Natural Estolides Produced by *Pseudomonas* sp. 42A2 Grown on Oleic Acid: Production and Characterization

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ABSTRACT: Estolides are a group of FA polyesters resulting from ester bond formation between a hydroxyl or olefinic group of one FA and the terminal carboxyl group of a second FA. These products are commonly found in trace amounts, forming tetraglycerides in several oil seed plants, and have been produced by acid clay and enzymatic catalysis *in vitro*. In this study, natural estolides produced by a bacterial culture are presented for the first time. *Pseudomonas* sp. 42A2 produced (*E*)-10-hydroxy-8-octadecenoic acid and (*E*)-7,10-dihydroxy-8-octadecenoic acid when grown on oleic acid. It is suggested that these FA were polymerized in culture by a lipase produced by the bacterial strain, resulting in a mixture of estolides. These compounds amounted to 3.8 g/L after 72 h of incubation. LC–MS analysis indicated that the types of estolides formed were dimers (*m/z* 560–610), trimers (*m/z* 845–906), tetramers (*m/z* 1122–1202), pentamers (*m/z* 1328–1424), and hexamers (*m/z* 1554–1788), with a relative abundance of 27.5, 19.4, 15, 9.7, and 11%, respectively. This is the first report in which hexamers were detected in a bacterial culture.

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KEY WORDS: Biotransformation, estolides, lipases, oleic acid, polyesters, *Pseudomonas.*

Estolides are a group of FA polyesters resulting from ester bond formation between a hydroxyl or olefinic group of one FA and the terminal carboxyl group of a second FA (1). Estolides are commonly found in trace amounts as tetraglycerides in several oilseed plants such as the Cruciferaceae *Heliophilia amplexicaulis* or the Brassicaceae *Lesquerella* and related genera. The oil of the Cruciferaceae has a high content of lesquerolic acid (14-hydroxy-*cis*-11-eicosenoic acid) (2), whereas that of the Brassicaceae has a high content of densipolic acid (15-hydroxy-9,15-octadecadienoic acid), auricolic acid (14-hydroxy-11,17-eicosadienoic acid), and lesquerolic acid (3).

Estolides were first synthesized by high-pressure condensation of oleic acid over acidic clays (4,5). Other unsaturated FA monomers containing various functionalities on the carbon chain such as petroselenic acid (*cis*-6-octadecenoic acid), linoleic acid [9(*Z*),12(*Z*)-octadecadienoic acid], and hydroxy-FA have been used for estolide synthesis (4–6). Recently, lipases isolated from *Candida rugosa*, *Rhizomucor miehei*, *Pseudomonas,* and *Alcaligenes* were found suitable for estolide synthesis *in vitro* using δ-lactones or hydroxy FA of various chain lengths. The largest monomers described as estolide components have been ricinoleic acid and 12-hydroxystearic acid (7,8).

Asadauskas *et al.* (9) claimed great potential for specific applications of estolides owing to their high viscosity indices and biodegradability; they may replace certain components of wax polyesters, lubricants, coating agents, inks, cosmetics, and surfactants.

Here we report detecting and characterizing new bacterial estolides, isolated from a submerged culture of *Pseudomonas* sp. 42A2 grown on oleic acid, the monomers being the hydroxy FA derivatives (*E*)-10-hydroxy-8-octadecenoic acid (MHOD) and (*E*)-7,10-dihydroxy-8-octadecenoic acid (DHOD) formed in the same culture (10).

MATERIALS AND METHODS

Chemicals. Technical-grade oleic acid was a gift from Lasem (Terrassa, Spain). All chemicals and solvents of analytical grade were purchased from Panreac (Barcelona, Spain), SDS (Peypin, France), or Merck (Darmstadt, Germany) and used as received. The microbiological medium, Trypticase Soy Agar, was supplied by Pronadisa (Barcelona, Spain).

Organism and growth conditions. Pseudomonas 42A2 NCIMB 40045 was originally isolated in our laboratory from oil-contaminated water. The microorganisms, after being grown on Trypticase Soy Agar medium for 24 h at 30°C, were kept at 4°C, subcultured fortnightly, and preserved frozen in cryovials (EAS Laboratoire, Combourg, France) at –20°C.

Natural estolide production and purification. An overnight culture (2% vol/vol) was used to inoculate the mineral salt medium (MSM) of the following composition (g/L): KCl: 0.1; KH_2PO_4: 1; K_2HPO_4: 2; $CaCl_2$: 0.01; $MgSO_4 \cdot 7H_2O$: 0.5; $FeSO_4 \cdot 7H_2O$: 0.012; and $NaNO_3$: 7. As stated below, 2% (vol/vol) of technical-grade oleic acid (70% oleic acid by GC), MHOD, or DHOD was alternatively used as carbon source. Carbon source and mineral salt solutions were autoclaved separately. Final pH was adjusted to 6.8 with 1 N NaOH if necessary. Submerged microbial cultures were incubated at 30°C for 72 h on a reciprocal rotary shaker (150 rev/min) in 1-L baffled Erlenmeyer flasks containing 150 mL of medium. Samples were withdrawn from the culture and centrifuged at 12,000 × *g,* 30 min at 4°C, and the cell pellet

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was discarded. The supernatant was acidified with HCl to pH 2, and the precipitated lipids were lyophilized. The lyophilized sample was extracted with chloroform (3×) and evaporated to dryness. The combined extracts were filtered under vacuum to remove the insoluble fraction and concentrated under vacuum at 40°C in a rotary evaporator to give a brown oily residue. To purify the extract and isolate the polymer fraction, the oily residue was dissolved in chloroform and precipitated with chilled methanol (11). The precipitate was separated from the monomers by centrifugation at 10,000 × *g*, 10 min at 4°C. The remaining solvent was removed from the precipitate under a nitrogen stream, and the purified estolides were prepared for further analysis.

MHOD and DHOD production and recovery. Pseudomonas 42A2 was incubated for 23 h in MSM as described above with oleic acid as carbon substrate. After this period, the culture was centrifuged at 12,000 × *g,* 30 min at 4°C, and the pellet was discarded. The supernatant was acidified to pH 3 with 2 N HCl, and subsequently lipids were extracted twice with 2 vol of chloroform (1:2 vol/vol). The organic phases were collected and dried with anhydrous Na_2SO_4, and the solvent was removed to dryness in a rotary evaporator. To obtain MHOD and DHOD, the crude lipid extract was purified by column chromatography on a 50 × 3 cm i.d. column packed with 60 g of silica gel G 60A C.C. (SDS, Peypin, France). The column (retention volume = 140 mL) was successively eluted with 140 mL of the following hexane/diethyl ether (vol/vol) mixtures: 100:15, 100:25, 100:35, 100:45, 100:60, 100:75. Two retention volumes (i.e., 280 mL) of a 100:100 (vol/vol) hexane/diethyl ether solution were then used to conclude the gradient elution. All hexane diethyl ether solutions were acidified by adding 1 mL of acetic acid per 140 mL mixture. Finally, the column was eluted with methanol. A total of 40 fractions was collected and analyzed by TLC. TLC analyses were performed on 20 × 20 cm silica gel 60 (0.25 mm thickness) analytical plates (Panreac, Spain). Plates were developed with 80:50:1 chloroform/methanol/acetic acid (by vol), and spots were visualized by spraying with a solution of phosphomolybdic acid in absolute ethanol, followed by charring with a heat gun. Those fractions containing compounds corresponding to the R_f similar to the MHOD and those corresponding to the R_f of the DHOD were combined and analyzed in a Shimadzu LC 9A gradient system (Shimadzu, Kyoto, Japan). Pure MHOD was found in fractions eluted with mixtures of 50:50 hexane/diethyl ether, whereas pure DHOD eluted in methanol fractions.

Lipase production and recovery. *Pseudomonas* 42A2 was incubated in MSM as described above with oleic acid as carbon substrate for 72 h. After centrifugation at 12,000 × *g* for 30 min at 4°C the cells were discarded. The enzyme was purified from the culture media by 80% ammonium sulfate precipitation with constant stirring for 10 min, and the mixture was then centrifuged at 17,000 × *g* for 15 min at 4°C. Immediately after, the sample was resuspended in 5 mM Tris-HCl, pH 7.5, and subsequently dialyzed against the same buffer for 24 h at 4°C. The sample was applied to a column of Q-Sepharose Fast Flow (Amersham, Cerdanyola, Spain) (25 × 2.6 cm) pre-equilibrated in a 5 mM Tris-HCl buffer, pH 7.5, and eluted with a linear gradient of NaCl from 0 to 1 M. Fractions containing lipase activity were eluted at a NaCl concentration of about 0.6 M. The specific activity was determined as 2.5 U/mg and the purity was checked by SDS gel electrophoresis.

In vitro *oleic estolides production*. A polymer from oleic acid was formed *in vitro* by incubating 85 µg of oleic acid with 30 µg of the partially purified lipase, obtained from *Pseudomonas* 42A2, in 10 mL of 5 mM Tris, pH 9.2, with 0.78 mg of sodium deoxycholate and 1 mg of $CaCl_2$. Incubation was carried out for 72 h at 30°C. The mixture was acidified to pH 2 with HCl, and the polymer was recovered from the reaction medium with chloroform.

Analytical methods. Microbial growth was measured by the protein content of the cultures following the method of Lowry *et al.* (12) against a standard of BSA (Sigma Chemical Co., St. Louis, MO).

Lipase activity was determined in an *in vitro* assay that was based on the decrease in turbidity when a lipase hydrolyses emulsified triolein. The decrease in turbidity was monitored by following the changes of A_{340nm}. The reaction mixture comprised: 26 mmol/L Tris buffer, pH 9.2; 19 mmol/L sodium desoxycholate; 0.1 mmol/L calcium chloride; and 0.30 mmol/L triolein. The standard reaction mixture also contained 3.0 mg/L colipase. The assay was carried out at 37°C. Lipase activity (U/L) of a sample was calculated by comparing the rate of change of A_{340nm} of the sample to that of a standard lipase sample of known activity, as described in the manual of the LIP MPR 1 (Cat. No. 159697; Boerhinger-Mannheim, Mannheim, Germany). Residual nitrate content of the medium was measured using a specific nitrate test (Aquamerck 8032; Merck).

Purified estolides were subjected to hydrolysis with 0.5 N NaOH at room temperature for 24 h. Before GC analysis, samples were methylated with diazomethane (13) and diluted with ether.

Methyl esters of the monomers were analyzed by GC analysis on a Shimadzu GC-14A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a split-splitless injector and a FID. A Supelco Omegawax™ 250, 30 m × 0.25 mm × 0.25 µm capillary column was used (Supelco, Bellefonte, PA). The oven temperature was kept at 210°C for 3 min, followed by an increase of 10°C/min to 240°C, held for 8 min, before initiating a second program of 2°C/min to 248°C, and then held for 20 min. Peaks were recorded on a Shimadzu C-R4 Chromatopac Integrator. Analyses were carried out on the methyl esters of the organic extract of the cell-free culture. Methyl ricinoleate (Sigma) of known concentration was used as standard. Retention times of MHOD and DHOD were 11.2 min and 25.3 min, respectively.

IR spectra were recorded on a Nicolet model 510 with a Fourier transform spectrometer (Madison WI).

^1H and ^{13}C NMR spectra were obtained in $CDCl_3$ solutions on a Varian spectrometer, operating at 250 and 300 MHz for ^1H and 62.5 and 75 MHz for ^{13}C, and the values are expressed in δ scale relative to internal tetramethylsilane. For the 250 MHz spectrometer the following conditions were used:

temperature 27°C, acquisition time 6,396 s, pulse 39.37, and spectral width 5123 Hz. In the case of the 300 MHz spectra, the conditions were: temperature 27°C, pulse 39.37 for ^{1}H and 43.27 for ^{13}C, acquisition time 1,998 s, spectral width 4500.5.

EI-MS were run on the trimethylsilyl ethers of the methyl ester derivatives (14) using a Hewlett-Packard mass spectrometer coupled to a gas chromatograph. The GC was equipped with a HP-5MS, 30 m × 0.25 mm × 0.25 mm capillary column (Hewlett-Packard, Palo Alto, CA). The oven temperature was kept at 140°C for 3 min, followed by a program of 5°C/min to 210°C, held for 8 min, and a second program of 10°C/min to 240°C, kept for 8 min; a final program of 2°C/min raised the oven temperature to 248°C, which was maintained for 10 min.

HPLC was performed on a Shimadzu LC9A gradient-capable system equipped with a Hypersil WP-300 C8 150 × 0.46 mm reversed-phase column (Teknokroma, S. Cugat, Spain). The solvent gradient was programmed from a 1:1 mixture of acetonitrile/water containing 0.1% acetic acid to pure acetonitrile at a flow rate of 1 mL/min. The chromatograph was coupled to an ELSD (Sedere, Alfortville, France) set at 50°C and 2 bar. Standards used were oleic acid, MHOD, and DHOD.

In the HPLC–electrospray (ES–MS) system, postcolumn addition of acetone at 200 µL/min was carried out using a syringe pump. Mobile phase and acetone were mixed in a tee valve, and a split system (1:50) was used to introduce the effluent into the ES. MS was performed using a quadrupole mass spectrometer equipped with a pneumatically assisted electrospray (ES) ion source. The positive ion mode was used. Full scan data were obtained by scanning from *m/z* 100 to 2900 in compressed centroid mode using a cycle time of 3.3 s and an interscan time of 0.34 s. The working conditions for the ES were as follows: dry nitrogen was heated to 100°C and introduced into the capillary region at a flow rate of 400 L/h. The capillary was held at a potential of 3.5 kV and the extraction voltage was −30 V.

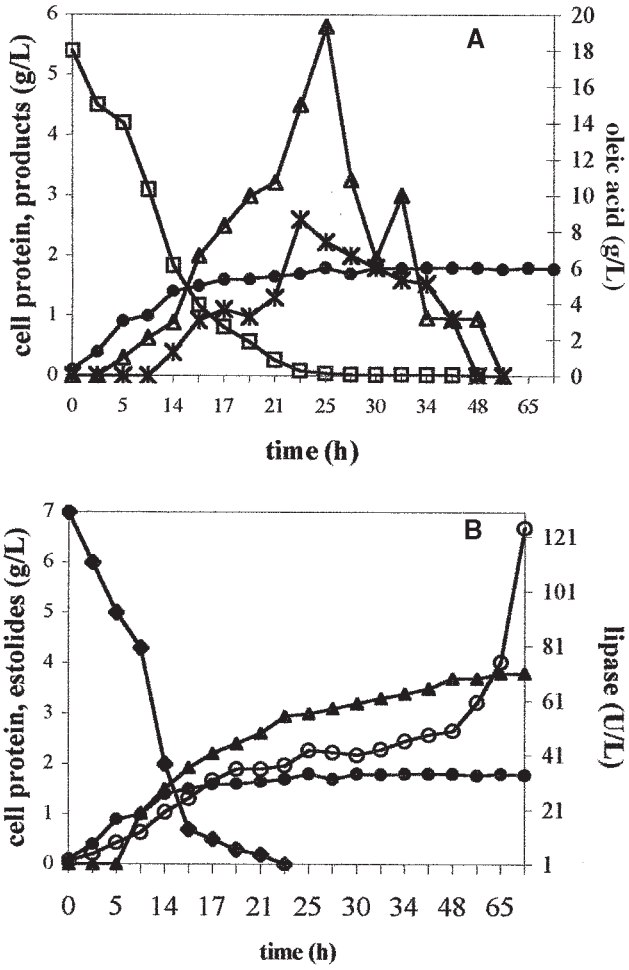


FIG. 1. Time course of submerged culture of *Pseudomonas* sp. 42A2 in mineral medium with 2% oleic acid as carbon source incubated at 30°C for 70 h. (A) cell protein (●), (*E*)-10-hydroxy-8-octadecenoic acid (MHOD) (△), (*E*)-7,10-dihydroxy-8-octadecenoic acid (DHOD) (✱), oleic acid (□). (B) extracellular lipase (○), estolides (▲), cell protein (●), residual $NaNO_3$ (◆).

RESULTS AND DISCUSSION

Time course of estolide production. As reported earlier, when *Pseudomonas* 42A2 was cultivated in MSM with oleic acid as the sole carbon source, high amounts of the hydroxy FA derivatives MHOD and DHOD were produced in the extracellular medium (10,15). The time course of the process is shown in Figure 1. After 24 h of incubation, oleic acid (Fig. 1A) and the nitrogen source (Fig. 1B) were depleted from the culture medium; thereafter, no significant bacterial growth was observed. Total cell protein reached 1.8 g/L. Analysis of the culture supernatant products showed that the main hydroxy FA formed were MHOD (maximum yield: 5.8 g/L at 25 h) with a cellular yield of $Y_{\text{MHOD/cell protein}} = 3.2$ (w/w) and a maximum of 2.6 g/L of DHOD at 23 h of incubation, with a cellular yield of $Y_{\text{DHOD/cell protein}} = 1.4$ (w/w). Nevertheless, after 50 h of incubation, neither MHOD nor DHOD was detected in the culture supernatant.

As previously reported, an extracellular lipase was detected in the cultures of this strain when incubated in mineral medium with frying oils as carbon source (16). Although no report has been found in the literature, these data suggested that the secreted lipase might potentially catalyze polymerization of the oleic acid and the hydroxy FA accumulated in the supernatant with the consequent formation of new natural estolides. On these premises, we studied the presence of estolides in the culture medium. Lipase activity was detected early in the culture, increasing sharply to 124 U/L at the end of the experiment (Fig. 1B). Estolides were detected after 5 h of incubation, and the amount of polymer increased with the lipase content in the culture, reaching 3.8 g/L at the end of the culture. They were isolated by organic extraction from the culture medium, yielding a white powder. This hypothesis of lipase-catalyzed estolide formation was reinforced by the fact that estolides from oleic acid were obtained when purified lipase from *Pseudomonas* 42A2 culture supernatant was used, as stated above.

Characterization of natural estolides. At the end of the culture (72 h) of *Pseudomonas* 42A2 on technical-grade oleic acid, cells were collected by centrifugation and removed. After acidification, solvent extraction of the supernatant and

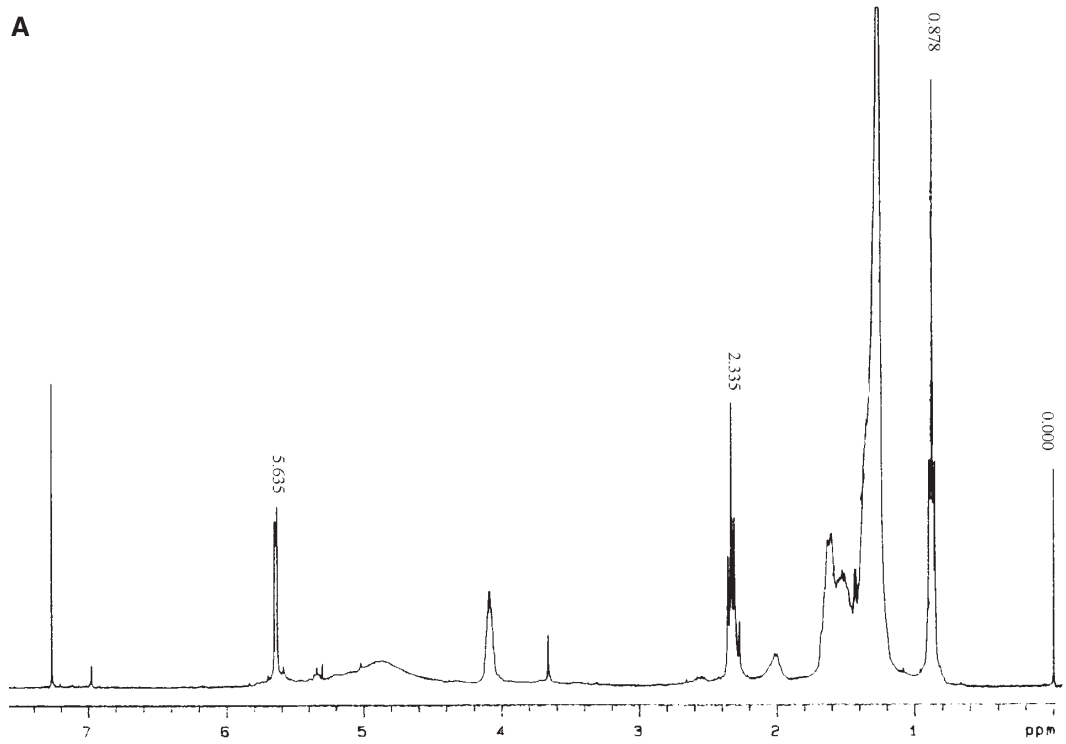
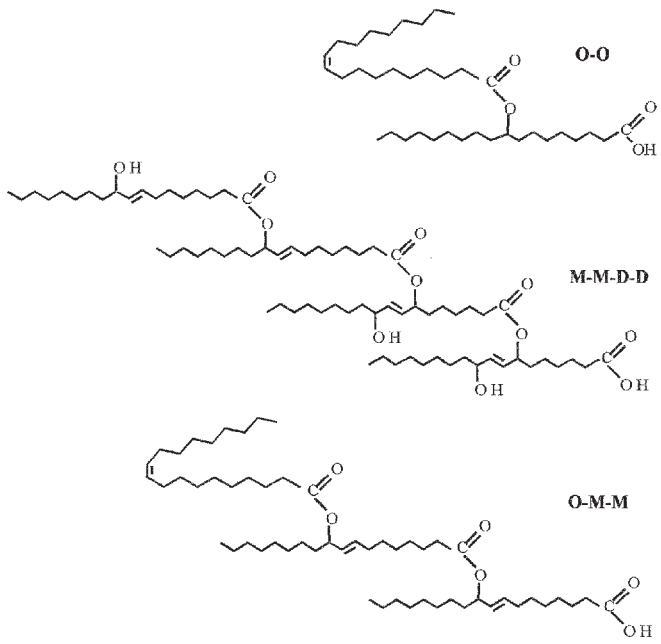


FIG. 2. ^{1}H (A) and (B) ^{13}C NMR (300 MHz) spectra of estolides produced by *Pseudomonas* sp. 42A2 when grown in mineral media with oleic acid as carbon source.

subsequent methanol precipitation furnished the natural estolide, whose purity was determined by HPLC (see the Material and Methods section). The IR spectrum revealed the presence of ester and acid groups as well as a *trans* unsaturation. IR bands (cm^{-1}) observed were: 3648, 3432, 2946, 1741, 1712, 1231, and 970 (*trans* alkene) cm^{-1}. The polymeric nature of the estolide was confirmed by its NMR spectra presented in Figures 2A and 2B. ^{1}H NMR peaks (δ) were: 5.62 (*m,* CHOH–C<u>H</u>=C<u>H</u>–CHOH), 4.90 (*b,* CHOCOR), 4.05 (*m,* C<u>H</u>OH–CH=CH), 2.32 (*t, J* = 7.2 Hz, C<u>H</u>$_2$CO$_2$R). ^{13}C NMR (ppm): δ 178.0 (CO_2H), 169.0 (CO_2R), 72.4 (<u>C</u>HOH), 70.8 (<u>C</u>HOCO). A fraction of the purified estolides was subjected to base hydrolysis (0.5 M KOH/CH_3OH), and the resulting monomers were silylated and methylated. GC–MS analysis confirmed the presence of the expected monomers in the following ratio: oleic acid, 10–20%; MHOD, 44–56%; and DHOD, 17–24%.

Owing to the presence of the three monomers in the culture medium (oleic acid, MHOD, and DHOD), several classes of estolides can be formed, and thus a wide range of polymers with various M.W. were found in each type of estolide. As an example, in Scheme 1 are represented the possible types of estolides that can be found in the culture supernatant for *Pseudomonas* 42A2 grown in mineral medium with 2% oleic acid as carbon source at 30°C for 75 h. The purified organic extract from *Pseudomonas* 42A2 culture after 72 h of incubation was analyzed by HPLC–MS (Fig. 3). Peak assignments were based on M.W. in the MS spectra considering the possible combinations of the M.W. of the corresponding monomers, i.e., O = oleic acid, M = MHOD, and D = DHOD. As shown in the chromatogram, estolides with higher M.W. eluted at longer retention times. The first section of the chromatogram (elution time 5.6 to 12.0 min) corresponded to dimers (E^1) of M.W. between *m/z* 560 (i.e., O-D) and 610 (i.e, D-D). The second part of the chromatogram (elution time



SCHEME 1

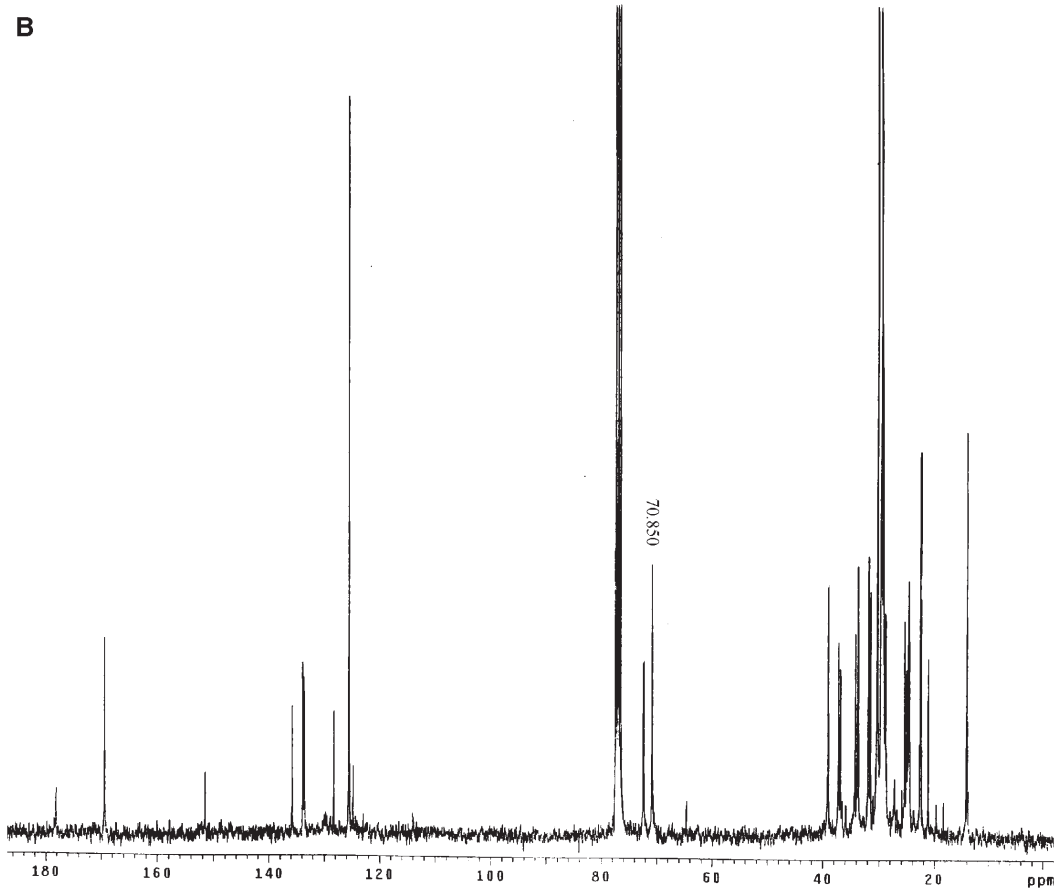


FIG. 2. (continued).

14.0–16 min) corresponded to trimers (E^2) of M.W. between *m/z* 845 and 906 (i.e., M-D-D; O-M-M; O-O-D; or O-D-D). In the third section of the chromatogram, tetramers (E^3) of molecular mass between *m/z* 1122 and 1202 (i.e., O-O-D-D; O-D-D-D; M-M-D-D; or D-D-D-D) were eluted between 16.2 and 18.6 min. Between 18.8 and 21.8 min, the fourth part of the chromatogram, pentamers (E^4), eluted with a range of M.W. between *m/z* 1328 and 1498 (i.e., O-O-O-O-O; O-O-D-D-D; M-M-D-D-M; M-M-M-M-M). In the last section of the chromatogram (elution time 22.0 to 23.2 min) appeared the largest estolide (E^5) hexamer-type (Fig. 4) with an apparent M.W. of approximately *m/z* 1554 to 1788 (i.e., O-M-M-O-M-M or O-D-D-O-D-D; M-M-D-M-M-D; M-D-D-M-D-D). The calculated relative abundance of estolides was: dimers 27.5%, trimers 19.4%, tetramers 15.0%, pentamers 9.7%, and hexamers 11.0%. It should be noted that an average of tetramers has been obtained by enzymatic synthesis (5,10), whereas the largest analytes detected in *Pseudomonas* 42A2 culture were hexamers. To our knowledge, no other reports to date have described natural polymers composed of six monomers.

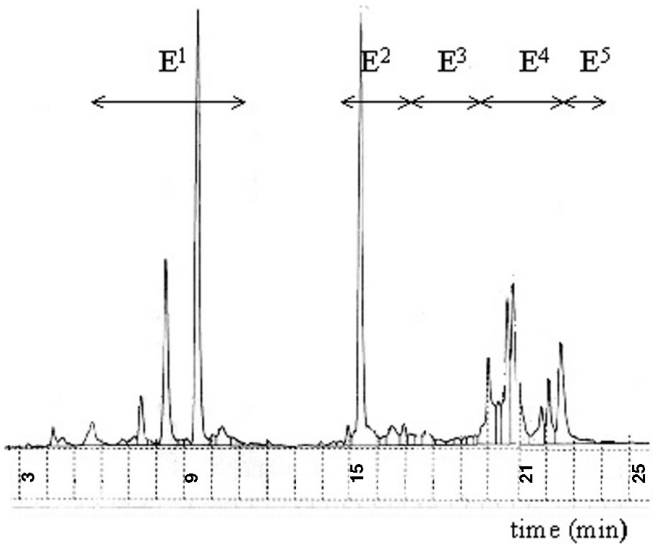


FIG. 3. LC-MS chromatogram of the purified organic extract from the supernatant of *Pseudomonas* sp. 42A2 grown in mineral salt medium with oleic acid at 30°C for 75 h. E^1: section of dimers; E^2: section of trimers; E^3: section of tetramers; E^4: section of pentamers; E^5: section of hexamers.

The polymerization of MHOD was carried out by incubating *Pseudomonas* 42A2 for 72 h using MHOD as carbon substrate in MSM. After acidic precipitation (HCl, pH 2) of the culture supernatant and subsequent lyophilization, the residual white powder was extracted with chloroform and purified by methanol precipitation. The purified polymer was analyzed by IR and the following bands were observed: 3412, 2926, 1710, 1242, 1184, 1096, 969 (*trans* alkene), and 724 cm^{-1}. ^1H NMR ($CDCl_3$) peaks (δ) were as follows:

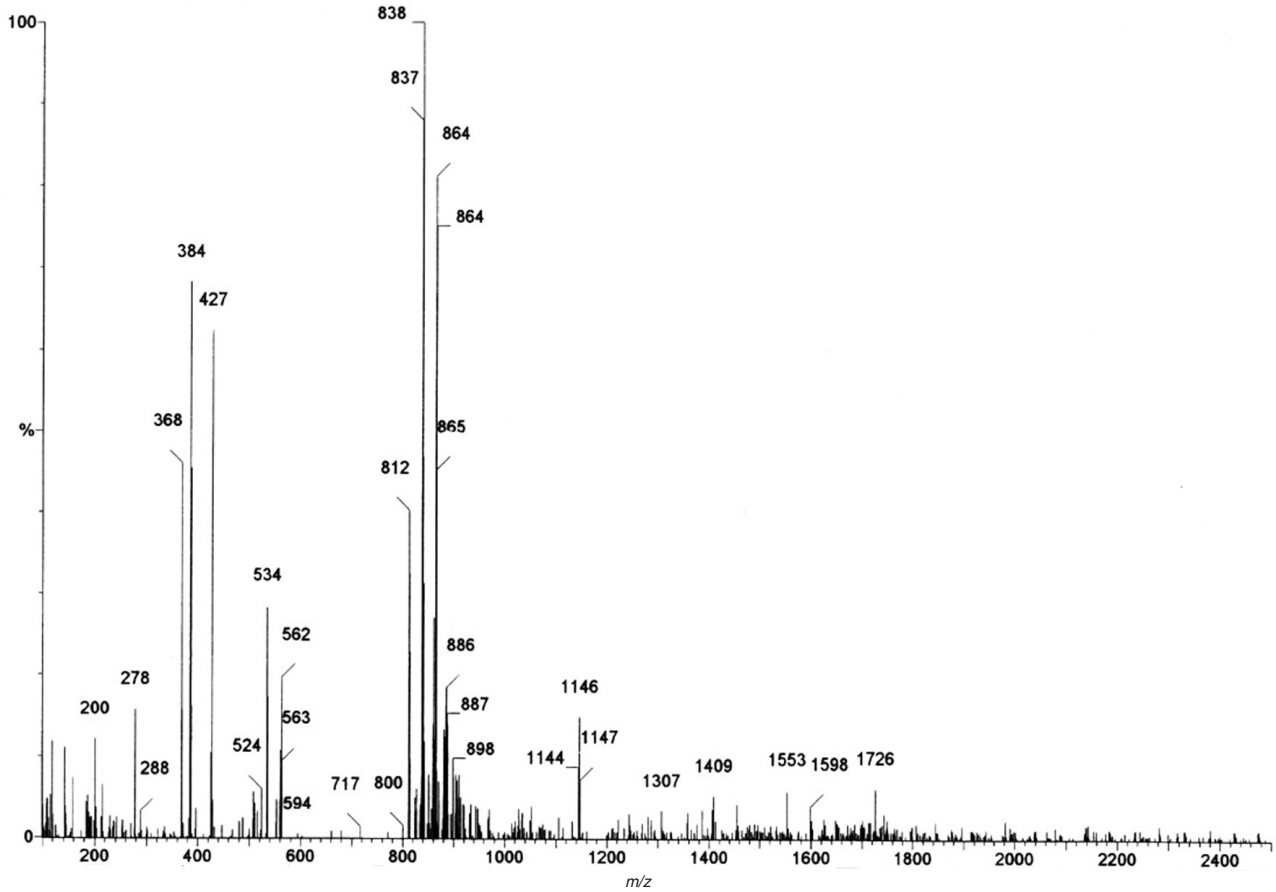


FIG. 4. MS of the estolides fraction E^5 obtained from *Pseudomonas* sp. 42A2 culture grown in mineral medium with technical-grade oleic acid as carbon source. For abbreviation see Figure 3.

5.8–5.0 (complex absorption with two multiplets at δ 5.35 and 5.67 ppm corresponding to CHOHC$\underline{H}_a$=C$\underline{H}_b$ surpassed), 2.35 (*t,* CH_2CO_2R), 2.04 (*m,* CH_2C=C) (Fig. 5). The following ^{13}C NMR signals (δ) were observed: ($CDCl_3$): 179.1 (COOH), 173.2 (COOR), 125.4, 136.0–128.0 (olefinic C clusters), and 70.4 ppm. Basic hydrolysis (0.5 M KOH/EtOH) of the polymer followed by methylation and silylation gave two major peaks, one of which was identified as methyl 10-trimethylsilyloxy-8-octadecenoate by the characteristically intense fragments at *m/z* (%) 271 (64) and 241 (17), corresponding to $[Me_3SiOCHCH{=}CH(CH_2)_6CO_2CH_3]^+$ and $[HC{=}CHCH(OSiMe_3)(CH_2)_7CH_3]^+$, respectively. A fragment of *m/z* 129 (27) may be assigned to $[(CH_2)_5CO_2CH_3]^+$ from allylic cleavage of the molecular ion, and/or to $[CH_2{=}CHCHOSiMe_3]^+$ from further cleavage of ions at *m/z* 241 or 271.

Likewise, when DHOD was used as substrate, a new polymer was obtained from the culture medium. The IR spectrum showed bands at ν 3426, 2926, 1712, 1235, 1155, 1072, and 971 (*trans* alkene) cm^{-1}. The ^1H NMR spectrum (Fig. 6) had the following main features: δ 5.62 (*m,* CHOH–C$\underline{H}$=C$\underline{H}$-CHOH), 5.18 (*m,* CH=CHC$\underline{H}$OCO), 4.10 (C$\underline{H}$OH–CH=CH–C$\underline{H}$OH), and 2.34 (*t,* C$\underline{H}_2$–CO_2R). The ^{13}C NMR showed bands at δ 178.6 (CO_2H), 170.0 (CO_2R), 72.3 (CHOH), and 70.9 (C$\underline{H}$OCO). The poly-DHOD was similarly hydrolyzed (0.5 M KOH/CH_3OH), rendering back the expected monomer, which was characterized by its ^1H NMR spectrum. GC–MS of the silylated methyl ester of the monomer was also identical [main fragments at *m/z* (%) 359 (11), 343 (10), 269 (15), 253 (14), 231(7), and 215 (7)] to that described elsewhere (10), confirming the structure of the DHOD monomer.

In an attempt to understand or explain the behavior of the lipase in the bulk of the culture, we suggest that mixed micelles or supramolecular aggregates are formed in the culture due to the presence of oleic acid, MHOD, and DHOD (which have surfactant properties) in which the lipase may be trapped within the hydrophobic domain of such aggregates.

Enzymatic preparation of estolides. Oleic acid estolide was obtained by the enzymatic action of the lipase obtained from *Pseudomonas* 42A2 culture on pure oleic acid. After purification and methylation, the methylated polyoleic acid showed IR bands at 2929, 1728, 1275, 1123, and 958 cm^{-1}. The presence of the characteristic bands for polyoleic acid in the ^1H NMR spectrum at δ 4.8 ppm, corresponding to the methine proton C$\underline{H}$OCOR, at δ 2.25 of the α-carboxylic protons, the 74.0 ppm of the methine carbon in the ^{13}C NMR, and the two carbonyl carbons at 179.7 ppm of the acid and 173.7 ppm of the estolide, identical to the main features of the

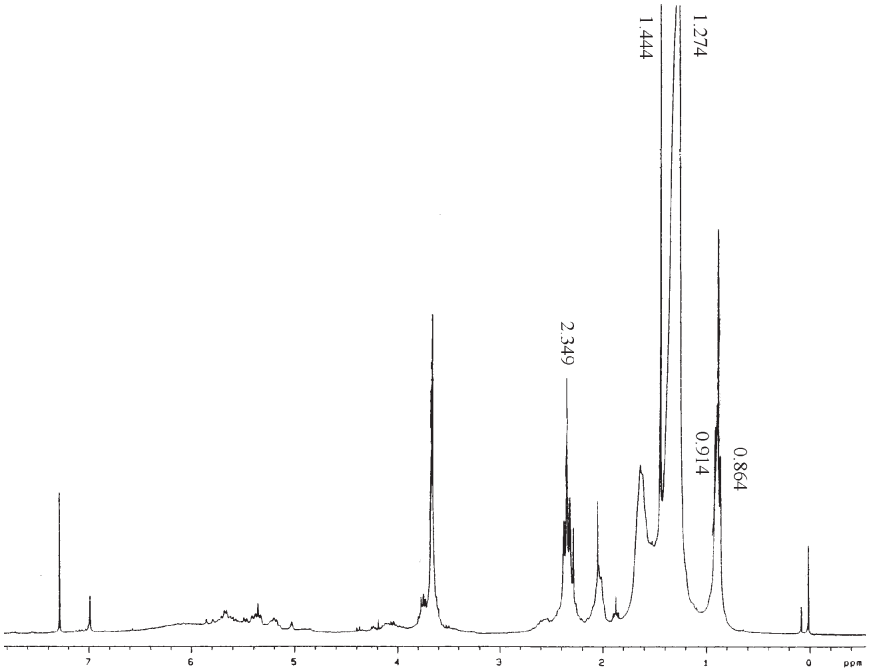


FIG. 5. [1]H NMR spectrum of estolides produced by *Pseudomonas* sp. 42A2 when grown in aerated culture at 30°C in mineral medium with MHOD as carbon source. For abbreviation see Figure 1.

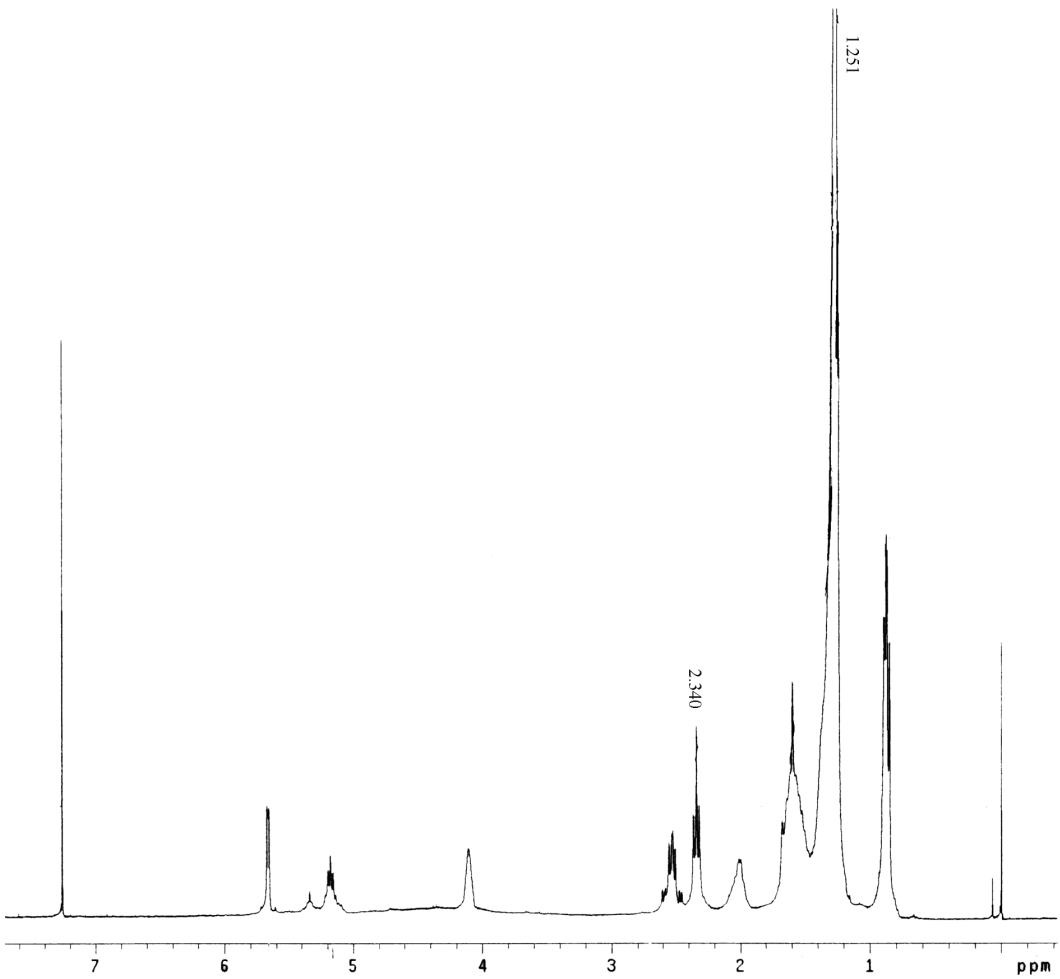


FIG. 6. [1]H NMR spectrum of estolides produced by *Pseudomonas* sp. 42A2 when cultivated in aerated culture at 30°C in mineral medium with DHOD as carbon source. For abbreviation see Figure 1.

polyestolide reported by Isbell *et al*. (18), indicated that our crude lipase catalyzed the polymerization of oleic acid *in vitro*. Other ^{1}H NMR bands appeared at δ 5.34 (*t,* 2H, CH=CH), 4.89 (*t,* CHOCOR), 3.66 (*s,* 3H, CO_2CH_3), 2.4–2.2 (*m,* CH_2COOR), and 2.00 (*m,* $CH_2C=C$), whereas in the ^{13}C NMR, the remaining peaks resonated at δ 174.7, 130.8, 130.0, 129.7, 128.7, 73.1, 71.9, 51.5, 48.2, 47.3, and 42.0 ppm.

From the results presented above it is obvious that estolide formation has occurred. The route of this reaction may lie in the autoxidation of oleic acid in the aqueous and aerated reaction system. These oxidized derivatives of oleic acid are then postulated to form estolides in an enzymatic reaction.

In a similar fashion, enzymatic formation of estolides from ricinoleic acid, linoleic acid, palmitic acid, stearic acid, or dihydroxystearic acid has been described using free lipases from *Candida* and *Pseudomonas* sp. in an aqueous system or reverse micelles (8,17). On the other hand, Yoshida *et al.* (8), when using immobilized lipase in an organic medium, reported a strong inhibition of the enzyme activity due to the presence of water.

With few exceptions, 1,3-positional-specific lipases cannot form estolides because they can rarely utilize secondary alcohols as substrate (18). However, the lipases from *Pseudomonas* species are non-positional-specific and may catalyze esterification reactions using aliphatic acids or lactones in aqueous environments (19).

This is the first report of natural estolides being detected and characterized in a bacterial culture and characterized. Probably owing to the presence of an extracellular lipase, when grown on oleic acid *Pseudomonas* 42A2, induced the formation of several species of estolides. The monomers found in these estolides were: oleic acid, MHOD, and DHOD. When the carbon substrates were MHOD or DHOD, the estolides produced were composed of the unique monomer. The degree of polymerization, up to a hexamer, is the largest reported so far. The precipitated lipase catalyzed the formation of estolide *in vitro*.

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