

Microbial Polyhydroxyalkanoates and Nonnatural Polyesters

So Young Choi, In Jin Cho, Youngjoon Lee, Yeo-Jin Kim, Kyung-Jin Kim, and Sang Yup Lee*

Microorganisms produce diverse polymers for various purposes such as storing genetic information, energy, and reducing power, and serving as structural materials and scaffolds. Among these polymers, polyhydroxyalkanoates (PHAs) are microbial polyesters synthesized and accumulated intracellularly as a storage material of carbon, energy, and reducing power under unfavorable growth conditions in the presence of excess carbon source. PHAs have attracted considerable attention for their wide range of applications in industrial and medical fields. Since the first discovery of PHA accumulating bacteria about 100 years ago, remarkable advances have been made in the understanding of PHA biosynthesis and metabolic engineering of microorganisms toward developing efficient PHA producers. Recently, nonnatural polyesters have also been synthesized by metabolically engineered microorganisms, which opened a new avenue toward sustainable production of more diverse plastics. Herein, the current state of PHAs and nonnatural polyesters is reviewed, covering mechanisms of microbial polyester biosynthesis, metabolic pathways, and enzymes involved in biosynthesis of short-chain-length PHAs, medium-chain-length PHAs, and nonnatural polyesters, especially 2-hydroxyacid-containing polyesters, metabolic engineering strategies to produce novel polymers and enhance production capabilities and fermentation, and downstream processing strategies for cost-effective production of these microbial polyesters. In addition, the applications of PHAs and prospects are discussed.

1. Introduction

Polymers derived from microorganisms have been attracting increasing attention due to their superior properties such as sustainability, biodegradability, biocompatibility, and diverse material properties suitable for industrial and medical applications.^[1,2] There are six major types of biopolymers which are naturally synthesized in microorganisms: polynucleotides (such as DNA and RNA), polysaccharides (such as alginate, xanthan, and cellulose), polyesters [such as polyhydroxyalkanoates (PHAs) and polymalic acid], polythioesters, inorganic polyanhydrides (such as polyphosphates), and polyamides (such as polypeptides, cyanophycin, ϵ -poly-L-lysine, and poly- γ -glutamate).^[1,2] Among them, PHAs and bacterial polyesters are biodegradable and biocompatible and naturally produced in many gram-positive and gram-negative bacteria including *Cupriavidus*, *Pseudomonas*, *Alcaligenes*, *Bacillus*, and *Aeromonas* (Figure 1).^[1–3] These bacteria intracellularly accumulate PHAs in the form of distinct granules as storage material of carbon, energy, and reducing power, when they are cultured


in a medium limited in growth components such as nitrogen, phosphorus, or potassium in the presence of excess carbon source. PHAs exhibit various properties from hard plastics to flexible elastomers depending on the types and compositions of the monomers. Thus, PHAs have been studied as promising substitutes for petroleum-based plastics and a few members of PHAs are currently commercialized for packaging, agricultural, and medical applications with an annual production of up to 270 000 tons year⁻¹.^[3,4] As several companies are actively planning to expand their production volumes to meet the increasing demand for bioplastics, the production is predicted to reach over 932 000 tons year⁻¹ in 2020.^[5]

Numerous natural PHA producing bacteria were isolated and the genes encoding polymerizing enzymes (PHA synthases), metabolic enzymes, and auxiliary proteins involved in PHA biosynthesis were identified and characterized at molecular level. Also, our understanding on PHA biosynthesis in the context of entire metabolism has advanced significantly.^[1–5] In addition, various metabolic engineering approaches have been adopted to develop PHA overproducing bacteria with higher PHA titer, yield, and productivity. Furthermore, microbial strains have

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been engineered to produce PHAs with altered monomer compositions and different molecular weights for better thermal and mechanical properties.^[3–5,7] Several non-native PHA producers such as *Escherichia coli* and *Corynebacterium glutamicum* have been engineered to produce PHAs as well. These host strains have shown several advantages in PHA production such as ultra efficient accumulation of PHAs by engineered *E. coli* and endotoxin-free PHA production by engineered *C. glutamicum*.^[8,9] Recent advances in systems-level studies are enabling more comprehensive understanding of the metabolic network and efficient strain development. For example, in silico genome-scale metabolic analysis and transcriptome profiling in *Pseudomonas putida* KT2440 and *Cupriavidus necator* H16 (previously known as *Alcaligenes eutrophus*, and then *Ralstonia eutropha*) provided detailed information on metabolic capabilities, genetic and physiological changes in response to altered environmental conditions, which ultimately led to the development of engineered strains with desirable PHA production performance.^[10–14] Also, much effort has been exerted to identify and evolve the PHA synthases which catalyze the polymerizing reaction in PHA biosynthesis and influence PHA productivity, monomer type and composition, and molecular weight.^[15–20] One good example is the development of evolved PHA synthases capable of accepting nonnatural substrates such as 2-hydroxyacyl-coenzyme A (CoA), which made it possible to produce nonnatural PHAs.^[19,20] Recently, the crystal structures of PHA synthases have been elucidated, which allows our better understanding on detailed polymerization mechanisms and suggests protein engineering strategies.^[21–24]

Herein, we review the current state of microbial production of PHAs and other nonnatural polyesters as promising alternatives to the petroleum-based synthetic polymers. We provide an overview on PHA metabolism in the context of entire metabolism together with enzymes and proteins involved in. We then discuss how microorganisms have been metabolically engineered to produce PHAs and nonnatural polyesters having desired monomer compositions with high-performance indices (titer, yield, and productivity). Also, strategies for fermentation and downstream processing are considered for developing more competitive bioprocess for the production of microbial polyesters. Finally, the material properties and applications of microbial polyesters are presented.

2. Diversity of Polyhydroxyalkanoates

In 1926, lipid-like inclusions in *Bacillus megaterium* were identified as poly(3-hydroxybutyrate) [poly(3HB)], which was the first discovered member of PHAs.^[25] About 50 years later, 3-hydroxyvalerate (3HV) and 3-hydroxyhexanoate (3HHx) were also identified as monomers of PHAs.^[26] Poly(3HB-co-3HV), showing much better material properties, was produced in large amounts in industrial scale and was sold under the trade-name Biopol. Such initial success led to the burst period for discovering and identifying possible monomer constituents of PHAs by feeding related carbon sources and analyzing the monomer constituents of PHAs produced. With such effort, more than 90 monomers of PHAs were discovered until 1995 (Figures 2 and 3).^[27] Currently, more than 150 monomers are



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identified as the constituents of PHAs, which can be classified according to the number of carbons (C2–C20), the position of hydroxyl-group [2-, 3-, 4-, 5-, and 6-hydroxycarboxylic acids (HAs)], and functional groups in the side chains, such as phenoxy, phenyl, benzyl, cyclohexyl, epoxy, thio, fluoro, bromo, cyano, and acetoxy groups (Table S1, Supporting Information).^[27,28] Generally, PHAs are divided into short-chain-length (SCL) and medium-chain-length (MCL) PHAs comprising C3–C5 and C6–C14 monomers, respectively.^[1–3] Most microorganisms accumulating PHAs in nature produce one of these two types since the polymerizing enzyme (PHA synthase) prefers either SCL- or MCL-hydroxyacyl-CoAs as substrates; the details are discussed next. Some PHA synthases can accept both SCL- and MCL-hydroxyacyl-CoAs to produce SCL-MCL-PHAs. In some cases, long-chain-length (LCL) PHAs comprising even longer carbon chain lengths (C15–C20) can be produced.

Such huge diversity of PHAs is possible due to the promiscuity of PHA synthases which can accept a broad range of substrates. According to the monomer types and compositions, various PHA homopolymers and copolymers can be synthesized to exhibit different material properties such as chemical, mechanical, biodegradable, and biocompatible properties. For example, SCL-PHAs are generally hard and stiff thermoplastics with high crystallinity while MCL-PHAs are flexible and elastomeric rubber-like polymers.^[1–5] PHAs comprising both SCL and MCL monomers can possess the intermediate properties depending on their monomer compositions as detailed later. Such diversity of material properties obtainable is one of the

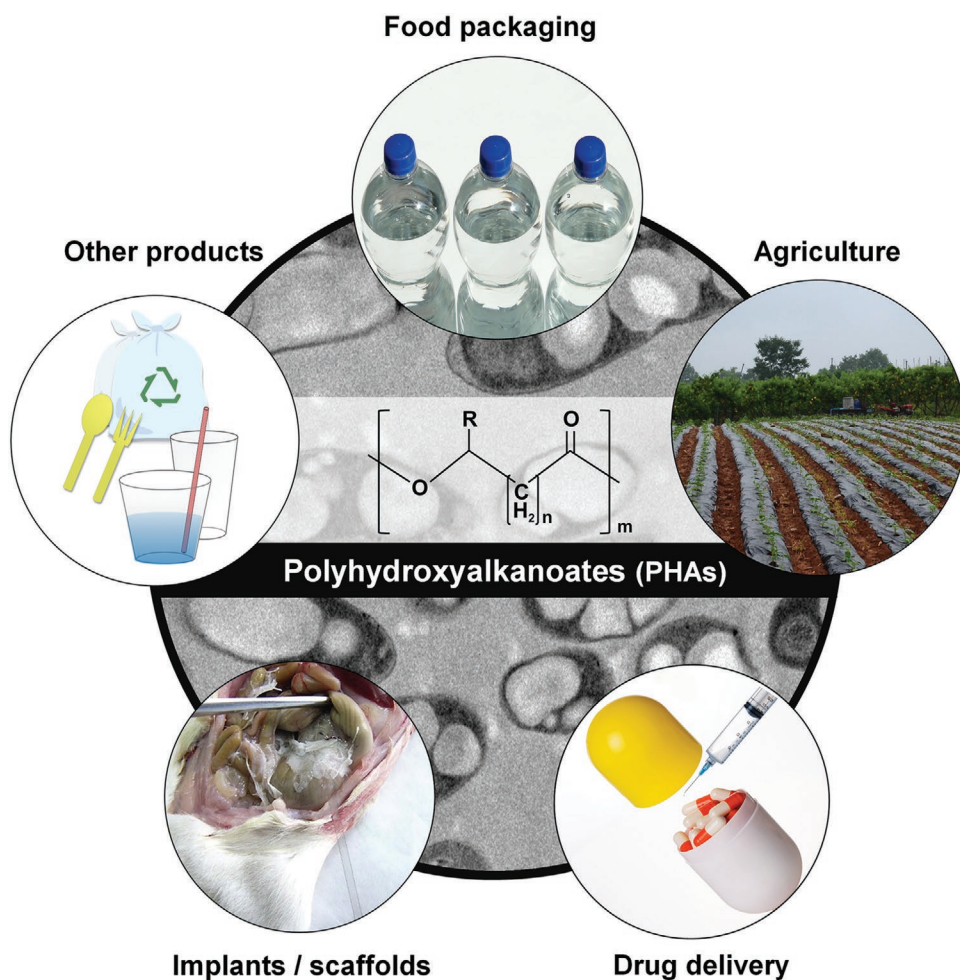


Figure 1. General chemical structure and various applications of PHAs. The image of the PHA implant is adapted with permission.^[6] Copyright 2009, Elsevier.

attractive characteristics of PHAs enabling synthesis of customized polymers for desired applications. PHAs have been extensively explored for various applications such as food and general packaging, plastic bag, agricultural mulching film, medical sutures, and others.

3. PHA Synthase and Other Enzymes Involved in PHA Biosynthesis

Considerable advances have been made on understanding the metabolic pathways and the enzymes involved in PHA biosynthesis in different bacteria, which consequently led to the elucidation of the metabolisms of PHA biosynthesis and degradation. PHAs exist as cytoplasmic inclusions (e.g., PHA granules), which are surrounded by several different types of granule-associated proteins (Figure 3). On the PHA granules, many proteins involved in biosynthesis, degradation, mobilization, and stabilization of PHAs are attached. These proteins include PHA synthase (PhaC) which polymerizes hydroxyacyl-CoAs into PHAs, PHA depolymerase (PhaZ), and PHA-oligomer hydrolase (PhaY) which degrade PHAs,^[29,30] phasins

(PhaP, PhaI, PhaF, ApdA, GA14, and Mms16),^[31–36] regulatory proteins (PhaR and PhaQ),^[29,37] and a PHA synthase activating protein (PhaM).^[38] A schematic model of *C. necator* in vivo PHA granule based on the previous reports is shown in Figure 3. The PhaY found in such bacteria including *C. necator* and *Ralstonia pickettii* is a PhaZ-like enzyme with a higher enzyme specificity toward 3HB oligomer than poly(3HB).^[30,39,40] For example, *C. necator* PhaY (PhaZc) showed 100-fold higher specific activity toward 3HB-pentamer than that toward artificial amorphous poly(3HB).^[39] Noticeably, some studies reported that *C. necator* PhaYs were mostly found in the soluble fraction and only a negligible amount of PhaYs in the PHA granule fraction.^[29,39] Thus, more research is required to understand the state of actual in vivo PHA granule. Phasins, a group of amphiphilic proteins (14–24 kDa),^[41,42] have both structural and regulatory functions in PHA biosynthesis. Several phasins from different microbial strains have been characterized: PhaPs from *C. necator* and *B. megaterium*,^[29,37] PhaI from *P. putida*,^[31] PhaFs from *P. oleovorans* and *P. putida* KT2442,^[32,33] ApdA from *Rhodospirillum rubrum*,^[34] GA14 from *Rhodococcus ruber*,^[35] and Mms16 from *Magnetospirillum gryphiswaldense*.^[36] The details of the granule-associated proteins have been thoroughly reviewed elsewhere,

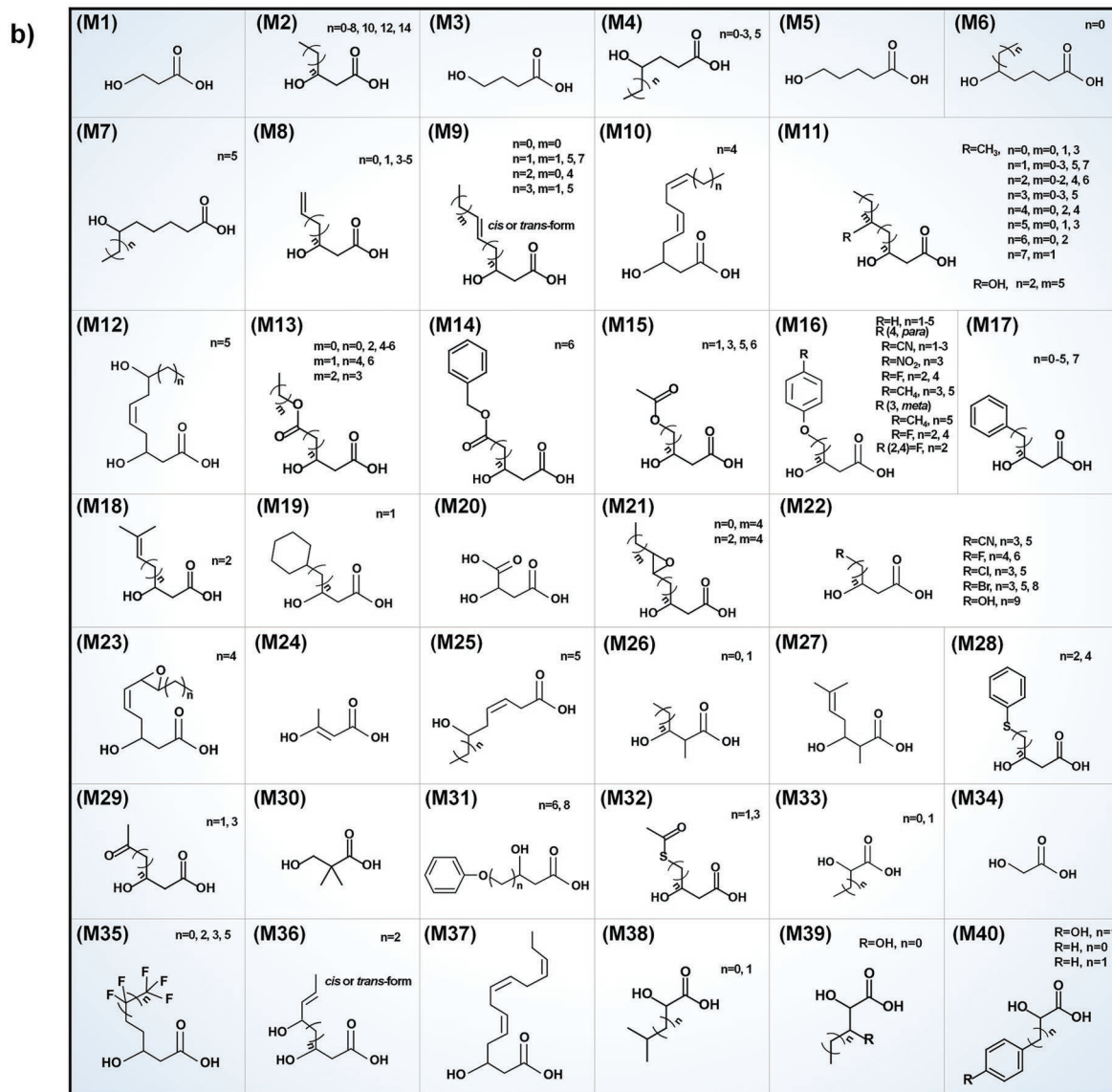
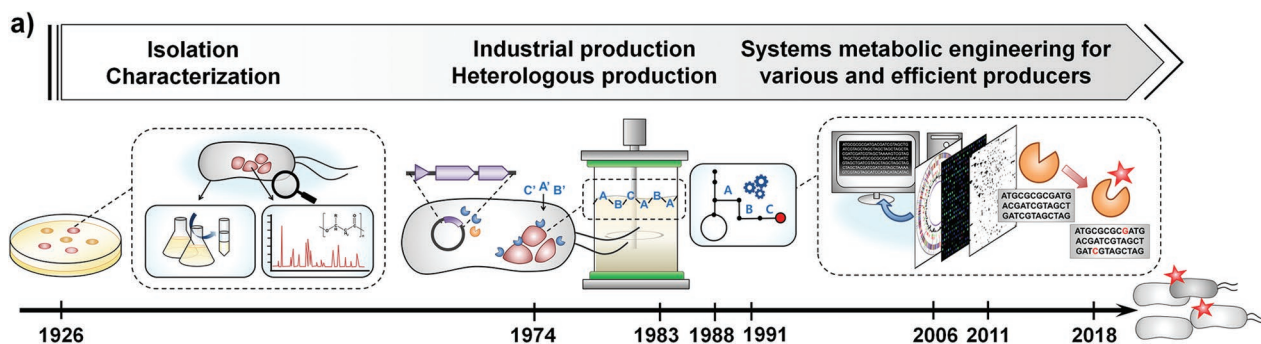


Figure 2. The diversity of monomers in PHAs. a) Schematic illustration of the development in PHA production. The representative years are indicated: 1) 1926, the first identification of PHA [poly(3-hydroxybutyrate)]; 2) 1974, the discovery of second set of PHA monomers (3-hydroxyvalerate and 3-hydroxyhexanoate); 3) 1983, the discovery of MCL-PHA (3-hydroxyoctanoate monomer); 4) 1988, the discovery of 4-hydroxybutyrate-containing PHA; 5) 1991, the discovery of 3-hydroxypropionate-containing PHA; 6) 2006, the first incorporation of 2-hydroxyacid monomer (lactate); 7) 2011, the first incorporation of glycolate; 8) 2018, the first incorporation of aromatic 2-hydroxyacid monomers (phenyllactate and mandelate). b) The chemical structures of monomers identified as the constituents of PHAs and nonnatural polyesters. The n and m indicate the number of repeating units in the chemical structure, while "R" represents a side group. For convenience, the chemical structures are numbered with (M1)–(M40). All chemical structure names and references are provided in Table S1 (Supporting Information), together with the matching monomers (M1)–(M40) shown here.

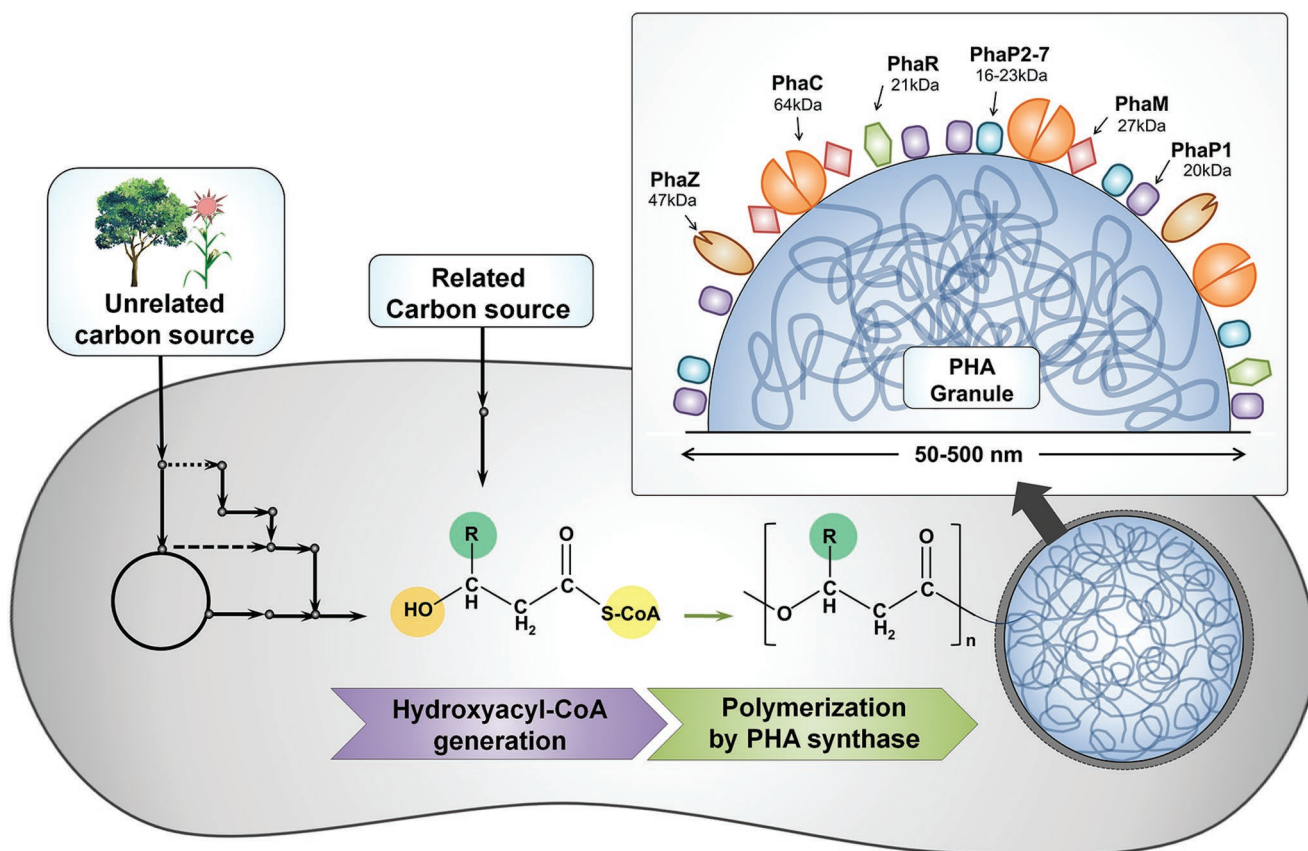


Figure 3. A schematic representation of PHA granule biosynthesis. A PHA granule of the representative PHA-producing strain, *C. necator*, is shown with the granule-associated enzymes and proteins; PhaC (PHA synthase), PhaZ (PHA depolymerase), PhaP1-7 (phasins, PhaP1 is the most abundant protein in the granule), PhaR (regulatory protein), and PhaM (granule-associated protein).

see refs. [29,43] Phasin is not essential for the polymerization activity but is involved in determining the size and number of PHA granules by stabilizing the hydrophobic surface of granules in cytoplasmic condition and in segregating PHA granules during cell division.^[32,33,41,42] In *C. necator* and *B. megaterium*, the expression level of PhaP has been reported to be regulated by PhaR and PhaQ, respectively.^[37,44] Similar to phasins, PhaM is a small protein (26 kDa) found in *C. necator* and functions as an activator of PHA synthase.^[38,45] Recent studies have revealed that *C. necator* PhaM might play an important role in inheriting PHA granules to daughter cells during cell division based on the presence of nucleoid-binding PAKKA motifs at the C-terminal part of PhaM.^[38,45] Other enzymes and metabolic pathways involved in PHA biosynthesis with respect to the generation of various hydroxyacyl-CoAs will be discussed for each monomer in Section 4.

Among these PHA granule-associated proteins, PHA synthase is the key enzyme responsible for actual polymerization, affecting monomer composition, molecular weight, and productivity of PHA.^[46] Thus, much effort has been exerted to understand polymerization mechanisms and to develop superior PHA synthases. The details regarding the engineering of PHA synthase are described in Section 5.3. As mentioned above, one of the key characteristics of PHA synthase is the promiscuity allowing the use of a broad range of substrates for

the synthesis of various polymers. Another characteristic is the stereospecificity of PHA synthases which accept only the monomers of (*R*) configuration.^[24,46]

PHA synthases can be classified into four different classes according to the enzyme subunit configuration and favorable substrate carbon length.^[24,47–55] Class I PHA synthases comprise one type of subunit (PhaC) having a molecular weight of 60–65 kDa in most cases, and are suggested to be active as a form of homodimer.^[21–23,48,49] *C. necator* and *Aeromonas* spp., including *A. caviae*, *A. hydrophila*, and *A. punctata*, are representative bacteria possessing class I PHA synthases.^[48–50] Class I PHA synthases prefer SCL monomers (C3–C5) as substrates and have negligible or low activity for MCL monomers (C6–C16).^[48,49] On the other hand, class II PHA synthases utilize MCL-hydroxyacyl-CoAs (C6–C14) as substrates. Class II PHA synthase comprises a single-subunit PhaC of about 60 kDa and is active as a form of homodimer.^[47,50–52] There are two different PhaCs encoded by *phaC1* and *phaC2* genes.^[50,51] Class II PHA synthases are mainly found in *Pseudomonas* species such as *P. putida* and *P. aeruginosa*.^[50,52]

Class III PHA synthase comprises two different subunits, PhaC (≈40 kDa) and PhaE (≈20 or 40 kDa), and are found in bacteria such as *Allochrochromatium vinosum* and *Desulfococcus multivorans*.^[48,53,54] Class III PHA synthase synthesizes SCL-PHAs and both subunits (PhaC and PhaE) are required for the

polymerization activity. The PhaC subunit of class III PHA synthase exhibits high amino acid sequence similarity of 21–28% with the PhaC subunits of class I and II PHA synthases, and thus, is considered a catalytic subunit. On the other hand, the PhaE subunit has no similarity with other known PHA synthases and its exact role is undisclosed. Class IV PHA synthases comprise PhaC subunit (≈ 40 kDa) and PhaR subunit (≈ 20 kDa) and require both subunits for PHA synthesis.^[55,56] Class IV PHA synthases favor SCL-hydroxyacyl-CoAs as substrates like class I and III PHA synthases, and are mostly found in *Bacillus* species such as *B. megaterium* and *B. cereus*.^[55,56] Several PHA synthases possess exceptionally broad substrate utilization range, and are capable of polymerizing both SCL- and MCL-hydroxyacyl-CoAs at the same time. Such PHA synthases have been found in several bacterial species including *Thiocapsa pfennigii*, *A. caviae*, *Pseudomonas* sp. 61-3, and *Pseudomonas* sp. MBEL 6-19.^[20,57–59]

All PHA synthases possess three highly conserved residues (cysteine, histidine, and aspartate) which function as a covalent nucleophile, a general base, and an electron donor, respectively, forming a Cys-His-Asp catalytic triad (Figure 4).^[21–23] Site-directed mutagenesis of any of these residues in *C. necator* PhaC to alanine resulted in the complete loss of enzyme activity both in vivo and in vitro.^[21] The crystal structure of PHA synthase could not be determined for almost three decades because the protein crystal could not be made. Thus, several models have been proposed for the polymerization mechanism of PHA synthase based on the computational structure prediction and modeling with related biochemical studies. Recently, the crystal structure of the catalytic domain

of *C. necator* PHA synthase was determined. The protein crystal of the C-terminal catalytic domain of PHA synthase was serendipitously obtained through unintended proteolysis during the storage of the purified protein.^[21] Similarly, the crystal structure of the C-terminal domain of PHA synthase from *Chromobacterium* sp. USM2, which also belongs to class I PHA synthase, has been determined.^[23] Based on the crystal structures of PHA synthases from *C. necator* and *Chromobacterium* sp. USM2, the polymerization reaction mechanisms have been proposed.^[21–24] Each PhaC monomer possesses one catalytic site comprising Cys-His-Asp catalytic triad and the two catalytic sites of the PhaC dimer structure are positioned 28.1 and 33.4 Å apart for the *C. necator* and *Chromobacterium* sp. USM2, respectively. These structures indicate that the polymerization reaction occurs independently at each site. Based on the crystal structures, different enzyme models have been suggested with respect to the existence of entrance and egress routes for the substrates and products. This is because the crystal structures of the active state PHA synthases bound to their actual substrates could not yet be determined. Thus, further studies are needed to elucidate more detailed mechanism of PHA polymerization. A review paper by Sagong et al.^[24] can be consulted for the details of enzyme structures and mechanisms.

In addition, the 3D reconstructed models of full-length PhaC from *C. necator* and its complex with PhaM from *C. necator* (PhaC/PhaM) were determined by small angle X-ray scattering (SAXS) analyses.^[17] The models showed that, in the PhaC dimer, the C-terminal domains are located at the center and the N-terminal domains are located opposite of the dimerization domain of PhaC dimer. This indicates that the N-terminal

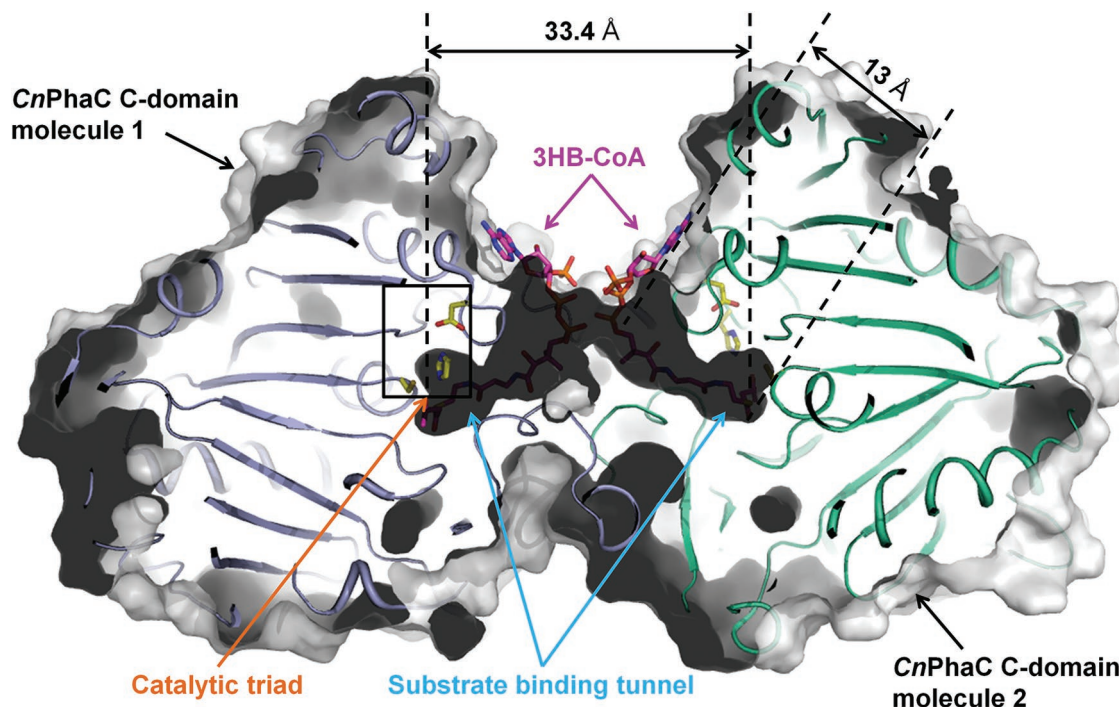


Figure 4. The 3D structure of the C-terminal domain (C-domain, Gly193-Ala589) of *C. necator* PHA synthase (CnPhaC; PDB ID: 5HZ2). A dimeric structure of the C-domain of PhaC is presented as a cartoon diagram in a surface cross-section view. Two C-domain molecules are distinguished in purple and light cyan, respectively. The catalytic triad (Cys, His, and Asp) is shown as a yellow-colored stick model at box. The substrate-binding tunnels are labeled. The bound 3-hydroxybutyryl-CoA (3HB-CoA) molecules are also shown as a stick model in magenta.

Table 1. Key pathways and enzymes involved in the biosynthesis of PHAs and nonnatural polyesters.

Pathway and monomer ^{a)}	Key enzymes	Related enzymes	Representative polymers	Ref.
Glycolysis				
3HB	PhaA	β -Ketothiolase	Poly(3HB)	[8,60]
	PhaB	Acetoacetyl-CoA reductase		
LA	LdhA	LA dehydrogenase	Poly(LA)	[61]
	Pct	Propionyl-CoA transferase	Poly(LA-co-3HB)	
3HP	AccABCD	Acetyl-CoA carboxylase	Poly(3HP)	[62]
	Mcr	Malonyl-CoA reductase		
	PrpE	Propionyl-CoA synthetase		
Glycerol pathway				
3HP	DhaB	Glycerol dehydratase	Poly(3HP)	[63]
	PduP	Propionaldehyde dehydrogenase		
Dahms pathway (xylose)				
GA	XylB	Xylose dehydrogenase	Poly(LA-co-GA)	[64]
	XylC	Xylonolactonase		
	Pct	Propionyl-CoA transferase		
Fatty acid de novo biosynthesis and degradation (β-oxidation)				
3HA	FadA	3-Ketoacyl-CoA thiolase	MCL-PHA	[65–69]
	FadB	Acyl-CoA dehydrogenase	SCL-MCL-PHA	
	FadG	Acyl-CoA dehydrogenase		
	FadD	Acyl-CoA synthetase		
	FadE	Acyl-CoA dehydrogenase		
	FadR	Fatty acid metabolism regulator		
	PhaJ	(<i>R</i>)-specific enoyl-CoA hydratase		
	TesA	Thioesterase		
	AccABCD	Acetyl-CoA carboxylase		
	PhaG	3-Hydroxyacyl-CoA:ACP transacylase		
	FabG	3-Ketoacyl-ACP reductase		
	FabA	3-Ketoacyl-ACP dehydrase		
	FabB	3-Ketoacyl-ACP synthase I		
	FabF	3-Ketoacyl-ACP synthase II		
	FabH	3-Ketoacyl-ACP synthase III		
FabI	Enoyl-ACP reductase			
FabD	Malonyl-CoA:ACP transacylase			
TCA cycle				
GA	AceA	Isocitrate lyase	Poly(LA-co-GA-co-3HB)	[70]
	YcdW	Glyoxylate reductase		
	Pct	Propionyl-CoA transferase		
4HB	SucD	Succinate semialdehyde dehydrogenase	Poly(4HB)	[71]
	4HbD	4HB dehydrogenase	Poly(4HB-co-3HB)	[72]
	OrfZ	4-Hydroxybutyryl-CoA transferase		
	Pct	Propionyl-CoA transferase		
2,3DHBA	BktB	β -Ketothiolase	Poly(2,3DHBA-co-3HB)	[73]
3HV	PhaB	Acetoacetyl-CoA reductase	Poly(3HV-co-3HB)	[74]
	Sbm	Methylmalonyl-CoA mutase		
	YgfG	Methylmalonyl-CoA decarboxylase		
	BktB	β -Ketothiolase		
	PhaA	β -Ketothiolase		
PhaB	Acetoacetyl-CoA reductase			
Propionate-dependent pathway				
2HB	PrpE	Propionyl-CoA synthetase	Poly(2HB-co-3HB-co-LA)	[75]
	PDHc	Pyruvate dehydrogenase		
	PanE	2-Hydroxyacid dehydrogenase		
	Pct	Propionyl-CoA transferase		

Table 1. Continued.

Pathway and monomer ^{a)}	Key enzymes	Related enzymes	Representative polymers	Ref.
Amino acid biosynthesis				
2HB	CimA	Citramalate synthase	Poly(2HB-co-3HB-co-LA)	[76]
	LeuCD	Isopropylmalate isomerase		
	LeuB	3-Isopropylmalate dehydrogenase		
	PanE	2-Hydroxyacid dehydrogenase		
	Pct	Propionyl-CoA transferase		
2HIV	AlsS	Acetolactate synthase	Poly(2HIV-co-LA)	[77]
	IlvC	Ketol-acid reductoisomerase		
	IlvD	Dihydroxyacid dehydratase		
	PanE	2-Hydroxyacid dehydrogenase		
	Pct	Propionyl-CoA transferase		
2H4MV	LdhA	2H4MV dehydrogenase	Poly(2H4MV-co-3HB-co-LA-co-2HIV-co-PhLA)	[78]
	HadA	Isocaproate CoA-transferase		
PhLA 4HPhLA	PheA	Chorismate mutase/prephenate dehydratase	Poly(PhLA-co-3HB)	[79]
	FldH	PhLA dehydrogenase		
	HadA	Isocaproate CoA-transferase		
	FldA	Cinnamoyl-CoA:PhLA CoA-transferase		
	LdhA	2H4MV dehydrogenase	Poly(3H4MV-co-3HB)	
HadA	Isocaproate CoA-transferase			
HadIBC	2H4MV-CoA dehydratase (HadBC) and activator (HadI)			
PhaJ	(R)-specific enoyl-CoA hydratase			

^{a)}3HB, LA, 3HP, GA, 3HA, 4HB, 2,3DHBA, 3HV, 2HB, 2HIV, 2H4MV, 4HPhLA, PhLA, and 3H4MV indicate 3-hydroxybutyrate, lactate, 3-hydroxypropionate, glycolate, 3-hydroxycarboxylate, 4-hydroxybutyrate, 2,3-dihydroxybutyrate, 3-hydroxyvalerate, 2-hydroxybutyrate, 2-hydroxyisovalerate, 2-hydroxy-4-methylvalerate, 4-hydroxyphenyllactate, phenyllactate, and 3-hydroxy-4-methylvalerate, respectively.

accumulating poly(3HB). The poly(3HB) biosynthetic pathway comprising PHA synthase, β -ketothiolase, and reductase (encoded by the *phaCAB* operon) from *C. necator* has first been characterized at molecular level through three independent pioneering works by Sinskey, Steinbuchel, and Dennis, which was similarly performed for other poly(3HB) biosynthesis genes in different bacteria as detailed in a review paper.^[60] In this pathway, two molecules of acetyl-CoA are condensed to acetoacetyl-CoA by β -ketothiolase (*phaA*). Acetoacetyl-CoA is reduced to 3-hydroxybutyryl-CoA by NADPH-dependent acetoacetyl-CoA reductase (*phaB*). Finally, 3-hydroxybutyryl-CoA is polymerized into the growing chain of poly(3HB) by PHA synthase (*phaC*) (Figure 5). Again, it should be noted that hydroxyacyl-CoAs (e.g., 3-hydroxybutyryl-CoA above) described in this paper are all in (R) configuration, and thus is not indicated separately.

This biosynthetic pathway of poly(3HB) is relatively simple compared to other PHAs and starts from acetyl-CoA which is an essential metabolite of organisms. As these characteristics are attractive to the metabolic engineers, several PHA nonproducers have been engineered to produce poly(3HB) by introducing the *phaCAB* genes from the native PHA producers. Among them, engineered *E. coli* strain showed superior production performances; poly(3HB) can be produced up to 141.6 g L⁻¹ with a productivity of 4.63 g L⁻¹ h⁻¹.^[8] Also,

engineered *E. coli* was able to synthesize poly(3HB), having molecular weight as high as 20 million Da when cultured at low pH.^[81] In addition to bacteria, plants including *Arabidopsis thaliana* and tobacco plant have been successfully engineered to produce poly(3HB).^[82-85] Thanks to much effort exerted for a long time, industrial production of poly(3HB) is the most cost-competitive compared with other PHAs.

4.2. PHAs Containing 3-Hydroxyvalerate as a Monomer

3HV was found as a monomer of poly(3HB-co-3HV), which was produced by *C. necator* grown in a medium supplemented with propionic acid, pentanoic acid, or *n*-pentanol.^[86,87] The 3HV-containing PHAs are generally synthesized from the propionyl-CoA-dependent pathway. In this pathway, acetyl-CoA and propionyl-CoA are condensed to 3-ketovaleryl-CoA by PhaA, and then 3-ketovaleryl-CoA is reduced to 3-hydroxyvaleryl-CoA by PhaB (Figure 5).^[88] It should be noted that the same enzymes are used for the generation of 3-hydroxybutyryl-CoA and 3-hydroxyvaleryl-CoA by using different precursors, two acetyl-CoAs for the former and acetyl-CoA and propionyl-CoA for the latter. Due to such enzyme characteristics, 3HV containing PHAs produced by this pathway always contain 3HB as a comonomer.

The first 3HV incorporation without the addition of related precursors was reported in a mutant *C. necator* R3.^[88] Since this *C. necator* strain has mutations related to the branched-chain amino acid biosynthetic pathways, it has high metabolic fluxes toward the amino acids L-valine and L-isoleucine. As propionyl-CoA can be synthesized from the intermediates of L-valine and L-isoleucine biosynthetic pathways, the mutant strain could produce poly(3HB-co-3HV) having 4–7 mol% of 3HV from the unrelated carbon sources such as fructose or gluconate.^[88]

Another pathway was first developed in recombinant *Salmonella enterica* serovar Typhimurium.^[74] In this pathway, succinyl-CoA derived from TCA cycle is converted to propionyl-CoA by two heterologous enzymes, methylmalonyl-CoA mutase, and methylmalonyl-CoA decarboxylase encoded by the *E. coli sbm* and *ygfG* genes, respectively. In addition, the *Acinetobacter* poly(3HB) biosynthesis operon (comprising *phaBCA* genes) was introduced. When the whole pathway was constructed in the PrpC mutant *S. enterica*, which has high intracellular propionyl-CoA pool, the engineered *S. enterica* strain accumulated poly(3HB-co-16 mol% 3HV) to a polymer content of about 45 wt% in a medium containing glycerol as a carbon source and vitamin B₁₂ as a supplement.^[74]

Propionyl-CoA can also be converted from 2-ketobutyrate, which can be synthesized from L-threonine. By engineering this pathway, the engineered *E. coli* strain produced poly(3HB-co-3HV) from glucose or xylose.^[89] To achieve this, three major metabolic engineering strategies were implemented. First, the feedback resistant *thrABC* operon was introduced to enhance L-threonine biosynthesis. Second, the *C. glutamicum ilvA* gene was overexpressed to promote the conversion of L-threonine to 2-ketobutyrate. Finally, the endogenous propionyl-CoA catabolic pathway encoded by *scpC*, *pta*, and *prpC* genes was blocked by knocking out these three genes. Using the resulting strain, poly(3HB-co-3HV) having 3HV fraction up to 17.5 mol% could be produced from xylose as a carbon source.^[89]

4.3. Medium-Chain-Length PHAs

MCL-PHAs are synthesized via the β -oxidation pathway and/or fatty acid de novo biosynthesis pathway by various bacteria, including *Pseudomonas*.^[65,66,90,91] The metabolic pathways with relevant enzymes are summarized in Figure 5 and Table 1. The β -oxidation pathway catabolizes fatty acids to acetyl-CoA by iterative cycles. In each cycle, the chain length of the fatty acids is reduced by two carbon units. The first step is the conversion of fatty acid into its CoA thioester by acyl-CoA synthetase (FadD), which is followed by the oxidation of acyl-CoA to enoyl-CoA by acyl-CoA dehydrogenase (FadE). Enoyl-CoA is then converted into acyl-CoA shortened by two carbons with the release of acetyl-CoA by the complex of acyl-CoA dehydrogenase (FadB) and 3-ketoacyl-CoA thiolase (FadA). This β -oxidation pathway provides 3-hydroxyacyl-CoAs from enoyl-CoAs or 3-ketoacyl-CoAs by enoyl-CoA hydratase (PhaJ) or MaoC) or acyl-CoA dehydrogenase (FadG), respectively. Thus, various hydroxyacyl-CoAs having different carbon lengths such as 3-hydroxyhexanoyl-CoA and 3-hydroxyoctanoyl-CoA are generated and polymerized into PHAs.^[66,67] Similarly, the fatty acid de novo biosynthesis

pathway is also an iterative cycle, and the carbon length of fatty acids is increased by two per cycle.^[67–69] It starts with carboxylation of acetyl-CoA by acetyl-CoA carboxylase (Acc), forming malonyl-CoA which is subsequently converted to malonyl-acyl carrier protein (ACP) by malonyl-CoA:ACP transacylase (FabD). There are three β -ketoacyl-ACP synthases generating 3-ketoacyl-ACPs from malonyl-ACP: FabH (synthase III), which mediates the condensation of malonyl-ACP and acetyl-CoA to form acetoacetyl-ACP; FabB (synthase I) and FabF (synthase II), which mediate the condensation of malonyl-ACP and acyl-ACP which is generated from 3-ketoacyl-ACP including acetoacetyl-ACP through the metabolic pathway comprising 3-ketoacyl-ACP reductase (FabG), 3-hydroxyacyl-ACP dehydrase (FabA), and enoyl-ACP reductase (FabI) as shown in Figure 5. Various hydroxyacyl-ACPs with different carbon lengths are generated and converted into hydroxyacyl-CoAs by acyl-CoA:ACP transacylase (PhaG). Thus, it is common that MCL-PHAs are generally composed of 3-HA monomers of different carbon lengths simultaneously.

To produce homo MCL-PHAs, the β -oxidation pathway has been blocked so that fatty acids could not be further metabolized to fatty acids of shorter carbon lengths.^[65,92] For example, mutant *P. putida* KT2442 lacking acyl-CoA dehydrogenases (FadB, FadB2x, PP2047, and PP2048), thiolases (FadA, FadAx), and 3-hydroxyacyl-CoA:ACP transacylase (PhaG) which provides hydroxyacyl-CoAs via the fatty acid de novo biosynthesis pathway could synthesize poly(3-hydroxydecanoate) [poly(3HD)] and poly(3HD-co-84 mol% 3-hydroxydodecanoate) [poly(3HD-co-84 mol% 3HDD)] when grown on decanoic acid and dodecanoic acid, respectively.^[92]

In addition, the fatty acid de novo biosynthesis pathway can be linked with the β -oxidation pathway.^[93] The acyl-ACPs generated from the fatty acid de novo biosynthesis pathway could be converted to free fatty acids by thioesterase, and then these fatty acids could be metabolized through the β -oxidation pathway. To produce MCL-PHAs in *E. coli* by employing this strategy, the *E. coli* chromosomal genes, *fadR* (encoding a regulator of fatty acid metabolism) and *fadB* (encoding acyl-CoA dehydrogenase) were deleted. Also, the endogenous *tesA* gene encoding thioesterase (TesA) and *P. oleovorans phaC1* gene encoding PHA synthase were overexpressed. This strain produced 2.3 wt% of MCL-PHA from gluconate.^[93]

More recently, a synthetic pathway called reverse β -oxidation pathway has been developed and employed for the production of various chemicals such as alcohols, fatty acids, and also MCL-PHAs.^[94–96] This pathway allows production of MCL-PHAs without supplementation of fatty acids similar to the de novo fatty acid biosynthesis pathway. To establish this pathway in *E. coli* for the production of MCL-PHAs from glucose, *E. coli ydiO* (encoding enoyl-CoA reductase) and the *fadBA* genes and *P. aeruginosa* PAO1 *phaJ1* [encoding (R)-specific enoyl-CoA hydratase] and *phaC2* (encoding PHA synthase) genes were overexpressed in *E. coli* LS5218. As the *E. coli* LS5218 strain contains mutations in the *fadR* and *atoC* genes, this strain was used to constitutively express the enzymes of the β -oxidation pathway without being regulated by the multifunctional regulator encoded by *fadR*. After deletion of the *ptsG* gene encoding glucose-specific phosphotransferase system enzyme IICB component and major thioesterases genes (*tesA*, *tesB*, and *yciA*), the

engineered *E. coli* strain produced MCL-PHA to 6.62 wt% of DCW.^[96] In addition, the use of PHA synthase with broad substrate specificity resulted in the production of SCL-MCL-PHAs. When *P. stutzeri* 1317 *phaC2* gene instead of the *P. aeruginosa* PAO1 *phaC2* gene was overexpressed with *C. necator phaAB* genes in the above engineered strain, poly(21 mol% 3HB-co-MCL-3-HA) was synthesized with a polymer content of 12 wt% of DCW.^[96]

4.4. Poly(4-hydroxybutyrate)

In 1988, 4-hydroxybutyrate (4HB) was first discovered as a constituent of poly(3HB-co-4HB), which was produced by cultivating *C. necator* in a medium supplemented with 4HB or 4-chlorobutyric acid.^[97] Previously, 4HB containing PHAs were only produced by feeding a structurally related precursor such as 1,4-butanediol or γ -butyrolactone in addition to 4HB and 4-chlorobutyric acid.^[97,98] To produce 4HB containing PHAs from unrelated carbon sources, an anaerobic succinate degradation pathway of *Clostridium kluyveri* was constructed in *E. coli*.^[99,100] In this pathway, succinyl-CoA is converted to succinate semialdehyde by *C. kluyveri* succinate semialdehyde dehydrogenase (SucD), and then succinate semialdehyde is converted to 4HB by *C. kluyveri* 4HB dehydrogenase (4HbD). The resulting 4HB is converted to 4-hydroxybutyryl-CoA by *C. kluyveri* 4-hydroxybutyryl-CoA transferase (OrfZ), and then polymerized by *C. necator* PHA synthase (PhaC) (Figure 5).^[100] Although poly(3HB-co-4HB) was successfully produced from glucose for the first time, the 4HB fraction was only 2.8 mol% due to the weak metabolic flux toward 4HB.^[100] To further increase the 4HB mole fraction, endogenous *sad* and *gabD* genes both encoding semialdehyde dehydrogenases were deleted to block the conversion of succinate semialdehyde into succinate. This resulted in an increased 4HB fraction up to 11 mol%.^[71] Furthermore, by overexpressing *C. necator* phasin protein (PhaP1), poly(4HB) production was significantly enhanced. The homo poly(4HB) content and titer reached 68.2 wt% and 7.8 g L⁻¹, respectively, by the fed-batch fermentation of the *sad* and *gabD* deficient *E. coli* expressing *sucD*, *4hbD*, *orfZ*, *phaC*, and *phaP1* genes on glucose.^[72]

4.5. Poly(3-hydroxypropionate)

PHAs containing 3-hydroxypropionate (3HP) were first synthesized by *C. necator* cultivated in a medium supplemented with structurally related precursors such as 1,5-pentanediol, 1,7-heptanediol, and 3HP.^[101] Also, feeding 1,3-propanediol or acrylic acid resulted in production of 3HP-containing PHAs.^[102,103] Aside from a monomer of PHAs, 3HP itself is also an industrial platform chemical.^[104] 3HP was selected as the third most important building block chemical derived from biomass by the U.S. Department of Energy (DOE) in 2004. Thus, there have been many studies to develop efficient 3HP producers using inexpensive carbon sources.^[105–107] Until now, three different 3HP biosynthetic pathways have been developed: pathways based on glycerol metabolism and pathways via malonyl-CoA and β -alanine, respectively.^[105–107]

The first biosynthesis of PHAs containing 3HP from unrelated carbon sources was achieved by engineered *C. necator* employing the 3HP biosynthetic pathway via malonyl-CoA.^[108] In this study, malonyl-CoA reductase (Mcr) and 3-hydroxypropionyl-CoA synthetase domain of trifunctional propionyl-CoA synthase (Acs) from *Chloroflexus aurantiacus* were introduced into *C. necator* (Figure 5). The strain could produce poly(3HB-co-2.1 mol% 3HP) to 0.775 g L⁻¹ with a polymer content of 31 wt% of DCW from fructose.^[108] This pathway was also constructed in the engineered *E. coli* overexpressing the genes of *C. aurantiacus* Mcr, *E. coli* propionyl-CoA synthetase (PrpE), and acetyl-CoA carboxylase (AccABCD), and *C. necator* PhaC.^[62] As a result, poly(3HP) was successfully produced from glucose as a sole carbon source. The titer and content of poly(3HP) were 1.32 g L⁻¹ and 0.98 wt% of DCW, respectively.

Another biosynthetic route based on glycerol metabolism has been extensively studied; the highest 3HP titer (102.6 g L⁻¹) in the literature was obtained by engineered *Klebsiella pneumoniae* employing this route.^[109] Also, this route facilitated the first homo poly(3HP) biosynthesis without supplementing any precursor.^[63] Here, the three enzymes, glycerol dehydratase (DhaB1) from *C. butyricum*, propionaldehyde dehydrogenase (PduP) from *S. enterica* serovar Typhimurium LT2, and PhaC from *C. necator* were introduced in *E. coli* (Figure 5). This engineered *E. coli* strain produced 1.42 and 0.27 g L⁻¹ of poly(3HP) by fed-batch fermentation using pure glycerol and crude glycerol, respectively.^[63]

In addition, production of 3HP in engineered *Saccharomyces cerevisiae* and *E. coli* was reported by using the pathway via β -alanine.^[106,107] β -Alanine generated from oxaloacetate^[106] or fumarate^[107] is converted into malonic semialdehyde by β -alanine pyruvate transaminase, and then malonic semialdehyde is converted into 3HP by 3HP dehydrogenase. The engineered *S. cerevisiae* and *E. coli* strains could produce 13.7 and 31.1 g L⁻¹ of 3HP from glucose, respectively. This β -alanine pathway has only been studied for the production of 3HP monomer and can be potentially used for 3HP-containing polymer production.

5. Strain Development for Efficient Production of PHAs and Nonnatural Polyesters

Although PHAs are promising biomaterials to substitute the petroleum-based synthetic plastics, high production cost hinders the commercialization of PHAs. Over the last decade, much effort has been made to enable economically viable production of PHAs by considering the entire bioprocess: strain development (upstream), fermentation (midstream), and separation/purification (downstream) (Figure 6).^[7,60,110] In this section, we focus on the strategies for the upstream process showing how microbial strains have been engineered for better PHA production performance. The strategies can be divided into three main categories: metabolic flux engineering, cell morphology engineering, and PHA synthase engineering. We illustrate these strategies with representative case studies below. In addition, we discuss the integrated strategies, which have been used to facilitate the development of microbial strains producing nonnatural polyesters such as PLA.

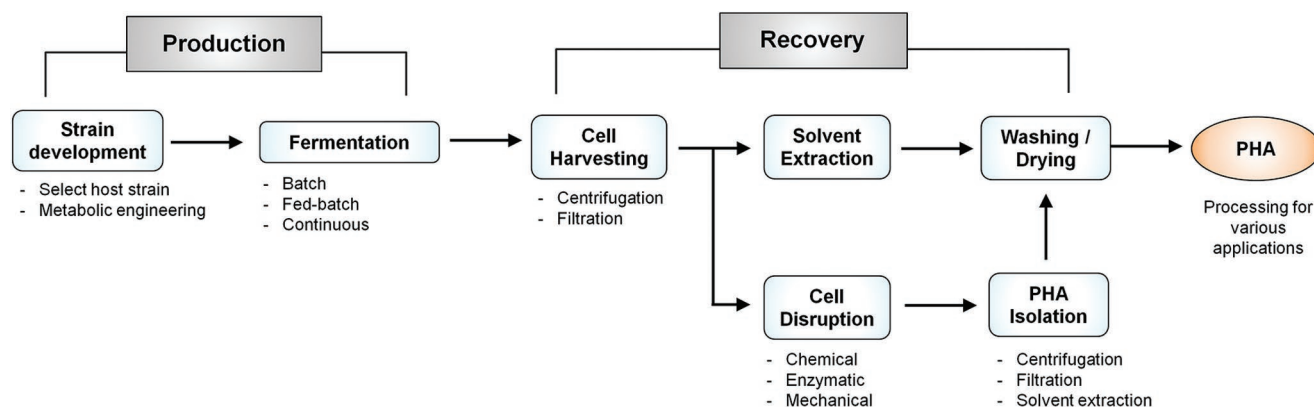


Figure 6. The schematic diagram of overall PHA production process. The strain developed is used for PHA production by fermentation, typically in fed-batch mode. Cells are harvested after fermentation, and are subject to one of the recovery processes depicted in the figure.

5.1. Metabolic Flux Engineering

5.1.1. Traditional Metabolic Engineering

The traditional metabolic engineering involves rational and intuitive genetic manipulations such as amplification of a handful of biosynthetic genes and inactivation of the competing metabolic pathways including those involved in byproducts formation. Despite being relatively simple and straightforward compared to the latest system-wide strategies based on computational and omics technologies, it has effectively improved the production performance of numerous strains. The natural PHA producers, such as *C. necator*, *Alcaligenes latus*, and *A. hydrophila*, can accumulate large amounts of PHAs amounting to about 90 wt% of DCW through the optimization of the culture conditions and feeding strategies for fed-batch cultures, rather than extensive metabolic engineering^[60,111] (for details about the cultivation strategies, see Section 6). On the other hand, traditional metabolic engineering has been effectively adopted for converting PHA nonproducers to overproducers. Among the bacterial strains, the engineered *E. coli* producing poly(3HB) has been most extensively studied. *E. coli* has been engineered to produce poly(3HB) by introducing a poly(3HB) biosynthesis operon from native producers such as *C. necator* and *A. latus*.^[8,112] The production performance has been optimized by using different copy-number plasmids harboring *phaCAB* genes,^[112] enhancing the plasmid stability by using the *hok/sok* system,^[8,112] and suppressing cell filamentation caused by PHA accumulation by overexpressing *ftsZ* gene.^[113] Through appropriate fed-batch cultures, these engineered *E. coli* strains could produce poly(3HB) up to 141.6 g L⁻¹ with the polymer content up to 86 wt% of DCW,^[8,113] which are comparable with poly(3HB) native producers. Other characteristics such as cell fragility upon accumulation of a large amount of poly(3HB) led to more efficient and cost-effective purification of polymer.

For poly(3HB) production, several engineering strategies were implemented to increase the pool of NADPH, which is required for the conversion of acetoacetyl-CoA into 3-hydroxybutyryl-CoA by PhaB.^[114–116] For example, the intracellular NADPH pool in *E. coli* could be increased by overexpressing the *zwf* gene encoding glucose-6-phosphate dehydrogenase, as it is the first enzyme of the pentose phosphate pathway generating NADPH.

The overexpression of the *zwf* gene together with *C. necator phaCAB* genes increased poly(3HB) content by 41% compared to that obtained by overexpressing only *phaCAB* genes.^[114] Also, overexpressing the *yjfB* gene encoding NAD kinase, which catalyzes the phosphorylation of NAD to NADP,^[115] or the *udhA* gene encoding soluble pyridine nucleotide transhydrogenase, which transfers the reducing equivalents between NAD and NADP,^[116] was effective in enhancing poly(3HB) production in *E. coli*. The *yjfB*-overexpressing *E. coli* strain harboring poly(3HB) biosynthesis pathway produced 14 g L⁻¹ poly(3HB), while the control strain without *yjfB* overexpression produced 7.14 g L⁻¹ by fed-batch fermentation using glucose as a sole carbon source.^[115]

In another study, the formation of byproducts such as acetate, lactate (LA), ethanol, and formate was prevented by knocking out the corresponding genes including *ackA-pta* (encoding acetate kinase-phosphate acetyltransferase), *poxB* (encoding pyruvate oxidase), *ldhA* (encoding LA dehydrogenase), and *adhE* (encoding acetaldehyde dehydrogenase).^[117] This engineered *E. coli* overexpressing the *C. necator phaCAB* genes showed 3.5 times higher poly(3HB) titer (0.45 g L⁻¹) than that obtained with the control strain.^[117]

E. coli is also used as a workhorse for MCL-PHA and SCL-MCL-PHA production by engineering the β -oxidation pathway and/or fatty acid de novo biosynthesis pathway together with the introduction of suitable PHA synthase. By overexpressing the *phaC1* gene from *P. aeruginosa* in *E. coli fadB* mutant, which accumulates the intermediates of the β -oxidation pathway, MCL-PHA comprising 3HHx, 3-hydroxyoctanoate (3HO), 3HD, and 3HDD monomers was produced from decanoic acid.^[118] To produce MCL-PHA without fatty acid supplementation, *E. coli* was engineered to overexpress *P. putida phaG* gene encoding 3-hydroxyacyl-CoA:ACP transacylase, which converts 3-hydroxyacyl-ACP generated through the fatty acid de novo biosynthetic pathway to 3-hydroxyacyl-CoA. The engineered *E. coli* coexpressing *P. putida phaG* and *phaC1* genes produced MCL-PHA with a polymer content of 3 wt% from gluconate.^[119] In another study, poly(66.9 mol% 3HB-co-MCL-3-HA) was produced from glucose by overexpressing endogenous *fabH* and *fabG* genes with *Pseudomonas* sp. 6-13 *phaC1* gene in *E. coli*.^[120] The *fabH* and *fabG* genes encode 3-ketoacyl-ACP synthase III (FabH) and 3-ketoacyl-ACP reductase (FabG), respectively. The 3-ketoacyl-ACP generated from

the fatty acid de novo biosynthesis pathway is converted into 3-ketoacyl-CoA by FabH and the 3-ketoacyl-CoA is further converted into 3-hydroxyacyl-CoA by FabG. Then, the 3-hydroxyacyl-CoAs with different carbon chain lengths are polymerized by *Pseudomonas* sp. 6-13 PHA synthase yielding SCL-MCL-PHA since this particular PHA synthase can accept both SCL and MCL-hydroxyacyl-CoAs as substrates even though it prefers MCL monomers.^[120]

The engineered *E. coli* strains have several advantages as PHA production hosts including high polymer content, fast growth rate, and easy cell lysis owing to the fragility of *E. coli* cells accumulating large amounts of PHAs. Since these characteristics reduce the overall PHA production cost, *E. coli* has been widely examined for the production of various PHAs and also nonnatural polyesters.^[121]

5.1.2. Systems and Synthetic Biology with Metabolic Engineering

Systems biology allows us to better understand cellular characteristics at a system level by collecting and integrating a wide range of biological data and also by analyzing data with various mathematical and computational technologies.^[110,122] As a systems biology approach, several omics studies have been performed to understand the metabolic and physiological changes related to PHA synthesis. The early attempts were made by comparing the proteomic profiles of wild-type and engineered *E. coli* producing poly(3HB).^[123,124] The two strains showed remarkable differences in expression levels of several proteins including heat shock proteins (GroEL, GroES, and DnaK) and glycolytic pathway enzymes (FbaA and TpiA).^[123] Based on the results,^[123] FbaA and TpiA were selected for engineering targets to enhance the production of poly(3HB).^[14] When the *E. coli* strains overexpressed *tpiA* and/or *fbaA* genes, the strains showed enhanced production of poly(3HB).^[14]

Transcriptome and metabolome analyses have also been conducted to understand the metabolic characteristics more precisely. These studies were performed for several PHA native producers such as *C. necator*,^[125] *P. putida*,^[126,127] *Haloarcula hispanica*,^[128] and *Azotobacter vinelandii*.^[129] The omics analyses were conducted under different nutrient conditions (PHA accumulating and PHA nonaccumulating),^[128] growth phases (exponential and stationary),^[130] and carbon sources (styrene, biodiesel-derived fatty acids, and glycerol).^[126,131,132] Also, the PHA producers were compared with their mutant strains such as PHA nonaccumulating mutant or superior producing mutant.^[125,129] These studies have elucidated several important features of PHA producing microorganisms and offered a comprehensive insight into PHA metabolism. For example, alterations in RNA transcripts (transcriptome), protein profiles (proteome), and the metabolome including 233 intracellular metabolites in key metabolic pathways including the Entner–Doudoroff pathway, TCA cycle, and amino acid biosynthetic pathway were analyzed for *P. putida* KT2442 strain grown under different nutrient limitation conditions.^[133] By integrating these omics results, the metabolic responses of *P. putida* KT2442 to a shift in resource distribution were explained at a system level considering the physiological changes of cells during PHA accumulation.^[133]

With comprehensive measurements of biological data, there have been several attempts to quantify the intracellular metabolic fluxes based on the reaction equations and mass balances. Understanding the changes of fluxes upon genetic and environmental perturbations provides engineering directions for the enhanced production performance of microorganisms.^[134] Initial metabolic flux analyses were performed for *C. necator* and recombinant *E. coli* producing poly(3HB) using only limited sets of reactions.^[135–138] For example, the flux model for the engineered *E. coli* harboring poly(3HB) biosynthesis genes consisted of a total of 12 fluxes of key pathways related to poly(3HB) biosynthesis.^[135] With further progress, a metabolic network model of *E. coli* producing poly(3HB) comprising a total of 304 intracellular reactions was constructed and used to analyze the distribution of metabolic fluxes.^[10] Simulation of this metabolic network suggested that the Entner–Doudoroff pathway was highly activated under the poly(3HB) producing condition compared with the poly(3HB) nonproducing condition. To validate the simulation results, the *eda* mutant *E. coli* that is not able to utilize the Entner–Doudoroff pathway was investigated for poly(3HB) production and the mutant showed lower poly(3HB) production, as expected.^[10]

As the tools and strategies for in silico metabolic modeling and simulation become available, genome-scale metabolic network models for the representative PHA producers including *P. putida* and *C. necator* have been reconstructed.^[11,13,139,140] Based on in silico simulation of the metabolic model, *P. putida* was rationally engineered to produce more MCL-PHAs from glucose.^[141] The metabolic model and FluxDesign approach^[142] predicted several beneficial gene deletion targets including *gcd* gene encoding glucose dehydrogenase. Knockout of the *gcd* gene resulted in 80% and 100% increases in final PHA yield and titer, respectively.^[141]

Recently, metabolic pathways for acetyl-CoA formation alternative to the classical pathway comprising the Embden–Meyerhof–Parnas pathway and pyruvate dehydrogenase have been employed for the production of poly(3HB).^[143,144] First, L-threonine bypass was newly suggested as an optimal metabolic pathway for poly(3HB) by flux balance analysis (FBA) of an *E. coli* genome-scale model (ijO1366).^[143] This pathway encompasses L-threonine synthesis and degradation to L-glycine, conversion of L-glycine to L-serine, and finally to pyruvate. Theoretically, the L-threonine bypass generates two moles of acetyl-CoA from one mole of phosphoenolpyruvate (PEP) and one mole of CO₂, while the classical pathway generates one mole of acetyl-CoA from one mole of PEP. To construct the L-threonine bypass in *E. coli*, the metabolic flux toward L-threonine biosynthesis was strengthened by introducing a mutation on the *thrA* gene (Ser345Phe) encoding feedback resistant aspartokinase I and overexpressing the *ppc* gene encoding PEP carboxylase. Also, L-threonine degradation to pyruvate was promoted by overexpressing the genes involved in the degradation pathway, which are *kbl*, *tdh*, *glyA*, and *sdaA* genes encoding 2-amino-3-ketobutyrate lyase, L-threonine 3-dehydrogenase, serine hydroxymethyltransferase, and serine deaminase, respectively. To generate NADPH required in the L-threonine bypass, *pntAB* genes encoding a transhydrogenase were also overexpressed. The engineered strain showed 3.3- and 2.3-fold increases of poly(3HB) titer and yield, respectively.^[143]

More recently, FBA of the extended *E. coli* genome-scale metabolic network model including the nonoxidative glycolysis (NOG) pathway revealed that introduction of the NOG pathway can increase the theoretical poly(3HB) yield from 0.48 to 0.64 g_{poly(3HB)} g⁻¹_{glucose}.^[144] The synthetic NOG pathway was first designed and constructed in *E. coli* by introducing phosphoketolase from *Bifidobacterium adolescentis* to split the fructose-6-phosphate (F6P) molecule into acetyl phosphate and erythrose 4-phosphate (E4P).^[145] Three molecules of E4P then undergo carbon rearrangement to make two molecules of F6P.^[145] Through this pathway, hexoses, pentoses, and triose phosphates can be catabolized into acetyl-CoA without carbon loss. As guided by the FBA results, *E. coli* was engineered to enhance the NOG pathway flux by introducing the *B. adolescentis* phosphoketolase gene and overexpressing *tktA* and *talA*. Further engineering by overexpressing *pntAB* and deleting *fumC* increased the poly(3HB) yield to 0.31 g g⁻¹, which was 93% higher than that (0.16 g g⁻¹) of the control strain.^[144]

Various synthetic biology techniques have also complemented traditional metabolic engineering strategies by providing useful tools for fine control and regulation of gene expression, multiple targeting, genome engineering, among others.^[122] For example, poly(3HB) production in *E. coli* was improved by adjusting the expression levels of *C. necator phaA*, *phaB*, and *phaC* genes with rationally designed ribosomal binding sites (RBSs).^[146] To fine-tune the expression levels of these genes, several RBSs of different strengths were designed by RBS Calculator and libraries consisting of different RBSs for each of the three poly(3HB) biosynthesis genes were constructed in 1000 different combinations. Superior poly(3HB) producers were first selected by primary screening based on the white color of colonies. The positive colonies were then cultivated in 96-well plates subjected to fluorescence staining and selection by fluorescent activated cell sorting (FACS). Finally, about 20 variants were further analyzed via gas chromatographic analysis, which showed increased poly(3HB) content up to 91.6 from 70.9 wt% and 52.9 from 11.3 wt% by using high-copy plasmid and low-copy plasmid, respectively.^[146]

In another example, synthetic clustered regularly interspaced short palindromic repeats interference (CRISPRi) was used to modulate the metabolic fluxes by regulating several chromosomal genes at the same time. CRISPRi comprises catalytically inactive Cas9 (dCas9) and single guide RNA (sgRNA). Since dCas9 binds to the target DNA guided by sgRNA in a sequence-specific manner, this system can block the transcription of target gene.^[147] The CRISPRi was applied to an engineered *E. coli* strain producing poly(3HB-co-4HB) to repress expression of five genes in the succinate metabolic pathway, *sdhAB* (encoding succinate dehydrogenase), *sucCD* (encoding succinyl-CoA synthetase), and *sad* (encoding succinate semialdehyde dehydrogenase).^[148] When all five genes were appropriately repressed by CRISPRi, the engineered strain produced poly(3HB-co-18.4 mol% 4HB), representing the highest mole fraction of 4HB from an unrelated carbon source.^[148] More recently, CRISPRi has also been employed for engineering *Halomonas* sp. TD01, a promising industrial PHA producer capable of growing on sea water.^[149] By using the CRISPRi system, the expression levels of chromosomal genes, *ftsZ* (encoding a bacterial fission ring formation protein),

prpC (encoding 2-methylcitrate synthase), and *glcA* (encoding citrate synthase), were down-regulated to different extents. This strategy allowed increase of the 3HV monomer ratio in poly(3HB-co-3HV) and polymer titer with elongated cells.^[149]

5.1.3. Block Copolymer Synthesis

Block copolymers such as A-b-B diblock, A-b-B-B-C triblock, and (A-b-B)_n multiblock copolymers can yield polymer properties different from random copolymers.^[150,151] Based on individual properties of each block, new properties can be obtained in block copolymers. Several approaches have been taken to produce PHA block copolymers. Naturally, PHA copolymers produced by microorganisms are random copolymers because PHA synthesis occurs in the cell cytoplasm where various hydroxyacyl-CoA monomers are randomly accessible to PHA synthase. Thus, it is evident that metabolic fluxes need to be controlled to supply each monomer at a specified time to produce PHA block copolymer.

As a simple and traditional approach, a block copolymer can be produced by adding the precursors for monomers into the culture medium sequentially or periodically.^[150,151] There have been several reports on PHA block copolymers biosynthesized in this manner, including poly(3HB)-*b*-poly(3HV-co-3HHp),^[152] poly(3HB)-*b*-poly(3HHx),^[153] poly(3HB)-*b*-poly(4HB),^[154] and poly(3HP)-*b*-poly(4HB).^[151] As an example, to produce poly(3HP)-*b*-poly(4HB), engineered *E. coli* overexpressing five genes, *C. kluyveri orfZ*, *C. aurantiacus pcs'* (encoding the ACS domain of trifunctional propionyl-CoA ligase), *P. putida* KT2442 *dhaT* (encoding alcohol dehydrogenase), *aldD* (encoding aldehyde dehydrogenase), and *C. necator phaC*, was grown first in the presence of 1,4-butanediol, a precursor of 4-hydroxybutyryl-CoA. This strain synthesized poly(4HB). The cells were then grown in the presence of 1,3-propanediol, a precursor of 3-hydroxypropionyl-CoA, for the second poly(3HP) block synthesis.^[151] The block copolymer having 29–37 mol% of 4HB showed higher molecular weight, yield strength, and maximum tensile strength and Young's modulus compared with poly(3HP-co-4HB) having a similar monomer composition.^[151]

In another approach, block copolymers, poly(3HB)-*b*-poly(3HP), were synthesized in *E. coli* by modulating the synthetic pathways for each monomer, 3HB and 3HP, under different induction systems using isopropyl β-1-thiogalactopyranoside (IPTG) and arabinose, respectively.^[155] The genes involved in 3-hydroxypropionyl-CoA synthesis, *K. pneumonia dhaB* and *gdrAB* encoding glycerol dehydratase and its reactivatase and *Salmonella typhimurium pduP* encoding propionaldehyde dehydrogenase, were expressed under the T7 promoter, which is induced by IPTG.^[155] The genes involved in 3-hydroxybutyryl-CoA synthesis, *phaAB* from *C. necator*, were expressed under the P_{BAD} promoter with the regulator gene *araC*. This P_{BAD}-AraC system has been reported to be tightly regulated according to the L-arabinose concentration and inhibited by glucose.^[156] When L-arabinose was added into the culture medium, the 3-hydroxybutyryl-CoA biosynthetic genes were actively expressed together with *C. necator phaC* gene, which was expressed constitutively, and poly(3HB) block was produced using fructose as a sole carbon source. After

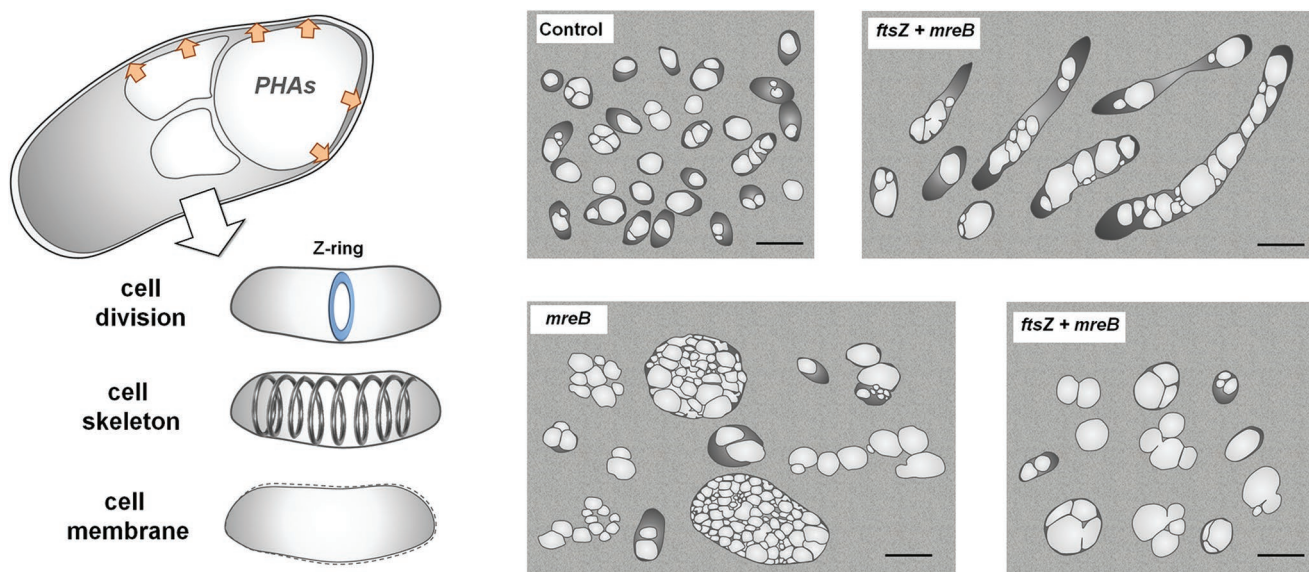


Figure 7. Cell morphology engineering. Expression levels of proteins involved in cell division process and cell wall biosynthesis can be modulated to change cell morphology for achieving better cell growth or enhanced PHA production.

several hours, glucose and glycerol were added to switch off the 3-hydroxybutyryl-CoA biosynthetic pathway and then IPTG was added to start poly(3HP) block formation. By changing the concentration of L-arabinose and IPTG, the block compositions could be altered from homo poly(3HB) to poly(3HB)-b-poly(3HP) having 25 mol% of 3HB.^[155]

5.2. Cell Morphology Engineering

As PHAs are inclusion bodies accumulated inside cells, several studies have focused on cell morphology changes upon accumulation of PHA granules (Figure 7).^[157–159] When a recombinant *E. coli* harboring *C. necator phaCAB* genes was cultured to produce poly(3HB), significant morphological changes represented by filamentous cells were observed.^[157] Since cell filamentation can prevent cell growth and cell division, it leads to decreased cell concentration and overall PHA production. To solve this problem by suppressing cell filamentation, the *ftsZ* gene encoding the earliest acting cell division protein forming a Z-ring at the division site was overexpressed to facilitate cell division.^[113,160] This strategy effectively suppressed cell filamentation and enhanced the production of poly(3HB)^[113,160,161] to 157 g L⁻¹ with a productivity of 3.2 g L⁻¹ h⁻¹ by fed-batch fermentation.^[162]

On the other hand, a different approach was taken to increase PHA accumulation by enlarging the intracellular space as a storage room of PHAs. By overexpressing the *sulA* gene encoding a cell division inhibitor, the engineered *E. coli* elongated and accumulated more poly(3HB) and poly(3HB-co-4HB).^[163,164] The morphological changes also enabled the cells to be precipitated by letting them stand for only 20 min, which provides an energy-saving way of separating cells from culture broth.^[164] Deletion of *envC* and *nlpD* or *minCD* was also effective to make filamentous *E. coli* cells with enhanced poly(3HB) production.^[165] The *envC* and *nlpD* genes

can regulate the peptidoglycan hydrolases, which are responsible for the splitting of cell wall material between daughter cells.^[166] MinCD can block the formation of the FtsZ ring at all sites, and thus inactivation of MinCD changes the division pattern from binary division into multiple fission.^[167] When *E. coli sulA* gene and *C. necator phaCAB* genes were expressed in the *envC* and *nlpD* deleted *E. coli* and the *minCD* deleted *E. coli* strains, both strains showed long filamentous shapes and increased poly(3HB) content relative to those obtained by wild-type *E. coli* strain overexpressing *sulA* and *phaCAB* or *phaCAB* alone.^[165]

MreB is an actin-like protein that plays a role in determining cell shape in *E. coli*. Thus, MreB was also tested for the enhanced production of poly(3HB).^[159,168,169] Overexpressing *mreB* in an *mreB* deletion mutant under inducible expression of *sulA*, the shape of *E. coli* changed from rod-type to larger spherical shapes with enhanced poly(3HB) accumulation.^[159] Through the finely controlled repression of *ftsZ* and *mreB* using the CRISPRi tool, the poly(3HB) production could be improved by having longer and larger cell shapes.^[169] In addition, the new target *mtgA* gene encoding a monofunctional peptidoglycan glycosyltransferase was selected by genome-wide mutagenesis followed by a plate-assay based on Nile red staining. Deletion of *mtgA* increased production of poly(3HB-co-LA) by enlarging the *E. coli* cells.^[170] The *mtgA* deleted strain showed increased cell size (1.4-fold) only when cells produced polymers.^[170] These results suggest that cell morphology engineering can be employed for enhanced PHA production in several different ways.

5.3. PHA Synthase Engineering

There has been much effort to develop PHA synthases that enable tailor-made PHA production with enhanced PHA titer and productivity. As the crystal structures of PHA synthases have been determined only recently,^[21–23] most previous

studies on engineering PHA synthases employed random and semirational approaches through library construction and screening the variants. To generate the libraries, several methods including in vivo random mutation using a mutator *E. coli* strain,^[171] error-prone PCR,^[172,173] site-specific saturation mutagenesis, localized semirandom mutagenesis,^[174] and gene shuffling^[175] were employed. The libraries were transformed into strains capable of supplying the substrates, hydroxyacyl-CoAs. Since the colonies of PHA-accumulating strain are distinguishable from those of PHA nonaccumulating strain due to the different transparency and whiteness, the high polymer accumulating variants can be selected by visual agar plate screening.^[146,174] For colorimetric and fluorescent screening of PHA producers, lipophilic dyes staining the intracellular PHAs have been widely employed as well. Some of the most widely used ones are Nile red, Nile blue A, Sudan Black B, and BODIPY.^[176–178] When cells are cultured on a Nile red containing agar plate, the colonies appear bright red the extent of which correlates with the amount of PHA accumulated.^[176] Using the flow cytometer, the fluorescence signals can be measured at the single-cell level.^[179–183] This flow-based quantification enables monitoring of the population heterogeneity in cell size and PHA accumulation within a short time (10 min for about 10^6 cells) with high sensitivity.^[183] Thus, FACS can be used to isolate the desired variants of poly(3HB) producing *E. coli*.^[182] This type of screening is simple and powerful, but it only depends on the amount of PHA accumulation regardless of the monomer compositions.^[184] Therefore, after the first screening, the positive variants are further examined by high-pressure liquid chromatography (HPLC) and/or gas chromatography (GC) for accurate quantification and determination of monomer compositions of PHAs.

Once the positive amino acid residues in PHA synthase variants are identified, those residues are further optimized by site-specific saturation mutagenesis and combinations of the mutations.^[185,186] The beneficial mutations observed in one PHA synthase can also be applied to other PHA synthases of different strains or different classes based on the sequence alignments,^[20,187] although the positive outcome is not always guaranteed. By error-prone PCR mutagenesis and plate screening using Nile red, Ser325 and Gln481 residues of *Pseudomonas* sp. 61-3 PHA synthase (Class II PHA synthase) were found to be important for enhanced production of poly(3HB) in *E. coli*.^[188] Based on the site-specific saturation mutagenesis of Ser235 and Gln481, several double mutants were constructed. Among them, mutations Ser325Thr and Gln481Lys showed drastically enhanced poly(3HB) production.^[188] These mutant enzymes were also employed for the production of poly(3HB-co-MCL-3-HA), which increased the polymer content from 13 to 35 wt%.^[189] In particular, the mutations at Gln481 altered the substrate specificity of the enzyme toward C4 and C6 monomers.^[189] As the residue corresponding Gln481 is conserved as alanine for class I PHA synthase, the saturation mutagenesis of *C. necator* PHA synthase was conducted.^[187] The site saturation mutagenesis of Ala510 showed little effect on the activity but a remarkable effect on the substrate specificity.^[187] Additionally, Glu130,^[190] Ser477,^[15] and Gln508^[191] residues of *Pseudomonas* sp. 61-3 PHA synthase have been revealed to affect the production of poly(3HB) and poly(3HB-co-MCL-3-HA) by a similar process.

Interestingly, the engineered PHA synthases harboring these mutations showed significantly enhanced activity toward a non-natural substrate, lactyl-CoA.^[19,20,186,192] The previous attempts to produce LA-containing PHA had not been successful because of very low or negligible activity of PHA synthase toward lactyl-CoA. Various PHA synthases of different classes from *C. necator*, *Allochromatium vinosum*, *Ectothiorhodospira shaposhnikovii*, and several *Pseudomonas* strains showed negligible activities toward 2-hydroxyacyl-CoAs including lactyl-CoA and 2-hydroxybutyryl-CoA.^[19,48,186,193,194] The first production of LA-containing polymer, poly(3HB-co-LA) was reported by using *C. necator* PHA synthase, but the LA fraction was rather small.^[195] The use of *Pseudomonas* sp. MBEL 6-19 PHA synthase (PhaC1) having combinatorial mutations of Glu130, Ser325, Ser477, and Gln481 increased the LA fraction in poly(3HB-co-LA) up to 86 mol% and also homo poly(LA) [PLA] production.^[186,192] The detailed metabolic engineering strategies for LA containing polymers will be discussed in Section 5.4. By the site-directed mutagenesis of the four residues, Class II PHA synthases from *Pseudomonas* sp. 61-3, and MBEL 6-19, *P. putida* KT2440, *P. chlororaphis*, *P. resinovorans*, and *P. aeruginosa* PAO1 were also engineered to synthesize PLA.^[20] Mutation of Gln481 was also effective for the class I PHA synthase.^[196] *C. necator* PHA synthase (class I PHA synthase) harboring mutation at Ala510, which corresponds to Gln481, produced poly(3HB-co-7 mol% LA).

As semirandom approaches, PHA synthases have been evolved based on the sequence analysis, multiple alignment, and comparison with similar well-known enzymes. The localized semirandom mutagenesis targeting the conserved regions of a PHA synthase family based on multiple sequence alignment resulted in the development of several mutant *P. putida* GPo1 PHA synthase with altered substrate specificities.^[174] As another approach, N-terminal truncation and mutation have been conducted since the N-terminal region of PHA synthase is highly variable while the C-terminal region represents conserved blocks of α/β hydrolase fold.^[16,17,197,198] Although the N-terminal region is not directly involved in enzyme catalysis, it has been shown to influence PHA productivity, substrate specificity, and molecular weights of PHAs.^[16,17,197] Several chimeric PHA synthases have been developed by exchanging conserved α/β hydrolase fold portions of PhaC1 and PhaC2 of *P. resinovorans*,^[199] or by mixing two different PHA synthases from *C. necator* and *A. caviae*.^[200] These approaches showed some effect in altering substrate specificities.^[199–201] These attempts herein have yielded remarkable achievements, especially the evolved PHA synthases accepting the novel substrate, lactyl-CoA. The 3D structures of recently disclosed PHA synthases^[21–23] can lead to rational engineering and accelerate the development of tailor-made polymerase.

5.4. Engineering Microbial Strains for Nonnatural Polyester Production

The integrated approaches termed as systems metabolic engineering^[110,122] resulted in transforming microorganisms into nonnatural polyester producers that had not existed before. To develop nonnatural polyester producers, there are two

major challenges to be addressed: the absence of the metabolic pathway generating nonnatural hydroxyacyl-CoAs and the lack of PHA synthase possessing high enough activity to polymerize these nonnatural substrates. In the past few decades, breakthroughs were made in nonnatural polyester production by systems metabolic engineering. In particular, popular bioplastics, PLA and poly(LA-co-glycolate) [poly(LA-co-GA)], could be produced by one-step direct fermentation of engineered bacterial strains. In this section, metabolic engineering strategies employed for the production of these nonnatural polymers are discussed in detail.

5.4.1. Polylactate

PLA is currently a popular polymer possessing high strength, high modulus, biocompatibility, and low toxicity to organisms.^[202,203] PLA has been used in various applications including containers, bottles, bags, clothes, packaging, and biomedical materials such as absorbable sutures and drug delivery carriers.^[203,204] Currently, the largest PLA production company, NatureWorks, produces PLA under the trade name of Ingeo in a commercial scale of 140 000 tons year⁻¹.^[204] The company developed a bio and chemical hybrid process which produces PLA from renewable biomass such as cassava, corn starch, and sugar cane. This process includes the microbial fermentation for producing LA, followed by chemical polymerization of lactide, which is a ring form of LA dimer.

To develop a one-step fermentative PLA production process instead of the complex hybrid process, several attempts were made to employ PHA synthase for polymerizing lactyl-CoA into PLA as described in Section 5.3. Although the natural PHA synthases have shown negligible activities toward lactyl-CoA, the engineered PHA synthases from *Pseudomonas* sp. MBEL 6-19 and *Pseudomonas* sp. 61-3 harboring amino acid mutations at Glu130, Ser325, Ser477, and Gln481 showed significantly increased activities toward lactyl-CoA.^[19,20,61,186,192] PHA synthase variants used for lactyl-CoA polymerization have been thoroughly examined previously.^[193]

For the one-step direct fermentative production of PLA, *E. coli* was engineered to convert LA to lactyl-CoA by engineered propionyl-CoA transferase (Pct) from *C. propionicum*. Lactyl-CoA is then subsequently polymerized into PLA by an engineered PHA synthase from *Pseudomonas* sp. MBEL 6-19^[192] (Figure 3). In more detail, the *C. propionicum* Pct mutant, Pct532 (Ala243Thr and one silent nucleotide mutation of A1200G) and *Pseudomonas* sp. MBEL 6-19 PHA synthase (PhaC1) mutant (PhaC1400, Glu130Asp, Ser325Thr, Ser477Arg, and Gln481Met) were overexpressed in *E. coli*.^[192] To strengthen the metabolic flux toward PLA, two chromosomal genes (*ldhA* and *acs*, encoding LA dehydrogenase and acetyl-CoA synthetase, respectively) were overexpressed by replacing the native promoters with strong *trc* promoter. Also, three chromosomal genes (*ackA*, *ppc*, and *adhE*, encoding acetate kinase, PEP carboxylase, and acetaldehyde/alcohol dehydrogenase, respectively) were deleted based on rational selection and in silico genome-scale metabolic flux analysis.^[192] This strategy resulted in microbial production of PLA to a polymer content of 11 wt%. When the engineered *E. coli* strains additionally expressed *C. necator phaAB* genes, the

strains produced poly(3HB-co-LA) having high LA mole fractions (up to 70 mol%) from glucose. This PLA biosynthesis platform has been successfully moved to other host strains, which are PHA native and non-native producers including *Sinorhizobium meliloti*,^[205] *C. glutamicum*,^[9] and *C. necator*.^[206]

5.4.2. Poly(lactate-co-glycolate)

The engineered PHA synthases from *Pseudomonas* sp. MBEL 6-19 and *Pseudomonas* sp. 61-3 were found to utilize glycolyl-CoA as a substrate as well.^[64,207] In addition, some Pcts can convert GA into glycolyl-CoA in addition to conversion of LA to lactyl-CoA.^[64,207] Thus, the PLA biosynthesis system can be expanded to produce poly(LA-co-GA) [PLGA], which is a US FDA-approved important medical polymer. Production of GA containing polymers from unrelated carbon sources was achieved by constructing two different GA biosynthetic pathways, the glyoxylate shunt pathway associated with the TCA cycle^[70] and the Dahms pathway starting from xylose^[64] (Figure 5). To produce GA via glyoxylate shunt, the *E. coli aceA* and *aceK* genes encoding isocitrate lyase and isocitrate dehydrogenase kinase/phosphatase, respectively, were overexpressed with the *E. coli ycdW* gene encoding glyoxylate reductase. The generated GA was converted into glycolyl-CoA by *M. elsdenii* Pct and then polymerized by *Pseudomonas* sp. 61-3 PHA synthase (PhaC1) mutant (Ser325Thr, Gln481Lys). By coexpressing the *C. necator phaAB* genes, poly(3HB-co-17 mol% LA-co-16 mol% GA) was produced.^[70]

Another pathway, the Dahms pathway is a xylose metabolizing pathway found in *Caulobacter crescentus*.^[208] In the Dahms pathway, xylose is converted to pyruvate and glycolaldehyde, and then glycolaldehyde is further converted into GA by aldehyde dehydrogenase (Figure 5). When *E. coli* XL1-Blue strain was equipped with the Dahms pathway by expressing the *C. crescentus xylBC* genes encoding xylose dehydrogenase and xylonolactonase, GA was produced but cells grew poorly.^[64] In silico genome-scale metabolic simulation revealed that the insufficient metabolic fluxes of lower glycolysis caused by the introduction of the Dahms pathway resulted in growth retardation. Based on the simulation result, *E. coli* was engineered to utilize xylose and glucose simultaneously, which was accomplished by deleting the *ptsG* gene encoding glucose-specific phosphotransferase system enzyme IIBC component. Then, *E. coli* was further engineered to concentrate the metabolic flux toward LA and GA by knocking out several chromosomal genes including *adhE*, *poxB*, *frdB*, *dld*, *aceB*, and *glcDEFGB* and by replacing the native *ldhA* promoter with strong *trc* promoter. The resulting *E. coli* strain expressing *xylBC*, *pct540* (encoding *C. propionicum* Pct mutant, which is Pct540 with Val193Ala change and four silent mutations of T78C, T669C, A1125G, and T1158C), and *phaC1437* (encoding *Pseudomonas* sp. MBEL 6-19 PhaC1 mutant which is PhaC1437 with the changes of Glu130Asp, Ser325Thr, Ser477Gly, and Gln481Lys) produced poly(70.5 mol% LA-co-29.5 mol% GA) to a polymer content of 36.2 wt% by fed-batch fermentation.^[64] This was the first demonstration of one-step fermentative production of PLGA by engineered bacterium using carbohydrates (glucose and xylose) as carbon sources.

This engineered *E. coli* strain also produced various GA containing copolymers including poly(LA-co-GA-co-2-hydroxybutyrate) [poly(LA-co-GA-co-2HB)], poly(LA-co-GA-co-3HB), poly(LA-co-GA-co-4HB), poly(LA-co-GA-co-5-hydroxyvalerate), poly(LA-co-GA-co-6-hydroxyhexanoate), and poly(LA-co-GA-co-2-hydroxyisovalerate) [poly(LA-co-GA-co-2HIV)] by establishing additional pathways generating the third monomer or by feeding the monomer precursors into the culture medium.^[64] As a follow-up study, the Dahms pathway was further engineered by using synthetic biology tools.^[209] By employing synthetic promoters having different strengths, the metabolic flux toward the Dahms pathway was delicately manipulated, which resulted in production of PLGA and poly(LA-co-GA-co-2HB) having a wider range of GA fractions (8.8–60.9 mol%) with enhanced PLGA titer of up to 6.93 g L⁻¹, which was about threefold higher than that reported previously.^[64,209]

5.4.3. Polymers Containing Other 2-Hydroxyacids

The Pcts from *C. propionicum* and *M. elsdenii* can convert 2HB and 2HIV into their CoA thioesters, which can subsequently be polymerized by the evolved PHA synthases from *Pseudomonas* sp. MBEL 6-19 and *Pseudomonas* sp. 61-3.^[64,76,210] To produce 2HB containing polymers from unrelated carbon sources, the 2HB biosynthetic pathway has been constructed. The first production of 2HB containing polymers from glucose has been reported by an engineered *E. coli* strain utilizing the citramalate pathway.^[76] In this pathway, pyruvate and acetyl-CoA are condensed to citramalate by citramalate synthase (CimA) from *Methanococcus jannaschii*. The citramalate is then converted into 2HB by sequential reactions mediated by 3-isopropylmalate dehydrogenase and isopropylmalate isomerase (LeuBCD) from *E. coli* and 2-HA dehydrogenase (PanE) from *Lactococcus lactis* subsp. *lactis* I11403.^[76] When the engineered *E. coli* strain expressed *cimA*, *leuBCD*, *panE*, *pct540*, *phaC1437*, and *C. necator phaAB* genes, the strain produced poly(3 mol% 2HB-co-96 mol% 3HB-co-LA) to a polymer content of 74 wt% from glucose as a sole carbon source.^[76]

Later, it was found that 2HB can be generated through the endogenous *E. coli* L-isoleucine biosynthesis pathway.^[64] Here, 2-ketobutyrate is produced from L-threonine and is converted to 2HB by an unidentified endogenous keto-acids dehydrogenase. Thus, in the above study on LA and GA containing polymers, a small amount of 2HB (about 4 mol%) was also incorporated to result in poly(LA-co-GA-co-2HB) production unless necessary engineering was performed to prevent 2HB incorporation.

Similarly to 2HB described above, 2HIV can be synthesized from 2-ketoisovalerate, a precursor of L-valine, by *L. lactis* subsp. *lactis* I11403 PanE.^[64,77,211] The related branched chain amino acid biosynthetic pathway was metabolically engineered to produce 2HIV-rich polymer from glucose.^[77] The feedback resistant *E. coli ilvBN* genes encoding acetohydroxyacid synthase, *E. coli ilvCD* genes encoding ketol-acid reductoisomerase and dihydroxyacid dehydratase, and *L. lactis* subsp. *lactis* I11403 *panE* gene were overexpressed in *E. coli* to efficiently produce 2HIV. Then, the *E. coli* chromosomal genes, *poxB*, *adhE*, *pflB*, and *frdB* were deleted to increase the pyruvate pool, a common

precursor of 2HIV and LA. As a result, poly(20 mol% 2HIV-co-LA) was produced to a polymer content of 9.6 wt%.^[77]

More recently, 2-hydroxy-3-methylvalerate (2H3MV) and 2-hydroxy-4-methylvalerate (2H4MV) have been newly identified as monomers of PHAs.^[78] They are intermediates of L-isoleucine and L-leucine biosynthesis and can be converted to 2-hydroxy-3-methylvaleryl-CoA and 2-hydroxy-4-methylvaleryl-CoA by two subsequent enzymatic reactions catalyzed by LdhA (2-HA dehydrogenase) and HadA (CoA transferase) from *C. difficile*. Then, the engineered *Pseudomonas* sp. 61-3 PHA synthase variant having Ser325Thr and Gln481Lys mutations can polymerize both 2-hydroxyacyl-CoAs as monomers.^[78] When L-valine, L-isoleucine, and L-leucine were supplemented into the culture medium at 1 g L⁻¹, copolymers having 8.3 mol% 2HIV, 0.6 mol% 2H3MV, and 38.1 mol% 2H4MV, respectively, were produced.^[78] Examination of these polymers produced by the engineered *E. coli* strain expressing *ldhA*, *hadA*, *phaC* mutant, and *C. necator phaAB* genes revealed that about 1.4 mol% of phenyllactate (PhLA) was also incorporated. This was possible since LdhA can convert phenylpyruvate, an intermediate of L-phenylalanine biosynthesis, to PhLA, which is then converted to phenyllactyl-CoA by HadA. The engineered PhaC can incorporate phenyllactyl-CoA into the polymer (Figure 5). When 1 g L⁻¹ of L-phenylalanine was supplemented to the culture medium, the mole fraction of PhLA in the produced polymer was increased up to 17.2 mol%.^[78]

In addition to PhLA, other aromatic 2-HAs, 4-hydroxyphenyllactate [4HPhLA, 2-hydroxy-3-(4-hydroxyphenyl)propionate] and mandelate (MA, 2-hydroxy-2-phenylacetate) can also be incorporated into polymers by employing HadA from *C. difficile* and the engineered PHA synthase from *Pseudomonas* sp. MBEL 6-19.^[79] The *C. difficile* HadA showed a broad substrate utilization spectrum covering various aromatic and aliphatic HAs including 4HPhLA, PhLA, MA, 3HB, 4HB, 2HB, and LA. Through systems metabolic engineering of the aromatic amino acid biosynthetic pathway described next, PhLA containing polyesters could be produced by an engineered *E. coli* strain using glucose as a carbon source (Figure 5). To strengthen the metabolic flux toward PhLA, the *tyrR* gene encoding a transcriptional dual regulator involved in aromatic amino acid biosynthesis was deleted from the *E. coli* chromosome. The *aroG* (Asp146Asn) and *pheA* (Thr326Pro) mutant genes encoding feedback inhibition resistant 3-deoxy-7-phosphoheptulonate synthase and chorismate mutase/prephenate dehydratase, respectively, and *fldH* gene encoding D-LA dehydrogenase from *C. botulinum* were overexpressed. Based on the in silico genome-scale metabolic flux analysis, the chromosomal *tyrB* and *aspC* genes were additionally deleted to increase the metabolic flux toward PhLA. The resulting *E. coli* strain expressing *hadA*, *phaC1437*, and *C. necator phaAB* produced 13.9 g L⁻¹ of poly(61.9 mol% 3HB-co-38.1 mol% PhLA) by fed-batch cultivation.^[79]

6. Fermentation and Downstream Processes

6.1. Fermentation

Although the engineered cells serve as microbial cell factories, actual production of polymers cannot be realized without

fermentation. Fermentation process should be optimized to maximize the product titer (polymer concentration; g polymer per liter), yield (g polymer per g carbon source), and productivity (g polymer per liter per hour). When performing strain development described above, fermentation should be performed to ensure the strain being developed to give high enough titer, yield, and productivity, and also ensure reduced byproducts formation to increase the product yield and reduce recovery and purification costs.^[212,213] Fermentation performance can be affected by many factors including temperature, pH, carbon-to-nitrogen (C:N) ratio in the feed, substrate concentration, trace elements, dissolved oxygen (DO), ionic strength (IS), agitation intensity, and nutrient feeding strategy.^[212,214–216]

Fermentation can be performed in three different modes: batch, fed-batch, and continuous cultures.^[217] Among them, fed-batch culture is the most preferred cultivation mode in industry. For the production of PHA with high titer, yield, and productivity by fed-batch fermentation, factors including the nutrient compositions of initial growth medium and feeding solution, nutrient feeding strategy, aeration rate, DO, and pH control need to be optimized.^[212,216,218] In this section, we discuss the characteristics of each mode of fermentation for PHA production with representative operating strategies.

6.1.1. Batch Cultures

For the products like PHAs, batch culture is often performed at research stage. In batch culture, the addition of feeding solution and removal of culture broth do not occur during the fermentation. Due to this simplicity of operation, batch cultures have been extensively studied for various PHA producing strains with different carbon sources as feedstocks. Recently, sequential batch cultures and mixed cell cultures using renewable cheap feedstocks have also been reported.^[219,220]

Sequential batch fermentation is a mode of batch culture where multiple rounds of batch cultures are performed using a single bioreactor. One of the biggest drawbacks of traditional batch culture is the fermentation downtime and associated costs for preparing new fermentation upon the completion of the previous fermentation; the bioreactor should be emptied and sterilized after each round of fermentation. To overcome this problem, sequential batch culture can be performed by leaving a certain amount of culture broth containing cells after a round of batch fermentation, and the remaining volume can be filled with fresh culture medium for another round of batch culture.^[221–223] For example, *Azohydromonas australica* DSM 1124 produced 20.6 g L⁻¹ poly(3HB) with a productivity of 0.31 g L⁻¹ h⁻¹, when about 20% of culture broth was left in the bioreactor and supplemented with a new medium after a round of batch culture.^[223] Of course, this process can be repeated as many times as desired as long as contamination is avoided and cell performance does not decrease.

Mixed microbial batch culture is another mode of sequential batch culture where more than one bacterial strain is cultivated

in a single bioreactor.^[220] Mixed microbial batch culture for PHA production has been performed without maintaining sterile condition.^[224] For the initial stage of mixed microbial batch fermentation, a nonsterile condition permits various strains including both PHA producing and nonproducing strains to grow. At the later stage, PHA accumulating cells are sorted through a cyclic feast and famine feeding strategies. While PHA accumulating strains can withstand the famine phase by utilizing the intracellular PHAs stored during the feast phase, PHA nonaccumulating strains have less chance to survive during the famine phase. Another advantage of mixed microbial cell culture is the possibility of employing diverse feedstocks. PHAs have been produced from molasses,^[225] olive oil mill effluents,^[226] and cheese whey by employing mixed microbial batch cultures.^[227] Using such inexpensive feedstocks is favorable for reducing PHA production costs and reducing environmental concerns. Due to the characteristics of employing mixed population of different cells, however, PHAs produced are often blends of various homopolymers and copolymers that might not be suitable to be used in actual applications.^[228]

6.1.2. Fed-Batch Cultures

Fed-batch culture is a process where additional nutrients are fed into the culture broth during the fermentation. Batch culture has an inherent limitation in achieving high concentration and productivity of PHA due to the depletion of carbon source; it is not possible to have a high concentration of carbon source in the initial batch medium as it can inhibit cell growth. In fed-batch operation mode, additional feeding of carbon sources facilitates a prolonged growth phase and PHA accumulation, thereby resulting in enhanced PHA production.^[229] Fed-batch culture can employ various feeding strategies to maximize cell growth and PHA production. In general, a nutrient-rich feed is provided at the early phase of culture to promote cell growth, while nitrogen- or phosphorus-limited feeding solution is later added to promote PHA production, as can be seen from many examples summarized in **Table 2**.^[230–232]

Some PHA producers do not require a nutrient limited condition for PHA accumulation. A good example is engineered *E. coli* strains lacking in cellular regulatory mechanisms for PHA biosynthesis and PHA degradation enzymes. For the fed-batch culture of such strains, high-cell-density cultivation is performed while pursuing to obtain high PHA content. For example, an engineered *E. coli* strain overexpressing *ftsZ* and *C. necator phaCAB* genes could grow to a high cell density of 153.7 g DCW L⁻¹, producing 101 g L⁻¹ of poly(3HB) with a productivity of 2.8 g L⁻¹ h⁻¹ in a 50 L bioreactor.^[162] In another fed-batch study, engineered *E. coli* harboring the *A. latus phaCAB* operon produced 141.6 g L⁻¹ of poly(3HB) with a productivity of 4.63 g L⁻¹ h⁻¹ when grown to a high cell density of 194.1 g DCW L⁻¹.^[8]

Optimization of nutrient feeding strategy is the most important for successful fed-batch cultivation.^[251] An additional carbon source is fed into the bioreactor when the carbon source in the culture broth is depleted or its concentration is reduced below a certain value.^[251] For aerobic fed-batch

Table 2. Summary of production of PHAs and nonnatural polyesters.

Polymer type ^{a)}	Host	Carbon source	Polymer content [wt%]	Titer [g L ⁻¹]/productivity[g L ⁻¹ h ⁻¹]	Culture mode	Culture strategy	Ref.
Poly(3HB)	<i>Vibrio proteolyticus</i>	Fructose	54.67	2.7/0.038	Batch	Unsterilized culture	[233]
Poly(3HB)	<i>C. necator</i> ATCC 17697	Syn gas	67.8	61.9/1.55	Batch	Oxygen limited condition	[234]
Poly(3HB)	<i>Alcaligenes</i> sp.	Cane molasses, urea	74.6	8.8/0.17	Batch	Statistical optimization used	[214]
Poly(3HB)	<i>B. megaterium</i> R11	Pulp industry waste	51.6	12.48/0.26	Batch	Statistical optimization used	[215]
Poly(3HB)	<i>A. australica</i>	Sucrose	0.82	20.6/0.31	Batch	Repeated batch	[223]
Poly(3HB-co-22%3HHx)	Recombinant <i>C. necator</i> Re2133	Coffee waste oil	69	0.64/0.009	Batch	C:N ratio of 20 used	[235]
PLA	Recombinant <i>E. coli</i> XL1-Blue	Glucose	11	–/–	Batch	–	[192]
Poly(3HB)	<i>C. necator</i>	Glucose	82.5	232/3.14	Fed-batch	High-cell-density culture with 60 L bioreactor	[236]
Poly(3HB)	Recombinant <i>E. coli</i>	Glucose	73	141.6/4.63	Fed-batch	pH-stat pulse feeding	[8]
Poly(3HB)	Recombinant <i>E. coli</i>	Glucose	65.9	101/2.8	Fed-batch	50 L bioreactor pH-stat pulse feeding with oxygen limitation	[162]
Poly(3HB)	<i>C. necator</i> H16	Soybean oil	76	95.76/0.998	Fed-batch	Manual feeding of carbon source, phosphorus limitation	[230]
Poly(3HB)	<i>Zobellella denitrificans</i>	Glycerol	66.9	54.3/1.09	Fed-batch	42 L bioreactor, continuous feeding	[237]
Poly(3HB)	<i>Methylobacterium extorquens</i>	Methanol	43	46.1/0.48	Fed-batch	Methanol feeding proportional to DO	[238]
Poly(3HB-co-5.6%3HV)	<i>C. necator</i> H16	Mixed volatile fatty acids	83.1	93.5/2.13	Fed-batch	pH-stat pulse feeding with DO stat pulse feeding	[239]
Poly(3HB-co-1.6%4HB)	<i>Burkholderia sacchari</i> DSM 17165	Saccharose, γ -butyrolactone	72.6	55.8/1.87	Fed-batch	Add nitrogen source as base (NH ₄ ⁺) to keep pH during growth phase and NaOH used as base in PHA accumulation phase, manual feeding of carbon source	[231]
Poly(3HB-co-16.1%4HB)	Recombinant <i>H. bluephagenesis</i> TD01	Glucose, γ -butyrolactone, crude corn extract	60.62	50.1/1.04	Fed-batch	1000 L bioreactor in non-sterile condition, flow rate of feed solution altered based on glucose concentration	[240]
Poly(3HB-co-10.8%3HHx)	Recombinant <i>E. coli</i>	Dodecanoic acid	50	21.5/0.53	Fed-batch	DO was maintained at 40% during lag/initial phase, then reduced to 5% when DCW reached 40 g L ⁻¹	[241]
Poly(3HB-co-5%3HHx)	Recombinant <i>C. necator</i> PHB-4	Soybean oil	74	102.1/1.06	Fed-batch	Manual feeding of carbon source, phosphorus limitation	[230]
MCL-PHA	<i>P. putida</i> KT2442	Oleic acid	51.4	72.6/1.91	Fed-batch	Phosphorus limitation, manual feeding of oleic acid and KH ₂ PO ₄ by the change of pH occurred by oleic acid depletion	[232]
Poly(3HB)	Recombinant <i>E. coli</i>	Glucose	17.5	11.11/0.309	Fed-batch	Exponential feeding limited by pH stat, $\mu = 0.3$ h ⁻¹	[251]
MCL-PHA	<i>P. putida</i> KT2440	Glucose, nonanoic acid	32	31.35/2.85	Fed-batch	Exponential feeding at $\mu_{max} = 0.67$ h ⁻¹ followed by linearly increased feeding rate with oxygen limitation	[243]

Table 2. Continued.

Polymer type ^{a)}	Host	Carbon source	Polymer content [wt%]	Titer [g L ⁻¹]/ productivity [g L ⁻¹ h ⁻¹]	Culture mode	Culture strategy	Ref.
MCL-PHA	Recombinant <i>P. putida</i> KT2440	Glucose	67	41.5/0.83	Fed-batch	Exponential feeding at $\mu = 0.2 \text{ h}^{-1}$ followed by pH-stat pulse feeding	[244]
PLGA	Recombinant <i>E. coli</i> XL1-Blue	Glucose, xylose	36.2	1.95*/0.023*	Fed-batch	pH-stat pulse feeding	[64]
PLGA	Recombinant <i>E. coli</i> XL1-Blue	Xylose	33.4	6.93/0.099*	Fed-batch	pH-stat pulse feeding	[209]
Poly(2HB-co-LA-co-GA)	Recombinant <i>E. coli</i> XL1-Blue	Glucose, xylose	40.4	–/–	Fed-batch	pH-stat pulse feeding	[64]
Poly(2HB-co-LA-co-GA)	Recombinant <i>E. coli</i> XL1-Blue	Xylose	32.3	6.8*/0.09*	Fed-batch	pH-stat pulse feeding	[209]
Poly(PhLA-co-3HB)	Recombinant <i>E. coli</i> XL1-Blue	Glucose	55	13.9/0.145*	Fed-batch	pH-stat pulse feeding	[79]
Poly(3HB)- <i>b</i> -poly(4HB)	<i>P. putida</i> KTOY08ΔGC	γ -Butyrolactone, glucose	49.58	2.71/0.025*	Fed-batch	Glucose added at 0, 12, 36, 60, 84 h time points while γ -butyrolactone was added at 12 and 36 h of culture	[154]
Poly(3HB)	<i>A. beijerinckii</i> NCIB 9067	Glucose	44.6	–/–	Single-stage continuous	$D = 0.049\text{--}0.252 \text{ h}^{-1}$, oxygen limited culture	[245]
Poly(3HB-co-18.5%3HV)	<i>C. necator</i> DSM 545	Sucrose, pentanoic acid	43	1.9*/0.285*	Two-stage continuous	$D = 0.15 \text{ h}^{-1}$	[246]
Poly(3HB-co-79%3HV)	<i>C. necator</i>	Fructose, pentanoic acid	17.6	0.3/0.096	Single-stage continuous	$D = 0.3 \text{ h}^{-1}$	[247]
Poly(3HB)	<i>C. necator</i> WSH3	Glucose	47.6	11.9/1.43	Two-stage continuous	$D = 0.12 \text{ h}^{-1}$	[248]
Poly(3HB)	<i>C. necator</i> DSM 545	Glucose	77	63/1.85	Five-stage continuous	$D = 0.13\text{--}0.17 \text{ h}^{-1}$	[249]
Poly(3HB)	<i>C. necator</i> DSM 545	Glucose	77	2.14/62.4*	Five-stage continuous	Statistical optimization used	[216]
MCL-PHA	<i>P. oleovorans</i> ATCC 29347	Octane	63	11.7/1.06	Two-stage continuous	$D = 0.16\text{--}0.22 \text{ h}^{-1}$	[250]

^{a)}The monomer composition is presented as mol%. The “*” indicates that the value is obtained through simple calculation from data of the reference paper.

culture, depletion of carbon source such as glucose causes the rise of pH and DO concentration, which can be used as a signal to automatically add a preset amount of nutrient feeding solution.^[220,251] Other feeding strategies such as pulsed feeding, exponential feeding, linearly increased feeding, and others can be employed for high-cell-density fed-batch cultures (Table 2).^[242–244] In the case of exponential feeding, the feeding volume is exponentially increased according to the specific growth rate (μ) of the cells. Using this feeding strategy in the fed-batch culture of *P. putida* KT2440, 31.35 g L⁻¹ of MCL-PHA could be produced with a productivity of 2.85 g L⁻¹ h⁻¹.^[243]

Controlling the DO concentration is also important as it can affect the cell physiology and growth rate, eventually affecting PHA production as well.^[252] The DO concentration is commonly controlled by changing the agitation speed and supplying air. For the high-cell-density culture of aerobic microorganisms, ensuring enough DO concentration is critical. When cell density becomes higher, however, supplying air

alone no longer supports enough oxygen supply and thus pure oxygen gas needs be supplemented at additional cost. In this sense, *A. chroococcum* that shows enhanced PHA production by fed-batch culture under nitrogen and oxygen-limited condition does not have oxygen supply problem.^[253] It was also shown in another study that monomer composition can be manipulated by changing the DO concentration.^[186] In the case of fed-batch culture of an engineered *E. coli* strain producing poly(LA-co-3HB), the polymer produced contained 8.7 mol% LA when the DO concentration was controlled above 30% of air saturation, while the LA mole fraction increased to 27.0 mol% when the DO concentration was controlled at a lower level (10%) to promote LA synthesis.^[186]

Changing the carbon source and its concentration during fed-batch culture can also affect the monomer composition of the polymer produced. When the monomers are supplied from different substrate origins, the concentrations of those substrates in the feed can obviously alter the monomer composition of the polymer produced.^[254] In addition, sequential feeding of

different substrates with temporal separation allows production of diverse block copolymers such as poly(3HB)-*b*-poly(4HB), poly(3HB)-*b*-poly(3HV-*co*-3HHp), and poly(3HB)-*b*-poly(3HB-*co*-3HV)^[150,152,154,255] as already discussed in Section 5.1.3.

6.1.3. Continuous Cultures

Continuous culture is a mode of cultivation where a substrate solution is continuously fed into the reactor while the culture broth is taken out from the bioreactor at the same volumetric flow rate. This process is a standard operation mode in petrochemical industry because continuous process can give high productivity and uniform products.^[256] However, a continuous process is typically considered risky in microbial fermentation. When the reactor is contaminated, the entire operation should be shut down and the overall production line should be sterilized. Also, long-term cell stability can be a problem due to the occurrence of low-performance mutant cells. Thus, continuous cultivation has not been widely used for PHA production. Continuous culture for PHA production therefore is less frequently employed compared to other modes of fermentation. Nonetheless, there have been some continuous cultivation studies to better understand cellular physiological characteristics and PHA production under various controlled growth conditions.

As chemostat condition of continuous culture allows precise and controlled manipulation of the bioprocess parameters including the cell growth rate through setting the dilution rate,^[257,258] PHAs with consistent monomer fractions can be achieved by providing a constant growth condition.^[247,259] For example, the monomer composition of MCL-PHA produced by *P. putida* changed over time in batch culture, but it could be maintained fairly consistent during the continuous culture.^[260]

Several types of continuous cultures have been performed for the production of PHAs. Single-stage continuous culture using one continuous stirred tank reactor (CSTR) has been widely applied for the production of PHAs using bacteria such as *C. necator* and *A. beijerinckii*.^[245–247] However, single-stage continuous culture is not usually efficient since it is not easy to apply nutrient limited condition required for PHA accumulation in many natural producers. On the other hand, two-stage continuous culture facilitates cell growth at the first nutrient-rich stage and PHA accumulation at the second nutrient-limited stage (e.g., oxygen, nitrogen, and phosphorus). For example, two-stage continuous culture of *C. necator* allowed poly(3HB) production with a productivity of 1.23 g L⁻¹ h⁻¹ (see Table 2 for comparison).^[248] In another study, two-stage continuous fermentation of *P. oleovorans* ATCC 29347 produced MCL-PHA with a productivity of 1.06 g L⁻¹ h⁻¹.^[250] A multistage process can adopt multiple fermentors with different conditions to mimic a continuous plug flow tubular reactor (CPFTR). A good example is the five-stage continuous fermentation of *C. necator* DSM 545 for the production of poly(3HB). With the first stage allowing good cell growth, the following stages operated under high C:N ratio (using glucose as a carbon source) allowed production of poly(3HB) with a productivity of 1.85 g L⁻¹ h⁻¹ for over 200 h.^[249]

6.2. Recovery

Since PHAs are intracellularly accumulated as distinct granules, it is necessary to break open the cells for their recovery and purification. After fermentation, cells containing PHAs are separated from the culture broth by conventional methods such as centrifugation, filtration, or flocculation-centrifugation.^[60] Then, PHAs can be extracted from the cells by using solvents such as chlorinated hydrocarbons (chloroform, methylene chloride, and 1,2-dichloroethane) and cyclic carbonates (ethylene carbonate and 1,2-propylene carbonate).^[261,262] Such solvent extraction technique has been the most widely used method to obtain highly purified PHAs with negligible degradation of PHAs.^[261,262] In addition, this method allows removal of bacterial endotoxins during the purification. However, the solvent extraction method is not appropriate for large-scale purification of PHAs. The extracted polymer solution becomes highly viscous due to the high molecular weight of PHAs, and consequently complicates further purification process.^[60] Thus, the solvent extraction method often requires a large amount of solvent. Furthermore, those solvents employed are known to be highly toxic and harmful for humans and the environment, which compromises the environmental benefits of producing biodegradable and bio-based PHAs.^[60,261,262]

Alternatively, several nonsolvent based techniques have been developed. Cells are digested by mechanical, chemical, and/or enzymatic methods, and then PHAs are subsequently separated from cell debris by a method such as centrifugation or filtration. A well-known method uses sodium hypochlorite for differential digestion of non-PHA cellular materials.^[263] Although this method is effective in the digesting non-PHA cellular materials, it also results in severe degradation of PHAs causing reduced molecular weights of PHAs purified.^[263] Thus, various acids, alkalis, and surfactants have been examined to digest non-PHA cellular materials.^[264,265] When the recombinant *E. coli* cells accumulating poly(3HB) were treated by 0.2 N of sodium hydroxide (NaOH) for ≈30 min, poly(3HB) could be recovered with a purity of 98.5%.^[264] This simple digestion using mild alkaline solution such as NaOH and KOH can significantly reduce the recovery and purification. It should be noted that this mild alkaline digestion method is useful for those cells that become fragile upon PHA accumulation, which is the case for engineered *E. coli* cells.^[261,264]

The use of surfactants such as sodium dodecyl sulfate (SDS), Triton X-100, and LAS-99 resulted in less degradation of PHAs, but the recovered PHAs were of low purity and the costs of surfactants were high.^[264,265] In addition, combination methods of sodium hypochlorite-chloroform or surfactant-sodium hypochlorite were developed to take advantages of both methods. When sodium hypochlorite was used with chloroform, chloroform immediately dissolves the isolated PHAs released from the cells by sodium hypochlorite. This method prevented PHAs from further degradation by sodium hypochlorite.^[266]

Another option is enzymatic digestion under mild condition. Various enzymes such as cellulases, lysozymes, and proteases such as trypsin and papain have been evaluated for this purpose.^[267,268] Extraction of PHAs by this method resulted

in high purity and yield of PHAs with good polymer quality. However, the high costs of enzymes and relatively complex process including heat treatment make this process economically unattractive.^[268]

Moreover, several mechanical methods such as bead milling and high-pressure homogenization have been employed to disrupt cells containing PHAs. Since these methods are free of chemicals, PHA can be purified with less degradation. However, the methods generally require high capital investment costs and long processing time.^[261]

7. Properties and Applications of PHAs

7.1. Properties of PHAs

7.1.1. Biodegradability and Biocompatibility

The biodegradability of PHAs has been attracting much attention to replace nondegradable petrochemical plastics to be discarded in our environment, such as one-time use plastics. In nature, PHAs are efficiently degraded into H₂O and CO₂ by mixed microbial consortia including the families, *Pseudomonadaceae*, *Micromonosporaceae*, *Thermomonosporaceae*, *Streptosporangiaceae*, and *Streptomyetaceae*, which are found in various environments such as river, seawater, soil, sludge, and compost under either aerobic or anaerobic condition.^[269,270] These microorganisms degrade PHAs by using their extracellular PHA depolymerases as PHAs cannot be transported into the cell. Those microorganisms naturally accumulating PHAs also contain intracellular PHA depolymerases for mobilization of PHA when needed. Most of the intracellular PHA depolymerases exist in cytoplasm, but as a special case, *R. rubrum* PHA depolymerase was found in the periplasm.^[271] PHAs are degraded to low-molecular-weight oligomers and monomers through their inherent metabolism. Some microorganisms also produce dimer hydrolase which accelerates the degradation by breaking down the oligomers into the corresponding monomers.^[272]

To date, about 600 PHA depolymerases from different microorganisms have been reported.^[273] The intracellular and extracellular PHA depolymerases can be further categorized according to their substrate type (SCL or MCL-PHAs).^[273] Intracellular PHA depolymerases degrade the endogenous PHA granules accumulated by the cells to obtain carbon, energy, and reducing power under carbon starvation condition. Extracellular PHA depolymerases are secreted from the cells and degrade the extracellular PHAs released from the lysed cells or materials made of PHAs. The intracellular PHA depolymerases target in vivo native PHA granules, which exist in amorphous state and have a surface layer of several proteins (see Section 3), while extracellular PHA depolymerases act on the denatured extracellular PHAs, which are partially crystalline.^[273,274] There are a few exceptions: extracellular PHA depolymerase (PhaZ7) from *Paucimonas lemoignei*, for example, is active only for native PHA granules.^[275]

The PHA biodegradation rate obviously depends on the accessibility of PHA depolymerases (or other enzymes) to the ester bonds in the polymers, which are the enzymatic attack

points, and thus varies with the physicochemical properties of the polymer material to be degraded. Monomer type and composition, crystallinity, molecular weight, physical form, surface shape, and surface area of the polymer material to be degraded, and also the environmental conditions including temperature, moisture, and pH all affect the degradation rate.^[269,271,275,276] In addition, PHA depolymerases from different strains exhibit different activities and substrate specificities.^[276,277] For example, *P. stutzeri* PHA depolymerase showed higher degradation rate for poly(3HP) compared to *A. faecalis* PHA depolymerase, while the latter showed higher degradation rate for poly(4HB).^[277] One of the crucial factors affecting biodegradability is the crystallinity of polymers, as the enzymatic hydrolysis initially occurs in the amorphous region of polymer. Several studies reported that the PHA degradation rate decreased with the increased crystallinity.^[276,278] For example, poly(3HB-co-3HV) was hydrolyzed faster than poly(3HB), and the degradation rate increased with the increasing 3HV fraction in the polymers since the crystallinity of poly(3HB-co-3HV) decreases with increasing 3HV fraction to a certain level.^[279] In another study, poly(3HB) and poly(3HB-co-3HV) having similar crystallinity were subjected to the degradation by an extracellular PHA depolymerase, which showed lower degradation rate for poly(3HB-co-3HV).^[280] The accessibility of the depolymerase to the ester linkage was suggested to be a possible reason as the side chain of 3HV in the copolymer is bulkier than that of 3HB.^[280]

As PHAs attracted much attention for their applications in the medical fields, their degradation characteristics have been extensively studied under various biologically relevant conditions using human blood, animal tissues, calf serum, pancreatin, and synthetic gastric juice.^[281–283] Together with the biodegradability, the biocompatibility of PHAs has also been examined by observing immune and other physiological responses upon their complete degradation in a host organism. As 3HB is a natural metabolite of animals produced via fatty acid oxidation in liver, it is detected in blood (normal concentration of 0.3–1.3 × 10⁻⁶ M in human), urine, and cerebrospinal fluid, and also in tissues and organs.^[284] The biocompatibility assays have been conducted in vitro and in vivo on poly(3HB), poly(3HB-co-3HV), poly(3HB-co-3HHx), poly(3HHx-co-3HO), poly(3HB-co-4HB), and poly(4HB).^[285,286] In vitro assays suggested that PHAs have good biocompatibility with several different cell lines including osteoblasts, epithelial cells, and chondrocytes.^[283,287] The results of in vivo studies on PHAs in various animals such as dogs,^[288] rats,^[6,289] sheep,^[290] rabbits,^[291] and humans^[292] also suggested that PHAs are highly biocompatible. Biocompatibility will be further discussed with examples of medical applications in Section 7.2.4.

7.1.2. Thermal Properties

Thermal properties are important factors in industrial processing and applications of polymers. Generally, SCL-PHAs and MCL-PHAs show different thermal properties. The material properties including thermal properties for natural and nonnatural polyesters are summarized in Table 3. Poly(3HB) has a high melting temperature (T_m) of about 170 °C and

Table 3. Material properties of various PHAs and nonnatural polyesters.

Polymer type ^{a)}	Mechanical properties			Molecular weight properties			Thermal properties [°C]		Ref.
	σ_t^b [MPa]	E^c [MPa]	ε_b^d [%]	M_n [kDa]	M_w [kDa]	PDI ^{e)}	T_m	T_g	
Poly(3HB)	43	3500	5	350	700	2.0	178	4	[294]
Poly(3HB)	62	1100	58	6000	11 000	–	–	–	[295]
Poly(3HV)	31.2	– ^{g)}	14	128	888	6.93	104.2–109.7	–16.5	[87]
Poly(3HB-20%3HV)	20	–	50	–	–	–	145	–1	[296]
MCL-PHA 15%3HDD ^{f)}	8.7	3.6	188.7	80	100	1.25	53	–44	[297]
MCL-PHA 28%3HDD ^{f)}	12.6	6.0	180.0	95	134	1.41	58	–45	[297]
MCL-PHA 39%3HDD ^{f)}	16.3	11.5	125.0	108	157	1.45	65	–43	[297]
Poly(3HD)	12.0	19.9	312.9	248.6	361.4	1.45	72.2	–37.2	[92]
Poly(16%3HD-3HDD)	5.2 3.9	103.1	88.3	119.4	155.5	1.3	77.6	–32.5	[92]
Poly(7%3HHx-3HO)	7.4	1.2	346.3	160	230	1.46	61.0	–37.8	[298]
Poly(8%3HO-3HD)	7.1	1.7	261.6	104	128	1.24	69.4	–42.0	[298]
Poly(44%3HD-3HDD)	5.9	2.0	188.3	125	190	1.50	74.9	–43.0	[298]
Poly(3HB-10%3HHx)	21	–	400	117	304	2.6	127	–1	[299]
Poly(3HB-17%3HHx)	20	–	850	510	1122	2.2	120	–2	[299]
Poly(3HB-21%3HHx)	1.8	23.6	75.3	81	202.5	2.5	55.4	–18.1	[153]
Poly(4HB)	13.8(y) 34.7(m)	180.9	696.6	332	389	1.17	61	–47	[103]
Poly(4HB)	32.55	12.8	1014	104	220	2.11	–	–	[72]
Poly(4HB)	104	–	1000	339	780	2.3	53	–48	[300]
Poly(3HB-23%4HB)	13	–	626	590	1062	1.8	152	–7	[301]
Poly(3HB-36%4HB)	4	–	400	540	1026	1.9	164	–11	[301]
Poly(3HB-45%4HB)	3	–	268	410	820	2.0	162	–16	[301]
Poly(3HB-66%4HB)	9	–	446	320	800	2.5	163	–43	[301]
Poly(3HB-75%4HB)	16	–	526	260	780	3.0	51	–45	[301]
Poly(3HB- <i>b</i> -80%4HB)	13.3(y) 19.9(m)	–	438	50	155	3.1	54 161.4	–47.3	[154]
Poly(3HP)	33.8(y) 21.5(m)	2889.3	497.6	110	163	1.48	78.1	–17.9	[103]
Poly(3HP-12%4HB)	13.0(y) 48.8(m)	3.9	1248.3	221	284	1.29	61.7	–24.4	[103]
Poly(3HP-25%4HB)	1.7(y) 6.4(m)	14.5	962.9	196	259	1.32	62.7	–31.3	[103]
Poly(3HP-38%4HB)	0.9(y) 0.5(m)	4.4	1611.0	215	280	1.30	63.5	–36.1	[103]
Poly(3HP-67%4HB)	0.6(y) 0.3(m)	1.8	429.2	213	278	1.31	64.8	–41.9	[103]
Poly(3HP-82%4HB)	2.6(y) 6.3(m)	18.5	594.7	236	302	1.28	35.7	–29.5	[103]
Poly(3HP- <i>b</i> -29%4HB)	20.0(y) 44.7(m)	177.0	876.7	217	548	2.52	55.0 67.6	–20.2 –46.2	[151]
Poly(3HP- <i>b</i> -37%4HB)	7.4(y) 25.3(m)	113.2	1031.5	209	547	2.62	53.4 66.8	–21.6 –45.2	[151]
Poly(3HB- <i>b</i> -42%3HHx)	1.4	7.6	207.3	81	202.5	2.5	172.1	2.7 –16.1	[153]
Poly(3HB-4%LA)	30	905	7	160	740	4.6	160 174	–6	[302]
Poly(3HB-15%LA)	10	194	75	342	820	2.4	149 167	–9 19	[302]

Table 3. Continued.

Polymer type ^{a)}	Mechanical properties			Molecular weight properties			Thermal properties [°C]		Ref.
	$\sigma_t^{b)}$ [MPa]	$E^c)$ [MPa]	$\epsilon_b^{d)}$ [%]	M_n [kDa]	M_w [kDa]	PDI ^{e)}	T_m	T_g	
Poly(3HB-29% LA)	7	154	156	40.9	90	2.2	141 158	-8 25	[302]
Poly(3HB-40%LA)	6	148	64	20	70	3.5	140 156	-8 30	[302]
Poly(3HB-47%LA)	7	153	84	30	70	2.3	140 157	-8 34	[302]
Poly(2HB)	8.5	75	173	15	27	1.8	98.7	29.7	[210]
Poly(LA-86%2HB)	18.5	466	115	28	39	1.4	98.5	31.0	[210]
Poly(17%LA-16%GA-3HB)	6.8	–	95	74	147	2.0	159.8	14.8	[70]
Poly(63%LA-13%GA-3HB)	8.3	2.8	33.9	16	35	2.2	–	38.2	[64]
Poly(67%LA-24%GA-4HB)	0.75	1.63	769.5	19	36	1.9	–	32.5	[64]

^{a)}3HB, 3HV, 3HHx, 3HO, 3HD, 3HDD, 4HB, 3HP, LA, GA, and 2HB indicate 3-hydroxybutyrate, 3-hydroxyvalerate, 3-hydroxyhexanoate, 3-hydroxyoctanoate, 3-hydroxydecanoate, 3-hydroxydodecanoate, 4-hydroxybutyrate, 3-hydroxypropionate, lactate, glycolate, and 2-hydroxybutyrate, respectively. The monomer composition is presented as mol%. The “Poly(A-b-B)” represents block copolymer, poly(A)-*b*-poly(B); ^{b)}Tensile strength at yield (*y*), at maximum (*m*); ^{c)}Young’s modulus; ^{d)}Elongation at break; ^{e)}Polydispersity index, M_w/M_n ; ^{f)}Poly(3HHx-co-3HO-co-3HD-co-3HDD); ^{g)}Data not available.

a decomposition temperature (T_d) of about 200 °C.^[60] The narrow temperature range between T_m and T_d can lead to thermal instability during polymer processing. In the melting step of poly(3HB) near 180 °C, some poly(3HB) chains can be degraded into its oligomers, which makes the processing difficult. On the other hand, poly(3HP), another homo SCL-PHA, has a T_g of -20 °C and a T_m of 80 °C.^[103,104] The thermal stability and high crystallization speed make poly(3HP) competitive with other synthetic plastics such as polyethylene for industrial applications.^[103,104] Poly(4HB) also shows thermal stability with a T_g of -50 °C and a T_m of 60 °C.^[103,293] The thermal properties of nonnatural polyesters are summarized in a recent review.^[193]

Several SCL-PHA copolymers such as poly(3HB-co-3HV), poly(3HB-co-4HB), and poly(3HB-co-3HP) have been investigated for the changes of their thermal properties according to the varying monomer composition. Also, much effort has been exerted to improve the thermal stability of poly(3HB) by incorporating second monomers such as 3HP and 4HB. As expected, the T_g of 3HB copolymers decreased as the fractions of comonomers such as 3HV, 3HP, and 4HB increased.^[303] On the other hand, the changes of T_m showed a different tendency from T_g . As the 3HP fraction in poly(3HB-co-3HP) increased from 0 to 67 mol%, the T_m of the copolymer decreased from about 170 to 50 °C. When the 3HP fraction became higher than 67 mol%, however, T_m rather increased up to about 70 °C.^[279] Similar tendency of T_m change was observed for poly(3HB-co-3HHx) and poly(3HB-co-4HB) as well.

In contrast to SCL-PHAs, MCL-PHAs possess relatively low crystallinity below 40%.^[304] MCL-PHAs have T_g ranging from about -52 to -25 °C and T_m ranging from about 38 to 80 °C (Table 3).^[92,305] As the T_g values are lower than room temperature, MCL-PHAs exhibit elastomeric properties resembling natural rubber within a narrow range of temperatures because

of their low T_m . At temperatures above or close to the T_m , MCL-PHA becomes completely amorphous and sticky.^[305,306] Several studies showed that the T_g of MCL-PHA decreased with an increase of carbon length of the side chain, while the T_m increased with it.^[307] Similar studies were performed on homo MCL-PHAs and various MCL-PHAs comprising >95 mol% of single 3-HA monomers having a side chain length of C3–C9. The T_m values decreased with the increasing the side chain length from C3 to C4, and increased with further increasing the side chain length from C4 to C9.

Due to the low T_m and low crystallization rate of MCL-PHAs, which are unfavorable for polymer processing, applications of MCL-PHAs have been rather limited. Several methods such as crosslinking, blending with other polymers, and grafting have been studied to improve the processability and crystallization rate.^[308]

7.1.3. Mechanical Properties

Poly(3HB) is highly crystalline with 60–80% crystallinity and exhibits high tensile strength of 43 MPa and a Young’s modulus of 3.5 GPa, which are similar to some conventional plastics such as polypropylene and polyethylene (Table 3, **Figure 8**).^[294,300] Poly(3HB) is stiff and brittle upon aging with a low elongation at break of about 5%. To improve the elasticity and toughness of poly(3HB), several different monomers have been incorporated to make copolymers, such as poly(3HB-co-3HV), poly(3HB-co-4HB), poly(3HB-co-3HP), poly(3HB-co-3HHx), and poly(3HB-co-LA) (the engineering strategies for the production are described in Sections 4 and 5), which successfully reduced polymer crystallinity, stiffness, and aging of poly(3HB).

As a special case of poly(3HB), an ultrahigh-molecular-weight (22 MDa) poly(3HB) was produced in an engineered

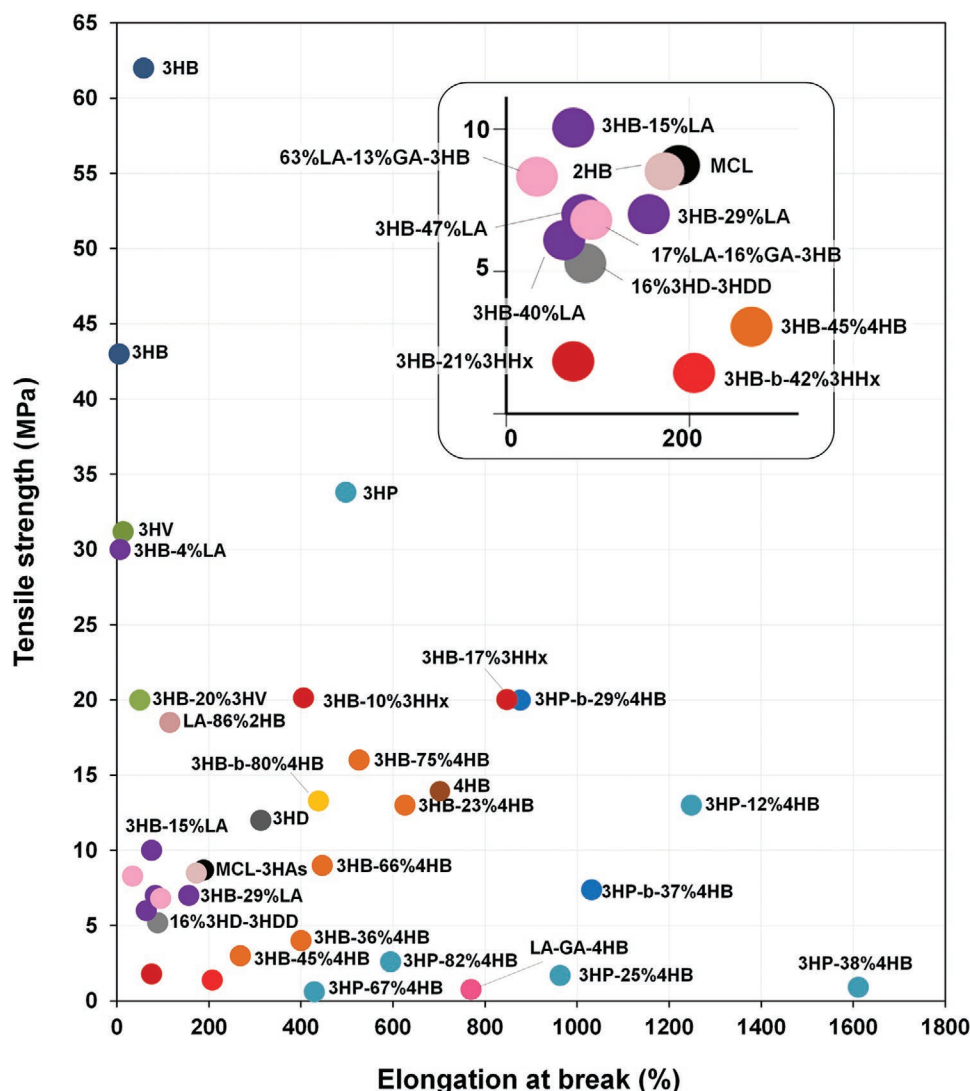


Figure 8. Mechanical properties of diverse PHAs. Two representative polymer properties, tensile strength and elongation at break, of various PHA homopolymers and copolymers are shown. Detailed information is provided in Table 3.

E. coli strain cultivated at a low pH (6.0) condition. This ultrahigh-molecular-weight poly(3HB) showed improved mechanical properties compared with the typical poly(3HB) having molecular weight of less than 1 MDa.^[81,295,309] More detailed studies on poly(3HB) having a molecular weight of 11 MDa showed that it can be processed to an oriented film by hot-drawing. The poly(3HB) film exhibited improved mechanical properties, such as a Young's modulus of 1.1 GPa, a tensile strength of 62 MPa, and an elongation at break of 58%.^[295]

Poly(3HP) has a Young's modulus of 0.3 GPa and a tensile strength of 27 MPa with an elongation at break of about 600%.^[310] Compared to poly(3HB), poly(3HP) is more flexible with similar tensile strength, which makes it attractive for various applications.

Poly(4HB) is a strong and elastic thermoplastic with a Young's modulus of 180 MPa, a tensile strength of 104 MPa, and an elongation at break of 1000%.^[103,294,300] The ductile characteristic of poly(4HB) has been used to improve the flexibility

of other polymers by copolymerizing with 4HB monomer. For example, the physical properties of poly(3HB-co-4HB) can vary from rigid crystalline plastic at low 4HB fractions to highly elastic polymer at high 4HB fractions. Poly(3HB-co-4HB) containing 82 mol% of 4HB showed an elongation at break of 1320%.^[294] As another example, when 4HB monomer was incorporated into PLGA at 9.1 mol%, the resulting poly(LA-co-GA-co-4HB) showed greatly improved elasticity; the elongation at break of poly(LA-co-GA-co-4HB) was about 800% while PLGA is less flexible with an elongation at break of 14%.^[64]

MCL-PHAs are elastomers and possess very different mechanical properties with those of SCL-PHAs. MCL-PHAs have a low degree of crystallinity (below 40%), low tensile strength, and high elongation at break.^[304,305] They can be soft and flexible or even sticky materials according to the monomer type and composition.^[304,305] MCL-PHAs, which are generally copolymers of 3HHx, 3HO, 3HD, and 3HDD, have elongation at break of 88–350%, Young's modulus of 1–103.1 MPa, and

tensile strength of 5–16.3 MPa.^[92,297,298] When small amounts of MCL monomers as low as 5 mol% are incorporated into SCL-PHAs such as poly(3HB), the SCL-MCL copolymers show much improved material properties including high toughness and ductility and good thermal stability under the conventional plastic processing condition, making them suitable for various applications.^[299,311] For example, as the 3HHx fraction in poly(3HB-co-3HHx) was increased from 0 to 17 mol%, the tensile strength of the copolymer decreased from 43 to 20 MPa while the elongation at break significantly increased from 6% to 850%.^[299] Studies on the mechanical properties of SCL-MCL-PHAs having MCL monomers at similar mole fractions showed a tendency of decreasing Young's modulus with an increase of side group carbon length for the MCL monomer.^[311] The values of Young's modulus of poly(3HB-co-10 mol% 3HHx), poly(3HB-co-10 mol% 3HO), and poly(3HB-co-10 mol% 3-hydroxyoctadecanoate) were \approx 510, 375, and 150 MPa, respectively.^[311] The use of different side groups resulted in different flexural modulus, and the extension of the side groups increased the flexibility of polymers.^[311]

7.2. Applications of PHAs

7.2.1. Prospects for PHAs: Promising Materials to Replace Some Nondegradable Plastics

To address severe problems arising from the accumulation of plastic wastes, governments around the globe have started tightening regulations against the use of nonbiodegradable plastics. One of the first regulations on thin plastic bags started in 2002 by the Bangladeshi government.^[312] Other countries have also taken different regulatory or legislative measures on nonbiodegradable plastics. For example, South Africa introduced plastic bag levies in 2004^[313] and Tanzania banned the manufacture and domestic distribution of plastic bags, applying the policy first to their citizens and eventually to tourists.^[314] The United States in 2019 introduced at least 95 bills that would ban or impose fines against the use of nonbiodegradable plastic bags.^[315] Moreover, Europe has established the most stringent regulations on the current usage of plastics. The new European Union (EU) Single-Use Plastics Directive and the Fertiliser Regulation were officially adopted in May 2019 to restrict the sales of disposable plastic products and other oxo-degradable plastics in the EU from 2021.^[316] According to this new regulation, single-used plastic bottles should contain recycled content of at least 25% by early 2025.

Recent legislations and regulations against the use of plastic commodities have been revitalizing the development of substitutes for conventional nonbiodegradable plastics. As PHAs are biodegradable and possess material properties comparable to conventional petroleum-based plastics, PHAs will become more widely employed to address such legislations and regulations. Today, a number of companies are involved in commercial production of PHAs and/or development of applications in the industrial, agricultural, and medical fields (Text S1, Figure S1, and Table S2, Supporting Information). PHAs have already been used to make a wide range of commodity products including disposable razors, dishes, cups, utensils, diapers,

feminine hygiene products, cosmetic containers, shampoo bottles, packaging films, bags, food containers, and paper coatings.^[317,318] One of the biggest challenges in large-scale commercialization of PHAs is high production cost. However, the increasing interest and demand for biodegradable plastics will lead to further scale-up and optimization of PHA production processes toward lowering the price of PHAs.

7.2.2. Food Applications

The packaging industry is responsible for consuming more than 40% of the overall plastics produced, in which food packaging accounts for about a half of that. As huge amounts of plastic wastes have been generated due to the heavy use and inappropriate disposal of single-use plastics including food packaging plastics, it is necessary to replace the packaging materials, especially those not easily recyclable, from nonbiodegradable plastics to biodegradable plastics such as PHAs.^[319,320] PHAs are hydrophobic, optically active, thermoplastic, piezoelectric, nontoxic to human and nature. Also, PHAs exhibit good barrier characteristics for gases and water.^[318,321,322] These material properties and characteristics make PHAs suitable for packaging materials. Also, PHAs can be used in food-related products such as straws and bottles. In 2018, the first fully biodegradable plastic straw made of Nodax PHA was developed by Danimer Scientific. In 2019, drink bottles made entirely of PHAs were developed by the Cove, which is launching in California. The containers can be degraded within about five years in the soil while conventional PET plastic bottles may take about 500 years to be broken down.^[323] More recently, the food and beverage production company Nestle started a partnership with Danimer Scientific to develop biodegradable plastic bottles. This partnership aims at making all packaging materials recyclable or reusable by 2025.^[324]

7.2.3. Cosmetic Applications

The best developed products utilizing PHAs in the cosmetic field are bio-based beauty masks and pads.^[318,321] Several casting films of PHA blends with other bio-based materials have been developed for manufacturing beauty masks and pads.^[325–328] In 2017, the EU launched Polybioskin research project,^[329] broadening the use of biopolymers in skin-contact applications and beauty masks containing beneficial molecules for the skin. Another application of PHAs in cosmetics is PHA microplastics. Petroleum-derived microplastics are big problems as they cannot be collected; they are generated due to our actual use of microplastics in our products including face cleanser and toothpaste, and also by weathering of already disposed waste plastics. These microplastics end up in the river and ocean, and come into our body through eating fish and seafood. Due to the increasing concerns on potential harmful impacts of micro- and nanoplastics on the environment and human health,^[330] regulations have been implemented. The European Commission began the regulation of utilizing microplastics in cosmetic applications in early 2018. Due to its unspecified ban regarding the range of microplastics (petroleum-based

nondegradable microplastics or biodegradable PHA microplastics), the feasibility of applying PHA microplastics as an alternative is yet to be seen. Nevertheless, various PHA microplastic techniques have been developed with firmly tested for the biodegradability and nontoxicity. Recently, Unilever developed sun protection products containing PHA micropowders. These micropowders are the first cosmetic ingredients derived from renewable biomass and are fully biodegradable. These products (the brand name of MyKAI) are now being sold in the first half of 2019.

7.2.4. Medical Applications

PHAs have received much attention in the medical and therapeutic materials industry due to their biocompatibility and biodegradability. PHAs are especially advantageous even compared with other popular medical polymers like PLA and poly(GA) since the in vivo degraded products of PHAs are less acidic than LA and GA, thus less likely to cause local necrosis and inflammation.^[331,332]

To date, many studies have reported the applications of PHAs for surgical sutures, stents, bone fracture fixation plates, repair patches, cardiovascular patches, orthopedic pins, adhesion barriers, guided tissue repair/regeneration devices, articular cartilage repair devices, nerve guides, tendon repair devices, heart valve scaffold, bone marrow scaffolds, wound dressings, and drug delivery carriers (Table 4 and Figure 9).^[283,333,334] Early attempt to apply PHA for medical use was in the surgical suture which was made of poly(3HB).^[335] Beyond poly(3HB), various types of PHAs such as poly(3HB-co-3HV), poly(3HB-co-3HHx), and poly(4HB) have been studied for their uses in medical products. When monofilament sutures made of poly(3HB) and poly(3HB-co-3HV) were intramuscularly implanted in animals, the inflammatory and other responses observed were similar to those of silk and less severe than catgut, two traditionally used surgical materials.^[289] Compared to poly(3HB) and poly(3HB-co-3HV), poly(3HB-co-3HHx) showed even better biocompatibility and hemocompatibility, and high affinity to a variety of cells as well as higher break strength and flexibility, which makes it even more advantageous for its application as a medical suture.^[336]

PHAs, in the form of PHA scaffolds, have also been extensively studied for their biocompatibility and capabilities of tissue regeneration and stem cell differentiation. For example, poly(3HB-co-3HV-co-3HHx) scaffold was demonstrated to promote differentiation of human bone marrow mesenchymal stem cell (BMSC) into nerve cells.^[332,347] Furthermore, poly(3HB-co-3HHx) scaffold was found to improve osteoblasts attachment, propagation, and differentiation compared with poly(3HB).^[347,366] In another study, poly(3HB-co-3HHx) also promoted the chondrogenesis of human BMSCs.^[348] A terpolymer, poly(3HB-co-4HB-co-3HHx), has shown even better performance than poly(3HB-co-3HHx) in MSC differentiation.^[349] Scaffolds made of poly(3HB-co-3HHx) with collagen were also successfully employed for growing MSCs.^[350] Recently, the highly open porous microspheres made of poly(3HB-co-3HV-co-3HHx) were employed as an injectable scaffold for tissue regeneration.^[367] These open porous PHA microspheres could

Table 4. Medical applications of PHAs.

Products ^{a)}	Applications	Ref.
Poly(3HB)	Nanoparticles for intracellular drug release Suture healing muscle-fascial wounds Bone tissue engineering	[337,340–346]
Poly(4HB)	Absorbable suture (TephaFLEX, the first commercial product of PHAs approved by the FDA)	[343]
Poly(3HB-co-3HHx)	Tissue engineering (cartilage, bone marrow cell, osteoblast, heart valves) Nanoparticles for intracellular drug release	[347–351]
Poly(3HB), poly(3HB-co-3HV), poly(3HB-co-3HHx)-hydroxyapatite composites	Marrow osteoblast, bone tissue engineering	[352–355]
Poly(3HO) film with layers of PGA	Vascular tissue engineering	[338]
Poly(3HB-co-3HV)	Antibiotic delivery system Drug delivery carrier for cancer therapy Topical and transdermal drug delivery carrier	[356–359]
Poly(3HB-co-4HB)	Antibiotic delivery system Implant with Sulperazone or Duocid Drug delivery carriers	[360,362]
Poly(3HO-co-3HHx)	Microspheres, tubes, and pellets	[363]
Poly(3HHx-co-3HO)/PGA blend Poly(3HO)/ α -poly(3HB) blend	Cardiovascular tissue engineering	[338,364]
Poly(3HB-co-3HV)/PCL blend	Drug delivery carrier for photodynamic therapy	[365]

^{a)}The abbreviations are: 3HB, 3-hydroxybutyrate; 4HB, 4-hydroxybutyrate; 3HHx, 3-hydroxyhexanoate; 3HV, 3-hydroxyvalerate; 3HO, 3-hydroxyoctanoate; α -poly(3HB), atactic poly(3HB); PLGA, poly(lactate-co-glycolate); PGA, polyglycolate; PCL, poly(ϵ -caprolactone).

protect the cells from stresses during injection and allow the cells to proliferate and migrate to a defected tissue. It also supported high viability for human BMSCs and good cell adherence on its surface and internal porous space. These results clearly demonstrated that PHAs have great potential for their use as cell and tissue scaffolds in diverse medical applications.

Utilization of PHAs in bone tissue engineering is focused on regenerating new bone with the aid of porous PHA scaffolds. Among various porous scaffolds made of poly(3HB), poly(3HB-co-3HV), and poly(3HB-co-3HHx), poly(3HB-co-3HHx) showed the best outcomes with respect to cell adhesion and bone tissue regeneration.^[352,368] Moreover, the porous scaffold made of poly(3HB)-hydroxyapatite or poly(3HB-co-3HHx)-hydroxyapatite was found to serve as a good material for bone reconstruction. The hydroxyapatite composites showed better mechanical properties, biocompatibility, and osteoconductivity.^[337,340–342,369]

PHAs were examined as a material in heart valve tissue engineering as well. Heart valve tissue engineering was first studied using a material made by putting polyglactin woven meshes between nonwoven poly(GA) meshes.^[370] Afterwards, various

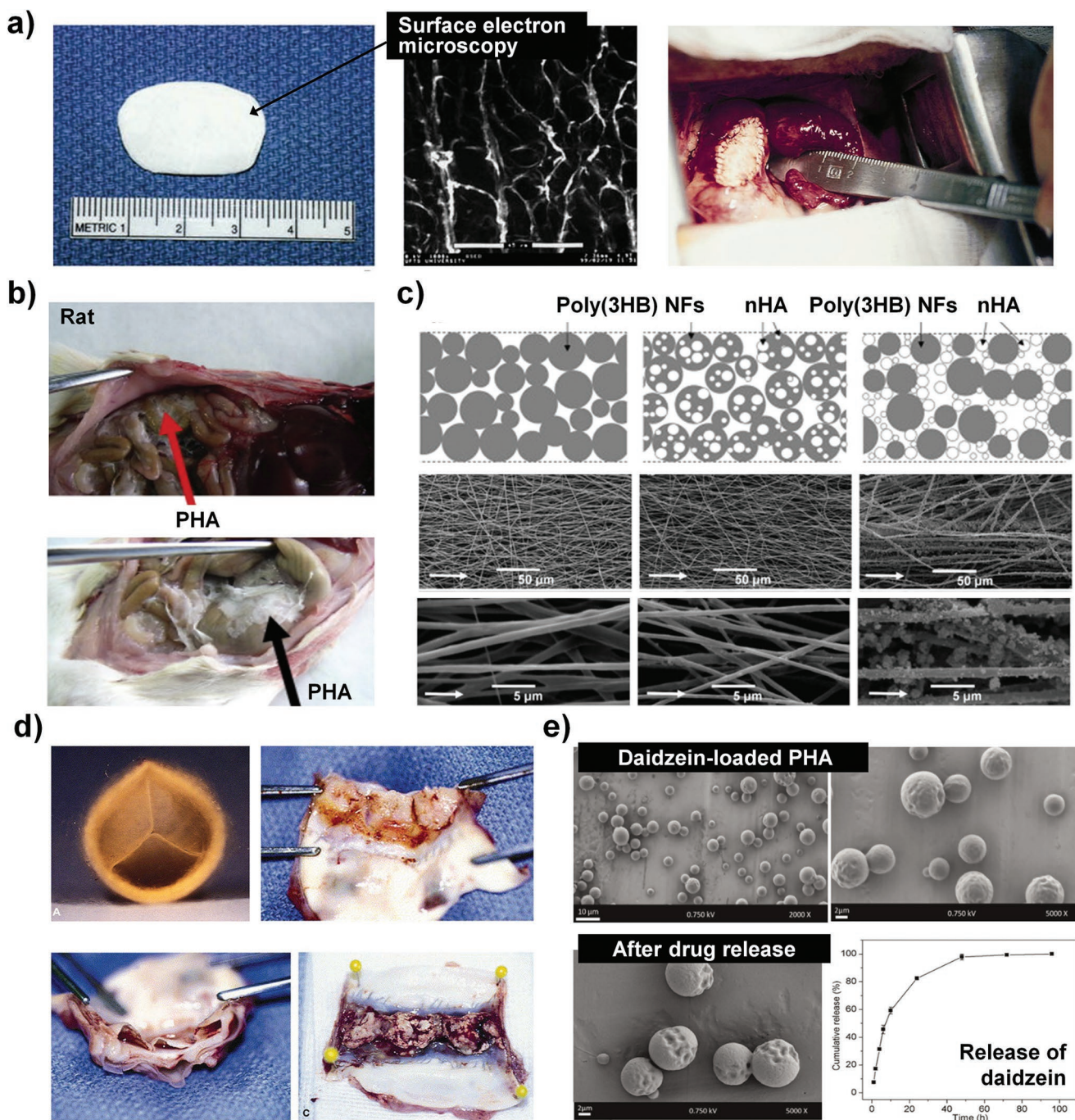


Figure 9. Various medical applications of PHAs. a) Implanted poly(4-hydroxybutyrate)-patch seeded with autologous vascular cells in the main pulmonary artery in a juvenile sheep model. Reproduced with permission.^[290] Copyright 2000, Elsevier. b) Films of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) as tissue adhesion prevention films for surgical operations. Reproduced with permission.^[6] Copyright 2009, Elsevier. c) Nanofibers (NFs) of poly(3-hydroxybutyrate) and hydroxyapatite nanoparticle (nHA) for bone tissue engineering. Reproduced with permission.^[337] Copyright 2014, Elsevier. d) Three-leaflet valve scaffold of poly(3-hydroxyoctanoate). Reproduced with permission.^[338] Copyright 2000, Elsevier. e) Drug (daidzein)-loaded microspheres of poly(3-hydroxybutyrate-co-3-hydroxyvalerate). Reproduced with permission.^[339] Copyright 2015, Elsevier.

biodegradable polymers were tested for their suitability as a tri-leaflet heart valve. In earlier days, porous poly(GA) and PLA were tested to replace all tri-leaflet heart valve but failed due to the stiffness and rigidity of the materials. To solve this problem, PHA based materials were tested in a sheep model.^[334,371] A

major limitation of PHA scaffolds in these studies was that relatively slow biodegradation prevented the replacement of the scaffold with the desired tissues.^[334,371,372] Thus, combination of poly(GA) with more flexible poly(4HB) was developed to improve the mechanical strength of poly(GA)-based scaffold.^[372-374]

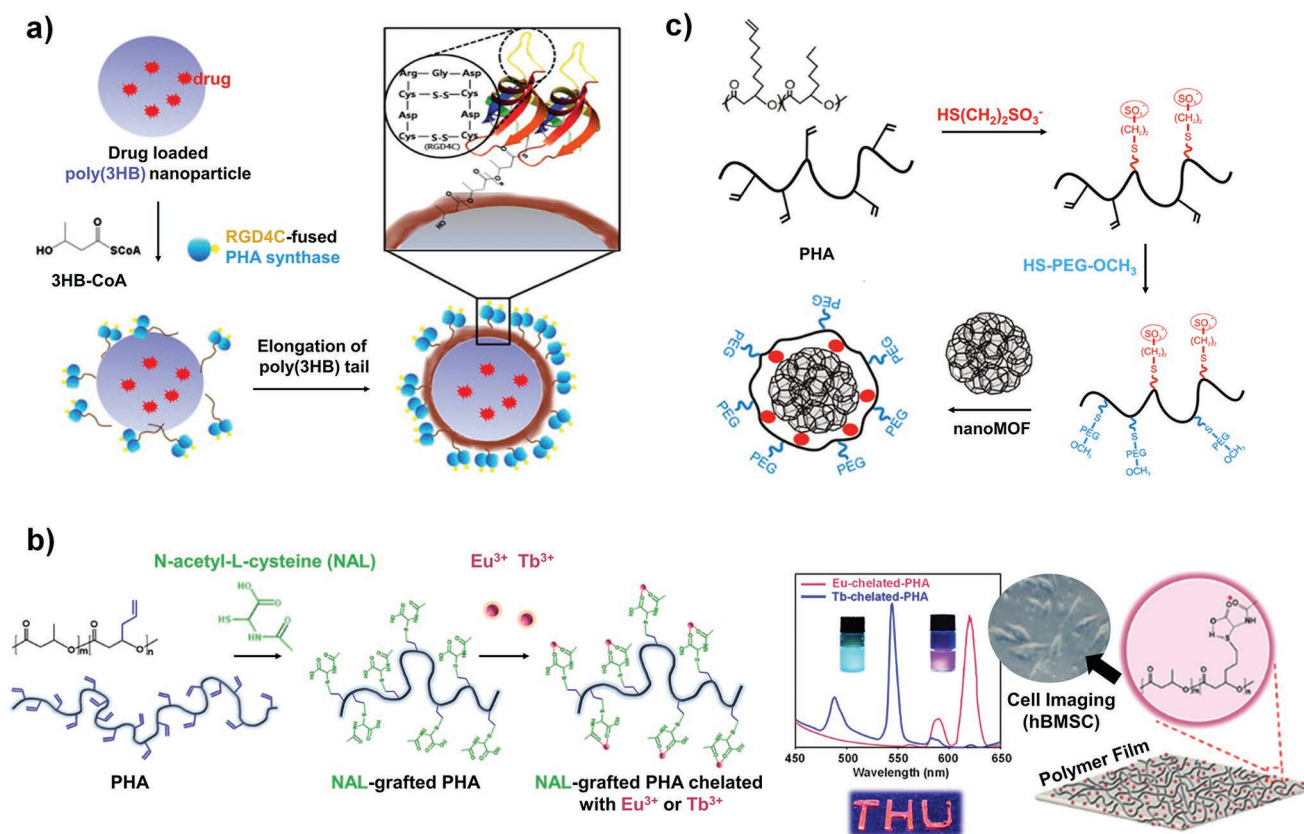


Figure 10. Examples of PHA functionalization. a) Surface functionalization of poly(3-hydroxybutyrate) nanoparticles through enzymatic reaction. Adapted with permission.^[376] Copyright 2011, Elsevier. b) Functional *N*-acetyl-L-cysteine (NAL)-grafted PHA chelated with rare earth metal ions (Eu³⁺ and Tb³⁺). Adapted with permission.^[388] Copyright 2019, American Chemical Society. c) Nanoparticle of PHA and metal organic framework (MOF) hybrid. Adapted with permission.^[389] Copyright 2019, American Chemical Society.

Implementation of this scaffold resulted in successful tissue remodeling in a sheep with complete biodegradation of the scaffold within 6 to 8 weeks.

PHAs are also used as delivery carriers for prolonged release of therapeutics such as anticancer drugs,^[375,376] hormones,^[339,377] and antibiotics.^[360,378,379] For example, poly(3HB-co-3HV) microspheres were applied for the sustained release of anticancer drug 5-fluorouracil.^[375] Microspheres of poly(3HB) have been tested for releasing the antibiotics rifampicin,^[380] gentamicin,^[381,382] and tetracycline.^[383–385] In another study, poly(3HB-co-3HV) microspheres and microcapsules containing tetracycline were shown to be effective against many of the periodontal disease-related bacteria. Furthermore, poly(3HB-co-3HV) and poly(3HB-co-4HB) were used as antibiotic-loaded implantable rods for the local delivery of antibiotics (Sulperazone and Duocid, Sulbactam-cefoperazone) in chronic osteomyelitis therapy.^[360–362] With advances in techniques of combining biomolecules with nanomaterials, PHAs can be processed into more diverse and customized forms including nanoparticles of desired sizes, and also functionalized with biomolecules.^[386] As an example, the surface of poly(3HB) nanoparticles could be functionalized with a tumor-specific ligand RGD4C for more accurately targeting MDA-MB 231 breast cancer cells.^[376] It was achieved via hydrophobic interaction between the surface of poly(3HB) nanoparticle and

growing poly(3HB) chain synthesized by RGD4C fused PHA synthase (Figure 10). In another study, poly(3HB-co-3HHx) was fabricated into nanoparticles encapsulating an antitumor agent, NVP-BEZ235 (BEZ).^[387] The poly(3HB-co-3HHx) nanoparticles loaded with BEZ allowed a sustained release of the antitumor agent without nanotoxicity in vitro. In addition, the delayed tumor growth and reduced tumor proliferation were observed by in vivo real-time imaging in human prostate cancer cell line PC3 tumor xenograft mouse.

The range of PHA applications in biomedical fields has been further extended through various chemical functionalization methods. For example, rare earth fluorescent elements Eu³⁺ and Tb³⁺ were chelated onto the *N*-acetyl-L-cysteine-grafted poly(3HB-co-3-hydroxyhex-5-enoate) (Figure 10).^[388] The rare-earth-modified PHA exhibited good photoluminescence properties and biocompatibility by in vitro assay using human BMSCs. Since the conventional fluorescent chemicals and materials such as organic dyes and quantum dots can be easily photobleached or can emit toxic free ions, the rare-earth-modified PHAs can be a promising alternative for various biomedical applications.

Water-soluble PHAs containing sulfonate ion (SO₃⁻) were also synthesized by two successive photoactivated thiol-ene reactions of sodium-3-mercapto-1-ethanesulfonate and poly(ethylene glycol) (PEG) on poly(3HO-co-3-hydroxyundecenoate)

[poly(3HO-co-3HUD)] (Figure 10).^[389] The functionalization of poly(3HO-co-3HUD) with sulfonate group was performed to promote ionic interaction with the metal–organic framework (MOF), which was followed by functionalization with PEG to improve hydrophilicity. The resulting grafted polymers poly(HO)/SO₃⁻/PEG and poly(3HO-co-3HUD)/SO₃⁻/PEG were successfully combined with MOF, which could be used as new biocompatible therapeutic nanoparticles for drug delivery.

8. Conclusion

Plastics have become an indispensable material in our everyday life. Due to the severe environmental pollution caused by the accumulation of carelessly disposed plastics, we need to act now to better recycle the used plastics and also develop biodegradable plastics. PHAs are biodegradable and bio-based polyesters, and thus are highly promising materials to replace the non-degradable plastics made from fossil resources. Since the first discovery of PHA accumulating bacteria, there have been remarkable achievements in PHA research. Numerous bacteria have been isolated as natural PHA producers and numerous genes related to PHA biosynthesis have been identified. Several enzymes involved in PHA biosynthesis and degradation have been studied in detail to better understand PHA metabolism and to develop superior producers and various applications. Also, numerous studies have been carried out to develop better fermentation and downstream processing technologies.

Two major challenges impeding commercialization of PHAs are the high production cost and poor material properties compared with petroleum-derived plastics. Numerous natural and engineered bacteria including *C. necator*, *A. latus*, *P. putida*, *E. coli*, and *Halomonas bluephagenesis* have been employed for the efficient production of PHAs from inexpensive carbon sources, and some of them are currently used for large-scale industrial production by several companies. Systems metabolic engineering will allow optimization of the metabolic fluxes at genome-wide scale, which will consequently lead to enhanced production of PHAs and nonnatural polyesters from inexpensive substrates with higher PHA titer, yield, and productivity for achieving overall cost-effectiveness of PHAs.

Recently, the 3D crystal structure of the catalytic domain of PHA synthase has been determined, solving the 30 year mystery of the key PHA polymerizing enzyme. Based on the crystal structure, PHA polymerization mechanism could be better understood to provide detailed information useful for rational enzyme engineering not only to enhance PHA production but also to produce PHAs comprising desired monomers and even nonnatural polyesters that have not yet been produced. Also, such enzyme engineering can be combined with systems metabolic engineering for the enhanced production of desired PHAs at high efficiencies.

To date, more than 150 monomers have been identified as the constituents of PHAs, which, when incorporated into PHAs, provide a wide range of material properties from hard and rigid thermoplastics to rubber-like elastomers depending on the monomer types and compositions. Although natural PHA synthases possess very little to no activities for 2-HA substrates such as GA and LA, the evolved PHA synthases were demonstrated to be able to polymerize 2-HA monomers, allowing

production of PLA and PLGA through one-step microbial fermentation from renewable resources. Thus, it is expected that the monomer spectrum will be further expanded to cover various natural and nonnatural monomers through enzyme and metabolic engineering, which will allow production of more diverse PHAs and nonnatural polyesters possessing material properties equivalent to or better than those of currently used petroleum-derived plastics for a wider range of applications.

When PHAs are discarded in the environment, they will be completely biodegraded. However, it is interesting to note that PHAs might also be used as a supplementary carbon source for some animals beyond microorganisms. In a recent study, it was demonstrated that feeding poly(3HB) allowed large yellow croaker fish to grow faster, gain more weight, and survive better,^[390] which provides additional benefits of using poly(3HB) and potentially other PHAs as plastics to be discarded in the ocean and river.

Considering the ever increasing demand for biodegradable and bio-based plastics, PHAs tailored to possess diverse material properties will serve as one of the major groups of polymers for replacing the current plastics made from fossil resources. To improve material properties further, compounding of PHAs can be performed similarly to the currently used plastics as well. Regarding the cost-competitiveness, it is to be seen how low the PHA production cost can go down through the development of an optimal production strain and optimization of fermentation and downstream processes. We believe that the overall PHA production cost will decrease significantly to an acceptable range with further research and development. Of course, the cost-competitiveness of PHAs is heavily affected by the oil price; when the oil price is high, PHA obviously becomes more cost competitive. As the fossil resources will ultimately be depleted or its use will be discouraged due to increasing environmental concerns, establishing the PHA-based polymer industry will not only serve to protect our environment, but also to prepare for the time without fossil resources. Toward this end, more effort needs to be exerted to develop superior production hosts by integrated enzyme engineering and metabolic engineering, together innovative fermentation and downstream processes.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

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bacterial polyesters, metabolic engineering, microorganisms, plastics, polyhydroxyalkanoates

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