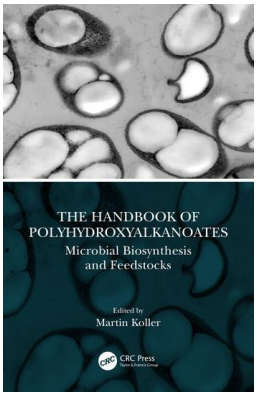


The Handbook of Polyhydroxyalkanoates Microbial Biosynthesis and Feedstocks

Martin Koller

Production and Modification of PHA Polymers Produced from Long-Chain Fatty Acids

Publication details
https://www.routledgehandbooks.com/doi/10.1201/9780429296611-13
Chris Dartiailh, Nazim Cicek, John L. Sorensen, David B. Levin
Published online on: 06 Nov 2020

11 Production and Modification of PHA Polymers Produced from Long-Chain Fatty Acids

Christopher Dartiailh, Nazim Cicek, John L. Sorensen, and David B. Levin

CONTENTS

11.1 INTRODUCTION

Polyhydroxyalkanoates (PHA) are microbial storage polyesters produced to sequester carbon in response to nutrient-limiting environments. Upon depletion of an external carbon source, the polymer may later be consumed as a source of energy (ATP) and reducing power (NADH/NADPH). Some PHA have characteristics similar to a range of petroleum-derived polymers [1] and can be produced using renewable resources. PHA are biodegradable due to a breadth of microorganisms that produce external PHA depolymerases or non-specific lipases, which result in environmental decomposition [2–6]. Another important characteristic of PHA is their biocompatibility, making them suitable for medical applications [7]. Taken together, PHA provide sustainable alternatives for current plastic materials. Formed by the enzymatic (*PhaC*-encoded) condensation of hydroxyalkanoate-CoA monomers, PHA polymers are polyesters consisting of a linear backbone, minimally composed of 3-carbon repeating units, with side chains of varying lengths [8]. Short-chain-length (*scl*-) PHA range in length from three to five carbons (C3–C5), and medium-chain-length (*mcl*-)PHA contain monomers from six to fourteen carbons (C6–C14) [9,10].

Long-chain-length (*lcl*-)PHA are longer than C14 but have only been produced in trace quantities during *mcl*-PHA production [11,12].

The production cost of PHA remains a limitation to broader commercial applications. While research has focused on reducing costs in all facets of the production, extraction, and purification processes, the costs and effects of various substrates have drawn the most attention. The poor substrate yield of *in vivo* systems drives up the associated production cost. Although the estimated substrate cost for *scl*-PHA production was reduced to 22% of the total cost when using methane at thermophilic temperatures [13], the estimates for elastomeric *mcl*-PHA production can exceed 50% of the total cost [14–16].

Long-chain fatty acids (LCFAs) are the main components of vegetable oils and a promising source of cheap, renewable substrates for PHA production [17]. LCFAs are highly reduced and provide ATP as well as reducing equivalents to the cell when metabolized. Worldwide vegetable oil production in 2012 eclipsed 150 million tons [18], and food processing has been estimated to produce over one million tons of waste vegetable oil [19]. LCFAs can be used as inexpensive substrates for *scl*-PHA production with high yield and intracellular content. However, the polymers remain saturated and do not contain any functional moieties [20,21]. Saturated PHA can be modified through the reaction of the polymer ends or free radical mechanisms [10], regardless of the substrate used for their synthesis. The literature has emphasized the functionalization of *mcl*-PHA from LCFAs, as the unsaturated moieties of some LCFAs can be retained to obtain olefinic *mcl*-PHA. The theoretical yield of *mcl*-PHA from LCFAs is relatively high. The estimated theoretical substrate yield from the LCFAs of canola oil was 0.72 g *mcl*-PHA per g substrate [22].

LCFAs can be composed of saturated fatty acids (SFAs) and unsaturated fatty acids (USFAs). The USFAs can further be classified as monounsaturated (MUFAs) or polyunsaturated (PUFAs) [23,24]. The fatty acid composition of vegetable oils varies with crops and cultivars [25,26]. However, since fatty acids are largely incorporated into *mcl*-PHA from fatty acid degradation while conserving the olefin position [27,28], they can be classified by their unsaturation level for the sake of predicting *mcl*-PHA composition. Vegetable oils can be highly comprised of SFAs (coconut, palm), MUFAs (olive, canola), or PUFAs (soybean, flax) [29]. The predominant monomers in *mcl*-PHA are C8 and C10, and, as a result, low incorporation of olefin moieties occur from predominant Δ9-monounsaturated fatty acids (oleic acid); the ω-3 unsaturation of linolenic acid found in polyunsaturated fatty acids can be expected in all monomers C8 and longer [30].

Mcl-PHA produced from octanoic, nonanoic, or decanoic acids have been described as elastomeric materials similar to polyethylene [31]. The incorporation of vinyl moieties reduces the crystallinity of these polymers, such that they become completely amorphous with sufficient unsaturation [32]. LCFAs cannot be considered a cheap substrate replacement if the polymer has drastically different properties. However, these novel polymers can be reassessed for alternative applications, and olefin moieties provide opportunities for polymer modification.

The objective of PHA production is to produce sustainable and renewable plastic products capable of replacing current petroleum-derived plastics that are nonbiodegradable and a major source of environmental pollution. The trends in *mcl*-PHA

production from LCFAs along with the implications of olefin moieties and their modifications on thermomechanical properties will be reviewed here.

11.2 STRATEGIES FOR PRODUCTION OF *MCL*-PHA

PHA are stored as intracellular granules, and high production rates of polymer require high cell titers. Strategies to improve cell titer include high mixing rates while maintaining high dissolved oxygen (DO) concentrations, often by sparging with pure oxygen or pressurizing the bioreactor [33,34]. The induction of PHA accumulation has been linked to increased intracellular NADH and acetyl-CoA when growth is limited [35]. Nitrogen, phosphate, sulfur, magnesium, and oxygen limitation have all been used to successfully promote PHA synthesis [36,37]. Further improvement of intracellular PHA content has been observed in the dual limitation of nitrogen and oxygen [38]. Contrary to two-phase growth and PHA accumulation models, growth-associated *mcl*-PHA production has been demonstrated at controlled specific growth rates, suggesting the requirement for nutrient limitation is strain- and substrate-dependent [39]. Approaches for reducing PHA production costs focus on maximizing PHA productivity and substrate yield.

11.3 STRATEGIES FOR MAXIMUM VOLUMETRIC PRODUCTIVITY

Stirred-tank bioreactors of various discontinuous or continuous operational configurations have been applied to maximize volumetric productivity [g PHA/(L·h)], thereby improving the cost of PHA production. High biomass titers are required for optimum production rates since PHA are intracellular products, and inhibitory concentrations of medium components limit the productivity of simple batch reactors [40]. Therefore, strategies for fed-batch and continuous PHA production have been developed, which will be briefly summarized here, with a focus on LCFA PHA production, as more comprehensive reviews of PHA bioreactor operations have recently been published [15,36,41,42].

Optimized fed-batch bioreactors have used various feeding regimes to provide the carbon substrate and other nutrients to maximize volumetric productivity, and have provided the highest volumetric productivities [15]. Fed-batch reactors have been more frequently operated using a two-phase feeding regime to first maximize the cell biomass titer before an induction phase for PHA accumulation. A significant challenge in fed-batch processes is determining nutrient delivery to maintain concentrations between limiting and inhibitory levels, as optimized feeding may reduce the impact of other fed-batch challenges (i.e., heat and mass transfer, foaming). Substrates have been delivered for biomass production based on predicted specific growth rates or calculated cumulative substrate consumption using predetermined yield coefficients [43,44]. Alternatively, response-based substrate delivery has been implemented for pH value [37], dissolved oxygen concentration [37,45,46], or carbon dioxide evolution [43,47]. Ultimately, due to the low solubility and diffusional limitations of oxygen into the medium, all these regimes will approach a maximum biomass titer for any reactor configuration due to limited dissolved oxygen [48].

Substrate and nutrient delivery modifications are required for the PHA induction phase as the maximum biomass titer is approached.

Pseudomonas species with growth-associated *mcl*-PHA biosynthesis have been optimized through carbon-limited control of the specific growth rate. In processes using medium-chain fatty acids (MCFAs), such as nonanoic acid, a reduced specific growth rate lowered the oxygen uptake rate such that higher biomass was achieved before the onset of oxygen-limiting conditions when nonanoic acid buildup became toxic [39]. Carbon-limited growth using a quadratic-decaying exponential feed strategy that switched to a linear feed rate, experimentally modeled to optimize growth rate while avoiding oxygen limitation, resulted in the highest reported volumetric productivity of *mcl*-PHA [2.3 g PHA/(L·h)] [44].

High cell density *mcl*-PHA production from LCFAs in fed-batch is limited to a few reports, all of which have distinct growth and PHA production phases. Concomitantly, two fed-batch systems were reported using oleic acid. The highest reported volumetric productivity of *mcl*-PHA from LCFAs was achieved using a combination of oleic acid delivery methods to maintain the maximum specific growth rate, first by monitoring optical density, then by switching to DO control followed by pH control. Following the depletion of phosphate, PHA accumulation rates increased sharply, leading to a final PHA content of 51.4 wt.-% in 141 g/L of biomass for final volumetric productivity of 1.91 g PHA/(L·h) [37]. A similar DO-control fed-batch reactor using oleic acid, but employing nitrogen limitation, resulted in a lower *mcl*-PHA volumetric productivity of 0.57 g PHA/(L·h), which is partly due to the lower biomass obtained, but also because of the much lower PHA content. Curiously, the PHA content reached its maximum early into the cultivation through growth-associated PHA synthesis, long before nitrogen limitation prevented further cell division. Importantly, oleic acid cultivation could be scaled-up into a 30-L reactor with results similar to those in a 2-L reactor [49]. A DO-control fed-batch system for high cell density using phosphorous limitation was used to promote PHA accumulation on corn oil hydrolysate. This resulted in a volumetric *mcl*-PHA accumulation rate of 0.68 g PHA/(L·h) [45]. Recently, a fed-batch approach with oleic acid controlled through nitrogen-limitation to maintain a low growth rate and couple growth to PHA accumulation was reported to improve the PHA content and carbon yield. In this system, biomass of 125.6 g/L containing 54.4 wt.-% *mcl*-PHA was obtained, but the productivity was lower than that reported by Lee et al. [37] due to the length of cultivation [47]. Finally, the monomer composition and thermal properties of *mcl*-PHA using waste rapeseed oil differed with the method of substrate delivery. Pulse-feeding resulted in higher intracellular polymer concentration but lower C12 monomer content, crystallinity, and molecular weights than a continuous feed [50].

Continuous-feed PHA production has been developed using a single bioreactor (single-stage continuous) or with the addition of subsequent reactors to separate the growth and PHA accumulation phases (dual-stage or multi-stage continuous) [41]. A continuous-feed bioreactor may not achieve the same maximum biomass titer or PHA content as a fed-batch reactor. However, by maintaining a high steady-state concentration of PHA long term, continuous PHA production can theoretically result in the highest PHA productivities with simpler feeding control and

lower operating costs [51,52]. Dilution rates between 0.1 and 0.3 1/h are optimal for maximizing volumetric productivity. Lower dilution rates increased the PHA content, while higher dilution rates could result in cell washout [40,51,53–56]. Nitrogen limitation is the most prevalent condition for controlling the growth rate and promoting PHA synthesis [54,55]. However, oxygen limitation and dual nutrient-limiting conditions have been reported [53,57,58]. Early single-stage, continuous-feed *mcl*-PHA cultivation resulted in volumetric productivity of 0.17 g PHA/(L·h) [51], and this was subsequently improved up to 0.76 g PHA/(L·h) by increasing the concentration of nitrogen and improving oxygen transfer rates to maintain higher cell density [55].

A two-stage continuous-feed process for *mcl*-PHA production from octane optimized high cell density production in the first stage, which fed into a second reactor with conditions suitable for PHA production. In this manner, the highest continuous volumetric productivity was reported at 1.06 g PHA/(L·h) [54]. From LCFAs, single-stage cultivation using oleic acid was optimized to 0.69 g PHA/(L·h) under oxygen limitation [53]. While the monomer composition of PHA was not affected by the growth rate from octanoate [38,55], the use of oleic acid resulted in a mild shift toward longer monomer composition at low dilution rates, without affecting the molecular weights of the PHA products [53]. While the theoretical potential of continuous cultivation has yet to be realized, continuous-feed PHA production has proven invaluable for its ability to study the effects of growth rate, substrate, and nutrient limitation on factors such as monomer compositions, PHA content, yields, molecular weight, and thermal properties.

11.4 STRATEGIES FOR IMPROVED SUBSTRATE YIELDS FROM MCFAS AND LCFAS

The substrate costs are estimated to account for the largest proportion of the overall techno-economic assessment [57,58]. High volumetric productivities must be balanced with a high substrate yield to minimize production cost. The maximal *mcl*-PHA yield from LCFAs varies depending on the substrate and monomer composition. However, estimates have been reported between 0.58 and 0.72 g/g, which compares to 0.98 g/g from octanoic acid [22,45,53]. The overall PHA yields are typically much lower when considering other process yields, such as biomass production and maintenance [32,38].

Optimizations for high intracellular PHA content were necessary to improve the overall *mcl*-PHA substrate yield, and reduce the PHA extraction and purification costs [53,59]. This was confirmed in a continuous-feed bioreactor operation as nitrogen-limiting conditions with lower dilution rates resulted in an improved *mcl*-PHA substrate yield [57]. The experimental carbon yield was improved by a stepwise decrease in nitrogen feed during the nitrogen-limitation phase, to couple growth with *mcl*-PHA production [47]. Furthermore, strategies to fulfill the substrate requirement for non-PHA biomass, maintenance, and respiration using cheaper substrates have been developed, greatly improving the overall *mcl*-PHA yield from the more expensive substrate [60,61]. The overall *mcl*-PHA yield from octanoate was

improved (0.4 g/g) by providing glucose during the exponential growth and adding octanoate during the nutrient limitation of the PHA accumulation phase, however, the PHA content was suboptimal [38].

A similar approach was scaled-up with glucose and nonanoic acid, which resulted in a higher PHA content to yield *mcl*-PHA at 0.56 g/g from nonanoic acid. PHA accumulation began without lag upon the addition of nonanoic acid, supporting the concept of phase-fed fatty acids [62]. Acrylic acid can be provided to inhibit the fatty acid degradation pathway preventing the use of fatty acids for biomass production and further improve on this approach. A continuous reactor containing glucose and nonanoic acid reported an increase in PHA yield from nonanoic acid from 0.15 to 0.90 g/g with the addition of acrylic acid [63]. Similarly, in a carbon-limited fed-batch, volumetric productivity of 1.8 g PHA/(L·h) was reported with an overall *mcl*-PHA yield from nonanoic acid of 0.78 g/g [64]. Since *mcl*-PHA production has a high affinity for C8, C9, and C10 monomers, the use of acrylic acid with fatty acids of the same length can effectively produce *mcl*-PHA [65]. Partial knockout of the fatty acid degradation pathway was effective at producing high C14 monomer content from tetradecanoic acid [66]. Ultimately, blocking the fatty acid degradation pathway may not be effective for increasing the *mcl*-PHA yield from LCFAs, certainly not without drastically changing the monomer composition and production kinetics. Two-reactor systems promise cost-savings through improved substrate yields since no growth occurs in the second PHA-accumulating reactor [52]. This was demonstrated by an overall *mcl*-PHA yield of 0.63 g/g from octane in the second stage, nearly reaching the maximum theoretical yield of 0.66 g/g [55].

11.5 EXTRACELLULAR LIPASE FOR TRIACYLGLYCERIDE CONSUMPTION

Many *Pseudomonads* do not have the extracellular lipase enzymes required for the metabolism of vegetable oils. The growth of these strains from vegetable or animal sources of triacylglycerides (TAGs) required chemical (hydrolysis) or enzymatic (lipases) pretreatment [60,67]. However, *P. aeruginosa*, *P. resinovorans*, *P. chlororaphis*, and other isolated *Pseudomonas* sp. strains have been reported to grow directly from TAGs [17,67–70]. Growth and PHA synthesis directly from TAGs is important because it reduces preprocessing steps. It has been reported that the lipase activity is relatively low in *P. resinovorans* [71]. Fed-batch cultivation of *P. resinovorans* using olive oil deodorizer distillate had a maximum specific growth rate of 0.19 1/h [72], compared to higher rates of 0.55 1/h reported by *P. putida* KT2440 [34]. The apparent decrease in the growth rate for *P. resinovorans* is not necessarily detrimental for PHA productivity when considering high productivities at set growth rates of 0.2 1/h [39] if the cost benefits of growth from TAGs offset productivity loss. Genes encoding the lipase precursor protein (LipA) and lipase chaperone protein (LimA), which together confer the ability to grow and synthesize PHA directly from TAG substrates, have been cloned and expressed in *P. putida* KT2442. The total biomass of the recombinant bacteria was the same, whether cultured with free fatty acids or TAGs, indicating that the lipase activity enabled direct catabolism of the TAGs [73].

11.6 BIOSYNTHESIS AND MONOMER COMPOSITION

Medium-chain-length polyhydroxyalkanoates can be synthesized from a wide range of substrates primarily by species of *Pseudomonas* [74]. *Mcl*-PHA synthesis relies on the microorganism's central fatty acid metabolism, in which fatty acid degradation is the major pathway for *mcl*-PHA production from LCFAs, although it has also been shown to work in concert with fatty acid biosynthesis pathways [75,76]. LCFAs are longer than the monomers typically incorporated into *mcl*-PHA, and, as such, undergo several rounds of fatty acid degradation. Each round of fatty acid degradation produces an $FADH_2$ and NADH and releases acetyl-CoA, which shortens the fatty acid by two carbons (see Figure 11.1). Therefore, continued fatty acid degradation is preferred for energy production while the cells are actively dividing, but PHA production rates increased upon onset of conditions limiting to cell growth [77].

Intermediates of fatty acid degradation can be converted to (*R*)-3-hydroxyacyl-CoA for polymerization, a process that requires no expenditure of ATP or NADH [47,78]. Thereby, LCFAs can be more directly converted into *mcl*-PHA than substrates that are unrelated to the 3-hydroxyalkanoate subunits that make up PHA polymers (i.e., carbohydrates), resulting in higher yields [79]. Table 11.1 summarizes the monomer compositions of *mcl*-PHA produced from various fatty acids and demonstrates that the activities and specificities of enzymes involved in PHA production vary considerably among *Pseudomonads* given similar substrate and culture conditions.

Three classes of monomer-supplying enzymes are hypothesized to convert fatty acid degradation intermediates to (*R*)-3-hydroxyacyl-CoA for PHA synthesis: hydratases, reductases, and epimerases. Hydratases, encoding at least four different *phaJ* genes, convert enoyl-CoA to (*R*)-3-hydroxyalkanoate, which have been confirmed to supply monomers in recombinant hosts (see Figure 11.1) [80–82]. Reductases, encoded by *fabG*, were confirmed to convert 3-ketoacyl-CoA to (*R*)-3-hydroxyacyl-CoA in recombinant hosts [83,84]. Epimerases, encoded by *fadB* in *E. coli*, as part of the multi-enzyme complex of fatty acid degradation, convert (*S*)-3-hydroxyacyl-CoA to (*R*)-3-hydroxyacyl-CoA [85]. The putative epimerase was proposed to become more active in response to a knockout of the (*S*)-3-hydroxyacyl-CoA dehydrogenase, which prevents further fatty acid degradation [86], and the existence of granule-bound epimerase activity is a possible explanation for *in vitro* polymerization of PHA from (*S*)-3-hydroxyacyl-CoA [87]. However, PHA monomer-supplying epimerase activity has not been observed in *Pseudomonas* spp. [78].

The activities and specificities of these monomer-supplying enzymes vary with acyl length [81], and since the expression levels of these monomers can be expected to change with microbial strain and stress response, the monomer composition of *mcl*-PHA may depend on the monomer-supplying enzymes to a greater extent than the specificity of the PHA synthase [88]. However, the PHA synthase genes, *phaC1* and *phaC2*, have been shown to have varying monomer specificities [89]. A knockout of the native PHA synthases of *Pseudomonas* sp. resulted in a combination of *scl*-PHA and *mcl*-PHA monomers when provided an alternative PHA synthase [90]. *E. coli* was provided with the *fabG* monomer-providing enzyme, which resulted in

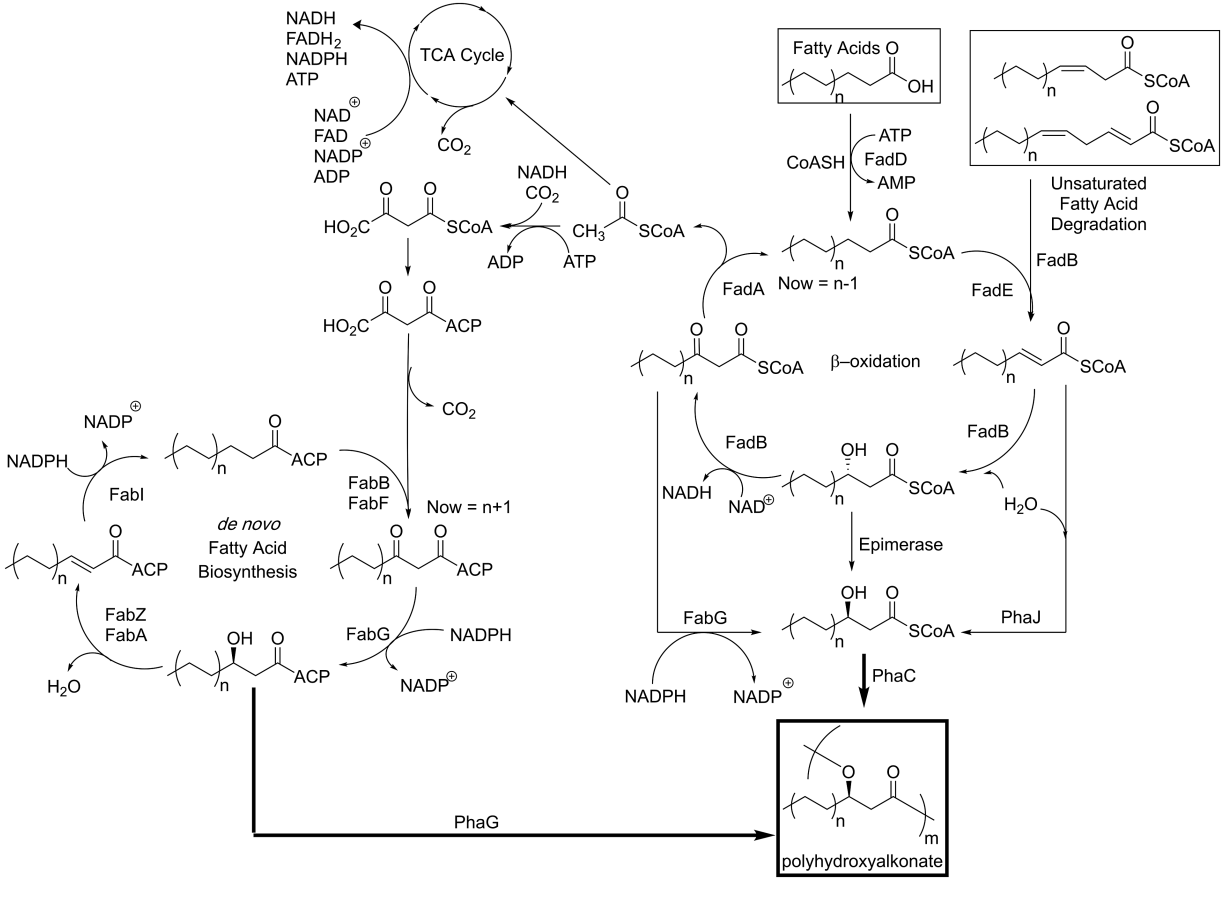


FIGURE 11.1 Biosynthetic pathway for *mcl*-PHA synthesis from fatty acid substrates.

TABLE 11.1
Monomer Composition of *mcl*-PHA Produced by *Pseudomonas* Spp. from Fatty Acids

		Monomer Composition (mol-%)												
Substrate	**Microorganism [Ref.]**	**C_4**	**C_6**	**C_8**	**$C_{8:1}$**	**C_{10}**	**$C_{10:1}$**	**C_{12}**	**$C_{12:1}$**	**$C_{12:2}$**	**C_{14}**	**$C_{14:1}$**	**$C_{14:2}$**	**$C_{14:3}$**
Octanoic Acid (C_8)	*P. putida* KT2442 [75]	–	6	92	–	2	–	–	–	–	–	–	–	–
Decanoic Acid (C_{10})	*P. putida* KT2442 [27]	–	5.3	52.3	–	42.3	–	–	–	–	–	–	–	–
Palmitic Acid (C_{16})	*Pseudomonas* sp. DR2 [17]	–	3.4	18.7	–	35.6	–	37.0	–	–	–	–	–	–
Petroselinic ($C_{18:1}$)	*P. putida* KT2442 [24]	–	5.1	45.2	–	33.1	–	12.7	Tr	–	3.9	Tr	–	–
Oleic Acid ($C_{18:1}$)	*P. putida* KT2442 [24]	–	4.4	33.5	–	32.2	–	14.4	Tr	–	–	15.5	–	–
Oleic Acid ($C_{18:1}$)	*P. aeruginosa* 27853 [64]	–	4	55	–	27	–	8	–	–	6	–	–	–
Oleic Acid ($C_{18:1}$)	*P. aeruginosa 42A2* (30°C) [124]	–	–	24.3	–	30.8	–	3.8	24.2	–	–	3.7	13.1	–
Oleic Acid ($C_{18:1}$)	*P. aeruginosa 42A2* (37 °C) [124]	–	–	2.2	–	39	–	4.7	25.6	–	–	2.1	26.5	–
Oleic Acid ($C_{18:1}$)	*P. resinovorans* B-2649 [71]	3.5	7	37	–	33	–	10	–	–	1	8	–	–
Linoleic Acid ($C_{18:2}$)	*P. putida* KT2442 [26]	–	5.6	38.9	–	22.7	–	–	16.9	–	–	–	15.9	–
Erucic Acid ($C_{22:1}$)	*P. aeruginosa* 27853 [68]	–	3	43	–	36	–	10	–	–	–	8	–	–
Nervonic Acid ($C_{24:1}$)	*P. aeruginosa* 27853 [68]	–	4	28	–	43	–	14	–	–	–	11	–	–
Coconut Oil	*P. resinovorans* B-2649 [69]	–	8	37	–	35	–	17	–	–	3	–	–	–
Canola Fatty Acids	*P. putida* LS46 [22]	–	5.4	41.4	–	26.7	–	9.1	3.6	–	9.3	8.6	–	–
Soybean Oil	*P. resinovorans* B-2649 [69]	Tr	8	29	Tr	30	2	5	9	2	2	2	8	–
Flax Fatty Acids*	*P. putida* KT2442 [30]	–	4.7	23.3	11.4	16.9	9.3	3.9	3.2	~5.5	Tr	2.7	~5.5	~13.6
Linseed Oil	*P. aeruginosa* 42A2 [125]	–	0.4	33.7	5.6	24.3	7.0	4.9	1.4	4.8	0.4	6.4	2.8	8.3

* Values have been assigned as approximate as $C_{12:2}$ and $C_{14:2}$ were combined as 11%, and the 13.6% of $C_{14:3}$ included some $C_{16:3}$.

mcl-PHA when provided an *mcl-phaC* but produced PHB-*co*-PHHx given an *scl-phaC* [84]. Both these examples indicate that the monomer-supplying enzymes provided a variety of monomer lengths, and the selectivity was due to the PHA synthase. Ultimately, the monomer composition is dependent on a variety of factors, but *mcl*-PHA is produced with a preference for C8 and C10 monomers due to enzyme specificities, as shown in Table 11.1.

Despite the aforementioned monomer preferences, the low substrate specificity of *mcl*-PHA synthases accounts for the incorporation of over 150 monomer types [12,91]. The monomer composition of *mcl*-PHA will differ by species and is determined both by the substrate and the culturing conditions. The length of the fatty acid influences the PHA monomer length [68], and functional groups may be incorporated into *mcl*-PHA from the substrate [12]. The functional moieties in LCFAs can be retained during *mcl*-PHA production, depending on their position in the substrate [28,68,73].

The double bonds of MUFAs and PUFAs are removed as the chain is shortened using the fatty acid degradation pathway. The enzyme cis-3,trans-2,enoyl-CoA isomerase is responsible for removing the odd-carbon double bonds, while even-numbered double bonds (such as the Δ12 olefin group of linoleic acid) are removed by 2,4-dienoyl-CoA reductase [28]. In this way, one may predict the position of double bonds in the PHA polymer based on the substrate provided and the monomer length. For instance, 15.5 mol-% of *mcl*-PHA synthesized by *P. putida* KT2442 grown with oleic acid (Δ9) was monounsaturated, as the olefin was maintained in the C14 monomers, compared with no retained olefins from petroselinic acid (Δ6). Growth on linoleic acid (Δ9, Δ12) tripled the unsaturation of PHA as both olefins were maintained in the C14, and one olefin remained in C12 [28]. Moreover, PHA produced from linolenic acid (Δ9, Δ12, Δ15) produced highly unsaturated PHA containing mono-(C8, C10), di-(C12), and poly-(C14) unsaturated monomers [30]. Table 11.1 illustrates that the average monomer length and mol-% of unsaturated monomers increased proportionally with the length and unsaturation of the substrate. Hydroxyl and epoxy moieties have also been observed in *mcl*-PHA when *P. aeruginosa* 44T1 was provided with castor or euphorbia oil [92]. *Mcl*-PHA have incorporated halogen, hydroxyl, carboxyl, thiol, epoxy, aromatic, and branched moieties, to name a few, using the appropriate fatty acids [11,12,91]. These functional properties provide the basis for modification of unsaturated *mcl*-PHA, but their inclusion also broadens the applicable chemical modifications.

Culture conditions affect polymer composition due to a shift in the central fatty acid metabolism. As the fatty acids in the bacterial membrane change in length and unsaturation in response to incubation temperature, so too does the *mcl*-PHA composition [76]. PHA production using LCFAs at a lower incubation temperature resulted in a shift toward longer monomers with higher unsaturation. As the unsaturated monomers were not consistent with the LCFAs, it was inferred that the lower temperature increased the incorporation of *mcl*-PHA monomers from *de novo* fatty acid synthesis. The same study also demonstrated a drastic shift in monomer composition based on changing from nitrogen limitation to phosphate limitation [93]. All the above factors ultimately affect the thermal and mechanical properties of *mcl*-PHA.

11.7 FUNCTIONAL MODIFICATIONS OF *MCL*-PHA

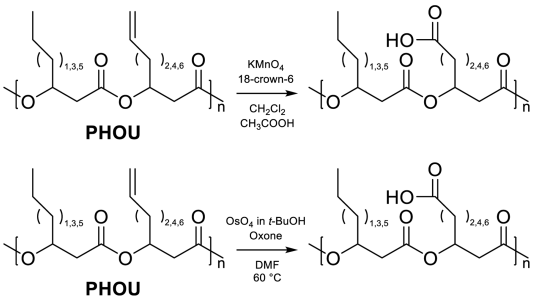
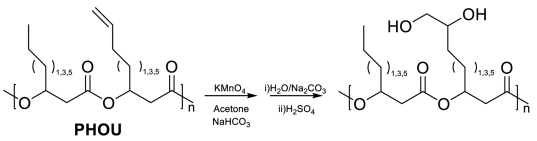
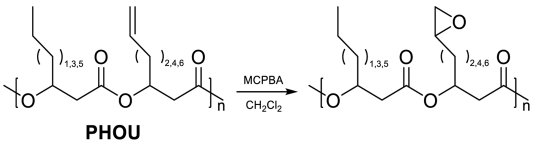
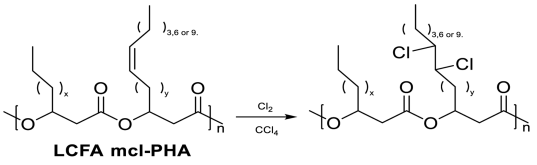
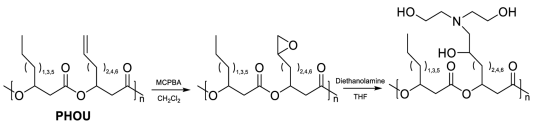
The incorporation of vinyl moieties into *mcl*-PHA reduces their crystallinity, ultimately weakening their mechanical properties, but also imparting functionality to the polymer. Chemical modifications have imbued unsaturated *mcl*-PHA with strength or with new properties targeted at niche biomedical applications. The remainder of this chapter discusses the modification of *mcl*-PHA to attain novel properties.

Mcl-PHA with vinyl moieties inherited from MUFAs and PUFAs can be tailored by choice of microorganism and culture conditions, but most simply by the substrate delivery (Table 11.1). Vegetable oils vary in their fatty acid composition, and co-feeding strategies can be applied to control the relative unsaturation [22]. LCFAs appear to increase chain termination during *mcl*-PHA polymerization resulting in lower molecular weights with increasing unsaturation [69]. The glass transition temperatures (T_g) and melting point (T_m) decrease with higher unsaturation in the LCFA substrate [94]. Commonly, 10-undecenoic acid is provided for *mcl*-PHA (PHU) production with terminal vinylic carbons in the side chains [95,96]. Varying the feed ratio of octanoic acid and 10-undecenoic acid resulted in polyhydroxyoctanoate-*co*-undecenoate (PHOU) with an unsaturated monomer concentration equal to the substrate ratio and no effect on molecular weight. When co-feeding octanoate with 10-undecenoate in a continuous steady-state bioreactor, lower dilution rates increased the relative concentration of aliphatic monomers while molecular weights were not affected by the growth rate [56]. Increased feed ratios of 10-undecenoic acid resulted in a decreased glass transition temperature and melting temperature of the *mcl*-PHA and became amorphous with high unsaturation [56]. The same trends were observed with octane:octene ratios, having a decreasing melt endotherm until completely amorphous at 15 mol-% unsaturation [32].

The modification of *mcl*-PHA containing side-chain vinyl groups has produced polymers with new properties (Table 11.2). Chlorination across the double bond drastically elevated the glass transition temperature from −50°C to +58°C with melting temperatures more consistent with *scl*-PHA than *mcl*-PHA, despite hydrolysis resulting in lower molecular weights. The observed changes in these polymers were from sticky to a soft, elastic polymer with moderate chlorine addition. The high chlorine content in the polymers was described as crystalline and brittle. While crystallinity was not measured in that study, these highly chlorinated polymers were observed well below their glass transition temperatures [97].

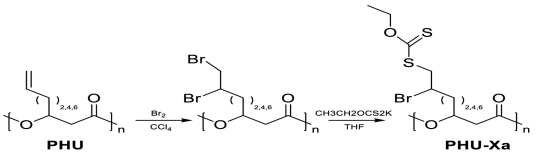
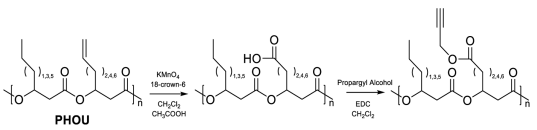
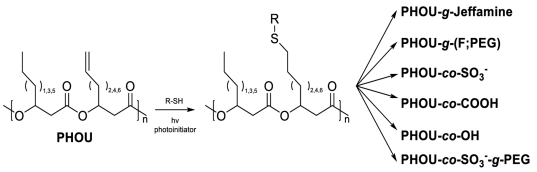
Carboxylation and hydroxylation at the terminal vinyl position of PHOU have both been demonstrated to change the solubility of the polymer with no reduction in molecular weight [98,99]. Increasing unsaturation content in the PHOU resulted in polymers with higher polarity after modification until the polymers were no longer soluble in the organic solvent [96,99,100]. Epoxidation of PHOU was performed without a reduction in molecular weight or cross-linking. The glass transition temperature was lower with increased epoxidation, but the polymer remained amorphous. The addition of epoxides provided an avenue for cross-linking or producing amphiphilic polymers [94,100,101]. Copolymer grafting has been achieved via free radical polymerization with the side-chain vinyl moieties of unsaturated *mcl*-PHA from soybean fatty acids.

TABLE 11.2
Functional Modification of Unsaturated *mcl*-PHA

Reaction	Described Effect
Carboxylation 	Increasing carboxylation results in higher hydrophilicity. This functional moiety is also a precursor for other modifications (see "click-ready") [97,99].
Hydroxylation 	Hydroxylation increased the hydrophilicity of the polymer, becoming insoluble in organic solvents [100].
Epoxidation 	Reduced glass transition temperature. Precursor to cross-linking, or for further modification (see "transamination") [96,102,103].
Halogenation 	Higher polymer chlorine content resulted in elevated glass transition and melting temperatures [99]. Halogenation provides the leaving group for further substitutions (see "RAFT").
Amination 	Complete change in solubility from hydrophobic to hydrophilic. Transamination resulted in a significant decrease in molecular weight due to PHA chain scission [126].

(*Continued*)

TABLE 11.2 (CONTNIUED)
Functional Modification of Unsaturated *mcl*-PHA

Reaction	Described Effect
RAFT Derivatization 	Xanthate substitution converts unsaturated PHA into macro RAFT agents. RAFT polymerization using *N*-isopropyl acrylamide produced thermo-responsive, amphiphilic polymers with glass transition temperatures between 58°C and 100°C [106].
Click-Ready Terminal Alkyne Derivatization 	CuAAC-ready *mcl*-PHA for diverse applications. Demonstrated improved PEG grafting compared with previous methodology, resulting in improved crystallinity [127].
Thiol-ene Addition 	Thiol-ene reaction with PHOU eliminates polymer premodification for "clickable" functionalization. Significantly broadens types of functional groups and grafting while avoiding chain scission of cross-linking [110,112–115].

Methyl methacrylate (MMA) initiated by benzoyl peroxide formed a copolymer graft with PHA (PHA-*g*-PMMA) in which cross-linking could be prevented with the addition of hydroquinone. Soybean *mcl*-PHA is not in a glassy state and exhibits no crystallinity. However, the grafted polymer became hard and brittle, a property inherited by the glass transition temperature of poly(MMA) (PMMA). Grafted copolymers were produced with higher tensile strength and elongation at break than either homopolymer [102]. Graft polymers were alternatively produced by first activating the MMA or styrene using an oligoperoxide to produce activated PMMA or polystyrene (PS), respectively. Mixing the activated polymers into unsaturated *mcl*-PHA produced PHA-*g*-PMMA and PHA-*g*-PS; however, the PHA-*g*-PS cross-linked [103]. Bromination across the vinyl moieties of unsaturated *mcl*-PHA followed by xanthate substitution, which resulted in macro reversible addition-fragmentation chain transfer (RAFT) agents. RAFT polymerization using N-isopropyl acrylamide produced thermo-responsive, amphiphilic polymers with glass transition temperatures between 58°C and 100°C [104].

Click chemistry can be applied to the vinyl groups of *mcl*-PHA derived from unsaturated fatty acid substrates, which drastically increases the number of modification

permutations. Click-ready *mcl*-PHA were first produced by converting PHOU to contain terminal *R*-group carboxylic acids, then by esterification with propargyl alcohol yielding a "clickable" terminal alkyne. The objective was to produce amphiphilic polymers through polyethylene glycol (PEG) grafting. PHA-*g*-PEG was produced using PEG-azide producing polymers using longer PEG oligomers than could be grafted with previous direct esterification methods, ultimately increasing the molecular weight and crystallinity of this copolymer [105]. The terminal alkyne produced enables copper-catalyzed azide-alkyne [3+2] cycloaddition (CuAAC) with molecules containing azide groups.

Instead of modifying the vinyl groups of *mcl*-PHA to introduce click moieties, *mcl*-PHA were cultured with terminal azide groups. The resulting *mcl*-PHA are CuAAC-ready without the need for polymer modification. This was achieved with ω-azido fatty acid substrates delivered to engineered *E. coli*, producing *mcl*-PHA with a yield, molecular weights, and thermal properties consistent with PHOU production [106]. *P. oleovorans* co-fed nonanoate and 11-bromoundecanoic acid produced polymers with terminal bromine groups, which allowed azide substitution. Instead of a terminal azide, the azide-alkyne reaction could be achieved with terminal alkyne *mcl*-PHA produced when co-fed nonanoate and 10-undecynoic acid [107]. Furthermore, a strain-promoted cycloaddition was demonstrated, eliminating the copper requirement of CuAAC [104,105].

Another approach that uses click chemistry without metallic catalysts is thiol-ene addition. In this case, the desired functional molecules contain pendant thiol moieties, which undergo anti-Markovnikov additions to the side-chain vinyl groups of unsaturated *mcl*-PHA. An increase in hydrophilicity without a reduction in the molecular weight of PHOU and unsaturated *mcl*-PHA from soybean LCFAs was achieved when hydroxylated and carboxylated in this manner [108]. Poly(3-hydroxybutyrate)-*co*-undecenoate (PHBU) was cross-linked using a polythiol [pentaerythritol tetrakis (3-mercaptopropionate)] for the thiol-ene click reaction to increase both the elongation and tensile strength compared with native PHBU [109].

A pendant sulfonate addition to PHOU resulted in a dramatic change in solubility, becoming insoluble in organic solvents above 5 mol-% sulfonated monomers and self-aggregated into nanoparticle micelles with sizes dependent on sulfonate concentration [110]. Furthermore, the use of thiol-ene reactions has produced various grafted copolymers from PHOU. Amphiphilic polymers have been designed for medical applications, such as drug delivery by grafting, using thiol-ene click reactions. Jeffamine® grafting onto PHOU increased the hydrophilicity of the polymer [111]. The sequential grafting of fluorinated chains and PEG onto PHOU produced multi-compartment micelles [112]. The same procedure substituting fluorinated chains with sulfonated chains produced amphiphilic polymers coated onto nanometal organic frameworks to produce stable hybrid nanoparticles that displayed no cytotoxicity [113].

11.8 CROSS-LINKING

Cross-linking of *mcl*-PHA involves radical propagation along olefinic moieties, forming a polymer network. Unsaturated *mcl*-PHA have been reported to auto-oxidize

and have had cross-linking initiated by reactive peroxides or radiation [32,114–116]. Cross-linking is the result of either C-C, ether, or peroxy bonds, although ether bonds appear to be dominant in cross-linking during auto-oxidation [117,118]. Hydrogen atom abstraction is most likely to occur in the allylic position, at which time the radical reacts with oxygen resulting in a peroxyl radical. The peroxyl radical can abstract another allylic hydrogen until the reaction is finally terminated [119]. The degradation of the hydroperoxide into an epoxide precedes ether bond linkages [118]. The rate of cross-linking can be increased with heat, irradiation, supplementation of oxygen, or with increasing unsaturation. Chain scission and cross-linking will occur simultaneously, but the ester cleavage requires much higher activation energy (almost three-fold), therefore, cross-linking is promoted before chain scission [116,120].

Cross-linking of *mcl*-PHA polymers resulted in markedly different physical and thermal properties. When the octane-based polymer was irradiated, only chain scission occurred and caused a reduction in molecular weight. Irradiation of a polymer with 15 mol-% unsaturated monomers (amorphous) caused cross-linking, and the polymer became solid and less sticky. The cross-linked polymers then showed a constant dynamic modulus from the glass transition temperatures (–15°C to –30°C) to the onset of thermal degradation (170°C), whereas the octane-based polymer showed a significant amount of variation around ambient temperature and softened at 40°C due to melting. The tear resistance of the cross-linked, amorphous polymer was poor, with much less tensile strength than the crystalline, saturated polymer. However, the cross-linked PHA was still biodegradable [32]. Irradiation of *mcl*-PHA with 11 mol-% monounsaturated monomers synthesized by *P. resinovorans* (NRRL B-2649) from tallow made it slightly stronger and more rigid than its crystalline counterpart. However, this further increased the cross-linking density resulting in a decrease in tear resistance, and the soluble fraction showed molecular weight reductions up to 70% [114]. *Mcl*-PHA synthesized from coconut oil (95% saturated), tallow (37% unsaturated oleic acid), and soybean (86% unsaturated or polyunsaturated) fatty acids all displayed increased tensile strength after irradiation.

Mcl-PHA polymers synthesized from soybean oil produced a solid film with a higher Young's modulus than the irradiated polymers produced from coconut or tallow feedstocks [120]. Linseed oil derived *mcl*-PHA were cross-linked by two methods: chemically induced cross-links with meta-chloroperoxybenzoic acid (m-CPBA) and naturally induced cross-links by exposure to air (auto-oxidation). Chemical treatment with m-CPBA resulted in cross-linking in less than 25 days, and by that time, 98% of the polymer was solid and insoluble. Cross-linking by auto-oxidation took between 50 and 75 days under ambient conditions. However, the auto-oxidized PHA had higher tensile strength and was more brittle than the chemically cross-linked polymers. It was suggested that m-CPBA treatment results in ether cross-links, whereas auto-oxidation resulted in carbon-carbon cross-links conferring more strength to the polymer [101].

PHA films were synthesized by *P. oleovorans* grown with various ratios of octanoic, 10-undecenoic acid, and soybean acids followed by cross-linking treatment. Large variations in tensile strength and elongation at break were observed, such that the film characteristics could be tailored by substrate feeding. In all cases, the films were biocompatible, but each elicited different magnitudes of inflammation. They

concluded that variation in film properties and degradation rates allow for diverse medical applications [121,122]. A cross-linked network of PHOU-*g*-PEG was produced by UV irradiation to determine the effect on swelling for drug delivery. The addition of PEG into the cross-linked polymer reduced the tensile strength and elongation to break with increasing PEG concentration and increased the degree of cross-linking and hydrophilicity. PEG further improved the biocompatibility of *mcl*-PHA by reducing platelet and protein interactions [123–127].

11.9 CONCLUSIONS AND OUTLOOK

The abundant supply of LCFAs from renewable and waste sources, coupled with high biomass production rates and substrate yields, improves the cost efficiency of sustainable PHA production. Furthermore, the use of unsaturated LCFAs for *mcl*-PHA production inserted functional moieties into the polymer. The content of these moieties can be controlled through cultivation conditions to tailor the composition of the *mcl*-PHA. These unsaturated *mcl*-PHA provide the opportunity to be modified at their vinyl groups (i.e., halogenation, hydroxylation, carboxylation, grafting, cross-linking), and platforms for the modification of these unsaturated *mcl*-PHA continue to emerge. Further exploration and characterization will enable tailored-made PHA for stronger films and amphiphilic polymers for medical applications.

REFERENCES

1. Madison LL, Huisman GW. Metabolic engineering of poly(3-hydroxyalkanoates): From DNA to plastic. *Microbiol. Mol. Biol. Rev.* 1999; 63(1): 21–53.
2. Jendrossek D, Handrick R. Microbial degradation of polyhydroxyalkanoates. *Annu. Rev. Microbiol.* 2002; 56(1): 403–432.
3. Knoll M, Hamm TM, Wagner F, et al. The PHA depolymerase engineering database: A systematic analysis tool for the diverse family of polyhydroxyalkanoate (PHA) depolymerases. *BMC Bioinform.* 2009; 10(89): 1–8.
4. Chuah JA, Yamada M, Taguchi A, et al. Biosynthesis and characterization of polyhydroxyalkanoate containing 5-hydroxyvalerate units: Effects of 5HV units on biodegradability, cytotoxicity, mechanical and thermal properties. *Polym. Degrad. Stab.* 2013; 98(1): 331–338.
5. Doi Y, Kanesawa Y, Kunioka M, et al. Biodegradation of microbial copolyesters: Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) and poly(3-hydroxybutyrate-co-4-hydroxy butyrate). *Macromolecules* 1990; 23(1): 26–31.
6. Jendrossek D, Knoke I, Habibian RB, et al. Degradation of poly(3-hydroxybutyrate), PHB, by bacteria and purification of a novel PHB depolymerase from *Comamonas* sp. *J. Environ. Polym. Degrad.* 1993; 1(1): 53–63.
7. Bonartsev AP, Myshkina VL, Nikolaeva DA, et al. Biosynthesis, biodegradation, and application of poly (3-hydroxybutyrate) and its copolymers – Natural polyesters produced by diazotrophic bacteria. *Commun. Curr. Res. Educ. Top. Trends Appl. Microbiol.* 2007; 1: 295–307.
8. Lageveen RG, Huisman GW, Preusting H, et al. Formation of polyesters by *Pseudomonas oleovorans*: Effect of substrates on formation and composition of poly-(*R*)-3-hydroxyalkanoates and poly-(*R*)-3-hydroxyalkenoates. *Appl. Environ. Microbiol.* 1989; 54(12): 2924–2932.
9. Lee SY. Bacterial polyhydroxyalkanoates. *Biotechnol. Bioeng.* 1996; 49: 1–14.

10. Hazer B, Steinbüchel A. Increased diversification of polyhydroxyalkanoates by modification reactions for industrial and medical applications. *Appl. Microbiol. Biotechnol.* 2007; 74(1): 1–12.
11. Steinbüchel A, Valentin HE. Diversity of bacterial polyhydroxyalkanoic acids. *FEMS Microbiol. Lett.* 1995; 128(3): 219–228.
12. Kim DY, Kim HW, Chung MG, et al. Biosynthesis, modification, and biodegradation of bacterial medium chain length polyhydroxyalkanoates. *J. Microbiol.* 2007; 45(2): 87–97.
13. Levett I, Birkett G, Davies N, et al. Techno-economic assessment of poly-3-hydroxybutyrate (PHB) production from methane – The case for thermophilic bioprocessing. *J. Environ. Chem. Eng.* 2016; 4(4): 3724–3733.
14. Choi JI, Lee SY. Process analysis and economic evaluation for poly(3-hydroxybutyrate) production by fermentation. *Bioprocess Eng.* 1997; 17(6): 335–342.
15. Blunt W, Levin DB, Cicek N. Bioreactor operating strategies for improved polyhydroxyalkanoate (PHA) productivity. *Polymers* 2018; 10(11): 1197.
16. Koller M, Maršálek L, Miranda de Sousa Dias M, et al. Producing microbial polyhydroxyalkanoate (PHA) biopolyesters in a sustainable manner. *New Biotechnol.* 2017; 37: 24–38.
17. Song JH, Jeon CO, Choi MH, et al. Polyhydroxyalkanoate (PHA) production using waste vegetable oil by *Pseudomonas* sp. strain DR2. *J. Microbiol. Biotechnol.* 2008; 18(8): 1408–1415.
18. Kerenkan AE, Béland F, Do TO. Chemically catalyzed oxidative cleavage of unsaturated fatty acids and their derivatives into valuable products for industrial applications: A review and perspective. *Catal. Sci. Technol.* 2016; 6(4): 971–987.
19. Chhetri AB, Watts KC, Islam MR. Waste cooking oil as an alternate feedstock for biodiesel production. *Energies* 2008; 1: 3–18.
20. Ciesielski S, Mozejko J, Pisutpaisal N. Plant oils as promising substrates for polyhydroxyalkanoates production. *J. Clean. Prod.* 2015; 106: 408–421.
21. Koçer H, Borcakli M, Demirel S, et al. Production of bacterial polyesters from some various new substrates by *Alcaligenes eutrophus* and *Pseudomonas oleovorans. Turk. J. Chem.* 2003; 27(3): 365–373.
22. Blunt W, Dartiailh C, Sparling R, et al. Carbon flux to growth or polyhydroxyalkanoate synthesis under microaerophilic conditions is affected by fatty acid chain-length in *Pseudomonas putida* LS46. *Appl. Microbiol. Biotechnol.* 2018; 102(15): 6437–6449.
23. Chowdhury K, Banu LA, Khan S, et al. Studies on the fatty acid composition of edible oils. *Bangladesh J. Sci. Ind. Res.* 2007; 42(3): 311–316.
24. Awogbemi O, Onuh EI, Inambao FL. Comparative study of properties and fatty acid composition of some neat vegetable oils and waste cooking oils. *IJLCT* 2019; 14: 417–425.
25. Gruzdienė D, Anelauskaitė E. Chemical composition and stability of rapeseed oil produced from various cultivars grown in Lithuania. *JFAE* 2010; 39(61): 10–13.
26. Ramos MJ, Fernández CM, Casas A, et al. Influence of fatty acid composition of raw materials on biodiesel properties. *Bioresour. Technol.* 2009; 100: 261–268.
27. Eggink G, de Waard PE, Huijberts GNM. The role of fatty acid biosynthesis and degradation in the supply of substrates for poly (3-hydroxyalkanoate) formation in *Pseudomonas putida*. *FEMS Microbiol. Rev.* 1992; 103(2): 159–163.
28. de Waard P, van der Wal H, Huijberts GN, et al. Heteronuclear NMR analysis of unsaturated fatty acids in poly(3-hydroxyalkanoates). Study of beta-oxidation in *Pseudomonas putida*. *J. Biol. Chem.* 1993; 268(1): 315–319.
29. Bart J, Palmeri N, Cavallero S. Feedstocks for biodiesel production. In: *Biodiesel Science and Technology*. Woodhead Publishing Series in Energy, 2010: 130–225.
30. Casini E, de Rijk TC, de Waard P, et al. Synthesis of poly(hydroxyalkanoate) from hydrolyzed linseed oil. *J. Environ. Polym. Degrad.* 1997; 5(3): 153–158.

31. Antonio RV, Steinbüchel A, Rehm BHA. Analysis of *in vivo* substrate specificity of the PHA synthase from *Ralstonia eutropha*: Formation of novel copolyesters in recombinant *Escherichia coli*. *FEMS Microbiol. Lett.* 2000; 182(1): 111–117.
32. de Koning GJM, van Bilsen HMM, Lemstra PJ, et al. A biodegradable rubber by cross-linking poly(hydroxyalkanoate) from *Pseudomonas oleovorans*. *Polymer* 1994; 35(10): 2090–2097.
33. Sun Z, Ramsay JA, Guay M, et al. Fermentation process development for the production of medium-chain-length poly-3-hyroxyalkanoates. *Appl. Microbiol. Biotechnol.* 2007; 75(3): 475–485.
34. Follonier S, Henes B, Panke S, et al. Putting cells under pressure: A simple and efficient way to enhance the productivity of medium-chain-length polyhydroxyalkanoate in processes with *Pseudomonas putida* KT2440. *Biotechnol. Bioeng.* 2012; 109(2): 451–461.
35. Ren Q, de Roo G, Ruth K, et al. Simultaneous accumulation and degradation of polyhydroxyalkanoates: Futile cycle or clever regulation. *Biomacromolecules* 2009; 10(4): 916–922.
36. Koller M, Braunegg G. Potential and prospects of continuous polyhydroxyalkanoate (PHA) production. *Bioengineering* 2015; 2(2): 94–121.
37. Lee SY, Wong HH, Choi JI, et al. Production of medium-chain-length polyhydroxyalkanoates by high-cell-density cultivation *Pseudomonas putida* under phosphorus limitation. *Biotechnol. Bioeng.* 2000; 68(4): 466–470.
38. Kim GJ, Lee IY, Yoon SC, et al. Enhanced yield and a high production of medium-chain-length poly(3-hydroxyalkanoates) in a two-step fed-batch cultivation of *Pseudomonas putida* by combined use of glucose and octanoate. *Enzyme Microb. Technol.* 1997; 20(7): 500–505.
39. Sun Z, Ramsay JA, Guay M, et al. Carbon-limited fed-batch production of medium-chain-length polyhydroxyalkanoates from nonanoic acid by *Pseudomonas putida* KT2440. *Appl. Microbiol. Biotechnol.* 2009; 82(4): 657–662.
40. Ramsay BA, Saracovan I, Ramsay JA, et al. Continuous production of long-side-chain poly-3-hydroxyalkanoates by *Pseudomonas oleovorans*. *Appl. Environ. Microbiol.* 1991; 57(3): 625–629.
41. Koller M. A review on established and emerging fermentation schemes for microbial production of polyhydroxyalkanoate (PHA) biopolyesters. *Fermentation* 2018; 4(2): 30.
42. Kaur G, Roy I. Strategies for large-scale production of polyhydroxyalkanoates. *Chem. Biochem. Eng. Q.* 2015; 29(2): 157–172.
43. Sun Z, Ramsay JA, Guay M, et al. Automated feeding strategies for high-cell-density fed-batch cultivation of *Pseudomonas putida* KT2440. *Appl. Microbiol. Biotechnol.* 2006; 71(4): 423–431.
44. Maclean H, Sun Z, Ramsay JA, et al. Decaying exponential feeding of nonanoic acid for the production of medium-chain-length poly (3-hydroxyalkanoates) by *Pseudomonas putida*. *Can. J. Chem.* 2008; 86(1): 564–569.
45. Shang L, Jiang M, Yun Z, et al. Mass production of medium-chain-length poly(3-hydroxyalkanoates) from hydrolyzed corn oil by fed-batch culture of *Pseudomonas putida*. *World J. Microbiol. Biotechnol.* 2008; 24(12): 2783–2787.
46. Kellerhals MB, Hazenberg W, Witholt B. High cell density fermentations of *Pseudomonas oleovorans* for the production of *mcl*-PHAs in two-liquid phase media. *Enzyme Microb. Technol.* 1999; 24(1): 111–116.
47. Andin N, Longieras A, Veronese T, et al. Improving carbon and energy distribution by coupling growth and medium chain length polyhydroxyalkanoate production from fatty acids by *Pseudomonas putida* KT2440. *Biotechnol. Bioprocess Eng.* 2017; 22(3): 308–318.

48. Vendruscolo F, Rossi MJ, Schmidell W, et al. Determination of oxygen solubility in liquid media. ISRN Chem. Eng. 2012; 2012: 1–5.
49. Kellerhals MB, Kessler B, Witholt B, et al. Renewable long-chain fatty acids for production of biodegradable medium-chain-length polyhydroxyalkanoates (*mcl*-PHAs) at laboratory and pilot plant scales. *Macromolecules* 2000; 33(13): 4690–4698.
50. Mozejko J, Ciesielski S. Pulsed feeding strategy is more favorable to medium-chain-length polyhydroxyalkanoates production from waste rapeseed oil. *Biotechnol. Prog.* 2014; 30(5): 1243–1246.
51. Preusting H, Hazenberg W, Witholt B. Continuous production of poly(3-hydroxyalkanoates) by *Pseudomonas oleovorans* in a high-cell-density, two-liquid-phase chemostat. *Enzyme Microb. Technol.* 1993; 15(4): 311–316.
52. Hartmann R, Hany R, Witholt B, et al. Simultaneous biosynthesis of two copolymers in *pseudomonas putida* GPO1 using a two-stage continuous culture system. *Biomacromolecules* 2010; 11(6): 1488–1493.
53. Huijberts GNM, Eggink G. Production of poly(3-hydroxyalkanoates) by *Pseudomonas putida* KT2442 in continuous cultures. *Appl. Microbiol. Biotechnol.* 1996; 46(3): 233–239.
54. Jung K, Hazenberg W, Prieto M, et al. Two-stage continuous process development for the production of medium-chain-length poly(3-hydroxyalkanoates). *Biotechnol. Bioeng.* 2001; 72(1): 19–24.
55. Hazenberg W, Witholt B. Efficient production of medium-chain-length poly(3-hydroxyalkanoates) from octane by *Pseudomonas oleovorans*: Economic considerations. *Appl. Microbiol. Biotechnol.* 1997; 48(5): 588–596.
56. Hartmann R, Hany R, Pletscher E, et al. Tailor-made olefinic medium-chain-length poly[(R)-3-hydroxyalkanoates] by *Pseudomonas putida* GPo1: Batch versus chemostat production. *Biotechnol. Bioeng.* 2006; 93(4): 737–746.
57. Durner R, Witholt B, Egli T. Accumulation of poly[(R)-3-hydroxyalkanoates] in *Pseudomonas oleovorans* during growth with octanoate in continuous culture at different dilution rates. *Appl. Environ. Microbiol.* 2000; 66(8): 3408–3414.
58. Durner R, Zinn M, Witholt B, Egli T. Accumulation of poly[(R)]-3-hydroxyalkanoates] in *Pseudomonas oleovorans* during growth in batch and chemostat culture with different carbon sources. *Biotechnol. Bioeng.* 2001; 72(3): 278–288.
59. Chanprateep S. Current trends in biodegradable polyhydroxyalkanoates. J. Biosci. Bioeng. 2010; 110(6): 621–632.
60. Jiang G, Hill D, Kowalczuk M, et al. Carbon sources for polyhydroxyalkanoates and an integrated biorefinery. *Int. J. Mol. Sci.* 2016; 17(7): 1157.
61. Choi J, Lee SY. Factors affecting the economics of polyhydroxyalkanoate production by bacterial fermentation. *Appl. Microbiol. Biotechnol.* 1999; 51(1): 13–21.
62. Davis R, Duane G, Kenny S, et al. High cell density cultivation of *Pseudomonas putida* KT2440 using glucose without the need for oxygen enriched air supply. *Biotechnol. Bioeng.* 2015; 112(4): 725–733.
63. Jiang X, Sun Z, Marchessault RH, et al. Biosynthesis and properties of medium-chain-length polyhydroxyalkanoates with enriched content of the dominant monomer. *Biomacromolecules* 2012; 13(9): 2926–2932.
64. Jiang XJ, Sun Z, Ramsay JA, et al. Fed-batch production of MCL-PHA with elevated 3-hydroxynonanoate content. *AMB Expr.* 2013; 3(1): 50.
65. Qi Q, Steinbüchel A, Rehm BH. Metabolic routing towards polyhydroxyalkanoic acid synthesis in recombinant *Escherichia coli* (fadR): Inhibition of fatty acid {β}-oxidation by acrylic acid. *FEMS Microbiol. Lett.* 1998; 167: 89–94.
66. Liu W, Chen GQ. Production and characterization of medium-chain-length polyhydroxyalkanoate with high 3-hydroxytetradecanoate monomer content by *fadB* and *fadA* knockout mutant of *Pseudomonas putida* KT2442. *Appl. Microbiol. Biotechnol.* 2007; 76(5): 1153–1159.

67. Povolo S, Romanelli MG, Fontana F, et al. Production of polyhydroxyalkanoates from fatty wastes. *J. Polym. Environ.* 2012; 20(4): 944–949.
68. Impallomeni G, Ballistreri A, Carnemolla GM, et al. Synthesis and characterization of poly(3-hydroxyalkanoates) from *Brassica carinata* oil with high content of erucic acid and from very long chain fatty acids. *Int. J. Biol. Macromol.* 2011; 48(1): 137–145.
69. Ashby RD, Foglia TA. Poly(hydroxyalkanoate) biosynthesis from triglyceride substrates. *Appl. Microbiol. Biotechnol.* 1998; 49(4): 431–437.
70. Sharma PK, Munir RI, de Kievit T, et al. Synthesis of polyhydroxyalkanoates (PHAs) from vegetable oils and free fatty acids by wild-type and mutant strains of *pseudomonas chlororaphis*. *Can. J. Microbiol.* 2017; 63(12): 1009–1024.
71. Lee JH, Ashby RD, Needleman DS, et al. Cloning, sequencing, and characterization of lipase genes from polyhydroxyalkanoate (PHA)-synthesizing *Pseudomonas resinovorans*. *Appl. Microbiol. Biotechnol.* 2012; 96: 993–1005.
72. Cruz MV, Araújo D, Alves VD, et al. Characterization of medium chain length polyhydroxyalkanoate produced from olive oil deodorizer distillate. *Int. J. Biol. Macromol.* 2016; 82: 243–248.
73. Solaiman DKY, Ashby RD, Foglia TA. Production of polyhydroxyalkanoates from intact triacylglycerols by genetically engineered *Pseudomonas*. *Appl. Microbiol. Biotechnol.* 2001; 56(5): 664–669.
74. López NI, Pettinari MJ, Nikel PI, et al. Polyhydroxyalkanoates: much more than biodegradable plastics. *Adv. Appl. Microbiol.* 2015; 93: 73–106.
75. Huijberts GNM, Eggink G, de Waard P, et al. 13C Nuclear magnetic resonance studies of *Pseudomonas putida* fatty acid metabolic routes involved in poly(3-hydroxyalkanoate) synthesis. *Synthesis* 1994; 176(6): 1661–1666.
76. Huijberts GNM, Eggink G, de Waard P, et al. *Pseudomonas putida* KT2442 cultivated on glucose accumulates poly(3-hydroxyalkanoates) consisting of saturated and unsaturated monomers. *Appl. Environ. Microbiol.* 1992; 58(2): 536–544.
77. Blunt W, Dartiailh C, Sparling R, et al. Microaerophilic environments improve the productivity of medium chain length polyhydroxyalkanoate biosynthesis from fatty acids in *Pseudomonas putida* LS46. *Process Biochem.* 2017; 59: 18–25.
78. Fiedler S, Steinbüchel A, Rehm BH. The role of the fatty acid β-oxidation multienzyme complex from *Pseudomonas oleovorans* in polyhydroxyalkanoate biosynthesis: Molecular characterization of the *fadBA* operon from *P. oleovorans* and of the enoyl-CoA hydratase genes *phaJ* from *P. oleovorans* and *P. putida*. *Arch. Microbiol.* 2002; 178(2): 149–160.
79. Blunt W, Lagasse A, Jin, Z. Efficacy of medium chain-length polyhydroxyalkanoate biosynthesis from different biochemical pathways under oxygen-limited conditions using *Pseudomonas putida* LS46. *Process Biochem.* 2019; 82: 19–31.
80. Davis R, Chandrashekar A, Shamala TR. Role of (R)-specific enoyl coenzyme A hydratases of *Pseudomonas* sp in the production of polyhydroxyalkanoates. *Anton. Leeuw.* 2008; 93(3): 285–296.
81. Tsuge T, Taguchi K, Taguchi S, et al. Molecular characterization and properties of (R)-specific enoyl-CoA hydratases from *Pseudomonas aeruginosa*: Metabolic tools for synthesis of polyhydroxyalkanoates via fatty acid B-oxidation. *Int. J. Biol. Macromol.* 2003; 31(4): 195–205.
82. Fukui T, Doi Y. Cloning and analysis of the poly(3-hydroxybutyrate-co-3-hydroxy hexanoate) biosynthesis genes of *Aeromonas caviae*. *J. Bacteriol.* 1997; 179(15): 4821–4830.
83. Ren Q, Sierro N, Witholt B, et al. FabG, an NADPH-dependent 3-ketoacyl reductase of *Pseudomonas aeruginosa*, provides precursors for medium-chain-length poly-3-hydroxyalkanoate biosynthesis in *Escherichia coli*. *J. Bacteriol.* 2000; 182(10): 2978–2981.

84. Taguchi K, Aoyagi Y, Matsusaki H, et al. Co-expression of 3-ketoacyl-ACP reductase and polyhydroxyalkanoate synthase genes induces PHA production in *Escherichia coli* HB101 strain. *FEMS Microbiol. Lett.* 1999; 176(1): 183–190.
85. DiRusso C.C. Primary sequence of the *Escherichia coli fadBA* operon, encoding the fatty acid-oxidizing multienzyme complex, indicates a high degree of homology to eucaryotic enzymes. *J. Bacteriol.* 1990; 172(11): 6459–6468.
86. Steinbüchel A. Perspectives for biotechnological production and utilization of biopolymers: Metabolic engineering of polyhydroxyalkanoate biosynthesis pathways as a successful example. *Macromol. Biosci.* 2001; 1(1): 1–24.
87. Kraak MN, Smits THM, Kessler B, et al. Polymerase C1 levels and poly (R-3-hydroxyalkanoate) synthesis in wild-type and recombinant *Pseudomonas* strains. *J. Bacteriol.* 1997; 179(16): 4985–4991.
88. Lageveen RG, Huisman G, Preusting H, et al. Formation of polyesters by *Pseudomonas oleovorans*: Effect of substrates on formation and composition of poly-(R)-3-hydroxyalkanoates and poly-(R)-3-hydroxyalkenoates. *Appl. Environ. Microbiol.* 1989; 54: 2924–2932.
89. Qi Q, Rehm BHA, Steinbüchel A. Synthesis of poly(3-hydroxyalkanoates) in *Escherichia coli* expressing the PHA synthase gene *phaC*2 from *Pseudomonas aeruginosa*: Comparison of PhaC1 and PhaC2. *FEMS Microbiol. Lett.* 1997; 157: 155–162.
90. Sharma P, Munir R, Blunt W, et al. Synthesis and physical properties of polyhydroxyalkanoate polymers with different monomer compositions by recombinant *Pseudomonas putida* LS46 expressing a novel PHA synthase ($PhaC1_{16}$) enzyme. *Appl. Sci.* 2017; 7(3): 242.
91. Steinbüchel A, Lütke-Eversloh T. Metabolic engineering and pathway construction for biotechnological production of relevant polyhydroxyalkanoates in microorganisms. *Biochem. Eng. J.* 2003; 16: 81–96.
92. Eggink G, de Waard P, Huijberts GNM. Formation of novel poly(hydroxyalkanoates) from long-chain fatty acids. *Can. J. Microbiol.* 1995; 41: 14–21.
93. Haba E, Vidal-Mas J, Bassas M, et al. Poly 3-(hydroxyalkanoates) produced from oily substrates by *Pseudomonas aeruginosa* 47T2 (NCBIM 40044): Effect of nutrients and incubation temperature on polymer composition. *Biochem. Eng. J.* 2007; 35(2): 99–106.
94. Park WHO, Lenz RW, Goodwin S. Epoxidation of bacterial polyesters with unsaturated side chains. II. Rate of epoxidation and polymer properties. *J. Polym. Sci. Pt. A Polym. Chem.* 1998; 36(13): 2381–2387.
95. Kurth N, Brachet F, Robic D, et al. Poly(3-hydroxyoctanoate) containing pendant carboxylic groups for the preparation of nanoparticles aimed at drug transport and release. *Polymer* 2001; 43(4): 1095–1101.
96. Follonier S, Riesen R, Zinn M. Pilot-scale production of functionalized *mcl*-PHA from grape pomace supplemented with fatty acids. *Chem. Biochem. Eng. Q.* 2015; 29(2): 113–121.
97. Arkin AH, Hazer B, Borcakli M. Chlorination of poly(3-hydroxyalkanoates) containing unsaturated side chains. *Macromolecules* 2000; 33(9): 3219–3223.
98. Lee MY, Park WH, Lenz RW. Hydrophilic bacterial polyesters modified with pendant hydroxyl groups. *Polymer* 2000; 41(5): 1703–1709.
99. Stigers DJ, Tew GN. Poly(3-hydroxyalkanoate)s functionalized with carboxylic acid groups in the side chain. *Biomacromolecules* 2003; 4(2): 193–195.
100. Bear MM, Leboucher-Durand MA, Langlois V, et al. Bacterial poly-3-hydroxyalkenoates with epoxy groups in the side chains. *React. Funct. Polym.* 1997; 34(1): 65–77.
101. Ashby RD, Foglia TA, Solaiman DKY, et al. Viscoelastic properties of linseed oil-based medium chain length poly (hydroxyalkanoate) films: Effects of epoxidation and curing. *Int. J. Biol. Macromol.* 2000; 27: 355–361.

102. Ilter S, Hazer B, Borcakli M, et al. Graft copolymerisation of methyl methacrylate onto a bacterial polyester containing unsaturated side chains. *Macromol. Chem. Phys.* 2001; 202(11): 2281–2286.
103. Cakmakli B, Hazer B, Borcakli M. Poly(styrene peroxide) and poly(methyl methacrylate peroxide) for grafting on unsaturated bacterial polyesters. *Macromol. Biosci.* 2001; 1(8): 348–354.
104. Hazer B. Synthesis and characterization of the novel thermoresponsive conjugates based on poly (3-hydroxyalkanoates). *J. Polym. Environ.* 2014; 22: 159–166.
105. Babinot J, Renard E, Langlois V. Preparation of clickable poly(3-hydroxyalkanoate) (PHA): Application to poly(ethylene glycol) (PEG) graft copolymers synthesis. *Macromol. Rapid Commun.* 2010; 31(7): 619–624.
106. Pinto A, Ciesla JH, Palucci A, et al. Chemically intractable no more: *In vivo* incorporation of 'click'-ready fatty acids into poly-[(*R*)-3-hydroxyalkanoates] in *Escherichia coli*. *ACS Macro Lett.* 2016; 5(2): 215–219.
107. Nkrumah-Agyeefi S, Scholz C. Chemical modification of functionalized polyhydroxyalkanoates via 'Click' chemistry: A proof of concept. *Int. J. Biol. Macromol.* 2017; 95: 796–808.
108. Hazer B. Simple synthesis of amphiphilic poly (3-hydroxy alkanoate)s with pendant hydroxyl and carboxylic groups via thiol-ene photo click. *Polym. Degrad. Stab.* 2015; 119: 159–166.
109. Levine AC, Heberlig GQ, Nomura CT. Use of thiol-ene click chemistry to modify mechanical and thermal properties of polyhydroxyalkanoates (PHAs). *Int. J. Biol. Macromol.* 2016; 83: 358–365.
110. Modjinou T, Lemechko P, Babinot J, et al. Poly(3-hydroxyalkanoate) sulfonate: From nanoparticles toward water soluble polyesters. *Eur. Polym. J.* 2015; 68: 471–479.
111. Le Fer G, Babinot J, Versace DL, et al. An efficient thiol-ene chemistry for the preparation of amphiphilic PHA-based graft copolymers. *Macromol. Rapid Commun.* 2012; 33(23): 2041–2045.
112. Babinot J, Renard E, Droumaguet L, et al. Facile synthesis of multicompartment micelles based on biocompatible poly(3-hydroxyalkanoate). *Macromol. Rapid Commun.* 2013; 34(4): 362–368.
113. Jain-Beuguel C, Li X, Houel-Renault L, et al. Water-soluble poly(3-hydroxyalkanoate) sulfonate: Versatile biomaterials used as coatings for highly porous nano-metal organic framework. *Biomacromolecules* 2019; 20(9): 3324–3332.
114. Ashby RD, Cromwick AM, Foglia TA. Radiation crosslinking of a bacterial medium-chain-length poly(hydroxyalkanoate) elastomer from tallow. *Int. J. Biol. Macromol.* 1998; 23(1): 61–72.
115. Hazer B, Demirel SI, Borcakli M, et al. Free radical crosslinking of unsaturated bacterial polyesters obtained from soybean oily acids. *Polym. Bull.* 2001; 46(5): 389–394.
116. Schmid M, Ritter A, Grubelnik A. Autoxidation of medium chain length polyhydroxyalkanoate. *Biomacromolecules* 2007; 8(2): 579–584.
117. Muizebelt WJ, Hubert JC, Venderbosch RAM. Mechanistic study of drying of alkyd resins using ethyl linoleate as a model substance. *Prog. Org. Coatings* 1994; 24(1): 263–279.
118. Mallegol J, Gardette JL, Lemaire J. Long-term behaviour of oil-based varnishes and paints. Fate of hydroperoxides in drying oils. *JACS* 2000; 77(3): 249–255.
119. Simic MG. Free radical mechanisms in autoxidation processes. *J. Chem. Educ.* 1981; 58(2): 125.
120. Ashby RD, Foglia TA, Liu C, et al. Improved film properties of radiation-treated medium-chain-length poly(hydroxyalkanoates). *Biotechnol. Lett.* 1998; 20(11): 1047–1052.

121. Hazer DB, Hazer B, Kaymaz F. Synthesis of microbial elastomers based on soybean oily acids. Biocompatibility studies. Biomed. Mater. 2009; 4(3): 1–9.
122. Hazer B, Hazer DB, Çoban B. Synthesis of microbial elastomers based on soybean oil. Autoxidation kinetics, thermal and mechanical properties. *J. Polym. Res.* 2010; 17(4): 567–577.
123. Chung CW, Kim HW, Kim YB, et al. Poly(ethylene glycol)-grafted poly(3-hydroxyundecenoate) networks for enhanced blood compatibility. *Int. J. Biol. Macromol.* 2003; 32(1): 17–22.
124. Fernández D, Rodriguez E, Bassas M, et al. Agro-industrial oily wastes as substrates for PHA production by the new strain *Pseudomonas aeruginosa* NCIB 40045: Effect of culture conditions. *Biochem. Eng. J.* 2005; 26(2): 159–167.
125. Bassas M, Diaz J, Rodriguez E, et al. Microscopic examination *in vivo* and *in vitro* of natural and cross-linked polyunsaturated mclPHA. *Appl. Microbiol. Biotechnol.* 2008; 78(4): 587–596.
126. Sparks J, Scholz C. Synthesis and characterization of a cationic poly(β-hydroxyalkanoate). *Biomacromolecules* 2008; 9(8): 2091–2096.
127. Babinot J, Guigner JM, Renard E, et al. A micellization study of medium chain length poly(3-hydroxyalkanoate)-based amphiphilic diblock copolymers. *J. Colloid Interface Sci.* 2012; 375(1): 88–93.