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# Novel Description of *mcl*-PHA Biosynthesis by *Pseudomonas chlororaphis* from Animal-Derived Waste

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ABSTRACT

A novel description of *mcl*-PHA biosynthesis by *Ps. chlororaphis* from tallow-based biodiesel as an inexpensive carbon feed stock is presented. Fermentation protocols, kinetic analysis, an efficient product recovery strategy, and product characterization are included. Maximum specific growth rates ($\mu_{max.}$) of 0.08 $h^{-1}$, 0.10 $h^{-1}$ and 0.13 $h^{-1}$, respectively, were achieved in three different fermentation set-ups. Volumetric productivity for *mcl*-PHA amounted to 0.071 g/L h, 0.094 g/L h and 0.138 g/L h, final intracellular PHA contents calculated from the sum of active biomass and PHA from 22.1 to 29.4 wt.-%, respectively. GC-FID analysis showed that the obtained biopolyester predominantly consists of 3-hydroxyoctanoate and 3-hydroxydecanoate, and, to a minor extent, 3-hydroxydodecanoate, 3-hydroxynonanoate, 3-hydroxyhexanoate, and 3-hydroxyheptanoate monomers. The overall distribution of the monomers remained similar, regardless to working volumes, biodiesel concentrations and pre-treatment of the inoculum.



## 1. Introduction

Polyhydroxyalkanoates (PHAs) attract increasing attention as biobased and biodegradable "green plastics" due to their promising material properties and sound integration of their life cycle into nature's closed carbon balance (Keshavarz and Roy, 2010). These polyesters of hydroxyalkanoic acids biologically act as microbial reserve compounds for carbon and energy. They are accessible from renewable resources by the biosynthetic action of selected prokaryotes, displaying diverse properties. Depending on the carbon source and the strain, the material obtained for industrial applications can range from thermoplasts to elastomers, latexes, and even high-performance, functional polymers (Chen, 2010a). From the plastic-industrial point of view, they exhibit the potential to replace their petrol-based competitors in several bulk and niche segments of the plastic market in the foreseeable future (Zinn et al., 2001).

For a break-through on the market, "green plastics" like PHAs must compete against well-established, mainly low-priced petrol-based polymers. This is especially valid in economic terms, not only regarding material performance (Braunegg et al., 1998). PHA biosynthesis is reported from renewable carbon feedstocks like carbohydrates, lipids, alcohols, organic acids, or methane (Koller et al., 2010a). Up to date, the lion's share of the expenses for the entire PHA production process is attributed to the carbon feedstock, since mainly prized substrates of high nutritional value like pure starch, sugars, and edible oils are used for this purpose. In addition, it has to be taken into account that PHA biosynthesis occurs under aerobic conditions. Consequently, a considerable share of the carbon source is metabolized towards $CO_2$ and minor by-products. However, many inexpensive carbon-rich industrial by-products and waste can be applied as feedstock for a variety of PHA-producing microbes. This approach can make PHAs economically competitive and removes ethical concerns arising from the interference with human nutrition or animal feeding. This has of course to go in parallel with the economic improvement of other production steps, mainly process design, product recovery, and closing of water-, material- and energy cycles.

Depending on their monomeric composition, PHAs are distinguished into short chain length (*scl*) and medium chain length (*mcl*) PHAs. *scl*-PHA monomers consist of 3 to 5 carbon atoms and mainly constitute *R*-configured chiral 3-hydroxyalkanoates. Due to their physical characteristics, *scl*-PHAs mainly feature properties of classical thermoplasts. Therefore, they compete on the market with Poly(ethylene) or Poly(propylene) and, regarding other "green

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plastics", also with biobased Poly(lactic acid). The eubacterial strain *Cupriavidus necator*, a member of the Burkholderiaceae family, constitutes the best investigated bacterial *scl*-PHA producer. In contrast, *mcl*-PHAs are by far less crystalline than their *scl*-relatives; their monomeric building blocks are mainly *R*-configured chiral 3-hydroxyalkanoates and, to a minor extent, *R*-configured chiral 3-hydroxyalkenoates consisting of 6 to 14 carbon atoms (Chen at al., 2001). Sometimes, these *mcl*-building blocks possess functionalities that allow post-synthetic chemical modification of *mcl*-PHA for fine-tuning of the material properties (Bear et al., 1997). Characteristics of *mcl*-PHA resemble those of elastomers, latexes and resins. Due to their low glass transition temperature, *mcl*-PHAs do not become brittle even at temperatures far below the frosting point, making them interesting as biological rubber-like materials.

*Pseudomonas putida* GPo1 (formerly known as *Ps. oleovorans* GPo1) was the first investigated *mcl*-production strain; it constitutes the most widely investigated organism for biosynthesis of these materials (Furrer et al., 2007; Van Beilen et al., 2001). This organism is well known to convert various fatty substrates like n-alkanes, n-alcohols, and n-fatty acids (Lenz et al., 1992). It was isolated from oil-based cooling fluids already in 1941 (Lee and Chandler, 1941). Due to the intensive research devoted to this strain during the last decades, high amounts of *mcl*-PHA (up to 60% and more) can already be obtained, both in continuous and discontinuous mode (Jung et al., 2001; Kim, 2000). Using this strain, various tailor-made *mcl*-PHAs with defined proportions of the building blocks are accessible by dual-nutrient-limited chemostat cultivations (Durner et al., 2001; Hartmann et al., 2006). It has to be emphasized that the substrates used for these studies are purified fatty acids and hence rather cost-demanding. Further, most applied substrates display toxic effects on *Ps. putida* already at considerably low concentrations, complicating the feeding regime. In contrast to other *mcl*-PHA producers like *Ps. aeruginosa* or *Ps. resinovorans*, strains of *Ps. putida* are not able to directly convert cheap triacylglycerides like various plant oil or tallow (Ashy et al., 2001; Cromwick et al., 1996; Zinn, 2010). As an additional drawback, *Ps. putida* GPo1does not possess the metabolic requisites of fatty acid *de novo* synthesis, hence it cannot convert structurally unrelated carbon sources like sugars to precursors for *mcl*-PHA biosynthesis as it is the case e.g. for *Ps. fluorescens* BM07, a strain that produces *mcl*-PHA containing high shares of unsaturated building blocks like 3-hydroxy-cis-5-dodecenoate or 3-hydroxy-cis-7-tetradecenoate from simple unrelated substrates like fructose or succinic acid up to intracellular *mcl*-PHA contents of around 25% (Lee et al., 2001).

Similar to the decoding of the genome of *scl*-PHA producers (Pohlmann et al., 2006), the genome of *Ps. putida* KT2442, a close relative of *Ps. putida* GPo1, is completely sequenced (Nelson et al., 2002). *Ps. putida* KT2440 is widely investigated and known as a producer of *mcl*-PHA containing as well saturated as unsaturated building blocks. Also in the case of this strain, the feeding strategy renders itself rather complicated if polyesters of constant composition shall be produced (Sun et al., 2009).

In contrast to the huge number of publications reporting the production of *scl*-PHA from carbon-rich industrial waste (Albuquerque et al., 2010; Koller et al., 2013, Koller et al., 2010b, Koller et al., 2005; Lee, 1996; Ng et al., 2011; Titz et al., 2012), information on *mcl*-PHA production from such inexpensive feedstocks is still scarce (Cromwick et al., 1996; Muhr et al., 2013; Solaiman et al., 2006a). This is contradictory to the fact that *mcl*-PHAs are more and more being considered as potential candidates to act as sustainable basic polymers for several special applications (rubbers, smart latexes, basic materials for post-synthetic functionalization by chemical or enzymatic means, thermo sensitive adhesives and glues, and others) (Chen, 2010a; Zinn, 2010). Therefore, *mcl*-PHA production from saturated biodiesel fractions (SFAE) stemming from animal waste lipids was investigated. In Europe, such animal lipids from slaughtering and animal-processing industry amount to more than 500,000 t per year. Using them for biodiesel production by alkaline transesterification, the available SFAE is estimated with annually 50,000 t. SFAE antagonizes the biodiesel properties as engine fuel, but, if separated, it can be used as feedstock for the biotechnological production of PHA, whereas the remaining unsaturated biodiesel fraction performs as excellent 2nd generation biofuel.

*Ps. chlororaphis* was selected as production strain due to some encouraging reported features (Solaiman et al., 2006b), partially published during the duration of this work (Chung and Rhee, 2012). Additionally to reported *mcl*-PHA production, *Ps. chlororaphis* produces, like *Ps. aurantiaca*, green insoluble chlororaphin (Peix et al., 2007). It is a complex of reduced and oxidised phenanzine-1-carboxyamids (Kanner et al., 1978), which are nitrogen-containing heterocyclic secondary metabolites. These phenanzines are acting as antibiotics, electron shuttle, influence growth in plants and can influence the cellular response (Laursen and Nielsen, 2004). This feature of the strain was of additional interest and should be further investigated in the future. Reliable experimental data on growth and PHA production are necessary since, up to date, no profound information is reported neither for kinetics of *mcl*-PHA production by this strain under controlled conditions in bioreactors, nor for detailed characterization of the produced biopolyesters or their use for processing to marketable products.

In the study at hand, the strain *Ps. chlororaphis* was used for *mcl*-PHA production from inexpensive substrates derived from animal waste, namely SFAE, for the first time. Additionally, this is the first study reporting reliable kinetic data for *mcl*-PHA biosynthesis by this organism. Moreover, during the experimental work, a sufficient amount of product was obtained, which will further be used for polymer characterization regarding composition, thermoanalysis and molecular mass distribution. Future points of interest are the formation of composites and blends with compatible organic and inorganic fillers as well as post-synthetic modifications. The biodegradation in different environments and its biocompatibility should also be investigated. Furthermore, future work could include the analysis of chlororaphin production from SFAE as substrate.

## 2. Materials and Methods

### 2.1. Microorganism and culture conditions

A sample of *Pseudomonas chlororaphis* DSM 50083 was obtained from the reference stocks of Graz University of Technology, Austria. The cells were transferred to solid agar plates (nutrient agar) according to DSMZ instructions containing (per litre) peptone 5.0 g, meat extract 3.0 g, agar-agar 15.0 g and were incubated at 30 °C for proliferation. Subsequently, colonies were cultivated in minimal media according to Küng (1982) containing saturated biodiesel as sole carbon source for adaptation to this substrate.

A chemically defined mineral medium according to Küng (1982) was used for cultivations in shaking flasks (SF) and laboratory bioreactor containing (per litre): $Na_2HPO_4$, 7.17 g; $KH_2PO_4$, 3 g; $MgSO_4{*}7H_2O$, 0.2 g; $(NH_4)_2SO_4$, 1 g; $CaCl_2{*}2H_2O$, 0.02 g; $NH_4Fe$(III)citrate, 0.05 g; trace element solution SL6, 1 mL; saturated biodiesel, 5 to 10 g. The trace element solution SL6 was composed as follows (per liter): $ZnSO_4{*}7H_2O$, 100 mg; $H_3BO_3$, 300 mg; $CoCl_2{*}6H_2O$, 200 mg; $CuSO_4$, 6 mg; $NiCl_2{*}6H_2O$, 20 mg; $Na_2MoO_4{*}2H_2O$, 30 mg; $MnCl_2{*}2H_2O$, 25 mg.