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Conversion of Fatty Acids by *Bacillus sphaericus*-Like Organisms

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Abstract. *Bacillus sphaericus* species are mesophilic round-spored organisms that readily utilize fatty acid-based surfactants during growth, but their ability to modify fatty acids is unknown. Among 57 *B. sphaericus*-like strains tested for fatty acid transformation activity in Wallen fermentation (WF) medium, ten converted oleic acid to a new product determined by gas chromatography – mass spectrometry (GC-MS) to be 10-ketostearic acid (10-KSA). Additionally, a few other strains converted ricinoleic acid and linoleic acid to new products that remain to be characterized. Unlike most microbial hydrations of oleic acid, which produce a mixture of 10-KSA and 10-hydroxystearic acid, the conversion of oleic acid by *B. sphaericus* strains was unique in that 10-KSA was the sole reaction product. By replacing dextrose with sodium pyruvate in WF and adjusting to pH 6.5, conversion of oleic acid to 10-KSA by strain NRRL NRS-732 was improved from about 11% to more than 60%. Using the defined optimal conditions, the conversion reaction was scaled up in a stirred-batch reactor by using technical-grade oleic acid as substrate. This is the first report on the characterization of fatty acid conversions by *B. sphaericus* species.

Hydroxy- and ketofatty acids are useful industrial chemicals applied in plasticizer, surfactant, lubricant, and detergent formulations. Such hydroxy saturated fatty acids as 14-hydroxyeicosanoic acid and 10-hydroxystearic acid are used as commercial grease thickeners [6]. Also, ketofatty acids or derivatives of ketofatty acids are ingredients of multipurpose greases [12, 13].

It is notable that specific microorganisms can be used to hydrate such fatty acids as oleic acid to form 10-hydroxystearic acid (10-HSA) and/or 10-ketostearic acid (10-KSA). Wallen et al. [25] first described a *Pseudomonas* sp. (NRRL B-2994) possessing an intracellular enzyme that converts oleic acid to 10-HSA under anaerobic conditions with a 14% yield. Other bioconversions of oleic acid to 10-HSA have been found with *Corynebacterium* sp. S-401 [24], *Rhodococcus rhodochrous* [22], *Nocardia cholesterolicum* [14], *Saccharomyces cerevisiae* NRRL Y-2034 and *Candida intermedia* UI 5159 [4], *Micrococcus luteus* [5], *Sphingobacterium thalpophilum* NRRL B-14797 [9], *Staphylococcus* sp. NRRL B-14813 [10], *Selenomonas ruminantium* and *Enterococcus faecalis* [8], and *Nocardia paraffinae* CBS 255.58 [21].

10-Ketostearic acid is a major product of oleic acid bioconversion of one *Mycobacterium* and two *Nocardia* species [4], *Aspergillus terreus* strains UI 58 and ATCC 11156 [4], *Staphylococcus warneri* sp. [20], *Flavobacterium* sp. strain DS5 [7] and strain 12-4A [11], and *S. thalpophilum* strain O22 [16]. Among these oleic acid bioconverters, *S. cerevisiae* NRRL Y-2034, *C. intermedia* UI 5159 [4], and *S. thalpophilum* NRRL B-14797 [9] produce 10-HSA exclusively, whereas *Flavobacterium* sp. 12-4A [11] produces solely 10-KSA under the usual, aerobic conditions.

Bacillus sphaericus-like bacteria are mesophilic, round-spored organisms that readily utilize fatty acid-based surfactants during growth. They display various degrees of mosquito pathogenicity owing partially to genetic heterogeneity [23]. Phylogenetic analyses based on 16S rDNA sequences revealed seven distinct groups, each displaying characteristic substrate utilization patterns and fatty acid composition [23]. In this study, we examined the ability of *B. sphaericus* strains to convert

Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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Table 1. Culture media used by *B. sphaericus* strains in fatty acid conversions

Ingredients	WF (pH 7.3)[a] (g/L)	SM6 (pH 7.2)[a] (g/L)	PF (pH 7.3)[a] (g/L)	PF6 (pH 6.5)[a] (g/L)
1. Dextrose	4.0	4.0	—	—
2. Sodium pyruvate	—	—	2.5	2.5
3. Yeast extract	5.0	1.0	5.0	5.0
4. K_2HPO_4 (anhydrous)	4.0	4.0	4.0	1.6
5. KH_2PO_4 (anhydrous)	—	—	—	2.4
6. $(NH_4)_2HPO_4$	—	1.0	—	—
7. $MgSO_4 \cdot 7H_2O$	0.5	0.1	0.5	0.5
8. $FeSO_4 \cdot 7H_2O$	0.015	0.02	0.015	0.015
9. $MnSO_4 \cdot H_2O$	—	0.02	—	—

[a] Abbreviations: WF, Wallen fermentation medium [9, 25]; SM, screening medium [15]; PF, pyruvate fermentation medium; PF6, modified pyruvate fermentation medium.

fatty acids to products with increased functionality. Using strain NRRL NRS-732 as a model system, we further defined the optimal conditions for large-scale production of 10-KSA in a stirred-batch reactor. Herewith, we report the first characterization of fatty acid conversions by *B. sphaericus* species.

Materials and Methods

Chemicals. High-purity (99+%) oleic acid, linoleic acid, and ricinoleic acid were purchased from Nu-Chek-Prep, Inc. (Elysian, MN). Technical-grade oleic acid was obtained from Fisher Scientific (Pittsburgh, PA). A potent silylating reagent (BSA+TMCS+TMSI) was obtained from Supelco (Bellefonte, PA). 10-KSA, used as a GC standard, was from previous studies [19]. All other chemicals were reagent-grade and used without further purification.

Microorganisms. The *B. sphaericus* strains used in this study, originally isolated from a variety of environments throughout the world, are maintained by the Agricultural Research Service Culture Collection (NRRL), National Center for Agricultural Utilization Research, Peoria, Illinois. The source and the history of 58 strains were described in detail in a previous study [23]. All of these strains were transferred from freeze-dried culture onto tryptone–glucose–yeast extract (TGY), pH 7.0 [3] liquid medium to grow at 28°C for 24–48 h and were subsequently maintained on TGY agar slants at 4°C with monthly transfer. All 58 strains, except for NRRL NRS-1198, were tested for their ability to transform fatty acids.

Bioconversion analysis and product identification. Cultures maintained on TGY slants were transferred to fresh TGY broth and incubated at 28°C for 1 day with shaking at 200 rpm. One-tenth ml of this growth was used to inoculate 10 ml Wallen fermentation (WF) medium in 50-ml Erlenmeyer flasks, or to inoculate the other culture media described in Table 1. Inoculated broths were shaken for about 24 h under the same conditions, followed by the addition of 0.1 ml high-purity fatty acids. The reaction was allowed to proceed an additional 48 h. Lipids were recovered from the acidified broth by two extractions with an equal volume of methanol/ethyl acetate (1:9, vol/vol) [4, 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. A portion of the extract was dried immediately under a nitrogen stream for further analysis.

Bioconversion was monitored by gas chromatography (GC). Lipid extracts were esterified with diazomethane and analyzed by an HP (Hewlett Packard; Palo Alto, CA) Model 5890 Series II Gas Chromatograph that was 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 under the conditions described previously [15]. HP ChemStation software was used for data acquisition and integration. Palmitic acid was added as an internal standard prior to solvent extraction for quantitative estimation of the product. A linear relationship was established for the peak area ratios of products versus methyl palmitate.

The chemical structure of the methylated and TMS-derivatized products was confirmed by GC-mass spectrometry (GC–MS), with authentic 10-KSA as a standard. The GC retention time and chemical structure of 10-KSA standard were characterized by GC, GC–MS, nuclear magnetic resonance, and Fourier transform infrared spectroscopies in a previous study [16].

Reaction in bioreactor. The conversion reaction was carried out in a 2-L Applikon (Schiedam, The Netherlands) stirred bioreactor system equipped with sensors for controlling pH, temperature, and dissolved oxygen (DO), and two 6-bladed Rushton impellers. The vessel, containing 1 L PF6 medium (Table 1) and 100 ppm SO-25 antifoam (Sigma, St. Louis, MO), was inoculated with 10 ml 24-h-old strain NRRL NRS-732 culture at 30°C, 500 rpm, and 40% DO saturation level. After 17 h of culture growth, technical-grade oleic aicd (12 ml, equivalent to 10 g oleic acid) was added to the culture, and the culture pH was immediately adjusted to 8.0 with sterile 6 *N* KOH to initiate the conversion reaction. Aliquots (10 ml) in three replications were withdrawn from the reactor at 24-h intervals for analysis of products as described above.

Results and Discussion

Bioconversion reactions. Of the 57 *B. sphaericus* strains examined for ability to convert oleic acid, ten strains produced milligram quantities of 10-KSA in 10-ml WF culture broths. Strains NRRL NRS-732, NRRL BD-119, NRRL NRS-866, and NRRL NRS-111 were the best converters, producing 10.8, 4.7, 2.2, and 1.9 mg of 10-KSA, respectively, which corresponded to a respective yield of 10.8, 4.7, 2.2, and 1.9%. Gas chro-

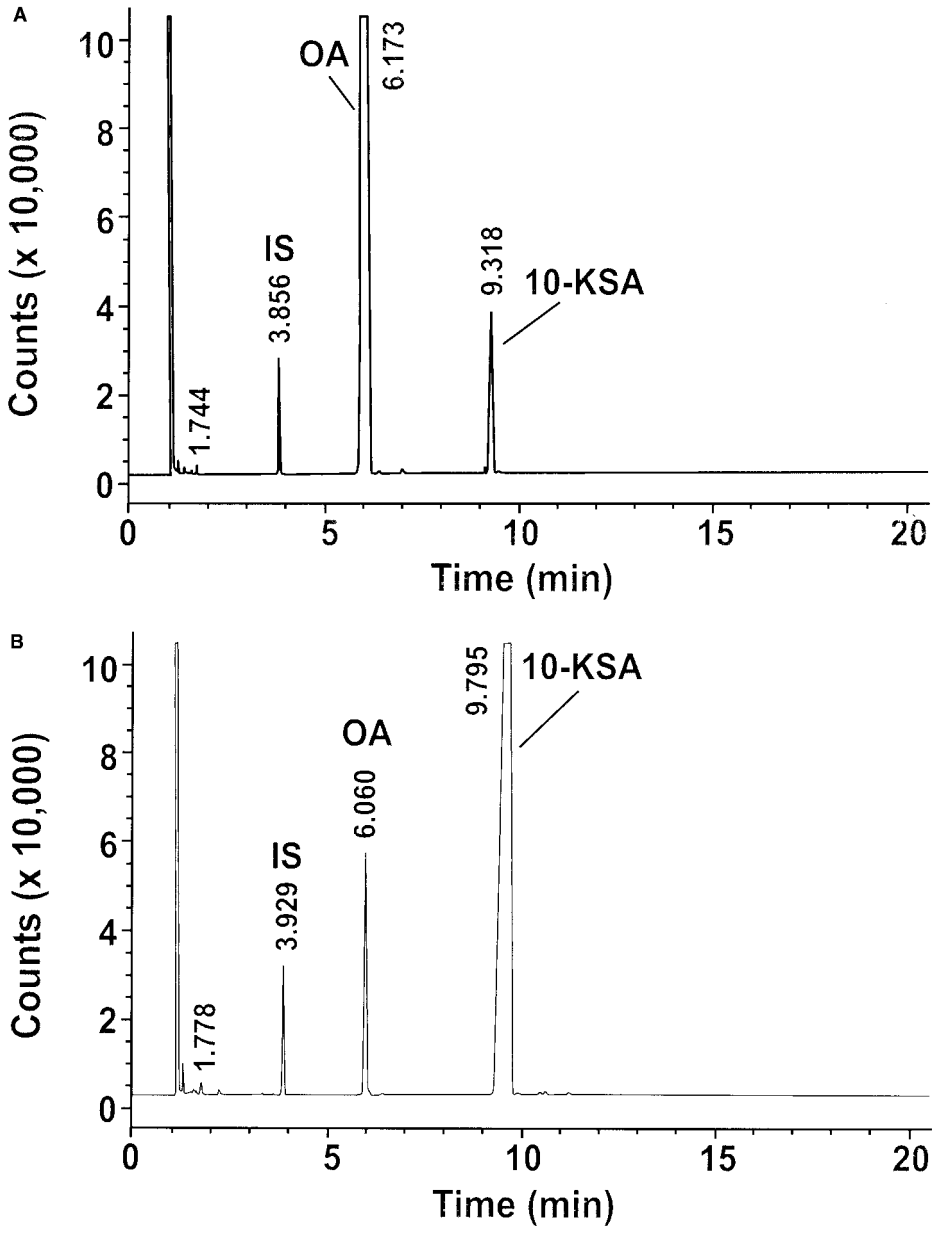

Fig. 1. Gas chromatographic analysis of conversion products produced by *B. sphaericus* strains with oleic acid as substrate. (**A**) The conversion reaction was carried out in Wallen fermentation medium at 28°C and 200 rpm for 48 h. (**B**) The reaction was carried out in the pyruvate fermentation medium at pH 6.5 under the same conditions by strain NRRL NRS-732. IS stands for internal standard (palmitic acid) added to the sample prior to lipid extraction for identification and quantitation. OA is oleic acid, and 10-KSA is 10-ketostearic acid.

matographic analysis of the conversion products revealed a single, new GC peak that was determined to be 10-KSA (Fig. 1A). There were no other appreciable conversion products, such as 10-HSA, that could be detected under the experimental conditions. Therefore, unlike most microbial hydrations of oleic acid, which produce a mixture of 10-KSA and 10-HSA [18], the conversion of oleic acid by *B. sphaericus* strains was unique in that 10-KSA was the sole reaction product. Previous findings in our laboratory with *Staphylococcus* sp. [19], *Flavobacterium* sp. strain DS5 [7], and strains of *S. thalpophilum* [17] showed conversion of oleic acid predominantly to 10-KSA, but with 6–13% 10-HSA also present. Other major 10-KSA producers are *Corynebacterium* [24], *Sarcina lutea* [2], *Mycobacterium* and *Nocardia* strains [4]. On the other hand, 10-HSA is the main conversion product from oleic acid by *Pseudomonas* [25], *R. rhodochrous* [22], *No-*

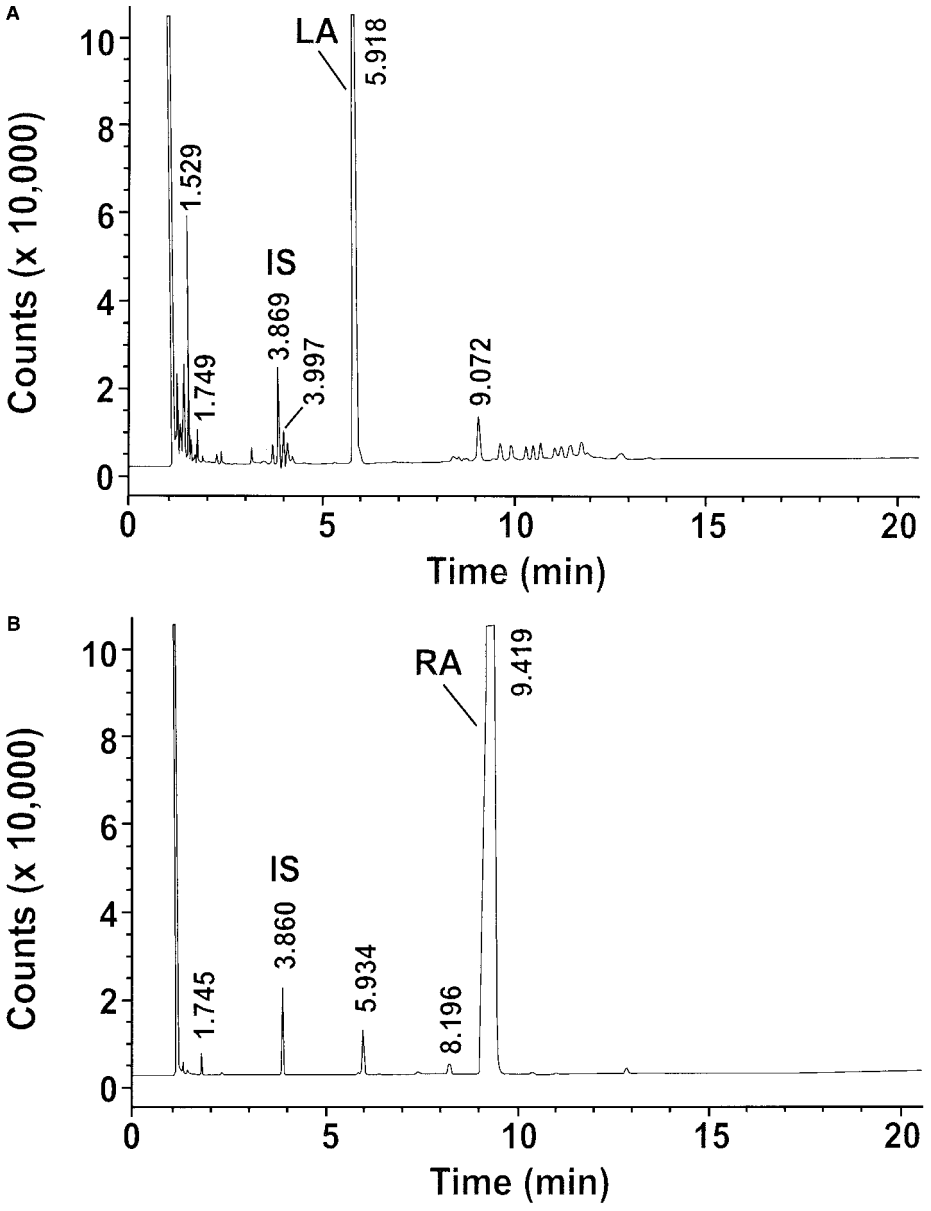

Fig. 2. Gas chromatographic analysis of conversion products produced by *B. sphaericus* strains using (**A**) linoleic acid and (**B**) ricinoleic acid as substrate in Wallen fermentation medium at 28°C and 200 rpm for 48 h. LA is linoleic acid, and RA is ricinoleic acid. A few unknown product peaks are indicated by their GC retention times.

cardia species [14, 21], and several *Staphylococcus* species [20].

The ability of microorganisms to convert oleic acid to either 10-KSA or 10-HSA as a main product was thought to be strongly related to the cellular regulation of both hydratase and secondary alcohol dehydrogenase activities [7]. Therefore, it is plausible that cell-free extracts of aerobic NRRL B-14797 cultures, which convert oleic acid to 10-HSA exclusively [9], may be devoid of secondary alcohol dehydrogenase activity. Furthermore, *Flavobacterium* sp. 12-4A [11] and the *B. sphaericus* strains examined in this study, which produce only 10-KSA from oleic acid conversions, are likely to possess high activities of secondary alcohol dehydrogenase for converting 10-HSA to 10-KSA.

Two *B. sphaericus* strains (NRRL NRS-718 and NRRL NRS-866) were found to convert linoleic acid to produce milligram quantities of a new GC peak with retention time of about 9.1 min (Fig. 2A). Additionally, a number of small GC peaks were also present in the

Table 2. Conversion of oleic acid by strain NRRL NRS-732 to produce 10-ketostearic acid at different pH in pyruvate fermentation medium[a]

PF[b] medium no.	K_2HPO_4 (g/L)	KH_2PO_4 (g/L)	Apparent pH of medium	10-KSA produced[c] (mg/10 ml)
1–3	3.6–2.8	0.4–1.2	7.3–6.9	n.d.
4	2.4	1.6	6.8	48.8 ± 10.2
5	2.0	2.0	6.7	51.8 ± 5.0
6	1.6	2.4	6.5	60.8 ± 6.0
7	1.2	2.8	6.3	50.1 ± 3.0
8	0.8	3.2	6.1	39.7 ± 3.5
9	0.4	3.6	5.9	39.2 ± 2.2

[a] High-purity oleic acid, 0.1 ml (100 mg), was added to 24-h-old microbial cultures in 10-ml fermentation broth, and the bioconversion continued for 48 h at 28°C and 200 rpm prior to lipid extraction. The pH of pyruvate fermentation medium described in Table 1 was adjusted by using different amounts of anhydrous K_2HPO_4 and KH_2PO_4 as shown above.
[b] Abbreviations: PF, pyruvate fermentation; 10-KSA, 10-ketostearic acid; n.d., not determined.
[c] The amount of product produced is the average of triplicate analyses ± standard error.

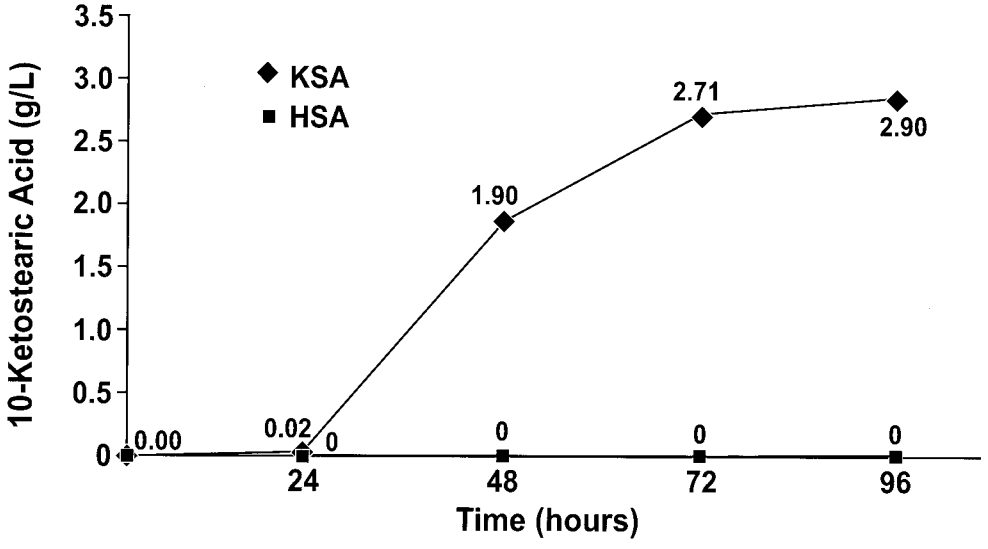

Fig. 3. Time course for the production of 10-ketostearic acid by strain NRRL NRS-732 in a bioreactor. KSA stands for ketostearic acid and HSA stands for hydroxystearic acid. Technical-grade oleic acid, 12 ml (equivalent to 10 g oleic acid), was added to a 24-h-old culture under the conditions as described in Materials and Methods. The bioconversion reaction was allowed to proceed for a given time as shown. Each data point is the average of triplicate analyses.

conversion products. When ricinoleic acid was the conversion substrate, three *B. sphaericus* strains (NRRL BD-94, NRRL NRS-719, and NRRL NRS-111) were found to produce milligram quantities of a new GC peak with a retention time of about 5.9 min (Fig. 2B). These bioconversion reactions need to be further characterized.

Improvement of oleic acid conversion. The ability of *B. sphaericus* strains to produce solely 10-KSA from oleic acid, instead of a mixture of 10-KSA and 10-HSA, makes this bioconversion an ideal system for developing large-scale production of 10-KSA in a reactor. In order to develop such a reactor process, we studied conditions for an improved oleic acid conversion to 10-KSA. Strain NRRL NRS-732 was chosen for further characterization because it was the best 10-KSA producer found among *B. sphaericus* strains in the initial tests in WF medium, as discussed above.

Conversion of oleic acid by strain NRRL NRS-732 was compared in WF and in screening medium no. 6 (SM6), in addition to the pyruvate fermentation (PF) medium (Table 1). WF and SM6 were routinely used in our laboratory and showed great influence on the production of TOD and DOD from ricinoleic acid and oleic acid, respectively, by *Pseudomonas aeruginosa* strain PR3 [15, 17]. Because *B. sphaericus* strains metabolized common hexoses, pentoses, or disaccharides only poorly,

but preferentially grew on pyruvate, amino acids, purine or pyrimidine compounds as their energy or carbon sources [23], we devised the PF medium by simply replacing dextrose in WF with sodium pyruvate. The preliminary results showed that the ability of strain NRRL NRS-732 to convert oleic acid to 10-KSA was in the order of PF > WF ≫ SM6 (data not shown). The PF medium was subsequently subjected to further study.

The pH of PF medium affected the ability of strain NRRL NRS-732 to convert oleic acid to 10-KSA. In a preliminary experiment in which the medium pH was adjusted to above 6.8, there was a drastic decrease in the conversion activity. By varying the amount of monobasic and dibasic potassium phosphate used in the preparation of culture medium to control pH in the range of 5.9–6.8, the conversion of oleic acid exhibited a peak activity in PF medium around pH 6.5 (Table 2). Therefore, by simply replacing dextrose in WF with sodium pyruvate and adjusting pH to 6.5, conversion of oleic acid to produce 10-KSA by strain NRRL NRS-732 was improved from 11 mg (an 11% yield) in WF medium to 61 mg (a 61% yield) in PF6 medium (Table 2). A GC analysis of the conversion products showed that the 10-KSA peak was greatly enhanced, and no other oxygenated fatty acids, such as 10-HSA, were present (Fig. 1B).

Time course of 10-KSA production in bioreactor. The improved bioconversion conditions discussed above were applied to a stirred reactor study with PF6 medium and technical-grade oleic acid as substrate. A time course study on 10-KSA production by strain NRRL NRS-732 in a reactor indicated that accumulation of 10-KSA did not occur in the first 24 h, increased rapidly in the next 24 h, and slowed down substantially thereafter. The reaction could eventually reach a yield of 3 g 10-KSA, or a 30% yield, when it was allowed to continue beyond 96 h (Fig. 3). This is a relatively low yield in comparison with what was achieved at a 60% yield in shaking flasks containing small volumes of culture medium. Previous studies on oleic acid conversion by strains of *S. thalpophilum* [16], *Flavobacterium* sp. strain DS5 [7], and *Staphylococcus* sp. [19] showed that the 10-KSA produced was not further metabolized in the culture. The low conversion yield of 10-KSA in a stirred batch reactor could, therefore, be attributed to factors directly related to the formation of 10-KSA. In a study of oleic acid conversion to produce hydroxy monounsaturated C18 fatty acid in a stirred bioreactor, Bastida et al. [1] demonstrated that the growth rate of the microbial culture was directly related to the oxygen solution transfer coefficient, which in turn exhibited an exponential relationship to product formation.

In summary, the results showed that *B. sphaericus*-like organisms could readily convert fatty acids to new products of industrial use. The ability of certain strains of *B. sphaericus* to uniquely convert oleic acid solely to 10-KSA warranted further characterization of the conversion reaction. As such, the conversion of oleic acid to produce 10-KSA by strain NRRL NRS-732 was improved from 11% to more than 60% yields by simply modifying the composition of the culture medium. Initial attempts to produce 10-KSA in a bioreactor achieved a yield of only 30%. It is imperative that various factors, such as substrate concentration, culture growth, oxygen transfer rate, reaction pH, and temperature, etc., be evaluated and further optimized in a reactor process to obtain large quantities of 10-KSA. This is the first report on the characterization of fatty acid conversions by *B. sphaericus*-like organisms.

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Literature Cited

1. Bastida J, de Andrés C, Culleré J, Busquets M, Manresa A (1999) Biotransformation of oleic acid into 10-hydroxy-8*E*-octadecenoic aicd by *Pseudomonas* sp. 42A2. Biotechnol Lett 21:1031–1035
2. Blank W, Takayanagi H, Kido T, Meussdoerffer F, Esaki N, Soda K (1991) Transformation of oleic acid and its esters by *Sarcina lutea*. Agric Biol Chem 55:2651–2652
3. Difco Laboratories (1984) DIFCO manual, 10th edn. Detroit: Difco Laboratories, p 679
4. El-Sharkawy SH, Yang W, Dostal L, Rosazza JPN (1992) Microbial oxidation of oleic acid. Appl Environ Microbiol 58:2116–2122
5. Esaki N, Ito S, Blank W, Soda K (1994) Biotransformation of oleic acid by *Micrococcus luteus* cells. Biosci Biotechnol Biochem 58:319–321
6. Hagemann JW, Rothfus JA (1991) Thermal behavior of prospective hydroxy acid grease thickeners. J Am Oil Chem Soc 68:139–143
7. Hou CT (1994) Production of 10-ketostearic acid from oleic acid by *Flavobacterium* sp. strain DS5 (NRRL B-14859). Appl Environ Microbiol 60:3760–3763
8. Hudson JA, MacKenzie CAM, Joblin KN (1995) Conversion of oleic acid to 10-hydroxystearic acid by two species of ruminal bacteria. Appl Microbiol Biotechnol 44:1–6
9. Kaneshiro T, Huang JK, Weisleder D, Bagby MO (1994) 10(*R*)-Hydroxystearic acid production by a novel microbe, NRRL B-14797, isolated from compost. J Ind Microbiol 13:351–355
10. Kaneshiro T, Nakamura LK, Bagby MO (1995) Oleic acid transformations by selected strains of *Sphingobacterium thalpophilum* and *Bacillus cereus* from composted manure. Curr Microbiol 31: 62–67
11. Kawashima H (1995) Production of 10-ketostearic acid from oleic acid by a strain of *Flavobacterium* sp. 12-4A. Biotechnol Lett 17:493–496
12. Kenney HE, Donahue ET, Maerker (1974a) Lithium polycyanoethylated keto fatty soap based greases. US Patent 3,832,368

13. Kenney HE, Donahue ET, Maerker (1974b) Greases thickened with keto fatty acid lithium soaps or keto cyanoethylated fatty acid lithium soaps. US Patent 3,836,468
14. Koritala S, Hosie L, Hou CT, Hesseltine CW, Bagby MO (1989) Microbial conversion of oleic acid to 10-hydroxystearic acid. Appl Microbiol Biotechnol 32:299–304
15. Kuo TM, Manthey LK, Hou CT (1998) Fatty acid bioconversions by *Pseudomonas aeruginosa* PR3. J Am Oil Chem Soc 75:875–879
16. Kuo TM, Lanser AC, Nakamura LK, Hou CT (2000) Production of 10-ketostearic acid and 10-hydroxystearic acid by strains of *Sphingobacterium thalpophilum* isolated from composted manure. Curr Microbiol 40:105–109
17. Kuo TM, Kim H, Hou CT (2001) Production of a novel compound, 7,10,12-trihydroxy-8(*E*)-octadecenoic acid from ricinoleic acid by *Pseudomonas aeruginosa* PR3. Curr Microbiol 43:198–203
18. Kuo TM, Kaneshiro T, Hou CT (2002) Microbiological conversions of fatty acids to value-added products. In: Kuo TM, Gardner HW (eds) Lipid biotechnology. New York: Marcel Dekker, pp 605–628
19. Lanser AC (1993) Conversion of oleic acid to 10-ketostearic acid by a *Staphylococcus* species. J Am Oil Chem Soc 70:543–545
20. Lanser AC, Nakamura LK (1996) Identification of a *Staphylococcus warneri* species that converts oleic acid to 10-ketostearic acid. Curr Microbiol 32:260–263
21. Latrasse A, Paitier S, Lachot B, Bonnarme P, Féron G, Durand A, Le Quéré JL (1997) Conversion of oleic acid to 10-hydroxystearic acid by *Nocardia paraffinae*. Biotechnol Lett 19:715–718
22. Litchfield JH, Pierce GE (1986) Microbiological synthesis of hydroxy-fatty acids and keto-fatty acids. U.S. Patent 4,582,804
23. Nakamura LK (2000) Phylogeny of *Bacillus sphaericus*-like organisms. Int J Syst Evol Microbiol 50:1715–1722
24. Seo CW, Yamada Y, Takada N, Okada H (1981) Hydration of squalene and oleic acid by *Corynebacterium* sp. S-401. Agric Biol Chem 45:2025–2030
25. Wallen LL, Benedict RG, Jackson RW (1962) The microbiological production of 10-hydroxystearic acid from oleic acid. Arch Biochem Biophys 99:249–253