Efficient Production of (*R*)-3-Hydroxycarboxylic Acids by Biotechnological Conversion of Polyhydroxyalkanoates and Their Purification

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An efficient method to prepare enantiomerically pure (*R*)-3-hydroxycarboxylic acids from bacterial polyhydroxyalkanoates (PHAs) accumulated by *Pseudomonas putida* GPo1 is reported in this study. (*R*)-3-Hydroxycarboxylic acids from whole cells were obtained when conditions were provided to promote in vivo depolymerization of intracellular PHA. The monomers were secreted into the extracellular environment. They were separated and purified by acidic precipitation, preparative reversed-phase column chromatography, and subsequent solvent extraction. Eight (*R*)-3-hydroxycarboxylic acids were isolated: (*R*)-3-hydroxyoctanoic acid, (*R*)-3-hydroxyhexanoic acid, (*R*)-3-hydroxy-10-undecenoic acid, (*R*)-3-hydroxy-8-nonenoic acid, (*R*)-3-hydroxy-6-heptenoic acid, (*R*)-3-hydroxyundecanoic acid, (*R*)-3-hydroxynonanoic acid, and (*R*)-3-hydroxyheptanoic acid. The overall yield based on released monomers was around 78 wt % for (*R*)-3-hydroxyoctanoic acid. All obtained monomers had a purity of over 95 wt %. The physical properties of the purified monomers and their antimicrobial activities were also investigated.

Introduction

Polyhydroxyalkanoates (PHAs) are biodegradable and biocompatible polyesters that are produced by a wide variety of microorganisms.[1,2] PHAs are stored in intracellular granules and used by bacteria as a carbon and energy source.[2] Many bacteria accumulate PHAs when they are cultured in an excess carbon source and when growth is limited by the lack of one or more essential nutrients.[1,3] Under carbon starvation the accumulated PHAs can be degraded to monomers that can be utilized for better cell survival.[4] To date, more than 140 types of 3-, 4-, 5-, or 6-hydroxycarboxylic acids have been found to be incorporated into the polymer.[5,6] All monomers analyzed so far have absolute (*R*)-configuration.[7,8]

(*R*)-3-Hydroxycarboxylic acids (*R*3HAs) have been reported to be valuable synthons and can be used as starting materials for the synthesis of antibiotics, vitamins, flavors, and pheromones.[9−13] It was also reported that some (*R*)-3-hydroxycarboxylic acids exhibit important biological activities, such as antimicrobial and/or antiviral potential.[14,15] For example, (*R*)-3-hydroxy-*n*-phenylalkanoic acids can be used to effectively attack *Listeria monocytogenes*, which is a ubiquitous microorganism, able to multiply at refrigeration temperatures, and is resistant to both high temperatures and low pH values.[14] This food pathogen has become an important issue in many countries during the past several decades.

Different methods were described to produce *R*3HAs: They can be chemically synthesized by enantioselective reduction of the corresponding 3-keto acids.[16] For certain products, this process requires the synthesis of precursor molecules, which complicates the synthetic procedure and may reduce the product yield.[17,18] Other synthetic approaches include stereoselective functionalization through Sharpless' asymmetric epoxidation and hydroxylation or through Brown's asymmetric allyboration.[19] These approaches require chiral, often expensive metal-complex catalysts that might contaminate the final products. For some conversions vigorous reaction conditions have to be applied such as high pressure, flammable reaction media, or cryogenic conditions.[19,20] Furthermore, products often have lower enantiomeric excesses than those obtained by biochemical processes.[21] Other approaches include microorganisms such as recombinant *Escherichia coli*[22] or *Saccharomyces cerevisae* as biocatalysts to introduce the chiral center.[23] Product inhibition of enzyme activity and moderate yields of product isolation can be limitations of these procedures.[9,21]

Alternative to chemical synthesis, bacterial PHA could be used as an important source of *R*3HAs.[10] To obtain *R*3HAs from PHAs, in vivo depolymerization has been investigated and efficiently accomplished for poly(3-hydroxybutyrate).[21] (*R*)-3-Hydroxybutyrate has been produced with a yield of 96%.[21] *R*3HAs with chain lengths of 6−11 carbon atoms can also be produced with a high yield (90%) by in vivo depolymerization of PHA from *Pseudomonas putida* GPo1.[24] However, bacterially produced PHA is often a copolymer containing *R*3HA with the chain length differing by one or more ethylene units.[6] The corresponding monomers derived from one type of PHA have very similar chemical properties, and therefore, they are difficult to separate.

One approach to purify a mixture of different *R*3HAs generated from PHA has been reported by de Roo and co-workers:[8] *R*3HA methylesters were produced by acid-catalyzed hydrolysis of the extracted PHA. The distinct methyl-

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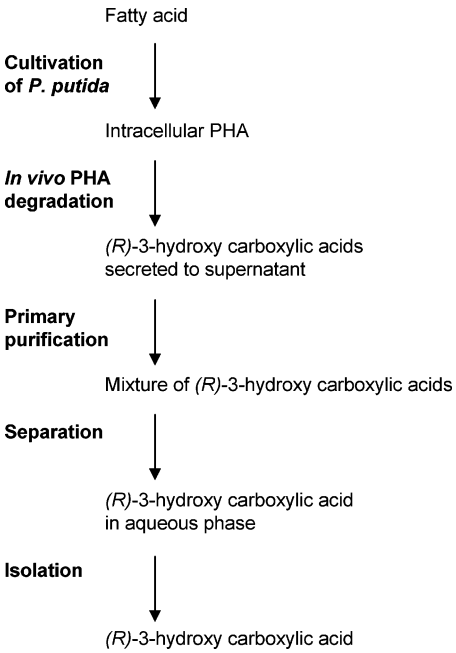


Figure 1. Schematic representation of the process to isolate (*R*)-3-hydroxycarboxylic acids (*R*3HAs).

esters were separated by fractional distillation, and the free acids were obtained by subsequent saponification. Even though high yields were obtained (~80%), this procedure is rather complicated and tedious. It takes 7 days for distillation.[8] Furthermore, considerable amounts of organic solvents are needed for the extraction of PHAs.

A satisfying process to provide *R*3HA on a preparative scale has not yet been achieved. For future applications of *R*3HAs as synthons or antimicrobial agents, it is not only important to be able to produce *R*3HAs but also to isolate them to a high degree of purity. In this study we developed an easy process to produce various chemically pure *R*3HA in milligram to gram quantities as shown in Figure 1. *P. putida* GPo1 in continuous growth culture under dual-nutrient-limited conditions (C- and N-limitation) was used to accumulate PHA. Afterward, the bacteria were exposed to an environment where enzymatic depolymerization of intracellular PHA took place. PHA monomers are secreted; thus extraction of PHA with organic solvents is not necessary, which renders this step environmentally friendly. The monomeric units of the PHA were collected and separated by reversed-phase preparative column chromatography followed by solvent extraction. The overall yield based on released monomers was around 78 wt % for (*R*)-3-hydroxyoctanoic acid. The physical properties of the purified monomers and their antimicrobial activities were also investigated.

Materials and Methods

Continuous Cultivation. To produce PHA, the wild-type strain *P. putida* GPo1 (ATCC 29347) was cultivated in chemostat cultures at a dilution rate (D) of 0.1 h^{-1} under dual-nutrient-limited growth conditions.[25,26] Continuous cultivation was performed as previously reported:[24,27] 40 L of continuous culture medium supplied with mineral trace elements (CCMT) was filter-sterilized (0.22 μm filter) into γ-sterilized 50 L medium bags (Flexboy, Stedim S. A., Aubagne Cedex, France). The carbon source (octanoic, undecanoic, or 10-undecenoic acid (Fluka, Buchs, Switzerland)) was pumped directly into the culture vessel by using a dosimat (Metrohm, Herisau, Switzerland). A 3.7 L laboratory bioreactor (KLF 2000, Bioengineering, Wald, Switzerland) with a working volume of 2.8 L was used. Cells grew in CCMT supplied with octanoic acid or 10-undecenoic acid at a carbon-to-nitrogen ratio (C/N) of 15.0 g g^{-1} or with undecanoic acid at a C/N ratio of 10.8 g g^{-1}. The cultures were kept at 30 ± 0.1 °C, and the pH was maintained at 7.0 ± 0.05 by automated control. The dissolved oxygen tension was monitored continuously with an oxygen probe (Mettler Toledo, Greifensee, Switzerland), and care was taken that it remained above 35% air saturation. The culture volume was kept constant with a balance that controlled the harvest pump. The harvested cell broth was collected in a 10 L tank kept on ice.

Ammonium was determined by a photometric ammonium assay (Spectroquant, Merck, Darmstadt, Germany). The carbon content of the medium was controlled with a total organic carbon analyzer (TOC-5050A, Shimadzu, Reinach, Switzerland). The cell dry weight (CDW) was recorded as described previously.[26]

Monomer Production. Cells collected during steady-state conditions were resuspended in 50 mM phosphate buffer at pH 10 to a concentration of 3−10 g L^{-1} depending on the CDW and PHA content to achieve a final PHA concentration of approximately 1.5 g L^{-1}. The suspension was incubated at 30 °C for 8−10 h. Subsequently, the supernatant was obtained by centrifugation (4500*g*, 4 °C, 20 min; Multifuge 3 S-R, Osterode, Germany) or filtration (filters LS14 1/2 270 mm, Schleicher & Schuell, Feldbach, Switzerland). Finally, the supernatant containing the PHA monomers was acidified to pH 1 with concentrated HCl. The monomers remained in the supernatant and were subjected to the separation process. It was possible to store the monomer-containing solution at 4 °C for several months without degradation of monomers (controlled by high-performance liquid chromatography mass spectrometry (HPLC-MS)).

Monomer Separation. Separation of various *R*3HAs was first attempted by solvent extraction methods. However, the amphiphilic character of *R*3HA led to formation of an emulsion. Complete phase separation did not occur, and *R*3HA accumulated in the interphase. Several anion exchange resins for column chromatography were tested as well, but the separation of *R*3HAs with these resins was not satisfactory. The reason for this presumably is that ionic properties of all *R*3HAs are too similar.

Monomer separation was accomplished with a glass column (length, 50 cm; diameter, 1.5 cm) packed with 40 g of silica gel 100 C18 reversed-phase (particle size, 0.040−0.063 mm; maximum surface coverage, 17−18% C or ±4 μmol m^{-2}; Fluka). During operation, the column was cooled to <10 °C and set under N_2 pressure (~80 kPa). After the column was washed with 0.1 M HCl (>5× bed volume), the mixture of monomers was fractionated using a stepwise elution with 0.1 M HCl/acetonitrile mixtures as the mobile phase. First, 100−150 mL of 0.1 M HCl/acetonitrile with a composition of 85:15 (vol %) was applied, second 300−400 mL of 0.1 M HCl/acetonitrile with a composition of 50:50 (vol %), and finally 100 mL of pure acetonitrile. The collected fractions were analyzed for the presence of monomers by HPLC.

Solvent Extraction. The fractions containing only one type of *R*3HA were pooled, and the acetonitrile was removed by rotary evaporation (150 mbar; 60 °C). Subsequently, the solutions were further acidified by addition of 1 M HCl (acid/sample ≈ 1:1 (vol %)), saturated with KCl, and extracted three times with an equal volume of *tert*-butyl methyl ether (aqueous phase/organic phase = 1:1 (vol %)). The combined organic phases were dried over Na_2SO_4 and filtered, and the solvent was evaporated (450 mbar; 40 °C). The purified monomers were stored under vacuum at room temperature.

Antimicrobial Assay. The antimicrobial activity of *R*3HA was tested by measuring the effect caused by several concentrations of the purified *R*3HA (ranging from 100 μM to 10 mM) on the growth of different species of bacteria (*L. innocua*, *L. monocytogenes*, *L. ivanovii*, *E. coli* MG1655, *Salmonella enterica*, and *Staphylococcus aureus* RN4220). (*R*/*S*)-3-Hydroxyoctanoic acid (Larodan, Malmö, Sweden) (ranging from 100 μM to 10 mM) and octanoic acid (ranging from 100 μM to 100 mM) were also investigated for comparison. Other racemic mixtures such as (*R*/*S*)-3-hydroxy-6-heptenoic acid, (*R*/*S*)-3-hydroxy-8-nonenoic

acid, and (*R*/*S*)-3-hydroxy-10-decenoic acid were not commercially available; thus the experiments using these compounds could not be carried out. The bacteria were cultured in TSB media (17 g/L casein peptone, 3 g/L soy peptone, 5 g/L NaCl, 2.5 g/L K_2HPO_4, 2.5 g/L glucose) in the presence or in the absence of the molecule to be tested. The pH was adjusted to pH 7.0 with sodium hydroxide. The conditions were 37 °C and shaking at 150 rpm. Precultures were grown in TSB to the exponential phase. For inoculation, they were diluted 1:30 (vol/vol) into the test solution to start the assay. Their growth was determined by measuring the optical density of the cultures at 550 nm over 24 h, using a microtiter plate procedure.[14,28] Minimal inhibitory concentration (MIC) was determined as the lowest concentration where no bacterial growth was observed.

Analytical Methods. *Gas Chromatography.* Quantitative analysis of *R*3HA was carried out by applying the following method adapted from a procedure for fatty acids:[29] For monomer measurement, 8 mL of monomer-containing solution or 5−15 mg of isolated monomers was filled into a 10 mL Pyrex tube. In the former case the liquid phase was evaporated by a nitrogen stream at 175 mL min^{-1}. Approximately 1 mL of 2-ethyl-2-hydroxybutyric acid (Fluka), dissolved in dichloromethane, was added as an internal standard. Its concentration was set to be similar to the estimated monomer content (1−10 mg/mL). Subsequently, 1 mL of BF_3 (∼10% in methanol; Fluka) was added, and the sample was tightly capped and heated to 80 °C for 20 h. Afterward, 2 mL of dichloromethane and 2 mL of saturated NaCl solution were added, and after intense shaking the upper phase was discarded, and the lower one was dried over Na_2SO_4 and subsequently analyzed on a gas chromatograph (Fisons Instruments, Rodano, Italy) equipped with a polar fused silica capillary column (Supelcowax-10; length, 30 m; inside diameter, 0.31 mm; film thickness, 0.5 μm; Supelco, Buchs, Switzerland). The injection temperature was 250 °C; the injection volume was 1 μL with a split ratio of 1:10. Helium was used as the carrier gas (3 mL min^{-1}), and detection was performed with a flame ionization detector (FID) at 285 °C. The temperature was increased from 80 to 280 °C at a rate of 10 °C min^{-1} to record a gas chromatography (GC) spectrum. The content was calculated with regard to the signal intensity of the internal standard. The calibration was accomplished with racemic 3-hydroxycarboxylic acids of varying chain lengths (Biotrend, Köln, Germany). The detected response factors were used to calculate absolute concentrations of the samples.

For intracellular PHA measurements, the cell suspension was centrifuged (10 000*g*; 4 °C; 15 min), and the cell pellet was lyophilized for 48 h. Approximately 50 mg of dry biomass were filled into a 10 mL Pyrex tube and treated in the same manner as the monomers.

Chiral GC Analysis. Purchased (*R*,*S*)-3-hydroxycarboxylic acids (Biotrend, Köln, Germany) and *R*3HA purified in this study were methylated.[7] Separation of the enantiomers was performed on a GC equipped with a Beta-DEX 120 column (fused silica capillary column; length, 30 m; inside diameter, 0.25 mm; film thickness, 0.25 μm; Supelco, Buchs, Switzerland). Absolute configurations of saturated *R*3HA were determined by comparison with reference materials. Since there was no standard material available for the *R*3HAs with terminal double bonds, their configurations were deduced from saturated *R*3HA. The injection volume was 1 μL with a split ratio of 1:10. The temperature was increased from 100 to 130 °C at a rate of 1 °C min^{-1} for optimal peak separation. The carrier gas was helium, and the compounds were detected by FID.

HPLC. Samples were diluted to 0.1−10 ppm in acetonitrile/water/acetic acid (50.0/49.9/0.1 (vol %)). Separation of compounds was performed on a reversed-phase C18 column (Gemini 5 μm C18 110 Å, 250 × 2.00 mm, Phenomenex, Macclesfield, U. K.) applying a linear gradient of 100% diluted acetic acid (0.1 vol % in water) to 100% acetonitrile as the mobile phase. The flow rate was 0.2 mL min^{-1}. The gradient was completed within 11 min. After each run the column was equilibrated at starting conditions for 8 min. The injection volume was 7.5 μL. Peaks were detected in negative mode by electrospray ionization mass spectrometry (ESI-MS) (Esquire HCT, Bruker Daltonics, Bremen, Germany).

DSC. Differential scanning calorimetry (DSC) was performed to determine the melting points (T_m) and enthalpies of the purified *R*3HA. Samples of 8−14 mg PHA were weighed into aluminum pans and analyzed with a differential scanning calorimeter 30 (Mettler Toledo, Greifensee, Switzerland). The samples were cooled to −80 °C within 10 min. After equilibration of the temperature, they were heated to 100 °C at a heating rate of 10 °C min^{-1}. Melting points were evaluated based on maximum peak heights. Data were determined with a standard deviation of ±13%.

^{1}H NMR. Proton nuclear magnetic resonance experiments in $CDCl_3$ (deuterated chloroform) solution were performed on a Bruker AV-400 spectrometer. Chemical shifts are given in ppm relative to the remaining signals of chloroform as an internal reference (^{1}H NMR, 7.26 ppm).

Abbreviations. Three different copolymers were produced by continuous cultivation of *P. putida* GPo1. They were used to isolate eight different *R*3HAs. For ease of readability, special abbreviations for the obtained polymers and monomers were used, as listed in Table 1.

Results and Discussion

In this study, *P. putida* GPo1 was selected for the biosynthesis of PHA due to its broad carbon substrate spectrum and its ability to accumulate PHA up to 63% of cell dry weight (CDW).[30] *P. putida* GPo1 was grown in continuous cultivation under carbon and nitrogen (C,N) limitation as described in the Materials and Methods section. These well-defined dual-nutrient-limited growth conditions ensured optimal and reproducible production of homogeneous PHA polymers.[31]

Production of Intracellular PHA. Three types of intracellular PHA were synthesized in this study based on the cultivation parameters listed in Table 2. When octanoic acid was used as the sole carbon source, 1.47 g L^{-1} CDW and 45.5 wt % PHA were obtained. The monomer composition C8-0/C6-0 was found to be 92/8 (wt %). A copolymer of PHUA was produced with undecanoic acid as the carbon substrate. The CDW and PHA content were 6.70 g L^{-1} and 16.6 wt %, respectively. The monomer ratio of C11-0/C9-0/C7-0 was 9/51/40 (wt %). The third type of polymer, PHUE, was synthesized when 10-undecenoic acid was applied as the single carbon source. The CDW of 1.36 g L^{-1} and the PHA content of 13.5 wt % were detected. The monomer composition of C11-1/C9-1/C7-1 was found to be 14/69/17 (wt %). To investigate a potential effect on monomer release patterns, different conditions were applied for PHA accumulation. However, the state of the continuous cultures during PHA accumulation in this context did not seem to play a significant role for subsequent monomer release.

The monomer compositions measured here by GC (e.g., C8-0/C6-0 = 92/8 wt %) were slightly different from those previously reported (e.g., C8-0/C6-0 = 87/13).[24,26] This might be caused by different analytical methods that mainly vary in the sample preparation for GC analysis. We compared the previously reported derivatization, namely, the formation of propylesters catalyzed by HCl, with the method used in this study, namely, the formation of methylesters catalyzed by BF_3. With the former method the monomer composition of C8-0/C6-0 was detected to be 87/13, which was the same as what was reported.[24,26] In this study the latter method showed better reproducibility and fewer side products; hence it was used further throughout subsequent experiments.

Production of (*R*)-3-Hydroxycarboxylic Acids. For in vivo depolymerization, bacterial cells containing various types of PHA were collected and suspended in 50 mM phosphate buffer

Table 1. Abbreviations Used for Polymers and Monomers Investigated in This Study

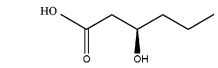
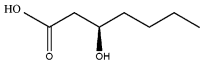
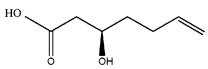
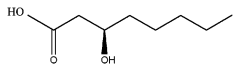
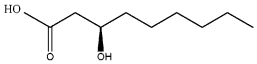
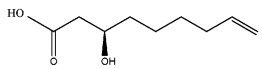
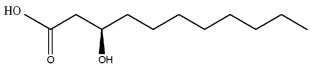
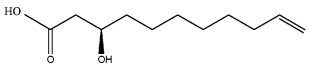
substance	abbreviation
polymers	
poly((*R*)-3-hydroxyoctanoate-*co*-3-hydroxyhexanoate)	PHO
poly((*R*)-3-hydroxyundecanoate-*co*-3-hydroxynonanoate-*co*-3-hydroxyheptanoate)	PHUA
poly((*R*)-3-hydroxy-10-undecenoate-*co*-3-hydroxy-8-nonenoate-*co*-3-hydroxy-6-heptenoate)	PHUE
monomers	
(*R*)-3-hydroxyhexanoic acid 	C6-0
(*R*)-3-hydroxyheptanoic acid 	C7-0
(*R*)-3-hydroxy-6-heptenoic acid 	C7-1
(*R*)-3-hydroxyoctanoic acid 	C8-0
(*R*)-3-hydroxynonanoic acid 	C9-0
(*R*)-3-hydroxy-8-nonenoic acid 	C9-1
(*R*)-3-hydroxyundecanoic acid 	C11-0
(*R*)-3-hydroxy-10-undecenoic acid 	C11-1

Table 2. PHA Synthesis with Different Carbon Sources in *P. putida* GPo1[a]

cultivation parameters	PHO	PHUA	PHUE
carbon substrate	octanoic acid	undecanoic acid	10-undecenoic acid
C/N ratio (g g^{-1})	15.0	10.8	15.0
feed nitrogen (mg (NH_4^+) L^{-1})	150	800	150
carbon supply (mg (C) L^{-1})	1750	6720	1750
dilution rate (h^{-1})	0.1	0.1	0.1
pH	7.00 ± 0.02	7.00 ± 0.01	7.00 ± 0.02
CDW (g L^{-1})	1.47 ± 0.23	6.70 ± 0.51	1.36 ± 0.07
PHA content (wt % of CDW)	45.5 ± 2.2	16.6 ± 1.2	13.5 ± 0.6
monomer composition (wt %)	C8-0/C6-0 = 92/8	C11-0/C9-0/C7-0 = 9/51/40	C11-1/C9-1/C7-1 = 17/69/14

[a] Data for CDW and PHA content were derived from at least four independent measurements.

at pH 10. In the cell suspension the final PHA concentrations were always approximately 1.5 g L^{-1}. The cells were then incubated for 8−10 h at 30 °C. In all cases, PHA degradation started immediately, and monomers were secreted into the supernatant as described previously.[24] The efficiency of the intracellular PHA degradation reached over 70 wt % after 8 h when testing the cell pellet for remaining PHA content by GC. At the same time, the supernatant was tested for monomer content, and the corresponding yields also achieved over 70 wt %. Different CDW and PHA contents obtained during continuous cultivation had no significant influence on either PHA degradation or monomer release under the tested conditions.

The monomer yield obtained in this study was lower (∼70 wt %) than what we obtained before (∼90 wt %).[24] One explanation for this observation is that the concentration of PHA in the phosphate buffer was higher (≥2 times) than what was tested in previous studies.[24] Higher initial PHA concentrations lead to theoretically higher monomer concentrations. Previous studies showed that the release of monomers strongly depended on the extracellular pH and was optimal under alkaline conditions.[24] Released monomers constantly lowered the extracellular pH, which can lead to an unfavorable condition for monomer release.[24] Thus, higher PHA concentrations could result in a lower monomer yield when the extracellular pH was not controlled at the optimum. A system to regulate pH during monomer release might be appropriate to avoid this influence. However, we cannot rule out the possibility that the released monomers were reutilized by cells as carbon and energy sources.

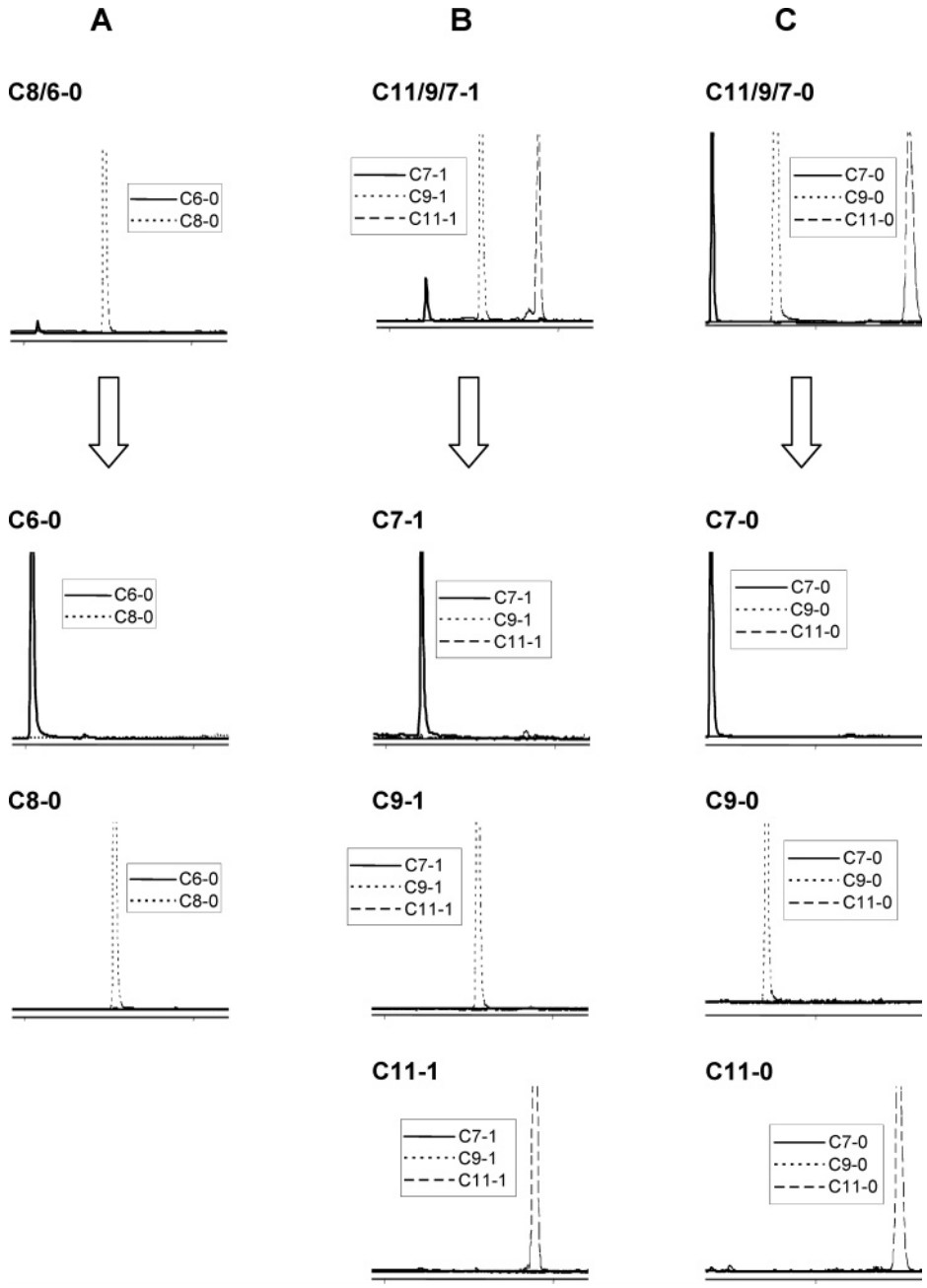


Figure 2. Separation of several *R*3HAs analyzed by HPLC. The masses *m*/*z* of all the relevant monomers are plotted in each figure as extracted ion chromatograms of the corresponding HPLC-MS spectra. Since measurement was performed with ESI in negative mode, the masses of the corresponding anions could be detected without fragmentation: panel A, monomers from cell growth on octanoic acid, *m*/*z* 131 = 3-hydroxyhexanoate, *m*/*z* 159 = 3-hydroxyoctanoate; panel B, monomers from cell growth on 10-undecenoic acid, *m*/*z* 143 = 3-hydroxy-6-heptenoate, *m*/*z* 171 = 3-hydroxy-8-nonenoate, *m*/*z* 199 = 3-hydroxy-10-undecenoate; panel C, monomers from cell growth on undecanoic acid, *m*/*z* 145 = 3-hydroxyheptanoate, *m*/*z* 173 = 3-hydroxynonanoate, *m*/*z* 201 = 3-hydroxyundecanoate. Arrows indicate the monomers before and after separation using column chromatography. All possible *R*3HA anions are plotted in each picture.

Cellular debris and some impurities precipitated at pH 1 were further removed by centrifugation. The amount of *R*3HA in the pellets was found to be negligible (≤2 wt % of the total released monomers). All kinds of *R*3HAs investigated in this study remained soluble. This was rather surprising as carboxylic acids are protonated at low pH and thus show low water solubility. An explanation might be that the hydroxy group in the β-position contributes to the solubility at low pH. The presence of complexing ions in the supernatant might also prevent precipitation of *R*3HA. In addition to free *R*3HA, the solutions contained salts and other cellular components that do not precipitate either at pH 1 or at pH 10.

Separation and Purification of (*R*)-3-Hydroxycarboxylic Acids. Since the obtained monomers from one type of PHA differ in one or more ethylene units of the carbon chain, the chemical properties of these compounds were very similar. We were able to separate these monomers by utilizing their unequal hydrophobic properties through column chromatography with a C18 reversed-phase (RP) packing material (for details see the Materials and Methods section).

The separation of *R*3HA was verified by HPLC-MS (Figure 2). Since measurements were performed with ESI in the negative mode, the masses of the anions corresponding to the distinct *R*3HAs would be detected without fragmentation. Therefore,

Table 3. Yields of Consecutive Isolation Steps[a]

purification step	C8-0 (mg L^{-1})	yield (%)
1. monomer released in supernatant	436 ± 16	*b*
2. after column separation	345 ± 2	79 ± 0.4
3. after solvent extraction	341 ± 2	78 ± 0.4

[a] The concentrations of 3-hydroxyoctanoic acid after the corresponding isolation step were analyzed by gas chromatography. The concentrations were calculated with respect to 2-ethyl-2-hydroxybutyric acid as an internal standard and (*R*,*S*)-3-hydroxyoctanoic acid for calibration. Data originated from 4 independent measurements. [b] The monomer concentration measured in the supernatant after in vivo PHA degradation was considered as 100%.

Table 4. Melting Points and Enthalpies of *R*3HA Measured by DSC[a]

R3HA	T_m (°C)	ΔH_f (J g^{-1})	non-hydroxylated couterparts	T_m[b] (°C)
C8−0	22 ± 2	−96 ± 11	octanoic acid	15−17
C9−1	12 ± 2	−199 ± 20	8-nonenoic acid	*c*
C9−0	53 ± 1	−159 ± 1	nonanoic acid	9
C11−1	39 ± 3	−110 ± 9	10-undecenoic acid	23−25
C11−0	60 ± 1	−108 ± 5	undecanoic acid	28−31

[a] Data originated from at least 2 independent measurements. [b] T_m values were obtained from the Sigma-Aldrich catalog. [c] Value cannot be found in the literature.

incomplete separation of *R*3HA could have been discovered by detection of the corresponding masses of the anions. In Figure 2, panels A, B, and C represent spectra of monomers obtained from cells grown on octanoic acid, 10-undecenoic acid, and undecanoic acid, respectively. *R*3HA, being produced by in vivo degradation of PHA, appears as a mixture of monomers. Pure products were subsequently obtained through the purification process. In this study, we were able to purify *R*3HA monomers in milligram to gram quantities.

During the sample collection, fractions with low product concentrations were discarded to reduce the working volume for the following purification steps. This explains why a yield of only ~80 wt % for *R*3HA C8-0 was reached (Table 3). The isolation of other *R*3HAs gave similar yields (data not shown). Further purification was carried out by extracting the respective *R*3HAs from combined fractions with *tert*-butyl methyl ether. This step was accomplished with high yields, e.g., ~98 wt % based on monomer content after column chromatography and 78 wt % based on total released monomer (Table 3). After extraction, small amounts of *R*3HA, e.g., less than 1 μg mL^{-1} 3-hydroxyoctanoic acid, were detected in the aqueous phase.

Previously, several *R*3HAs have been separated by using fractional distillation of the corresponding *R*3HA methyl esters and their subsequent saponification.[8] This process is quite complicated because the free acids had to be derivatized in an additional step. Sample preparation took more than 3 days, and distillation itself took 7 days. In addition, large amounts of organic solvents had to be used. The separation process described in this paper (including column chromatography and solvent extraction) involved only a few steps under mild conditions; therefore handling was much easier and could be accomplished within 2 days. Furthermore, less technical equipment, energy, and organic solvent were used. The organic solvent applied in this study could be recycled. The procedure developed here can easily be transferred to a larger scale.

Characterization of the Purified (*R*)-3-Hydroxycarboxylic Acids. To determine purities and structures of the produced *R*3HAs, the isolated products were analyzed by ^{1}H NMR spectroscopy (Figure 3). Ratios of the respective peak integrals

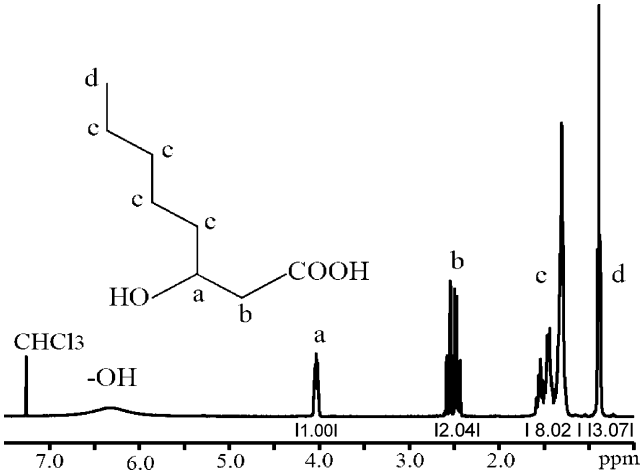


Figure 3. ^{1}H NMR spectrum of (*R*)-3-hydroxyoctanoic acid.

corresponded well with the chemical structures, and no side products were detected. Purities of all *R*3HAs were in a similar range and at least higher than 95 wt % as confirmed by both ^{1}H NMR and GC (data not shown). The configuration of the purified monomers was examined by chiral GC, as illustrated for C8-0 in Figure 4. The racemic standard (*R*,*S*)-3-hydroxyoctanoate methyl ester showed two peaks with the retention times (t_R) of 27.3 and 27.7 min that correspond to the two enantiomers. As previously reported, the later peak can be assigned to the (*R*)-enantiomer.[24] Only one peak was detected with the prepared 3-hydroxyoctanoate methyl ester from this study, revealing the high enantiomeric purity of this substance. By mixing 3-hydroxyoctanoate methyl ester and the racemic standard, the area of the later peak at 27.7 min increased, confirming the absolute (*R*)-configuration of purified *R*3HAs. Similar results were obtained for C6-0, C7-0, C9-0, and C11-0. Since it is unlikely that the *R*3HAs with terminal double bonds would be (*S*)-enantiomers, they were deduced to have (*R*)-configurations. Thus, in vivo depolymerization and subsequent isolation is a satisfactory approach to produce enantiomerically pure *R*3HA.

The melting points (T_m) and enthalpies of the isolated *R*3HAs were analyzed by DSC measurements (Table 3). The melting points measured for C8-0, C9-1, C9-0, C11-1, and C11-0 were 22, 12, 53, 39, and 60 °C, respectively. Melting points of non-hydroxylated counterparts are listed for comparison. C8-0 seemed to have a less crystalline, i.e., a less ordered, solid-state structure than its homologues with longer carbon chains. The weaker intermolecular interactions require less energy to be overcome, which results in a lower melting temperature of C8-0. Melting points of *R*3HA with chain lengths shorter than eight carbon atoms were below 0 °C. Enthalpies of C8-0, C9-1, C9-0, C11-1, and C11-0 were found to be between −199 and −96 J g^{-1}.

Antimicrobial Activity of Purified *R*3HAs. Previously, it was reported that aromatic (*R*)-3-hydroxy-*n*-phenylalkanoic acids have antimicrobial activity against food pathogens such as *L. monocytogenes*.[14] In this study, we tested whether some of the purified *R*3HAs also have antibacterial effects on different *Listeria* (*L. innocua*, *L. monocytogenes*, *L. ivanovii*) and other bacterial species (*E. coli* MG1655, *S. enterica*, and *S. aureus* RN4220). When C8-0, C9-1, and C11-1 were used, the minimal inhibitory concentration (MIC) for all tested *Listeria* species and *S. aureus* RN4220 ranged between 1 and 5 mM. For *E. coli* and *S. enterica* no growth inhibition was detected at the investigated concentrations (up to 10 mM for all tested *R*3HAs).

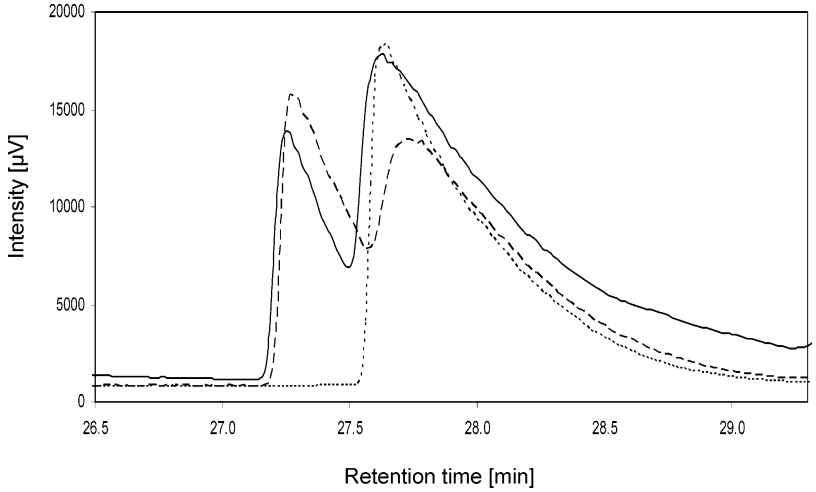


Figure 4. Chiral gas chromatography analysis of purified (*R*)-3-hydroxyoctanoic acid (C8-0): dashed line, racemic (*R*,*S*)-3-hydroxyoctanoic acid methyl esters; dotted line, 3-hydroxyoctanoic acid methyl ester prepared from C8-0 purified in this study; solid line, mixture (1/1 vol %) of the racemic standard (dashed line) and purified (*R*)-3-hydroxyoctanoic acid methyl ester (dotted line).

As a comparison, the commercially available racemic mixture (*R*/*S*)-3-hydroxyoctanoic acid and octanoic acid were also investigated for their antibacterial activities. It was observed that the former had a lower inhibitory effect (MIC above 10 mM on all tested strains) than its (*R*)-enantiomer, and the latter did not cause any effect on the bacterial growth of the different species unless the concentration was higher than 50 mM. It suggests that the antibacterial activity is mainly caused by the (*R*)-enantiomer.

The results that C8-0, C9-1, and C11-1 inhibited the growth of *Listeria* species and *S. aureus* with a MIC of 1−5 mM were similar to what was reported recently that (*R*)-3-hydroxyphenylalkanoates could inhibit bacterial growth with a MIC of 3−6 mM.[14] These data suggest that not only aromatic but also aliphatic (*R*)-3-hydroxycarboxylic acids are effective against *Listeria* species. Thus, the work described here opens a new route for the preparation of various enantiomerically pure (*R*)-hydroxycarboxylic acids for antimicrobial applications.

Conclusions

In this study, we have demonstrated that various enantiomerically pure (*R*)-3-hydroxycarboxylic acids can be produced and purified via in vivo depolymerization, column chromatography, and solvent extraction. Preparative liquid column chromatography with reversed-phase material is proven to be an efficient method for good separation and high recoveries of *R*3HAs. Scaleup of the separation process might be accomplished if larger columns and automated systems could be used. In comparison to previously described methods,[8,10] the procedure developed here leads to effective cost reduction, easy downstream processing, and an environmentally friendly approach. The procedure is not restricted to the *R*3HAs tested in this study. It can probably be applied to other *R*HAs that are accumulated in bacterial PHA, i.e., approximately 140 potential candidates.

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