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Synthesis of polyhydroxyalkanoates through the biodegradation of poly(*cis*-1,4-isoprene) rubber

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The search of alternative substrates for the synthesis of polyhydroxyalkanoates (PHA) has become an important factor in order to decrease the production costs. Therefore, the use of industrial by-products or waste materials as carbon and energy sources for different PHA-producing microorganisms has been evaluated during the last decades. Recombinant strains of *Gordonia polyisoprenivorans* VH2 harboring plasmid pAK68, which contains *phaCAB* from *Ralstonia eutropha* and plasmid pAK71 comprising *phaC1* from *Pseudomonas aeruginosa* were evaluated for PHA production. Cultivations were performed in shake flasks, using different carbon sources under an N-starvation condition. Having in consideration the rubber degrading capability of the actinomycete, poly(*cis*-1,4-isoprene) was utilized as sole carbon source. After twenty days of cultivation the PHA content was analyzed using GC-MS. In cultures of *G. polyisoprenivorans* harboring pAK68, the detection of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) monomer units indicated the accumulation of the copolyester poly(3HB-*co*-3HV). This study proposes a recycling method for rubber waste through its biotransformation into bioplastic.

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[**Key words:** Bioconversion of rubber; Biopolymer; Polyhydroxyalkanoates; Rubber recycling]

Polyhydroxyalkanoates (PHAs) are a group of bacterial polyesters produced by a variety of prokaryotic microorganisms under unbalanced nutrition conditions as carbon and energy storage materials (1). These polymers have attracted extensive interest as environmental-friendly, biodegradable alternatives to petroleum-based plastics. Unlike petroleum-based plastics, PHAs can be obtained from renewable resources and microorganisms present in the soil, sea and sewage degrade them into carbon dioxide and water under aerobic conditions and into methane under anaerobic conditions (2,3).

An important factor for the high production cost of PHAs is related to the costs of the substrates which account for 30–40 % of total production costs. Apart from glucose, several other carbon sources including lactic acid (4), acetic acid (5), oleic acid (6), plant oils (7) and waste glycerol (8) have been used as the sole carbon source to produce PHA. In order to reduce the production costs, industrial by-products have been used for PHA production using different microorganisms (9–12). However, the expanding PHA industry has raised concerns, since most PHAs are produced primarily from food crops, sugar cane and vegetable oils and the production of these carbon sources competes with food supply production. As an example, 34,000 tons of PHAs would utilize about 126,000 tons of corn (13). Therefore, it is essential to exploit non-food-based carbon sources for a sustainable production.

The physicochemical properties of these polymers vary depending on the size of the monomer. Depending on the number of carbon atoms, PHAs are divided into two groups: short-chain length PHAs (scl-PHA), which consist of 3–5 carbon atoms and have thermoplastic properties similar to polypropylene, and medium-chain-length PHAs (mcl-PHA) which consist of 6–14 carbon atoms and have elastomer-like properties (14). Normally mcl-PHAs are synthesized from fatty acids or other aliphatic carbon sources and the composition of the resulting PHA depends on the growth substrate used (15). Furthermore, it is known that PHAs are accumulated mainly in response to unbalanced growth conditions such as a lack of nitrogen, phosphorous or oxygen, which links PHAs accumulation to the stringent response (16).

PHA biosynthesis was previously reported in a recombinant strain of *Gordonia polyisoprenivorans* VH2 harboring the genes encoding for PHA synthase from *Pseudomonas aeruginosa* (17). Using pentadecane or hexadecane as carbon sources a total PHA content of 8.3% and 7.4%, respectively, of cell dry weight was reached. Substrates of PHA mcl synthase (3-hydroxyacyl-coenzyme A) are available from β-oxidation when the cells grow on *n*-alkanes (17). Based on the fact that the enzymes involved in the metabolic pathway of *G. polyisoprenivorans* strain VH2 growing on poly(*cis*-1,4-isoprene) (IR) (18) could potentially provide precursors for PHA synthesis derived from the fatty acid oxidation pathway, rubber or rubber containing material could be an interesting carbon source for PHA production.

The degradation of rubber via the β-oxidation pathway was proposed in detail by Hiessl et al. (18) by analyzing the genome of *G. polyisoprenivorans* VH2 together with the generation of mutant strains. The oligo(*cis*-1,4-isoprene) molecules, generated by the oxidative cleavage of the polymer in the presence of the latex

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clearing protein (Lcp), are incorporated inside the cell via an ATP-dependent, Mce protein-driven mechanism. The intermediates are further metabolized by β-oxidation based on the literature evidence involving the formation of specific intermediates, inhibition approaches of the pathway and the utilization of the pathway for similar compounds (19–23).

It is known that large amounts of rubber-containing materials are discarded, representing a serious environmental problem. One recycling method for rubber waste is the utilization of old rubber material as substrate for obtaining new products (24). In this study, the bioconversion of different carbon sources including IR into PHA was analyzed.

MATERIALS AND METHODS

Bacterial strains and cultivation conditions Precultures of *G. polyisoprenivorans* VH2 were cultivated at 30°C in Standard-I (St-I) medium (Merck, Darmstadt, Germany) for 18 h. For the inoculation of liquid cultures, cells were washed twice with saline solution (0.9% NaCl w/v). Main cultures of *G. polyisoprenivorans* VH2 were cultivated in 250-ml shake flasks containing 50-ml of mineral salts medium (MSM) (25). Carbon sources (gluconate, propionate, hexadecane) were sterilized by filtration and added to the autoclaved cultivation medium (121°C for 20 min) in a concentration of 0.5% w/v. IR was autoclaved at 121°C for 20 min together with MSM medium in a concentration of 0.5% w/v. Liquid cultures were agitated at 150 rpm on a horizontal rotatory shaker. Solid media were prepared by the addition of 1.8% (w/v) agar in St-I medium. Before inoculation, 50 μg ml^{-1} of thiostrepton was added for strains containing plasmids. Cell growth was measured by optical density at 600 nm (OD_{600}). Since IR is an insoluble solid substrate in aqueous solutions, OD_{600} was measured without the IR particles to avoid disturbances. For this, samples were filtered using a glass filter porosity 3 (nominal pore size: 16–40 μm).

Cells were harvested by centrifugation at 7700 × *g* at 4°C for 15 min. The cell pellet was washed twice with saline solution (0.9% w/v NaCl), the supernatant was discarded and the cell pellet was freezed at −20°C and lyophilized. Cell dry weight (CDW) was determined by the difference of weight of the dried tubes containing the cells and the empty dried tubes before adding the cells.

Plasmids Plasmids pAK68 and pAK71 were constructed as described by Kalscheuer et al. (26) (Fig. 1). The plasmid pAK68 harbors the genes *phaA*, *phaB* and *phaC* encoding the enzymes acetoacatyl-CoA reductase, β-ketothiolase, and PHA synthase, respectively, from *Ralstonia eutropha*, while pAK71 harbors the genes encoding *phaC1* from *P. aeruginosa*. Both vectors were introduced into *G. polyisoprenivorans* VH2 using an optimized electroporation protocol as described by Arenskötter et al. (17).

Rubber material preparation Purification of IR was performed as described previously (18). After freezing the IR in liquid nitrogen, the material was milled using an ultra-centrifugal mill (model ZM 200, Retsch GmbH, Haan, Germany). According to the method of Warneke et al. (27), the IR particles were separated to define size ranges. The fraction size between 63 and 500 μm was used in this study.

PHA analysis An amount of 5–10 mg of dried cells were subjected to acid methanolysis in the presence of 85% (v/v) methanol and 15% (v/v) sulfuric acid for 4 h at 100°C. The resulting methyl esters of hydroxyalkanoic acids and fatty acids were analyzed by gas chromatography according to Brandl et al. (28). For gas chromatographic-mass spectrometry (GC-MS) analysis, an Agilent 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a BP21 capillary column (50 m by 0.22 mm; film thickness, 250 nm; stationary phase: polyethylene glycol [PEG]), and a flame ionization detector (Agilent Technologies) were used. GC was coupled to a mass selective detector HP 5973 (Hewlett–Packard, Palo Alto, CA, USA). For the analysis of the data, the software Agilent Cerity QA-QC was used. As a reference, retention times of commercially available 3-hydroxy fatty acid standards were used for the identification and quantification of the fatty acids present in the samples.

RESULTS AND DISCUSSION

Growth curves of PHA producer strains of *G. polyisoprenivorans* VH2 Since pAK68 contained the genes encoding the enzymes acetoacetyl-CoA reductase, β-ketothiolase and PHA synthase from *R. eutropha*, short chain carbon sources (gluconate, propionate) were added to the corresponding cultivation media. Cultivation of *G. polyisoprenivorans* VH2 harboring pAK71, containing the PHA synthase from *P. aeruginosa*, hexadecane was selected as carbon source. Whereas pAK68 was designed for obtaining short chain length PHA (scl-PHA), pAK71 will provide medium chain length PHAs (mcl-PHA).

Cultivations in MSM with the investigated carbon source under conditions of N-starvation were performed. The growth was monitored by measuring OD_{600} until the late stationary phase. Previous studies in our laboratory (29) described propionate as a suitable carbon source for the rapid growth of *G. polyisoprenivorans* VH2 in shake flasks. On the other hand, gluconate was already described for the production of PHB in *Rhodococcus opacus* PD630 (26), while hexadecane was examined for the synthesis of mcl PHA in *R. opacus* PD 630 as well as in *G. polyisoprenivorans* VH2 (17). As a control for the GC-MS analysis, cells of *G. polyisoprenivorans* VH2 were grown in parallel without the plasmids.

Arenskötter et al. (17) concluded that *G. polyisoprenivorans* did not produce a lac repressor and *lacZ* promoter dependent genes are constitutively expressed. The *lacZ* promoters of the vectors pAK68 and pAK71 are located upstream of the *phaCAB* and *phaC1* genes, respectively. Consequently, the biosynthesis of PHA did not depend on isopropyl-β-D-thiogalactopyranoside (IPTG) addition. Because of this, in our study no IPTG was added to promote the induction.

The cell growth rate and the maximum cell density was depending on the carbon source (Fig. 2). Only 36 h were necessary to reach the stationary phase by using propionate. This condition was reached only after 14 days by growing on IR. Higher cell densities during the stationary phase by cells harboring the plasmids are supposed to come from the intracellular accumulation of PHAs.

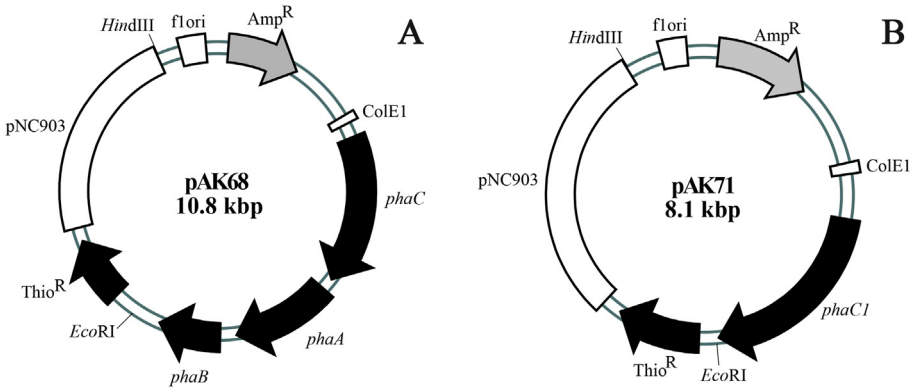

FIG. 1. Restriction maps of plasmids pAK68 and pAK7 modified from Kalscheuer et al. (26). Relevant structural genes and other elements are indicated: *amp*, ampicillin resistance gene; *thio*, thiostrepton resistance gene; pNC903, fragment from pNC903 comprising the *ori* for replication in *Rhodococcus*; *phaB*, *phaA*, *phaC* encoding β-ketothiolase, acetoacetyl-CoA reductase and PHA synthase from *R. eutropha*, respectively; and *phaC1* encoding PHA synthase from *P. aeruginosa*.

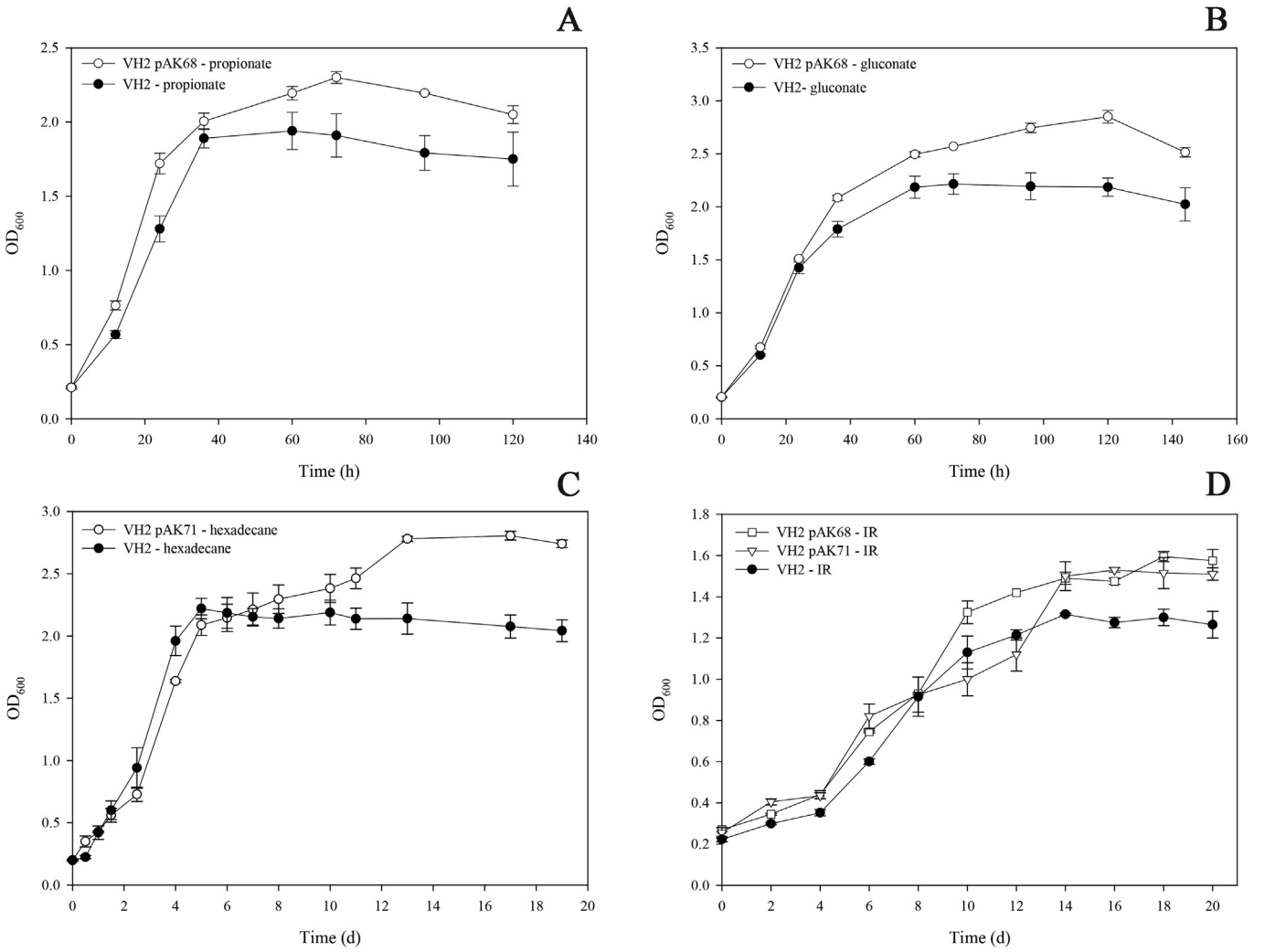

FIG. 2. Growth curves of *G. polyisoprenivorans* VH2 strains containing pAK68 and pAK71 plasmids. The selected carbon sources are indicated in the figure. As a control, *G. polyisoprenivorans* VH2 without plasmids was cultivated at the same conditions.

In fact, after 17 days of cultivation a 35% higher cell density was reached by *G. polyisoprenivorans* VH2 harboring pAK71 growing on hexadecane, compared to the strain without the plasmid.

Cells of *G. polyisoprenivorans* growing on IR as sole carbon source reached final optical densities (OD_{600}) of 1.2 for wild type and 1.6 for the mutants. The difference would suggest PHA accumulation as described above. Additionally, it is interesting to remark that growth yields were not as in controls with gluconate, propionate and hexadecane, yielding a 50% less of final biomass. The explanation is based on the difficulties that present the cleavage mechanism and further metabolism of an insoluble solid substrate as IR for bacteria. Since IR particles were still visible in the end of cultivations, mass quantification of the remaining particles was done. After filtration and drying the IR particles, it was possible to calculate a mean value of 57.2% of IR degradation, which is in accordance to the cell growth.

Metabolism of poly(*cis*-1,4-isoprene) and the synthesis of PHA The proposed pathway for the degradation of poly(*cis*-1,4-isoprene) (18) releases acetyl-CoA and propionyl-CoA, which are metabolized by the central metabolism (Fig. 3). Moreover, acetyl-CoA and propionyl-CoA are the precursors for the synthesis of poly(3HB) and poly(3HV), respectively. Initially, the backbone of IR is cleaved by Lcp in the extracellular space. As a result, different sizes of oligo-isoprenoid molecules containing 2 to 18 isoprene units in their structure (29) are accumulated and transported inside the cell. Those oligo(*cis*-1,4-isoprene) molecules are oxidized via β-oxidation and provide the necessary precursors for the synthesis of PHA.

The recombinant biosynthesis of PHA in *G. polyisoprenivorans* VH2 is summarized in Table 1. The expression of the genes *phaA*, *phaB* and *phaC* from *R. eutropha* located on pAK68 resulted in the synthesis of scl-PHA in recombinant *G. polyisoprenivorans* VH2. Cells growing on gluconate led to the formation of poly(3HB), while by using propionate the copolyester poly(3HB-*co*-3HV) was synthesized. A similar result was reached when cells were cultivated on IR as sole carbon source, detecting 3HB and 3HV monomer units. The availability of acetyl-CoA and propionyl-CoA resulting as end-degradation products from the β-oxidation in *G. polyisoprenivorans* VH2 plays a relevant role regarding cell growth and as precursors for the synthesis of scl-PHA (Fig. 3). GC-MS analysis did not detect the formation of PHAs in controls (without the plasmids). The results show that the native promoter of the *R. eutropha pha* operon (in pAK68) and the *lacZ* promoter (in pAK71) of *P. aeruginosa phaC1* were recognized by *G. polyisoprenivorans* RNA polymerase and confirmed the results from a previous study (26).

Some microorganisms accumulate PHAs containing 3HV even in the absence of typical 3HV precursors such as propionate or

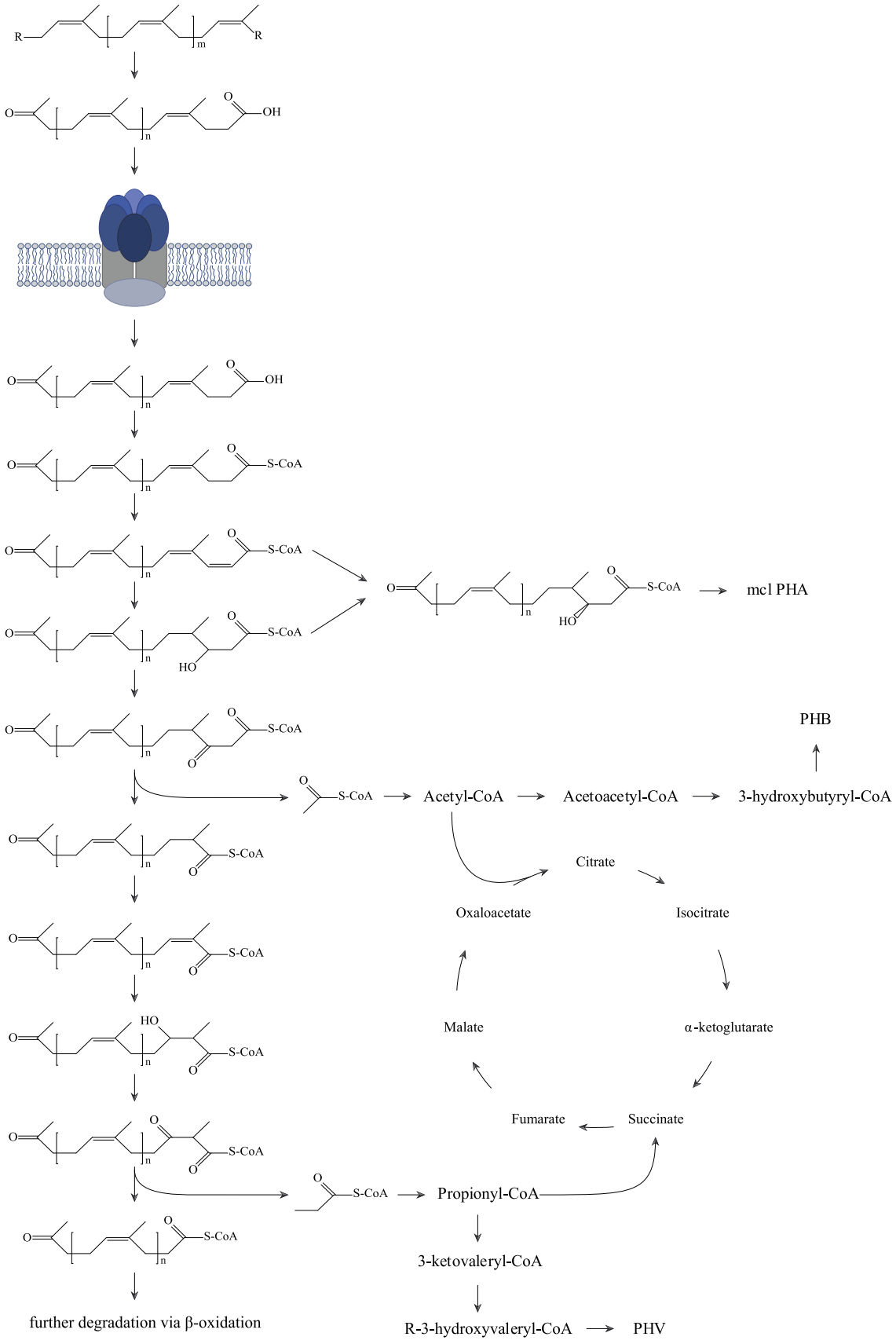

FIG. 3. Metabolism of poly(*cis*-1,4-isoprene) by *G. polyisoprenivorans* VH2 and the hypothetical pathways for the synthesis of PHAs.

TABLE 1. PHA accumulation by recombinant strains of *G. polyisoprenivorans* VH2.

Plasmid	Carbon source	PHA content	PHA composition (mol %)					
		(% CDW)	3HB	3HV	3HHx	3HO	3HD	3HDD
pAK68	Propionate	18.9	82.7	17.3	nd	nd	nd	nd
None	Propionate	nd						
pAK68	Gluconate	22.5	100	nd	nd	nd	nd	nd
None	Gluconate	nd						
pAK68	IR	10.2	67.2	32.8	nd	nd	nd	nd
None	IR	nd						
pAK71	Hexadecane	25.8	nd	nd	17.6	40.3	28.5	13.6
None	Hexadecane	nd						
pAK71	IR	6.4	nd	nd	nd	nd	nd	nd
None	IR	nd						

Cells were cultivated in MSM medium containing 0.5% w/v of the carbon sources indicated in the table. For each sample an amount of 5–10 mg of CDW was analyzed. 3HB, 3-hydroxybutyrate; 3HV, 3-hydroxyvalerate; 3HHx, 3-hydroxyhexanoate; 3HO, 3-hydroxyoctanoate; 3HD, 3-hydroxydecanoate; 3HDD, 3-hydroxydodecanoate; CDW, cell dry weight; nd, not detected.

valerate in the feed (30). An explanation can be that the 3HV monomer is derived from acetyl-CoA and propionyl-CoA, where the latter is a product of the methylmalonyl-CoA pathway (31). In this pathway, succinyl-CoA is converted to methylmalonyl-CoA, which is decarboxylated to propionyl-CoA. Propionyl-CoA and acetyl-CoA are converted to poly(3HB-*co*-3HV) by PhaA, PhaB and PhaC. However, the presence of C_{odd} oligo-isoprenoids might form propionyl-CoA as shown in Fig. 3.

G. polyisoprenivorans VH2 harboring pAK71 comprising *phaC1* from *P. aeruginosa* synthesized a copolyester consisting of even-numbered 3-hydroxyalkanoates (3HHx, 3HO, 3HD, 3HDD) growing on hexadecane as sole carbon source. The accumulation of this mcl-PHA represented 25.8% w/w of the CDW. Compared to Arenskötter et al. (17), our results showed a 3.5-fold higher PHA accumulation working with the same strain and under similar conditions. This improvement is related to the harvesting time. In Fig. 2C, a significant difference in OD_{600} is shown between *G. polyisoprenivorans* VH2 harboring pAK71 and *G. polyisoprenivorans* VH2 wild type, mainly after 12 days of cultivation. Since the cells were harvested after 19 days of cultivation, the PHA accumulation was higher than after 10 days of cultivation. Further results showed no significant differences in PHA accumulation between 14 and 19 days.

The co-monomer composition of mcl-PHA depends mainly on the carbon source, the cultivation conditions, and the metabolic routes leading to PHA formation (32). In most of the instances to date, the carbon source is an obvious precursor of the additional hydroxyacid monomer (33). When cells were cultivated on hexadecane, mcl-PHA were accumulated. This suggests the presence of precursors of *R*-3-hydroxyacyl-CoA and the bioconversion of *trans*-2-enoyl-CoA into *R*-3-hydroxyacyl-CoA by an enoyl-CoA hydratase, by converting the (*S*)-isomer of 3-hydroxyacyl-CoA into the (*R*)-isomer by epimerases or by a specific 3-ketoacyl-CoA reductase. The results showed that *G. polyisoprenivorans* VH2 containing pAK71 growing on IR as sole carbon source did not produce any of the 3-hydroxyalkanoates listed in Table 1. However, GC-MS analysis detected three major peaks that were not present in the control presenting a molecular mass of 486, 554 and 622 g mol^{-1}, which might correspond to PHA-monomers coming from the metabolism of IR. The uptake and the β-oxidation of oligo-isoprenoid molecules might provide precursors for the synthesis of PHA. The presence of *R*-3-hydroxyacyl-CoA when cells were cultivated on IR and an active PHA polymerase might lead to the synthesis of PHA based on the structure of the IR intermediates (Fig. 3). Nevertheless, further characterization studies are necessary to confirm the occurrence of such a polymer.

Conclusions This study was focused on the biosynthesis of PHA utilizing IR as sole carbon source in order to find recycling methods for rubber waste materials and alternative carbon sources for the synthesis of PHA. Cultivations with recombinant *G. polyisoprenivorans* VH2 harboring pAK68 led to the intracellular accumulation of known scl-PHA, as the copolymer poly(3HB-*co*-3HV).

ACKNOWLEDGMENTS

The authors declare no conflicts of interest.

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