Journal of Biotechnology 165 (2013) 45–51

Contents lists available at SciVerse ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

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

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ARTICLE INFO

*Article history:*
Received 11 January 2013
Received in revised form 4 February 2013
Accepted 6 February 2013
Available online xxx

*Keywords:*
Biodegradable resin
Biodiesel
*mcl*-PHA
Polyhydroxyalkanoate
*Pseudomonas chlororaphis*

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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http://dx.doi.org/10.1016/j.jbiotec.2013.02.003

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.

### 2.2. Feedstock analysis and purification

Saturated biodiesel from waste animal fat was produced by ARGENT Energy Ltd, UK. The fatty acid methyl esters (FAME) were purified *via* distillation under reduced pressure (0.95 mbar) at 140–150 °C.

Fatty acid composition of the biodiesel samples was determined by gas liquid chromatography on a HP 7890 GC apparatus equipped with a flame ionization detector, according to a standard procedure (American Chemists Society, 2009). Chromatographic separation of the individual fatty acid esters was done *via* DB wax column (30 m × 0.25 mm × 0.15 μm), helium was used as carrier gas (0.7 mL $min^{-1}$) and 1 μL of the sample was injected. The peak identification was done by comparison of the retention times with a reference material (GLC-462, Nu Check Prep Inc).

### 2.3. mcl-PHA biosynthesis

Three aerobic cultivations were carried out in a laboratory bioreactor (Labfors 3, Infors, CH, working volume 2 or 5 liter respectively, consisting of a glass vessel stirred from the upper side) under controlled conditions for pH-value (7.1 ± 0.1), temperature (30 °C) and dissolved oxygen concentration $pO_2$ (controlled by agitation speed of one or two axial stirrers respectively and air flow rate; during growth phase, $pO_2$ amounted to 40%, during the phase of predominant PHA-accumulation to 20% of the oxygen saturation concentration). Glanapon 2000 (undiluted) (Bussetti, Austria) was used as antifoam agent and was added automatically by the bioreactor upon alert from the antifoam probe. However no serious foaming was encountered during the experiments.

The volumes of prepared media and according inocula are described for each experiment in "Results and Discussion".

SFAE was added by substrate pulses, according to the consumption rate by the cells, in order to provide a sufficient concentration of carbon source during the entire process. For enhanced distribution of the hydrophobic SFAE in the aqueous fermentation broth and, therefore, enhanced susceptibility of the carbon source for the cells, the emulsifier Grinsted Citrem SP70 was added in small amounts together with SFAE.

During the first phase of cultivation, pH-value was kept constant by automatic supply of aqueous $NH_4OH$ solution (25%), which also kept the concentration of the nitrogen source $NH_4^+$ at a constant level. When a desired concentration of biomass was obtained, the $NH_4OH$ solution was exchanged by aqueous NaOH solution (20%) in order achieve restricted concentrations of nitrogen. This initiated the stop of biomass growth and the shift of carbon flux towards *mcl*-PHA accumulation.

After stopping of the fermentations, the cells were *in situ* pasteurized for 1 hour at 70 °C, centrifuged for 20 minutes at 4 °C and 5000 rpm (Sorvall RC-5B Refrigerated Superspeed centrifuge), frozen and lyophilized for 48 h (Lyophilisator Christ Alpha 1-4 B, Germany).

### 2.4. Cell dry mass determination

A gravimetric method was used to determine the biomass concentration expressed as cell dry mass (CDM) in fermentation samples. 5 mL of culture broth was centrifuged in pre-weighed glass screw-cap tubes for 10 minutes at 10 °C and 4000 rcf in a Heraeus Megafuge 1.0 R refrigerated centrifuge. The supernatant was decanted and used for substrate analysis. The cell pellets were washed with an ethanol / distilled water solution (1:1), re-centrifuged, frozen and lyophilized to a constant mass. CDM was determined as the mass difference between the tubes containing cell pellets and empty tubes. The determination was done in duplicate. The lyophilized pellets were subsequently used for determination of intracellular *mcl*-PHA as described below.

### 2.5. Optical density determination and nitrogen source ($NH_4^+$) rapid test

To monitor the bacterial growth, the samples were diluted if necessary, and the optical densities measured at λ = 420 nm with deionized water as zero reference utilizing a Genesys 10S UV-VIS Spectrophotometer (Thermo Scientific, USA). For fast determination of the ammonium concentration, a visual semi quantitative test (Merckoquant 110024, Merck Millipore, Germany) was used. After sampling and centrifugation, 1 mL of the supernatant was mixed with 2 drops of reagent NH4-1. A test strip was immersed into the sample for 3 sec. After 10 sec the reaction zone of the test strip was compared with the colour scale.

### 2.6. Exact determination of nitrogen source ($NH_4^+$)

A commercially available test (Merck, Spectroquant, 1.00683.0001) was used, following the principle of ammonia reacting with hypochlorite ions to monochloramine, which further reacts with substituted phenol to form a blue indophenol derivative. This complex can be determined photometrical at λ = 690 nm. The measuring range of the test for ammonium is 6–193 mg/L. As reference ammonium sulphate was used.

After the centrifugation of the samples, the supernatants of the parallel tubes were combined and an aliquot was filtrated for the ammonium test. First, 5 mL of $NH_4$-1 reagent were mixed with 0.1 mL of sample. Second, 1 level blue micro spoon $NH_4$-2 was added and the solution was mixed until the reagent was dissolved. The initial solution was left to stand for 15 min (reaction time) and measured in a 10-mm cell at λ = 690 nm. Deionized water was used as zero reference.

### 2.7. mcl-PHA recovery from biomass

After degreasing the biomass by overnight extraction with the 20 fold amount of $C_2H_5OH$ at room temperature by continuous stirring, the biomass was filtrated and the remaining $C_2H_5OH$ was finally removed of by air drying. *mcl*-PHA was isolated from the degreased biomass by overnight Soxleth extraction with $CHCl_3$. The volume of this solution was decreased by removing the major part of $CHCl_3$ by a rotary evaporator. Finally, *mcl*-PHA was precipitated as slightly brownish resin by removing the remaining $CHCl_3$ by air drying. Completeness of the isolation was determined by GC-FID analysis of the residual biomass.

### 2.8. GC-FID analysis of mcl-PHA

PHA in lyophilized biomass samples was transesterificated by acidic methanolysis (Braunegg et al., 1978). The gas chromatographic analysis was performed with a 6850 Network GC System (Agilent Technologies), equipped with a 25 m × 0.32 mm × 0.52 μm HP5 capillary column and a flame ionization detector (FID). Helium (*Linde*; purity = 4.6) was used as carrier gas with a split- ratio of 1:5, hydrogen (*Linde*; purity = 5.0) and synthetic air (*Linde*; purity = "free of hydrocarbons") as detector gases and nitrogen (*Linde*; purity = 5.0) as auxiliary gas.

Two temperature programs were used per sample in order to determine as well *mcl*- as eventual *scl*-PHA building blocks. For *scl*-PHA, the following protocol was used: Initial temperature: 50 °C; rate 1: 15 °C/min; final temperature 1: 60 °C; rate 2: 2 °C/min; final temperature 2: 80 °C; final temperature 3: 300 °C; final time 3: 5 min. *mcl*-PHA were determined as follows: Initial temperature: 50 °C; rate 1: 15 °C/min; final temperature 1: 200 °C; final time 1:

**Table 1**
Fatty acid distribution of feedstock sample according AOCS Ce 1-62.

| Fatty Acid | [% m/m] |
|---|---|
| Lauric | 0.11 |
| Myristic | 2.79 |
| Pentadecanoic | 0.55 |
| Pentadecenoic | 0.21 |
| Palmitic | 26.7 |
| Palmitoleic | 2.63 |
| Heptadecanoic | 0.65 |
| Stearic | 17.9 |
| Oleic | 38.5 |
| Linoleic | 4.47 |
| Linolenic | 0.91 |
| Arachidic | 0.09 |
| Gadoleic | 0.08 |
| Not identified | 2.85 |

10 min; rate 2: 15 °C/min; final temperature 2: 240 °C; final time 2: 4 min final temperature 3: 300 °C; final time 3: 5 min. The determination of all samples was done in duplicate. The methyl esters of PHA constituents were detected by a flame ionization detector (FID); carrier gas: helium (split- ratio of 1:5), injection volume 2 μL.

As reference materials, *mcl*-PHA samples containing 3-hydroxyhexanoate (HHx), 3-hydroxyoctanoate (HO), 3-hydroxydecanoate (HD), 3-hydroxydodecanoate (HDD) (purchased from Metabolix Inc. USA), 3-hydroxyhepenoate (HHp) and 3-hydroxynonenoate (HN) (purchased from Versamer™, Polyferm Canada) were used. In addition, poly(3HB-*co*-15.6%-3HV) (BIOPOL™, ICI, UK) and poly(3HB-*co*-11.2%-4HB) (GreenBio™, Tianjin Green Bioscience & DSM, PR China) were used as *scl*-PHA references; hexanoic acid acted as internal standard. The concentration of *mcl*-PHA was defined as the sum of the concentrations of all detected building blocks. The *mcl*-PHA content (wt.-%) was defined as the percentage of *mcl*-PHA concentration in dry cell mass (CDM). Residual biomass (g/L) was defined as the difference between CDM (g/L) and *mcl*-PHA (g/L).

### 2.9. Protein Quantification

Due to the high amount of ethanol soluble extra cellular material produced by the strain, an additional protein quantification of the final cultivation samples was accomplished. Therefore, the cell suspension was quenched four times applying a French press with 100 bar pressure. Afterwards a commercially available Protein Quantification Assay (Macherey-Nagel, Germany) was used for determination of protein content in the suspension. The extinction at λ = 570 nm was measured utilizing a Genesys 10S UV-VIS Spectrophotometer (Thermo Scientific, USA).

## 3. Results and Discussion

### 3.1. Feed stock analysis (Fatty acid composition)

Table 1 provides the data for 13 different fatty acids that were identified as components of the applied biodiesel. Oleic acid (38.5%), palmitic acid (26.7%) and stearic acid (17.9%) constitute the predominant acids.

### 3.2. mcl-PHA Biosynthesis

The scope of this work was to use the strain *Ps. chlororaphis* for the production of *mcl*-PHA. The strain has been basically reported to produce PHA (Timm and Steinbüchel, 1990), but has not been acknowledged as industrially relevant production strain before the study at hand. Prior to aerobe cultivations in a bioreactor, several experiments on shaking flask scale have been done. The achieved

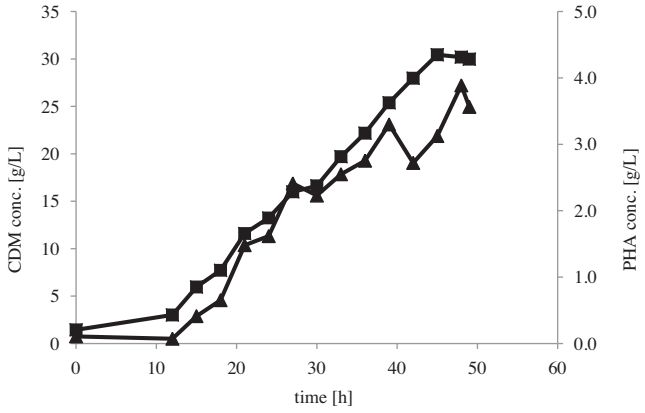


**Fig. 1.** CDM [■] of the first cultivation is drawn *versus* time using the left scale for concentration [g/L]. The *mcl*-PHA concentration [▲] is also drawn *versus* time and is using the right scale to enhance visibility.

results (data not shown) provided information about optimized media composition, the general behaviour of *Ps. chlororaphis* in presence of biodiesel as carbon source, the most efficient emulsifier (Grinsted Citrem SP70) and the toxicity of different concentrations of saturated biodiesel. Not only that the strain adapted really fast to saturated biodiesel as sole carbon source, there were no observable inhibiting effects regarding the applied biodiesel concentrations in the investigated range (1 to 15 g/L). Based on these promising data, the conditions for the *mcl*-PHA biosynthesis on bioreactor scale were chosen.

Continuous chemostat processes for PHA production on laboratory scale are described in literature for single, two- and multistage set-ups; such process-engineering approaches enable an optimal nutrient supply, higher volumetric productivities and a high uniformity of the product (Atlić et al., 2011; Du et al., 2001a, Du et al., 2001b; Jung et al., 2001; Mothes and Ackermann, 2005; Yu et al., 2005; Zinn et al., 2003). Nevertheless, fed-batch mode of cultivation still constitutes the state of the art in (semi)industrial biotechnological polymer production (Chen, 2010b; Nonato et al., 2001), mainly because such discontinuous processes are more robust against microbial contamination, as well as cheaper and easier to install and to handle. Hence, discontinuous fed-batch mode was also used for this study.

The first cultivation for *mcl*-PHA biosynthesis was carried out using a 2 L setup with 1 L minimal medium containing the double concentration for all ingredients as described in chapter 2.1. The bioreactor was inoculated with the same volume of a dense culture (cultivated for 24 h) of the production strain *Ps. chlororaphis*. A concentration of 5 g/L saturated biodiesel as carbon source was used, in accordance to the excellent growth at this concentration in fluid cultures on shaking flask scale. The mean $OD_{420}$ at the start of the cultivation was 7.3. After an initial phase of 12 hours, sampling was performed every 3 hours. The growth phase lasted for 27 hours and achieved a mean $OD_{420}$ of 148.5. Afterwards the process was switched from growth to accumulation phase by a change of bases for pH-value control. The sampling after 42 hours showed a reduction of nitrogen and the PHA accumulation was continued until 49 hours, when a mean $OD_{420}$ of 223.5 was reached (Fig. 1). At this point, the optical density was already reduced, which is an indicator of having exceeded the maximum *mcl*-PHA content possible under given circumstances and the cells started the intracellular degradation of the PHA. Therefore, the cultivation was ended by pasteurization and followed by further downstream processing of the cell suspension for polymer recovery.

A second aerobic cultivation was started with 2.5 L of the previously described minimal medium with double ingredient

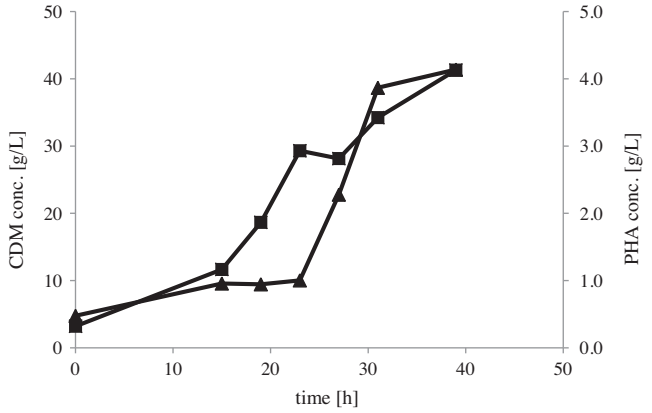


**Fig. 2.** CDM [■] of the second cultivation is drawn *versus* time using the left scale for concentration [g/L]. The *mcl*-PHA concentration [▲] is also drawn *versus* time and is using the right scale to enhance visibility.

concentration and was inoculated with the same volume of a dense culture of *Ps. chlororaphis*. A higher level of saturated biodiesel, namely 10 g/L was used as carbon source and a mean $OD_{420}$ of 9.9 was measured. After a starting phase of 15 hours, sampling was done every 4 hours. This time the growth phase was terminated after 23 hours by stopping the supply with nitrogen source at a mean $OD_{420}$ of 157.5. The next sampling at 27 hours already revealed nitrogen depletion and the cultivation was stopped after 39 hours, when a mean $OD_{420}$ of 369 was measured (Fig. 2). The higher concentrations of saturated biodiesel resulted in an increase of growth rate and accumulation concerning time and $OD_{420}$. However, more details were provided later on by exact determination of nitrogen source ($NH_4^+$) and CDM as well as by GC-FID analysis.

The third aerobic cultivation used a volumetrically reduced inoculum of *Ps. chlororaphis*, which was grown in 1.5 L medium. The main reason for this treatment was enabling measurements of the adaption to the reactor without interfering metabolites. The inoculum was then centrifuged for 20 minutes at 4 °C and 5000 rpm in a Sorvall RC-5B Refrigerated Superspeed centrifuge. Afterwards, the supernatant was discarded and the pellet suspended in 1 L of minimal medium. This inoculum was added to the 4 L minimal medium already present in the bioreactor. The mean $OD_{420}$ at the start of the cultivation was 1.9 and a concentration of 10 g/L SFAE as carbon source was used. Sampling was done every 3 hours. After 24 hours, a mean $OD_{420}$ of 123.5, the $NH_4OH$ solution was exchanged by aqueous NaOH solution, in order to switch to accumulation phase. The next measurement revealed nitrogen depletion and the cultivation

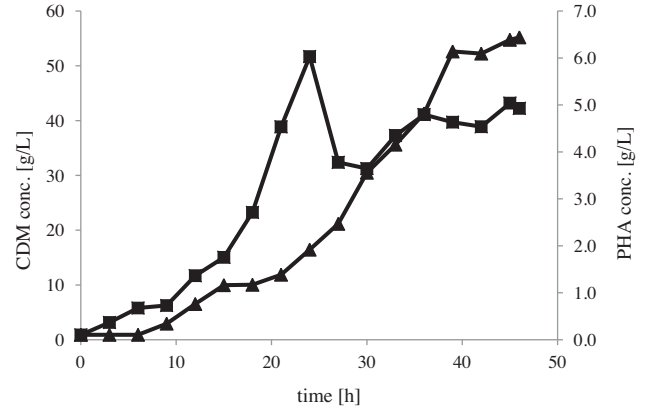


**Fig. 3.** CDM [■] of the third cultivation is drawn *versus* time using the left scale for concentration [g/L]. The *mcl*-PHA concentration [▲] is also drawn *versus* time and is using the right scale to enhance visibility.

was stopped after 46 hours at $OD_{420}$ 218 (Fig. 3). After having collected all data, the analysis provided even deeper insight into the described cultivations.

To support the results of CDM determination and GC-FID analysis, the nitrogen concentrations were measured first verifying the rapid test results as well as the time points of $NH_4^+$ depletion for the cultivations. Afterwards the CDM determination completed the necessary data for the kinetic analysis of the growth behaviour, while the GC-FID analysis provided the information about *mcl*-PHA content and composition. The first cultivation achieved a maximum specific growth rate $\mu_{max.}$ of 0.08 $h^{-1}$, the volumetric productivity for *mcl*-PHA amounted to 0.071 g/L h. During this experiment, the volumetric productivity during the accumulation phase kept nearly at the same rate of 0.070 g/L h. Furthermore, a final CDM of 30 g/L with 11.9% *mcl*-PHA content was obtained, with a SFAE to mcl-PHA transformation yield of 0.075 g/g and a SFAE to biomass transformation yield of 0.62 g/g. Due to the small bioreactor and content (2 L), the second bioreactor experiment was initiated with a bigger working volume (5L) and higher biodiesel concentration (10 g/L) to verify these results. This cultivation performed with a maximum specific growth rate $\mu_{max.}$ of 0.10 $h^{-1}$ and a volumetric productivity for *mcl*-PHA of 0.094 g/L h. During accumulation, the volumetric productivity strongly increased to 0.155 g/L h. The final CDM resulted in 41.3 g/L with a *mcl*-PHA content of 10.0%. The SFAE to mcl-PHA yield remained approximately at the same level with 0.070 g/g while the SFAE to biomass transformation yield increased to 0.73 g/g. These values clearly confirmed the assumption of a positive impact of higher saturated biodiesel concentrations on the biomass growth, keeping the *mcl*-PHA content at approximately the same level. However, due to quite large amounts of extracellular material, which could not be separated from the cellular biomass by centrifugation, additional protein quantification was done for the final samples of both cultivations. The results of this pure protein *versus mcl*-PHA content revealed a protein mass of 16.1 g/L and, therefore, a correlated *mcl*-PHA content of 22.1% for the first cultivation. The second cultivation resulted in 14.1 g/L protein mass and 29.4% *mcl*-PHA content. Taking these results into account, the production of extra cellular material continues in the accumulation phase and increases with a higher carbon source. As a result, the cellular *mcl*-PHA content is higher than assumed at first glance. Therefore, the third cultivation was done with the same volume and biodiesel concentration but utilizing the previously centrifuged inoculum. This experiment achieved an even higher maximum specific growth rate $\mu_{max.}$ of 0.13 $h^{-1}$ with a volumetric productivity for *mcl*-PHA of 0.138 g/L h. During the accumulation phase, the volumetric productivity increased to 0.209 g/L h and a final CDM of 42.2 g/L with 15.2%. *mcl*-PHA content was achieved. The transformation yield of SFAE to *mcl*-PHA increased to 0.101 g/g while the SFAE to biomass transformation yield resulted between the values from the prior cultivations at 0.66 g/g. The impact of the smaller but very dense inoculum exceeded the expectations resulting in a high growth rate and increased *mcl*-PHA production.

The spent fermentation broth remaining after stop of the cultivations was analysed for the residual concentrations of SFAE, which amounted to 2.69 g/L (1st cultivation), 3.28 g/L (2nd cultivation), and 10.79 g/L (3rd cultivation), respectively.

The analysis of accumulated PHAs revealed further information. While no *scl*-PHA building blocks (3-hydroxybutyrate, 4-hydroxybutyrate, 3-hydroxyvalerate) were detected, the composition of the *mcl*-PHA content was even more interesting. The main part of the obtained *mcl*-PHA consisted of 3-hydroxyoctanoate (HO) and 3-hydroxydecanoate (HD). A smaller portion referred to 3-hydroxyhexanoate (HHx) and hydroxydodecanoate (HHD), while only traces of 3-hydroxyheptanoate (HHp) and 3-hydroxynonanoate (HN) were detected. As most remarkable example, Fig. 4 displays the course of PHA content during the third

**Table 2**
Comparison of *mcl*-PHA compositions in percentages to total PHA at the end of the experiments.

| [%] of total *mcl*-PHA | HHx[a] [%] | HHp[b] [%] | HO[c] [%] | HN[d] [%] | HD[e] [%] | HHD[f] [%] |
|---|---|---|---|---|---|---|
| Shaking flask | 15.17 | 2.99 | 50.51 | 5.09 | 20.83 | 5.41 |
| Cultivation 1 | 15.45 | 1.12 | 50.64 | 1.67 | 26.14 | 4.98 |
| Cultivation 2 | 14.60 | 2.20 | 45.69 | 3.48 | 27.32 | 6.71 |
| Cultivation 3 | 10.18 | 1.60 | 47.91 | 2.69 | 31.39 | 6.22 |

[a] 3-hydroxyhexanoate.
[b] 3-hydroxyhepenoate.
[c] 3-hydroxyoctanoate.
[d] 3-hydroxynonenoate.
[e] 3-hydroxydecanoate.
[f] 3-hydroxydodecanoate.

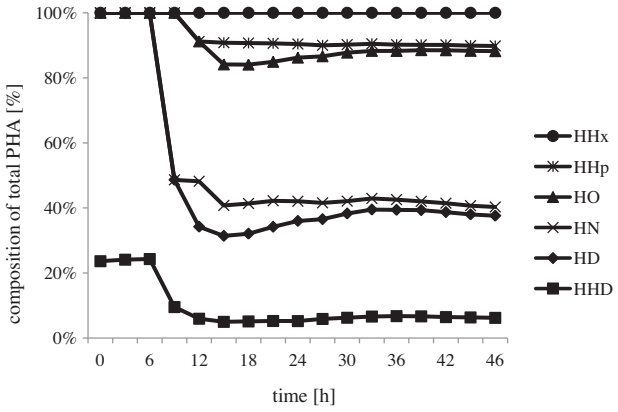


**Fig. 4.** The course of the *mcl*-PHA composition of the third cultivation in percentages of total PHA content. The graphs represent the upper limit of the corresponding area and include 3-hydroxyhexanoate (HHx ●), 3-hydroxyhepenoate (HHp *), 3-hydroxyoctanoate (HO ▲), 3-hydroxynonenoate (HN × ), 3-hydroxydecanoate (HD ♦) and 3-hydroxydodecanoate (HHD ■).

cultivation set-up, clearly underlining the difference between lag phase, growth phase (up to 24 hours) and the phase of predominant *mcl*-PHA accumulation during nitrogen limitation. Especially the shift from HD and HDD towards a similar distribution of the building blocks in the other cultivations indicates a very stable composition of the final *mcl*-PHA content. Some of these effects were also observed by analysing the first and second cultivation (data not shown), but not that clearly visible, especially during the lag phase. To visualize the mentioned very stable distribution of the *mcl*-PHA components, the final values of a preliminary shaking flask experiment as well as from the first, second and third cultivation are summarized in Table 2. While the shaking flask experiment and cultivation 1 were performed using a carbon source concentration of 5 g/L, the increase in cultivations 2 and 3 to 10 g/L during the experiments did not result in significant differences of the *mcl*-PHA composition. This fact and the high growth rates make this strain especially interesting for industrial production of *mcl*-PHA from saturated biodiesel fractions.

## 4. Conclusions

The study demonstrates the high potential of the strain *Ps. chlororaphis* for *mcl*-PHA production from by-products of the animal-based biodiesel industry. Compared to *Ps. citronellolis* DSM 5033, another strain producing *mcl*-PHA from animal-derived biodiesel, the biomass production was about equal, but the *mcl*-PHA content was much higher in the work at hand (Muhr et al., 2013). Referring to *Ps. putida* GPo1 (ATCC 29347), *Ps. chlororaphis* as well as *Ps. citronellolis* surpassed the capabilities of *Ps. putida* GPo1 in a fed-batch process (data not shown) using the same inexpensive carbon source. Furthermore, the obtained results are already in a reasonable range if compared to similar processes for *mcl*-PHA production on expensive carbon sources. From the biotechnological point of view, future experiments on bioreactor scale have to focus on the increase of the intracellular *mcl*-PHA content. This could be accomplished by investigating growth-limiting factors others than the nitrogen source, such as restricted phosphate supply (Lee et al., 2000). Finally, genetic engineering for knocking-out the PHA-depolymerase gene might be a suitable tool to achieve higher productivities by preventing the degradation of already accumulated *mcl*-PHA. Another option for genetic engineering could be the increase of the number of gene copies for the *mcl*-PHA synthases, or the introduction of an inducible promoter that can increase the polymerase level as shown for *Ps. putida* GPp104 (Ren et al., 2005). In addition, genetic engineering might also facilitate the recovery of *mcl*-PHA after cell disruption (Boynton et al., 1999). In this context, high amounts of nucleic acids are liberated, resulting in increased viscosity of the cell lysate that complicates PHA recovery. Excretion of high nuclease quantities right after cell disruption results in lower viscosities that facilitate the separation of PHA granules from the surrounding liquid phase. This could be achieved by integrating nuclease-encoding genes into the genome of *Ps. chlororaphis*.

## Acknowledgement

The authors gratefully acknowledge the financial support provided by the European Commission by granting the 7th Framework project "*Biotechnological conversion of carbon containing wastes for eco-efficient production of high added value products*", Acronym ANIMPOL (Contract No: 245084).

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