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Biodegradable latexes from animal-derived waste: Biosynthesis and characterization of *mcl*-PHA accumulated by *Ps. citronellolis*

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ABSTRACT

Background: *mcl*-PHA biosynthesis by *Pseudomonas citronellolis* from tallow-based biodiesel as inexpensive carbon feed stock was accomplished. Fermentation protocols, kinetic analysis, an efficient product recovery strategy, and a detailed product characterization are presented.
Results: A maximum specific growth rate, $\mu_{max.}$ of 0.10 and 0.08 h^{-1}, respectively, was achieved in two different fermentation set-ups. Volumetric productivity for *mcl*-PHA amounted to 0.036 g/L h and 0.050 g/L h, final intracellular PHA contents calculated from the sum of active biomass and PHA to 20.1 and 26.6 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. This was confirmed by ^{1}H- and ^{13}C NMR, also evidencing the occurrence of low quantities of unsaturated and 3-hydroxyvalerate building blocks. High purity of the recovered materials was proofed by elemental analysis. Regarding the results from thermogravimetric analysis, differential scanning calorimetry and molecular mass determination, results were in a range typical for this type of PHA (1st fermentation: decomposition temperature T_d = 296 °C, peak of melting range T_m = 48.6 °C; glass transition temperature T_g = −46.9 °C, degree of crystallinity X_c = 12.3%, M_w = 66,000, M_n = 35,000, dispersity index P_i = 1.9; 2nd fermentation: T_d = 295 °C, T_m = 53.6 °C, T_g = -43.5 °C, X_c = 10.4%, M_w = 78,000, M_n = 196,000, P_i = 2.5).

1. Introduction

The excellent material properties and integration of their life cycle into naturés carbon balance, has led to Poly(hydroxyalkanoates) (PHAs) attracting attention as "green plastics" [1]. Biologically they act as microbial reserve compounds; as plastics, they might soon replace their petrol-based competitors in several bulk and niche market segments [2]. PHAs displaying desired properties are accessible from renewable resources by selected prokaryotes, opening the door for substituting petrol-based thermoplasts, elastomers, latexes, and even functional polymers [3].

For the break-through on the market, PHAs must compete against well-established petrol-based polymers in terms of material performance and economically [4]. PHA biosynthesis is reported from renewable feedstocks like carbohydrates, lipids, alcohols, acids, or methane [5]. The major share of the expenses is attributed to these mainly pure, prized feedstocks of nutritional value. Further, PHA biosynthesis occurs under aerobic conditions; hence, a considerable share of the carbon source is metabolized towards CO_2 and minor by-products. As an alternative, inexpensive carbon-rich industrial by-products can act as feedstock, making PHAs economically competitive without interfering with human nutrition. These efforts have to go in parallel with improving all process steps, mainly process design, product recovery, and closing of material- and energy cycles.

Depending on their monomers, short chain length (*scl*) and medium chain length (*mcl*) PHAs are distinguished. Low glass transition temperatures (around −40 °C), low crystallinity (X_c not exceeding 40%), broad melting ranges at low melting temperatures (around 60 °C), and low degrees of polymerization (molecular masses typically below 100,000 Da) discriminate *mcl*-PHAs from *scl*-PHAs. In the latter case, high glass transition temperatures around 0 °C, sharp melting points of up to 180 °C, and high

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molecular masses (up to 4 MDa) are observed [6–8]. Regarding polydispersities (P_i) of molecular masses, *mcl*-PHAs generally feature lower values than *scl*-PHAs [9].

Concerning physical properties, *mcl*-PHA display high elongation to break of some 100%; for poly(3-hydroxybutyrate) (PHB), the most important *scl*-PHA, this value is about 6%. Comparing tensile strength, the values for most *scl*-PHAs are reported with 30–40 MPa in contrast to about 10 MPa for *mcl*-PHAs. Poly(4-hydroxybutyrate) constitutes an exception with an elongation to break of about 1000%; its glass transition temperature of about −50 °C is in the same range as for *mcl*-PHAs [7,8].

Monomers of *scl*-PHAs consist of 3–5 carbon atoms. According to the physical features discussed before, *scl*-PHAs resemble classical thermoplasts; hence, they compete with Poly(ethylene), Poly(propylene), or biobased Poly(lactate). *Cupriavidus necator* from the *Burkholderiaceae* family constitutes the best investigated *scl*-PHA producer.

mcl-PHAs with monomers of 6–14 carbon atoms are by far less crystalline [10]. Sometimes, the building blocks possess functionalities allowing post-synthetic modification for fine-tuning of polymer properties [11]. *mcl*-PHA can be regarded as biological rubbers or latexes that do not become brittle even far below the freezing point. As the most prominent *mcl*-PHA producer, *Pseudomonas putida* (a.k.a. *Ps. oleovorans*) is well-described by the scientific community [12].

In contrast to numerous reports for *scl*-PHA production from industrial waste [13–19], information on *mcl*-PHA production from inexpensive feedstocks is scarce [20,21]. Therefore, *mcl*-PHA production from saturated biodiesel fractions (SFAE) originating from animal waste lipids was investigated. In Europe, such lipids from animal-processing industry amount to 500.000 t per year. Using them for biodiesel production, the available SFAE is estimated with annually 50.000 t. SFAE deteriorate biodiesel properties as engine fuel due to poor cold temperature behaviour; if separated, it can be used as feedstock for PHA biosynthesis, whereas the remaining unsaturated biodiesel fraction performs as 2nd generation biofuel.

The strain *Pseudomonas citronellolis* was selected due to encouraging properties. The broad range of converted carbon substrates, mainly linear mono- and dicarboxylic acids, and the variety of monomers assembling the produced *mcl*-PHAs attract interest in this organism [22,23]. Substrates used in these studies, e.g. citronellol, 3-methyl-n-valerate, 3-methyl-n-butanol, 3-methyladipate, octanoate, or 5-phenylvalerate are expensive, and only mg quantities were obtained by the reported cultivations. This is a typical feature for most processes described for *mcl*-PHA production and has to be overcome in order to achieve cost-efficiency. *Ps. citronellolis* is quite comprehensively described for its potential ecological benefit as a "bio-remediator" due to its ability to eliminate recalcitrant hazardous hydrocarbons from spoiled environments [24–27]. In the presented study, *Ps. citronellolis* was for the first time used for *mcl*-PHA production from inexpensive substrates, providing reliable kinetic data for *mcl*-PHA biosynthesis by this organism and basic characterization of the obtained polymer.

2. Materials and methods

2.1. Materials

2.1.1. Microorganism and culture conditions

A lyophilized sample of *Ps. citronellolis* DSM 50332 was purchased from DSMZ culture collection, Germany. 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 revitalization. Subsequently, colonies were cultivated in minimal media according to Küng [28] containing saturated biodiesel as the sole carbon source for adaptation to this substrate.

A chemically defined mineral medium according to Küng [28] was used for cultivations in shaking flasks (SFs) 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, 5–10 g. The trace element solution SL6 was composed as follows (per litre): $ZnSO_4{*}7H_2O$, 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.

2.1.2. 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 (GC-FID), according to a standard procedure [29]. Chromatographic separation of the individual fatty acid esters was done using a DB wax column (30 m × 0.25 mm × 0.15 μm), helium was used as carrier gas (0.7 mL min^{-1}) injecting 1 μL of the sample. The peak identification was done by comparison of the retention times with a reference material (GLC-462, Nu Check Prep Inc.).

2.1.3. mcl-PHA biosynthesis

Two cultivations were carried out aerobically in a laboratory bioreactor (Labfors 3, Infors, CH, working volume 5 L; glass vessel stirred from the upper side) under controlled conditions of pH (7.1 ± 0.1), temperature (30 °C), and dissolved oxygen concentration pO_2 (controlled by agitation speed of two axial stirrers 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).

In both set-ups, 2.5 L of a minimal medium containing the double concentration for all ingredients as described for shaking flask cultures (in order to compensate nutrient limitations in the inoculum) was inoculated with the same volume of a dense culture (cultivated for 24 h) of the production strain *Ps. citronellolis.*

According to the consumption rate by the cells, SFAE was added by substrate pulses if needed in order to provide a sufficient concentration of carbon source during the entire process. For enhanced solubilisation of the hydrophobic SFAE in the aqueous fermentation broth, hence for enhanced utilization of the carbon source for the cells, 120 μg/L of the emulsifier Grinsted Citrem SP70 was added together with SFAE.

During the first phase of cultivation, the pH-value was kept constant by automatic supply of aqueous NH_4OH solution (25%) that, at the same time, kept the concentration of the nitrogen source NH_4^+ at a constant level. After a desired concentration of biomass was obtained, NH_4OH solution was exchanged by aqueous NaOH solution (20%) in order achieve restricted concentrations of nitrogen to initiate the stopping of biomass growth and the shift of carbon flux towards *mcl*-PHA accumulation.

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

2.1.4. 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, subsequent filtration and final removal of remaining C_2H_5OH by air drying, *mcl*-PHA was isolated by overnight Soxhlet

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 latex by adding the ten-fold volume of ice-cooled C_2H_5OH. Completeness of the isolation was determined by GC-FID.

2.1.5. Optical density and nitrogen source (NH_4^+) determination

To monitor the bacterial growth, the samples were diluted if necessary, and the optical densities measured at $\lambda = 420$ 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 s. After 10 s the reaction zone of the test strip was compared with the colour scale.

For exact determination of NH_4^+, a commercially available test (Merck, Spectroquant, 1.00683.0001) was used. The kit follows 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 photometrically at $\lambda = 690$ nm. The measuring range of the test for ammonium is 6–193 mg/L. Ammonium sulphate was used as reference.

After the centrifugation of the tubes from sampling, the supernatants of the parallel tubes were combined and frozen at −20 °C. The samples were thawed and an aliquot was filtrated for the ammonium test. First, 5 mL of NH4-1 reagent were mixed with 0.1 mL of sample. Second, 1 level blue micro spoon NH4-2 were added and the solution was mixed until the reagent was dissolved. The initial solution was left to stand for 15 min (reaction time) and the absorbance measured in a 10-mm cell at λ = 690 nm. Deionized water was used as zero reference.

2.1.6. 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 min at 10 °C and 4000 rpm 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.

2.1.7. Protein quantification

Due to the high amount of ethanol soluble extra cellular material, an additional protein quantification of the final cultivation samples was accomplished. Therefore, the cell suspension was quenched four times applying a French pressure cell 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 570 nm was measured utilizing a Genesys 10S UV–VIS Spectrophotometer (Thermo Scientific, USA).

2.2. Physicochemical characterization

2.2.1. GC-FID analysis of mcl-PHA

PHA in lyophilized biomass samples was transesterificated by acidic methanolysis [30]. 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 different temperature programs were used per sample: one for *mcl*- and one for 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 1:10 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 in order to compensate evaporation of the solvents during transesterification. 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 to dry cell mass (CDM). Residual biomass (g/L) was defined as the difference between CDM (g/L) and *mcl*-PHA (g/L).

2.2.2. ^1H NMR and ^{13}C NMR characterization of mcl-PHA

The ^1H and ^{13}C NMR spectra were recorded using a Bruker - Avance II 600 MHz equipped with Ultrashield Plus Magnets. The ^1H and ^{13}C NMR spectra were run in $CDCl_3$ using tetramethylsilane (TMS) as an internal standard. ^{13}C NMR spectra were recorded on a Bruker–Avance II 150.9 MHz.^{13}C NMR spectra were obtained with 20,480 scans, a 9.40 μs pulse width, and a 0.9088 s acquisition time.

2.2.3. Elemental analysis

Elemental analysis as a measure for product purity were performed using a Perkin Elmer Analyzer 2400.

2.2.4. Thermoanalysis: thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC)

TGA was done using a TGA/DSC 1 Mettler Tolledo equipment at the temperature range from 25 to 650 °C. The maximum decomposition temperature (T_{max}) was determined at a heating rate 10 °C/min in nitrogen atmosphere with a flow rate of 60 mL/min.

DSC measurements were performed with a TA DSC 2010 apparatus (TA Instruments, New Castle, DE) in the temperature range from −80 to +200 °C. The glass transition temperatures (T_g) were determined at a heating rate 20 °C/min. In this study, T_g was taken as the midpoint of the step-transition. The instrument was calibrated with high purity indium and gallium.

The degree of crystallinity (X_c) of the *mcl*-PHA samples was determined by considering the value of the melting enthalpy of 146 J/g for the 100% crystalline PHB [31].

2.2.5. Molecular mass determination

The number-average molecular mass (M_n) and molecular mass dispersity index P_i (M_w/M_n) were determined by gel permeation chromatography (GPC) conducted in $CHCl_3$ solution at 35 °C, at a

flow rate of 1 mL/min using a Spectra-Physic 8800 solvent delivery system with a set of two PLgel 5 μm MIXED-C ultra-high efficiency column and Shodex SE 61 refractive index detector. A 10 μL sample in $CHCl_3$ at a concentration of 1% w/v was injected. Polystyrene standards with narrow molecular mass distribution were used to generate a calibration curve.

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. The fatty acid composition is typical for animal fat containing about 45% of saturated fatty acids, the amount of odd-numbered margaric acid is lower than 1%.

3.2. mcl-PHA biosynthesis

The scope of this work was to use the strain *Ps. citronellolis* for the production of *mcl*-PHA. The strain has been basically reported to produce PHA [22], but has not been acknowledged as a possible production strain until this study. Prior to aerobic cultivations in a bioreactor, several experiments with shaking flasks have been performed. The achieved results (data not shown) provided information about optimized media composition, the general behaviour of *Ps. citronellolis* in the presence of biodiesel as a carbon source, the most efficient emulsifier (Grinsted Citrem SP70) and the toxicity of different saturated biodiesel (SFAE) concentrations. Not only that the strain adapted really fast to SFAE as sole carbon source, there were no observable inhibiting effects due to the applied biodiesel concentrations in the investigated range (1–15 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 [32–40]. Nevertheless, fed-batch mode of cultivation still constitutes the state of the art in (semi)industrial biotechnological polymer production [41,42], mainly because such discontinuous processes are more robust against microbial contamination, and cheaper and easier to install. Hence, discontinuous fed-batch mode was also used for this study. The first cultivation for *mcl*-PHA biosynthesis was carried out using a concentration of 5 g/L SFAE, 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 6.4. After an initial phase of 12 h, sampling was performed every 3 h. The growth phase lasted for 33 h and achieved a mean OD_{420} of 138. Afterwards the process was switched from growth to accumulation phase by a change of bases for pH-value control, thus provoking nitrogen limitation. The sampling after 39 h showed a depletion of nitrogen and the PHA accumulation continued until 72 h, when a mean OD_{420} of 244 was reached (Fig. 1). At this point, the optical density remained at approximately the same level, which is an indicator of having achieved the maximum *mcl*-PHA content possible under the given circumstances. Therefore, the cultivation was ended by pasteurization and followed by further downstream processing of the cell suspension.

A second aerobic cultivation was started with a higher level of SFAE, namely 7.5 g/L and a mean OD_{420} of 19.6. After a starting phase of 15 h, sampling was done every 4 h. This time the growth phase was terminated after 19 h by exchange of the base, thus provoking nitrogen limitation, at a mean OD_{420} of 98.5. For the accumulation phase, the concentration of SFAE was further increased to 10 g/L. The next sampling at 23 h already revealed nitrogen depletion and the cultivation was stopped after 45 h, where a mean OD_{420} of 312 was measured (Fig. 2). The higher concentrations of saturated biodiesel indicated an increase in growth rate and accumulation concerning time and OD_{420}.

To ensure the correct understanding of CDM determination and GC-FID analysis, the nitrogen concentrations were measured first; this verified the rapid test (see Section 2.1.5) results as well as the time points of NH_4^+ depletion for both 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 0.036 g/L h. However, the volumetric productivity during the accumulation phase nearly doubled to 0.067 g/L. Furthermore, a final CDM of 30.6 g/L with 9.2% *mcl*-PHA content was obtained. The second bioreactor experiment performed with a maximum specific growth rate $\mu_{max.}$ of 0.10 h^{-1} and a volumetric productivity for *mcl*-PHA of 0.050 g/L h. During accumulation, the volumetric productivity increased to 0.100 g/L h. The final CDM resulted in 42.0 g/L with a *mcl*-PHA content of 6.8%. These values clearly confirmed the assumption of a positive impact of higher saturated biodiesel concentrations. However, due to quite large amounts of extracellular material, which interfered with CDM determination, additional protein quantification was done for the final samples of both cultivations. The results revealed a protein concentration of 14.1 g/L and, therefore, a correlating *mcl*-PHA content (data from GC-FID analysis) of 20.1% for the first cultivation. The second cultivation resulted in a protein concentration of 11.2 g/L and a *mcl*-PHA content (data from GC-FID analysis) of 26.6%. Taking these results into account, the production of extra cellular material continues in the accumulation phase and increases with a higher concentration of provided carbon source. In any case, the intracellular *mcl*-PHA content is higher than assumed at first glance.

The analysis of stored PHAs revealed more information. While no *scl*-PHA was detected, the composition of the *mcl*-PHA content was even more remarkable. The main part of the stored *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-hydroxynonenoate (HN) were detected. As an example, Fig. 3a displays the normalized temporal variation and Fig. 3b the concentration of PHA content during the first cultivation set-up, clearly underlining the difference between growth phase (up to 39 h) and the phase of predominant *mcl*-PHA accumulation

Table 1
Fatty acid distribution of feedstock sample according to 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

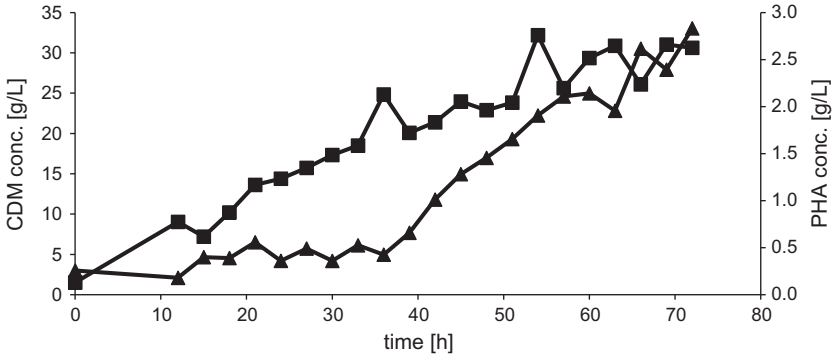

Fig. 1. CDM [■] of the first cultivation is drawn versus time using the left scale for concentration [g/L]. The *mcl*-PHA concentration [▲] is also drawn versus time and is using the right scale to enhance visibility. The total amount of added SFAE was 53.97 g/L.

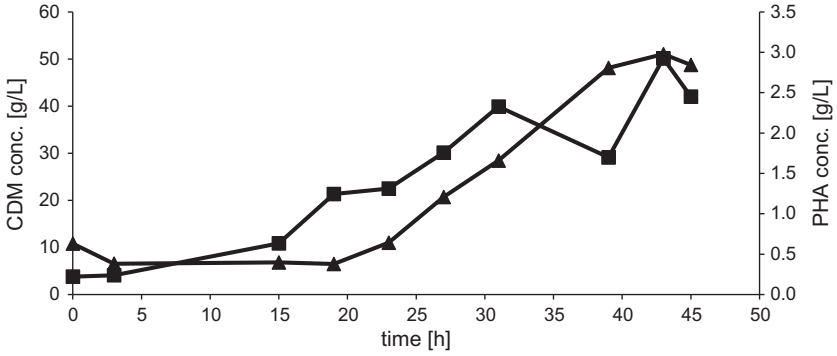

Fig. 2. CDM [■] of the second cultivation is drawn versus time using the left scale for concentration [g/L]. The *mcl*-PHA concentration [▲] is also drawn versus time and is using the right scale to enhance visibility. The total amount of added SFAE was 64.68 g/L.

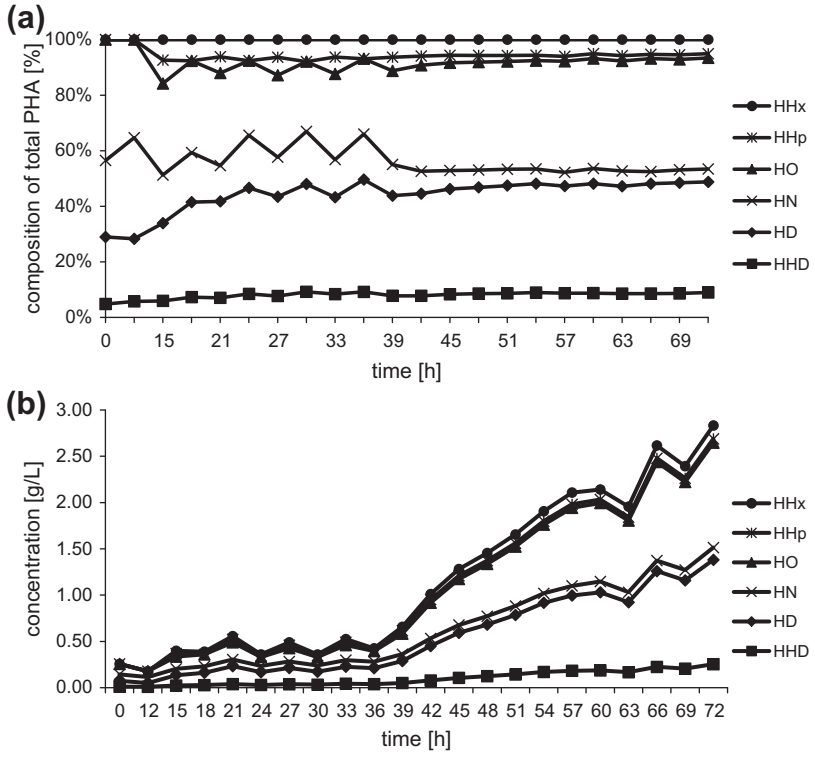

Fig. 3. (a) The course of the *mcl*-PHA composition of the first cultivation in percentages of total PHA content. The graphs include 3-hydroxyhexanoate (HHx ●), 3-hydroxyhepenoate (HHp*), 3-hydroxyoctanoate (HO ▲), 3-hydroxynonenoate (HN×), 3-hydroxydecanoate (HD ♦) and 3-hydroxydodecanoate (HHD ■). (b) The course of the *mcl*-PHA composition of the first cultivation in g/L added to total PHA content. The graphs include 3-hydroxyhexanoate (HHx ●), 3-hydroxyhepenoate (HHp*), 3-hydroxyoctanoate (HO ▲), 3-hydroxynonenoate (HN×), 3-hydroxydecanoate (HD ♦) and 3-hydroxydodecanoate (HHD ■).

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

(%) Of total *mcl*-PHA	HHx[a] (%)	HHp[b]	HO[c] (%)	HN[d] (%)	HD[e] (%)	HHD[f] (%)
Shaking flask	6.0	1.6	45.6	2.4	36.8	7.6
Cultivation 1	5.1	1.5	40.0	4.7	39.8	9.0
Cultivation 2	6.5	1.7	46.5	2.6	35.8	6.9

[a] 3-Hydroxyhexanoate.
[b] 3-Hydroxyhepenoate.
[c] 3-Hydroxyoctanoate.
[d] 3-Hydroxynonenoate.
[e] 3-Hydroxydecanoate.
[f] 3-Hydroxydodecanoate.

Table 3
Results from DSC and GPC analysis for *mcl*-PHA produced by two bioreactor cultivations.

mcl-PHA sample from	T_g (°C)	T_m (°C)	ΔH_m (J/g)	X_c (%)	T_{max} (°C)	M_w (g/Mol)	M_n (g/Mol)	P_i
Cultivation 1	−46.9	48.6	18.0	12.3	297	66,000	35,000	1.9
Cultivation 2	−43.5	53.6	15.2	10.4	295	196,000	78,000	2.5

T_g: glass transition temperature; ΔH_m; X_c: degree of crystallinity; T_{max}: temperature of onset of decomposition; M_w: weight average molecular mass; M_n: number average molecular mass; P_i: polydispersity index.

at nitrogen limitation. The same effect was observed after the analysis of the second cultivation (data not shown). To illustrate the very stable distribution of the *mcl*-PHA components during the accumulation phase, the final values of a preliminary shaking flask experiment as well as from the first and second cultivation are summarized in Table 2. While the shaking flask experiment and cultivation 1 had a carbon source concentration of 5 g/L, the increase in cultivation 2 to finally 10 g/L in the accumulation phase did not result in significant differences of the *mcl*-PHA composition. This makes this strain especially interesting for industrial production.

3.3. Determination of mcl-PHA purity with the aid of elemental analysis

Elemental analysis has the capability of being a rapid method for PHA purity determination by measurement of nitrogen content, eventually present due to the contamination by phasine proteins. Elemental analysis showed that the nitrogen content in the synthesized *mcl*-PHA by *Pseudomonas citroneollis* were very low, for the first sample 0.002% and for the second one 0.000%, confirming the appropriateness of the applied extraction method.

3.4. Thermal properties characterization via DSC and TGA

The thermal properties of the produced *mcl*-PHA were determined by means of differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA). The results presented in Table 3 for DSC analysis included values of glass transition temperature (T_g), melting temperature (T_m) and melting enthalpy (ΔH_m), while the TGA results are presented as the maximum decomposition temperature (T_{max}).

The T_g of the samples range from −43 °C to −48 °C and can be regarded as values typical for this type of PHA [43]. Samples obtained from both cultivations also showed a T_m in the range of 50 °C with a ΔH_m of about 20 J/g. Also these values are typical for *mcl*-PHAs [43] and indicate that the samples are not totally amorphous, but partially crystalline (X_c = 12.3% for the sample obtained by the first cultivation, and 10.4% for the second sample) with classical rubber- to latex like characteristics. This might be due to an assembling of both the main and the side chains of the polymer in a layered order as frequently observed for other polymers harbouring long side chains [42].

3.5. NMR analysis of mcl-PHA

Fig. 4a and b shows the ^{1}H and ^{13}C NMR spectra of the *mcl*-PHA obtained by the first fermentation. The NMR peaks assignment was carried out according to [43]. Fig. 4c shows the ^{13}C NMR spectrum of the *mcl*-PHA obtained by the second fermentation. In the ^{1}H NMR spectrum of Fig. 4a, peaks before 1.0 ppm (O,D), assigned to 3-hydroxyoctanoate (3HO) 3-hydroxydecanoate (3HD) are present. In the ^{13}C NMR spectrum of Fig. 4a and b, the signals at 14.5 and 38 ppm confirm the presence of the predominant 3HO and 3HD units, thus matching the results of GC-FID analyses. In addition, the NMR spectra give evidence to the presence of low amounts of 3-hydroxyvalerate (3HV) and even unsaturated building blocks.

3.6. Molecular mass distribution

The results of molecular mass measurement of the analyzed samples, estimated by GPC experiments with polystyrene standards, are depicted in Table 3. The number-average molecular mass in both cases is located at the range of high molecular masses, and the M_n of cultivation 2 is higher than that of cultivation 1. The unimodal distribution of the peaks was observed and the dispersity index for both cultivations is low and equal about 2.0.

4. Conclusions and outlook

The study demonstrates that the strain *Ps. citronellolis* is a prospective candidate for *mcl*-PHA production on a larger scale starting from by-products available from the animal-based biodiesel industry.

Considering the fact that the cultivations were accomplished in discontinuous fed-batch mode, the obtained results are already in a reasonable range if compared to similar, comparable processes for *mcl*-PHA production on expensive carbon sources. Considering the concentrations of active biomass, the novel process even surpasses the performance of comparable processes using related Pseudomonad strains. From the biotechnological point of view, future experiments on bioreactor scale have to focus on the increase of the intracellular *mcl*-PHA content. This can be accomplished by investigating growth-limiting factors others than nitrogen source, e.g. restricted oxygen supply or phosphate limitation [44]. Also genetic engineering for knocking-out the PHA-depolymerase gene will be a suitable tool to achieve higher productivities by

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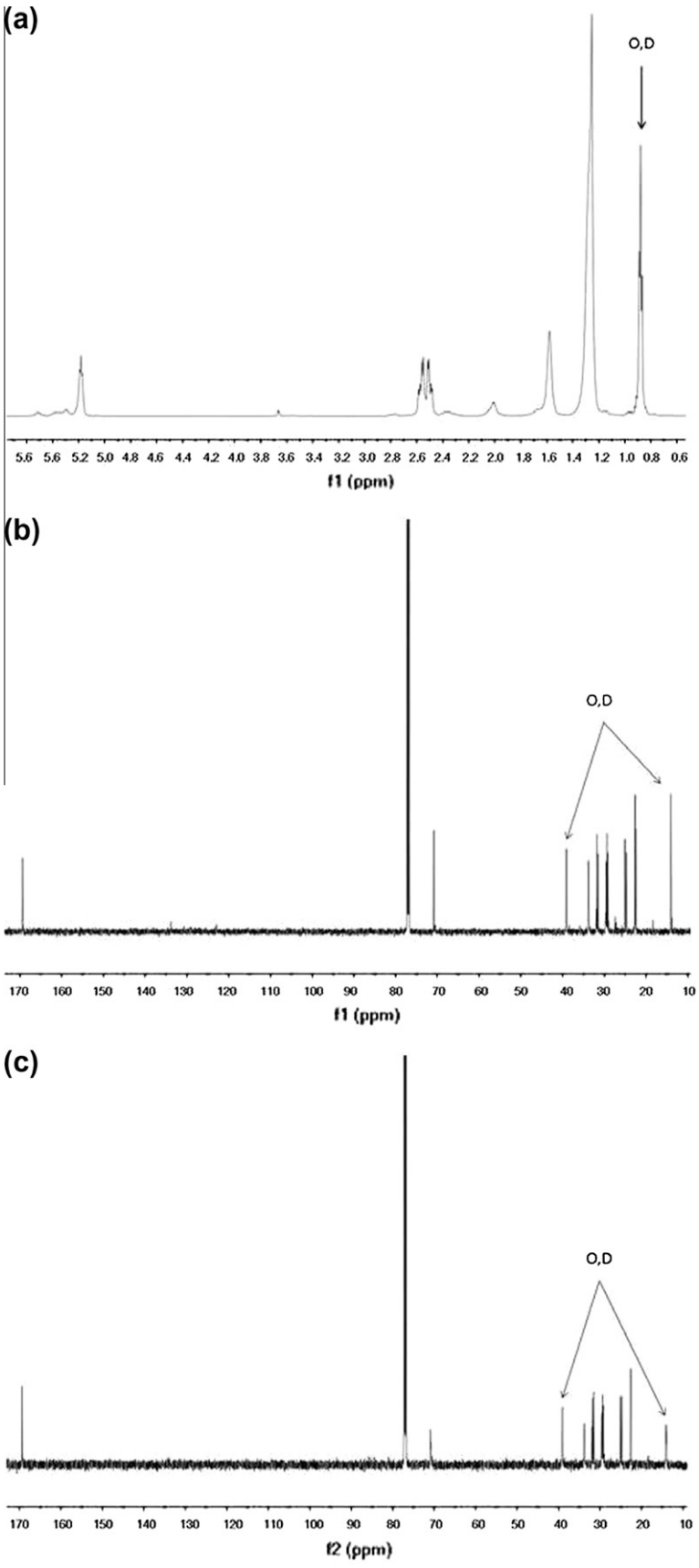

Fig. 4. (a) The ^{1}H NMR spectrum of the *mcl*-PHA sample obtained from the first cultivation. (b) The ^{13}C NMR spectrum of the *mcl*-PHA sample obtained from the first cultivation. (c) The ^{13}C NMR spectrum of the *mcl*-PHA sample obtained from the second cultivation.

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preventing the degradation of already accumulated *mcl*-PHA. Regarding the process design, higher productivities are probably obtained by a continuous two-stage chemostat process. This strategy turned out to be successful with other *mcl*-PHA production strains like *Ps. putida* GPo1 [36]. In addition, enhanced dissolved oxygen concentration available for the growing cells is possible by putting the microbes "under pressure". High pressure conditions resulting in elevated dissolved oxygen levels were recently demonstrated to be beneficial for *mcl*-PHA production by *Ps. putida* KTT2441 [45].

First results for product characterization indicate the high quality of the *mcl*-PHA latex obtained by this organism. It is very likely that the material displays a considerable market potential as biological latex or rubber. For this purpose, after preparation of prototype and test items, additional material characterization is needed especially to get knowledge about physical features of this biopolyester like tensile strength and elongation to break. This should be done with the native material as well as with its composites with compatible matters. Such tests will provide the required knowledge if post-synthetic modification, e.g. cross-linking or insertion of functional groups, is needed to meet the requirements for a defined commercial application. Biodegradation, eco-toxicity and cytotoxicity tests of the material have to be performed according to valid norms and standards in order to receive admission of *mcl*-PHA from *Ps. citronellolis* for application, especially in the medical field.

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References

[1] T. Keshavarz, I. Roy, Current Opinion in Microbiology 13 (2010) 321–326.
[2] M. Zinn, B. Witholt, T. Egli, Advanced drug delivery review 53 (2001) 5–21.
[3] G.-Q. Chen, Plastics completely synthesized by bacteria: polyhydroxyalkanoates, in: G.-Q. Chen, A. Steinbüchel (Eds.), Plastics from bacteria, Natural functions and applications, Springer, Münster, 2010, pp. 17–38.
[4] G. Braunegg, G. Lefebvre, K.F. Genser, Journal of Biotechnology 65 (1998) 127–161.
[5] M. Koller, A. Salerno, M. Miranda de Sousa Dias, A. Reiterer, G. Braunegg, Modern biotechnological polymer synthesis: a review, Food technology and biotechnology 48 (2010) 255–269.
[6] J. Helm, K.-D. Wendlandt, M. Jechorek, U. Stottmeister, Journal of Applied Microbiology 105 (2008) 1054–1061.
[7] S. Khanna, A.K. Srivastava, Process Biochemistry 40 (2005) 607–619.
[8] M. Williams, Applications of PHAs in medicine and pharmacy, in: Y. Doi, A. Steinbüchel (Eds.), Biopolymers 4, Polyesters III – Applications, Wiley-VCH, Weinheim, 2002, pp. 91–103.
[9] M. Zinn, Biosynthesis of Medium-Chain-Length Poly[(R)-3-hydroxyalkanoates], in: G.-Q. Chen, A. Steinbüchel (Eds.), Plastics from bacteria, Natural functions and applications, Springer, Münster, 2010, pp. 213–236.
[10] G.-Q. Chen, J. Xu, Q. Wu, Z. Zhang, K.-P. Ho, Reactive & Functional Polymers 48 (2001) 107–112.
[11] M.M. Bear, M.-A. Leboucher-Durand, V. Langlois, R.W. Lenz, S. Goodwin, P. Guérin, Reactive & Functional Polymers 34 (1997) 65–77.
[12] P. Furrer, S. Panke, M. Zinn, Journal of Microbiol Methods 69 (2007) 206–213.
[13] G. Braunegg, M. Koller, P.J. Hesse, C. Kutschera, R. Bona, C. Hermann, P. Horvat, J. Neto, L. Dos Santos Pereira, Production of plastics from waste derived from agrofood industry, renewable resources and renewable energy: a global challenge, CRC Press, Boca Raton, 2006. pp. 119–135.
[14] M. Koller, R. Bona, G. Braunegg, C. Hermann, P. Horvat, M. Kroutil, J. Martinz, J. Neto, P. Varila, L. Pereira, Biomacromolecules 6 (2005) 561–565.
[15] M. Koller, A. Atlić, M. Dias, A. Reiterer, G. Braunegg, Microbial PHA production from waste raw materials, in: G.-Q. Chen, A. Steinbüchel (Eds.), Plastics from bacteria, Natural functions and applications, Springer, Münster, 2010, pp. 85–119.
[16] S.Y. Lee, Trends in Biotechnology 14 (1996) 431–438.
[17] M.G.E. Albuquerque, C.A.V. Torres, M.A.M. Reis, Water Research 44 (2010) 3419–3433.
[18] K.S. Ng, Y.M. Wong, T. Tsuge, K. Sudesh, Process Biochemistry 46 (2011) 1572–1578.
[19] M. Titz, K.-H. Kettl, K. Shahzad, M. Koller, H. Schnitzer, M. Narodoslawsky, Clean Technologies and Environmental Policy 14 (2012) 495–503.
[20] A.M. Cromwick, T. Foglia, R.W. Lenz, Applied Microbiology and Biotechnology 46 (1996) 464–469.
[21] D.K.Y. Solaiman, R.D. Ashby, T.A. Foglia, W.N. Marmer, Applied Microbiology and Biotechnology 71 (2006) 783–789.
[22] M.H. Choi, S.C. Yoon, Applied and Environment Microbiology 60 (1994) 32–45.
[23] D.M. Chung, M.H. Choi, J.J. Song, S.C. Yoon, I.-K. Kang, N.E. Huh, International Journal of Biological Macromolecules 29 (2001) 243–250.
[24] D. Bhattacharya, P.M. Sarma, S. Krishnan, S. Mishra, B. Lal, Applied and Environment Microbiology 69 (2003) 1435–1441.
[25] R.R. Fall, J.L. Brown, T.L. Schaeffer, Applied and Environment Microbiology 38 (1979) 715–722.
[26] R.J.S. Jacques, E.C. Santos, R. Haddad, R.R. Catharino, M.N. Eberlin, F.M. Bento, F.A. de Oliveira Camargo, Brazilian Journal of Microbiology 39 (2008) 353–356.
[27] Z. Sadouk, A. Tazerouti, H. Hacene, World Journal of Microbiology & Biotechnology 25 (2009) 65–70.
[28] W. Küng, Wachstum und Poly-D-(-)-hydroxybuttersäure–Akkumulation bei Alcaligenes latus, Graz University of Technology, Austria, Diploma Thesis, 1982.
[29] American Chemists' Society: Fatty Acid Composition by Gas Chromatography, Official Methods and Recommended Practices of the American Oil Chemists' Society. 6th ed. American Oil Chemists' Society Champaign 2009 Method Ce 1–62.
[30] G. Braunegg, B. Sonnleitner, R.M. Lafferty, European Journal of Applied Microbiology and Biotechnology 6 (1978) 29–37.
[31] P.J. Barham, A. Keller, E.L. Otun, P.A. Holmes, Journal of Materials Science 19 (1984) 2781–2794.
[32] B.A. Ramsay, K. Lomaliza, C. Chavarie, B. Dubé, P. Bataille, J.A. Ramsay, Applied and Environment Microbiology 56 (1990) 2093–2098.
[33] N. Koyama, Y. Doi, Biotechnology Letters 17 (1995) 281–284.
[34] G. Du, J. Chen, J. Yu, S. Lun, Journal of Biotechnology 88 (2001) 59–65.
[35] G. Du, J. Chen, J. Yu, S. Lun, Process Biochemistry 37 (2001) 219–227.
[36] K. Jung, W. Hazenberg, M. Prieto, B. Witholt, Biotechnology and Bioengineering 72 (2001) 19–24.
[37] M. Zinn, H.-U. Weilenmann, R. Hany, M. Schmid, T. Egli, Acta Biotechnologica 23 (2003) 309–316.
[38] G. Mothes, J.-U. Ackermann, Engineering in Life Sciences 5 (2005) 58–62.
[39] S.T. Yu, C.C. Lin, J.R. Too, Process Biochemistry 40 (2005) 2729–2734.
[40] A. Atlić, M. Koller, D. Scherzer, C. Kutschera, E. Grillo-Fernandes, P. Horvat, E. Chiellini, G. Braunegg, Applied Microbiology and Biotechnology 91 (2011) 295–304.
[41] R.V. Nonato, P.E. Mantelatto, C.E.V. Rossell, Applied Microbiology and Biotechnology 57 (2001) 1–5.
[42] G.-Q. Chen, Industrial production of PHA, in: G.-Q. Chen, A. Steinbüchel (Eds.), Plastics from bacteria, Natural functions and applications, Springer, Münster, 2010, pp. 121–132.
[43] G. Impallomeni, A. Ballistreri, G.M. Carnemolla, S.P. Guglielmino, M.S. Nicolò, M.G. Cambria, International Journal of Biological Macromolecules 48 (2011) 137–145.
[44] S.Y. Lee, H.H. Wong, J. Choi, S.H. Lee, S.C. Lee, C.S. Han, Biotechnology and Bioengineering 68 (2000) 466–470.
[45] S. Follonier, B. Henes, S. Panke, M. Zinn, Biotechnology and Bioengineering 109 (2012) 451–461.