Renewable Long-Chain Fatty Acids for Production of Biodegradable Medium-Chain-Length Polyhydroxyalkanoates (mcl-PHAs) at Laboratory and Pilot Plant Scales

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ABSTRACT: Several types of mcl-PHAs were produced by *Pseudomonas putida* KT2442 at pilot and laboratory scales from renewable long-chain fatty acids (LCFAs) and octanoic acid. These and other mcl-PHAs are now available in sufficient amounts to carry out application and processing studies. We have isolated and purified these polymers in preparative amounts of 10−500 g by solvent recovery and selective enzymolysis. The molecular weights of mcl-PHA copolymers produced from LCFAs were generally similar to those found for octanoic acid based material, but the polydispersity was higher and the degree of polymerization was lower. The polymers showed thermal properties common for amorphous or semi-crystalline thermoplastic elastomers above their T_g, which decreased with increasing average pendant chain length. PHAs derived from LCFAs, which contained 3−12 new hydroxyacid comonomers compared to PHA produced from oleic acid, were amorphous, did not crystallize, and showed liquid properties at room temperature. As the number of comonomers and thus the degree of disorder increased in these PHAs, the polymers became more viscous and tacky. PHAs derived from octanoic acid and oleic acid were not affected by the production scale in terms of composition and physical properties. Although different production process control strategies used at lab and pilot scale did influence the process productivity, the substrate yield was not affected by the process control type applied and was always close to the theoretical PHA yield to be expected for fatty acid utilization through the β-oxidation pathway. Isolation and GC−MS analysis of the methanolyzed trimethylsilyl- (TMSI-) derivatives allowed the identification of a large number of previously unknown 3-hydroxy acid PHA components. All purified polymers were subjected to in vitro aerobic biodegradation using a compost isolate. The extent of mineralization varied from 15 to 60% of the theoretical biochemical oxygen demand (ThBOD). The polymer weight loss after 32 days ranged from 40 to 90% for the different mcl-PHAs.

Introduction

Poly(hydroxyalkanoates) (PHAs) are biological polyesters produced by a large variety of microorganisms. They are of scientific and industrial interest because of their fascinating properties and potential as renewable and specialty plastics.[1] There are two major groups of PHAs: those that contain short chain length hydroxyalkanoic acid monomers with five or fewer carbon atoms (scl-PHAs) and those that consist of medium-chain-length hydroxyalkanoic acid monomers with six or more carbon atoms (mcl-PHAs). Monomers are linked in ester bonds between the carboxyl group of monomer *I* and the hydroxyl group of monomer $I + 1$. The monomer hydroxy group is usually located on carbon 3, in the *R*-configuration, but hydroxy groups are also found at carbons 2−5. Thus far over 120 different hydroxyacids have been identified as PHA constituents at analytical scale.[2]

Of these, only poly(3-hydroxybutyrate) P(3HB) and its copolymers with 3-hydroxyvalerate (Biopol), which were manufactured by Monsanto, have been available on a scale of 500 tons per year. A few other scl-PHA compositions[3] and several mcl-PHAs[4−6] have been produced in gram to kilogram amounts. All other known polyhydroxyalkanoates, generally mcl-PHAs, which account for 95% of identified PHAs, have been produced only in analytical amounts. Obviously, development of mcl-PHAs applications in the area of nanocomposites[7] or drug delivery systems[8] or involving processing steps such as thermal polymer processing,[3] electron beam cross-linking,[9] or plasma surface treatment[10] will require more than analytical amounts of material. Thus, the limited availability of mcl-PHA copolymers represents a major bottleneck for application studies and the development of mcl-PHA based materials (Table 1).

Table 1. Present Availability of PHA Subclasses at Different Production Scales

PHA Type	Production scale: Analytical mg	Test 10 g	Application 10-1000 g	Pilot 1-1000 Kg	Technical > 1 t
scl-PHAs (PHB, BIOPOL™)	√	√	√	√	√
Aliphatic mcl-PHAs	√	√	√	√	
Olefinic mcl-PHAs	√	√	√		
Unnatural 3-OH- mcl-PHAs	√	√			
4-, 5-, and 6-OH- mcl-PHAs	√			next 5 to 10 years	

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Table 2. Production of mcl-PHAs by *P. Putida* KT2442: Composition of Substrate Fatty Acid Mixtures and of the Resulting PHAs

fatty acid	FA_{an}[a] (% w/v)	FA_{veg} (% w/v)	PHA_{an} (mol %)			PHA_{veg} (mol %)		
			X:0 [b]	*X*:1	*X*:2	*X*:0	*X*:1	*X*:2
C6[c]	0.6	-[d]	8.5	-	-	8.9	-	-
C8	0.6	-	40.5	-	-	40.7	-	-
C10	0.7	-	27.4	<0.1	-	25.6	0.1	-
C12	6.1	0.6	7.2	5.9	-	6.1	6.3	-
C14	2.6	1.3	0.9	2.8	6.6	0.5	4.6	7.1
C16	12.6	9.5	<0.1	-	-	<0.1	-	-
C18	9.1	2.9	-	-	-	-	-	-
C18:1	41.5	31.2	-	-	-	-	-	-
C18:2	22.5	48	-	-	-	-	-	-
C18:3	1.3	0.7	-	-	-	-	-	-
C20:1	0.5	0.1	-	-	-	-	-	-
C20	0.4	0.9	-	-	-	-	-	-
C22	0.5	2	-	-	-	-	-	-
C24	0.2	2.2	-	-	-	-	-	-
minor components	<0.5 [e]	<0.5 [e]	<0.1 [f]	<0.1 [f]	-	<0.1 [f]	<0.1 [f]	-

[a] Animal fatty acids (FA_{an}) and vegetable fatty acids (FA_{veg}) were analyzed by gas chromatography. [b] Molar ratio of saturated (*X*:0), monounsaturated (*X*:1) and diunsaturated (*X*:2) 3-hydroxy fatty acids in FA_{veg}-derived PHA (PHA_{veg}) and FA_{an}-derived PHA (PHA_{an}). [c] Key: C6, hexanoate; C8, octanoate; C10, decanoate; C12, laurate; C14, myristate; C16, palmitate; C18, stearate; C18:1, oleate; C18:2, linoleate; C18:3, linolenate; C20, arachidate; C20:1, eicosenate; C22, behenate; C24, lingnocerate [d] All hyphens indicate that nothing was detected. [e] Minor fatty acid components (<0.5%) included C14:1, C15:0, C16:1, C17, C17:1, C20:2, and C22:1. [f] Minor hydroxy fatty acid components in PHA (<0.1%) were identified as C7, C9, C11, C9:1, C10:1, and C11:1.

mcl-PHA copolymers can be produced using alkanes,[11] long-chain fatty acids,[12] glucose,[13] and a variety of unnatural substrates.[14] The substrates used for bacterial growth and production of mcl-PHAs contribute significantly to the overall mcl-PHA production costs.[15,16] Thus, carbon sources such as glucose,[17] purified long chain fatty acids, plant oils,[13,18] tallow free fatty acids (FFAs),[19] and beet molasses,[20] some of which may be derived from cheap agricultural waste streams, have been used for the laboratory scale production of PHAs. To effectively use these inexpensive and renewable carbon sources (such as different FFA mixtures) for the production of mcl-PHAs, it is necessary to evaluate the resulting polymers with respect to volumetric process productivity, substrate yield, novel polymer constituents, and resulting material properties. Accordingly, we have produced several mcl-PHAs from *Pseudomonas putida* KT2442 at the laboratory-scale (2 L) and pilot-scale (25 L), using technical oleic acid and FFA mixtures of both plant and animal origin. In this paper we compare the resulting purified polymers with respect to composition, physical properties and aerobic biodegradability.

Experimental Section

Microorganism and preculture conditions. *P. putida* KT2442[21] was used in all experiments. The organism was precultured in 100 mL of Luria Broth (LB) medium[22] for 5−7 h. For lab-scale experiments, 4 mL of LB culture were transferred into 200 mL of E* medium[11] containing 10 mM of the desired substrate. Oleic acid and FFA mixtures were added to a final concentration of 0.5% (v/v). Precultures were grown to an optical density (OD_{450}) of 2 and thereafter used to inoculate the bioreactor. For pilot scale experiments (25 L), 100 mL of LB culture was precultured in 1 L of E* medium supplemented with the desired substrates, as stated above.

Reactor Media. All reactor experiments were carried out using a minimum salt medium.[4] Carbon sources were added at initial concentrations of 10 mM Na−octanoate, 0.5% (v/v) technical oleic acid, Buchs, Switzerland (Aldrich) or 0.5% (v/v) fatty acid mixtures, Horn, Switzerland (SAIS). The fatty acid mixtures, obtained from SAIS, were prepared by steam distillation and subsequent saponification during the standard refining process of edible raw oils and are pooled byproducts of either animal (FA_{an}) or vegetable (FA_{veg}) origin, with compositions as shown in Table 2. Two separate feed lines were used for carbon feed and mineral salt feed. The latter consisted of 215 g of $(NH_4)_2SO_4$, 20 g of $MgSO_4 \cdot 7H_2O$, 0.5361 g of $FeSO_4 \cdot 7H_2O$, 0.0551 g of $ZnSO_4 \cdot 7H_2O$ and 0.0123 g of $CuCl_2 \cdot 2H_2O$ per liter of deionized water, sterilized by autoclaving. Fe^{2+}, Cu^{2+}, and Zn^{2+} were filter sterilized through a disposable filter (0.2 μm, Sterico, Dietikon, Switzerland) and added separately. Carbon feed solutions consisted of either FFAs as obtained from the supplier, technical oleic acid (80% or 90% v/v, Aldrich, Buchs, Switzerland) or 1 M Na−octanoate, depending on the process. PPG2000 (20% v/v, Fluka, Buchs, Switzerland) was added to the culture as antifoam agent when necessary. The basal reactor medium, the reactor, and peripheral devices were autoclaved at 121 °C for 35 min. All carbon substrates, solutions, and feed media were separately autoclaved at 121 °C for 25 min.

Laboratory-Scale Fermentation. All experiments were performed in a 2 L continuously stirred tank reactor (CSTR), equipped with pH, temperature, agitation, and airflow hardware controllers (University of Groningen, Groningen, The Netherlands). The pH was maintained at 7.00 by the addition of 2 M NaOH and 4 M H_2SO_4, the temperature was maintained at 30 °C, and the impeller speed was set at 2500 rpm in all experiments. The dissolved oxygen tension (DOT) was measured with an Ingold probe and maintained above 20% air saturation by a gradual increase of the airflow up to 3 L min^{-1}. Above 30 g L^{-1} of cell dry weight, if necessary, the sparging gas was gradually enriched with pure oxygen up to 50% (v/v) oxygen, to prevent oxygen transfer limitation. Process data monitoring (p_{O_2}, rpm, airflow, and pH) and control of both carbon and nitrogen supply rates were achieved by using a graphical programming environment (LabVIEW, Ver. 4.1, National Instruments, Austin, TX), running on a process computer (Deskpro590, Compaq). Data aquisition and carbon source concentration control have been described elsewhere.[23] Nutrient feed rates were open-loop controlled according to a feed algorithm described previously;[6] carbon source additions were controlled by a DO−STAT strategy.[24] Both feed solutions were added by means of two computer-controlled low-flow peristaltic pumps (Watson Marlow, Falmouth, U.K.). Consumption of feed media and the reactor weight were monitored gravimetrically and used for on-line feed rate control.

Pilot-Scale Fermentations. Pilot-scale fed-batch fermentations were performed in a 30 L CSTR (R110, New MBR, Zurich, Switzerland) using variable working volumes between 20 and 30 L. The reactor was equipped with pH, temperature, agitation, and airflow hardware controllers. The pH was kept at 7.00 with 2 M NaOH and 30% H_2SO_4, and the temperature

was kept at 30 °C. To avoid oxygen mass transfer limitations the impeller speed and the aeration rates were varied manually between 100 and 1000 rpm and between 0 and 30 L min^{-1}, respectively. On-line data aquisition and limited process control were performed by the computer software Caroline (PCS Process Control Systems AG, Wetzikon, Switzerland), running on an OS9 operating system. Platform compatibility was achieved by saving data in ASCII format and transferring it to a DOS compatible computer by serial communication. All medium streams were fed continuously with peristaltic pumps (Watson Marlow, Falmouth, U.K.) operating at preset values. Feed rates were determined according to an open-loop control strategy[6] and the profiles adjusted manually during the experiments.

PHA Isolation and Purification. PHA-containing cells were harvested by batch centrifugation (30 min, 8000*g* at lab scale; 30 min, 4000*g* at pilot scale). The resulting biomass slurry was processed either via conventional solvent recovery or by selective enzymolysis to an aqueous PHA latex.[26] Solvent recovery was carried out by lyophilization (1 mbar, 48−144 h) and subsequent Soxhlet extraction (CH_2Cl_2, 10% (w/v), 50 °C, 8 h) of dried cells. The resulting solution was filtered through a porous disperger (no. 4), and mcl-PHA was then precipitated in ice-cold methanol (10-fold excess) under vigorous stirring. After the methanol/CH_2Cl_2 solvent mixture was decanted, the resulting polymer was redissolved in CH_2Cl_2 and the precipitation step was repeated. Polymers were air-dried overnight and stored at 4 °C in the dark. Films were prepared by solvent casting from a 10−20% (w/v) solution in CH_2Cl_2. For latex production, cells (10% w/v) were resuspended in deionized water. The non-PHA biomass (R) was solubilized by addition of 0.4 U g^{-1} Alcalase (Novo Nordisk, DiHingen, Switzerland), 0.06 g g^{-1} EDTA, and 0.5 g g^{-1} sodium dodecyl sulfate (SDS) and incubation for 30 min at 60 °C, pH 8.5 under vigorous stirring. The resulting PHA suspension was washed by cross-flow diafiltration (KERASEP filter module, 19 channels, i.d. 2.5 mm × 400 mm, 0.1 μm cutoff, Ligakon AG, Topelswangen, Switzerland) with 10−12 volumes of deionized water. The latex was further concentrated by filtration to a final concentration of 15% (w/v). The washing step was carried out at pH = 8.5 and T = 60 °C in a CSTR operated in diafiltration mode and equipped with a recirculation loop.

Analytical Procedures. Samples were taken manually throughout the fermentation and optical densities (OD at 450 nm) were measured to estimate biomass concentrations. Ammonium $(NH_4)^+$ limitations were detected in the supernatant by the semiquantitative Merckoquant assay (Merck, Dietikon, Switzerland). Residual nitrogen concentrations in the supernatant were measured spectrophotometrically (CADAS 30 Photometer, Dr. Bruno Lange GmbH, Volketswil, Switzerland) using an Ammonium test kit (Ammonium LCK 304, Dr. Bruno Lange GmbH). Samples were washed in 25 mM $MgSO_4$ and filtered through 0.2 μm filters (Sterico, Dietikon, Switzerland) to gravimetrically determine the dry weight. The cellular PHA content was determined[11] with a gas chromatograph GC5200 (Fisons, USA) equipped with a 25-m CP−Sil5CB capillary column (Necherey-Nagel, Oensingen, Switzerland).

Identification of 3-Hydroxy Fatty Acids by GC−MS. Monomer identities were confirmed after trimethylsilyl (TMSI) derivatization of the methyl-3-hydroxyalkanoates and subsequent GC−MS according to the method of Lee et al..[25] mcl-PHA monomers were identified on a gas chromatograph GC8000 (Fisons) equipped with a 25-m CP-Sil5CB capillary column (Necherey-Nagel, Oensingen, Switzerland). Mass spectra were obtained by electron impact (EI) on a mass spectrometer MD800 (Fisons) at 70 eV. Base peaks at m/e = 73 or 89 and the peak at m/e = 175, which are typical for 3-hydroxy fatty acid TMSI derivatives, were used to screen the chromatograms for PHA monomers. The molecular ion-related mass fragments $m/e = [M - 15]$, $m/e = [M - 31]$, $m/e = [M - 47]$ and $m/e = [M - 73]$ were used to calculate the molecular weights of the TMSI derivatives. The double bond configurations and positions within the chain were assigned according to a detailed 2D-NMR study published by Huijberts et al.[24]

Physical Properties of mcl-PHAs. Molecular weights of purified mcl-PHAs were obtained by Gel Permeation Chromatography (GPC) (Knauer). Samples were dissolved in tetrahydrofuran (THF) and injected onto a PL-Gel mixed C column, 5 μm, 7.5 × 600 mm (Polymer Laboratories). The GPC system was equipped with a low-angle laser light scattering detector (Chromatix KMX-6 LALLS), a viscosity detector (H502, Viskotek) and a differential-refractive index detector (Knauer). Universal calibration by narrow-dispersed polystyrene standards (Polymer Laboratories) was used to calculate averaged molecular weights. Thermal properties were recorded on a DSC2000 differential scanning calorimeter (DSC) (Netzsch). For thermoanalysis, samples were heated from −80 to +100 °C (10 °C min^{-1}), rapidly cooled to −80 °C and heated again to +100 °C (10 °C min^{-1}). Tensile testing was performed on a Tensile Tester 4464 (Instron) at 25 °C and a displacement rate of 2 mm/min. The elastic modulus was calculated based on the first 5% displacement.

In Vitro Biodegradation of PHAs. Biodegradation studies were carried out in a Degramat incubation system (Rezzonico System Technology), which consisted of six gastight bottles equipped with automatic internal pressure monitoring. PHA polymers were incubated under stirring (20 rpm) for a maximum of 80 days at T = 27 °C in 100 mL of minimum medium containing 1.25 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.07 g of $CaCl_2 \cdot 2H_2O$, 0.6 g of KH_2PO_4 and 0.9 g of K_2HPO_4 per liter of deionized water and inoculated with 1 mL of bacterial compost enrichment culture (lab collection). Experiments always included one negative control (all assay components without addition of PHA), one positive control (all assay components and 0.1 g of PHB (Aldrich, Buchs, Switzerland) as the reference polymer), and four bottles containing two sample polymers (0.2−0.5 g, solvent cast thin films) in duplicate. To avoid influences on the measurement of O_2 partial pressure in the bottles, CO_2 evolved by the compost culture was absorbed by 2 mL of 10 M NaOH placed inside the bottle. The biodegradability of the test substance was calculated by subtracting biochemical oxygen demand (BOD) of the control blank from that of the averaged test medium and dividing the resulting experimental BOD by the theoretical biochemical oxygen demand (ThBOD)[27,28] of the test polymer. Maximal oxygen consumption rates were determined from the slope of BOD = $f(t)$; half-degradation times were calculated by curve fitting (exponential decay second order) and determining the time at which the BOD reached half of the ThOD.

Results

Production of mcl-PHAs from Octanoic Acid. *P. putida* KT2442 was grown on Na−octanoate, a reference carbon source for mcl-PHA production,[24,29−31] in a 2 L CSTR equipped with closed-loop octanoate concentration control. Biomass increased exponentially during the batch phase with a growth rate μ = 0.29 h^{-1}, until ammonium was depleted from the supernatant. Ammonium remained the growth limiting substrate from 17.8 to 23 h of cultivation, at which time the PHA production rate was 1.18 g of PHA L^{-1} h^{-1}, the highest measured in this experiment. The subsequent increase of the nitrogen concentration in the supernatant caused the volumetric PHA synthesis rate to decrease below 0.5 g of PHA L^{-1} h^{-1} (Figure 1A). At the end of the cultivation, after 43 h, the biomass and mcl-PHA concentrations reached 51.5 and 18 g L^{-1}, respectively, for an overall volumetric productivity of 0.41 g of PHA L^{-1} h^{-1} (Table 3).

To evaluate whether similar results could be achieved at the pilot-scale, *P. putida* KT2442 was cultured in a 30 L bioreactor, using open-loop control of the octanoate feed rate (Figure 1B). During the batch phase, the biomass increased with a maximum specific growth rate

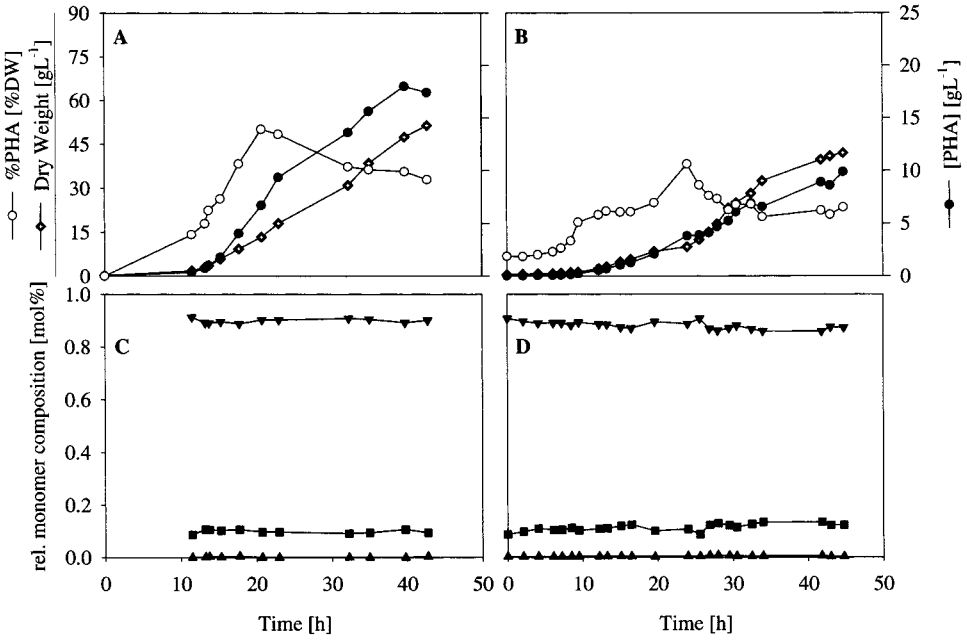

Figure 1. mcl-PHA production using octanoic acid as substrate. Panel A: Growth and mcl-PHA accumulation by *P. putida* KT2442 at lab scale. Panel B: Growth and PHA accumulation by *P. putida* KT2442 at pilot scale. Panel C: Monomer composition of mcl-PHA produced at lab-scale. Key: (▼) 3-hydroxyoctanoate; (■) 3-hydroxyhexanoate; (▲) 3-hydroxydecanoate. Panel D: Monomer composition of mcl-PHA produced at pilot scale; symbols are as shown in panel C.

Table 3. Fermentation Data of *P. Putida* Grown on Octanoic Acid and Renewable Long-Chain Fatty Acids in Laboratory- and Pilot-Scale Bioreactors

	growth				PHA			PHA production		
fatty acid substrate	scale (L)	time (h)	μ_{max} (h^{-1})	CDW (g L^{-1})	[PHA) (g L^{-1})	PHA (% CDW)	C=C (mol %)	$Y_{p/s}$ (g/g)	rP (g L^{-1} h^{-1})	rP_{max} (g L^{-1} h^{-1})
octanoic acid	2	42.75	0.29	51.5	18.0	35.8	0	0.21	0.41	1.18
octanoic acid	30	44.75	0.26	42.0	9.9	23.6	0	0.22	0.22	0.84
oleic acid[a]	2	38.75	0.35	67.5	18.9	28.0	6.2	0.49	0.49	1.42
oleic acid[b]	30	31.80	0.37	89.8	18.0	20.0	10.0	0.56	0.57	1.49
FA_{veg}[c]	2	45.00	0.26	73.0	25.0	34.2	18.0	0.46	0.56	0.86
FA_{an}[d]	2	46.00	0.20	28.5	15.4	54.0	15.3	0.38	0.33	1.14

[a] Technical oleic acid (80% w/v). [b] Technical oleic acid (90% w/v). [c] Vegetable fatty acids. [d] Animal fatty acids. [e] Nomenclature: μ_{max}, maximum growth rate; CDW, cell dry weight; $Y_{p/s}$, yield (product on substrate); rP and rP_{max} volumetric productivity and maximum synthesis rate.

Table 4. Molecular Weight and Physical Properties of Different mcl-PHAs

copolymers[a]	scale (L)	C=C[b] (mol %)	M_w (kDa)	x_w[c]	M_n (kDa)	x_n	d	T_g (°C)	T_m (°C)	E (MPa)
PHO_{lab}	2	0	187	1348	78	562	2.40	−31.0	56	11.0
PHO_{pil}	30	0	230	1658	98	706	2.35	−32.5	58	9.80
$PHOL_{80}$	2	6.2	135	878	49	321	2.76	−43.5	nd[d]	0.85
$PHOL_{90}$	30	10.0	125	798	46	294	2.71	−45.0	nd	0.80
PHA_{veg}	2	18.0	168	1017	65	394	2.68	−52.0	nd	na[e]
PHA_{an}	2	15.3	180	1175	71	465	2.53	−53.0	nd	na

[a] Substrates used: PHO_{lab}, octanoic acid (lab scale); PHO_{pil}, octanoic acid (pilot scale); PHOL, oleic acid (technical grade, 80 or 90% w/v, respectively); PHA_{veg}, vegetable-free fatty acids (FA_{veg}); PHA_{an}, animal-free fatty acids (FA_{an}). [b] Total amount of mono- and diunsaturated monomers in PHA. [c] Average degree of polymerization (i = w, weight, or i = n, number-averaged) is calculated based on copolymer composition. [d] Not detectable. [e] Not analyzable. [f] Nomenclature: M_w, weight-averaged molecular weight; x_w, weight-averaged degree of polymerization: M_n, number-averaged molecular weight; x_n, number-averaged degree of polymerization; d, polydispersity index; T_g, glass transition Temperature; T_m, melting point; E, elastic modulus.

of $\mu = 0.26$ h^{-1}, and nitrogen was depleted from the supernatant after 20.5 h. During the feed phase, we measured a maximum PHA synthesis rate of 0.84 g of PHA L^{-1} h^{-1}, and the biomass and cellular PHA content increased further to 42 and 9.9 g L^{-1}, respectively. The process was stopped at 45 h and showed an overall productivity of 0.22 g of PHA L^{-1} h^{-1} (Table 3). The mcl-PHA composition did not change significantly over time in either laboratory- or pilot-scale fermentations (Figure 1, parts C and D, respectively) and averaged 89.4 mol % 3-hydroxyoctanoic acid (C8:0), 10.0 mol % 3-hydroxyhexanoic acid (C6:0), and 0.6 mol % 3-hydroxydecanoic acid (C10:0) (Table 5).

Production of mcl-PHAs from Technical Oleic Acid. At laboratory scale, the culture grew exponentially on technical grade oleic acid (80% v/v) with a specific growth rate of $\mu = 0.35$ h^{-1} until nitrogen was depleted from the medium at $t = 14.5$ h (Figure 2A). The biomass and PHA concentrations increased to 67.5 and 18.9 g L^{-1}, respectively, during the feed phase, for a final cellular PHA content of 28% (w/w). The maximum PHA synthesis rate and the overall productivity

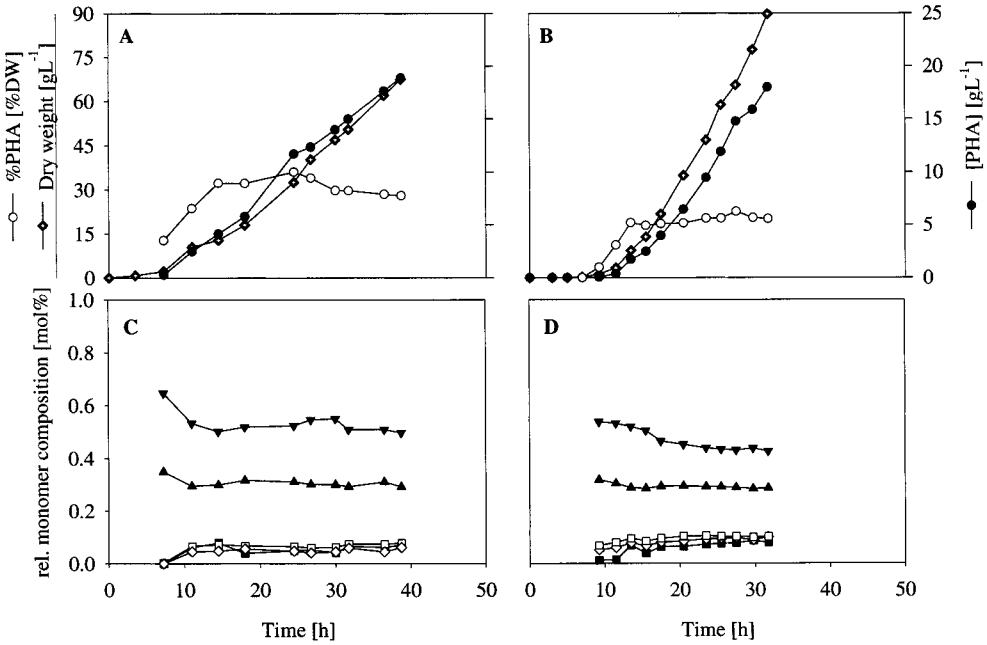

Figure 2. mcl-PHA production using technical oleic acid as substrate. Panel A: Growth and mcl-PHA accumulation of *P. putida* KT2442 at lab scale. Panel B: Growth and PHA accumulation by *P. putida* KT2442 at pilot scale. Panel C: Monomer composition of mcl-PHA produced at lab scale. Key: (▼) 3-hydroxyoctanoate; (▲) 3-hydroxydecanoate; (■) 3-hydroxyhexanoate; (□) 3-hydroxydodecanoate; (◊) 3-hydroxy-tetradecenoate. Panel D: Monomer composition of mcl-PHA produced at pilot scale; symbols are as shown in panel C.

Table 5. Copolymer Composition of Purified mcl-PHAs Produced by *P. Putida* KT2442 at Laboratory and Pilot Scale

	relative composition of purified mcl-PHA copolymers[a] (mol %)			
3-hydroxy fatty acid[b]	PHO_{lab}	PHO_{pil}	$PHOL_{80}$	$PHOL_{90}$
C6:0	10.0	10.0	7.1	7.9
C8:0	89.4	89.4	49.7	43.0
C10:0	0.6	0.6	29.2	28.9
C12:0			7.8	10.2
C14:1			6.2	10

[a] The polymers are named according to denominations used in the text. [b] Hydroxy fatty acyl monomers found in mcl-PHAs: C6:0, 3-hydroxyhexanoate; C8:0, 3-hydroxyoctanoate; C10:0, 3-hydroxydecanoate; C12:0, 3-hydroxydodecanoate; C14:1, 3-hydroxy-5-*cis*-tetradecenoate

were 1.42 and 0.49 g L^{-1} h^{-1}, respectively (Table 3). The PHA composition, as identified by GC/MS (data available on request), changed only slightly with time (Figure 2C). As shown in Table 5, the purified polymer consisted of five different monomers. 3-Hydroxyoctanoate (49.7 mol %) and 3-hydroxydecanoate (29.2 mol %) were the most abundant, with 3-hydroxy-5-*cis*-tetradecenoate (C14:1) (6.2 mol %), 3-hydroxyhexanoate (7.1 mol %), and 3-hydroxydodecanoate (C12:0) (7.8 mol %) as minor constituents.

P. putida KT2442 was also cultivated on technical oleic acid (90% v/v) for the 30 L pilot-scale experiment (Figure 2B). Biomass increased exponentially at a maximum growth rate of $\mu = 0.37$ h^{-1}, until nitrogen became the limiting nutrient at 13.5 h, when biomass and PHA concentrations reached 89.8 and 18 g L^{-1}, respectively. Major fermentation parameters are listed in Table 3. The PHA monomer composition changed slightly during the feed phase (Figure 2D), and the relative molar ratio of the major constituents 3-hydroxyoctanoate and 3-hydroxydecanoate decreased from 55 and 33 mol % to 43 and 28.9 mol %, respectively. Table 5 shows that the isolated and purified polymer contains three additional monomers, 3-hydroxyhexanoate (7.9 mol %), 3-hydroxy-5-*cis*-tetradecenoate (10 mol %), and 3-hydroxydodecanoate (10.2 mol %).

Laboratory Scale Production of mcl-PHAs from Fatty Acid (FA) Mixtures. As shown in Figure 3A, when a FA_{an} mixture pooled from different animal sources was used as the sole carbon substrate, the culture grew exponentially ($\mu = 0.20$ h^{-1}), and medium nitrogen was depleted at $t = 24$ h. During the feed phase the PHA concentration and cellular PHA content increased further to 15.4 g L^{-1} and 54.0% (w/w), respectively. At the end of the experiment a maximum PHA synthesis rate of 1.14 g of PHA L^{-1} h^{-1} and overall volumetric productivity of 0.33 g of PHA L^{-1} h^{-1} could be calculated (Table 3). The polymer consisted of 15 monomers of which 3-hydroxyoctanoate (40.5 mol %) and 3-hydroxydecanoate (27.4 mol %) were the most abundant (Figure 3C). Minor constituents are listed in Table 2.

When a FA_{veg} mixture derived from vegetable sources was used as a growth and PHA accumulating substrate, the biomass increased exponentially at $\mu = 0.26$ h^{-1}, nitrogen was depleted from the supernatant at $t = 11$ h and biomass and PHA concentrations increased to 73 and 25.0 g L^{-1} during the feed phase, respectively (Figure 3B). The cellular PHA content increased to 34.2% (w/w) and remained at that level until harvesting. The maximum mcl-PHA synthesis rate and final volumetric productivity were 0.86 and 0.56 g of PHA L^{-1} h^{-1}, respectively (Table 3). The PHA monomer composition changed only slightly over time and consisted of 15 monomers of which 3-hydroxyoctanoate (40.7 mol %) and 3-hydroxydecanoate (25.6 mol %) were the most abundant (Figure 3D). Minor constituents are shown in Table 2.

Physical Properties of Poly(3-hydroxyalkanoates). Physical properties of mcl-PHAs produced from octanoate and from different renewable carbon sources are shown in Table 4. The fraction of double bond-

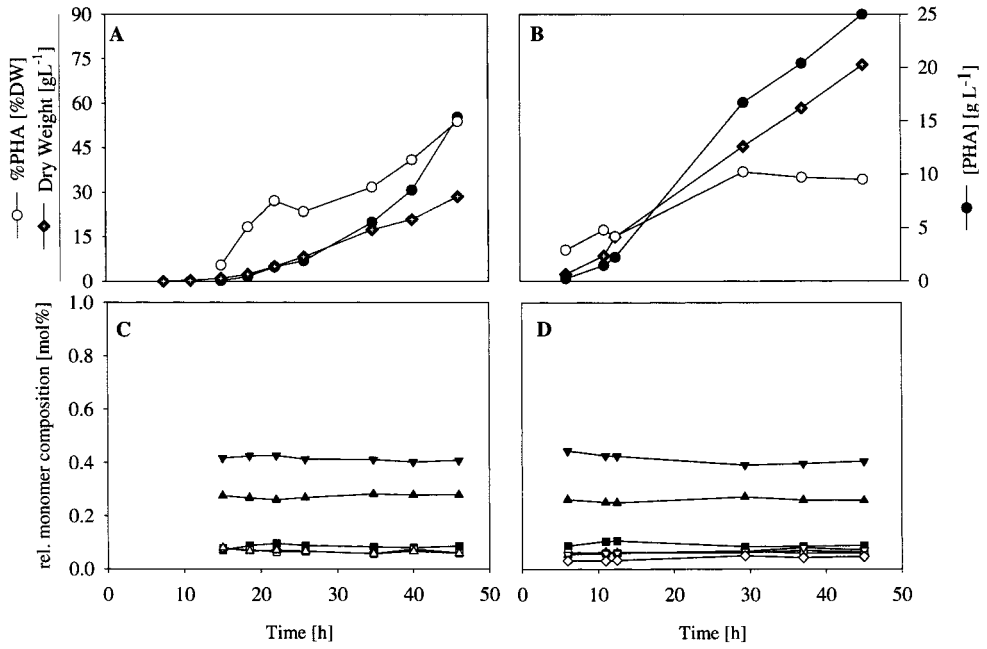

Figure 3. mcl-PHA production at lab scale using FA mixtures as renewable carbon sources. Panel A: Growth and mcl-PHA accumulation by *P. putida* KT2442 using FA_{an}. Panel B: Growth and PHA accumulation by *P. putida* KT2442 using FA_{veg}. Panel C: Monomer composition of mcl-PHA produced from FA_{an}. Key: (▼) 3-hydroxyoctanoate; (▲) 3-hydroxydecanoate; (■) 3-hydroxyhexanoate; (△) 3-hydroxydodecenoate; (□) 3-hydroxydodecanoate; (◇) 3-hydroxytetradecenoate; (▽) 3-hydroxytetradecadienoate. Minor components are not shown and are described in the text. Panel D: Monomer composition of mcl-PHA produced from FA_{veg}. Symbols are as shown in panel C.

containing monomers varied from 0 to 18.0 mol % and depended on the carbon substrate used. At both lab and pilot scale, mcl-PHAs produced from oleic acid showed significantly lower M_w and M_n values of ca. 130 and 48 KDa, respectively. mcl-PHAs from octanoic acid showed a somewhat lower polydispersity index of $d = 2.35-2.4$ as compared to polymers produced from long-chain fatty acids, which displayed values ranging from $d = 2.53$ to $d = 2.76$. Number- and weight-averaged degrees of polymerization ranged from 1658 and 706 for PHO to 878 and 294 monomers for PHOL, respectively, whereas FA-based polymers displayed intermediate values. Generally, the production scale did not significantly affect the molecular weight values. Glass transition points (T_g) of purified polymers decreased from −31 to −53 °C with increasing average pendant chain length and increasing number of double bond-containing PHA moieties. Only mcl-PHAs produced from octanoic acid showed a melting point around 57 °C. Furthermore, this polymer exhibited low crystallinity around 20% as could be calculated from the low values for ΔH_m (data not shown). All other polymers were completely amorphous and did not show any melting point. Tensile strength (E) varied from 11 MPa for PHO to 0.8 MPa for the much softer PHOL. Because of their liquid and incoherent state, no tensile strength could be measured for the FA-derived polymers. Mechanical properties were generally not affected by the production scale but changed dramatically as a function of the monomer composition.

In Vitro Biodegradation of mcl-PHAs. Different purified PHAs were used in vitro as sole carbon sources for growth of a bacterial compost enrichment culture. The polymers showed remarkable differences in terms of biodegradabilities (Figure 4). Growth on PHB resulted in the shortest induction time and the highest specific O_2 consumption seen in these experiments (data not shown). Surprisingly, only PHO_X (PHO isolated by selective enzymolysis) films were degraded, whereas

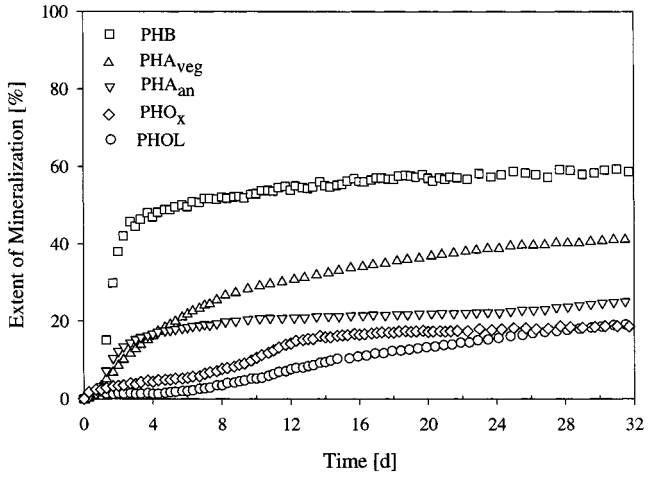

Figure 4. Biodegradation in vitro of mcl-PHA in a Degramat system. Extent of mineralization of various PHA samples, as described in the text. The extent of mineralization was calculated as shown in the text.

PHO_S (PHO isolated by solvent casting) films did not degrade at all. Metal content determination of 15 different trace metal species showed a very high (2500 ppm) Ni content in the PHO_S film (data not shown).

After 32 days (Figure 4), 60% of the PHB had been degraded, which was the highest biodegradability seen in these experiments. This was followed by PHA_{veg} at 40%, PHA_{an} at 20%, and PHO_X at 15% biodegradation after 32 days. PHOL biodegradation increased further to 27% after 60 days, when the experiment was stopped (data not shown). Since the general biodegradation equation[27] used for the calculation of ThBOD did not account for carbon fixation in biomass, the observed low biodegradation estimates of maximally 60% could be explained by this missing carbon balance term. Weight loss data are shown in Figure 5 and only PHB, PHA_{veg},

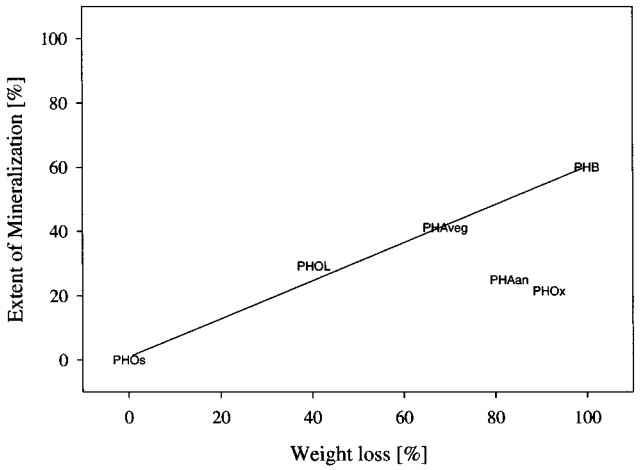

Figure 5. Biodegradation of PHAs: comparison of relative weight loss (as a measure for hydrolysis) to the achieved mineralization. Weight loss was calculated from remaining polymer samples after biodegradation experiments were stopped, extent of mineralization as shown in the text.

and PHOL weight losses matched that of ThBOD, implying complete mineralization to CO_2 and H_2O. Except for PHO_S films, all other polymers showed weight loss values ranging from 40% (PHOL) to 91.7% for PHO_X films. PHA_{veg} and PHA_{an} showed intermediate weight loss values of 69 and 83% of the initial weight, respectively.

Discussion

Renewable Substrates for the Production of mcl-PHAs. In this study, the application of a previously developed[23] closed-loop substrate control strategy yielded almost a 2-fold higher final PHA concentration and process productivity than by open-loop substrate control. Nevertheless, as expected, substrate yield and maximum growth and PHA synthesis rates did not change significantly when open-loop process control was used instead of closed-loop control. In fact, although temporary carbon limitations, induced by the open-loop carbon feeding strategy, affected overall process parameters, maximum PHA production rates were very similar to open- and closed-loop control. This suggests that mcl-PHAs can be produced routinely at pilot scale from octanoic acid with the same control systems used at lab scale.

Oleic acid is an interesting renewable substrate for PHA production, due to its high carbon yield.[5] The calculated yields were similar at the laboratory and pilot scales, at $Y_{p/s}$ of 0.49 and 0.56 g of PHA per g of oleic acid, respectively, and were close to the theoretical maximum yield of 0.62 g g^{-1} reported for fatty acid utilization via the β-oxidation pathway.[24] FA mixtures are clearly promising sources of oleic acid for mcl-PHA production from cheap substrates. PHA substrate yield was higher for FA_{veg} substrates than for FA_{an} substrates, which might be due to the higher amount of metabolizable oleic acid (C18:1) and linoleic acid (C18:2) in the former (79% w/v against 64% w/v). When the PHA yields of 0.38 and 0.46 g of PHA per gram of substrate are normalized to the amount of metabolizable fatty acids, the yields reach approximately 0.6 g g^{-1}, suggesting that both oleic acid and linoleic acid were completely metabolized. Because of a higher average fatty acid molecular weight and thus increased carbon chain length when using FA mixtures, yields (expressed as gram product per gram substrate) were generally higher than those measured for octanoic acid but in the same range as those obtained for technical oleic acid. These results suggest that, despite their complexity, FA mixtures may represent economical substitutes for oleic acid based mcl-PHA production.

Monomer Composition of mcl-PHA Produced from Renewable Resources. The final mcl-PHA composition depended on the substrate type used. When the purity of technical oleic acid was increased from 80% to 90% (v/v), the amount of C14:1 and C12:0 monomers also increased from 6.2 to 10.0 and 7.8 to 10.2 mol %, respectively. With fatty acid mixtures, the PHA monomer compositions were very similar to those of mcl-PHAs produced from either oleic or linoleic acid. This was expected, since FA_{an} and FA_{veg} contain large amounts of these unsaturated 18-carbon acids. Medium-chain length PHA monomers, such as C6:0, C8:0, C10:0, and C12:0, were not affected by changes in the substrate composition, which only influenced 14-carbon monomer levels. This can be understood given that long-chain fatty acids (LCFAs) such as C18:1 and C18:2 are degraded through different β-oxidation intermediates only to the level of 12-carbon atoms; further degradation occurs through common acyl−CoA intermediates containing 10, 8, 6, and 4 carbon atoms.[24] Traces of 3-hydroxydecenoic acid (C10:1), C-odd saturated and unsaturated monomers were probably due to traces of the related medium- or long-chain precursors within the FA mixtures. Furthermore, the supply of FA_{an} substrates increased significantly the relative 3-hydroxyhexadecanoic acid (C16:0) and 3-hydroxytetradecanoic acid (C14:0) monomer contents, due to a higher stearic (C18:0) and palmitic (C16:0) acid ratio. When linoleic acid was the predominant FA component, the 3-hydroxytetradecenedienoate and 3-hydroxydodecenoate molar ratios were higher than when oleic acid was the predominant FA. Except for the presence of diene-containing monomers and the large number of minor components, the monomer composition of FA-derived PHA did not differ significantly from oleic acid-derived PHAs. Thus, differences in the pattern of unsaturated PHA monomers are clearly related to differences in the 18-carbon FA mixture and are shown in Figure 6. In future, these copolymer compositions may be predicted by closely relating them to substrate FA compositions.

Physical Properties of mcl-PHAs. The number- and weight-averaged molecular weights of isolated mcl-PHA copolymers produced from LCFAs were generally similar to those found for octanoic acid based material. However, the polydispersity was higher and the degree of polymerization was significantly reduced for these mcl-PHAs produced from LCFAs. A wider molecular distribution of shorter molecules was observed suggesting that an increase in the array of possible "low-affinity"[12] β-oxidation intermediates might be a disturbing factor for high-molecular weight PHA synthesis.

The purified polymers displayed thermal properties common for amorphous or semicrystalline thermoplastic elastomers above their T_g. Only PHO-based materials showed a small melting endotherm, indicating the presence of a crystalline phase. PHO typically shows low ΔH_m values and crystallinity (X_c < 30%),[33] which is due to the relatively high degree of disorder of these statistical copolymers. As expected, PHA_{veg} and PHA_{an}, which contained an additional 3−12 hydroxy acid comonomers compared to PHOL, were amorphous co-

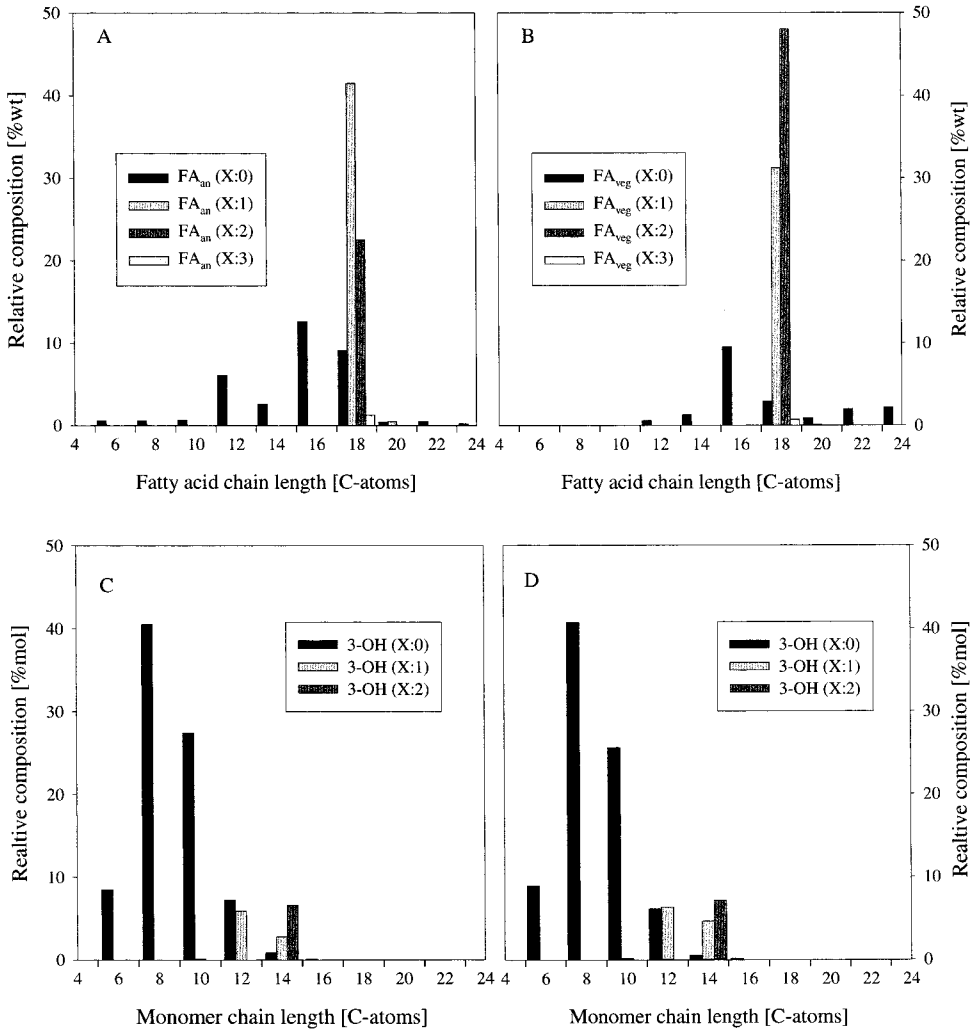

Figure 6. Relationship between fatty acid mixture substrate composition and mcl-PHA copolymers produced by *P. putida* KT2442. Panel A: Relative fatty acid composition of substrate mixture FA_{an}, grouped by amount of unsaturations. Panel B: Relative fatty acid composition of substrate mixture FA_{veg}, grouped by amount of unsaturations. Panel C: Relative 3-hydroxy fatty acid (3OH) composition in PHA_{an}, grouped by amount of unsaturation. Panel D: Relative 3-hydroxy fatty acid (3OH) in PHA_{veg}, grouped by amount of unsaturation. In all panels *X*:0, *X*:1, *X*:2 and *X*:3 indicate saturated, monounsaturated, diunsaturated and triunsaturated fatty acids, respectively.

polymers with an increasingly variable degree of disorder, which did not crystallize. Although the polymer main chain structure and flexibility, which are the principal factors governing T_g, remained constant within the range of examined copolymers, T_g decreased with increasing average pendant chain length. This is probably due to increased steric hindrance and consequent decrease of the main chain stiffness as monomer size increases.[34]

At room temperature, PHO displayed rubbery and slightly tacky mechanical properties. As the number of comonomers and thus the degree of disorder increased in PHOL, the polymer became more viscous and tacky. PHA_{veg} and PHA_{an} copolymers, which consisted of a large number of different comonomers and showed a low T_g, displayed liquid properties at room temperature. This can be best explained by the very high degree of disorder and by the long pendant chains acting as internal plastifiers.[34] Although the measured tensile strength was in the range of that of natural rubbers, unmodified mcl-PHA copolymers are unlikely to be useful as load bearing materials, due to their low elongation at break.

Biodegradation of mcl-PHAs. Both reference polymer PHB and 5 of the mcl-PHAs were degraded by one mixed culture, under conditions which comply with ISO 14851 biodegradability test methods.[39] Since PHA depolymerases are inducible by and specific for either scl- or mcl-PHAs polymers,[35] degradation was probably due to at least two depolymerases, showing different substrate specificities. Alternatively, biodegradation could have been due to a single depolymerase, since some strains produce a depolymerase of low specificity.[36]

Polymer surface area influences the hydrolysis rate and thus, through the availability of substrates, the oxygen consumption rate exerted by the degrading culture. PHB was supplied as a high surface-area powder. Since pulverization of mcl-PHAs was impossible due to the tackiness of the materials, PHO and PHOL were supplied in this assay as thin films, while PHA_{veg} and PHA_{an} were supplied as viscous liquids on a glass support, thus being accessible from one side only. As a result, the assays may not be directly comparable. Thus, the high O_2 consumption rate of the PHB reference might be due to its high surface area.[33] This hypothesis is supported by a 3-fold decrease in final cell concentration when *Pseudomonas delafieldii* was grown for 50 h on poly(hydroxybutyrate-*co*-hydroxyvalerate) (PHB−HV) films instead of PHB−HV powder (data not shown).

Biodegradability depended on the more or less efficient consumption of polymer monomers, which acted as carbon sources for growth and energy of the degrading culture. When weight loss (hydrolysis) and biodegradability (mineralization) data were plotted against each other, the polymers were situated either on or below the line between zero and maximum biodegradability/weight loss (PHB). This indicates that the mcl-PHA monomers were less efficiently oxidized to carbon dioxide and water than the positive control PHB. Differences as a function of the length and type of monomers are expected, since short-chain, medium-chain, and long-chain fatty acids are utilized via different uptake and degradation systems.[37] Furthermore, PHO_X was cast from PHO latex and contained ca. 5% of organic impurities, which under the present conditions could have induced the formation of cracks, leading to accelerated biodegradation and weight loss (Figure 5, lower part).

Production of mcl-PHAs from Various Carbon Sources at Different Scales. We produced various mcl-PHAs at 2 L lab and 25 L pilot scales using a single production process. No major a priori limitations for further scaleup were detected. Minor differences did arise from the water solubility of the substrates used, but generally the DO-stat method was very suitable for supplying water insoluble LCFAs as growth and polymer accumulation substrates.

The monomer composition and resulting physical properties of the PHAs produced was rather constant, even when a complex and undefined fatty acid mixture was used, rather than a single substrate such as oleic acid. This is undoubtedly due to the defined specificity of PHA polymerase in incorporating fatty acyl−CoA precursors into PHA.[12,38]

Screening and application of other specific precursors should permit production of mcl-PHAs bearing different hydroxylation and/or monomer patterns, which might enlarge the range of obtainable polymer properties. Finally, different processing techniques may be useful in obtaining mcl-PHAs with suitable material properties, particularly when the copolymers show liquid properties at room temperature. Future work in the area of mcl-PHA synthesis and processing should help expand the range of biodegradable polymers for the next century.

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