Pilot-scale Production of Functionalized *mcl*-PHA from Grape Pomace Supplemented with Fatty Acids

S. Follonier,[a] R. Riesen,[b] and M. Zinn[a,*]

[a]Institute of Life Technologies, University of Applied Sciences and Arts Western Switzerland Valais (HES-SO Valais-Wallis), Route du Rawyl 64, 1950 Sion, Switzerland

[b]Changins, Route de Duillier 50, Case postale 1148, 1260 Nyon 1, Switzerland

doi: 10.15255/CABEQ.2014.2251

Original scientific paper
Received: July 21, 2014
Accepted: June 5, 2015

Bioprocess optimization is a prime target to decrease the cost of functionalized medium-chain-length poly(3-hydroxyalkanoate) (*mcl*-PHA). We have already demonstrated at the laboratory scale that the pomace of white wine grapes is a promising growth substrate for the biosynthesis of *mcl*-PHA that is both cheap and does not compete with food and land use. Here, we report the scale-up of a 2-step, 100 L bioprocess with *Pseudomonas putida* KT2440 involving: (1) a batch growth phase on extract of Gewürztraminer grape pomace, and (2) a fed-batch polymer accumulation phase with a linear feed of 50 mol % octanoic acid and 50 mol % 10-undecenoic acid. With this approach, we achieved a cell dry weight of 14.2 ± 0.3 g L^{-1} containing 41.1 ± 1.3 wt % of poly(3-hydroxyoctanoate-*co*-3-hydroxy-10-undecenoate) with 53 mol % and 47 mol % of saturated and unsaturated monomers, respectively. The molecular weight (M_w) was 139 000 Da (PDI = 1.97) and DSC analysis showed a T_g of –45.5 °C but no T_m thus indicating a completely amorphous polymer.

Key words

polyhydroxyalkanoate, *mcl*-PHA, scale-up, pomace, functionalized PHA

Introduction

Amongst the polyhydroxyalkanoate (PHA) family, the class of medium-chain-length PHA (*mcl*-PHA) that contains C_6 to C_{14} monomers has attracted a lot of attention due to its extraordinary diversity. In particular, the possibility to adjust material properties by controlling the monomeric composition, and functionalize it via introduction of vinyl groups is of great interest[1]. Whereas short-chain-length PHAs (*scl*-PHA, C4-C5 monomers) are readily available on the market[2,3], large-scale commercialization of *mcl*-PHA is still to be established. Reasons for this are lower PHA yields, more costly substrates (e.g. fatty acids) as well as the difficulty to control precisely the monomeric composition and thus the material properties of the biopolymer produced. In general, the carbon substrates used for the bacterial fermentations contribute to at least 30 % of the production cost[4]. Therefore, any notable improvement in this respect would have a remarkable impact on the production cost.

Mcl-PHAs are produced as intracellular granules by *Pseudomonas* from either related substrates like fatty acids via the β-oxidation pathway[5] or by unrelated sources like carbohydrates via *de novo* fatty acid synthesis[6]. The polymer composition is nearly constant when using unrelated sources, since the monomers are always derived from acetyl-CoA. In contrast, when fatty acids are used as *mcl*-PHA precursors, it is directly related to their composition and thus can be quite easily adjusted provided a well-defined fermentation strategy is applied[7]. As a result, fatty acids are normally used to produce tailor-made *mcl*-PHAs in a reproducible, controlled way. Nevertheless, cheaper alternative carbon sources may be used for the growth phase in order to reduce the overall cost of the bioprocess. The strategy then consists of (1) producing large amounts of biomass from a non-costly substrate (= growth phase), and (2) adding the fatty acids that are necessary for the accumulation of tailor-made *mcl*-PHAs (= *mcl*-PHA accumulation phase). As examples, Kim and co-workers designed a 2-step fed-batch cultivation with *Pseudomonas putida* BM01 using glucose for the growth phase and octanoate for the biopolymer accumulation phase[8], and Sun and co-workers did a similar bioprocess with *P. putida* KT2440 growing on glucose and accumulating *mcl*-PHA from nonanoic acid[9]. More recently, Le Meur and co-workers genetically engineered *P. putida* KT2440 so that it could grow on xylose and produce *mcl*-PHA from octanoate during the accumula-

*Corresponding author: e-mail: manfred.zinn@hevs.ch

tion phase[10]. Replacing fatty acids by carbohydrates for the growth phase also allows achieving higher cell densities, since the former can cause growth inhibition from concentrations starting at around 15–20 mmol L^{-1}, whereas sucrose is not problematic up to about 230 mmol L^{-1} for Pseudomonas[11]. With respect to the *mcl*-PHA accumulation phase, one should note that, when working with *P. putida* KT2440, the growth medium should be designed in such a way that nitrogen depletion – a well-known boosting factor for *mcl*-PHA synthesis[12], occurs at around the same time as the complete uptake of sugars. Indeed, this strain produces *mcl*-PHA from unrelated sources under nitrogen-limited conditions, which is not desired if one wants a precise control of the *mcl*-PHA composition[13].

As mentioned above, glucose has often been investigated as a carbon source for *mcl*-PHA fermentations. Nevertheless, its increasing cost[14] and the fact that this limited substrate should be preferentially used for food applications led us to explore cheaper and more sustainable alternatives, such as food wastes. Like agro-wastes, food wastes are very diverse and readily available, which renders them very interesting candidates for biopolymer production[15–17]. In a previous work, we designed bioprocesses for the synthesis of *mcl*-PHA using hydrolyzed fruit pomace (pressing residues) for the growth phase, and waste frying oil for the *mcl*-PHA accumulation phase[18]. In addition, we identified the pomace of grapes used for the production of sweet white wine as a promising carbon source due to a surprisingly high content of residual sugars (> 60 g L^{-1} glucose). Thus, whereas other types of pomace require enzymatic hydrolysis of cellulosic compounds to generate sufficient amounts of fermentable sugars, this step was shown to be superfluous for the pomace of white wine grapes.

Here, we demonstrate the scalability of this approach by establishing a 2-step 100 L fermentation in a pilot bioreactor for the production of tailor-made *mcl*-PHA with a high degree of unsaturation with *P. putida* KT2440. A glucose- and fructose-rich extract from the pomace of Gewürztraminer grapes was used as growth substrate, while the *mcl*-PHA precursors consisted of an equimolar solution of octanoic acid (C8) and 10-undecenoic acid (C11:1). Care was taken to keep the bioprocess simple in order to make an industrial transfer as easy, efficient and cheap as possible. In addition, the use of a genetically engineered strain requiring costly antibiotics for growth was avoided.

Materials and methods

Bioprocess

Strain, substrates and media. *P. putida* KT2440 (ATCC 47054) was used in this work. It was stored at –80 °C in the preculture medium and glycerol at a concentration of 15 wt %.

Precultures were prepared in medium E with 2.9 g L^{-1} trisodium citrate dihydrate as carbon source[19], whereas the mineral medium for the fermentation contained per kg of medium: 5.61 g $(NH_4)_2SO_4$, 3 g KH_2PO_4, 0.8 g EDTA disodium dihydrate, 1 g $Mg_2SO_4 \cdot 7H_2O$, 0.28 g $Fe_2SO_4 \cdot 2H_2O$, 0.03 g $CaCl_2 \cdot 2H_2O$, and 0.5 g of a trace element solution. The trace element solution contained 40 g $MnCl_2 \cdot 4H_2O$, 4 g $CoCl_2 \cdot 6H_2O$, 40 g $CuCl_2 \cdot 2H_2O$ and 20 g $ZnSO_4 \cdot 7H_2O$ and 20 g $Na_2MoO_4 \cdot 2H_2O$ dissolved in 1 L HCl 1M. The sterilization of this medium is described below, in the section *Preparation of the growth medium*.

Pomace of Gewürztraminer grapes produced in Denens (Switzerland) was collected after harvest (22.10.2013), dehydration, and pressing. This dried, solid pomace was stored at –20 °C until use.

Two fatty acid solutions were used as carbon source for the *mcl*-PHA accumulation phase: a solution of potassium octanoate (K-C8) prepared by neutralizing 144.2 g of C8 to pH 8.18 with KOH 4M and an equimolar solution of C8 and C11:1 (50/50 mol %) prepared by mixing 578.8 g C8 with 736.6 g C11:1.

Bioreactor. The fermentation was carried out in a 300 L stirred tank bioreactor equipped with probes for temperature (Pt100), pH, pO_2, and antifoam (Bioengineering, Wald, Switzerland). In addition, a turbidity probe was used to monitor cell growth during the process (Optek AS16–05, Optek-Danulat GmbH, Essen, Germany). The concentration of O_2 and CO_2 in the off-gas was measured continuously with a gas analyzer (GA4, DasGIP, Jülich, Germany).

Preparation of the pomace extract. Residual sugars were recovered from the Gewürztraminer pomace by water extraction. For this purpose, 30 kg of Gewürztraminer pomace were added to 60 kg deionized water, and mixed for 5.25 h at a stirring rate of 800–1000 rpm with a stirrer equipped with a marine propeller (Ekato, Shopfheim, Germany). The liquid fraction was recovered by filtration through a 2 mm textile mesh and subsequent pressing through a <1 mm textile mesh. With this method, it was possible to discard the major part of the solid fraction that would otherwise disturb the bioprocess (e.g. stirring) and cell separation during downstream processing.

Preparation of the growth medium. Pomace extract (40 kg) was supplemented with mineral medium (60 kg) without trace element solution and pumped into the bioreactor. This growth medium (100 kg, pH 3.5) was sterilized *in situ* at 120–121 °C for 30 minutes (Maillard reaction does not occur at this pH). After cooling, the separately autoclaved trace element solution was added and the pH adjusted to 6.85 with KOH 4M.

Precultures. A first preculture was prepared by inoculating 100 mL of preculture medium in a 500 mL shake flask with baffles with 0.5 mL *P. putida* KT2440 glycerol stock. It was grown at 30 °C and 200 rpm in an orbital shaker (Multitron, Infors HT, Bottmingen, Switzerland) for 14 h (OD_{600} = 1.26). Five 2 L shake flasks with baffles containing each 1 L preculture medium were inoculated with 8 mL of the first preculture and incubated at 30 °C and 180 rpm for 10 h (final OD_{600} = 1.18).

Batch phase. The growth medium was inoculated with 5 L of the second preculture. Temperature was maintained at 30 °C and pH was controlled to 6.85 by automatic addition of KOH 4M and H_3PO_4 4M. Agitation was manually increased from 245 to max. 600 rpm to prevent oxygen limitation and the aeration (with air) was approximately 0.25 vvm. PPG2000 was used as antifoam and automatically added to the culture.

Fed-batch phase. The solution of K-C8 was added to fermentation broth after the end of the batch phase, which was identified based on large peaks visible in the O_2 and CO_2 off-gas signals (Fig. 1C). The feed of C8/C11:1 50/50 mol % was started after 4.2 h (t = 21.6 h), first with a flow rate of 21.2 g h^{-1} and then of 35.0 g h^{-1} after 15.7 h (t = 37.3 h).

Analytical methods. The samples taken throughout the bioprocess were analyzed with respect to cell dry weight (CDW), PHA content, and residual concentrations of ammonium nitrogen (NH_4-N), sugars (glucose and fructose), and fatty acids (C8 and C11:1). Determination of CDW, PHA content (by gas chromatography (GC) after methanolysis performed on freeze-dried cells), NH_4-N and sugars was performed as described previously[18]. The residual concentration of fatty acids was assessed by HPLC (Agilent 1100/1200 series, Agilent Technologies, Santa Clara, USA) using a 5 µm Zorbax Eclipse XDB-C18 150 × 4.6 mm column with a 5 µm ZORBAX Eclipse XDB-C18 12.5 × 4.6 mm guard (Agilent Technologies, Santa Clara, USA). Elution was performed at 25°C and 1 mL min^{-1}, first with a gradient going from 30 % eluent A (= acenonitrile + 0.1 v % formic acid)/70 % eluent B (= water + 0.1 % formic acid) to 100 % eluent A in 10 minutes, and then in isocratic mode with 100 % eluent A for 5 minutes. Fatty acids were identified by UV detection at 210 nm.

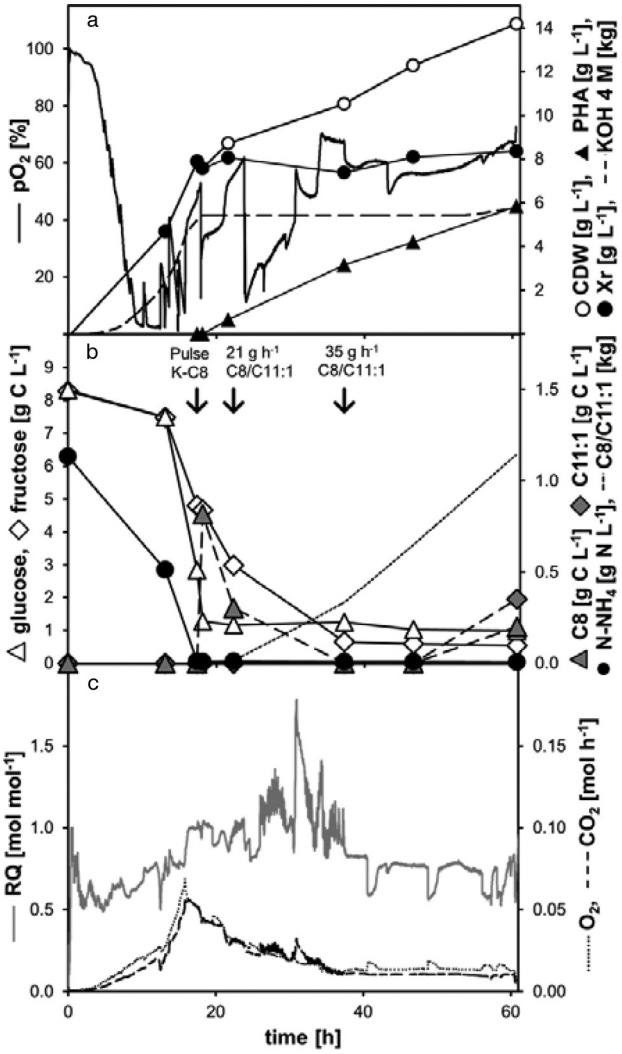

Fig. 1 – *Fermentation data for the 100 L bioprocess with P. putida KT2440 growing on Gewürztraminer pomace and synthesizing mcl-PHA from C8/C11:1 50/50 mol %. a) Biomass growth expressed as CDW, PHA-free residual biomass (Xr), PHA, pO_2 and base consumption (KOH 4M). b) Substrate consumption: residual glucose, fructose, C8, C11:1 are expressed in g C L^{-1} and residual N-NH_4 in g N L^{-1}. The amount of C8/C11:1 50/50 mol % fed to the culture broth is also indicated in kg, and the time of addition of C8, as well as the start of C8/C11:1 50/50 mol % feeding, and the change of flow rate are shown with arrows. c) Production of CO_2, consumption of O_2 and respiratory quotient (RQ) throughout the bioprocess.*

Downstream processing

Cell harvest. Cells were separated from the culture medium using a continuous centrifuge (Clara 20, Alpha Laval, Lund, Sweden) and subsequently a batch centrifuge in order to achieve better separation (HiCen XL, Herolab GmbH, Wiesloch, Germany). The biomass was then frozen at –80 °C and freeze-dried (Lyolab BII, LSL Secfroid, Aclens-Lausanne, Switzerland).

Polymer extraction and purification. The biopolymer was recovered from the freeze-dried biomass by solvent extraction using methylene chloride (CH_2Cl_2), and purified by precipitation in cold methanol. Briefly, the biomass was crushed into small pieces with a mortar and pestle, and added to CH_2Cl_2 (60 g of biomass for 1 L CH_2Cl_2). The particle size was further reduced using an Ultra-Turrax T25 equipped with the dispersing tool S25N-8G (IKA®-Labortechnik, Staufen, Germany) at maximum speed for 2–3 minutes, and the mixture stirred at ambient temperature for about 2 h. The residual cell biomass was separated from the *mcl*-PHA containing solvent phase by pressure filtration (1 L stainless steel filtration holder, Sartorius GmbH, Goettingen, Germany and 589[2] white ribbon filter papers, Schleicher & Schuell, Dassel, Germany). The filtrate was concentrated in a rotary evaporator (Laborota 4011-digital, Heidolf Instruments, Schwabach, Germany) and then added dropwise into ice-cold methanol under agitation (volumetric ratio methanol/*mcl*-PHA solution at least 3:1). The methanol phase was then discarded and the polymer recovered using CH_2Cl_2, which was subsequently evaporated in a vacuum dryer (Salvis Vacuum incubator KVTS 11, Renggli AG, Rotkreuz, Switzerland).

PHA characterization

Gas chromatography. The monomeric composition of the purified polymer was measured after methanolysis as described previously[18]. Two samples of purified polymer were methanolyzed and each injected in duplicate; thus, the value given is the average of 4 measurements.

Nuclear magnetic resonance (NMR). NMR spectra were measured with a Bruker UltraShield 400 MHz NMR spectrometer (Bruker Biospin AG, Fällanden, Switzerland) at 297 K using a 5 mm BBI probe. Between 10 and 20 mg of polymer were dissolved in 0.5 mL of $CDCl_3$. Chemical shifts are given in ppm relative to the remaining signals of chloroform as internal reference (^{1}H NMR: 7.26 ppm; ^{13}C NMR: 77.0 ppm). ^{1}H NMR spectrum was recorded at 400.13 MHz with the following parameters: 8.00 μs 90° pulse length, 6’983 Hz spectral width, 55 864 data points, 16 scans, and relaxation delay 10 s. ^{13}C NMR spectra were recorded at 100.62 MHz with ^{1}H WALTZ 16 decoupling. Other parameters were chosen as follows: 14.50 μs 90° pulse length, 25’063 Hz spectral width, 50’122 data points, 29’504 scans, 1.0 s relaxation delay, and field 1’800 Hz decoupling.

Gel permeation chromatography (GPC). The weight average molecular weight (M_w) and the polydispersity index (PDI) of the polymer were measured by GPC (1260 Infinity refractive index detector and 1220 Infinity liquid chromatography system, Agilent Technologies, Santa Clara, USA). About 5 mg of polymer were dissolved in 1 mL tetrahydrofuran (THF) and 10 μL of this solution were injected in duplicate in the system. Separation was achieved at 40 °C in a GPC column (PLgel 5 μm MiniMIX-C 250 × 4.6 mm, Agilent Technologies, Santa Clara, USA) coupled to a precolumn (PLgel 5 μm MiniMIX-C Guard 50 × 4.6 mm, Agilent Technologies, Santa Clara, USA) using THF as mobile phase with a flow rate of 0.3 mL min^{-1}. Calibration was done with 9 polystyrene bead standards having a M_w ranging from 580 to 990 500 (GPC/SEC Calibration Kit PL2010–0100, Agilent Technologies, Santa Clara, USA).

Differential scanning calorimetry (DSC). The polymer was analyzed by DSC (Mettler Toledo DSC 823e, Mettler Toledo GmbH, Giessen, Germany) to determine the glass transition temperature (T_g), melting temperature (T_m), and degradation temperature (T_d). After being kept 5 minutes at 25 °C, the sample (16.144 mg) was first heated to 125 °C at a rate of 10 °C min^{-1} in order to suppress memory effect, kept 5 minutes at 125 °C, then cooled down to –100 °C at a rate of –20 °C min^{-1}, kept 5 minutes at –100 °C, and finally heated to 350 °C at a rate of 10 °C min^{-1}.

Results and discussion

Sugar extraction from the pomace of Gewürztraminer grapes

The goal of the work presented here is to design a large-scale (100 L) bioprocess for the production of tailor-made *mcl*-PHA with a high degree of unsaturation using cheap, renewable and sustainable carbon substrates for the initial growth phase. The pomace of Gewürztraminer grapes was chosen for this purpose because of two interesting features. First, these grapes are used for the production of white wine, *i.e*. pressing is performed before the alcoholic fermentation, and as a result, residual concentrations of glucose and fructose are naturally present in the pomace. Second, the processing of these grapes involved a drying step before pressing which further concentrated the sugars in the pomace.

Water extraction was performed in order to recover glucose and fructose from the dried, solid pomace. The deionized water to pomace ratio applied was 3:1, which was high enough to obtain a semi-liquid solution easy to mix while being sufficiently low not to dilute the extracted sugars too much. After 5.15 h of mixing, the sugar content in

Table 1 – *Amount of sugars extracted from the Gewürztraminer pomace*

Time	Pomace extract		Growth medium	
	Glucose (g L^{-1})	Fructose (g L^{-1})	Glucose (g L^{-1})	Fructose (g L^{-1})
End of extraction phase	26.0[a]	26.0[a]	10.4[b]	10.4[b]
Fermentation start	52.0[b]	51.8[b]	20.8[a]	20.7[a]

[a]Values measured.

[b]Values calculated from the corresponding measured values knowing that 40 kg of pomace extract were diluted with 60 kg mineral medium to form 100 kg of growth medium.

the liquid fraction amounted to 26.0 g L^{-1} of glucose and 26.0 g L^{-1} of fructose (Table 1). Interestingly, these concentrations doubled during the following 3.5 h long separation step (filtration and pressing), as indicated by the values measured in the growth medium before the start of the fermentation (Table 1). This means that sugar extraction was strongly time-dependent, but also that the cumulative concentration of glucose and fructose recovered (> 100 g L^{-1}, equivalent to 40 g C L^{-1}) was more than sufficient for standard *mcl*-PHA-producing fermentations. For high-cell-density cultivations, the extraction procedure may be further optimized (extraction time, temperature, etc.) or the liquid extract concentrated. Moreover, if cost analyses show it to be pertinent, one may even add a hydrolysis step for converting cellulosic compounds of the pomace into fermentable sugars.

100 L fermentation for the production of tailor-made *mcl*-PHA

The 100 L fermentation was designed in the following way: (1) batch growth phase on glucose and fructose from the pomace extract, and (2) *mcl*-PHA accumulation phase starting with a pulse of K-C8 and followed by a linear feed of C8/C11:1 50/50 mol %. The mineral medium was designed so that nitrogen and sugar depletions would occur simultaneously. The calculation was based on the growth yields for glucose and nitrogen determined for *P. putida* KT2440 by Sun and co-workers (1 g CDW (g C)$^{-1}$ and 7 g CDW (g N)$^{-1}$, respectively)[20] and on the sugar concentrations measured at the end of the extraction phase (Table 1). Nevertheless, these values were underestimated for the reason explained in the previous section and therefore the sugars (in particular fructose) were depleted about 10 h later than nitrogen (Fig. 1b). Otherwise, the growth phase proceeded as expected and its end after about 16 h (see the PHA-free residual biomass X_r values in Fig. 1a) coincided with nitrogen depletion, peaks in the signals of O_2 and CO_2 in the off-gas and stop of the consumption of base for pH control (Fig. 1a-c). During this initial phase, the cells grew at a maximum specific growth rate of 0.43 h^{-1} and reached a biomass of about 8 g L^{-1} (Table 2). Growth yields and specific uptake rates were in quite close agreement with values reported in the literature for *P. putida* or the closely related strain *P. fluorescens*[13,20–22].

Table 2 – *Production data for the 100 L bioprocess with P. putida KT2440 growing on Gewürztraminer pomace and synthesizing mcl-PHA from C8/C11:1 50/50 mol %*

	Symbol	Value
Growth phase		
Maximum specific growth rate[a]	μ_{max}	0.43 ± 0.01 h^{-1}
Final residual biomass[b]	$X_{r,f}$	7.9 ± 0.4 g L^{-1}
Growth yield for nitrogen	$Y_{Xr/NH_4\text{-}N}$	6.99 g X_r (g N)$^{-1}$
Growth yield for glucose + fructose	$Y_{Xr/glu+fru}$	0.88 g X_r (g C)$^{-1}$
Specific nitrogen consumption rate	$q_{NH_4\text{-}N}$	0.06 g N (g X_r h)$^{-1}$
Specific glucose consumption rate	$q_{glucose}$	0.38 g C (g X_r h)$^{-1}$
Specific fructose consumption rate	$q_{fructose}$	0.20 g C (g X_r h)$^{-1}$
***mcl*-PHA accumulation phase**		
Final *mcl*-PHA content	PHA_f	41.1 ± 1.3 wt % 5.8 g PHA L^{-1}
mcl-PHA yield for C8/C11:1	$Y_{PHA/C8/C11:1}$	0.79 g PHA (g C)$^{-1}$
mcl-PHA yield for Gewürztraminer pomace	$Y_{PHA/Gew}$	0.02 g PHA (g pomace)$^{-1}$
mcl-PHA volumetric productivity[c]	P_{PHA}	0.10 g PHA L^{-1} h^{-1}
mcl-PHA specific productivity[d]	q_{PHA}	0.017 g PHA (g X_r h)$^{-1}$

[a]calculated from the CO_2 signal in the off-gas, error estimated on the linear regression

[b]averaged over the *mcl*-PHA accumulation phase

[c]calculated for the overall process (growth phase and *mcl*-PHA accumulation phase)

[d]calculated over the *mcl*-PHA accumulation phase

At the end of the batch phase, a pulse of 144.2 g C8 neutralized with KOH 4M was added to the fermentation broth (final concentration of 10 mmol L^{-1} or 0.96 g C L^{-1}). This strategy was chosen based on the findings of Kim and co-workers, who observed a lag phase when changing the carbon source from glucose to C8, and suggested that cell adaptation was required to metabolize the new substrate[8]. Four hours after the C8 pulse, a linear feed of C8/C11:1 50/50 mol % was started. The initial flow rate was set to 21 g h^{-1} and later increased to 35 g h^{-1} to avoid underfeeding. The choice of the feeding rate was based on two main considerations: on the one hand, sufficient amounts of carbon must be available to the cells to cover their maintenance needs and form a pool of precursors for *mcl*-PHA synthesis, while on the other hand, accumulation of fatty

acids to growth-inhibiting levels (about 15–20 mmol L^{-1}) must be avoided. As can be seen in Fig. 1a-b, the amount of fatty acids fed was large enough to trigger *mcl*-PHA production from the start of the fed-batch, and no accumulation occurred until the very end of the bioprocess, which confirms that the feeding rates chosen were adequate. With this fermentation strategy we achieved a final biopolymer concentration of 5.8 g L^{-1} *mcl*-PHA, with a cellular content of 41.1 ± 1.3 wt %, a volumetric productivity of 0.10 g PHA L^{-1} h^{-1} and a specific productivity of 0.017 g PHA (g X_r h)$^{-1}$ (Table 2). These values are quite typical for medium-cell-density cultivations with *Pseudomonas* strains producing mcl-PHA from related sources and could be further increased by optimizing the bioprocess, e.g. by carefully increasing the fatty acid feeding rate and/or targeting higher cell densities. Another important result of this study was that it was possible to produce 583 g of biopolymer by using 40 kg of Gewürztraminer pomace as growth substrate (and 1.1 kg of the C8/C11:1 50/50 mol % solution as polymer substrate), which corresponds to a yield of 0.02 g PHA (g pomace)$^{-1}$ (Table 2). This demonstrates that wastes from wine production processes, whose disposal is costly and may have a negative impact on the environment if not done properly[23], can be successfully valorized to synthetize high added value products such as biodegradable functionalized polymers. In addition, one should note that other valuable products such as phenolic compounds (e.g. pigments, antioxidants) or grape seed oil could be extracted as well from the pomace to exploit their full potential[24–25].

Finally, a simple cost estimation can be made to evaluate the savings potential of this strategy. A total amount of 900 g C (equivalent to 2.25 kg glucose) were consumed during the growth phase of the 100 L fermentation to produce 583 g of biopolymer. Knowing that the price of glucose on the market is about 500 $ per ton[26], this means that 1.93 $ per kg of polymer produced can be saved by using free pomace instead of glucose. It should be noted that, in reality, the savings would be slightly less since this calculation does not consider the cost of preparing sugar extracts from the pomace.

Fatty acids are much more expensive than carbohydrates, the price of C8 and C11:1 being around 5000 and 2000 $ per ton, respectively[27–28]. This proves that restricting the use of fatty acids to the polymer accumulation phase while using another cheaper growth substrate is a good strategy. Depending on the type of polymer that is desired, fatty acids may also be replaced as polymer substrate by carbohydrates or waste frying oil for instance, leading to a further reduction in costs. Nevertheless, most of the time, very specific monomeric compositions are required so that the material properties will fit with the applications foreseen for the *mcl*-PHA produced. As a result, it is not possible at this time to suppress completely the use of fatty acids in most cases. Yet, an optimal combination of cheap and more costly substrates, together with a careful design of the fermentation strategy and advances in molecular biology and metabolic engineering will allow producing *mcl*-PHA with a well-defined composition at a competitive cost in the future.

Characterization of the functionalized *mcl*-PHA produced

The freeze-dried biomass obtained from the 100 L fermentation was treated with CH_2Cl_2 in order to extract the biopolymer, which was subsequently purified by precipitation in cold methanol. This procedure is the most efficient with respect to recovery yield so far[28] and in addition involves a non-explosive solvent, which is a major advantage for industrial scale-up. Yet, chlorinated solvents – even if recycling is performed, are not desirable due to both environmental and health reasons. Therefore, efforts should be spent on developing alternative purification methods, either based on greener solvents, enzymatic digestion of the cell material or autolysis systems[2,15,29].

The *mcl*-PHA synthetized in this work was a copolymer of 3-OH-C6, 3-OH-C8, 3-OH-C7:1, 3-OH-C9:1 and 3-OH-C11:1 with 53.3 mol % and 46.7 mol % of total saturated and unsaturated monomers, respectively. This ratio is in good agreement with the relative concentrations of C8 and C11:1 (50/50 mol %) fed to the cell culture. Nevertheless, the content of 3-OH-C10 (5.5 mol %) was larger than expected, since *P. putida* KT2440 is known to synthesize polymer with less than 1 mol % 3-OH-C10 on octanoic acid only[31–32]. The additional 3-OH-C10 monomers most probably arose from the residual amounts of fructose still present after nitrogen limitation that could be metabolized into *mcl*-PHA via *de novo* fatty acid synthesis. To avoid this unwanted "contamination", one may choose to work with another production strain, such as *P. putida* GPo1, which is unable to produce biopolymers from unrelated sources[33]. The presence of the monomers identified by GC was confirmed by NMR (Fig. 2). In particular, the presence of 3 types of saturated and 3 types of unsaturated monomers was clearly visible on the ^{13}C NMR spectrum (Fig. 2b).

Remarkably, the biopolymer produced was completely amorphous with no melting point detected, a low glass transition temperature of –45.5 °C, and a high degradation temperature of 294.2 °C (Table 3). With respect to thermal properties, this polymer was similar to the ones derived from oils[34]

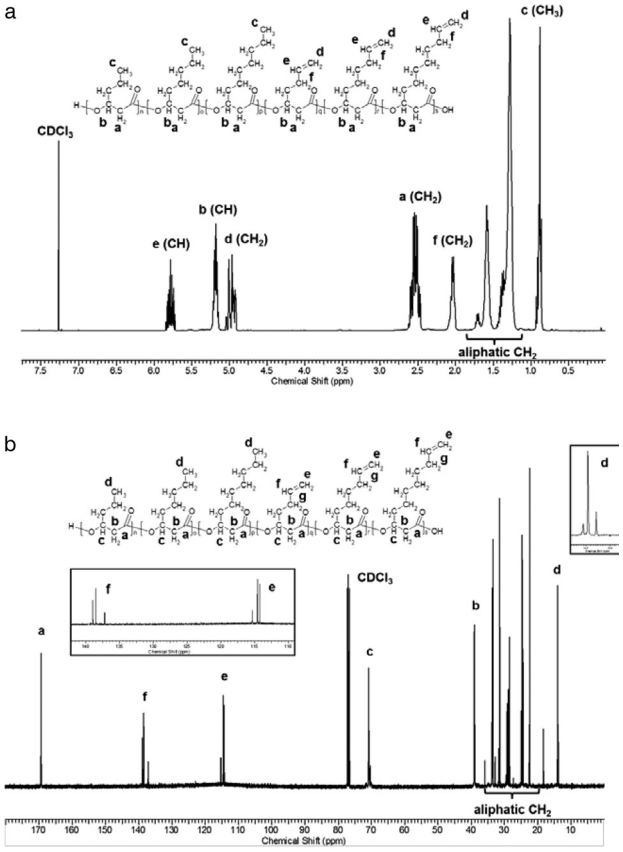

Fig. 2 – *1H (a) and 13C (b) NMR spectra of the purified mcl-PHA polymer. The depicted structure of polymer is based on GC analysis and confirmed by NMR. The hydrogen and carbon atoms identified in spectra (a and b, respectively) are labelled with a low-case letter.*

Table 3 – *Characteristics of the purified functionalized mcl-PHA*

	Symbol	Value
Monomeric composition		
3-hydroxyhexanoate	3-OH-C6	7.2 ± 0.9 mol %
3-hydroxyoctanoate	3-OH-C8	40.6 ± 0.3 mol %
3-hydroxydecanoate	3-OH-C10	5.5 ± 0.1 mol %
Total saturated monomers		*53.3 mol %*
3-hydroxy-10-heptenoate	3-OH-C7:1	7.0 ± 0.1 mol %
3-hydroxy-10-nonenoate	3-OH-C9:1	24.1 ± 0.2 mol %
3-hydroxy-10-undecenoate	3-OH-C11:1	15.5 ± 0.3 mol %
Total unsaturated monomers		*46.7 mol %*
Molecular weight	M_w	139 000 Da
Polydispersity index	PDI	1.97
Thermal properties		
Glass transition temperature	T_g	–45.5 °C
Melting temperature	T_m	–
Degradation temperature	T_d	294.2 °C

but it had a much larger degree of unsaturation that can be used for further functionalization[35–36]. Oils, and in particular, waste frying oils, are promising, cost-effective alternatives to fatty acids. Nevertheless, they have a quite narrow range of composition, which in addition is not very well defined. Moreover, the presence of glycerol may have a negative effect on the molecular weight of the polymers synthesized. Therefore, there are still a few challenges that need to be overcome in order to establish a bioprocess completely or partially based on oil for the production of *mcl*-PHAs with a well-controlled composition.

Conclusion

With this work, we have successfully established the scale-up of a 2-step bioprocess for the production of functionalized *mcl*-PHA production to the pilot scale (100 L) using Gewürztraminer pomace during the initial growth phase, and a mixture of fatty acids during the following biopolymer accumulation phase. We have demonstrated that it was possible to synthesize *mcl*-PHA with a well-defined composition using food waste as cheap, sustainable growth substrates, thus proving the benefits of this strategy. It should be noted that other types of food waste may be used depending on local availability and seasonality. Finally, amongst the challenges that need to be addressed in order to fully optimize the whole bioprocess at industrial scale, downstream processing is of major importance and we are currently investigating this aspect more deeply.

ACKNOWLEDGMENTS

This work was granted by RealTech, which supports collaborative projects between different schools of HES-SO in Switzerland. We are grateful to Julien Pott for precious help during the preparation of the pomace solution and the 100 L fermentation. In addition, we would like to thank Fabrice Micaux and Monica Bassas, Antoine Fornage, Pascal Jacquemettaz, as well as Alexandra Amherd Hidalgo for NMR, GPC, GC and DSC analyses, respectively.

List of Abbreviations

3-OH-C6 – 3-hydroxyhexanoate
3-OH-C7:1 – 3-hydroxyhept-6-enoate
3-OH-C8 – 3-hydroxyoctanoate
3-OH-C9:1 – 3-hydroxynon-8-enoate
3-OH-C10 – 3-hydroxydecanoate
3-OH-C11:1 – 3-hydroxyundec-10-enoate

ATCC – American type culture collection
C8 – octanoic acid or octanoate
C11:1 – 10-undecenoic acid or 10-undecenoate
CDW – cell dry weight, g L^{-1}
CH_2Cl_2 – methylene chloride
DSC – differential scanning calorimetry
GC – gas chromatography
GPC – gel permeation chromatography
HPLC – high pressure liquid chromatography
K-C8 – potassium octanoate
mcl-PHA – medium-chain-length polyhydroxyalkanoate
μ_{max} – maximum specific growth rate, h^{-1}
NMR – nuclear magnetic resonance
OD_{600} – optical density at 600 nm
P_{PHA} – *mcl*-PHA volumetric productivity, g L^{-1} h^{-1}
P4HB – poly(4-hydroxybutyrate)
PDI – polydispersity index
PHA – polyhydroxyalkanoate
PHA_f – final *mcl*-PHA content
$q_{fructose}$ – specific fructose consumption rate
$q_{glucose}$ – specific glucose consumption rate
$q_{NH_4\text{-}N}$ – specific nitrogen consumption rate
q_{PHA} – *mcl*-PHA specific productivity
scl-PHA – short-chain-length polyhydroxyalkanoate
T_d – degradation temperature, °C
T_g – glass transition temperature, °C
T_m – melting temperature, °C
THF – tetrahydrofuran
$X_{r,f}$ – final residual biomass (PHA-free), g L^{-1}
$Y_{Xr/glu+fru}$ – growth yield for glucose and fructose, g g^{-1}
$Y_{Xr/NH_4\text{-}N}$ – growth yield for nitrogen, g g^{-1}
$Y_{PHA/C8/C11:1}$ – *mcl*-PHA yield for C8/C11:1, g g^{-1}
$Y_{PHA/Gew}$ – *mcl*-PHA yield for Gewürztraminer pomace, g g^{-1}

References

1. *Hazer, D. B., Kılıçay, E., Hazer, B.*, Poly(3-hydroxyalkanoate)s: diversification and biomedical applications: a state of the art review, Mater. Sci. Eng. **C 32** (2012) 637. doi: http://dx.doi.org/10.1016/j.msec.2012.01.021
2. *Jacquel, N., Lo, C.-W., Wei, Y.-H., Wu, H.-S., Wang, S. S.,* Isolation and purification of bacterial poly(3-hydroxyalkanoates). Biochem. Engin. J. **39** (2008) 15. doi: http://dx.doi.org/10.1016/j.bej.2007.11.029
3. *Koller, M., Salerno, A., Braunegg, G.,* Polyhydroxyalkanoates: Basics, Production and Applications of microbial biopolyesters, in Kabasci, S. (Ed.), Bio-based Plastics: Materials and Applications, Wiley, New York, 2013, pp 137–170. doi: http://dx.doi.org/10.1002/9781118676646.ch7
4. *Du, C., Sabirova, J., Soetaert, W., Ki Carol Lin, S.*, Polyhydroxyalkanoates production from low-cost sustainable raw materials, Curr. Chem. Biol. **6** (2012) 14.
5. *Kessler, B., Witholt, B.,* Factors involved in the regulatory network of polyhydroxyalkanoate metabolism, J. Biotechnol. **86** (2001) 97. doi: http://dx.doi.org/10.1016/S0168–1656(00)00404–1
6. *Rehm, B. H. A., Krüger, N., Steinbüchel, A.*, A new metabolic link between fatty acid *de novo* synthesis and polyhydroxyalkanoic acid synthesis: The *phaG* gene from Pseudomonas putida KT2440 encodes a 3-hydroxyacyl-acyl carrier protein-coenzyme A transferase, J. Biol. Chem. **273** (1998) 24044. doi: http://dx.doi.org/10.1074/jbc.273.37.24044
7. *Zinn, M., Hany, R.,* Tailored material properties of polyhydroxyalkanoates through biosynthesis and chemical modification, Adv. Eng. Mater. **7** (2005) 408. doi: http://dx.doi.org/10.1002/adem.200500053
8. *Kim, G. J., Lee, I. Y., Yoon, S. C., Shin, Y. C., Park, Y. H.,* Enhanced yield and a high production of medium-chain-length poly(3-hydroxyalkanoates) in a two-step fed-batch cultivation of Pseudomonas putida by combined use of glucose and octanoate, Enzyme Microb. Technol. **20** (1997) 500. doi: http://dx.doi.org/10.1016/S0141–0229(96)00179–2
9. *Sun, Z., Ramsay, J. A., Guay, M., Ramsay, B.,* Increasing the yield of MCL-PHA from nonanoic acid by co-feeding glucose during the PHA accumulation stage in two-stage fed-batch fermentations of Pseudomonas putida KT2440, J. Biotechnol. **132** (2007) 280. doi: http://dx.doi.org/10.1016/j.jbiotec.2007.02.023
10. *Le Meur, S., Zinn, M., Egli, T., Thöny-Meyer, L., Ren, Q.,* Production of medium-chain-length polyhydroxyalkanoates by sequential feeding of xylose and octanoic acid in engineered Pseudomonas putida KT2440, BMC Biotechnol. **12** (2012) 53. doi: http://dx.doi.org/10.1186/1472–6750–12–53
11. *Chirife, J., Herszage, L., Joseph, A., Kohn, E. S., In vitro* study of bacterial growth inhibition in concentrated sugar solutions: microbiological basis for the use of sugar in treating infected wounds, Antimicrob. Agents Chemother. **23** (1983) 766. doi: http://dx.doi.org/10.1128/AAC.23.5.766
12. *Lageveen, R. G., Huisman, G. W., Preusting, H., Ketelaar, P., Eggink, G., Witholt, B.,* Formation of polyesters by Pseudomonas oleovorans: Effect of substrates on formation and composition of poly-(*R*)-3-hydroxyalkanoates and poly-(*R*)-3-hydroxyalkenoates, Appl. Environ. Microbiol. **54** (1988) 2924.
13. *Follonier, S., Panke, S., Zinn, M.,* A reduction in growth rate of Pseudomonas putida KT2442 counteracts productivity advances in medium-chain-length polyhydroxyalkanoate production from gluconate, Microb. Cell Fact. **10** (2011) 25. doi: http://dx.doi.org/10.1186/1475–2859–10–25
14. *Wang, Y., Yin, J., Chen, G.-Q.,* Polyhydroxyalkanoates, challenges and opportunities, Curr. Opin. Biotechnol. **30** (2014) 59. doi: http://dx.doi.org/10.1016/j.copbio.2014.06.001
15. *Gomez, J. G., Méndez, B. S., Nikel, P. I., Pettinari, M. J., Prieto, M. A., Silva, L. F.*, Making Green Polymers Even Greener: Towards Sustainable Production of Polyhydroxyalkanoates from Agroindustrial By-products, in Petre, M. (Ed.), Advances in Applied Biotechnology, InTech, Rijeka, 2012. doi: http://dx.doi.org/10.5772/31847
16. *Koller, M., Atlić, A., Dias, M., Reiterer, A., Braunegg, G.,* Microbial PHA Production From Waste Raw Materials, in Chen, G. Q. (Ed.), Plastics from Bacteria, Springer, Berlin and Heidelberg, 2010, pp 85–119. doi: http://dx.doi.org/10.1007/978–3-642–03287–5_5
17. *Sanchez-Vazquez, S. A., Hailes, H. C., Evans, J. R. G.,* Hydrophobic polymers from food waste: Resources and synthesis, Polym. Rev. **53** (2013) 627. doi: http://dx.doi.org/10.1080/15583724.2013.834933

18. *Follonier, S., Goyder, M. S., Silvestri, A.-C., Crelier, S., Kalman, F., Riesen, R., Zinn, M.,* Fruit pomace and waste frying oil as sustainable resources for the bioproduction of medium-chain-length polyhydroxyalkanoates, Int. J. Biol. Macromol. **71** (2014) 42.
19. *Zinn, M., Durner, R., Zinn, H., Ren, Q., Egli, T., Witholt, B.,* Growth and accumulation dynamics of poly(3-hydroxyalkanoate) (PHA) in Pseudomonas putida GPo1 cultivated in continuous culture under transient feed conditions, Biotechnol. J. **6** (2011) 1240.
doi: http://dx.doi.org/10.1002/biot.201100219
20. *Sun, Z. Y., Ramsay, J. A., Guay, M., Ramsay, B. A.,* Automated feeding strategies for high-cell-density fed-batch cultivation of Pseudomonas putida KT2440, Appl. Microbiol. Biotechnol. **71** (2006) 423.
doi: http://dx.doi.org/10.1007/s00253–005–0191–7
21. *del Castillo, T., Ramos, J. L., Rodriguez-Herva, J. J., Fuhrer, T., Sauer, U., Duque, E., J.,* Convergent peripheral pathways catalyze initial glucose catabolism in *Pseudomonas putida*: Genomic and flux analysis, Bacteriol. **189** (2007) 5142.
doi: http://dx.doi.org/10.1128/JB.00203–07
22. *Fuhrer, T., Fischer, E., Sauer, U., J.* Experimental identification and quantification of glucose metabolism in seven bacterial species, Bacteriol. **187** (2005) 1581.
doi: http://dx.doi.org/10.1128/JB.187.5.1581–1590.2005
23. *Van Dyk, J. S., Gama, R., Morrison, D., Swart, S., Pletschke, B. I.,* Food processing waste: Problems, current management and prospects for utilisation of the lignocellulose component through enzyme synergistic degradation, Renew. Sust. Energ. Rev. **26** (2013) 521.
doi: http://dx.doi.org/10.1016/j.rser.2013.06.016
24. *Zhao, Y.,* Utilization of Berry Processing By-products, in *Zhao, Y.* (Ed.), Berry Fruit: Value-added Products for Health Promotion, CRC Press, Boca Raton, 2007. pp 387.
doi: http://dx.doi.org/10.1201/9781420006148.ch14
25. *Bustamante, M. A., Moral, R., Paredes, C., Pérez-Espinosa, A., Moreno-Caselles, J., Pérez-Murcia, M. D.,* Agrochemical characterisation of the solid by-products and residues from the winery and distillery industry, Waste Manage. **28** (2008) 372.
doi: http://dx.doi.org/10.1016/j.wasman.2007.01.013
26. http://www.alibaba.com/showroom/glucose-price.html (9.7.2014).
27. http://www.alibaba.com/showroom/caprylic-acid.html, (20.10.2014).
28. http://www.alibaba.com/showroom/undecenoic-acid-112--38--9.html (20.10.2014).
29. *Furrer, P., Panke, S., Zinn, M.,* Efficient recovery of low endotoxin medium-chain-length poly([*R*]-3-hydroxyalkanoate) from bacterial biomass, J. Microbiol. Methods **69** (2007) 206.
doi: http://dx.doi.org/10.1016/j.mimet.2007.01.002
30. *Martínez, V., García, P., García, J. L., Prieto, M. A.,* Controlled autolysis facilitates the polyhydroxyalkanoate recovery in Pseudomonas putida KT2440, Microb. Biotechnol. **4** (2011) 533.
doi: http://dx.doi.org/10.1111/j.1751–7915.2011.00257.x
31. *Follonier, S., Henes, B., Panke, S., Zinn, M.,* Putting cells under pressure: A simple and efficient way to enhance the productivity of medium-chain-length polyhydroxyalkanoate in processes with *Pseudomonas putida* KT2440, Biotechnol. Bioeng. **109** (2012) 451.
doi: http://dx.doi.org/10.1002/bit.23312
32. *Elbahloul, Y., Steinbüchel, A.,* Large-scale production of poly(3-hydroxyoctanoic acid) by Pseudomonas putida GPo1 and a simplified downstream process, Appl. Environ. Microbiol. **75** (2009) 643.
doi: http://dx.doi.org/10.1128/AEM.01869–08
33. *Hoffmann, N., Steinbüchel, A., Rehm, B. H. A.,* Homologous functional expression of cryptic *phaG* from Pseudomonas oleovorans establishes the transacylase-mediated polyhydroxyalkanoate biosynthetic pathway, Appl. Microbiol. Biotechnol. **54** (2000) 665.
doi: http://dx.doi.org/10.1007/s002530000441
34. *Witholt, B., Kessler, B.,* Perspectives of medium chain length poly(hydroxyalkanoates), a versatile set of bacterial bioplastics, Curr. Opin. Biotechnol. **10** (1999) 279.
doi: http://dx.doi.org/10.1016/S0958–1669(99)80049–4
35. *Hany, R., Hartmann, R., Böhlen, C., Brandenberger, S., Kawada, J., Löwe, C., Zinn M., Witholt, B., Marchessault, R. H.,* Chemical synthesis and characterization of POSS-functionalized poly[3-hydroxyalkanoates], Polymer **46** (2005), 5025–5031.
doi: http://dx.doi.org/10.1016/j.polymer.2005.04.020
36. *Ramier, J., Boubaker, M. B., Guerrouache, M., Langlois, V., Grande, D., Renard, E.,* Novel routes to epoxy functionalization of PHA-based electrospun scaffolds as ways to improve cell adhesion, J. Polym. Sci. A Polym. Chem. **52** (2014) 816.
doi: http://dx.doi.org/10.1002/pola.27063