Synthesis, recovery and possible application of medium-chain-length polyhydroxyalkanoates: A short overview

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Abstract

Bacterial polyhydroxyalkanoates (PHAs) are polyesters of 3-hydroxyacids produced as intracellular granules by a large variety of bacteria, currently receiving much attention because of their potential as renewable and biodegradable plastics. The monomer units in these microbial polyesters are all in the *R*-configuration due to the stereospecificity of biosynthetic enzymes. Pseudomonads synthesise mainly medium-chain-lenght PHAs, formed of monomers of 6 to 14 carbons. The PHA monomer composition is influenced by the substrate added to the growth media and determines the physical properties of the plastic material. The capability of Pseudomonads to incorporate many different functional groups into the PHAs does extend their physical properties and potential applications, and suggests various possibilities to produce tailor-made polymers. The mcl-PHAs are of major interest for specific uses, where chirality and elastomeric property of the polymers are important. In this report we will focus on the biotechnological production, recovery and possible applications of mcl-PHAs.

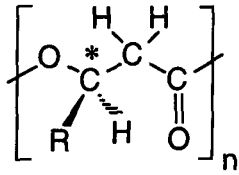
Introduction

Conventional plastics produced by the chemical industry comprise a very important group of materials, as their high molecular weight and low reactivity make them especially suited for applications where durable, inert materials are required. However, the annual disposal of several million tons of plastics worldwide has raised the demand for means of managing this non-

degradable waste stream. Next to recycling projects, selective use of biodegradable polyesters in certain applications might help to reduce the environmental impact of these plastic materials. One family of biopolymers, called poly-3-hydroxyalkanoates (PHAs) are currently receiving much attention because of their potential as renewable and biodegradable plastics.

The first example of polyhydroxyalkanoates to be discovered was polyhydroxybutyrate (PHB) in the year 1926 [1]. Since then PHB accumulation was found in various microorganisms, representatives of Gram-negative and Gram-positive species (i.e., autotrophs, heterotrophs, phototrophs, aerobes, anaerobes) and archaebacteria (as reviewed elsewhere [2-4]). Bacteria synthesize and accumulate PHAs as carbon and energy storage materials under conditions of limiting nutrients in the presence of excess carbon source. When the supply of the limiting nutrient is restored, the PHA can be degraded by intracellular depolymerases and subsequently metabolized as carbon and energy source [5]. The general structure of PHAs is shown in Figure 1. The monomer units in these microbial polyesters are all in *R*-configuration due to the stereospecificity of biosynthetic enzymes. The molecular weights of the polymers range from 2 x 10^5 to 3 x 10^6, depending on the specific polymer, the microorganism and growth conditions.

The discovery of a polyester consisting mainly of hydroxyoctanoate monomers by de Smet *et al.* [6] was the first example of a new group, so called medium-chain-length (mcl) PHAs which can contain a wide variety of different monomers. To date, more than 90 different monomers were found in the polymers [7]. Among those are 3-hydroxy acids of 6 to 14 carbon atoms with a large variety of saturated, unsaturated, straight or branched-chains



$R = CH_3$	Polyhydroxybutyrate (PHB)
$R = C_2H_5$	Polyhydroxyvalerate (PHV)
$R = C_3H_7 - C_{14}H_{29}$	Medium-chain-length PHAs

Figure 1. Structural formula of polyhydroxyalkanoates.

containing aliphatic or aromatic side groups. Furthermore, monomers with various different functional groups in the side chain such as halogen atoms, hydroxy-, epoxy-, cyano-, carboxyl- and esterified carboxyl groups have been introduced into mcl-PHAs (see review [3,7,8]). The mcl-PHAs are of interest for specific uses, where chirality and elastomeric property of the polymers are important. In addition, the monomers of PHAs that contain different functional groups in their side chain receiving more and more attention as source of chiral synthons. In this report, we will focus on the production, recovery and possible applications of mcl-PHAs (see for overview Figure 2).

Production of mcl-PHAs

The ability to accumulate mcl-PHA polymers is unique to the group of Pseudomonads. *P. oleovorans*, *P. putida*, *P. aeroginosa* and *P. resinovorans* are mostly used for production of mcl-PHAs [9-11]. Batch, fed-batch and continuous cultivation techniques have been extensively investigated at lab scale [12-14], but PHA production up to a 200 L scale have also been carried out [15]. Efficient PHA production requires rapid formation of biomass and product. The batch and fed-batch fermentations are offen carried out in a two-step process. During the first step, the cells are grown in a mineral salt medium with fatty acids as sole carbon and energy source, and a calculated amount of nitrogen based on the known requirements of the organism to allow production of a given amount of biomass. As the culture grows, nitrogen is depleted from the medium, and during the second step when nitrogen is limiting, the cells start to store polymer. During this nutrient limitation stage the residual

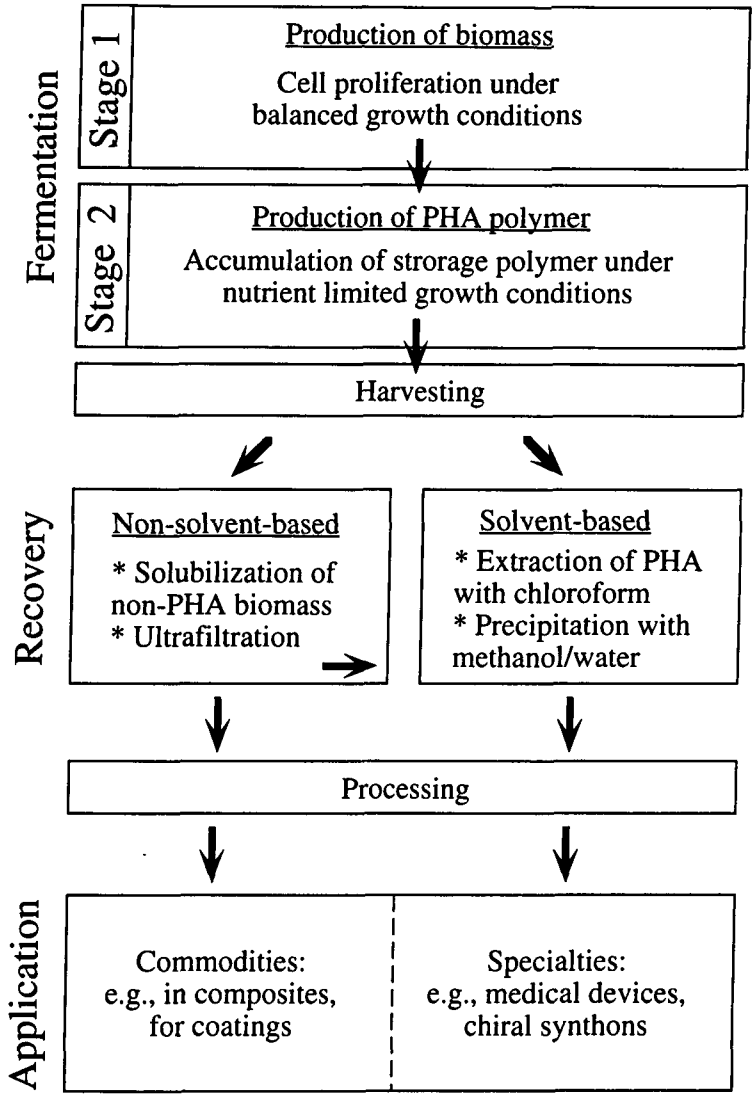


Figure 2. General overview of the mcl-PHA production process.

biomass (defined as the biomass minus the PHA mass) remains almost constant, and the biomass increases only because of the intracellular accumulation of PHA.

P. oleovorans is used to produce mcl-PHAs in two-liquid phase cultivations [16,17]. The organism is cultured in media consisting of an aqueous phase containing all minerals necessary for growth, and a second liquid phase containing *n*-alkanes as sole carbon and energy source. Two-liquid phase single stage chemostat and fed-batch cultivations of. *P. oleovorans* using *n*-octane as carbon source resulted in highest volumetric productivities of 0.58 g l^{-1} h^{-1} [12] and 0.25 g l^{-1} h^{-1} [13], respectively. In a two-liquid phase, two stage continuous cultivation a volumetric productivity of 1.06 g l^{-1} h^{-1} with a final cellular PHA content of 66% per total cell dry weight has recently been reported [18]. The monomer composition of PHAs produced from different alkanoates using *P. oleovorans* is shown in Table 1 [9]. The monomer composition of the synthesized polymer is mainly dependent on the strain used and the applied carbon source. The cultivation conditions only have a minor influence on the composition of the polymer. However, it was shown that temperature and concentration of the substrate have an influence on the molecular weight of the polymer [19]. Recently, it was reported that *Pseudomonas putida* U grown on octanoic acid as sole carbon source accumulated a homopolymer of poly-3-hydroxyoctanoate [20].

Different *Pseudomonas* strains have been used to produce mcl-PHAs from renewable resources, such as fatty acids [9,21] and carbohydrates [11,22,23]. In fed-batch cultivations, *P. putida* KT2442 was cultivated to high cell densities (92 g/l) with oleic acid as a substrate. With this process an overall volumetric productivity

Table 1. Composition of PHAs produced by *Pseudomonas oleovorans* grown on different alkanoates as sole carbon source

	Composition (mol% of total 3-hydroxy fatty acids)						
	HC	HH	HO	HN	HD	HUD	HDD
Hexanoate	95		5				
Heptanoate		100					
Octanoate	8		91		1		
Nonanoate		35		65			
Decanoate	8		75		17		
Undecanoate		28		59		13	
Dodecanoate	6		57		32		5
Tridecanoate		32		48	5	14	
Tetradecanoate	7		59		30		4
Pentadecanoate		32		47	8	13	
Hexadecanoate	8		50		30		12

HC: 3-hydroxyhexanoate; HH: 3-hydroxyheptanoate; HO: 3-hydroxyoctanoate; HN: 3-hydroxynonanoate; HD: 3-hydroxydecanoate; HUD: 3-hydroxyundecanoate; HDD: 3-hydroxydodecanoate.

of 1.6 g l^{-1} h^{-1} was achieved with a final cellular PHA content of 40% per total cell dry weight [24]. Furthermore, mcl-PHAs can be produced from glucose, fructose and glycerol using *P. putida* KT2442 [23]. In addition to the major constituent 3-hydroxydecanoate, six other monomers have been identified: 3-hydroxyhexanoate, 3-hydroxyoctanoate, 3-hydroxydodecanoate, 3-hydroxydodecenoate, 3-hydroxytetradecanoate, 3-hydroxydecenoate. The degree of unsaturation of this PHA is slightly influenced by the cultivation temperature [23].

Recently, it was shown that mcl-PHAs can be produced by specific recombinant *E. coli* strains which are deficient in the fatty acid β-oxidation, and in addition contain the PHA polymerase encoding gene of *P. aeroginosa* or *P. oleovorans* [25,26]. The recombinant strains were cultured on LB medium or mineral salt medium supplemented with yeast extract, and alkanoates as additional carbon source. Up to 21% mcl-PHA per cell dry weight could be achieved by these recombinant *E. coli* strains [25].

Recovery of mcl-PHAs

The cells containing the polymer have to be separated from the broth by conventional procedures such as centrifugation, filtration or flocculation-centrifugation. After the biomass is harvested, cells have to be disrupted to recover the polymer. A number of different methods have been developed for the recovery of PHAs. The first method that has most often been used involves extraction of the polymer from the biomass with solvents (e.g., chloroform or methylene chloride) and polymer precipitation by the addition of a methanol/water mixture [16]. A polyester with 99% purity can be

obtained with this process, however, apart from being expensive, these method generally involves working with large quantities of toxic, volatile solvents.

Therefore, an alternative, non-solvent-based extraction process was developed to make the overall production process more attractive [15,27,28]. The temperature of the fermentor is raised to 121°C for one minute immediately after termination of the cultivation. The cells are separated from the medium by centrifugation, and treated with a protease cocktail and a detergent to solubilize all cell components. Removal of the solubilized cell material and concentration of the resulting PHA suspension is achieved by crossflow microfiltration. In our hands, this technique is better than centrifugation because the submicron mcl-PHA granules display a bouyant density close to that of water (1.05 g cm^{-3} [29]). Thus, a mcl-PHA suspension does not settle [30], and is in fact a highly stable polymer latex. The overall purity of the latex amounts to 95%. Mcl-PHAs produced for applications which require higher purity, such as those in biomedicine, have to be further purified by solvent extraction of PHA from the latex.

Properties of mcl-PHAs

The mcl-PHA chains have a molecular weight in the order of 2 x 10^5 g/mol [17,19,22,31], and since the chiral centres of the monomers are all in the *R*-configuration, the polymers are fully isotactic. This allows mcl-PHAs to achieve some crystallinity, which can be as high as 25% [32]. The glass transition temperature is usually well below room temperature, ranging from -43°C to -25°C for different mcl-PHAs [17] and the melting temperatures varies

between 39°C and 68°C [17,22,31,32] dependent both on the thermal history and on the nature of the pendant chain.

Since the low melting temperature and crystallization rate hamper the applicability of mcl-PHAs as thermoplasts, crosslinking was anticipated to overcome both limitations [33]. Crosslinking was achieved by conventional techniques, namely electron-beam irradiation [33], peroxide crosslinking [34] or vulcanisation [35]. In all three studies, the presence of olefin side chains was shown to enhance chemical crosslinking. Sufficient crosslinking appeared to prevent polymer crystallization, so a true rubber could be produced. Properties were therefore constant over a wide temperature range from the glass transition temperature (T_g -30°C) up to the decomposition temperature (ca. 180°C) [33].

Possible Applications of mcl-PHAs

No applications for mcl-PHAs have been reported yet. There are probably at least two reasons for this. There has been little material available for development work, and applications of mcl-PHAs as commodities are primarily limited by their high cost. A preliminary economic study for the production of mcl-PHAs showed that the price for octanoate-based PHAs would be in the order of 20 US$ kg^{-1} for an annual production of 1000 tons [15,28]. Optimizing the polymer content in the cells, application of cheaper carbon sources as substrates, reduction of the chemicals used during downstream processing and reduction of energy consumption is expected to lower the mcl-PHA price to an ultimate minimum of 5 US$ kg^{-1} [15,28]. Further decreases in production costs are to come from the production of mcl-PHAs in recombinant plants.

Even at the relatively high prices anticipated for microbial PHAs, application of mcl-PHAs for coatings, such as paper coatings with moisture barrier characteristics in food/drink cartons and in sanitary napkins would result in total product biodegradability for little extra cost. In addition, polymer blending offers another interesting possibility to prepare inexpensive (biodegradable) materials with useful mechanical properties. In this context, PHAs could form an important source of biodegradable additives.

Furthermore, a notable feature of biopolyesters such as PHB is that they are biocompatible, producing mild foreign body responses to implants. Like PHB, the flexible elastomeric mcl-PHA might also be biocompatible, and hence useful in biomedical applications, such as vascular grafts, surgical swabs, wound dressing, breast prostheses, etc. [15]. In these applications, the properties of the material are of major importance, while the price is secondary.

Conclusions

Polyhydroxyalkanoates display an unique combination of features. First, in contrast to synthetic polymers, the natural PHAs have the fundamental advantage of being renewable resources not dependent on the supply of petroleum. Second, they are genuinely biodegradable, i.e. they can be completely metabolized into harmless, naturally occurring molecules. Cheaper biodegradables, such as the starch-based plastics, are available however. Therefore, the most important feature of these polyesters may well be their hydrophobicity, and hence they exceed their biodegradable competitors in moisture resistance.

The capability of bacteria to incorporate many different monomer units into mcl-PHAs suggests various possibilities to produce tailor-made polymers. Incorporation of unsaturated pendant groups to enhance crosslinking is one example [33]. A recent study reports that nitrophenoxy groups have been introduced aiming to create chiral polymers for nonlinear optical applications [36]. Specific functionalities for surface modifications could be introduced as well. In the long run, it should be feasible to custom design new polymers and then program the bacteria to produce them.

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