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Production of a new compound, 7,10-dihydroxy-8-(E)-octadecenoic acid from oleic acid by *Pseudomonas* sp. PR3*

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SUMMARY

Sixty-two cultures from the ARS Culture Collection and 10 cultures isolated from soil and water samples collected in Illinois were screened for their ability to convert agricultural oils to value-added industrial chemicals. A new compound, 7,10-dihydroxy-8-(E)-octadecenoic acid (DOD) was produced from oleic acid by a new strain, *Pseudomonas* sp. PR3 isolated from a water sample in Morton, IL. Strain PR3 is a motile, small rod-shaped, Gram-negative bacterium. It has multiple polar flagellae and is oxidase-positive. Strain PR3 grows aerobically and cannot grow anaerobically. The strain produces white, smooth colonies on agar plate and no water-soluble pigment. The yield of the product was greater than 60%. The optimum time, pH and temperature for the production of DOD were: 2 days, 7.0, and 30 °C, respectively. Glycerol and dextrose support the growth of strain PR3, but the cells grown from the former failed to catalyse the conversion of oleic acid to DOD. The production of DOD is unique in that it involves a hydroxylation at two positions and a rearrangement of the double bond of the substrate molecule.

INTRODUCTION

Vegetable oils, particularly soybean oil, are in great surplus. It is important to convert these surplus oils into new value-added products [1]. Surprisingly, most microbial R & D activities on hydrophobic compounds have concentrated on hydrocarbon substrates and not on agricultural oils. Hou [5] reviewed microbial transformations of important industrial hydrophobic compounds including aliphatic and aromatic hydrocarbons. Several microbial/enzymic approaches for modifying agricultural oils or fats have been reported in the recent years, including commercial application of lipases for fat splitting [2]. *Nocardia corallina*, *Pseudomonas* sp. and *Puccinia graminis* are known to biosynthesize epoxy groups [3,11,12]. Oleic acid serves as substrate for *Pseudomonas* sp. and *P. graminis* to form 9,10-epoxy stearic acid [11,12]. The cytochrome p_{450} system from *Bacillus megatherium* appears to serve as a common enzyme for both epoxidation and hydroxylation of a variety of mono-unsaturated fatty acids [13]. Marsh and James [9] showed that yeast biosynthesizes hydroxy stearic acid from stearic acid. Microbial conversion of oleic acid to 10-hydroxystearic acid was first reported by Wallen et al. in our laboratories [16]. They found that a pseudomonad isolated from a fatty material hydrated oleic acid at the double bond with a 14% yield. In a patent disclosure, *Rhodococcus rhodochrous* was reported to convert oleic acid to 10-hydroxystearate and minor amounts of 10-*keto*-stearic acid [8]. Recently, olive oil was converted to a new surfactant, an unsaturated hydroxy fatty acid [10]. We have identified several microorganisms that hydrate oleic acid to 10-hydroxystearic acid at greater than 90% yield [7].

In our continuing screening program for new industrial chemicals from vegetable oils, we have discovered that a new bacterial strain PR3, isolated from a water sample taken at a pig farm in Morton, IL, converts oleic acid to a new compound, 7,10-dihydroxy-8-(E)-octadecenoic acid (DOD). This paper describes the identification of strain PR3, the production and structure determination of this new compound.

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MATERIALS AND METHODS

Microorganisms

Sixty-two microbial cultures (23 bacteria, 12 fungi, 14 actinomycetes and 13 yeasts) from the ARS Culture Collection and 10 cultures from soil and water samples in Illinois were screened for their ability to modify oleic acid. Each culture was grown at 30 °C aerobically in a 125-ml Erlenmeyer flask (shaker at 150 rpm) containing 30 ml of medium with the following composition (per liter): Dextrose, 4 g; $(NH_4)_2HPO_4$, 10 g; yeast extract, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; $MnSO_4 \cdot H_2O$, 0.008 g; $ZnSO_4 \cdot 7H_2O$, 0.014 g; and nicotinic acid, 0.1 g. The pH of the medium was adjusted to 7.0 with dilute phosphoric acid. Flagella stain was performed with modified Ryu reagent [4]. Oxidase activity was assayed with the method of Steel [15].

Chemicals

All chemicals were reagent grade and were used without further purification. Thin-layer precoated Kieselgel 60 F_{254} plates were obtained from EM Science (Cherry Hill, NJ).

Bioconversion reaction

Oleic acid (0.4 ml) was added to a 36- to 48-h-old culture in the above medium and the flasks were shaken at 150 rpm at 30 °C for 2 days. At the end of the reaction, the culture broth was acidified to pH 2 with 6N hydrochloric acid. Then, the culture broth was extracted with an equal volume of ethyl acetate and with an equal volume of diethyl ether. The solvent was removed from the combined extracts with a rotary evaporator.

Analysis of products

The isolated reaction products were analysed by thin-layer chromatography (TLC) and gas-liquid chromatography (GC). TLC was developed with toluene : dioxane : acetic acid = 79 : 14 : 7 (v/v). The chromatograms were visualized first by iodine vapor and then by spraying the plate with a 50% sulfuric acid and heating in a 100 °C oven for 10 min. For GC, the samples were methylated with diazomethane. The methyl esters, dissolved in diethyl ether, were injected into a Hewlett Packard model 5890 gas chromatograph equipped with flame ionization detector, a Supelco SPB-1™ capillary column 15 m, i.d. 0.32 mm, 0.25 μm thickness and a Hewlett Parkard 3392A integrator. GC was run isothermally at 200 °C. For quantitative analysis, palmitic acid was added as an internal standard prior to the solvent extraction. A linear relationship was established on the peak area ratios of product vs. methyl palmitate.

Analytical methods

Infrared (IR) spectra of the free acid were obtained as KBr discs with a Mattson Polaris IR Spectrometer. Electron impact mass spectra were obtained with a Hewlett-Packard 5890 GC coupled to a Hewlett-Packard 5970 Series Mass Selective Detector. Chemical ionization mass spectra were obtained on a Finnigan TSQ mass spectrometer. Details of GC-MS analytic methods were reported previously [6]. Proton nuclear magnetic resonance spectra were determined in deuterated chloroform with a Bruker WM-300 spectrometer operating at a frequency of 300 MHz.

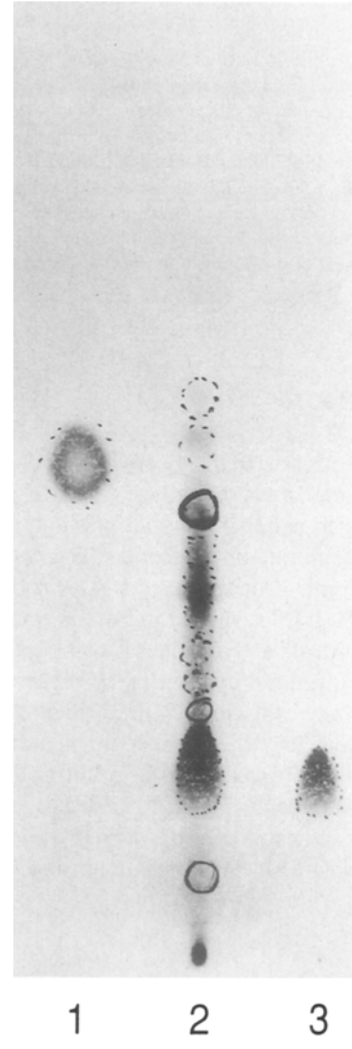

Fig. 1. Thin-layer chromatography of DOD. Solvent system = toluene : dioxane : acetic acid = 79 : 14 : 7 (v/v). Spots with dotted line were visualized with iodine vapor. 1. Oleic acid. 2. 48-h reaction products. 3. Purified DOD.

Purification of products

For larger scale production, the bioconversion of oleic acid (0.8 g) was carried out as described above but in a 3.8-l Fernbach flask containing 1 l of medium. The reaction products were extracted with solvents as described previously and purified with solvent partitioning and silica gel column chromatography. Solvent was evaporated in vacuo from the combined extracts, and the residue was partitioned between hexane : acetonitrile (30 ml : 30 ml). White material which appeared at the interface of these solvents was carefully collected and then washed twice with hexane. The nitrogen stream-dried white material (95 mg) showed one spot by TLC (Fig. 1, Rf 0.34). GC showed a major peak (97% of the total area) at 19.93 min. The acetonitrile fraction containing the major product was washed once with fresh hexane and then dried with a rotary evaporator. The solid residue was dissolved in diethyl ether and separated on a silica gel G column (35 × 2.2 cm i.d.) which was pre-equilibrated with diethyl ether. The column was washed with 200 ml ether and then eluted with ether : methanol (70 : 30 v/v). 5-ml portions were collected and assayed with TLC. Fraction numbers 45 to 55 contained the major product. These fractions were combined and evaporated to dryness. The solid residue was washed with hexane and then dried under a nitrogen stream to obtain 250 mg white powder. TLC and GC analyses of this white powder showed that it was identical to that collected from the interface above. The melting point of this white powder is 64 °C.

RESULTS AND DISCUSSION

Identification of strain PR3

Of the many cultures tested, only a bacterial strain PR3, isolated from a water sample at a pig farm in Morton, IL, was found to convert oleic acid to products with increased polarity. Strain PR3 formed a smooth, round, white colony on agar plate. The microorganisms were motile, short rod-shaped bacteria (Fig. 2). Flagella stain with modified Ryu reagent [4] showed multiple polar flagellae (Fig. 3). Strain PR3 grew aerobically and could not grow anaerobically. The oxidase activity of the cells was positive. PR3 produced no water-soluble pigment.

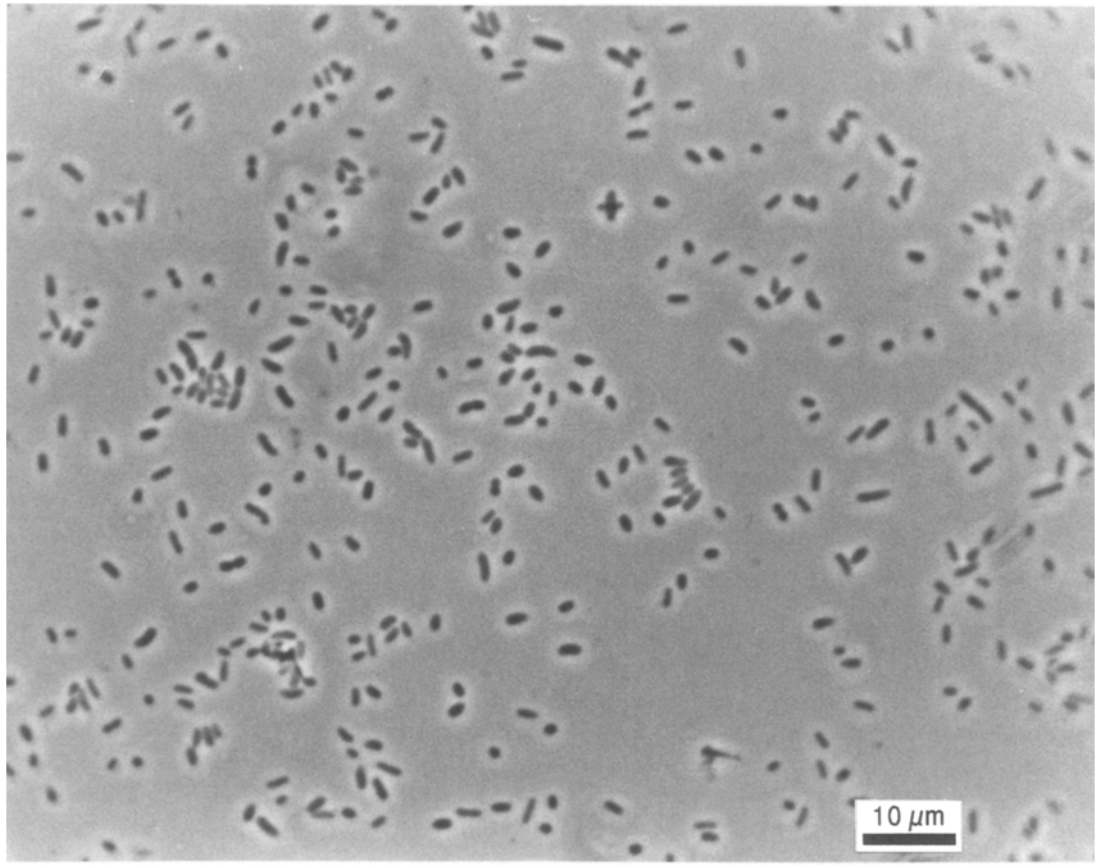

Fig. 2. Phase contrast microscopy of strain PR3.

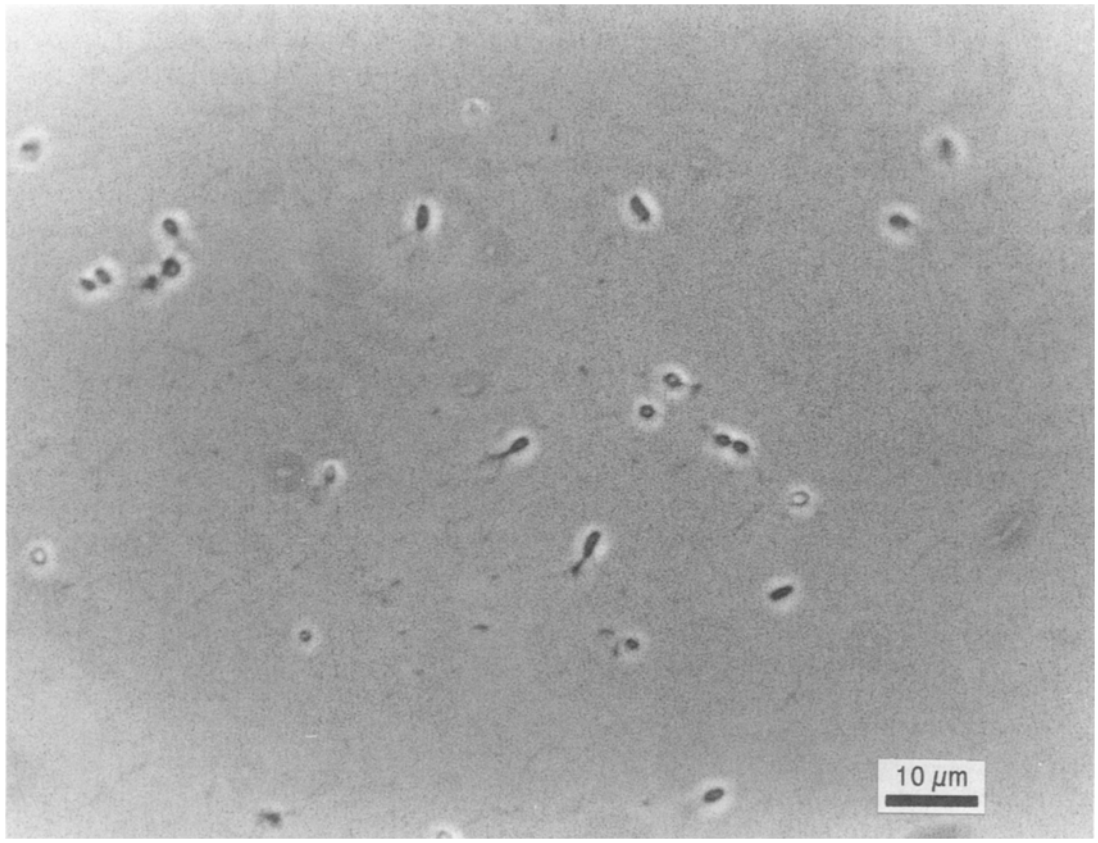

Fig. 3. Modified Ryu stain showing flagellae of strain PR3.

Based on these observations, strain PR3 tentatively belongs to the genus Pseudomonas.

Identification of product

Preliminary results on the identification of the reaction product were reported previously [6]. Chemical ionization (CI) mass spectrum of the methylated product showed a typical behavior of hydroxy fatty acid esters [14]. Instead of a molecule-ion peak, a peak at m/e = 327 ($M\text{-}1^+$; relative intensity 6), followed by m/e = 311 (MH-18; 100) and 295 ($M\text{-}32^+$; 18) were observed. Electron impact (EI) mass spectrum of the methylated product showed a peak at m/e = 310 ($M\text{-}18^+$; 0.5). These ions indicated that the product is a dihydroxy C_{18} monoene ester. Fragments observed from the EI spectrum of the TMS derivative of the methylated product established the locations of the hydroxyl groups and the double bond (Fig. 4). Ion peaks at m/e 343 (42%) and 359 (28%) represent fragments containing both TMS groups and the double bond which derived from cleavage alpha to the derivatized hydroxyl groups. These fragments located the hydroxyl groups at C7 and C10 and the double bond between the two

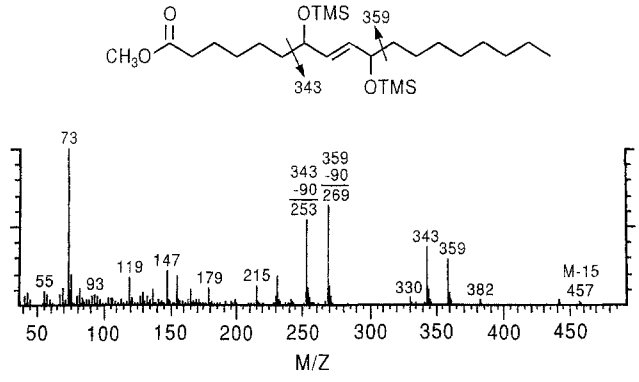

Fig. 4. EI mass spectrum of TMS derivative of methylated DOD.

hydroxyl groups at C8. Based on these MS data, the reaction product is 7,10-dihydroxy-8-octadecenoic acid.

Proton NMR of DOD showed the presence of the following resonance signals: $-CH = CH-$ group at 5.63 ppm ($J_{AA'}$ = 14.3 Hz); two tertiary protons $-CH-O-$ at 4.08 ppm; $-CH_2-COOH$ at 2.32 ppm;

11 methylene groups from 1.2 to 1.6 ppm and a $-CH_3$ group at 0.86 ppm. The coupling constant for the olefinic protons indicates *trans* unsaturation.

IR analysis of DOD confirmed the presence of the following groups: hydroxyl groups at 3400 cm^{-1}; carbonyl at 1712 cm^{-1}; and *trans* unsaturation at 975 cm^{-1}. Therefore, the structure of the product is 7,10-dihydroxy-8(E)-octadecenoic acid.

DOD is insoluble in hexane, slightly soluble in toluene and benzene, and soluble in diethyl ether, chloroform, ethyl acetate, acetone, methanol and acetonitrile.

Effect of carbon sources on the production of DOD

Strain PR3 utilizes dextrose, glycerol, and oleic acid as carbon sources for growth. However, the cells grown on dextrose were more active for conversion of oleic acid to DOD (Table 1). Therefore, dextrose-grown cells were selected for optimization studies.

Time course of the production of DOD

Oleic acid (0.4 ml) was added to 2-day-old cultures (30-ml) in a 125-ml Erlenmeyer flask to start the reaction. The reaction was carried out at 30 °C for the time specified. The amount of the product DOD in the culture media increased with time and reached a maximum (63%) after 48 h of reaction (Fig. 5). Because 1 mol of DOD was produced from 1 mol of substrate oleic acid, the maximum yield calculated was 63%. Further incubation reduced DOD content in the medium, thus strain PR3 metabolizes DOD. Therefore, production of DOD was standardized at 48 h whenever other variables were evaluated.

Effect of substrate concentration

Various amounts of oleic acid were added to 2-day-old cells of strain PR3. The production of DOD was assayed after 48 h of reaction. The amount of DOD produced was dependent on the amount of substrate initially added. An oleic acid concentration of 0.4 ml per 30 ml reaction medium supported maximum DOD production (Fig. 6).

TABLE 1

Effect of carbon sources for cell growth on subsequent DOD production

Carbon sources	DOD produced (mg)
Glucose	220
Glycerol	0
Oleic acid	120

The amount of carbon source used for growth of strain PR3 was 4 g per liter. The amount of DOD produced was in mg per 30 ml of reaction mixture. Details see MATERIALS AND METHODS.

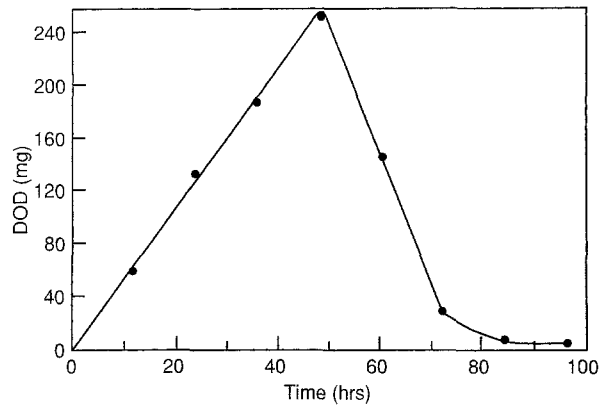

Fig. 5. Time course for the production of DOD by strain PR3. Reaction conditions: substrate oleic acid (0.4 ml) was added to a 36-h-old culture in a shake flask at 30 °C. The production of DOD was assayed at 12 h interval of incubation.

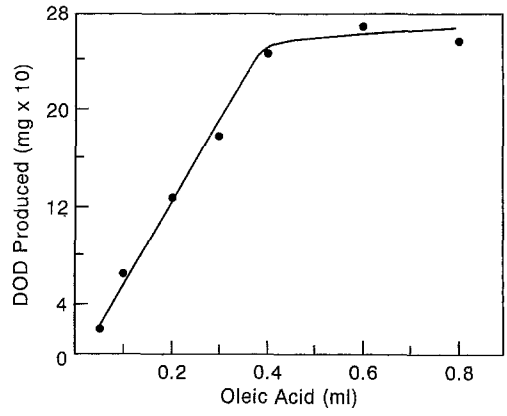

Fig. 6. Effect of substrate concentration on the production of DOD. The reaction conditions were the same as described in Fig. 5 except that various amount of oleic acid were added and the production of DOD was assayed at 48 h of incubation.

Effect of pH

The effects of pH on both the growth of strain PR3 and the production of DOD were studied. The medium was adjusted with either 2N NaOH or 3N HCl to the desired pH values (from pH 5.5 to 8.5) before inoculation with strain PR3. Cell density was measured at 600 nm after 2 days of growth. Fig. 7 shows cell dry weight calculated from a standard curve that correlates cell dry weight with optical density at 600 nm. Optimum pH for growth of strain PR3 was 6 to 7.

The effect of pH on production of DOD was studied with 2-day-old cells of strain PR3 grown on a pH 7.0 medium. Immediately before adding reaction substrate (oleic acid), the culture medium was adjusted to the desired pH (from 5.5 to 8.5) with either 2N NaOH or 3N

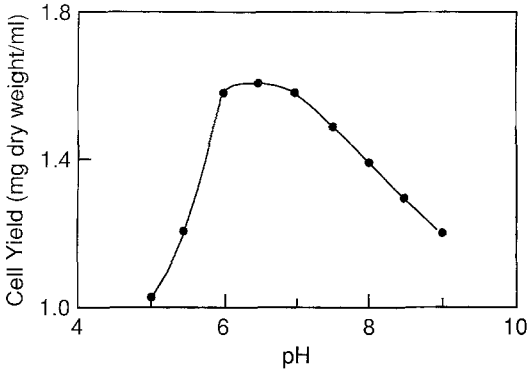

Fig. 7. Effect of pH on the growth of strain PR3. The medium was adjusted to the desired pH values before innoculation of strain PR3. Cell yields were measured after 2 days of growth.

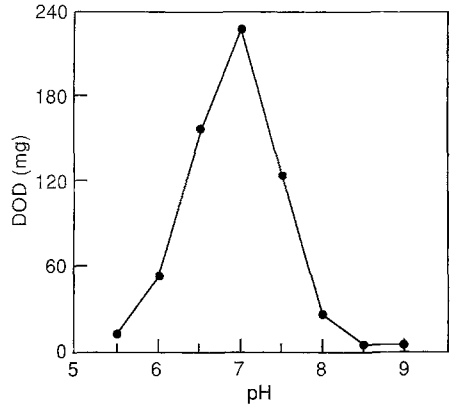

Fig. 8. Effect of pH on the production of DOD by strain PR3. Reaction conditions: Two-day-old cultures grown on pH 7.0 media were used. Prior to the addition of substrate oleic acid (0.4 ml), the pH of the culture media was adjusted as desired by the addition of 3N HCl or 2N NaOH. The reaction was carried out at 30 °C for 2 days.

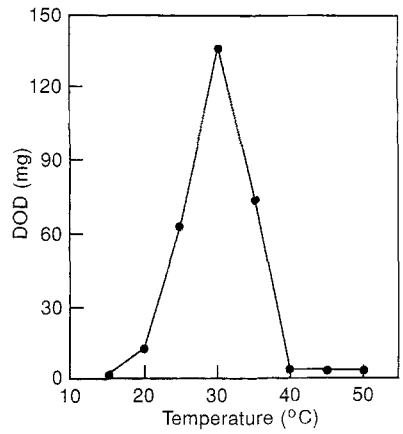

Fig. 9. Effect of temperature on the production of DOD by strain PR3. Cells were grown at 30 °C in pH 7.0 dextrose media for 2 days. After the addition of substrate oleic acid (0.2 ml), the flasks were incubated at the temperature indicated for an additional 2 days. The production of DOD was determined by GC analysis.

TABLE 2

Substrate specificity of bioconversion by strain PR3

Substrates	TLC R_f values	
	Substrate	Products
$C_{16:0}$ Palmitic acid	0.58	0.44
$C_{14:1}$ Myristoleic acid	0.45	0.31, 0.56
$C_{16:1}$ Palmitoleic acid	0.43	0.18, 0.55
$C_{18:1}$ Oleic acid	0.54	0.34[a], 0.43
$C_{18:2}$ Linoleic acid	0.43	0.55
Corn oil	0.69	0.23, 0.52, 0.54
Soybean oil	0.66	0.22, 0.51, 0.53

[a] Product identified is DOD.

HCl. At harvest time, each culture medium had become more acidic by 0.3 pH units. The maximum yield of DOD occurred at pH 7.0 (Fig. 8).

Effect of temperature

The temperature optimum for the production of DOD by cells of strain PR3 was about 30 °C (Fig. 9).

Substrate specificity

Possible substrates including saturated and unsaturated fatty acids as well as corn oil and soybean oil were tested for bioconversion by cells of strain PR3. The bioconversion activity was measured by the formation of products detectable by TLC. Although not in significant quantity, all substrates tested were modified by strain PR3 (Table 2). Therefore, strain PR3 has a broad substrate specificity. The exact chemical structures of these reaction products as well as the industrial uses of DOD are currently under investigation.

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REFERENCES

1 Bagby, M.O. and K.D. Carlson. 1989. Chemical and biological conversion of soybean oil for industrial products. In: Fat for the Future. (Cambie, R.C., ed.), Ellis Horwood Limited Press. pp. 301–306.

2 Brockerhoff, H. and R.G. Jensen, 1974. Lipolytic Enzymes. Academic Press, New York, NY.

3 Furuhashi, K. 1980. Production of 1,2-epoxides by microorganisms. Japanese Patent, Kokai Sho 55-13.

4 Heimbrook, M.E., W.L.L. Wang and G. Campbell. 1986. Easily made flagella stains. Abstract R22, ASM National Meeting, Washington, DC.

5 Hou, C.T. 1982. Microbial transformation of important industrial hydrocarbons. In: Microbial Transformations of Bioactive Compounds. (Rosazza, J.P., ed.), CRC Press, Boca Raton, FL. pp. 81–107.

6 Hou, C.T., M.O. Bagby, R. Plattner and S. Koritala. A novel compound, 7,10-dihydroxy-8(E)-octadecenoic acid from oleic acid by bioconversion. J. Am. Oil Chem. Soc. (in press).

7 Koritala, S., L. Hosie, C.T. Hou, C.W. Hesseltine and M.O. Bagby. 1989. Microbial conversion of oleic acid to 10-hydroxystearic acid. Appl. Microbiol. Biotechnol. 32: 299–304.

8 Litchfield, J.H. and G.E. Pierce. 1986. Microbiological synthesis of hydroxy-fatty acids and keto-fatty acids. U.S. Patent 4, 582, 804.

9 Marsh, J.B. and A.T. James. 1962. The conversion of stearic to oleic acid by liver and yeast preparations. Biochim. Biophys. Acta 60: 320.

10 Mercade, E., M. Robert, M.J. Espuny, M.P. Bosch, M.A. Manresa, J.L. Parra and J. Guinea. 1988. New surfactant isolated from *Pseudomonas* 42A2. J. Am. Oil Chem. Soc. 65: 1915–1916.

11 Nihaus, W.G. and G.J. Schroepfer. Jr. 1967. Enzymatic stereospecificity in the hydration of epoxy fatty acids.

12 Powell, R.G., C.R. Smith Jr. and I.A. Wolff. 1967. *cis*-5,*cis*-9,*cis*-12 Octadecatrienoic and some unusual oxygenated acids in *Xeranthemum annuum* seed oil. Lipids 2: 172–177.

13 Ruettinger, R.T. and A.J. Fulco. 1981. Epoxidation of unsaturated fatty acids by a soluble cytochrome p-$_{450}$-dependent system form *Bacillus megaterium*. J. Biol. Chem. 256: 5728–5734.

14 Ryhage, R. and E. Stenhagen. 1960. Mass spectrometric studies. VI. Methyl esters of normal chain oxo-, hydroxy-, methoxy-, and epoxy-acids. Arkiv fur Kemi 15: 545–560.

15 Steel, K.J. 1961. The oxidase reaction as a taxonomic tool. J. Gen. Microbiol. 25: 297–306.

16 Wallen, L.L., R.G. Benedict and R.W. Jackson. 1962. The microbiological production of 10-hydroxystearic acid from oleic acid. Arch. Biochem. Biophys. 99: 249–253.