nitrogen. In the same bacterium, the formation of exclusively mcl-PHA (48 %CDM) was observed when it was cultured on octanoic acid in the absence of nitrogen [87].

Despite generally accumulating lower amounts of PHA, Gram-positive bacteria are advantageous over Gram-negative bacteria owing to their lack of LPS which may make them a better source of PHA raw material for biomedical applications [89]. However, some Gram-positive bacteria are known to produce lipidated macroamphiphiles including lipoglycans and lipoteichoic acids (LTA), which have immunogenic properties similar to LPS [102]. The bacterial genera *Corynebacterium*, *Nocardia*, *Rhodococcus* reportedly produce lipoglycans [106–108]; and while LTA production occurs in the genera *Bacillus*, *Clostridium*, and *Staphylococcus* [109,110], some PHA-producing strains from these genera lack LTA [111,112]. Further investigation will be required to verify if there are alternative lipidated macroamphiphiles in Gram-positive and PHA-producing bacteria. At present, the immunogenic effects of lipidated macroamphiphiles in PHA remain unknown. Future *in vitro* or *in vivo* evaluation studies would be imperative to evaluate the suitability of PHA, derived from Gram-positive bacteria, for biomedical applications [89].

3.3. Archaea

PHA is also found in archaea but to date however, its discovery has been limited to haloarchaeal species, specifically the genera *Haloferax*, *Halalkalicoccus*, *Haloarcula*, *Halobacterium*, *Halobiforma*, *Halococcus*, *Halopiger*, *Haloquadratum*, *Halorhabdus*, *Halorubrum*, *Halostagnicola*, *Haloterrigena*, *Natrialba*, *Natrinema*, *Natronobacterium*, *Natronococcus*, *Natronomonas*, and *Natronorubrum* [95]. Haloarchaea are the extremely halophilic members of the archaea domain, which require high salt concentrations for normal enzyme activity, growing at saturation conditions of up to 6 M NaCl [113]. Haloarchaea have been reported to synthesize PHA from glucose, volatile fatty acids and more complex carbon sources such as starch, whey hydrolysate, vinasse and crude glycerol from biodiesel production [92–95]. The type of PHA synthesized appeared to be exclusively scl-PHA homopolymer containing either 3HB or 3HV monomers, and/or scl-PHA heteropolymer containing both 3HB and 3HV monomers [42,95]. Many PHA-producing haloarchaeal cultures are currently available from culture collections but most of them produce PHA at low cellular contents between 0.8 to 22.9 %CDM (Table 2) [95].

At present, the best PHA producer is *Haloferax mediterranei* (DSM 1411), which requires 2 to 5 M NaCl for growth and can accumulate high PHA levels between 50 and 76 %CDM [92–94]. *H. mediterranei* (DSM 1411) could be an attractive candidate for PHA production as the hypersaline conditions, required for its growth and PHA cultivation, meant that that very few contaminating organisms can survive thereby reducing the sterility requirements and its associated cost (*i.e.*, process piping, instrumentation and insulation, electricity for steam generation, *etc.*) [92]. However, when compared to moderately halophilic bacteria such as *H. boliviensis* LC1 (DSM 15516), the extreme salinity required by haloarchaea can be a bane to PHA production as the high salt concentration incur higher chemical cost and accelerates the corrosion of stainless steel fermentors [66]. Nevertheless, haloarchaea are advantageous over halophilic bacteria in the ease of PHA recovery. PHA recovery from halophilic bacteria typically requires the use of chemical, enzymatic or mechanical method for cell wall disruption to release intracellular PHA granules, and these methods could account for up to 50% or more of the overall PHA production cost [114]. Extraction solvents such as chloroform and acetone also posed potential environmental hazards if their utilization and disposal are mismanaged.

Conversely, haloarchaea undergo cell lysis in distilled water and release PHA granules that can be recovered by low speed centrifugation [42]. This makes PHA recovery from haloarchaea a relatively easy, less chemical- and energy-intensive process, which translates into lower extraction cost, and has lower ecological footprint.

3.4. Formulation of Defined Co-Cultures Using Deposited Microbial Strains

It has been estimated that at least 30% or more of PHA cost is attributed to carbon, nutrients and aeration cost [115]. This has prompted intensive research to diversify PHA production from cheaper carbon sources and waste carbon as a means to lower PHA cost [45]. Using waste resources for PHA production is particularly challenging as wastestream is often a complicated mixture of carbon substrates, some of which cannot be assimilated for PHA production or are inhibitory to a single microbial culture. Defined co-culture on the other hand, is a culture involving two or more microbes, and has been successfully applied to bioconvert more complex carbon feed into PHA [86,116]. The commercial availability of culture collection microbes enables the rapid formulation of defined co-culture using deposited microbial strains in accordance to the characteristics of the carbon feedstock for PHA production.

Co-culturing of microbial strains, with different carbon utilization, enables the synergistic bioconversion of carbon substrate mixture into PHA. This was exemplified in the bioconversion of synthetic plastic pyrolysis oil, containing toxic compounds BTEX and styrene, into PHA by a co-culture system consisting of three *P. putida* strains (*i.e.*, strains F1, mt-2 and CA-3) [86]. On their own, *P. putida* F1 (DSM 6899) could metabolize BTE to form mcl-PHA while *P. putida* mt-2 (NCIMB 10432) and *P. putida* CA-3 (NCIMB 41162) could do so with TX and styrene, respectively. Monoculture cultivations using single assimilable monoaromatic substrate led to bacterial growth of between 0.3 and 0.8 g L^{-1} and cellular PHA yields of between 0.048 and 0.26 g L^{-1} while no bacterial growth was observed for non-assimilable monoaromatic substrate. As a co-culture growing on BTEX and styrene mixture however, higher biomass yield (1 g L^{-1}) was attained and overall PHA yield (0.25 g L^{-1}) was equivalent to that observed in the best-performing monoculture. This suggests that under co-culture conditions, the cooperative metabolism amongst bacterial members not only maximizes the utilization of various carbon substrates for PHA production but may also enhance the robustness of individual strains through the removal of inhibitory compounds, providing co-culture an advantage over monoculture systems.

Co-culturing of microbial strains can be applied to expand the repertoire of carbon substrates for PHA formation by using one microorganism to convert carbon substrate into a metabolite which can be efficiently consumed by a second microorganism for PHA production. This was demonstrated for co-cultures of *C. necator* H16 (ATCC 17699) with lactate-producing bacteria. While *C. necator* H16 (ATCC 17699) is capable of high PHA accumulation, it can only readily metabolize and accumulate PHA from organic acids such as acetate, butyrate and lactate, and was unable to do so for common sugars such as glucose and xylose [117]. To overcome this problem, *Lactobacillus delbrueckii* (IAM 1928) and *Lactococcus lactis* IO-1 (JCM 7638) were used to convert glucose and xylose, respectively, into lactate which can be easily converted to P3HB by *C. necator* H16 (ATCC 17699) [116,118]. While a two-stage fermentation system was used for PHA production from xylose-derived lactate [118], the

feasibility of using single-stage fermentation system has also been demonstrated by Ganduri *et al.* [116] for PHA production from glucose-derived lactate, achieving up to 36.6 g L^{-1} of P3HB and displaying notably higher PHA productivity over monoculture system [50].

Co-culture system may also have the potential to mitigate biogas, produced from anaerobic digesters or landfills, while achieving aerobic PHA production without aeration supply. In a proof-of-concept study by van der Ha *et al.* [119], a gas mixture of 60% CH_4 and 40% CO_2 was photosynthetically fixed by an algal *Scenedesmus* sp. monoculture to produce 60% CH_4 and close to 40% O_2. The resultant gas components O_2 and CH_4 provided the aerobic condition and carbon substrate, respectively, required for P3HB accumulation by a second monoculture of methane-oxidizing bacteria *Methylocystis parvus* (NCIMB 11129). Under three cycles of feast-and-famine regime, cellular PHA content in *M. parvus* (NCIMB 11129) reached a maximum of 29.5 %CDM with 243 mg P3HB produced for every 1 g of CH_4–C consumed. Co-culturing of *Scenedesmus* sp. and *M. parvus* (NCIMB 11129) led to a conversion of 98% of CH_4–C and CO_2–C as algal and bacterial biomass but the PHA yield was not reported. Hence, the efficacy of the co-culture system for simultaneous biogas treatment and PHA production remains to be verified.

At present, the application of defined co-cultures for PHA production is still in its infancy. While the existing knowledge and commercial availability of deposited microbial strains facilitates the rapid formulation of defined co-cultures, there are still technical challenges that need to be circumvented. For single-stage co-culture fermentation systems, one of the main challenges is providing cultivation parameters for efficient and effective bioconversion of carbon substrates into PHA. Parameters such as inoculum concentration, dissolved oxygen, pH, temperature, cultivation time, carbon and nutrients feed rate, and secondary metabolites production rate would need to be fine-tuned in order to maximize the bioconversion process. For an example, *L. delbrueckii* (IAM 1928) requires anaerobic conditions for conversion of glucose into lactate while *C. necator* H16 (ATCC 17699) requires aerobic conditions for conversion of lactate into PHA. To overcome this problem, Ganduri *et al.* [116] employed an imperfectly mixed bioreactor to create non-uniform spatial distribution of dissolved oxygen, achieving 91.5% of P3HB theoretical yield (*i.e.*, 36.6 g L^{-1} of P3HB) within 30 h.

Another challenge is the harvesting and separation of PHA-containing biomass from non-PHA-containing biomass, particularly for co-cultures comprising of PHA-accumulating and non-PHA-forming microorganisms as the presence of non-PHA-containing biomass would increase the extraction cost of PHA. Compared to single-stage fermentation approach, two-stage fermentation approach may be more advantageous as it enables finer control over cultivation parameters and harvesting of PHA-accumulating biomass. However, higher capital and operation cost are associated with two-stage fermentation system. Ultimately, the type of systems chosen would greatly depend on the microbial characteristics of the co-culture as well as the economic viability of the bioprocess.

4. Techniques for Detecting PHA and PHA Production Potential in Microbes

Various methods are available for the detection and analysis of intracellular microbial PHA. These methods are useful in identifying novel PHA-producing microbes or for routine monitoring of PHA production bioprocesses. Table 3 provides a summary of these methods, sample characteristics and preparation, method execution, as well as their strengths and limitations.

Table 3. Methods for detection of PHA in biomass and PHA production capacity.

Method	Characteristic	Sample	Sample preparation	Typical conditions	Advantage	Limitation	Reference
Polymerase chain reaction (PCR) gene detection	*phaC* gene encoding enzyme PHA synthase	50–500 ng of DNA material or a single bacterial colony	DNA extraction or freeze/thaw cells to release DNA material	PCR thermal cycler temperature program for specific primer sets	Requires small sample size, high sensitivity and specificity, high throughput	Primers are inadequate for detection of all *phaC* genes, and prone to detection errors	[120,121]
Nile red and Nile blue A staining	Intracellular PHA granule structures	Bacterial colonies on agar medium	Add 0.5 μg mL^{-1} of Nile red or Nile blue A to sterilized agar growth medium	Expose the agar plates to ultraviolet light (312 nm) after appropriate cultivation periods	Enables direct observation of live and actively-growing cells, requires small sample size, rapid analysis, allows differentiation between scl- and mcl-PHA under flow cytometry analysis, high throughput	Method cannot discriminate between lipids and PHAs, and is also less effective at distinguishing between PHA-negative and PHA-positive strains of Gram-positive bacteria	[122–125]
		Microscope slide containing heat-fixed bacterial cells smear	Stain slide with 1% Nile blue A at 55 °C for 10 min. Remove excess stain with tap water before staining with 8% acetic acid for 1 min. Rinse slide with tap water and blot dry with bibulous paper	Examine slide with an epifluorescence microscope with an excitation wavelength of 460 nm			
		1 mL of cell culture with optical density at 600 nm (OD_{600}) of 1.0 or less	Add 2.0–10 μg mL^{-L} of Nile red to 1 mL cell culture and incubate in the dark for 15 min	Epifluorescence microscopy imaging with FITC filter with an excitation wavelength of 470–490 nm and an emission wavelength of 505 nm or fluorescence spectroscopy analysis at excitation wavelength of 488 nm and an emission wavelength of 590 nm and 575 nm for scl-PHA and mcl-PHA, respectively			

Table 3. *Cont.*

Transmission electron microscopy (TEM)	Intracellular PHA granule structures	1–3 mL of exponential or stationary phase cell culture	Cell fixation with glutaraldehyde in phosphate buffer, followed by post-fixation with osmium tetroxide. Dehydrate fixated cells through a graded acetone series before acetone-resin infiltration and resin polymerization. Cut resins into ultrathin sections (70–100 nm thickness) with an ultramicrotome	View with an accelerating voltage of 200 kV and perform imaging at magnifications of 25,000–40,000×	High magnification enables direct visualization and size measurements of PHA granules	Tedious sample preparation involving radioactive and hazardous chemicals, cells are killed during sample preparation	[43]
Crotonic acid assay	Quantitative determination of P3HB	5–50 µg P3HB	Add 10 mL concentrated H_2SO_4, and heat at 100 °C for 10 min to form crotonic acid	Measure UV absorbance at 235 nm	Easy operation, inexpensive per analysis, specific to P3HB determination	Result can be interfered by other endogenous components and matrix interferences can result in overestimation of P3HB content. Method is limited to P3HB determination	[89,105,126]
Fourier transform infrared spectroscopy (FTIR)	Cellular PHA content	0.4–10 mg biomass	Spread cells on thallium bromoiodide (KRS-5) window and air-dry	FTIR was used to record the PHA spectrum at ambient temperature (25 °C), at a spectra range of 400–4000 cm^{-1}, for 10–64 scans and a resolution of 4 cm^{-1}	Requires small sample size, short analysis time, solvent usage is optional, can provide quantitative information, enables online and real-time PHA analysis, high throughput	Method cannot discriminate between different PHA monomeric units, unable to distinguish between homogenous PHA and PHA copolymer, low sensitivity, quantification limited to scl-PHA	[127,128]

Table 3. *Cont.*

Liquid chromatography (LC)	PHA monomeric units	0.01–500 mg biomass or 0.01–14 µg P3HB	Hydrolytic digestion with concentrated sulfuric acid 90 °C for 30 min, cool on ice before adding 0.014 N of sulfuric acid with rapid mixing to yield crotonic acids	High performance liquid chromatography (HPLC) analysis with an ion-exclusion organic acid analysis column and a UV detector at 210 nm	Does not require cell lyophilization, requires small sample size, short sample preparation time, provides both quantitative and qualitative information. Coupling with mass spectrometer (MS) detector enables tentative identification of novel PHA monomers, applicable for quantitative and qualitative analysis of mcl-PHA monomers	Low separation power that is currently limited to analysis of scl-PHA monomers unless coupled to MS detector, unable to distinguish between homogenous PHA and PHA copolymer	[129,130]
		10–25 mg biomass or 2 mg PHA	Propanolic digestion with propanol and concentrated sulfuric acid at 90 °C for 1 h to yield a mixture of monomeric acids and propionyl esters	Ion chromatography (IC) analysis with an anion trap column and a conductivity detector			
		2 mg PHA	Reductive depolymerization by dissolution of PHA in toluene, followed by addition of lithium aluminum hydride in tetrahydrofuran (THF) with 15 min of gentle agitation at room temperature to yield 1,3-diols	HPLC-MS analysis with a C18 column			
Gas chromatography (GC)	PHA monomeric units	5–15 mg biomass or 0.15–15 mg PHA	Methanolysis with either sulfuric acid/methanol or boron trifluoride/methanol at 100 °C for 2 h–4 h to yield methyl esters or propanolysis with hydrochloric acid/propanol at 80 °C for 20 h to yield propyl esters	Analysis with a Supelco SPB-35 or DB-5 column using a flamed ionization detector (FID), or with a HP-5MS column using a MS detector	High separation power, high sensitivity, provides both quantitative and qualitative information, and can be applied for tentative identification of novel PHA monomers when coupled to MS detector	Requires cell lyophilization, long sample preparation time requiring the use of hazardous and volatile solvents, unable to distinguish between homogenous PHA and PHA copolymer	[43,131–134]

4.1. Detection of PHA in Biomass and PHA Production Capacity

Methods which detects for PHA or PHA-producing capacity in microbes include colony/cell staining, polymerase chain reaction (PCR) gene detection, and transmission electron microscopy (TEM). Colony/cell staining and gene detection are often used as front-line methods for high throughput screening and identification of novel microbes with PHA production potential owing to the relative ease of sample preparation and short analysis time. In colony/cell staining method, Nile red or Nile blue A dye is directly added into the solid growth medium [122], liquid cell cultures [123,124] or onto heat-fixed smeared cells [125]. Under UV illumination, Nile red and Nile blue A dye stains PHA to give a pink/red/yellow/orange appearance enabling the PHA-producing microbes to be identified and isolated. In PCR gene detection, primer pairs are designed to specifically amplify *phaC* gene, which encodes for PHA synthase, an enzyme responsible for PHA synthesis. The gene is typically present in PHA-accumulating microbes and absent in non-PHA-accumulating microbes, serving as the basis for identifying potential PHA-producers [120]. A review on the current research status of *phaC* gene detection and the available PCR primers is provided by Solaiman and Ashby [121].

Although staining and genetic detection methods provide a simple way to screen for PHA-producing microbes efficiently, these methods are also prone to detection errors. False positives may arise from the staining of other non-PHA lipid storage compounds and non-specific PCR amplification while false negatives could result from unfavorable conditions for biomass PHA accumulation and unsuitable detection primers or PCR conditions used in PCR amplification [120,122]. Hence, these two methods could only be employed as a presumptive test of PHA production potential. TEM on the other hand, enables direct visualization of PHA that appears as intracellular granules, providing affirmation of PHA bioacummulation [43]. However, TEM sample preparation is time-consuming and involves the use of radioactive chemicals [43], making it unsuitable for screening purposes. While Nile red/Nile blue staining, *phaC* gene detection and TEM are effective at providing evidence for PHA production or PHA-producing capacity, the downside of these methods is that they could neither quantify PHA nor provide qualitative information about PHA monomeric composition.

4.2. Quantification and Characterization of PHA in Biomass

Traditionally, crotonic acid assay was commonly used as a viable method for quantitative determination of P3HB [126,135,136]. In crotonic acid assay, P3HB is dissolved in concentrated sulphuric acid and converted into crotonic acid. As crotonic acid has a strong UV absorption at 235 nm in concentrated sulfuric acid, it can be measured by UV spectrophotometer. While this method is an easy and fast way to quantify P3HB, this method tends to overestimate P3HB content and is limited to P3HB determination [89,105]. Currently, quantification and characterization of various microbial intracellular PHAs can be achieved using modern analytical techniques, including fourier transform infrared spectroscopy (FTIR), liquid chromatography (LC) and gas chromatography (GC).

FTIR has been applied to detect and distinguish between the different types of PHA (*i.e.*, scl-PHA, mcl-PHA, and scl-mcl-PHA), present within intact cells or as purified polymers. Characteristic ester carbonyl band for intracellular scl-PHA, mcl-PHA, and scl-mcl-PHA were observed at 1732 cm^{-1}, 1744 cm^{-1} and 1739 cm^{-1}, respectively whereas the same band for purified polymer scl-PHA, mcl-PHA, and scl-mcl-PHA were observed at 1728 cm^{-1}, 1740 cm^{-1} and 1732 cm^{-1}, respectively [127].

FTIR has also been proposed as a quantification tool for scl-PHA. Arcos-Hernandez *et al.* [137] showed that biomass scl-PHA content, between 0.03 and 0.58 weight/weight (w/w), could be coupled to FTIR spectra using a partial least squares model, allowing scl-PHA content determination within a standard error of prediction value of 0.023 w/w. The solvent-less nature of the FTIR technique and short analysis time eliminates risk exposure to hazardous chemicals while providing fast data output. However, FTIR-based methods have lower detection sensitivities, are inapt at describing or detecting changes in PHA monomeric composition, and cannot discriminate between PHA blends and copolymers [127]. Hence, FTIR-based methods tend to be more suitable for routine monitoring of PHA production for standard bioprocesses with well-characterized PHA products.

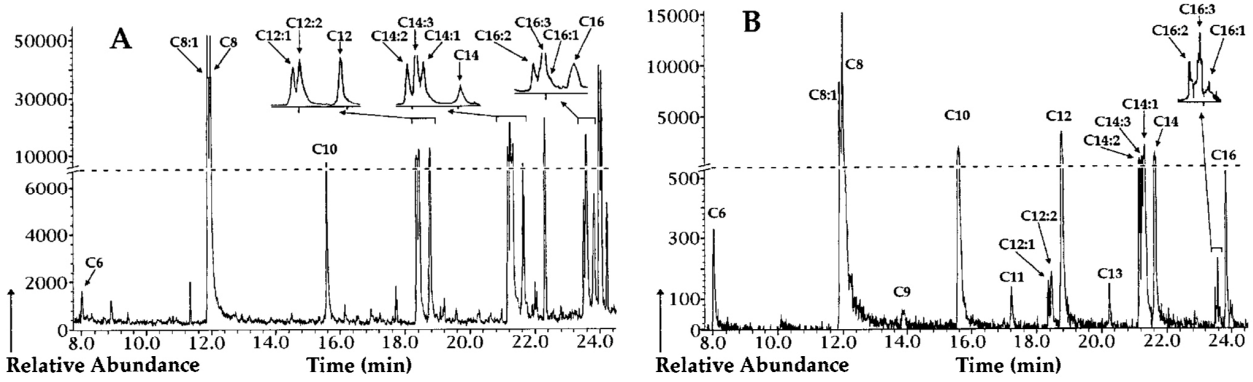
LC- and GC-based methods are the most frequently used analytical techniques due to automated sample analysis, and ability to provide accurate PHA quantification and qualitative information about PHA monomeric composition. Compared to FTIR-based methods, chromatography-based methods have higher detection sensitivities ranging from 0.014 to 14 μg for HPLC and 0.05 pg to 15 mg for GC depending on the type of detectors and chemical derivatization methods used [138]. The advent of automated LC platforms has enhanced scl-PHA analysis. Improvement in the measurement accuracy of P3HB-derived crotonic acid was made possible with ion-exchange HPLC coupled with UV detection [105,139,140]. Simultaneous analysis and quantification of 3HB and 3HV monomers could also be achieved through ion chromatography (IC) equipped with an anion trap column and a conductivity detector [129]. The advanced liquid chromatography-mass spectrometry (LC-MS) technique, using a combination of higher pressure and small diameter particles as column packing for separation with specific MS detection, could also be a sufficiently rapid and robust approach for the routine analysis of PHA monomers. However, to date, LC-MS has been applied only to a limited extent as a complementary technique to analyze PHA monomers [65]. There remains much potential to expand the capabilities of LC platforms beyond quantitative analysis of scl-PHA through utilizing LC-MS for quantification of other PHA monomers.

At present, GC remains the preferred method for qualitative and quantitative analysis of PHA monomers owing to its high separation power and detection sensitivity [138]. One of the earliest works on GC determination of PHA was reported by Braunegg *et al.* [134], who developed a method for accurate and reproducible determination of P3HB content in bacterial biomass using GC with flamed ionization detector (GC-FID). They showed that after subjecting P3HB-containing bacterial biomass to methanolysis, P3HB could be completely recovered in the form of its methyl esters derivatives and quantified to levels as low as 10^{-5} g L^{-1}. GC-FID analysis was subsequently expanded to P3HV and mcl-PHA [81,141]. The robustness of GC-FID determination however, is dependent on the inclusion of appropriate PHA analytical standards. Conversely, coupling GC to mass spectrometry detector (GC-MS) ensures more reliable detection, identity confirmation and quantification of PHA monomers, as well as enables tentative identification of novel PHA monomers in the absence of analytical standards [133]. Figure 4 shows representative GC-MS chromatograms of PHA monomers [37]. A recent study by Tan *et al.* [132] showed that PHA monomers with carbon number between 4 and 16 have strong linear correlations with their respective retention times and response factors ($R^2 \geq 0.987$) under GC-MS, which enabled the retention time and response factor of other PHA monomers to be predicted. This method allowed a wide series of PHA, ranging from scl-PHA to lcl-PHA, to be reliably detected and quantified in the absence of reference standards. It is also expected that the coupling of GC to tandem

mass spectrometry (GC-MS/MS) could offer improvements in sensitivity and specificity for determination of PHA monomers [142].

Despite their advantages, chromatography-based methods require PHA to be depolymerized and chemically converted into acids, diols or methyl ester derivatives prior to analysis [105,130,132]. This meant that chromatography-based methods are unable to distinguish if different PHA monomers are part of several homogenous PHA polymers or if they are part of a single PHA copolymer.

Figure 4. (**A**) Gas chromatography-mass spectrometry (GC-MS) total ion chromatogram of PHA after acidic methanolysis; (**B**) GC-MS extracted ion chromatogram of m/z 103. (Adapted from [37], with permission; Copyright 1998, National Academy of Sciences, USA). (C6, 3-hydroxyhexanoate; C8, 3-hydroxyoctanoate; C8:1, 3-hydroxyoctenoate; C9, 3-hydroxynonanoate; C10, 3-hydroxydecanoate; C11, 3-hydroxyundecanoate; C12, 3-hydroxydodecanoate; C12:1, 3-hydroxydodecenoate; C12:2, 3-hydroxydodecadienoate; C13, 3-hydroxytridecanoate; C14, 3-hydroxytetradecanoate; C14:1, 3-hydroxytetradecenoate; C14:2, 3-hydroxytetradecadienoate; C14:3, 3-hydroxytetradecatrienoate; C16, 3-hydroxyhexadecanoate; C16:1, 3-hydroxyhexadecenoate; C16:2, 3-hydroxyhexadecadienoate; C16:3, 3-hydroxyhexadecatrienoate)



5. PHA Polymer Extraction Methods

Microbial PHA is stored as insoluble intracellular granules. Methods to recover PHA would typically involve cell wall/cell membrane lysis, solubilization and purification of PHA component, and precipitation of PHA polymer. Common methods for PHA polymer recovery from microbial biomass are solvent extraction methods, chemical- and enzyme-based digestions methods, which will be briefly reviewed herein. A comprehensive review on this topic is provided by Kunasundari and Sudesh [143].

For scl-PHA, various PHA isolation methods have been developed using P3HB as a model polymer. A summary of the common extraction methods for scl-PHA is provided in Table 4. Among all recovery methods, solvent extraction is the most well-established and commonly used to obtain PHA polymer from biomass due to its high purity. In a study by Ramsay *et al.* [144], the solvents chloroform, methylene chloride or 1,2-dichloroethane were evaluated for P3HB recovery under various conditions (chloroform: 61 °C, 1 h; methylene chloride: 40 °C, 24 h; and 1,2-dichloroethane: 83 °C, 0.5 h). After solvent extraction, cellular debris was removed via filtration and the solution was concentrated by rotary evaporation before the P3HB polymer was precipitated by dropwise addition of ice-cold ethanol. Chloroform and 1,2-dichloroethane were observed to achieve high P3HB recovery

(68%) with high purity (96% to 98%) compared to methylene chloride (recovery: 25%, purity: 98%). A novel method was developed for extraction of scl-PHA by "scl-PHA anti-solvent" acetone at elevated temperature and pressure in a closed system combining components for extraction, filtration, and product work-up. The quality of acetone-extracted polyesters showed no significant difference from chloroform-extracted ones, providing a promising substitute in terms of higher recyclability of the solvent without negatively impacting the structural features of the biopolyester [145]. The preponderance of solvent extraction lies on its high P3HB recovery purity, however there are much concerns about the high cost of operation as well as negative environmental impact caused by the generation of hazardous waste. One of the ways to minimize this problem is by using waste solvents for P3HB extraction. This has been demonstrated in Brazil where a pilot-scale P3HB production plant was integrated with a sugarcane mill and the solvent by-products of ethanolic fermentation from the mill was subsequently used as extraction solvents for P3HB recovery [146], thus presenting a feasible solution for achieving high P3HB purity at lower environmental cost.

Digestion method is a well-established alternative to solvent extraction for PHA recovery. In chemical-digestion, sodium hypochlorite is used to solubilize non-P3HB biomass thus achieving separation of P3HB content which can be recovered by centrifugation [147]. While the method is simple and effective, P3HB polymers obtained through hypochlorite digestion are generally of lower molecular masses due to the severe polymer degradation [147]. To resolve this problem, a combination approach was developed using dispersions of sodium hypochlorite solution as cell solubilizer, and chloroform to protect P3HB from further degradation after its release from cells, thus taking advantage of both hypochlorite digestion and solvent extraction [148].

Compared to solvent extraction and chemical digestion, enzymatic digestion requires milder operating conditions while achieving negligible product degradation [149]. Biomass was suspended in a specialized buffer and incubated at a specific temperature, which were optimized for enzymatic activity. After enzymatic hydrolysis, P3HB polymer was recovered by centrifugation. A polymer purity of up to 90% could be attained with this method [149]. Enzyme-based PHA recovery methods are safer in terms of operation, posed lower health risks, and has lower environmental footprint. Nevertheless, the high cost of enzymes may drive up the overall extraction cost.

For mcl-PHA, the recovery methods are based on similar principles and procedures as those for scl-PHA. Table 5 provides a summary of the common extraction methods for mcl-PHA. Like scl-PHA, mcl-PHA could also be recovered through solvent extraction and enzyme digestion, albeit with modifications to optimize mcl-PHA recovery. For solvent extraction of mcl-PHA, chloroform is commonly used according to procedures similar to those for scl-PHA. The main difference is in the final polymer precipitation step where ethanol is replaced with ice-cold methanol [150]. Apart from chloroform, dichloromethane is a less hazardous choice as an extraction solvent and together with ice-cold methanol as precipitant, up to 98% purity of mcl-PHA could be attained [151]. Solvent extraction however, tends to result in significant polymer degradation [144]. Jiang *et al.* [152] showed that this issue could be circumvented. Using *P. putida* biomass sample, the combination of biomass pretreatment with methanol and using acetone as the extraction solvent resulted in no detectable molecular mass loss of mcl-PHA with an overall purity of 94% [152].

Table 4. Poly(3-hydroxybutyrate) (P3HB) recovery methods (modified from [143]).

Method	Chemical	Species	Conditions	Purity and recovery	Reference
Solvent extraction	Chloroform	*Cupriavidus necator* (DSM 545)	Mixing continuously at 25 °C for 12 h	Purity: 94.0%–96.0% Recovery: 65.0%–70.0%	[144]
	Methylene chloride	*Cupriavidus necator* (DSM 545)	Mixing continuously at 25 °C for 12 h	Purity: 95.0%–98.0% Recovery: 24.0%–25.0%	
	1,2-Dichloroethane	*Cupriavidus necator* (DSM 545)	Mixing continuously at 25 °C for 12 h	Purity: 93.0%–98.0% Recovery: 66.0%–70.0%	
	Acetone	*Haloferax mediterranei* (DSM 1411)	Mixing continuously at 120 °C, 7 bar for 20 min under anaerobic conditions followed by filtering hot solution and cooling it down at 4 °C to precipitate polymer	Purity: 98.4% Recovery: 96.8%	[145]
	Medium-chain-length alcohols	*Cupriavidus necator* and *Burkholderia* sp.	Multi-stage extraction process in continuous-stirred tank reactors. Remove cell debris from the extract and cool extract to recover polymer	Purity: > 98.0% Recovery: 95.0%	[146]
Hypochlorite digestion	Sodium hypochlorite	*Cupriavidus necator* (DSM 545)	Biomass concentration: 10–40 g/L; pH: 8–13.6; Temperature: 0–25 °C; Digestion time: 10 min–6 h; Hypochlorite concentration: 1%–10.5% weight/volume (w/v)	Purity: 90.0%–98.0% Recovery: 90.0%–95.0%	[147]
	Sodium hypochlorite and chloroform	*Cupriavidus necator* (NCIMB 11599) and recombinant *Escherichia coli*	Biomass concentration: 1% (w/v); Temperature: 30 °C; Digestion time: 1 h; Hypochlorite concentration: 3%–20% volume/volume (v/v)	Purity: 86.0% Recovery: NG Purity: 93.0% Recovery: NG	[153]
Enzyme digestion	Trysin, bromelain, pancreatin	*Cupriavidus necator* (DSM 545)	Digestion with 2% trypsin (50 °C, pH 9.0, 1 h) or 2% bromelain (50 °C, pH 4.75, 10 h) or 2% pancreatin (50 °C, pH 8.0, 8 h), followed by centrifugation and washing with 0.85% saline solution	Purity: 87.7%–90.3% Recovery: NG	[149]

NG, not given.

Table 5. Medium-chain length PHA (mcl-PHA) recovery methods.

Method	Chemical	Species	Conditions	Purity and recovery	Reference
Solvent	Chloroform	*Pseudomonas oleovorans* (strains NRRL B-14682, NRRL B-14683, and NRRL B-778)	30 °C overnight at 250 rpm	NG	[150]
	Chloroform	*Pseudomonas oleovorans* (NRRL B-14683), *Pseudomonas resinovorans* (NRRL B-2649), *Pseudomonas citronellolis* (NRRL B-2504), and *Pseudomonas putida* KT2442	Soxhlet extraction for 24 h	NG	[154,155]
	Chloroform	*Pseudomonas putida* IPT 046	Soxhlet extraction for 6 h	NG	[156]
	Chloroform	*Pseudomonas aeruginosa* 42A2 (NCIMB 40045)	100 °C for 3 h in screw cap tubes for small quantities or in a soxhlet apparatus for large amounts of cell material	NG	[157]
	Dichlorome-thane	*Pseudomonas oleovorans* (ATCC 29347)	Soxhlet extraction at 60 °C for 5 h	Purity: > 98.0% Recovery: NG	[151]
	Acetone	*Pseudomonas putida* KT2440 (ATCC 47054)	22 °C for 24 h at 170 rpm	Purity: 80.0%–90.0% Recovery: 60.0%–80.0%	[152]
Enzyme digestion	Alcalase, SDS, EDTA, lysozyme	*Pseudomonas putida*	Digestion with alcalase and SDS at pH 8.5 and 55 °C followed by further treatments with EDTA and lysozyme at pH 7 and 30 °C	Purity: 92.6% Recovery: nearly 90.0%	[158]
		Pseudomonas putida KT2442	Digestion with excess alcalase, EDTA and SDS at pH 8.5 and 55 °C followed by diafiltration	Purity: > 95.0% Recovery: NG	[159,160]

NG, not given.

Table 6. Techniques for PHA polymer characterization.

Characteristic	Index	Method	Sample	Sample preparation	Typical conditions	Reference
PHA monomeric composition	Chemical derivative of PHA monomers	LC	Refer to Table 3			
		GC	Refer to Table 3			
PHA polymeric composition	Topology and functional groups of PHA molecule	1D-Nuclear magnetic resonance (NMR)	5–10 mg PHA for ^{1}H-NMR and 20– 30 mg PHA for ^{13}C-NMR	Dissolution of PHA polymer in 0.7 mL deuterated chloroform ($CDCl_3$) containing 0.03% (v/v) tetramethylsilane (TMS)	^{1}H-NMR at 200 or 300 MHz and ^{13}C-NMR measurements at 75.4 MHz at 20 °C with a sampling pulse of 3 s. Chemical shifts were referenced to the residual proton peak of $CDCl_3$ at 7.26 ppm and to the carbon peak of $CDCl_3$ at 77 ppm	[82]
		2D-NMR	10 mg PHA for homonuclear 2D-NMR and 40–50 mg PHA for heteronuclear 2D-NMR	Refer to above "1D-NMR"	For homonuclear COSY and TOCSY, 16 scans were accumulated per increment over a spectral width of 7.8 ppm. For heteronuclear HSQC, 48 scans were accumulated per increment over a spectral width of 7.8 ppm for ^{1}H and 75 ppm for ^{13}C. For heteronuclear HMBC spectrum, 64 scans were acquired with the long-range coupling delay set for 8 Hz	[161]

Table 6. *Cont.*

PHA polymeric composition	Topology and functional groups of PHA molecule	Matrix assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS)	1 μg–1 mg PHA	The matrix used was either dithranol or dihydroxybenzoic acid (DHB) at a concentration of 10 mg mL^{-1} in THF. 1 mg mL^{-1} PHA solution (in chloroform) was mixed with equal volume THF. The matrix solution and the PHA solution were subsequently mixed in a 5:2 ratio (matrix/sample). 1 μL mixture was deposited onto the stainless steel sample holder. The solvent was allowed to air-dry before loading the sample plate into the MALDI ion source	MALDI-TOF-MS with 25 kV acceleration and detection in the positive-ion high-resolution reflection mode	[162]
Molecular distribution	Polydispersity, molecular mass and molecular mass distribution	Gel permeation chromatography (GPC)	0.1–1 mg PHA	Dissolution of PHA polymer in 1 mL of THF	Analysis conducted with a refractive index detector (47 °C, 2.0 bar) and a solvent-compatible GPC column. THF, containing 250 ppm of 2,6-di-tert-butyl-4-methylphenol (BHT) as inhibitor, was used as an eluent at a flow rate of 0.5 mL min^{-1} and 40 °C	[43]
				Dissolution of PHA polymer in chloroform	Analysis conducted with a differential refractive index detector (30 °C), a UV dual wavelength absorbance detector, and a combination of four GPC columns series. Chloroform was used as an eluent with a flow rate of 1.0 mL min^{-1}	[163]
		MALDI-TOF-MS	Refer to above "PHA polymeric composition"			

Table 6. *Cont.*

Thermal properties	Glass transition temperature and melting temperature	Differential scanning calorimetry (DSC)	10 mg PHA	-	Heat sample from −100 °C– 400 °C at a heating rate of 10 °C min^{-1} under purified air or nitrogen gas with a flow rate of 80 mL min^{-1}	[43]
		Differential thermal analysis (DTA)	5 mg PHA	-	Crystallization was carried out isothermally by abruptly quenching the samples from melt to the crystallization temperature, at which the samples were annealed for 10 min. Melting of semicrystalline samples was performed by heating at a rate of 5 °C min^{-1}	[164]
	Thermodegradation temperature	Thermogravimetric analysis (TGA)	10 mg PHA	-	Heat sample from room temperature to 700 °C at a heating rate of 10 °C min^{-1} under purified air or nitrogen gas with a flow rate of 50 mL min^{-1}	[43]
Crystallinity	Melting enthalpy	DSC	Refer to above "Thermal properties"			
	Infrared absorption bands correlated to crystallinity	FTIR	5–10 mg PHA	Dissolve PHA in chloroform, apply onto KRS-5 window and blow dry to evaporate solvent. Alternatively, mix PHA with potassium bromide (KBr) powder and pelletize	Refer to Table 3	[127,165]
				Place PHA sample between two pieces of barium fluoride slides	Melt sample at 100 °C for 2 min in FTIR hot stage under the protection of dry nitrogen gas. Quench the amorphous sample to 58 and 28 °C by a flow of liquid nitrogen and maintain at these temperatures for 30 min for isothermal melt-crystallization before re-heating at 1 °C min^{-1}	[166]

Table 6. *Cont.*

Crystallinity	Diffraction intensity correlated to crystallinity	X-ray diffraction	Dry polymer powder	-	Diffractogram of the sample powder were measured at room temperature by an imaging plate diffractometer with Cu-Kα radiation (wavelength = 0.1542 nm) as an incident X-ray source emitted by a X-ray generator with a Ni filter. The scattering angle range of 2θ = 10°–40° at a scan speed of 3° min^{-1}	[156]
Mechanical properties	Tensile strength, tensile stress, percent elongation, modulus of elasticity	Mechanical testing machine of the constant-rate-of-crosshead-movement type with extensometer and micrometers	Polymer thickness 1–14 mm, width 19–29 mm, length 165–246 mm	Test samples were prepared using a hydraulic press at 150 °C and conditioned at a relative humidity of 50% ± 5% for 24 h prior to measurements	Perform stress-strain test at room temperature with a strain rate of 20 mm min^{-1}	[167]

For mcl-PHA recovery through enzyme digestion, enzymes alcalase and lysozyme, together with sodium dodecyl sulfate (SDS) and ethylenediaminetetraacetic acid (EDTA) were used. This combination of enzymes and chemicals were successfully applied to mcl-PHA isolation for fed-batch fermentations of up to 200 L, where the cells were first ruptured by thermal treatment and the resultant debris was exposed to excess protease (alcalase), EDTA and SDS for solubilization. After cross-flow microfiltration, the final mcl-PHA latex had a purity exceeding 95%, demonstrating potential commercial applicability [159,160]. In another study [158], the PHA granules, present in water suspension after enzymatic treatment, were recovered by removing the solubilized non-PHA cell material through ultrafiltration system and purified through continuous diafiltration process. The final purity of PHA was 92.6% and recovery was nearly 90% [158]. While enzyme digestion is a more environmental-friendly approach than solvent extraction, the purity of polymer attained is lower. Biomedical application requires a final purity of 99% or more which is currently not achievable through enzymatic mcl-PHA recovery and a second purification using solvent extraction is necessary [160].

6. Techniques for PHA Polymer Characterization

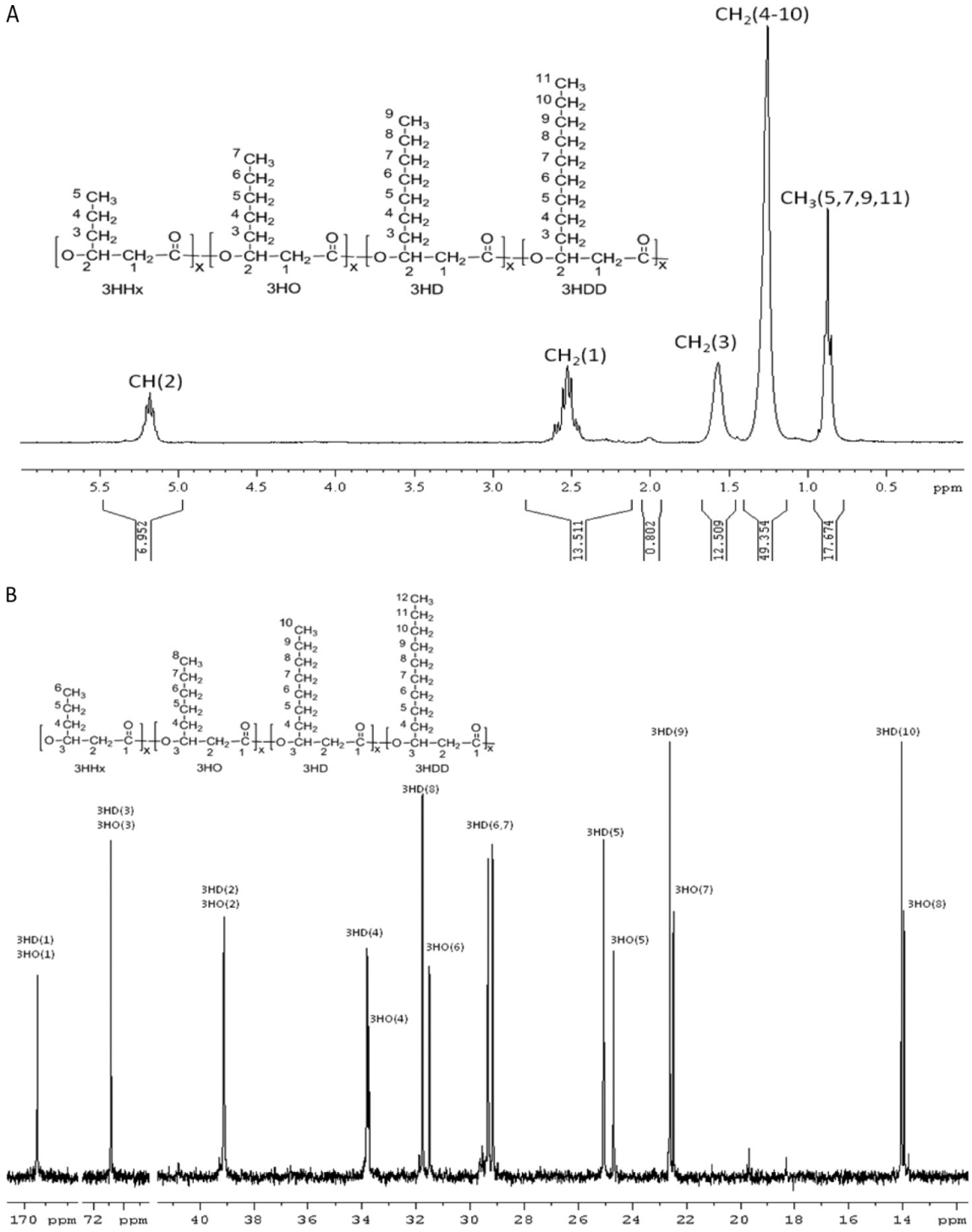
Purified PHA polymers are diverse in their chemical composition and material properties due to the myriad of PHA monomeric units available as well as the incorporation of these monomers at varying amounts. To identify suitable downstream applications for PHA, characterization of the biomaterial is imperative. A summary of these techniques and their execution is provided in Table 6.

6.1. Monomeric Composition and Distribution

The monomeric composition and distribution of PHA polymer could be determined from GC, LC and nuclear magnetic resonance spectroscopy (NMR). For chromatography-based methods, the analysis of PHA polymer is similar to that for intracellular PHA, requiring depolymerization of the polymer, usually combined with chemical derivatization before it can be analyzed [43,130,132], which meant that chromatography methods cannot analyze PHA as an intact polymer. NMR on the other hand, could study the chemical makeup of an intact PHA polymer and differentiate between PHA blends and PHA copolymer though providing details on the topology and functional group in molecules [150,153,154,168]. Typically, two types of NMR techniques are available and they are ^{1}H-NMR and ^{13}C-NMR. The high proton abundance in nature meant that ^{1}H-NMR is more sensitive and requires shorter analytical time (within one hour). In contrast, owing to low sensitivity and natural abundance of ^{13}C, it may require longer analysis (up to 24 h) to accumulate enough signal intensity for ^{13}C-NMR spectrum. Despite its shortcoming, ^{13}C-NMR performs better than ^{1}H-NMR at the analysis of macromolecule as well as long carbon chain of monomers. As PHA polymers contain hydrogen and carbon, ^{1}H-NMR and ^{13}C-NMR are usually applied in combination to provide a more comprehensive analysis of the polymer. NMR is broadly used for saturated and unsaturated PHA analysis. Functional groups such as methane protons, methylene protons, –CH=CH– can be identified from both NMR spectra while microstructures like 3-hydroxypropionate (3HP) and 4-hydroxybutyrate (4HB) can be obtained by analyzing both ^{1}H-NMR and ^{13}C-NMR spectra [156,169]. Quantitative estimation of PHA monomers can also be performed with NMR using the intensity ratio of the signals [156]. NMR is also a powerful non-destructive tool that could be applied to the analysis of novel functionalized PHAs for

which analytical standards are currently unavailable [170,171]. Typical ^{1}H-NMR and ^{13}C-NMR spectra of mcl-PHA are shown in Figure 5 [172].

Figure 5. (**A**) Typical ^{1}H-NMR spectrum of PHA. Protons in the polymers are numbered and assigned to the peaks in the spectrum; (**B**) Typical ^{13}C-NMR spectrum of PHA. Carbon atoms in polymers are numbered and assigned to peaks in the spectrum. (Adapted from [172], with permission). (3HHx, 3-hydroxyhexanoate; 3HO, 3-hydroxyoctanoate; 3HD, 3-hydroxydecanoate; 3HDD, 3-hydroxydodecanoate).



In addition, two-dimensional (2D) NMR methodologies (such as correlation spectroscopy [COSY], nuclear Overhauser effect spectroscopy [NOESY], heteronuclear multiple-quantum correlation [HMQC] and heteronuclear multiple bond coherence [HMBC]) are very useful in the characterization of all kinds of specialized PHA, such as unsaturated, branched, halogenated or acetylated PHA [43,56,161,173,174]. 2D-NMR provides information about the environment where each carbon/hydrogen is positioned. With the aid of 2D homonuclear or heteronuclear NMR techniques, the exact position of double bonds and the *cis*/*trans* configuration of monomers can be determined [43,173]. ^{1}H-NMR and 2D homonuclear NMR were used to unambiguously identify the position of the hydroxyl group and the positions of the double bonds in mcl-PHA [174]; while 2D heteronuclear COSY NMR was successfully applied to show that 4-hydroxyvaleric acid was a constituent of the PHA polymer [56]. Moreover, 2D heteronuclear HMBC NMR could also clearly reveal the presence of the block microstructure in PHA [175].

Besides, it is notable that MS techniques, such as fast-atom bombardment (FAB)-MS [176], pyrolysis/MS [177] and matrix assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS) [162,178,179] have been applied for the characterization of PHA composition. Among them, MALDI-TOF-MS offers a cost-effective, straightforward and high-throughput alternative to the well-established GC-MS methods [162,179]. Compared with GC-MS, MALDI-TOF-MS is a more straightforward method, and no chemical derivatization is required during sample preparation. As the differences in monomer composition and detailed PHA structural information can easily be identified by MALDI-TOF-MS, it can be used as complementary technique to NMR [178]. The total amount of sample deposited onto the target can be in the pico- to femtomole range, which makes MALDI-TOF-MS extremely sensitive with minimal sample requirement. The significant advantages provided by MALDI-TOF-MS technology therefore could facilitate routine analysis of PHA with high accuracy and precision.

6.2. Molecular Mass (M_w), Molecular Mass Distribution (M_n), and Polydispersity Index (PDI)

A PHA polymer's average molecular mass (M_w), molecular mass distribution (M_n), and polydispersity index (PDI; M_w/M_n) could be determined through a gel permeation chromatography (GPC) system, calibrated with polystyrene standards [150]. The M_w of PHA spans over a wide range from 50 kDa to 10,000 kDa and depending on M_n value, PDI could be between 1.1 and 6.0 [13,97,180]. GPC columns such as Styragel HMW 6E (5 to 10,000 kDa) [150] and PLgel MIXED-C (0.2 to 2,000 kDa) [181] have been applied for PHA analysis. Often, the broad M_w range of PHA makes the analysis of an unknown polymer more challenging particularly when the biomaterial is a mixture of several PHA molecules with vastly different M_w and M_n values. Two or more GPC columns, connected in series, are therefore necessary to fully reveal the polymer's M_w and M_n [153].

MALDI-TOF-MS, on the other hand, is the new and promising method for PHA characterization. It could potentially be used for evaluating the M_w and M_n of PHAs and their oligomers [162,178,179]. Unlike GPC that can only be used in determining apparent M_n values, MALDI-TOF-MS can offer accurate mass measurement of PHA [182]. Because of its high molecular resolution, excellent accuracy, reproducibility, and automation properties, MALDI-TOF-MS can make a significant contribution to the study of PHA.

6.3. Thermal Properties

Thermal properties such as glass transition temperature (T_g), melting temperature (T_m), and thermodegradation temperature (T_d) are commonly examined for PHA material to determine the temperature conditions at which the polymer can be processed and utilized. The T_g, T_m, and T_d values for PHA are usually in the range of −52 to 4 °C, non-observable to 177 °C, and 227 to 256 °C, respectively [7,180]. Information on T_g and T_m could be obtained using differential scanning calorimetry (DSC) and differential thermal analysis (DTA). The difference which sets them apart is that DTA is able to measure mass loss and qualitatively provide thermal information [183] while direct heat flow measurement enables DSC to provide not only qualitative results, but also quantitative thermal information, making DSC the preferred method in PHA studies [153,156]. The T_d value of PHA is obtained using thermogravimetric analysis (TGA), a technique where a sample is heated in a controlled atmosphere at a defined rate while sample mass loss is measured [153,156]. The development of the simultaneous thermal analysis (STA) combines TGA and DSC/DTA measurement techniques, enabling T_g, T_m, and T_d values determination on a single instrument, which provides a more productive and simpler means to analyze PHA [156].

6.4. Crystallinity

PHA polymers can range from non-crystalline to highly crystalline with crystallinity values between 0% and 70% [7,97]. Crystallinity could be measured by structural analysis instruments including FTIR, DSC and X-ray diffraction. In FTIR analysis, PHA displays characteristic infrared absorption bands at certain wavenumbers which can be correlated to crystallinity. The exact band locations vary according to the chemical composition of the polymer. For scl-PHAs such as P3HB and P3HB3HV, bands around 1279, 1228, and 1185 cm^{-1} are sensitive to the degree of crystallinity [184,185]. Band 1725 cm^{-1} and bands in the range of 1500 to 1300 cm^{-1}, 1300 to 1000 cm^{-1} and 1000 to 800 cm^{-1} are revealing of the conformational changes of mcl-PHA and scl-mcl PHA in both the crystalline phase and amorphous phase [166]. In DSC analysis, melting enthalpy (ΔH_m) provides an estimated value for heat of fusion (ΔH_f) under the analysis conditions, which could be related to PHA's crystallinity. PHA polymers with very low crystallinity typically have low to non-observable ΔH_f while highly-crystalline polymers such as P3HB can have ΔH_f values up to 146 J g^{-1} [7,186]. On their own, both FTIR and DSC are only adequate at measuring relative crystallinities within a given material. Measurement of the absolute crystallinity using FTIR and DSC can only be performed for PHA polymer with known crystallinity [187,188]. Absolute crystallinity could be obtained using methods based on X-ray diffraction. X-ray diffraction analysis is able to shed light on the polymer's rate of crystallinity, as well as atomic structures such as chemical bonds, their disorder [189]. Crystallinity percentage can be calculated according to semi-crystalline and amorphous polymer areas in the diffractogram using Lorentzian and Gauss functions [156].

6.5. Mechanical Properties

Young's modulus, elongation at break and tensile strength are mechanical properties commonly evaluated for PHA polymers. The Young's modulus provides a measure of PHA's stiffness and ranges

from the very ductile mcl-PHA (0.008 MPa) to the stiffer scl-PHA (3.5×10^3 MPa) [7]. Elongation at break measures the extent that a material will stretch before it breaks and is expressed as a percentage of the material's original length. PHA polymers can take the form of a hard rigid material or a soft elastomeric material, displaying a wide elongation at break values of between 2% and 1000% [180]. Tensile strength measures the amount of force required to pull a material until it breaks, and is typically in the range of 8.8 to 104 MPa for PHA polymers [7]. Measurement of the aforementioned mechanical properties can be performed with tensile tester instrument by standardized test methods such as the ASTM standards [167].

7. Conclusions

This review paper provided a summary of PHA biosynthetic pathways, PHA-producing microbial strains commercially available from culture collections and their application, as well as techniques for PHA analysis and polymer extraction. It is evident that there are many avenues through which PHA could be produced depending on the type of microorganisms employed, choice of carbon source, and cultivation conditions. These aforementioned factors also influenced the type of PHA produced, which in turn determines their downstream applications. Using this wealth of knowledge, future development in commercial PHA production could adopt a more "top-down" approach where the targeted carbon source and desired PHA product are decided *a priori* together with economic considerations before the appropriate microorganism or group of microoganisms is selected for the purpose as a means to achieve economic viability for the bioprocess. The formulation of microbial co-cultures for PHA production is largely considered as unexplored territories but may have the potential to produce PHA cheaply from organic waste streams. A fast-growing area in PHA research is the biosynthesis of tailored PHA for specific application needs. Existing microbial strains from culture collections serve as an excellent platform for genetic modification to produce specialized PHAs and enhancing PHA yield. The elucidation of PHA biosynthetic pathways is also likely to complement such research efforts. Many well-established methods are currently available for PHA analysis but each of them come with their own strengths and limitations. On the basis of the reports that have been gathered to date, GC-MS in conjunction with NMR remains a pre-eminent analytical tool in PHA investigations. Well-established analytical methods such as FTIR, GPC and X-ray diffraction can provide general information on the overall structures, molecular mass distribution and rate of crystallinity, respectively. However, it is important to further develop efficient technologies (e.g., LC-MS and MALDI-TOF-MS) for characterization of PHA. It is expected that the advanced analytical approaches will provide us with further insights about the physical properties and degradation mechanisms of PHA.

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Conflicts of Interest

The authors declare no conflict of interest.

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