Conversion of Linoleic Acid to 10-Hydroxy-12(*Z*)-Octadecenoic Acid by *Flavobacterium* sp. (NRRL B-14859)

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A new microbial isolate, *Flavobacterium* sp. strain DS5, converts linoleic acid into 10-hydroxy-12(*Z*)-octadecenoic acid (10-HOA) with 55% yield. The product was characterized by gas chromatography (GC), GC/mass spectrometry, nuclear magnetic resonance and Fourier transform infrared spectroscopy. The specific optical rotation of 10-HOA is $[\alpha]_D^{24} = -5.58$ (methanol). The optimum time, pH and temperature for the production of 10-HOA were 36 h, 7.5 and 20–35°C, respectively. The enzyme(s) that converts linoleic acid to 10-HOA is soluble and located intracellulary in strain DS5. Two minor products, 10-methoxy-12-octadecenoic acid and 10-keto-12-octadecenoic acid, were also identified. 10-HOA was further metabolized by strain DS5. Among the unsaturated fatty acids studied, the order of reactivity for the DS5 enzyme(s) is oleic > palmitoleic > linoleic > linolenic > γ-linolenic > myristoleic acid.

KEY WORDS: Bioconversion, *Flavobacterium*, hydroxy fatty acids, 10-hydroxy-12(*Z*)-octadecenoic acid, linoleic acid, unsaturated fatty acids.

Surplus vegetable oils represent attractive renewable feedstocks for the production of useful chemicals. We are investigating microbial conversion of vegetable oils and their component fatty acids to value-added products. Linoleic acid is the major component of soybean oil and corn oil, representing 54 and 59%, respectively, of the fatty acids present in the triglycerides from these sources.

Microbial conversions of unsaturated fatty acids have been widely exploited, especially at the National Center for Agricultural Utilization Research (Peoria, IL). The literature reveals that oleic acid is converted to ricinoleic acid by a soil bacterium (1) and to 10-hydroxystearic and 10-ketostearic acids by *Rhodococcus rhodochrous (Nocardia aurantia)* (2), *N. cholesterolicum* (3), *Pseudomonas* sp. (4–6), *Mycobacterium fortuitum* (7) and *Staphylococcus* sp. (8). Oleic acid is also converted to 15-, 16- and 17-hydroxy-9-octadecenoic acids by *Bacillus megaterium* (9) and *B. pumilus* (10). Also, oleic acid was converted to a new compound, 7,10-dihydroxy-8(*E*)-octadecenoic acid (11–13), *via* a 10-hydroxy-8(*Z*)-octadecenoic acid intermediate (14,15). In contrast to oleic acid, reports of microbial conversions of linoleic acid are rare. Linoleic acid was converted to lactones by *Sporobolomyces odorus* (16) and to 10-hydroxy-12(*Z*)-octadecenoic acid (10-HOA) by resting cell suspensions of three *Nocardia* strains (17).

For this report, we describe the isolation of a new microbial isolate, *Flavobacterium* sp. DS5, which converted linoleic acid to 10-HOA with 55% yield. 10-HOA is an analogue of ricinoleic acid, an important material used in greases and lubricants and for the production of sebacic acid. The latter is used commercially in the synthesis of lubricants, plasticizers and resins. This is the first report on this type of reaction catalyzed by microorganisms in the genus *Flavobacterium*. The use of growing cultures in this study also differs from the resting cell suspensions used in studies of *Nocardia* (17). Identification of the microorganism and products as well as the optimum conditions for product formation are reported.

MATERIALS AND METHODS

Microorganisms. Microorganisms from soil and water samples were routinely screened for their ability to modify linoleic acid. Each isolate from a single colony on TGY (for information on TGY, see Ref. 18) agar plates was grown at 30°C aerobically in a 125-mL Erlenmeyer flask (shaker at 200 rpm), containing 50 mL of SMD medium, which had the following composition (per liter): dextrose, 10 g; K_2HPO_4, 5 g; yeast extract, 5 g; soybean meal, 5 g; $FeSO_4 \cdot 7H_2O$, 0.5 g; $ZnSO_4$, 0.014 g; $MnSO_4 \cdot H_2O$, 0.008 g; and nicotinic acid, 0.01 g. The pH of the medium was adjusted to 7.0 with dilute phosphoric acid. Cultures were maintained on agar slant with the above mentioned medium except for the addition of 3% agar. Microbial isolates were identified by the Biolog automated bacteria and yeast identification system (Microstation, Hayward, CA).

Chemicals. Linoleic and oleic acids [purity >99% by gas chromatography (GC)] were purchased from Nu-Chek-Prep Inc. (Elysian, MN). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). All solvents used were ACS-grade and were obtained from commercial sources. Kieselgel 60 and thin-layer precoated Kieselgel $60F_{254}$ plates were obtianed from EM Science (Cherry Hill, NJ).

Bioconversion. Bioconversions were carried out by adding 0.3 mL (0.27 g) linoleic acid to a 24-hour-old culture, and the flasks were shaken again at 200 rpm at 30°C for 2 d. At the end of this time, the culture broth was acidified to pH 2 with 6N hydrochloric acid. The culture broth was then extracted twice with an equal volume of diethyl ether. The solvent was removed from the combined extracts with a rotary evaporator.

Isolation of products. Crude extracts that contained reaction products were subjected to high-performance liquid chromatography (HPLC) to isolate pure material for further identification. A Dynamax-60A silica column (25 cm × 21.4 mm i.d.) with methylene chloride/methanol (97:3, vol/vol) as solvent was used with a DuPont Instruments (Wilmington, DE) chromatographic pump equipped with a Waters Model 403 refractive index detector (Milford, MA) and an ISCO Inc. (Lincoln, NE) V^4 variable wavelength detector. Purity of fractions was determined with thin-layer chromatography (TLC) and GC.

Analyses of products. The reaction products were analyzed by TLC and GC as described previously (11,12). Toluene/dioxane/acetic acid (79:14:7, vol/vol/vol) was the TLC solvent system. For GC, the samples were methylated with diazomethane. GC of these methyl esters was carried isothermally at 200°C. For quantitative analyses, palmitic acid was added as internal standard prior to the solvent extraction. A linear relationship was established for the peak area ratios of product vs. methyl palmitate. Experiments were run in triplicate, and the averages of the replicates were reported.

Chemical structure of the product was identified through GC/mass spectrometry (GC/MS), nuclear magnetic resonance (NMR) and Fourier transform infrared (FTIR) spectroscopy. Electron impact mass spectra were obtained with a Hewlett-Packard 5890 GC (Palo Alto, CA) coupled to a Hewlett-Packard 5970 Series mass selective detector. The column outlet was connected directly to the ion source. Separations were effected in a methylsilicone column (15 m × 0.25 mm) with a temperature gradient of 8°C per min from 160 to 250°C after initially holding at 160°C for 3 min. Proton and ^{13}C NMR spectra were determined in deuterated chloroform with a Bruker WM-300 spectrometer (Rheins, Tepten, Germany) operated at frequencies of 300 and 75.5 MHz, respectively. FTIR analyses of both free acid and methyl ester as films on KBr were run on a Perkin-Elmer FTIR Model 1750 spectrometer (Perkin-Elmer, Inc., Oak Brook, IL). Optical rotation measurements were performed with a Perkin-Elmer 241 polarimeter.

RESULTS AND DISCUSSION

Identification of microorganism. Of the many water and soil samples screened, only one culture, DS5, isolated from a dry soil sample collected from Peoria, Illinois, converted linoleic acid to more polar compounds at greater than trace amounts. Strain DS5 is a gram-negative nonmotile rod (0.5 μm × 2 μm). It produces yellowish-brown pigment. Further identification with the Biolog automated bacteria identification system showed that strain DS5 belongs to the genus *Flavobacterium* and has a 50% similarity to the closest species, *gleum*. Therefore, strain DS5 is assigned as *Flavobacterium* sp. DS5 (NRRL B-14859).

Structure determination. The main reaction product purified from HPLC is a colorless oily liquid. It showed a single spot (R_f = 0.38) on TLC and 98.9% purity on GC analysis.

The electron impact spectrum of the methyl ester gave a molecular ion of *m/z* 312. Ions formed from alpha cleavage with respect to the hydroxy group give characteristic fragmentation patterns that provide sufficient information to determine the position of the hydroxy group (19). Large fragments corresponding to alpha cleavage with ions *m/z* 201 and 169 (relative intensity 35 and 100%, respectively) place the hydroxy group at the C-10 position (Fig. 1). This was further confirmed by GC/MS of the trimethylsilyl (TMS)-derivative of the product, which gave the largest fragment at *m/z* 273. Therefore, the product is likely 10-hydroxy-12-octadecenoic acid.

FTIR of the free acid showed absorption of the acid hydroxy group around 2800–3200 cm^{-1} and the alkyl hydroxy group at 3420 cm^{-1}. Upon methylation, the absorption for acid hydroxy group at 2800–3200 cm^{-1} disappeared, but the alkyl hydroxy group at 3420 cm^{-1} remained. As expected, the carbonyl at 1711 cm^{-1} for the acid shifted to 1742 cm^{-1} for the ester. No keto carbonyl was detected. In the absence of a significant absorbance at 970 cm^{-1}, which would be evidence of *trans* double bonds, the unsaturation seen at 3008 cm^{-1} is *cis*.

The reaction product was also analyzed by proton and ^{13}C NMR. Resonance signals (ppm) and corresponding molecular assignments, given in Table 1, further confirmed the identity of the bioconversion product as HOA. The olefinic coupling constant of 10.9 Hz confirmed our infrared data that the unsaturation is in the *cis* configuration. The specific optical rotation value of the product was $[\alpha]_D^{24} = -5.58$ (methanol).

Two minor products, GC retention times 11.1 (2.6% of the main product) and 12.6 min (25% of the main product), were noted. These minor products were further analyzed. GC/MS of the former showed the heaviest mass at *m/z* 324 and the following fragments at *m/z* 215, 183 and 151 (relative intensity 100, 18 and 25%, respectively). The

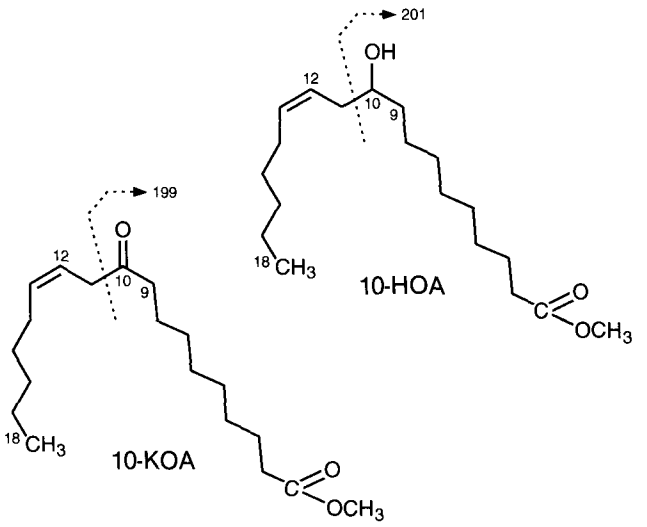

FIG. 1. Microbial conversion products from linoleic acid by strain DS5; 10-HOA, 10-hydroxy-12(Z)-octadecenoic acid, 10-KOA, 10-keto-12(Z)-octadecenoic acid.

TABLE 1

Proton and ^{13}C Nuclear Magnetic Resonance Signals and Molecular Assignments for Bioconversion Product

Type	Resonance chemical shifts (ppm)/coupling (Hz)	Carbon number
Proton	2.30 *t* (2.3 = 7.4)[a]	2
	1.59 *m*	3
	1.27 *m*	4–8, 15–17
	1.43 *m*	9
	3.60 *m*	10
	2.19 *m*	11
	5.38 *m* (12,13 = 10.9)[a]	12
	5.53 *m*	13
	2.01 *m*	14
	0.85 *t* (17,18 = 6.9)[a]	18
^{13}C	179.4	1
	34.0	2
	24.6	3
	28.9–29.5	4–7,15
	25.6	8
	36.6	9
	71.6	10
	35.1	11
	125.0	12
	133.4	13
	27.3	14
	31.5	16
	22.5	17
	14.0	18

[a]Coupling constant (*J* in Hz).

methyl ester did not form a TMS derivative. These characteristic fragments are identical to those reported for methyl 10-methoxy-12-octadecenoate (20). From literature (2,3,21,22) on the mechanism of microbial hydroxylation of unsaturated fatty acids, both hydroxy and keto acids might be expected. GC retention times of methyl 10-hydroxystearate and 10-ketostearate are 14.3 and 13.3 min, respectively. Therefore, the later compound [(retention time (RT) 12.6 min] might be the equivalent keto analog of methyl 10-hydroxy-12-octadecenoate (RT 13.7 min). GC/MS analyses of the compound (RT 12.6 min) showed the heaviest mass at *m/z* 310 and a large fragment at *m/z* 199 (relative intensity 100%). These data suggest that compound RT 12.6 min is 10-keto-12-octadecenoic acid (10-KOA).

To develop a production process, conditions were studied for the bioconversion of linoleic acid to 10-HOA.

Effect of media. The production of 10-HOA by strain DS5 was compared on four media: SMD, TGY, YM (18) and screening medium (12). Strain DS5 grows on all of these media; however, cells grown on SMD medium produced the highest bioconversion product. Relative activity on media SMD, TGY, YM and screening media for the production of 10-HOA by strain DS5 were 100, 81, 77 and 0%, respectively. Therefore, the optimum conditions for the production of 10-HOA from linoleic acid were studied further with SMD medium.

Time course. The reaction was carried out at 30°C for the time specified. The amount of product 10-HOA in the culture media increased with time and reached a maximum 36 h after addition of substrate to one-day-old culture (Fig. 2). Keto acid accumulation reached maximum at about the same time. Further incubation reduced 10-HOA and 10-KOA contents in the medium, which indicates that strain DS5 metabolizes both 10-HOA and 10-KOA. Therefore, production of 10-HOA was standardized at 36 h whenever other variables were evaluated.

Effect of pH. The effect of pH on the production of 10-HOA was studied with one-day-old cells of strain DS5 grown on a pH 7.0 medium. Immediately before adding reaction substrate (linoleic acid), the culture medium was adjusted to the desired pH (from 5.5 to 9.0) with either 2N NaOH or 3N HCl. At harvest, each culture medium had become more acidic by 0.3 pH units. The maximum yield of 10-HOA occurred at pH 7.5 (Fig. 3).

Effect of substrate concentration. Various amounts (0.1–0.8 mL) of linoleic acid were added to one-day-old cells of strain DS5 grown on pH 7.5 medium. The production of 10-HOA was assayed after 36 h of reaction. The amount of 10-HOA produced was dependent on the amount of substrate initially added. A linoleic acid concentration of 0.3 mL per 50 mL reaction medium supported maximum 10-HOA production.

Effect of temperature. Cells were grown at 30°C for 1 d, and 0.3 mL of linoleic acid was added. Immediately after the addition of substrate, the flasks were incubated

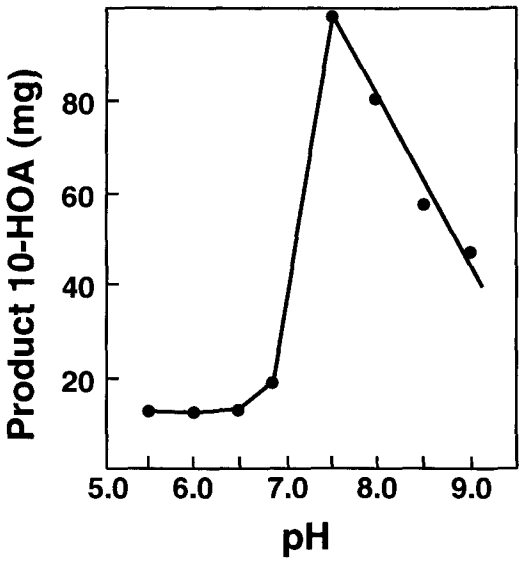

FIG. 3. Effect of pH on the production of 10-HOA from linoleic acid by strain DS5. See Figure 1 for abbreviation.

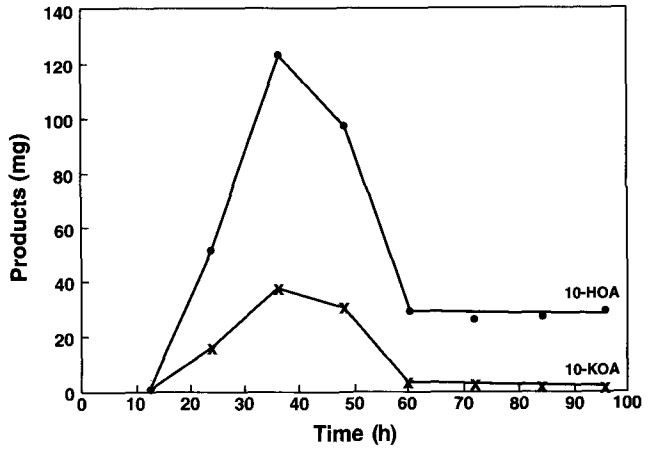

FIG. 2. Time course of the production of 10-HOA and 10-KOA from linoleic acid by strain DS5: 10-HOA (● — ● — ● — ●); 10-KOA (— X — X — X —). See Figure 1 for abbreviations.

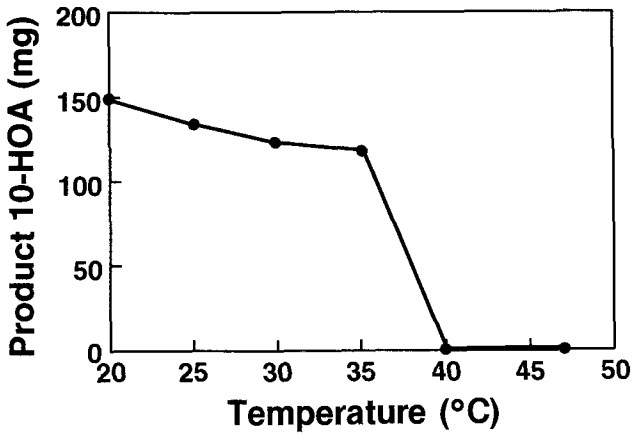

FIG. 4. Effect of temperature on the production of 10-HOA from linoleic acid by strain DS5. See Figure 1 for abbreviation.

at the desired temperatures at 200 rpm for 36 h. At the end of the incubation, the amount of 10-HOA in the culture broth was analyzed. It was to our surprise that the optimum temperature for the production of 10-HOA was 20–35°C (Fig. 4). The maximum yield calculated was 55%.

Nature of the enzyme(s). To determine whether the linoleic acid conversion enzyme(s) is either intra- or extracellular, strain DS5 was grown for 1 d and centrifuged at 8,000 rpm for 20 min to separate cells and supernatant. The cell pellet was washed twice with a 0.05 M potassium phosphate buffer of pH 7.0 and then resuspended in a small amount of the same buffer. The supernatant was filtered through a microfiltration (0.2 μ) unit to remove any remaining cells. Substrate linoleic acid was added to both the cell suspension and the cell-free supernatant, and the mixtures were incubated at 30°C for 18 h to study the bioconversion. Data obtained showed that only the cell suspension of DS5 converted linoleic acid to 10-HOA. Enzyme(s) activity was not found in the cell-free culture medium. Thus, the enzyme(s) is cell-bound. With the resting cells' suspension, the ratio of products, 10-HOA/10-KOA, was 97:3 (Table 2). Less 10-KOA was produced in comparison with that of the growing cells (Fig. 2). The cells in the cell suspension were disrupted with ultrasonic oscillation and centrifuged at 8,000 rpm for 60 min to separate into cell debris and cell-free crude extract. The linoleic acid converting enzyme(s) resided in the cell-free crude extract, and only 10-HOA (Table 2) was produced from linoleic acid. Therefore, the linoleic acid-converting enzyme(s) is soluble in nature in strain DS5.

Substrate specificity. Possible substrates were tested for bioconversion by incubation with the resting cell suspension of strain DS5 in 0.05M potassium phosphate buffer of pH 7.0 at 30°C, 200 rpm for 18 h. The bioconversion activity was measured by the formation of products detectable by GC and TLC. The products from oleic acid were purified and then identified with GC/MS and NMR as 10-ketostearic acid and 10-hydroxystearic acid (Hou, C.T., submitted for publication). Heat-killed cells produced no products from the various substrates tested. Activities, expressed relative to that against linoleic acid, are listed in Table 3. Strain DS5 oxidized unsaturated but not saturated fatty acids. The relative activities were in the following order: oleic > palmitoleic > linoleic > linolenic > γ-linolenic > myristoleic acid. The exact chemical structures of these products as well as their industrial applications are currently under investigation.

TABLE 2

Location of Linoleic Acid Conversion Enzyme(s) in Strain DS5

Fraction	Total products formed (mg)[a]	Ratio	
		10-HOA[b]	10-KOA[c]
Resting cell suspension	40	97	3
Cell-free culture medium	0	0	0
Cell-free crude extract	27	100	0

[a]Products formed from 90 mg linoleic acid in 5 mL reaction mixture in 18 h.
[b]10-Hydroxy-12(Z)-ocatadecenoic acid.
[c]10-Keto-12-octadecenoic acid.

TABLE 3

Substrate Specificity of Linoleic Acid Conversion Enzyme(s) in Strain DS5

Substrate	Gas chromatography retention time (min)		Relative activity (%)[a]
	Substrate	Products	
Myristoleic $C_{14:1}$	2.70	3.80 4.08	48
Palmitoleic $C_{16:1}$	4.21	6.85 7.31	161.8
Oleic acid	7.25	13.3 14.3	289.5
Petroselinic acid	7.24	12.50	0.9
Linoleic acid	6.92	12.6 13.7	100
Linolenic acid	6.99	11.84 12.61	97.2
γ-Linolenic acid	6.58	11.29	89.9
Elaidic acid	7.30	—	0
Palmitic acid	4.50	—	0
Stearic acid	7.81	—	0

[a]Relative activity is by comparison of the sum of these two products against that from linoleic substrate.

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