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Second-generation functionalized medium-chain-length polyhydroxyalkanoates: the gateway to high-value bioplastic applications

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Summary. Polyhydroxyalkanoates (PHAs) are biodegradable biocompatible polyesters, which accumulate as granules in the cytoplasm of many bacteria under unbalanced growth conditions. Medium-chain-length PHAs (mcl-PHAs), characterized by C6-C14 branched monomer chains and typically produced by *Pseudomonas* species, are promising thermoelastomers, as they can be further modified by introducing functional groups in the side chains. Functionalized PHAs are obtained either by feeding structurally related substrates processed through the β-oxidation pathway, or using specific strains able to transform sugars or glycerol into unsaturated PHA by *de novo* fatty-acid biosynthesis. Functionalized mcl-PHAs provide modified mechanical and thermal properties, and consequently have new processing requirements and highly diverse potential applications in emergent fields such as biomedicine. However, process development and sample availability are limited due to the toxicity of some precursors and still low productivity, which hinder investigation. Conversely, improved mutant strains designed through systems biology approaches and cofeeding with low-cost substrates may contribute to the widespread application of these biopolymers. This review focuses on recent developments in the production of functionalized mcl-PHAs, placing particular emphasis on strain and bioprocess design for cost-effective production. [**Int Microbiol** 2013; 16(1):1-15]

Keywords: *Pseudomonas* spp. · polyhydroxyalkanoates (PHAs) · medium-chain-length (mcl)-PHAs · functionalization of polymers · metabolism · low-cost substrates

The relevance of functionalized medium-chain-length-PHAs

From an industrial standpoint, polyhydroxyalkanoates (PHAs) are biopolyesters attracting extensive interest as technical-grade polymers due to their singular set of properties: (i) substitution potential for industrial thermoplastics such as polypropylene, polyethylene, polyvinylchloride and polyethylene terephthalate, (ii) biodegradability both in aerobic and anaerobic conditions, including aquatic environments, (iii) bio-based, renewable origin, (iv) biocompatibility with cells and tissues and (vi) structural diversity [4]. This last characteristic is critical to define potential applications, since the specific chemical monomer composition and molecular structure will determine the biological, thermal and mechanical properties of the resulting polymer.

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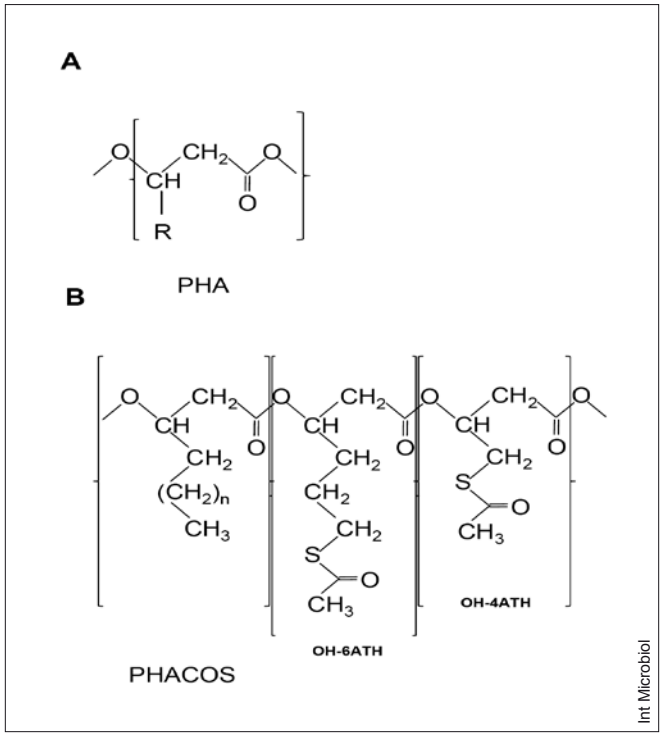


Fig. 1. Schematic representation of the chemical structure of PHAs. (**A**) PHAs are generally composed of 3-hydroxy fatty acids, where the pendant group (shown as R) varies from methyl (C1) to undecyl (C11). C3 carbon atom is asymmetric leading to pure *R* enantiomers as monomers. Thus, PHA monomers can be useful synthons in the pharmaceutical industry [9]. The best-known PHAs are PHB (R = methyl), P(HB-*co*-HV) (R = methyl or ethyl), and P(HO-*co*-HH) (R = pentyl or propyl). Second generation mcl-PHAs carry functionalized substitutions at pedant group. (**B**) Schematic representation of the chemical structure of the family of PHACOS, the acetylthioester-functionalized mcl-PHA [18]. OH-Alk 3-hydroxyalkanoate, OH-6ATH 3-hydroxy-6-acetylthiohexanoate, OH-4ATB 3-hydroxy-4-acetylthiobutanoate.

In this respect, PHAs have been classified attending to various criteria (Fig. 1): (i) their monomer size as short-chain-length PHAs (scl-PHAs), with C4-C5 monomers and medium-chain-length PHAs (mcl-PHAs) with C6-C14 monomers, (ii) their functional substituents found in the radical chain (such as double bonds or aromatic groups), and (iii) the structure of the polymer (formed by homogenous, random or block copolymers [46]). Although other added-value PHAs have been described, including in vivo protein-anchored, bioactive PHAs for biomedical applications [22,,49], this review will focus on functionalized mcl-PHAs as promising and versatile candidates for high added-value applications.

Short-chain-length-PHAs (scl-) such as poly-3-hydroxybutyrate (PHB) and its copolymers with poly-3-hydroxyvalerate (PHB-*co*-HV) are being produced on a commercial scale and have extensive application in packaging, moulding, fibres and other commodities [4]. Currently, mcl-PHAs are considered to be promising candidates for special bioplastic applications due to properties derived from their longer side-chains and altered crystalline structure, such as elasticity, hydrophobicity, low oxygen permeability, water resistance, and biodegradability. They can be moulded and processed into compostable packaging and resorbable materials for medical applications, and have also been used as food coatings, pressure-sensitive adhesives, paint binders and biodegradable rubbers [73].

Furthermore, unconventional mcl-PHAs bearing different functional moieties in their side chains can be produced through different biotechnological strategies, which will be reviewed in detail in the following sections of this article. These reactive groups enable tuning of the physical and chemical properties of the polymer, and they are also potential targets for post-biosynthetic modifications (Fig. 2). For example, the higher the molar fraction of unsaturated constituents in the monomers, the lower the resulting melting and glass transition temperatures due to crystallization inhibition by unsaturated side chains. Double bonds are also easily attacked in chemical reactions, allowing the polymer properties to be diversified even more [25]. A number of treatments have been described as responsible for crosslinking of unsaturated PHAs, namely electron-beam irradiation, UV-irradiation or even autoxidation and in some cases these PHAs are transformed into rubbers [3]. Chemical epoxidation of the pendant vinyl groups has also been applied to decrease melting temperature and increase glass transition temperature [48].

Thus, controlling monomer composition provides the chance to establish functionalized PHAs as tailor-made polymers for different applications. However, this scenario can be only achieved once we have gained a better understanding of how the incorporation of different monomers into PHA is controlled and how material properties are influenced by PHA composition.

Biochemical pathways for medium-chain-length-PHA synthesis

Chemically, PHAs are branched hetero-oxo-polyesters composed of (*R*)-3-hydroxy-alkanoic acid monomers (Fig. 1). Polyhydroxyalkanoates are synthesized as storage polymers by some *Archaea* and by a wide range of gram-

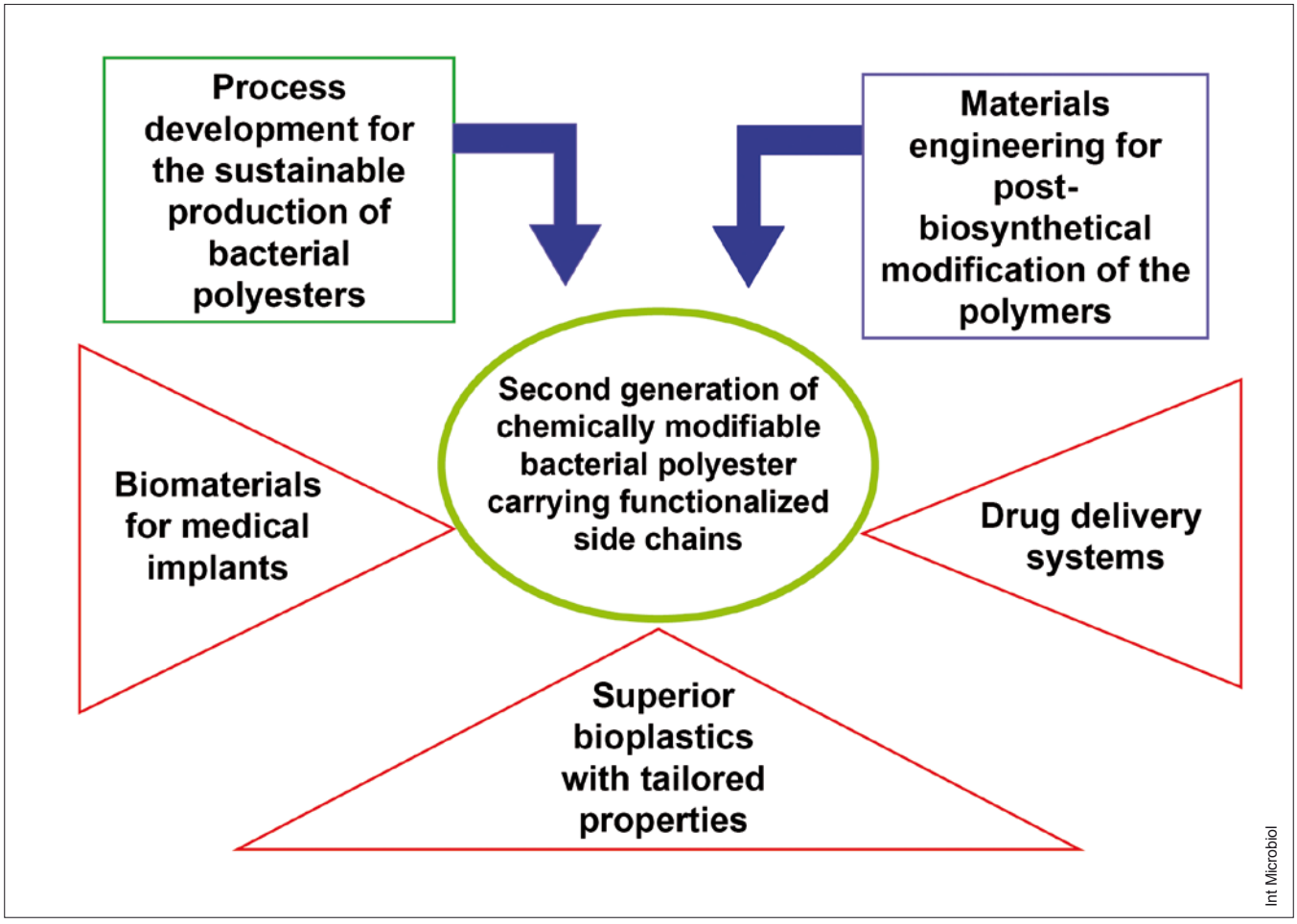
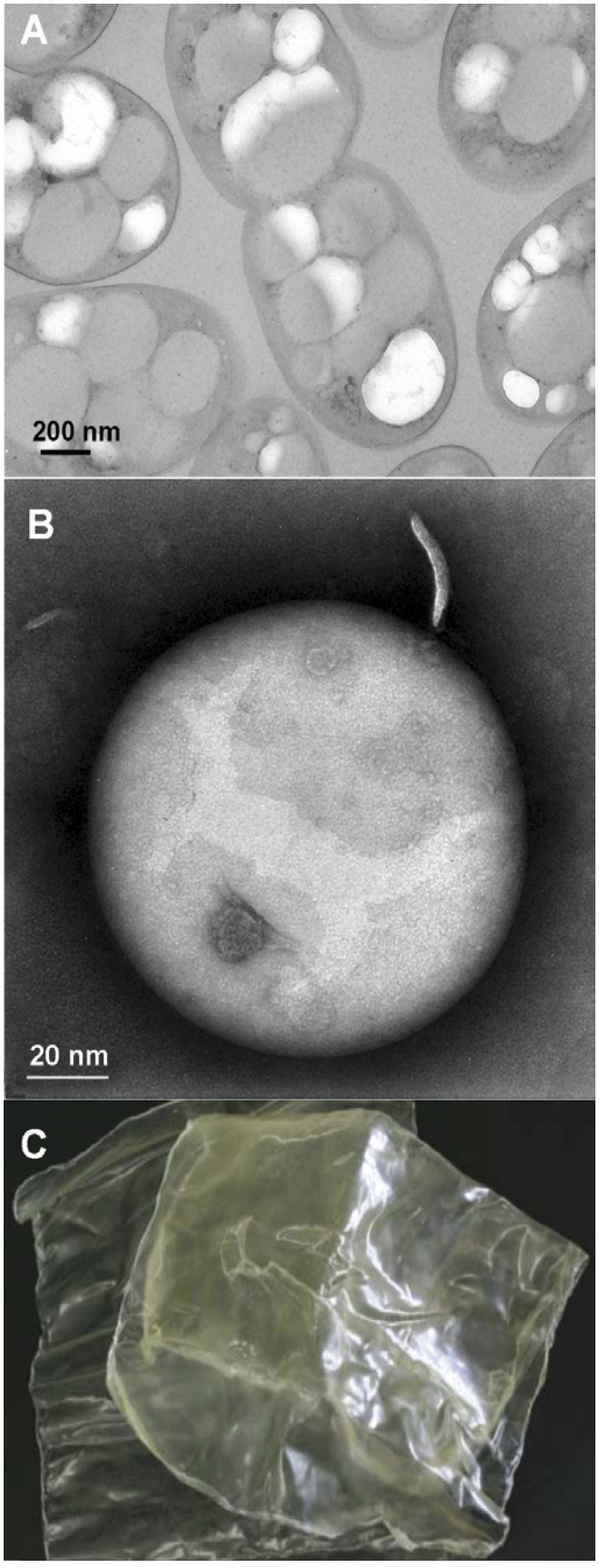


Fig. 2. Diagram of the key challenges for the production of added-value bacterial polyesters.

positive and gram-negative bacterial species, both in aerobic and anaerobic environments, although only a few strains produce PHAs at industrially significant rates. These biopolymers are accumulated as hydrophobic inclusions (PHA granules) (Fig. 3) in the bacterial cytoplasm, generally as a response to unbalanced growth conditions, such as inorganic nutrient limitations in the presence of excess carbon source [56].

A large array of bacterial species can produce PHB; however, mcl-PHAs are mainly, though not exclusively, produced by fluorescent Pseudomonads such as *Pseudomonas putida* [54] (Fig. 3). This division arises on account of differences in the in vivo substrate specificity of the PHA polymerase or synthase, the enzyme responsible for the assembly of PHA monomeric precursors, (*R*)-3-hydroxyacyl-CoAs, together with the evolved specialization of peripheral metabolic pathways and regulatory networks in each species. To date, the most widely studied and reference mcl-PHA producers are *P. putida* KT2440 (and its rifampicin-resistant mutant, KT24) and *P. oleovorans* GPo1 (ATCC 29347), reclassified and referred to herein as *P. putida* GPo1 [50,70].

In PHB producers, such as the paradigmatic *Ralstonia eutropha* H16 strain, the main enzymes involved in PHB synthesis are encoded in a gene cluster expressing: (i) a 3-ketothiolase, which condenses two acetyl-CoA molecules into acetoacetyl-CoA, (ii) a NADPH-dependent acetoacetyl-CoA reductase, which stereo-selectively reduces acetoacetyl-CoA to (*R*)-3-hydroxybutyryl-CoA, and (iii) a PHB synthase that finally converts (*R*)-3-hydroxybutyryl-CoA into PHB releasing free CoA [56]. Depending on the capacity of each strain to metabolize either simple or complex sugars, fats or oils into acetyl-CoA, PHB will be produced out of all, or only some of these substrates. Con-



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versely, Pseudomonads have a much more complex metabolism (Fig. 4). The *pha* cluster is formed in mcl-PHA producers by the genes encoding two synthases (PhaC1 and PhaC2), a depolymerase (PhaZ) [11] and other PHA-related proteins, including phasins, involved in formation, maintenance and segregation of PHA granules (PhaF and PhaI) [19]. The substrates for PHA synthesis, (*R*)-3-hydroxyacyl-CoAs, are supplied by two central pathways, β-oxidation pathway and *de novo* fatty acid synthesis, fed by fatty acids or other non-PHA-related substrates such as carbohydrate intermediates, respectively. In addition, these central pathways are supplemented by strain-dependent peripheral routes that transform non-conventional precursors into PHA.

Structurally related substrates, such as fatty acids, are processed by Pseudomonads through β-oxidation cycle [39]. The resulting acyl-CoAs are sequentially oxidized into enoyl-CoA, (S)-3-hydroxyacyl-CoA and (*R*)-3-ketoacyl-CoA. All of these intermediates may then be converted into (*R*)-3-hydroxyacyl-CoA by a stereospecific *trans*-enoyl-CoA hydratase (PhaJ), an epimerase, or a specific (*R*)-ketoacyl-CoA reductase (FabG), respectively [61]. *Pseudomonas putida* KT2440, in particular, has a large array of β-oxidation enzymes [43]. Concerning the fatty acids β-oxidation protein complex (FadAB), two sets of *fadAB* genes have been described in the strain *P. putida* KT24 [5,47]: *fadB* and *fadA* (PP_2136 and PP_2137), and *fadBx* and *fadAx* (PP_2214 and PP_2215). The former set would appear to play a role in fatty acid degradation, since although *fadB* and *fadA* deletion mutants do not show completely blocked β-oxidation, they produce PHA with a higher content of longer chain monomers, possibly due to their defective β-oxidation pathway [5,18,47].

Non-fatty acid precursors can be oxidized to acetyl-CoA and channelled into PHA by the *de novo* synthesis pathway, via (*R*)-3-hydroxyacyl-acyl carrier protein (ACP) intermediates. In this process, malonyl-CoA and its precursor, acetyl-CoA, are activated by transacylation to acyl-carrier protein (ACP). Malonyl and acyl-ACP derivatives

Fig. 3. (**A**) Transmission electronic microscopy (TEM) image of mcl-PHA-producing cells of *Pseudomonas putida* KT2442. (**B**) TEM image of a mcl-PHA granule isolated from *P. putida* KT2442. Granules are composed of mcl-PHA (shown as whitest core of the granule) coated by a monolayer of phospholipids and granule-associated proteins (gray layer at the surface of the granule). The most abundant proteins in the surface granules are phasins. Polymerases and intracelluar depolymerases are also associated to granules. (**C**) mcl-PHA film isolated from *P. putida* KT2440 and octanoic acid as growth and mcl-PHA precursor.

are condensed by ketoacyl-ACP synthetase, reduced, losing a ketone group, dehydrated, and saturated to the corresponding (*R*)-3-hydroxyacyl-ACP chain (Fig. 4), which may be further elongated in two-carbon growing chains. Acyl-ACP intermediates can then be re-transformed into (*R*)-3-hydroxyacyl-CoAs by a specific transacylase, PhaG, present in most *Pseudomonas*. Only *P. putida* GPo1 and *P. fragi* are unable to synthesize mcl-PHA out of non-fatty acid substrates, such as gluconate, apparently due to deficiencies in PhaG transcription [29,55]. Unsaturated monomers such as 3-hydroxy-5-dodecenoate and 3-hydroxy-7-tetradecenoate are also generated in this process [30], by fatty acid *de novo* synthesis that introduces double bonds into acyl-CoA or acyl-ACP intermediates, a regulatory mechanism of membrane fatty acid composition that alters membrane fluidity in response to changes in ambient temperature [14]. Tables 1, 2 and 3 revise the precursors described in the literature to produced mcl-PHA with substitutions at the side chain.

Functionalized PHAs through β-oxidation pathway

Structurally related carbon sources, such as alkanoic and alkenoic acids are incorporated to PHA by mcl-PHA producers through the β-oxidation pathway without being completely oxidized to acetyl-CoA [54]. The resulting PHAs are generally copolymers, a consequence of the sequential degradation of acetyl-CoA units (e.g., poly[3-hydroxy-octanoic-*co*-3-hydroxy-hexanoic]), at around 95 mol% of 3-hydroxy-octanoic acid and 5 mol% of 3-hydroxy-hexanoic is produced when *P. putida* is fed with octanoic acid). Linear and branched n-alkanes and alkenes can also be channelled to polymer accumulation, when processed by the alkane oxidation pathway encoded on the octane (OCT) plasmid [70]. Fatty acids however are usually preferred for bioprocessing as they overcome the limitations derived from two-phase fermentation systems required for hydrocarbons and avoid risk of explosion [67].

The structure of the precursor, including unsaturations, is recovered in the resulting mcl-PHA. Early reports by Lageveen and co-workers [34] describe the generation of PHA containing 3-hydroxy-5-hexenoate, 3-hydroxy-7-octenoate, 3-hydroxy-8-nonenoate and 3-hydroxy-9-decenoate when octene, nonene or decene were supplied. Non-terminal unsaturations were also successfully introduced. The molar fraction of unsaturated monomers depends on both the carbon source supplied and the metabolic capabilities of the bacterial strain. For example, whereas *P. putida* GPo1 fed with octene produces PHA with 50 mol% 3-hydroxyalkenoate content [53], when 10-undecenoate is used, practically all monomers are unsaturated. The molar content of unsaturated monomers can then be controlled by co-feeding nonanoic acid, as it correlates with the amount of 10-undecenoate. When *P. putida* was fed with mixtures of octanoic acid and undecenoic acid, the monomeric composition seemed to depend on the growth rate both in batch and chemostat cultures, but also correlated linearly with the fatty acids fed [24].

This strategy can be extended to a large variety of organic compounds in order to generate functionalized PHAs [62]. Depending on their chemical structure and the specific bacterial species, the compounds will support both cell growth and PHA accumulation, growth alone, or will support neither cell growth nor PHA accumulation [31]. Simultaneous feeding strategies based on co-metabolism can be applied to address the last two cases, as it has been shown that precursors that do not enable PHA production or growth can be processed when accompanied by an efficiently processed substrate, such as octanoic or undecenoic acid [24]. The first examples of these strategies include co-polyesters obtained from mixtures of octanoic acid and methylalkanoic acids such as 7-methyloctanoate, which contained both the unsubstituted and the methyl-branched 3-hydroxyoctanoate and 3-hydroxyhexanoate units. Although no polymer was formed when *P. putida* GPo1 was grown on pure 5- and 6-methyloctanoates, the presence of methyl-branched units in the polymer obtained by co-metabolism was detected when 5- and 6-methyloctanoate were fed as mixture with octanoate [37]. There was a direct correlation between the variation in ratios of PHA repeating units and feeding mixture composition.

This concept has been exploited to produce a plethora of tailor-designed mcl-PHAs, with highly diverse structures that include acetylthioester, acetoxy, alkoxy, amino, cyano, cyclohexyl, epoxy, halogenated, hydroxy or propylthiol groups (Tables 1 and 2). Note that besides the precise functional moeity introduced, a larger variety of PHA compositions can be generated in most cases, with varying molar amounts of functional groups, by altering the ratio of co-substrates.

Together with unsaturations, functional groups prone to chemical modifications have been introduced, mainly in the canonical *P. putida* GPo1 and KT24 strains, such as thio, bromine, chlorine and fluorine radicals, cyano and

Table 1. Precursors used in the literature to produce functionalized mcl-PHA (branched alkyl, cyclohexyl, halogenated)

Precursor	% Mol functional groups	% PHA	*Pseudomonas* strains	Ref.
Group at mcl-PHA side chain: Branched alkyl				
Citronellol	>99.0	27.2	*P. citronellolis* ATCC 13674	[1-T1]
Alkylhydroxyoctanoates	5.0	16.0–22.0	*P. putida* GPo1	[2-T1]
Methyloctanoates	3.0–96.4	7.3–19.2	*P. putida* GPo1	[3-T1, 4-T1]
Group at mcl-PHA side chain: Cyclohexyl				
Cyclohexylbutyric acid	>99.0	21.0	*P. cichorii* YN2	[5-T1]
Cyclohexylvaleric/butytic acid	13.2–100.0	2.0–31.0	*P. putida* GPo1	[6-T1, 7-T1]
Unsaturated				
Alkenes (C7-C9)	45.0–55.0	5.0–13.4	*P. putida* GPo1	[8-T1]
Undecenoic acid	27.1–100.0	1.8–37.4	*P. putida* KCTC 2407	[9-T1]
Undecenoic acid	5.0–99.0	17.0–34.0	*P. putida* GPo1	[10-T1,11-T1, 12-T1]
Hydroxyoctenoic acids	63.5-81.6	10.3-12.5	*P. putida* GPo1	[13-T1]
Dicarboxylic acids (C4-C10)	4.7-11.7	7.3-14.4	*P. citronellolis* ATCC 13674	[14-T1]
Undecynoic acid	32.0-100.0	N/A	*P. putida* GPo1	[15-T1]
Undecynoic acid	22.0-100.0	7.5-22.5	*P. putida* KCTC 2407	[15-T1]
Group at mcl-PHA side chain: Halogens				
Bromoalkanoic acids (C6-C11)	3.7-38.0	4.5-38.2	*P. putida* GPo1	[16-T1, 17-T1]
Chlorooctane	69.0	5.0-19.0	*P. putida* GPo1	[18-T1]
Fluorohexanoic/nonanoic acids	1.9-8.8	N/A	*P. putida* GPo1	[19-T1]
Fluorohexanoic/nonanoic acids	1.0-17.3	N/A	*P. putida* KT2440	[19-T1]
Fluorophenoxyundecanoic acid	>99.0	8.5-13.9	*P. putida* 27N01	[20-T1]

[1-T1] Choi MH, Yoon SC (1994) Appl Environ Microbiol 60:3245-3254 · [2-T1] Scholz C, et al. (1994) Macromolecules 27:6358-6362 · [3-T1] Fritzsche K, et al. (1990) Int J Biol Macromol 12:92-101 · [4-T1] Lenz RW, et al. (1992) FEMS Microbiol Lett 103:207-214 · [5-T1] Honma T, et al. (2004) J Environ Biotechnol 4:49-55 · [6-T1] Andújar M, et al. (1997) Macromolecules 30:1611-1615 · [7-T1] Kim DY, et al. (2001) Int J Biol Macromol 29:145-150 · [8-T1] Lageveen RG, et al. (1988) App Environ Microbiol 54:2924-2932 · [9-T1] Kim DY, et al. (2000) Int J Biol Macromol 28:23-29 · [10-T1] Kim YB, et al. (1995) J Polym Sci Part A: Polym Chem 33:1367-1374 · [11-T1] Park WH, et al. (1998) Macromolecules 31:1480-1486 · [12-T1] Sparks J, Scholz C (2008) Biomacromolecules 9:2091-2096 · [13-T1] Fritzsche K, et al. (1990) Int J Biol Macromol 12:85-91 · [14-T1] Choi MH, Yoon SC (1994) Appl Environ Microbiol 60:3245-3254 · [15-T1] Kim DY, et al. (1998) Macromolecules 31:4760-4763 · [16-T1] Kim YB, et al. (1992) Macromolecules 25:1852-1857 · [17-T1] Lenz RW, et al. (1992) FEMS Microbiol Lett 103:207-214 · [18-T1] Doi Y, Abe C (1990) Macromolecules 23:3705-3707 · [19-T1] Kim O, et al. (1996) Macromolecules 29:4572-4581 · [20-T1] Takagi Y, et al. (2004) Eur Polym J 40:1551-1557

Table 2. Precursors used in the literature to produce functionalized mcl-PHA (acetoxy, ester, alkoxy, epoxy, thio, cyano, nitro)

Precursor	% Mol functional groups	% PHA	*Pseudomonas* strains	Ref.
Group at mcl-PHA side chain: Acetoxy				
Octanone , octylacetate	3.3–10.3	19.0–26.0	*P. putida* GPo1	[1-T2]
Ester, alkoxy, epoxy				
Alkylheptanoate	2.5–60.0	0.27–6.9	*P. putida* GPo1	[2-T2]
Alkxyhexanoic/octanoic/undecanoic acids	31–100.0	3.0–15.0	*P. putida* GPo1	[3-T2]
10-epoxyundecanoic acid	25.0–75.0	3.0–18.0	*P. putida* GPo1	[4-T2]
C7-C12 alkenes	4.2–20.0	25.0–35.0	*P. cichorii* YN2	[5-T2]
Soybean oil	63.0	63.0	*P. stutzeri* 1317	[6-T2]
Group at mcl-PHA side chain: Thio, sulfanyl				
Acetylthiohexanoic acid	16.5–78.5	5.0–37.0	*P. putida* KT2442, KT24FadB	[7-T2]
Propylthiohexanoic acid	14.5–17.5	24.0–43.0	*Ralstonia eutropha* DSM541	[8-T2]
Propylthioundecanoic acid	6.02% w/w S	N/A	*P. putida* KT2440	[8-T2]
Methylsulfanylphenoxyvaleric acid	12.2–35.6	10.9–21.4	*P. cichorii* H45, YN2	[9-T2]
Methylsulfanylphenoxyvaleric acid	18.4	9.9	*P. jessenii* P161	[9-T2]
Thiophenoxyundecanoic acid	>99.0	9.6–19.5	*P. putida* 27N01	[10-T2]
Group at mcl-PHA side chain: Cyano, nitro				
Cyanoundecanoic acid	17.0–32.0	19.5–36.2	*P. putida* GPo1	[11-T2]
Cyanophenoxyhexanoic acid	0-2.2	N/A	*P. putida* GPo1	[13-T2]
Cyanophenoxyhexanoic acid	0.0–34.0	2.8–12.0	*P. putida* KT2440	[12-T2, 13-T2]
Nitrophenoxyhexanoic acid	4.2–5.1	N/A	*P. putida* GPo1	[13-T2]
Nitrophenoxyhexanoic acid	1.0–4.8	N/A	*P. putida* KT2440	[13-T2]
Dinitrophenylvaleric acid	1.2–6.9	15.0–40.0	*P. putida* GPo1	[14-T2]

[1-T2] Jung K, et al. (2000) Macromolecules 33:8571-8575 · [2-T2] Scholz C, et al. (1994) Macromolecules 27:2886-2889 · [3-T2] Kim DY, et al. (2003) J Microbiol Biotechnol 13:632-635 · [4-T2] Bear MM, et al. (1997) React Funct Polym 34:65-77 · [5-T2] Imamura T, et al. (2001) Int J Biol Macromol 29:295-301 · [6-T2] He W, et al. (1998) FEMS Microbiol Lett 169:45-49 · [7-T2] Escapa IF, et al. (2011) App Microbiol Biotechnol 89:1583-1598 · [8-T2] Ewering C, et al. (2002) Microbiology 148:1397-1406 · [9-T2] Kenmoku T, et al. (2002) EP1275727 B1. Priority date: 10-07-2002 · [10-T2] Takagi Y, et al. (1999) Macromolecules 32:8315-8318 · [11-T2] Lenz RW, et al. (1992) FEMS Microbiol Lett 103:207-214 · [12-T2] Gross RA, Kim O-Y, et al. (1996) Polym Int 39:205-213 · [13-T2] Kim O, et al. (1995) Can J Microbiol 41:32-43 · [14-T2] Aróstegui SM, et al. (1999) Macromolecules 32:2889-2895

epoxy groups (Table 2). The introduction of such groups also modifies the thermal properties, and thus the processing requirements of the resulting polymers, enabling higher melting and lower glass-transition temperatures, or modi-

fied biological activities. For example, different PHA compositions bearing acetylthioester groups in the side chain have been produced using *P. putida* KT24. These novel mcl-PHAs (PHACOS) can be obtained using decanoic acid as an inducer of growth and PHA synthesis, and 6-acetylthiohexanoic acid as PHA precursor, in one or two-stage cultivation strategies. The composition of PHACOS includes 6-acetylthio-3-hydroxyhexanoic acid (OH-6ATH) and the shorter derivative 4-acetylthio-3-hydroxybutanoic acid (Fig. 1). These polymers have tunable thermal properties as a result of different glass transition temperatures. Use of the derived strain KTFadB, mutated in the *fadB* gene from the β-oxidation pathway, gives rise to PHACOS overproduction and the polymer contains mainly OH-6ATH units [18] [Escapa IF, Morales V, García JL, Prieto MA (2012) Synthesis of polyhydroxyalkanoates (PHAS) with thioester groups in the side chain. International Patent WO 2012/038572].

Many reports have been devoted to the accumulation of aromatic radicals in *P. putida* strains (Table 3), in order to mimic widespread synthetic aromatic polyesters such as polyethyleneterephtalate (PET). Processing of aromatic substrates into PHA seems to depend both on the chemical structure of the precursors and the bacterial strain. Homopolymers with 100 mol% content of aromatic moieties, random copolymers or a blend of both have been produced. Bacterial isolates that are able to transform aromatic precursor into alkyl-PHAs have also been reported [72]. Hydrophilized PHAs bearing alkoxy, acetoxy or hydroxy groups are also of great interest, as they show enhanced solubility and biocompatibility [26].

Although diverse fermentation strategies based on co-metabolism have been extensively applied to produce functionalized mcl-PHAs, few works have been published connecting these findings to the molecular basis driving the carbon metabolism in *Pseudomonas* [10,20]. This is partially caused by the complex regulation of mcl-PHA metabolism, which is controlled: (i) at the enzymatic level, by cofactor inhibition and metabolite availability [17], (ii) at the transcriptional level, by specific and global transcriptional regulatory factors [9,10], and (iii) at the translational level, driven by global post-transcriptional regulators.

Recently, it has been shown that synthesis and degradation of mcl-PHA consist of a synchronous cycle in *P. putida* KT2440, as PHA synthase and depolymerase are simultaneously produced ensuring the PHA turnover. In *P. putida* KT2440, simultaneous production of PhaC (C1 and C2) and PhaZ enzymes is controlled by the protein PhaD [10], one of the few activators of the TetR-like family of transcriptional regulators. The *phaD* gene controls its own transcription and that of *phaIF* operon coding for phasins. Mutagenesis analyses and 3D structural models suggest that PhaD behaves as a carbon-source-dependent activator of the *pha* cluster, possibly induced by CoA intermediates of β-oxidation. The *pha* cluster would only be indirectly induced by fatty acids, such as octanoic acid, the true inducer being CoA derivatives of β-oxidation. This hypothesis supports co-metabolism effects and also the lower activation of PHA production on non-structurally related carbon sources, such as glucose, for which PHA cycle is only driven on basal activities of the *pha* promoters. However, differences in activation mechanisms are expected among the different mcl-PHA-producing strains because phylogenetically related strains, such as *Pseudomonas corrugata* and *Pseudomonas mediterranea*, bear vast differences in nucleotide sequences in the intergenic regions of *pha* cluster [66]. In addition to the intrinsic interest in PHAs due to their wide ranging biological, mechanical and thermal properties, much effort is still required to unravel the molecular and biochemical underpinnings of tuned PHA production.

De novo synthesis for functionalized PHAs

Although most mcl-PHA intermediates are obtained through fatty-acid β-oxidation, non-related carbon sources such as acetate, ethanol, fructose, glucose, gluconate or glycerol are also channelled to PHA by the *de novo* fatty acid pathway in *Pseudomonas* species such as *P. putida* or *P. aeruginosa* [68]. The main monomer found in these mcl-PHAs is 3-hydroxydecanoate, but unsaturated 3-hydroxy-5-dodecenoate and 3-hydroxy-7-tetradecenoate are also found [30]. The molar fraction of unsaturated monomers usually ranges from around 5 to 10 mol% depending on the strain [60,64], and it can be increased by reducing culture temperature [30]. This is consistent with the isolation of a psychrotrophic *P. fluorescens* strain able to accumulate mcl-PHA with up to 35 mol% of 3-hydroxy-5-dodecenoate content [36].

The use of carbohydrate-related sources to partially unsaturated PHA side chains is advantageous in terms of substrate cost and invariant monomer composition although PHA yields are generally lower in comparison to fatty acid

Table 3. Precursors used in the literature to produce functionalized mcl-PHA (aromatics)

Precursor	% Mol functional groups	% PHA	*Pseudomonas* strains	Ref.
Group at mcl-PHA side chain: Aromatics (benzoyl, methylphenoxy, phenoxy, phenyl)				
Benzoylalkanoic acids (C4–C8)	8.3–79.8	3.0–41.0	*P. cichorii* YN2	[1-T3]
Methylphenoxyalkanoic acids (C6, C8)	40.0–65.0	0.8–13.0	*P. putida* KCTC 2407	[2-T3]
Methylphenoxyalkanoic acids (C6, C8) (PVA, NA)	68.0–100.0	N/A	*P. putida* GPo1	[3-T3]
Methylphenoxyalkanoic acids (C6, C8)	24.0–100.0	0.8–23.7	*P. putida* KCTC 2407	[3-T3]
Phenoxyundecanoic acid	>99.0	>19.0	*P. putida* GPo1	[4-T3]
Phenoxyalkanoic acids (C6,C8,C11)	100.0	10.0–11.0	*P. putida* GPo1	[5-T3]
Phenoxyundecanoic acid	12.0–100.0	13.1–46.8	*P. putida* BM01	[6-T3]
Phenylvaleric acid	13.7–19.5	18.0–56.0	*P. putida* BM01	[7-T3]
Phenylvaleric acid	>99.0	N/A	*P. putida* GPo1	[8-T3]
Phenyl, tolylvaleric/octanoic acids	3.0–64.0	0.03–23.0	*P. putida* GPo1	[9-T3]
Phenylalkanoic acids (C4-C8)	>95.0	22.0–42.0	*P. jessenii* C8	[10-T3]
Phenylalkanoic acids (C4-C8)	>95.0	8.0–36.0	*P. putida* S12, CA-1, H4, F6, D5	[10-T3]
Phenylalkanoic acids (C6-C11)	>99.0	10.0–87.0	*P. putida* U fadA-, ΔFadBA-PhaZ	[11-T3, 12-T3, 13-T3]
Phenylvaleric acid	>99.0	25.0	*P. putida* GPo1	[14-T3]
Phenylvaleric acid	12.6–40.6	15.1–39.2	*P. putida* GPo1	[15-T3]

[1-T3] Honma T, et al. (2004) J Environ Biotechnol 4:49-55 · [2-T3] Kim DY, et al. (2000) Int J Biol Macromol 28:23-29 · [3-T3] Kim YB, et al. (1999) Macromolecules 32:6058-6064 · [4-T3] Ritter H, Spee von AG (1994) Macromol Chem Phys 195:1665-1672 · [5-T3] Kim YB, et al. (1996) Macromolecules 29:3432-3435 · [6-T3] Song JJ, Yoon SC (1996) App Environ Microbiol 62:536-544 · [7-T3] Song JJ, et al. (2001) J Microbiol Biotechnol 11:435-442 · [8-T3] Curley JM, et al. (1996) Int J Biol Macromol 19:29-34 · [9-T3] Curley JM, et al. (1996) Macromolecules 29:1762-1766 · [10-T3] Tobin KM, O'Connor KE (2005) FEMS Microbiol Lett 253:111-118 · [11-T3] Abraham GA, Get al. (2001) Biomacromolecules 2:562-567 · [12-T3] García B, et al. (1999) J Biol Chem 274:29228-292241 · [13-T3] Olivera ER, et al. (2001) Mol Microbiol 39:863-874 · [14-T3] Fritzsche K, et al. (1990) Macromol Chem Phys 191:1957-1965 · [15-T3] Kim YB, et al. (1991) Macromolecules 24:5256-5260

substrates. Nevertheless, the wide metabolic versatility of Pseudomonads, which do not prefer glucose over alternative carbon sources, can be exploited to generate functionalized PHAs out of a variety of substrates. Pseudomonads lack the main glycolytic enzyme, phosphofructokinase, but instead transform glucose into 6-phosphogluconate (6PG) through three convergent pathways. Then 6PG is metabolized by the Entner-Doudoroff (ED) enzymes into pyruvate to finally yield acetyl-CoA, which may be channelled into citric acid cycle or PHA cycle through *de novo* fatty acid synthesis (Fig. 4) [50].

The expression of the main metabolic steps of the phosphorylative branch of carbohydrate metabolism and ED pathways in *P. putida* KT2440 is tightly regulated by transcriptional repressors such as HexR, specifically induced by 2-keto-3-deoxy-6-P-gluconate (KDPG). Modified tran-

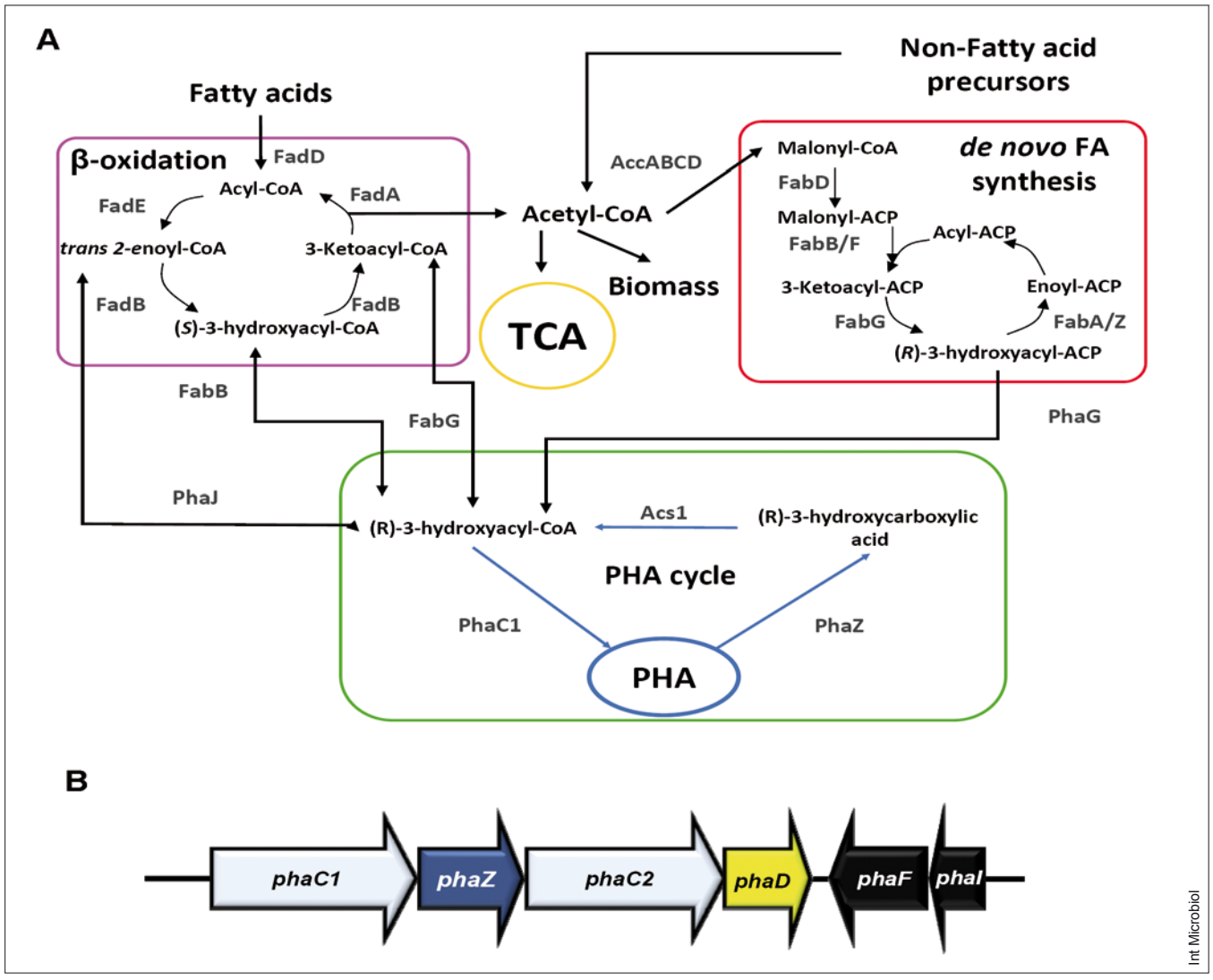

Fig. 4. (**A**) Metabolic pathways involved in PHA biosynthesis of Pseudomonads. In *Pseudomonas putida* synthesis and degradation of PHA were found to operate as a continuous cycle with 3-hydroxy-fatty acids released from PHA granules by PhaZ depolymerase and activated to 3-hydroxyacyl-CoAs by ACS1 acyl synthetase with the concomitant consumption of one ATP molecule. These activated monomers will be either metabolized via fatty acid degradation or re-incorporated into PHA by PHA synthase. The specific PHA metabolic pathways are interconnected with the main central pathways that converge in acetyl-CoA. (**B**) Genetic organization of *pha* cluster in *P. putida*. The open arrows indicate the directions of gene transcription. Phasins PhaI and PhaF encoding genes are shown in black. Enzymes-encoding genes are shown in blue; *phaC1* and *phaC2* genes (light blue) code for two synthases and are separated by the *phaZ* gene (dark blue) coding for an intracellular depolymerase. *phaD* gene (yellow) encodes a transcriptional regulator.

scription of HexR has been shown to improve the efficiency of the use of alternative carbon sources, such as xylose, in engineered *P. putida* S12 strain, balancing ED and pentose phosphate pathway activities [41].

Besides sugars, glycerol has also been considered to be an advantageous raw material for PHA production due to its availability as a by-product of the biodiesel industry [21]. In *Pseudomonas* strains, glycerol is converted into glycerol-3-phosphate and then to dihydroxyacetone phosphate, which is catabolized by a branch of the ED pathway [16,63]. Glycerol use is hindered by a prolonged lag phase, caused by the transcriptional repressor GlpR. However, GlpR repression can be overcome by adding small amounts of fatty acids as co-feeders, fully eliminating lag-phase in *P. putida* KT2440. Knock-out GlpR mutants also result in faster consumption of glycerol, together with improved PHA accumulation, possibly as a consequence of the larger availability of intermediates generated by *de novo* fatty acid synthesis [16].

Hence, to improve the utilization of non-structurally related carbon sources in bacterial PHA production re-

quires knowledge of the metabolic steps involved in their catabolism. It is also necessary to study their relationship with the central metabolic pathways, possibly through the cellular levels of key metabolic intermediates that may be directed to growth, energy generation or PHA synthesis. In-depth knowledge of the PHA cycle is therefore essential to coordinate global metabolism with resource availability in PHA producing microorganisms [9,17].

Functionalized PHAs from non-food raw materials

In addition to high-added value applications for functionalized PHAs, cost-efficient exploitation of these polymers can be approached through the use of different agro-industrial by-products [33]. Raw materials that do not compete or interfere with food supply are preferred in line with global sustainability strategies and biorefinery concepts.

The ability of different bacteria to use plant oils or animal fats to produce PHA has been widely described [7,23,27,40,65]. The same concept of controlling PHA composition in functionalized PHAs by feeding mixtures of carbon sources can be readily translated to unsaturated plant or animal derived fatty acids.

Accordingly, by using oleic or linoleic acid, which are constituents of a large number of plant oils, the monomers 3-hydroxy-6-dodecenoate, 3-hydroxy-5-tetradecenoate and 3-hydroxy-5,8-tetradecenoate have been incorporated into the PHA produced by *P. putida* GPo1 [12]. Also 10-undecenoic acid can be derived from inexpensive castor oil, and used to produce PHA containing 3-hydroxy-10-undecenoate, 3-hydroxy-8-nonenoate and 3-hydroxy-6-heptenoate in this strain [32]. Ashby and co-workers [2] evaluated the production of mcl-PHA by *Pseudomonas resinovorans* from glucose, soybean and coconut oils. Culture on mixtures of these substrates led to monomer composition, and thermal and mechanical properties that were intermediate to those of PHAs obtained from pure glucose or plant oils. Also mixtures of linoleic and oleic acids as well as different plant oils were supplied to *P. putida* IPT046 and *P. aeruginosa* IPT171 to evaluate the contribution of unsaturated fatty acids to the insertion of unsaturated monomers into the polymer [64]. A non-linear relationship between the molar fractions of 3-hydroxy-6-dodecenoate detected in PHA and the linoleic acid supplied was observed, which is compatible with the ability of biosynthesis system saturation to channel intermediates of β-oxidation to PHA synthesis.

Some bacterial species have also been shown to produce functionalized mcl-PHA from non-related carbon sources. *P. aeruginosa* 44T1 cultured in *Euphorbia* and castor oils produced mcl-PHA containing epoxy groups, besides other constituents normally found in PHA produced from fatty acids. Additional hydroxyl groups not involved in ester bond and the unsaturated monomer 6-hydroxy-3-dodecenoate have also been detected [15]. Epoxy group formation from soybean oil and 1-alkenes has been also reported for *Pseudomonas stutzeri* and *Pseudomonas cichorii* [28]. Concomitant lipase-catalysed self-epoxidation of the unsaturated precursors may be hypothesized as the underlying mechanism.

Triglycerides present in plant oils and animal fats can also be used for mcl-PHA production although the ability to convert these substrates is associated to lipase production and therefore much more restricted [1,7,66]. Recombinant expression of lipase genes has been used to improve triglyceride use [66]; however, functional expression of lipase in *P. putida* GPo1 did not enable growth on soybean oil (JGC Gómez, personal communication).

In relation to sugars, the use of an engineered strain of *P. putida* KT2440 for PHA production out of xylose has been reported recently [35]. The hemicellulose derivate, second in abundance after glucose and important for the production of second-generation bioethanol [13], can be used to support growth whereas mcl-PHA production is sustained by fatty acid addition in a sequential feeding strategy. Although only 20% w/w PHA content was obtained, the scheme may possibly be extended to combine cheap hemicellulosic hydrolysate with more expensive precursors to produce functionalized mcl-PHAs.

Perspectives and challenges

Research into functionalized mcl-PHAs spans the last 25 years; nonetheless, opportunities remain open for the enhancement both of microbial strains and bioprocesses to produce optimal mcl-PHA compositions. *In silico* genome-scale analysis of *P. putida* KT2440 has unveiled several isoenzyme-coding genes involved in hydroxy-acyl-CoA generation [43], which supports the well known high metabolic diversity of this strain, enabling it to incorporate different monomers into biopolyester. A higher fraction of long-chain-length monomers was produced when the main set of genes *fadA* and *fadB* was knocked-out [45,47]. This could be due to lower efficiency of the alternative

β-oxidation routes on mcl-fatty acid precursors. In fact, when *fadAB* mutant cells were cultured with conventional aliphatic precursors, such as decanoic acid, a higher proportion of 3-hydroxydecanoic acid monomer was detected when compared to that of the wild type. However, this effect was less evident when unsaturated fatty acids, such as 10-undecenoic acid, were used as carbon sources [18]. It would be interesting to ascertain if this could be ascribed to a kinetic effect or to the substrate specificity of the active β-oxidation routes. The possibility of controlling PHA monomer composition has been demonstrated by the inactivation of various selected sets of those *fad* genes, and a collection of homopolymers has been produced [6,38,69, 71]. An interesting approach would be to verify the effect of those mutations on the incorporation of unsaturated monomers into the polymer when produced not only from pure fatty acids, but also from fats and oils. Unsaturated monomers have also been detected in scl-PHA producers, such as *Rhodospirillum rubrum* or *Burkholderia* sp. [57]. Although some evidence would suggest the existence of two PHA synthases in *Burkholderia* [58,59], the metabolic pathway supplying 3-hydroxy-4-pentenoic acid monomers remains unknown, thus hindering the performance of processes with controlled insertion of this monomer in the PHA.

The growing availability of omics data and increased understanding of model strains have facilitated bottom-up approaches to design specialized strains [17,44]. By combining transcriptomic, proteomic and metabolomic measurements under well-controlled nutrient limitations, Poblete and co-workers [52] have reported the global multi-omics analysis of the *P. putida* KT2440 response to various nutrient limitations. Such studies provide a tremendous amount of knowledge that will be of great assistance in metabolic engineering design, thereby enhancing and diversifying mcl-PHA production. Furthermore, in-depth modelling and computational analysis of both metabolic versatility and PHA biosynthesis pathways represent a valuable tool for the design and production of less common and/or new functional PHA monomers. One example is the use of a detailed genome-scale model of *P. putida* (iJN746) to identify *in silico* a large set of non-glycolytic substrates, such as aromatic compounds, which are highly suitable for the production of different PHA monomers [44]. Very recently, a systems metabolic engineering approach has been reported, driven by *in silico* modelling to tailor *P. putida* for mcl-PHA synthesis on glucose [51]. Using the physiological properties of the parent wild type as constraints, elementary flux mode analysis of a large-scale model of *P. putida* metabolism was used to predict genetic targets for strain engineering. Among a set of priority-ranked targets, glucose dehydrogenase (encoded by *gcd*) was predicted as the most promising deletion target. This study illustrates the power of computational prediction to tailor microbial strains for the enhanced biosynthesis of added-value compounds. Moreover, the control of unsaturated content when mcl-PHA is produced from carbohydrates and related carbon sources will depend on the selection of strains showing enzymatic systems with a greater ability to channel such monomers into PHA precursors. In this respect, directed evolution strategies should be applied to modify enzymes involved in directing intermediates from fatty acid biosynthesis to PHA biosynthesis.

With regard to low-cost substrates, unsaturated fatty acid constituents present in oils or fats offer an excellent opportunity to insert functional groups in PHA. However, fats and oils are mixtures of different fatty acids that imply an even more complex metabolic network for tailoring polymers. This represents a challenge to mcl-PHA production and will depend on acquiring comprehensive knowledge of the factors involved in fatty acid and PHA metabolism. To reduce PHA production costs, crops could be used as a raw material source, though preferably those not competing with food sources. However, the greatest challenge will be the use of waste and biowaste, mostly because of their substrate and contaminant contents. Some such tested wastes from fats, oils and fatty acids include residues from food processing, such as waste frying oil [7] and residues from oil processing, such as oil sludge [23]. Considering carbohydrates and related carbon sources, a number of biowastes have been tested as substrates to produce PHA, namely glycerol, rice chaff, coconut oil cake, cotton seed cake, wafer residue, citrus pulp waste. Glycerol is an important carbon source, since it is the main residue from biodiesel production [8,21]. Although some of those residues may have the potential to generate unsaturated monomers, PHA composition has not been reported for most of them.

Last but not least, it is essential to define which PHA compositions are the best. This should be done by performing processing and technological assays to further characterize emerging strategies. To achieve this goal requires precise and robust manufacturing processes, to scale-up and provide samples to consolidate the design of tailor-made PHAs. Data correlating the type and amount of precursors used with the molar fraction of monomers detected in the polymer, and the changes in physical and chemical

properties of the resulting PHA could be used in the future to establish mathematical models. The different variables involved in these processes could be defined in order to provide a robust basis for directed optimization strategies to produce tuned functionalized mcl-PHAs.

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