Biocatalytic Production of 10-Hydroxystearic Acid, 10-Ketostearic Acid, and Their Primary Fatty Amides

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ABSTRACT: The objective of this study was to develop scaleup bioprocesses for producing 10-hydroxystearic acid (10-HSA) and 10-ketostearic acid (10-KSA) as well as their primary amides for potential new uses. A reactor process was examined to obtain the mono-oxygenated FA using *Sphingobacterium thalpophilum* (NRRL B-14797) and *Bacillus sphaericus* (NRRL NRS-732), which solely produce 10-HSA and 10-KSA, respectively, from technical-grade oleic acid. By using an 8-h-old B-14797 culture grown in a manganese-containing WF6 medium, pH 7.3, at 28°C under 350 rpm agitation and 0–50% dissolved oxygen concentrations provided by a controlled sparger aeration, the production of 10-HSA reached 7 g/L with a 40% yield in 4 d. In using a 12-h-old NRS-732 culture grown in a pyruvate-containing PF6 medium, pH 6.5, at 30°C under 750 rpm agitation without any sparger aeration during the conversion reaction, 10-KSA production reached 7.9 g/L with a yield of more than 54% in 72 h. The scaleup reactor process provided crystalline 10-HSA and 10-KSA for producing new primary amides *via* a lipase-catalyzed amidation reaction with yields of 94 and 92%, respectively. The primary amides of 10-HSA and 10-KSA displayed m.p. of 115 and 120°C, respectively.

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KEY WORDS: *Bacillus sphaericus*, bioconversion, bioreactor, 10-hydroxystearic acid, 10-ketostearic acid, primary fatty amides, *Sphingobacterium thalpophilum*.

Hydroxy saturated FA such as 14-hydroxyeicosanoic and 9(10)-hydroxystearic acids have been proposed for use in the formulation of grease thickeners (1). Chemically prepared keto FA and derivatives of keto FA have been patented as ingredients for use in multipurpose greases (2,3). However, certain microorganisms could be used as biocatalysts to oxidize oleic acid (OA) to form 10-hydroxystearic acid (10-HSA) and 10-ketostearic acid (10-KSA). Wallen *et al*. (4) first described a *Pseudomonas* sp. (NRRL B-2994) that converted OA to 10-HSA under anaerobic conditions with a 14% yield. Litchfield and Pierce (5) patented a microbial process for synthesizing hydroxy- and keto-FA by use of *Rhodococcus rhodochrous*. The bioconversion reaction proceeded *via* oxidation of the double bond having *cis* configuration at the 9-position of OA (6,7). A variety of microbial species also were reported to be capable of aerobically converting OA to mono-oxygenated FA. These microorganisms included *Corynebacterium* sp., *Saccharomyces cerevisiae*, *Candida intermedia*, *Micrococcus luteus*, *Sphingobacterium thalpophilum*, *Selenomonas ruminantium*, *Enterococcus faecalis*, *Mycobacterium* sp., *Sarcina lutea*, *Nocardia* spp., *Aspergillus terreus, Staphylococcus warneri* sp., *Flavobacterium* sp., and *Bacillus sphaericus* (8). Most of the bioconversion reactions, however, led to the formation in small shake-fermentation flasks of a mixture of 10-HSA and 10-KSA, which complicated the purification process.

A strain of *S. thalpophilum* (9) and several strains of *B. sphaericus* (10) were found to convert OA aerobically to form exclusively 10-HSA and 10-KSA, respectively. To exploit these unique biocatalytic properties, we undertook a scaleup process study using stirred batch reactors to produce each compound in sufficient quantity for further structural modification *via* enzymatic reaction. This report describes the results of the scaleup bioreactor production of 10-HSA and 10-KSA using technical-grade OA as well as the enzymatic formation and initial characterization of their primary fatty amides.

MATERIALS AND METHODS

Chemicals. High-purity (99+%) OA was purchased from Nu-Chek-Prep, Inc. (Elysian, MN). Technical-grade OA was obtained from Aldrich Chemical (Milwaukee, WI), and reagent-grade 3-(*N*-morpholino) propane-sulfonic acid (MOPS) was from Research Organics (Cleveland, OH). Yeast extract and tryptone were from Difco Laboratories (Detroit, MI). 10-HSA and 10-KSA used as GC standards were prepared from previous studies (9,11). Biospumex 153 K (modified polyalcoxyethers) was kindly provided by Cognis Corporation (Cincinnati, OH). Immobilized *Candida antarctica* lipase B (Novozym 435), 2-methyl-2-butanol (*tert*-amyl alcohol), ammonium carbamate, and SO-25 (polyol: silicone polymer) antifoam were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were reagent-grade and used without further purification.

Microorganisms. *Bacillus sphaericus* strain NRRL NRS-732 was selected from the ARS Culture Collection (NRRL), Agricultural Research Service, U.S. Department of Agriculture in Peoria, Illinois (10). *Sphingobacterium thalpophilum* NRRL B-14797 was selected from commercial compost amended with OA (9). Both strains were transferred from freeze-dried culture onto tryptone-glucose-yeast extract (TGY), pH 7.0 (12) liquid medium to grow at 28°C for 24–48 h and subsequently

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TABLE 1
Culture Media Used for the Bioconversion of Oleic Acid

Ingredients	WF3 (pH 7.3)[a]	WF6Mn (pH 7.3)	PF (pH 7.3)	PF6 (pH 6.5)
		(g/L)		
1. Dextrose	4.0	4.0	—	—
2. Sodium pyruvate	—	—	2.5	2.5
3. Yeast extract	5.0	2.5	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. Tryptone	—	2.5	—	—
7. MOPS	—	3.0	—	—
8. $MgSO_4 \cdot 7H_2O$	0.5	0.5	0.5	0.5
9. $FeSO_4 \cdot 7H_2O$	0.015	0.01	0.015	0.015
10. $MnSO_4 \cdot H_2O$	—	0.02	—	—

[a]Abbreviations: WF, Wallen fermentation medium (9); PF, pyruvate fermentation medium (10); MOPS, 3-(*N*-morpholino) propane-sulfonic acid. For the medium preparation, items 1–7 were added and dissolved in the bulk of deionized and distilled water and items 8–10 were dissolved separately in small amounts of the water. The two solutions were then mixed and adjusted to a desired pH and the final volume. The medium in shake flasks and reactors was autoclaved at 120°C for 20 and 30 min, respectively.

maintained on TGY agar slants at 4°C and subtransferred monthly.

Reaction in stirred batch reactor. The scaleup process was carried out in a 2-L bench-top bioreactor system (Applikon Inc., Foster City, CA) equipped with sensors for monitoring pH, temperature, and dissolved oxygen (D.O.), and one or two three-bladed (45 mm dia.) marine impellers. Unless specified otherwise, the vessel contained 1 L of fermentation medium (Table 1) and 100 ppm SO-25 antifoam and was inoculated with 1% (vol/vol) 24-h-old culture at the specified temperature, impeller speed, and aeration rate and frequency during culture growth and the conversion reaction. After 8–12 h of culture growth, technical-grade OA (2%, wt/vol) was added to the culture, which was immediately adjusted to neutral pH with sterile 6 N KOH to initiate the conversion reaction. To achieve certain D.O. levels, the rate and frequency of airflow through a bottom sparger was controlled by an Aalborg (Orangeburg, NY) model 062-01S flowmeter and a timer, whereas a continuous airflow at 220 mL/min from two inlets on the headplate was controlled by a model 082-03S flowmeter (13). The readings of pH, temperature, and D.O. concentration were monitored by PC software and a biocontroller, model ADI 1030 (Applikon Inc.). Aliquots (10 mL) in triplicate were withdrawn from the reactor at 24-h intervals for analysis by GC using the conditions described previously (14). The product yield was calculated based on the internal standard, C17:0, added to the sample prior to lipid extraction.

Preparation of fatty amides. Amidation reactions, containing 100 mM 10-HSA or 10-KSA in 15 mL of 2-methyl-2-butanol, an appropriate amount of ammonium carbamate, and 150 mg (10 mg/mL) Novozym 435, were carried out at 55°C as described previously (15). Briefly, the mixture was equilibrated at 55°C overnight prior to addition of enzyme to start the reaction. Transformation reactions were performed in duplicate in septum-sealed 16 × 125 mm screw-capped test tubes under agitation provided by a Labquake test-tube rotator (Barnstead Intl., Dubuque, IA). Reactions were ended by filtering out Novozym 435. Products were recovered by crystallization in ethyl acetate after removing the reaction solvent by vacuum evaporation and weighed after air-drying. The amidation reaction was monitored by GC analysis and quantified by the percentage of substrate removed from the reaction using each of the hydroxylated FA as reference. For each substrate there was only one product peak evident on GC chromatograms. Furthermore, Novozym 435 being the only biocatalyst in the reaction devoid of any fermentation, it was assumed that all substrate used was transformed into the amide product. The FA substrates were converted to methyl esters with diazomethane prior to GC analysis using an HP 5890 Series II gas chromatograph equipped with a Model 18593B autosampler, HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm film thickness), and an FID. Analytical conditions were as follows: injector, 250°C; detector, 290°C; initial oven temperature 200ºC for 1 min ramping to 270°C at 10°C/min and holding for 10 min. The purified reaction products derived from 10-HSA and 10-KSA were directly analyzed by electron impact GC–MS at 70 eV under similar conditions to those described above.

RESULTS AND DISCUSSION

Specific microorganisms are known to oxidize unsaturated FA such as OA to form 10-HSA and 10-KSA (8). A majority of the reactions produce both FA, which makes purification of these homologous compounds a challenging task. Screening of soil-compost samples led to the isolation of a novel NRRL (Northern Regional Research Laboratory) microorganism, strain B-14797, which aerobically transformed OA exclusively to 10-HSA in the WF3 medium (Table 1) in small shake flasks with a yield of about 25% (9). In this study, different media compositions were found to affect the conversion reaction. The yield was improved from 19% of the total lipid fraction as 10-HSA in the WF3 medium to 56% in the WF6Mn medium that contained manganese and a biological buffer (Table 1). The WF6Mn medium was used for scaling up production of 10-HSA by NRRL B-14797 from shake flasks to a fermentor. Previous studies on selective screening

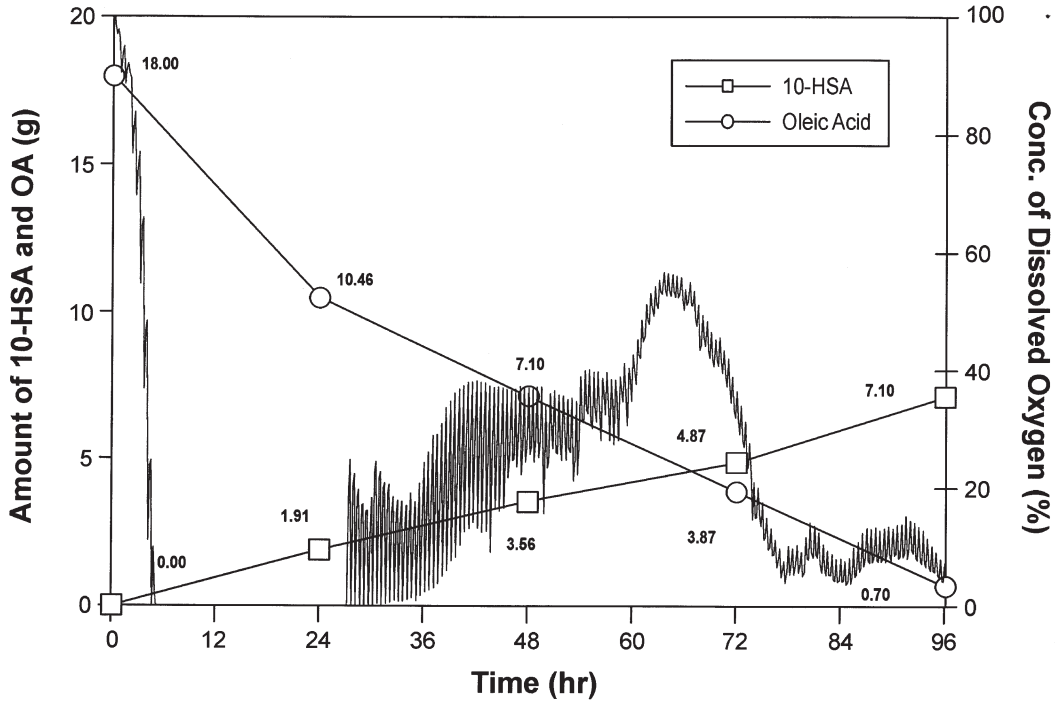

FIG. 1. A topographic illustration of changes in dissolved oxygen concentrations as related to the formation of 10-hydroxystearic acid (10-HSA) by *Sphingobacterium thalpophilum* NRRL B-14797 during the conversion of oleic acid (OA) in a 2-L stirred batch reactor. The conversion reaction was carried out in 1 L of WF6Mn medium (see Table 1 for description) containing 100 ppm SO-25 (polyol: silicone polymer) antifoam with an 8-h-old culture at 28°C under 350 rpm agitation provided by one marine impeller, 220 mL/min constant airflow from the top of the reactor headplate, and a regulated airflow through a bottom sparger. The sparger airflow was maintained for 15 min in every 30 min at 10 mL/min during the culture growth. The bioconversion was initiated by adding 18 g of OA and 400 ppm Biospumex 153 K (modified polyalcoxyethers) to the reactor culture that was subsequently adjusted to neutral pH. The airflow was increased to 15 mL/min from 10 mL/min after 20 h of conversion reaction and maintained at the same aeration rate and frequency thereafter. Each data point is the average of triplicate analyses.

of 58 *B. sphaericus*-like strains for OA conversion revealed that NRRL NRS-732 and a few others were capable of producing exclusively 10-KSA under aerobic conditions (10). The conversion reaction in different culture media (Table 1) was in the order of PF6 > PF > WF3 with yields being improved from 11% in the WF3 medium in small shake flasks to 60% in the PF6 medium using sodium pyruvate as carbon source (10). The PF6 medium was used for scaling up production of 10-KSA by NRRL NRS-732 from shake flasks to a fermentor. The chemical structures of both bioconversion products, 10-HSA and 10-KSA, were confirmed previously by GC–MS and NMR (9,10).

FA bioconversion in the batch reactor was accompanied by uncontrollable foaming during the reaction because of an actively growing microbial culture, the hydrophobic nature of FA substrates, and the surface-active property of the converted products. Adding antifoaming agents alone to the fermentation broth failed to control foaming. A new aeration mechanism, in which aeration was supplied continuously at 220 mL/min from the top of the reactor headplate and periodically at much low rates (15–75 mL/min) through a bottom sparger, in conjunction with the use of marine impellers (13), was designed to achieve the desired D.O. levels and control foaming. A defined amount of Biospumex 153 K defoamer, proven beneficial in preliminary experiments, was also added to the culture broth at the addition of FA mainly to enhance dispersion of the hydrophobic substrates in the aqueous culture medium.

A time course of 10-HSA production in the reactor process is shown in Figure 1. In the fermentor production of 7,10-dihydroxy-8(*E*)-octadecenoic acid by *P. aeruginosa* NRRL B-18602 (13) and 7,10,12-trihydroxy-8(*E*)-octadecenoic acid (TOD) by *P. aeruginosa* NRRL B-23260 (14), vigorous agitation using two impellers at 650 and 600 rpm, respectively, was required to increase the production yield. However, agitation above 450 rpm using only one marine impeller for 10-HSA production by *S. thalpophilum* NRRL B-14797 caused a decrease in yields of more than 80%. Although the level of D.O. continued to rise during TOD production (14), the increase in the level of TOD product ceased early in 30 h of reaction. In comparison, the level of 10-HSA produced by *S. thalpophilum* NRRL B-14797 continued to increase gradually during the fermentation (Fig. 1). Under moderate agitation and aeration conditions, D.O. levels began to rise after 24 h and declined sharply to approach zero after 65 h of reaction. At 96 h of reac-

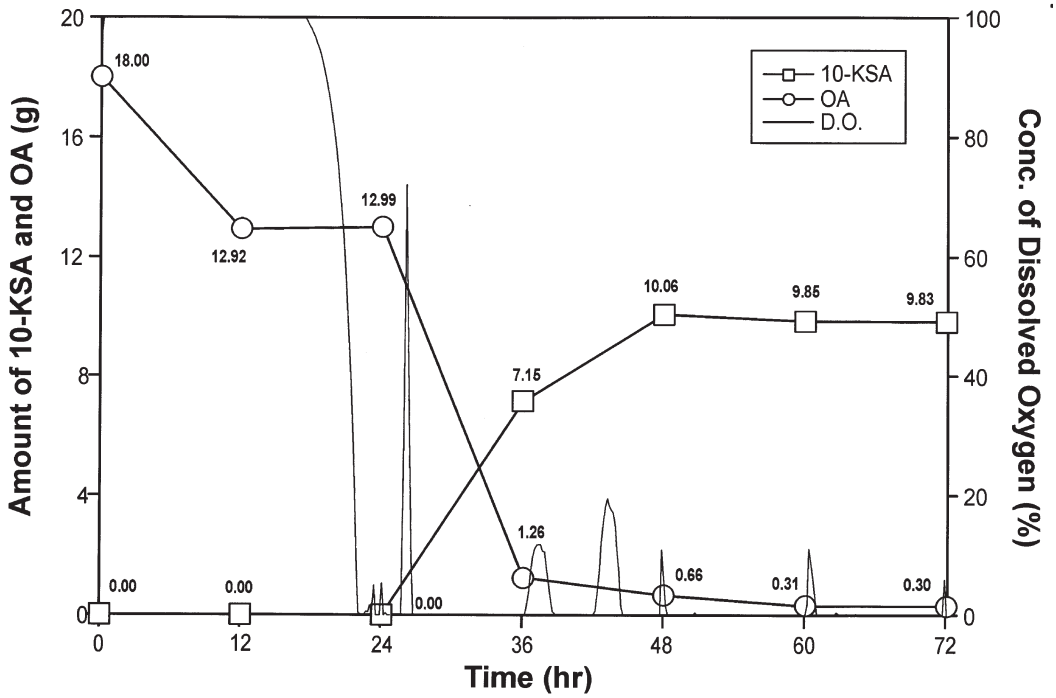

FIG. 2. The scaleup production of 10-ketostearic acid (10-KSA) from oleic acid (OA) conversion by *Bacillus sphaericus* NRRL NRS-732 in a 2-L stirred batch reactor. The bioconversion was carried out in 1.25 L PF6 medium (see Table 1 for description) with 12-h-old culture at 30°C under 750 rpm agitation provided by two marine impellers and a regulated airflow through a bottom sparger. The sparger airflow was maintained for 15 min in every 30 min at 10–15 mL/min during the culture growth. The bioconversion was initiated by adding 18 g of OA and 300 ppm Biospumex 153 K to the reactor culture that was subsequently adjusted to pH 8.0 as the sparger was turned off and stayed off for the entire course of conversion reaction. Conditions were the same as described in Figure 1.

tion, 10-HSA accumulated to more than 7 g in the reactor broth with a yield of about 40% (Fig. 1). 10-HSA subsequently was purified by a crystallization process from ethyl acetate at –20°C according to the basic procedures described previously (13) except that the volumes of culture broth and solvent were proportionally scaled down for use of 1–2 L separatory funnels. A total of about 3.5 g of the dried crystals (98% purity) was collected after filtration.

In the production of 10-KSA from OA by *B. sphaericus* NRRL NRS-732, the yields remained below 13.5% in a reactor broth containing 10-h-old cultures grown at 28 or 30°C in PF6 medium agitated with a marine impeller at 350 rpm, regardless of sparger airflow, sparging frequency, and the addition of Biospumex 153 K defoamer. Subsequent modifications of agitation speed and sparger aeration revealed that *B. sphaericus* favored vigorous agitation but little sparger aeration during production of 10-KSA (Fig. 2). As OA substrate and the defoamer were introduced to the culture broth, D.O. levels remained high (70–90%), then rapidly increased to 100%. Subsequently, the D.O. remained at 100% until about 18 h of reaction without sparger aeration. Thereafter, as 10-KSA began to accumulate the D.O. concentration sharply decreased and remained at low levels. The level of 10-KSA reached a maximal amount in the reactor broth after 48 h of reaction.

The influence of agitation and aeration on the conversion of OA to new products was strikingly different between *S. thalpophilum* NRRL B-14797 and *B. sphaericus* NRRL NRS-732 in the stirred batch reactor process. After 72 h of reaction, as OA substrate was depleted to a minimal amount, nearly 10 g of 10-KSA was produced, representing a yield of more than 54%. Therefore, the fermentor reached a production yield close to the 60% yield obtained in small shake flasks (10). 10-KSA was subjected to crystallization as described above and was fully recovered from ethyl acetate solution at greater than 97% purity. The crystallization of 10-HSA led to much lower recoveries, about 50%, probably owing to the presence of OA substrate in the solvent extract. This was because OA conversion for the 10-HSA production was much slower than that for 10-KSA (Figs. 1, 2).

Purified 10-HSA and 10-KSA were used as starting materials to produce the corresponding primary amides by known methods of lipase-catalyzed amidation with ammonia in an organic solvent (15–17). Using our purified 10-HSA and 10-KSA, we obtained initial rates of amide formation of 28.5 ± 0.0 and 31.3 ± 8.7 mmol min^{-1} g $enzyme^{-1}$, respectively. The yields for producing the putative 10-hydroxystearamide and 10-ketostearamide were 94.0 ± 0.03 and 92.1 ± 4.5%, respectively. These yields are comparable with the enzymatic production of oleamide, ricinoleamide, and lesquerolamide reported previously (15). The amide products of 10-HSA and 10-KSA were purified by crys-

tallization from ethyl acetate at about 35°C and subjected to GC–MS analysis.

Neither of the mass spectra of the purified products displayed significant molecular ion peaks. Both spectra showed strong characteristic primary amide peaks at *m/z* 59 and 72, resulting from McLafferty rearrangement and γ-cleavage, respectively (18). The spectrum of the 10-HSA product displayed prominent peaks at *m/z* 157 and 186. These fragments are consistent with cleavage on either side of the hydroxyl group (19). The spectrum also contained an abundant ion at *m/z* 169, possibly due to further decomposition of the *m/z* 186 peak by the loss of ammonia, analogous to the loss of methanol from methyl esters described by Murphy (19). In addition to the characteristic primary amide peaks, the spectrum of the 10-KSA product displayed a base peak at *m/z* 142, which was likely the result of β-cleavage to the keto group (19). Other peaks present in the spectrum at *m/z* 156 and 199 were consistent with McLafferty rearrangement ions resulting from cleavage on either side of the carbonyl group, while α-cleavage to each side of the carbonyl would result in peaks seen at *m/z* 141 and 184 (19). The results indicated the formation of 10-hydroxystearamide and 10-ketostearamide, respectively, by lipase-catalyzed direct amidation of 10-HSA and 10-KSA.

The melting point of 10-hydroxystearamide and 10-ketostearamide were 115 and 120°C, respectively. Both were substantially higher than their corresponding free FA of 84 and 83°C. This could make the newly prepared fatty amides useful in the formulation of special lubrication applications.

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