Conversion of Oleic Acid to 10-Ketostearic Acid by *Sphingobacterium* sp. Strain O22

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ABSTRACT: The conversion of oleic acid by a bacterium, tentatively identified as *Sphingobacterium thalpophilum* strain O22, was investigated. The microorganism was isolated as a stable culture from compost that was enriched with soybean oil outdoors and subsequently with oleic acid in the laboratory. Strain O22 converted oleic acid to products identified as 10-ketostearic acid (95% of the total conversion product) and 10-hydroxystearic acid (5%). This is in contrast to *S. thalpophilum* strain B-14797, which produces solely 10-hydroxystearic acid. Maximal conversion was reached in about 36 h after the addition of oleic acid to the fermentation broth. The yield of 10-ketostearic acid was approximately 75% from 0.26 g of oleic acid in 30 mL fermentation broth at 28°C and 200 rpm for 48 h. This is the first report on the major production of 10-ketostearic acid by a microorganism in the genus *Sphingobacterium*.

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KEY WORDS: Bioconversion, enrichment culture, 10-ketostearic acid, oleic acid, *Sphingobacterium thalpophilum*.

Hydroxy- and keto-fatty acids are useful industrial chemicals used in plasticizer, surfactant, lubricant, and detergent formulation. They are often manufactured from fats and oils by chemical reactions. Some of these products may be produced by enzymes or whole microbial cells for greater specificity and reduced environmental concerns.

Oleic acid is one of the major fatty acid components of corn and soybean oils. Microbial conversion of oleic acid to form value-added industrial products has been widely exploited. Among the bioconversions of oleic acid to monohydroxy- or keto-fatty acids, Wallen *et al*. (1) first reported that a *Pseudomonas* sp. converted oleic acid to 10-hydroxystearic acid (10-HSA) with a 14% yield. Similar bioconversions were subsequently found with *Rhodococcus rhodochrous* (2), *Nocardia cholesterolicum* (3), *Micrococcus luteus* and *Sarcina lutea* (4), and by *Sphingobacterium thalpophilum* NRRL B-14797 and *Staphylococcus* sp. NRRL B-14813 (5). Oleic acid is converted to 15-,16-, and 17-hydroxy-9-octadecenoic acid by *Bacillus megaterium* (6) and *B. pumilus* (7). It is also converted to hydroxy- (8,9) and hydroperoxy-octadecenoic acid (9) by *Pseudomonas* species. However, 10-ketostearic acid (10-KSA) is a major product of oleic acid bioconversion by a number of biological systems including a *Corynebacterium* sp. (10), one *Mycobacterium* and two *Nocardia* (11), a *Staphylococcus* sp. (12), and *Flavobacterium* sp. strain DS5 (13).

In our laboratory, we applied an enrichment culture technique to select stable microbial isolates able to effectively convert soybean oil and fatty acids to value-added products. The technique involved the use of composted plant and animal wastes that were mixed and enriched with soybean oil and subsequently with oleic acid to enhance the propagation of certain microbial species. In the study, we discovered that several isolates of *Sphingobacterium* sp. were able to convert oleic acid predominantly to 10-KSA. In this paper, we describe the isolation and identification of a specific strain, O22, and partial characterization of the bioconversion reaction including the structure determination of products. This is the first report on the predominant production of 10-KSA by a microorganism in the genus *Sphingobacterium*.

EXPERIMENTAL PROCEDURES

Isolation of microorganism. Microorganisms were isolated from an aerobic compost after subculturing in an enrichment medium (EM) and selection plates. The compost consisted of a loose top soil, horse manure, and decaying garden leaves–lawn grass mixture. Soybean oil (300 mL) was mixed onto the top layer of this outdoor compost heap at regular intervals, and samples for enrichment culture were taken from the top layer after 3, 10, and 12 mon. The compost sample (1.5–2.0 g) and oleic acid (0.5 mL) were added to 100 mL of the enrichment medium (pH 7.3), which contained (per liter) 5 g glucose, 0.3 g yeast extract, 4.0 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 15 mg $FeSO_4 \cdot 7H_2O$, and 1 mL trace minerals (5). The inoculated broths were incubated aerobically overnight on a Gump rotary shaker (orbital radius, 5.08 cm; New Brunswick Scientific, Edison, NJ) at 200 rpm, 28°C, and readjusted to pH ≥ 7.0 with dilute NaOH. After the fourth day, this regimen was repeated once or twice in fresh oleic acid-containing EM (2% inoculum) before dilution-plating onto a selective agar medium. The plates contained EM nutrient with (per liter) 17 g agar, 25 mg bromo-cresol green indicator, and 2.0 mL oleic acid (5). Discrete colonies were randomly selected to test their bioreactivity. All cultures were maintained on tryptone-glucose-yeast extract (TGY) (14)

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agar slants and stored at 4°C. Identity of the bioreactive cultures was established by Biolog System analysis using either a Gram-negative or Gram-positive data base (Biolog Inc., Heyward, CA).

Chemicals. Oleic acid (99+% purity) was purchased from Nu-Chek-Prep, Inc. (Elysian, MN). 10-KSA used as a GC standard was obtained from a previous study (12). All other chemicals were reagent-grade and used without further purification. Thin-layer precoated Silica Gel 60 plates were obtained from EM Separations Technology (Gibbstown, NJ).

Bioconversion assay. Cultures maintained on TGY slants were transferred to fresh TGY broth (pH 7.0) and incubated aerobically for 1-2 d before transfer (0.3 mL inoculum) into 30-mL assay broths in 125 mL Erlenmeyer flasks. The assay broths (pH 7.3) contained (per liter) 5.0 g yeast extract, 4.0 g glucose, 4.0 g K_2HPO_4, 500 mg $MgSO_4 \cdot 7H_2O$, and 15 mg $FeSO_4 \cdot 7H_2O$ (1,15). Inoculated broths were shaken overnight for 20 h at 200 rpm and 28°C and checked for pH ≥ 7.0 before adding 0.3 mL oleic acid (0.26 g). The conversion reaction of oleic acid by strain O22 was allowed for an additional 2–3 d under the same conditions. Lipids were recovered from the acidified broth by extracting twice with an equal volume of methanol/ethyl acetate (1:9; v/v) (11,15). The solvent was then removed from the combined extracts with a rotary evaporator. The concentrated lipid extracts were transferred to one-dram vials with methanol/chloroform (1:3; vol/vol) and ether and dried in an evacuated desiccator before weighing to obtain total lipid extracts. A portion of the extract was also dried immediately under a nitrogen stream for further analysis.

Analytical procedures. Bioconversion was monitored by gas chromatography (GC) and thin-layer chromatography (TLC). Lipid extracts were esterified with diazomethane. The methyl esters were injected into an HP (Hewlett Packard; Palo Alto, CA) model 5890 Series II gas chromatograph, equipped with a Supelco (Bellefonte, PA) SPB-1 capillary column (15 m × 0.32 mm, 0.25 μm film thickness), a flame-ionization detector and an HP 7673 autosampler, and HP ChemStation software was used for data acquisition and integration. The GC conditions were set up as described previously (15). For quantitative analysis, palmitic acid was added as an internal standard prior to solvent extraction. A linear relationship was established for the peak area ratios of products vs. methyl palmitate.

TLC analyses were carried out on Silica Gel 60 (0.25 mm thickness) plates (EM Science), developed in chloroform/methanol/acetic acid (9:1:0.1, vol/vol/vol). The chromatograms were visualized first with sulfuric acid spray, followed by charring with a heat gun, and then with vanillin/sulfuric acid spray, followed by brief heating (16).

Isolation and identification of products. The bioconversion product was isolated by high-performance liquid chromatography (HPLC). HPLC was performed with a Spectra-Physics (San Jose, CA) SP8800 solvent delivery system and a Dynamax 60-A silica column (25 cm × 21.4 mm i.d.; Rainin Instrument, Woburn, MA) as described previously (12). The chemical structure of the purified product was confirmed by GC–mass spectrometry (GC–MS) and by nuclear magnetic resonance (NMR) and Fourier transform infrared (FTIR) spectroscopy. Electron impact mass spectra were obtained with a Hewlett-Packard (Palo Alto, CA) 5890 GC coupled to a Hewlett-Packard 5970 Series Mass Selective Dectector. The column outlet was connected directly to the ion source. Separations were effected in a methylsilicone capillary column (15 m × 0.25 mm i.d.) with a temperature gradient of 20°C/min to 200°C and then 10°C/min to 270°C after initially holding at 120°C for 2 min. Proton and ^{13}C NMR spectra were determined in deuterated chloroform with a Bruker WM-300 spectrometer (Rheims Co., Tepten, Germany) operated at frequencies of 300 and 75.5 MHz, respectively. FTIR analyses of the methyl ester of the product were done on a Perkin-Elmer (Oakbrook, IL) FTIR spectrum RX II system.

RESULTS AND DISCUSSION

Enrichment culture selection. Commercial composted manure of unknown origin yielded *S. thalpophilum* (NRRL B-14797) and *Acinetobacter* (B-14920, B-14921, B-14923) cultures that converted oleic acid to 10-HSA and oleyl wax esters, respectively (5,17). In our present study, a compost mixture amended with soybean oil yielded similar microorganisms after enrichment with oleic acid in the laboratory. Nine of 74 randomly selected colonies from the agar plates gave positive bioconversions to a mixture of 10-KSA and 10-HSA identified by TLC and GC. Strain O22 was categorized by Biolog System analysis to be *S. thalpophilum.* The strain was chosen for further characterization because it was stable during maintenance, a fast grower, and a consistent 10-KSA producer.

Structure determination. 10-KSA isolated by HPLC was homogeneous on GC (data not shown). Its chemical structure was confirmed by FTIR, GC–MS, and NMR analyses. FTIR spectroscopy of the methyl ester showed carbonyl bond absorption at 1704 and at 1735 cm^{-1}, indicating the presence of saturated, acyclic ketone and ester groups, respectively (18). GC–MS analyses were performed both in electron impact ionization (EI) and chemical ionization (CI) (methane and isobutane) modes. Methane CI provided evidence for molecular weight of 312 at *m/z* 281 ($MH^+ - 32$, base peak), 313 (MH^+), 341 (M + 29) and 353 (M + 41). Isobutane CI confirmed the proposed molecular weight with a base peak at *m/z* 313 (MH^+) and a small ion at *m/z* 281. EI showed fragmentation ions consistent with cleavage on each side of the carbonyl ($C_{9,10}$, *m/z* 141 and 171; $C_{10,11}$, *m/z* 113 and 199) (Fig. 1). The two even ions (*m/z* 156 and 214) were from β-cleavage with respect to the oxo-group in the chain, a shifting of the double bond, and abstraction of a hydrogen atom (Fig. 1) (19). These fragments determined the position of the oxo-group to be at C-10. Proton and ^{13}C NMR further confirmed the identity of the main bioconversion product as being 10-KSA. Resonance signals (ppm) and the corresponding molecular assignments are given in Table 1.

One minor product of the bioconversion reaction (approximately 5% of 10-KSA) having a GC retention time of 7.67 min,

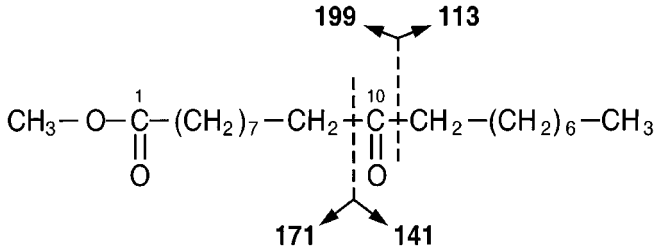

FIG. 1. Methyl ester and major mass spectral fragments of the conversion product (10-ketostearic acid) from oleic acid by *Sphingobacterium* sp. strain O22.

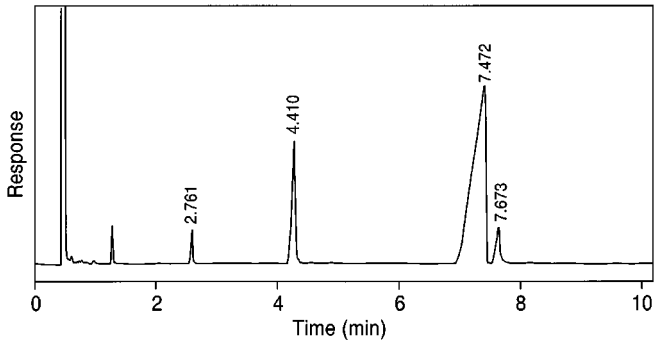

FIG. 2. Gas chromatograms of methyl esters recovered after the conversion of oleic acid by *Sphingobacterium* sp. strain O22. Peak with retention time (RT) 2.76 min is internal standard; RT of 4.41 min is oleic acid; RT of 7.47 min is 10-ketostearic acid; RT of 7.67 min is 10-hydroxystearic acid.

as opposed to the 7.47 min of 10-KSA, 4.41 min of substrate oleic acid, and 2.76 min of internal standard palmitic acid, was also present (Fig. 2). GC–MS analysis of the methyl ester of this minor product showed its molecular weight to be 314 with a base peak at *m/z* 315 (MH^+) and with prominent fragment ions at *m/z* 201 (M – 113; $C_{10,11}$ cleavage), 169 (201 – 32) and 143 (M – 171; $C_{9,10}$ cleavage). The data agree with those for 10-HSA, determined previously (13,20). Therefore, *S. thalpophilum* strain O22, much like a *Staphylococcus* sp. (12) and *Flavobacterium* sp. strain DS5 (13) discovered previously, produces mainly 10-KSA from oleic acid. Similar bioconversion also occurs with *Corynebacterium* (10), *Mycobacterium,* and *Nocardia* strains (11). However, 10-HSA is the main product in the bioconversion of oleic acid by *Pseudomonas* (1), *Rhodococcus rhodochrous* (2), and *Nocardia* species (3).

Bioconversion by strain O22. The maximal growth of strain O22 was reached at about 24 h after inoculation in 30 mL of culture medium. Therefore, the conversion reaction was generally initiated by adding oleic acid substrate to the fermentation broth after 20–24 h of strain O22 cell growth. A time course study on the production of 10-KSA from oleic acid by strain O22 indicated that the product increased linearly up to 24 h of reaction and reached its maximal level of about 200 mg per 30 mL of media in about 36 h (Fig. 3). The maximal yield of 10-KSA production generally was obtained in the range of 70–80%. The same product level was maintained as the reaction time was extended to 4 d. This is indicative that 10-KSA produced in the culture was not further metabolized. The characteristic was also observed previously for the bioconversion as catalyzed by a *Staphylococcus* sp. (12) and *Flavobacterium* sp. strain DS5 (13).

The ability of microorganisms to convert oleic acid to either 10-KSA or 10-HSA as a main product may strongly be related to the cellular regulation of both hydratase and secondary alcohol dehydrogenase activities (13). In *Nocardia* NRRL 5767, the product ratio of 10-KSA/10-HSA is influenced by different reaction conditions (3). Therefore, we compared the conversion reaction of strain O22 with the previously characterized *S. thalpophilum*, B-14797, and *Nocardia* NRRL 5767 under the same conditions. The results, as shown in Table 2, agree well with the previous report for

TABLE 1
Proton and ^{13}C NMR Signals and Molecular Assignments for the Methylated Bioconversion Product[a]

NMR type	Signal (ppm)	Hydrogen	Assignment
Proton	3.65	3	CH_3, methyl ester
	2.27–2.38	6	CH_2, α to carbonyls
	1.54–1.58	6	CH_2, β to carbonyls
	1.22–1.27	18	CH_2, C4–7, C13–17
	0.85–0.88	3	CH_3, terminal
^{13}C	211.56		C10
	174.21		C1
	51.36		C, methyl ester
	42.70–42.78		C9,11
	34.02		C2
	31.76		C16
	29.00–29.31		C4–7, C13–15
	24.85		C3
	23.77–23.84		C8,12
	22.57		C17
	14.00		C18

[a]NMR, nuclear magnetic resonance.

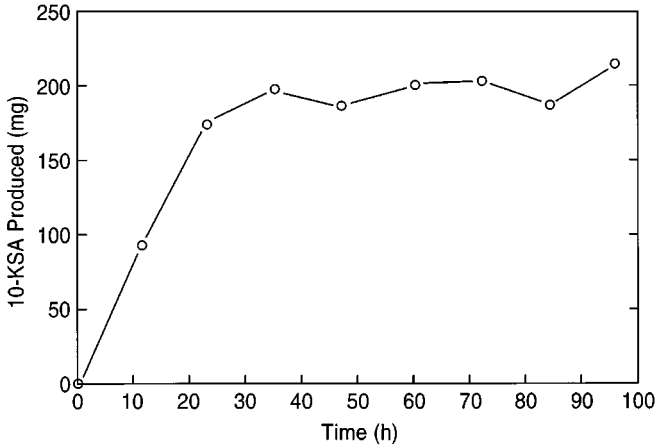

FIG. 3. Time course for the production of 10-ketostearic acid (10-KSA) from oleic acid by *Sphingobacterium* sp. strain O22. Reaction conditions: substrate oleic acid (0.3 mL) was added to a 24-h-old culture in 30-mL fermentation broths at 28°C and 200 rpm.

TABLE 2
Comparison of Oleic Acid Conversion by *Sphingobacterium thalpophilum* Strain O22 and Strain B-14797, and *Nocardia* NRRL 5767[a]

Microbe	10-KSA[b] (mg)	10-HSA (mg)	Total product (mg)	Total lipid[c] (mg)
Sphingobacterium				
Strain O22	185	10	195	238
Strain B-14797	0	180	180	236
Nocardia				
NRRL 5767	13	123	136	165

[a]Oleic acid (0.26 g) was added to 24-h-old microbial culture in 30-mL fermentation broth, and the bioconversion was allowed to proceed for 48 h at 28°C and 200 rpm prior to lipid extraction. (See Experimental Procedures section). Each figure is the average of duplicate analyses.
[b]Abbreviations: 10-KSA, 10-ketostearic acid; 10-HSA, 10- hydroxystearic acid.
[c]Total lipid included 10-KSA, 10-HSA, oleic acid (substrate), and palmitic acid (internal standard), as identified by gas chromatography.

NRRL 5767 (3) and confirm the observation that *S. thalpophilum* strain B-14797 is a sole 10-HSA producer (20). The result also demonstrates for the first time that a *S. thalpophilum* strain O22 converts oleic acid predominantly to 10-KSA. Hydroxy- and keto-fatty acids are useful industrial chemicals in the plasticizer, lubricant, and detergent formulation. Investigations are in progress to optimize the culture and reaction conditions for an increased production of 10-KSA by strain O22, as well as 10-HSA by strain B-14797.

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