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REVIEW

Bioplastics from Waste Glycerol Derived from Biodiesel Industry

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ABSTRACT: Polyhydroxyalkanoates (PHAs) are polyesters that can be biologically synthesized by many microorganisms and engineered plants that have been investigated by microbiologists, biochemists, polymer scientists, material engineers, and medical researchers for several decades. Research on microbial production of PHAs has been extensively focused on using pure carbon sources, such as sugars and fatty acids. Practical considerations of production costs of PHAs have resulted in research efforts to use alternative renewable and inexpensive feedstocks. One potential feedstock for the production of PHA polymers is the glycerol waste byproduct of biodiesel production. The major focus of this review is the production of PHA polymers from glycerol. A review of biosynthetic pathways for PHAs production from glycerol, current production of waste glycerol in biodiesel industry, physical and mechanical properties of PHAs, and applications of PHAs in the areas of packaging industry, implant materials, drug carrier, biofuels, are covered. © 2013 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 130: 1–13, 2013

KEYWORDS: biosynthesis of polymers; biopolymers & renewable polymers; degradation; biodegradable

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INTRODUCTION

Polyhydroxyalkanoates are Biobased, Biodegradable Plastics

Although petroleum-based plastics fulfill a multitude of uses, their extended use presents two major issues. Firstly, it is predicted that global oil reserves will eventually decline and therefore alternative methods to produce plastic products must be pursued. Secondly, petroleum-based plastics that are used for bulk-commodity products are non-biodegradable and present numerous waste disposal issues that result in a number of environmental problems. Polyhydroxyalkanoates (PHAs) are a family of polyesters which are biobased, biodegradable, biocompatible, and environmentally friendly thermoplastics and elastomers.[1] In nature, PHAs are accumulated inside of microbial cells during unbalanced growth conditions as carbon and energy reservoirs in a manner similar to starch in plants or fat for animals.[2] Thermal and mechanical properties of PHAs vary dramatically due to different sizes of the side chains in the repeating units of the polymer. Two main classes of PHA polymers can be defined on the basis of these repeating units. Short-chain length (scl) PHAs consist of 3–5 carbons per repeating unit and medium-chain-length (mcl) PHAs consist of 6–16 carbons per repeating unit (Figure 1). Scl-PHAs are highly crystalline and mcl-PHAs are elastomeric in nature.[3,4] PHA homopolymers, such as poly-3-hydroxybutyrate (P(3HB)),[5] poly-3-hydroxyvalerate (P(3HV)),[6] poly-4-hydroxybutyrate (P(4HB)),[7] poly-3-hydroxyhexanoate (P(3HHx)),[8] poly-3-hydroxyoctanoate (P(3HO)),[9] poly-3-hydroxydecanoate (P(3HD)),[9] and poly-3-hydroxydodecanoate (P(3HDD)),[9] have different physical properties, range from brittle and rigid to flexible and tough. PHA copolymers, such as P(3HB-*co*-3HV),[10] P(3HB-*co*-mcl-PHAs),[11] P(3HB-*co*-3HHx),[12] and P(3HB-*co*-3HV-*co*-4HB),[13] exhibit improved and favorable mechanical properties, conferred from the ratio of individual monomer units (Table I). The large number of potential monomeric subunits that can be incorporated into PHA polymers represents a valuable library of PHA polymers and expands their potential commercial applications based on their diverse properties. More than 150 different monomeric constituents that can be incorporated into PHA polymers are known.[19,20]

History of Polyhydroxyalkanoate Research

P(3HB) was first identified as an intracellular storage material in *Bacillus megaterium* by Lemoigne in 1926.[21] Due to limited

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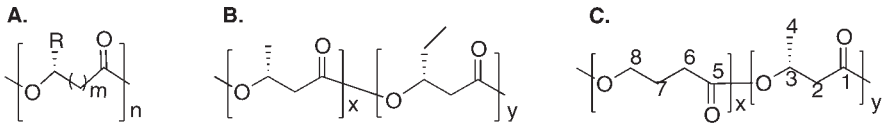

m=1,	R=H	poly-3-hydroxypropionate
	$R=CH_3$	poly-3-hydroxybutyrate
	$R=CH_2CH_3$	poly-3-hydroxyvalerate
	$R=(CH_2)_xCH_3$, x=2,3,…,11	mcl-poly-3-hydroxyalkanoates
m=2,	R=H	poly-4-hydroxybutyrate
m=3,	R=H	poly-5-hydroxyvalerate

Figure 1. General structure of PHAs (A) and copolymers (B) poly-(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (abbr. P(3HB-*co*-3HV)) and (C) poly-(3-hydroxybutyrate-*co*-4-hydroxybutyrate) (abbr. P(3HB-*co*-4HB)). R-groups and common names of polymers are indicated in the table.

methods for P(3HB) detection and qualification, further research on P(3HB) failed to spread until the late 1950s when microbial physiologists finally recognized the importance of P(3HB) in the overall metabolism of bacterial cells. Researchers at the University of Edinburgh in Scotland and at the University of California, Berkeley rediscovered P(3HB) and elucidated the function of P(3HB) in the cell metabolism independently in 1958 and 1959, respectively.[22,23] Marchessault and coworkers[24] at The State University of New York-College of Environmental Science and Forestry (SUNY-ESF) collected P(3HB) samples from various bacteria species and employed X-ray diffraction and infrared absorption analysis to explore the crystal and molecular structure of P(3HB).

Wallen and Rohwedder[25] reported that polyesters, isolated from activated sewage sludge, exhibited similar, yet not identical NMR and infrared spectra. Gas chromatographic analysis indicated a mixture of 3HB, 3HV, 3HHx, and possibly 3HHp (3-hydroxyheptanoate) monomeric units from these polyesters. The identification of a group of PHAs, in addition to P(3HB), greatly accelerated research on PHAs regarding their diverse material properties.

Promoted by the oil crisis in the 1970s, the first industrial production of P(3HB) was carried out in 1982 by Imperial Chemical Industries (ICI) in the UK. However, high production costs and high crystallinity resulted in a rather limited range of P(3HB) applications.[26] Based on the limitations of the originally produced P(3HB) homopolymer, P(3HB-*co*-3HV) copolymers (Trade name of Biopol®) with enhanced toughness and flexibility was first produced on a commercial scale in the late 1980s by ICI.[27] Eventually, Biopol was acquired by Metabolix (Cambridge, MA) in 2001.[28] In addition to Metabolix, which sells PHAs under trademark of Mirel™, Procter and Gamble (Cincinnati, OH) introduced novel PHA copolymers with 3HB as one monomer and mcl monomers such as 3HHx (C_6), 3HO (C_8) or 3HD (C_{10}) etc. under trademark of Nodax™.[29,30] Currently, the Nodax™ family of polymers are produced by Meredian, Inc. (Bainbridge, GA). Since the 1980s, BASF in Germany produced P(3HB) and

Table I. Physical and Mechanical Properties of Typical PHAs and Petroleum-Derived Plastics[14]

Polymers	Crystallinity[a] (%)	T_m[b] (°C)	T_g[c] (°C)	Tensile strength (MPa)	Elongation to break (%)
P(3HB)	60	177	4	43	5
P(3HV)[15]	/	130	−16	31	14
P(4HB)[16]	25	54	−48	104	1000
P(3HB-*co*-20%HV)	56	145	−1	20	50
P(3HB-*co*-84%HV)[15]	/	/	/	20	35
P(3HB-*co*-16%4HB)	45	150	−7	26	444
P(3HB-*co*-10%HHx)	34	127	−1	21	400
PLA[17,18]	56	170	57	41	4
Polypropylene	50–70	176	−10	38	400
Polyethylene (LDPE)[d]	20-50	130	−36	10	620

[a]Degree of crystallinity.
[b]Melting temperature.
[c]Glass transition temperature.
[d]LDPE: low density polyethylene.

Table II. Physical and Mechanical Properties of P(3HB-*co*-3HV) Copolymers[26]

Mol fraction (mol%)		T_m	T_g	Young's modulus	Tensile strength	Notched Izod impact strength
3HB	3HV	(°C)	(°C)	(GPa)	(MPa)	(J/m)
100	0	179	10	3.5	40	50
97	3	170	8	2.9	38	60
91	9	162	6	1.9	37	95
86	14	150	4	1.5	35	120
80	20	145	−1	1.2	32	200
75	25	137	−6	0.7	30	400

P(3HB-*co*-3HV) and blended these PHAs with its biodegradable polymer Ecoflex®.[31] With the expiration of the original ICI patents, TianAn Biologic Materials Co. in China have currently scaled up to commercial production of P(3HB-*co*-3HV) at 2000 metric tons per year.[31]

Physicochemical and Mechanical Properties of Polyhydroxyalkanoates

The physical and mechanical properties of PHA polymers resemble those of petroleum-derived polypropylene, polyethylene, and polystyrene, make PHAs potential substitutes to these non-biodegradable plastics.[32] P(3HB) is the most common type of PHAs produced by microorganisms. The P(3HB) homopolymer is a highly crystalline, stiff, yet relatively brittle material dependent on the molecular weight.[33] As shown in Table I, P(3HB) shows high tensile strength at 43 MPa and low elongation to break at 5%. The P(3HV) homopolymer exhibited tensile strength at 31 MPa and elongation to break at 14%, which demonstrated that P(3HV) showed less stiffness and higher flexibility than P(3HB).[15] Also in Table I, the copolymer P(3HB-*co*-20 mol % 3HV) exhibits lower crystallinity at 56%, less stiffness at 20 MPa, but much higher elasticity and flexibility, correspondingly to an elongation to break at 50%. When the mole fractions of 3HV in P(3HB-*co*-3HV) copolymers vary, the physical and mechanical properties of the PHA copolymers changes accordingly. In addition, the P(4HB) homopolymer demonstrated greatly enhanced flexibility, as shown in Table I with an elongation to break of 1000%.[16] Incorporation of different PHA monomeric units, such as 3-hydroxyhexanoate (3HHx), 4-hydroxybutyrate (4HB), 3-hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD), and 3-hydroxydodecanoate (3HDD), with 3-hydoxybutyrate (3HB) results in copolymers with varying material properties with numerous applications as packaging materials, textiles, plastics, fuel additives, medical implant materials, and drug delivery carriers.[31] Compared to polylactic acid (PLA) which is a popular and commercially available renewable and biodegradable polymer,[34] diverse combinations of PHA monomeric subunits offer a wide range of material properties as compared to PLA homopolymers.

It is feasible to manipulate material properties of PHAs by changing the mole fractions of the co-monomer in the copolymers. For instance, properties of the P(3HB-*co*-3HV) copolymers vary when the mole fraction of 3HV in the copolymers changes. As shown in Table II, when the mole fraction of 3HV repeating units increased from 0% (the homopolymer P(3HB)) to 25%, the melting temperature (T_m) and glass transition temperature (T_g) gradually decreased from 179°C to 137°C and from 10°C to −6°C, respectively. The P(3HB-*co*-3HV) copolymers also became more flexible (as indicated by a decrease in the Young's Modulus) and tougher (as indicated by the increase in impact strength) as the 3HV mole fraction increased (Table II).

The copolymer P(3HB-*co*-3HV) is unique among the PHA family of copolymers in that the size and structure of 3HB and 3HV monomers are similar. This similarity allows 3HB and 3HV to participate in a co-crystallization process, in which 3HV could be incorporated into the 3HB crystal lattice and vice-versa. This phenomenon is termed isodimorphism.[35,36] As a result, the melting temperatures of the P(3HB-*co*-3HV) copolymers decrease to a minimum point as the ratio of 3HV to 3HB repeating units increases and after this minimum melting temperature is reached, increases as the 3HV mole fraction further increases. Therefore, the isodimorphic phenomenon and the transition from the 3HB crystal lattice to the 3HV crystal lattice typically exhibits a V-shaped pattern.[35,36] PHA copolymers with lower melting temperatures have an important advantage for industrial applications that require melt processing at lower temperatures.

PHA polymers display a rather slow crystallization process due to high purity and limited heterogeneous nuclei,[37] which results in a longer manufacturing process time and less efficient industrial fabrication cycle for finished products. This has led to studies on the nucleation behavior of P(3HB) homopolymer and the P(3HB-*co*-3HV) copolymers.[37,38] The nucleation density of pure P(3HB) is often too low for efficient initiation of crystallization. The limited nuclei formed in pure P(3HB) leads to low numbers of spherulites so that the size of each P(3HB) spherulite is relatively large. The large size of these spherulites results in the relatively brittle nature of P(3HB) and makes the homopolymer subject to cracking.[39,40] P(3HB-*co*-3HV) also exhibited a slow crystallization behavior, which resulted in the films made from the copolymer with high 3HV mole fraction to remain tacky after cooling from the melting temperature.[41] A number of external nucleating agents, such as orotic acid,[42] boron nitride,[38] α-cyclodextrin,[43] talc,[38] saccharin and phthalimide,[44] have been studied to increase the crystallization rate of PHA polymers. These nucleating agents are also capable of increasing the number of spherulites. Inclusion of nucleating agents resulted in increased numbers of nuclei which led to the formation of spherulites of relatively small size, which resulted in improved material properties.[39] Nucleating agents should be considered as a supplement during hot melt processing of PHA biopolymers.

Polyhydroxyalkanoate Applications

PHAs have attracted much attention for their potential use in a variety of industries.[31] Like nylon, PHAs can be processed into fibers in for textiles.[45] PHAs are polyesters which can be easily

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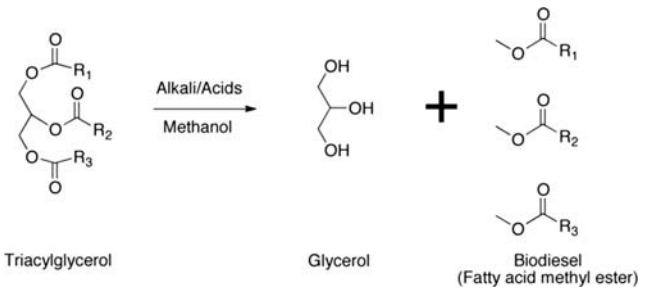

Figure 2. Production of biodiesel and glycerol. Typically, alkali or acid catalysts are combined with a triacylglycerol and a short chain alcohol such as methanol to generate fatty acid methyl esters (biodiesel) and glycerol.

stained and may be used in printing and photographic industry.[46,47] PHAs are biodegradable and biocompatible, therefore, can be developed into implant materials (cardiovascular patches, articular cartilage, bone marrow scaffolds, etc.)[48] and drug controlled-release matrices.[49,50] PHA oligomers has been studied as food supplements to obtain ketone bodies.[51] In addition, PHAs could be hydrolyzed into monomers, which can be converted to hydroxyalkanoate methyl esters for combustion as biofuels.[52] PHA monomers, especially *R*-type 3HB, also demonstrated clinical therapy on Alzheimer's and Parkinson's diseases, and memory improvement.[53–57]

Crude Glycerol from Biodiesel Manufacture

One of the main contributors to the high production costs of PHAs arises from feedstock consumption during the fermentation process. Pure carbon sources (i.e., sugars or fatty acids) as feedstocks for bacterial growth and biopolymer production can account for up to 50% of the entire production costs.[58,59] Identifying alternative feedstocks, which are inexpensive and renewable, would provide a promising solution to substantially reduce production costs of PHA polymers. One promising feedstock for PHA production that is garnering attention is waste glycerol from biodiesel production. Waste glycerol is generated in a large quantity as a major byproduct from biodiesel manufacturing. Although glycerol has been widely used in cosmetics, food, and pharmaceutical industry, it is expensive to refine crude glycerol to high purity for commercial applications.[60] Therefore, bacterial fermentation could be an economically viable route to utilize low-value waste glycerol and produce value-added bioproducts.

Glycerol is recognized as one of the top 12 building block chemicals from biomass by the US Department of Energy in 2004.[61] Biodiesel is traditionally produced from vegetable oils or animal fats through transesterification with methanol or ethanol. This process converts triacylglycerol and methanol into glycerol and fatty acid methyl esters (namely biodiesel) using alkali or acid catalysts (Figure 2). The amount of crude glycerol produced from this transesterification reaction accounts for approximately 10% of the final weight of biodiesel.[62] As biodiesel production has increased dramatically from 500,000 gallons in 1999 to 967 million gallons in 2011 in US,[5,63] crude glycerol generated from biodiesel manufacture has also been produced proportionally in large quantities.

The chemical composition of crude glycerol mainly varies with the type of catalysts, the transesterification efficiency, recovery efficiency of biodiesel, methanol and catalysts, and impurities from feedstocks.[64] Hansen et al.[65] studied the chemical compositions of 11 crude glycerol samples from different biodiesel producers and found that glycerol content ranged from 38 to 96%, with more than 14% methanol in some samples and up to 29% ash. Thompson and He[66] found that crude glycerol obtained from different oil seed (mustard, rapeseed, canola, crambe, soybean and waste cooking oil) stocks varied between 63 and 77%, accompanied with 23–38% methanol. Based on various industrial technologies for biodiesel production and recovery processes, it is reasonable to deduce that the chemical compositions in crude glycerol vary for different sources of waste glycerol. All of these variations should be considered for bioconversion of crude glycerol to value-added products using microorganisms as biocatalysts.

One major benefit of biodiesel boom resulted in market price of glycerol plummeting to approximately US $ 0.025–0.05/lb.[67,68] Conversion of crude glycerol into higher-value products improves the economic viability of biofuel industry by coupling the production of value-added products to the production of biodiesel and eliminating the cost of treatment for crude glycerol disposal. Fermentation of glycerol has been reported to produce many value-added bioproducts, such as 1,3-propanediol, dihydroxyacetone, succinic acid, propionic acid, ethanol,[69,70] butanol,[71,72] hydrogen, citric acid, lactic acid, glyceric acid, biosurfactants, pigments, and PHAs.[73,74] Among these bioproducts, 1,3-propanediol,[75] succinic acid,[76] lactic acid,[77–79] and glyceric acid[80,81] have been used as biomonomers for production of plastics, i.e. polyesters, polyethers, and polyurethanes, through chemical synthesis.[82] Compared to these plastics with biological origins and chemical catalysis, PHAs are a class of completely naturally occurring bioplastics.

PRODUCTION AND CHARACTERIZATION OF POLYHYDROXYALKANOATES FROM GLYCEROL

Biosynthesis and Characterization of Polyhydroxybutyrate from Glycerol

Biosynthesis of Polyhydroxybutyrate from Glycerol. A large number of microorganisms can uptake glycerol from ambient environment and metabolize it into building blocks for microbial growth and development. In native PHA producing bacteria, when abundant carbon sources (e.g., glycerol, sugars, fatty acids) are present and at least one of other nutrient is (e.g., nitrogen, phosphate, oxygen) depleted, PHAs are produced as carbon and energy reserves. Under such conditions, glycerol is generally converted into P(3HB) by various native PHA producing bacteria.[5,83–87] As shown in Figure 3, glycerol is converted to glyceraldehyde-3-phosphate (GAP) by three enzymes, glycerol kinase (GlpK), glycerol-3-phosphate dehydrogenase (GlpD), and triosephosphate isomerase (TPI). GAP is an intermediate in the glycolysis pathway and eventually metabolized to pyruvate. The pyruvate dehydrogenase complex contributes to transforming pyruvate into acetyl-CoA by a process called pyruvate decarboxylation. Microbial biosynthesis of P(3HB) starts with condensation of two molecules of acetyl-CoA by β-ketothiolase (PhaA)

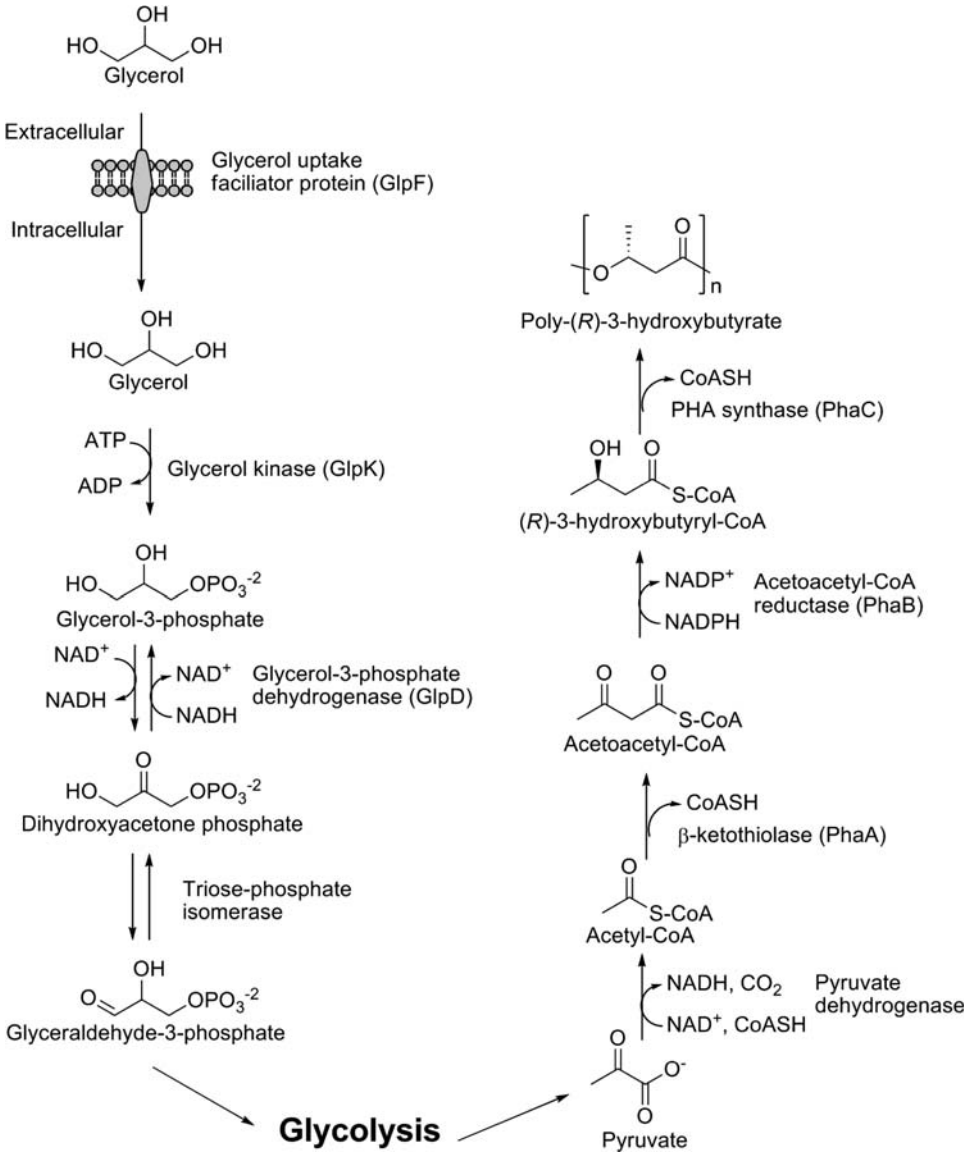

Figure 3. Proposed pathway for metabolism of glycerol and short-chain-length PHA production. Glycerol enters the cell via the glycerol facilitator protein (GlpF) and is phosphorylated by GlpK to produce glycerol-3-phosphate. Glycerol-3-phosphate is reduced by GlpD to produce dihydroxyacetone phosphate which enters into the glycolytic pathway to be converted to pyruvate, and eventually acetyl-CoA. Two molecules of acetyl-CoA are condensed by the *β*-ketothiolase (PhaA) enzyme to produce acetoacetyl-CoA. This molecule is reduced by acetoacetyl-CoA reductase (PhaB) to produce the substrate (*R*)-3-hydroxybutyryl-CoA, which is polymerized by the PHA synthase (PhaC).

into acetoacetyl-CoA, which is subsequently reduced to (*R*)-3-hydroxybutyryl-CoA by acetoacetyl-CoA reductase (PhaB). The last step of PHA biosynthesis is dependent on PHA synthase (PhaC) to polymerize 3-hydroxybutyryl-CoA moieties to P(3HB).[73,88,89]

The P(3HB) homopolymer is the most extensively studied PHA and has been produced by several species of bacteria using glycerol as a carbon source.[5,90–92] Teeka et al.[92] reported that P(3HB) accumulated to 45% of the cell dry weight (CDW) with biomass yield at 3.5 g/L in 72 h. Shrivastav et al.[93] isolated and identified two bacterial strains from soil and marine environments for producing P(3HB) from *Jatropha* biodiesel byproduct as a carbon source and found that P(3HB) was accumulated up to 76% of CDW in 4.0 g/L biomass in shake flasks. Compared to these shake flask experiments, fed-batch fermentation technology has been shown to dramatically increase cell density, up to 82.5 g/L CDW (Table III), using waste glycerol as a feedstock. In addition, P(3HB) production increased to 51 g/L using fed-batch reactors.[84] Improved fermentation processes and optimized fermentation conditions have the potential to be scaled-up for PHA industry to commercialize these biodegradable plastics with comparable market price while using renewable and inexpensive feedstocks, e.g., crude glycerol.[31]

Impurities found in crude glycerol, such as methanol and salts, can dramatically effect bacterial growth and PHA yield.[94] Mothes et al.[83] found that NaCl at concentration of 5.5% in the

Table III. Survey on Bioconversion of Glycerol to P3HB Using Various Bacteria

Strains	Glycerol purity	CDW (g/L)	PHB yield	References
Novosphingobium sp.	50%	3.5	45%	92
Halomonas hydrothermalis	95%	4.0	76%	93
Bacillus sonorensis	95%	2.8	72%	93
Pseudomonas oleovorans	≥99%	3.0	38%	87
Pseudomonas oleovorans	77%	2.5	32%	87
Pseudomonas oleovorans	47%	2.8	37%	87
Cupriavidus necator	88%	82.5	62%	84
Cupriavidus necator	93%	68.8	38%	84
Halomonas sp.	60%	1.6	39%	86
Halomonas sp.	≥99%	2.3	45%	86
Paracoccus denitrificans	85%	25.0	40%	83
Burkholderia cepacia	33%	5.0	82%	88
Burkholderia cepacia	85%	23.6	31%	5

crude glycerol resulted in a lower PHA yield due to osmoregulation. Ashby et al.[87] reported that the usage of untreated crude glycerol with 40% methanol affected microbial growth and P(3HB) production. Therefore, optimization of biodiesel production process for efficient catalysis and the following methanol recycling will not only reduce the production cost for biodiesel manufacturing, but also help subsequent utilization of crude glycerol during fermentation processes.

However, some impurities, such as free fatty acids and alkyl esters, could act as complementary carbon sources and be utilized by bacteria to enhance their growth. Ashby et al.[87] studied the compositions of crude glycerol and found that *Pseudomonas oleovorans* could use fatty acids and unrecovered alkyl esters as additional carbon sources for bacterial growth. *Burkholderia* sp. USM was identified to be capable of converting palm oil, fatty acids, and glycerol byproducts into P(3HB).[95] Zhu[88] used *Burkholderia cepacia* to grow on tall oil fatty acids, consisting of 52% oleic acid and 45% linoleic acid, for PHA production. The maximum CDW and P(3HB) yield reached 3.8 g/L and 50% of CDW, respectively, under these conditions. *B. cepacia* grown on crude glycerol generated by a small batch biodiesel processor at SUNY-ESF containing free fatty acids as a carbon source for comparable growth, yet much lower glycerol consumption, indicating that *B. cepacia* metabolized fatty acids, along with glycerol, for bacterial growth and PHA production.[88] Teeka et al.[96] isolated a previously unidentified strain, AIK7, which also demonstrated enhanced bacterial growth, even improved PHA yield, when using waste glycerol containing free fatty acid instead of pure glycerol.

Zhu et al.[5] studied the effects of glycerol concentration on P(3HB) production. High concentrations of glycerol (≥3%) in fermentation broth exhibited obvious inhibitory effects on bacterial growth due to osmotic stress on the cells. Maintenance of proper concentration of glycerol in the medium (1–3 wt %) should be considered for high cell density and PHA yields during fermentation of crude glycerol.[5,10,91]

The efficiency of converting glycerol to PHA varies due to the substrate concentration in the medium. Ibrahim and Steinbüchel[85] reported that glycerol concentration at 10 g/L gave the highest product yield at 0.31 g P(3HB)/g glycerol by *Zobellella denitrificans* MW1. Higher glycerol concentrations of 20, 30, and 50 g/L in the media resulted in the low product yield at 0.21, 0.12, and 0.03 g P(3HB)/g glycerol, respectively. The same group[97] studied the fed-batch fermentation for P(3HB) production. The product yield could be increased from 0.10 g P(3HB)/g glycerol to 0.25 P(3HB)/g glycerol after optimization of the fed-batch process. Cavalheiro et al.[84] used pure glycerol and waste glycerol as carbon sources in a two-stage fermentation by *Cupriavidus necator* (*Ralstonia eutropha*), and found that the product yield reached 0.36 and 0.34 g P(3HB)/g glycerol from pure and waste glycerol, respectively. Mixed microbial communities obtained from activated sludge in a municipal wastewater treatment plant could also efficiently utilize crude glycerol and the product yield reached 0.40 g PHA/g glycerol, which was comparable to the conversion efficiency of those using fatty acids as carbon sources.[98]

Characterization of Polyhydroxybutyrate from Glycerol. The P3HB homopolymer has been produced by many microorganisms utilizing carbon sources such as sugars and fatty acids. However, it is noteworthy that conversion of glycerol to P(3HB) results in polymers with relatively low molecular mass compared to P(3HB) polymers produced from sugars. Molecular mass analysis by gel permeation chromatography (GPC) showed significant decreases for PHA polymers isolated from strains utilizing glycerol as a carbon source compared to xylose.[5] The number average molecular weights (M_n) of P(3HB) produced from xylose and glycerol were 468 kDa and 175 kDa, respectively, indicating that the size of P(3HB) polymers produced from xylose was approximately three-fold larger than the size of P(3HB) polymers produced from glycerol. Several other reports have also shown that P(3HB) polymers produced from glycerol exhibited lower molecular mass than P(3HB) polymers produced from sugars.[90,91,99] ^{1}H-NMR detected that P(3HB) polymers produced from glycerol feedstocks were end capped with glycerol molecules through covalent esterification. Glycerol acts as a chain transfer agent resulting in early termination of P(3HB) polymerization, which led to low molecular mass of P(3HB).[5,87,91] High concentrations of glycerol in bacterial growth media inhibited bacterial growth and also resulted in lower M_n and M_w (number-average molecular weight and weight-average molecular weight, respectively). When concentrations of glycerol were increased from 3% to 9%, both M_n and M_w decreased gradually from 173 kDa and 304 kDa to 87 kDa and 162 kDa, respectively.[5] The polydispersity indices (PDIs) of all P(3HB) samples were between 1.9 and 2.1 in this research. However, P(3HB) produced from glycerol or xylose did not show any significant differences in thermal properties (T_m, T_g, and T_{decomp}) compared to P(3HB) polymers produced from glycerol.[5]

It has been proposed that PHA synthesis occurs within the active site of PhaC polymerase where two thiol groups are responsible for locating the PHA monomer unit and the other holding onto the propagating chain. The type of polymerization itself was also assumed to be a chain transfer polymerization.[100]

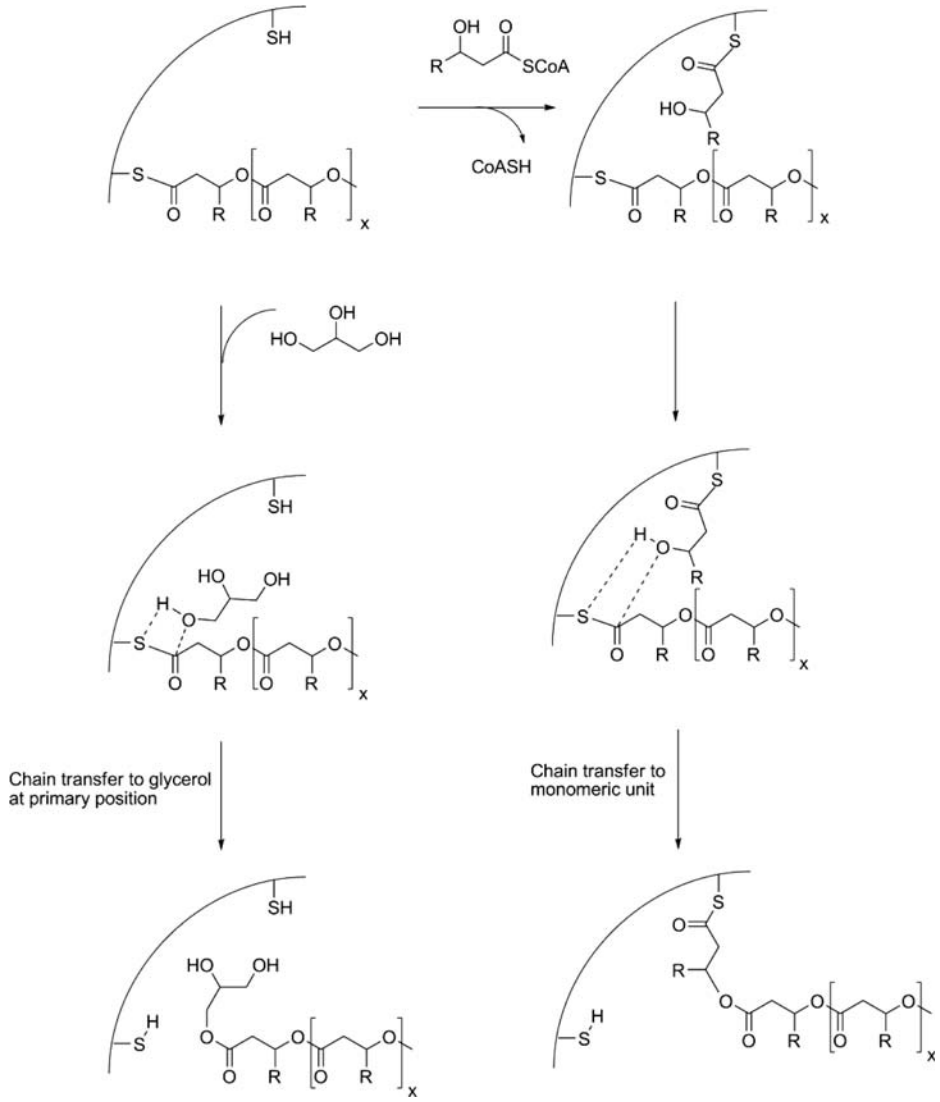

Figure 4. Proposed mechanism for chain transfer termination in PHA synthase enzymes by glycerol. A: If glycerol is present, it can enter the active site of the PHA synthase and prematurely terminate the extention of the PHA polymer, resulting in a glycerol end capped polymer. B: Under circumstances where chain transfer agents are not present, polymerization of PHA can proceed via transfer of the substrate (*R*-3-hydroxyacyl-CoA) and product between two active site cysteine residues.

Without any exogenous factors, high molecular weight PHAs are produced. With the addition of exogenous chain transfer agents with hydroxyl end groups such as glycerol and low molecular weight polyethylene glycol derivatives (PEG), PHA polymerization can be terminated early.[101,102] Since glycerol contains three hydroxyl groups per molecule and is much smaller than the PEG derivatives, it makes a good candidate as a chain transfer agent. By varying the concentration of glycerol in the starting media the molecular weight of PHAs can be controlled, due to the prevalence of glycerol terminating the PHA synthesis. Since glycerol is a small molecule it can find itself within the active site of the polymerase and covalently bond with the propagating chain.[5,87] A proposed mechanism is shown in Figure 4.

In addition to the effects of glycerol on molecular mass of P(3HB) polymers, residual methanol in the waste glycerol from the biodiesel process can also affect the size of P(3HB). Ashby et al.[87] reported that M_n (31 kDa) of P(3HB) produced from waste glycerol (47% glycerol and 40% methanol) was 10-fold lower than the M_n (314 kDa) of P(3HB) produced from pure glycerol as a carbon source. One-dimensional ^{1}H-NMR and two-dimensional DOSY (diffusion ordered spectroscopy) identified that the P(3HB) homopolymer was end-capped not only with glycerol, but also with methanol, which formed an ester linkage with P(3HB). The amount of methoxy group was found to be 100 times higher from the P(3HB) sample using waste glycerol than the P(3HB) sample using pure glycerol. Also, the PDI (M_w/M_n) of P(3HB) from pure glycerol and waste glycerol were 1.66 and 2.77, respectively. The higher PDI of P(3HB) produced from waste glycerol containing 40% methanol revealed that methanol in the crude glycerol may exacerbate premature chain termination by itself, which could serve as another chain termination agent. Previous research also showed poly(ethylene glycol) (PEG) could end

cap P(3HB).[103,104] In a word, addition of chain termination agents, such as glycerol, PEG, and methanol, results in the production of P(3HB) polymers with lower molecular mass during biosynthesis, and thus, needs to be taken into account for PHA production when using waste glycerol as a carbon source in fermentation process.

Biosynthesis and Characterization of Polyhydroxypropionate-Related Biopolymers from Glycerol

Andreeßen et al.[105] first reported in 2010 recombinant *Escherichia coli*, containing glycerol dehydratases (DhaB1, DhaB2), propionaldehyde dehydrogenase (PduP), and PHA synthase (PhaC1), produced homopolymer P(3HP) when using pure or waste glycerol as a sole carbon source. This new biosynthetic pathway converted glycerol to 3-hydroxypropionaldehyde and then to 3-hydroxypropionyl-CoA, which was finally polymerized to P(3HP). P(3HP) was accumulated to 12% (wt/wt) of the dry cell mass in this report.

The P(3HP-*co*-3HB) copolymer has been produced by several native and engineered strains using various carbon sources.[106–110] Fukui et al.[106] used engineered *C. necator* (more commonly known as *R. eutropha*), incorporating two enzymes, malonyl-CoA reductase and 3HP-CoA synthetase, for generation of 3-hydroxypropionyl-CoA from the building block acetyl-CoA, which is also the source for 3-hydroxybutyryl-CoA (Figure 3). The PHA synthase of *C. necator* polymerizes these two monomeric precursors into the copolymer P(3HP-*co*-3HB). Because this engineered *C. nector* used unrelated carbon sources (sugars or aliphatic acids) for copolymer production, it is deduced that glycerol may be used as a carbon source to generate the intermediate acetyl-CoA for production of P(3HP-*co*-3HB).

When the mole percentage of 3HP in the P(3HP-*co*-3HB) copolymer increased to approximately 65%, both crystallinity and T_m decreased from 62.1% and 177°C of the homopolymer P(3HB) to around 10% and 50°C, respectively.[111] Further increasing the mol % of 3HP in this copolymer resulted in elevated crystallinity and T_m, up to 61.7% and 77°C of the homopolymer P(3HP).[112]

Biosynthesis and Characterization of Polyhydroxyalkanoate Copolymers from Glycerol

Biosynthesis of P(3HB-*co*-3HV). As has been discussed, glycerol can be metabolized for bacterial growth, and under unbalanced nutritional conditions, be converted into the homopolymer P(3HB) by many microorganisms. However, to date, there have been no reports of PHA copolymer production in native PHA producing bacterial strains when glycerol is used to be sole carbon source.

Propionyl-CoA is known as the precursor for P(3HV) production. However, glycerol is generally metabolized into acetyl-CoA for P(3HB) production. Aldor et al.[113] engineered a *Salmonella enterica* strain, incorporating a novel propionyl-CoA biosynthesis pathway from *E. coli* and a PHA synthesis pathway from *Acinetobacter*, for production of P(3HB-*co*-3HV) from glycerol. The authors expressed two genes, *sbm* and *ygfG*, for (2*R*)-methylmalonyl-CoA mutase and (2*R*)-methylmalonyl-CoA decarboxylase from *E. coli*, respectively. These two enzymes enable conversion of succinyl-CoA derived from TCA (tricarboxylic acid) cycle to propionyl-CoA. In addition, the *prpC* (coding for 2-methylcitric acid synthase) mutation eliminated competition for propionyl-CoA in 2-methylcitric acid cycle and shunted this precursor for P3HV production. This recombinant *S. enterica* synthesized P(3HB-*co*-3HV) copolymer with up to 31 mol % 3HV when the cells were cultivated on glycerol as sole carbon source.

It is more common, however, that the P(3HB-*co*-3HV) copolymer is produced when using co-feedstocks, in which one carbon source, e.g., glycerol, is responsible to produce the 3HB monomer and the other feedstock acts as a supplementary donor for biosynthesis of the 3HV moiety. PHA copolymer production is accomplished when growth medium containing glycerol is supplemented with other structure-related carbon sources. The P(3HB-*co*-3HV) copolymers have been produced in many microorganisms when using glycerol and odd-number aliphatic organic acids (propionic acid, valeric acid, heptanoic acid, nonanoic acid, etc.) as carbon sources.[114–117] These odd-number acids could be converted into the precursor of 3HV monomer through β-oxidation or fatty acid *de novo* biosynthesis pathway for P(3HV) biosynthesis.

3HV mole fraction in the P(3HB-*co*-3HV) copolymer could be regulated based on the amount of 3HV-donor compounds in the medium. Zhu et al.[10] investigated the production of P(3HB-*co*-3HV) copolymers using *B. cepacia* using glycerol and levulinic acid (γ-ketovaleric acid) as co-substrates for production. Continuous feeding of levulinic acid into the medium resulted in the production of P(3HB-*co*-3HV) copolymers where the 3HV mole fraction increased from 5.6% to 32.6%. All copolymers produced under these growth conditions exhibited lower molecular masses as compared to the P(3HB) homopolymer. Ashby et al.[118] studied the effects of gradually increasing concentrations of levulinic acid on the 3HV fraction in the copolymer. Only P(3HB) homopolymer was produced when using glycerol as a sole carbon source. 3HV mole fraction gradually increased from 0 to 78%, eventually to 100% 3HV (P(3HV) homopolymer) when only levulinic acid was fed.

Characterization of P(3HB-*co*-3HV). The P(3HB-*co*-3HV) copolymers have attracted more attention than the P(3HB) homopolymer due to their better physical and mechanical properties, e.g. lower T_m and higher flexiblity.[88,118] One interesting characteristic of the P(3HB-*co*-3HV) copolymers with increasing 3HV mole fraction is the V-shaped curve for melting temperatures. When 3HV mole fraction increased, T_m started to decrease to a minimum point and then increased. This is a typical isodimorphic phenomenon for the copolymers P(3HB-*co*-3HV), which are statistically random copolymers containing closely related monomeric units 3HB and 3HV.[10,35,119] Both monomers can crystallize and one of the monomeric subunits can be included in the crystal lattice of the other and vice-versa.

Bluhm et al.[35] found that T_m started to decrease from 179°C to the minimum point, 84°C when the 3HV mole fraction increased from zero to approximately 30 mol % in the P(3HB-*co*-3HV) copolymer. Subsequently, after reaching this low melting point, the T_m increased when 3HV mol fraction increased beyond 30 mol %. Instead of bacterial produced P(3HB-*co*-

3HV), Bloembergen et al.[119] demonstrated that synthetic P(3HB-*co*-3HV) copolymers displayed a similar V-shaped pattern for melting temperature as described previously by Bluhm et al. Doi[26] also observed this isodimorphic phenomenon for the P(3HB-*co*-3HV) copolymers, and the minimum T_m at 75°C occurred at approximately 40 mol % of 3HV in the copolymer.

P(3HB-*co*-3HV) produced by *B. cepacia* using xylose and levulinic acid as carbon sources was reported to exhibit a similar isodimorphic behavior; however, the minimum threshold of T_m was close to 154°C at 25 mol % 3HV in the copolymer.[120] Interestingly, when using SUNY-ESF hemicellulosic hydrolysate (containing xylose, glucose and other trace amounts of sugars) instead of pure xylose for production of the P(3HB-*co*-3HV) copolymers by *B. cepacia*, the minimum T_m dropped to 81°C at 45 mol % 3HV.[121]

P(3HB-*co*-3HV) produced by *B. cepacia* using glycerol and levulinic acid as co-substrates showed a similar isodimorphic behavior and the minimum T_m occurred at 168°C for the P(3HB-*co*-3HV) copolymer comprised of 20 mol % 3HV.[10] Other two reports demonstrated that the P(3HB-*co*-3HV) copolymers displayed minimum T_m of 116°C at 20 mol % 3HV[122] and 131°C at 22 mol % 3HV,[123] respectively.

There is no obvious evidence as to which factor(s) of these P(3HB-*co*-3HV) copolymers may result in the differences for the minimum T_ms and their corresponding 3HV mole fractions. There is no direct linear relationship between T_m and molecular mass, based on melting temperatures of the copolymers with high molecular mass (average M_v = 687 kDa) produced from xylose and levulinic acid[121] and with low molecular mass produced from glycerol and levulinic acid (average M_w = 115 kDa).[88] Due to varying thermal history, aging process for these polymers and other potential differential background, it is reasonable to perform further research to find out the implication of the difference for these P(3HB-*co*-3HV) copolymers.

Biosynthesis of Other PHB-Related scl-Copolymers. Cavalheiro et al.[124] reported that *C. necator* could synthesize the copolymer P(3HB-*co*-4HB) (Figure 1) and terpolymer P(3HB-*co*-4HB-*co*-3HV) when using waste glycerol, γ-butyrolactone, and/or propionic acid as carbon sources. γ-Butyrolactone offers the precursor for 4HB monomer in this terpolymer. When glycerol and γ-butyrolactone were co-fed during fermentation, the maximum 4HB mole fraction reached at 21.5% in the P(3HB-*co*-4HB) copolymer. Once propionic acid was supplemented with glycerol and γ-butyrolactone, terpolymer P(3HB-*co*-4HB-*co*-3HV) was produced and the mole fractions of 4HB and 3HV varied from 11.4 mol % to 43.6 mol % and from 5.6 mol % to 9.8 mol %, respectively. By controlling the types of co-substrates and amounts of these substrates, the compositions and mole fraction of each monomer in the copolymers could be regulated at will. Meanwhile, their physical and mechanical properties could be controlled accordingly.

Biosynthesis and Modification of mcl-PHAs from Glycerol

Several native *Pseudomonas* species have been reported to produce mcl-PHAs solely using glycerol as a carbon source. Wang and Nomura[89] tested different carbon sources for mcl-PHA accumulation in *Pseudomonas putida* KT 2440. When using the fatty acid dodecanoate (lauric acid) as a carbon source, mcl-PHAs comprised of four monomers, 7 mol % 3HHx, 60 mol % 3HO, 23 mol % 3HD, and 8 mol % 3HDD were produced. However, switching to citrate as a carbon source, *P. putida* produced PHA copolymers with only two monomeric repeating units, 54 mol % 3HD and 46 mol % 3HDD. When *P. putida* was grown on glycerol as a sole carbon source, it synthesized mcl-PHAs composed of 24 mol % 3HO, 57 mol % 3HD, and 19 mol % 3HDD. Wang et al.[125] also engineered an *E. coli* strain with PHA biosynthetic pathway illustrating the function of a cluster of genes essential for production of mcl-PHAs from unrelated carbon sources, such as glucose and glycerol. The two major components of these mcl-PHAs produced from engineered *E. coli* consisted of 33–39 mol % 3HO and 57–65 mol % 3HD.

Ashby et al.[91] reported that *Pseudomonas corrugata* 388 grew on glycerol and produced mcl-PHAs consisting primarily of saturated 3HO ($C_{8:0}$; 13 mol %) and 3HD ($C_{10:0}$; 44 mol %), and unsaturated 3-hydroxydodecenoic acid ($C_{12:1}$; 31 mol %). The unsaturated side chains containing double bonds offer potentials for functionalizing these mcl-PHAs with improved properties or extended applications through chemical modification, such as chlorination,[126,127] crosslinking,[128–131] carboxylation,[131,132] hydroxylation,[133,134] and epoxidation.[135–141] Such modifications for the pedant groups make these mcl-PHAs functional with unusual physicochemical and mechanical properties.[142,143] Chlorination switched mcl-PHAs from sticky to hard, brittle and crystalline, depending on chlorine contents.[126] Crosslinking by gamma irradiation made these mcl-PHAs with higher tensile strength.[128] PHAs are well known to be hydrophobic, however, carboxylic (25%)[144,145] and hydroxylated (40–60%)[133] groups of mcl-PHAs were soluble in polar solvents, indicating that hydrophilicity of these mcl-PHAs is considerably enhanced. The epoxidated mcl-PHAs exhibited increased Young's modulus and tensile strength.[141]

The mcl-PHA with 2.7 mol % 3HHx, 31.3 mol % 3HO, 62.1 mol % 3HD, and 3.9 mol % 3HDD produced by *P. putida* using glycerol as the carbon source exhibited different thermal properties compared to scl-PHAs. This mcl-PHA has much lower T_m at 39.7°C, T_g at −50.7°C, and T_c at 11.0°C; however, T_{decomp} at 233.5°C was comparable to those of scl-PHAs. In addition, M_w and PDI of this mcl-PHA were 124 kDa and 1.9, respectively.[146]

Biosynthesis and Characterization of P(HB-*co*-mcl-PHAs) from Glycerol

Wang[146] engineered an *E. coli* strain with three genes *phaA*, *phaB*, and *phaC* for P(3HB) production (Figure 3) and two genes *phaG*, *alkK* for mcl-PHA production (Figure 5). These additions allow the strain to produce PHA copolymers from unrelated carbon sources like glucose and glycerol. Therefore, this *E. coli* strain showed the capacity to produce the P(3HB-*co*-mcl-PHAs) copolymer from glycerol. CDW and PHA% of CDW could reach up to 6.7 g/L and 60 wt %, respectively. Two copolymers were isolated and determined as P(3HB-*co*-2.7 mol % 3HO-*co*-2.5 mol % 3HD) and P(3HB-*co*-1.4 mol % 3HO-*co*-1.7 mol % 3HD-*co*-0.1 mol % 3HDD), which showed T_m, T_c

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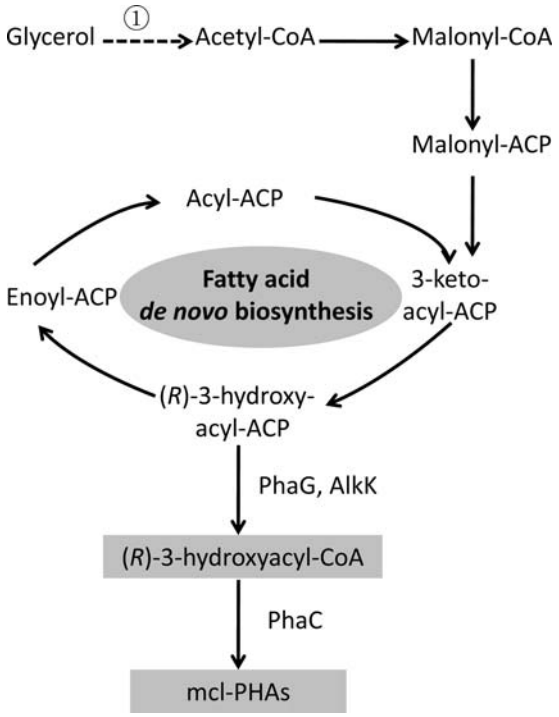

Figure 5. Pathway for mcl-PHAs biosynthesis from glycerol. The initial steps for the production of acetyl-CoA are the same as depicted in Figure 3. However, mcl-PHA production would proceed via fatty acid biosynthesis in the cell such that acetyl-CoA would be converted to malonyl-CoA and then malonyl-ACP to enter the fatty acid biosynthetic pathway. The (*R*)-3-hydroxyacyl-ACP intermediate could be converted to the (*R*)-3-hydroxyacyl-CoA substrate for polymerization by the PhaG and AlkK enzymes.

and T_{decomp} at 167°C, 176°C, and 78°C and 86°C, 219°C, and 234°C, respectively.

Incorporation of mcl-PHAs with P3HB for the P(3HB-*co*-mcl-PHAs) copolymer greatly enhanced mechanical properties of PHAs. P(3HB-*co*-14.7 mol % 3HO) and P(3HB-*co*-31.3 mol % 3HO) showed Young's modulus and elongation to break at 31.5 MPa and 110%, 12.0 MPa and 230%, respectively. Compared to P(3HB) at 185 MPa and 6.0%, the P(3HB-*co*-mcl-PHAs) copolymer demonstrated much higher flexibility.[147]

CONCLUSION

The application of fermentation technologies as an alternative solution for disposal of crude glycerol demonstrates a potentially significant advantage for biodiesel manufacturers to efficiently dispose of waste glycerol and gain profitable compensation from the value-added biodegradable plastics. Crude glycerol has been acknowledged as renewable and inexpensive feedstocks for bacterial growth and PHA production. It is promising that the production costs of PHA in the near future could be reduced significantly when using crude glycerol as a carbon source. The pool of PHAs, the P(3HB) homopolymer, and various copolymers, produced from glycerol demonstrated varying physical and mechanical properties, which offer diverse applications of PHAs in a number of industries.

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