Note

Production of Mannosylerythritol by *Candida* sp. KSM-1529Tohru KOBAYASHI, Susumu ITO
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It has been demonstrated that *Candida* sp. B-7 produces a unique glycolipid, mannosylerythritol lipid (MEL), during the fermentation of alkanes.¹⁾ This glycolipid is a mixture of the 2',6'-di-*O*-acyl ($C_8 \sim C_{14}$) esters of 4-*O*- β -D-mannosyl-D-erythritol (ME). The biological significance of MEL produced by the yeast may be related to the mechanism for the uptake of alkanes. In fact, *Torulopsis bombicola* produces sophorose lipid as a growth stimulant when it is grown on insoluble alkanes.^{2,3)}

Recently, we found that *Candida* sp. KSM-1529 produces a large amount of ME under certain growth conditions. In this paper, we describe these growth conditions and some biological characteristics of ME.

Candida sp. KSM-1529 (Strain b-1) is one of a series of mutants derived from the parental *Candida* sp. S-10⁴⁾ which produces itaconate from alkanes, and was kindly provided by Drs. T. Tabuchi and T. Nakahara of University of Tsukuba, Japan. *Candida* sp. KSM-1529 was recognized as a producer of MEL, at concentrations from 35 to 50 g/l, when it was grown in a broth which contained plant oils or alkanes (our laboratory, unpublished data). The production of ME by this yeast was first suggested by the observation that replacement of plant oils or alkanes with glucose caused the accumulation of an unknown polyol in the medium. The formation of ME was ascertained by thin-layer chromatography (Silica Gel G) with 1-butanol-acetic acid-ethyl ether-water (9:6:3:1, v/v) as a solvent. The ME spot was located by spraying the plates with Yoda's reagent,⁵⁾ and corresponded to an *R_f* value of 0.14. ME was measured by high-performance liquid chromatography on a Hitachi 655LC system with a TSK gel G-Oligo PW column (Toyo Soda Kogyo Co., Ltd.) and a Shodex RI-SE31 detector (Showa Denko Co., Ltd.). Elution was done with 5% (v/v) methanol at a flow rate of 0.7 ml/min, at room temperature.

The seed medium contained (per liter) 25 g of glucose, 5 g of casamino acids, 0.5 g of KH_2PO_4 , 0.5 g of $MgSO_4 \cdot 7H_2O$, and 0.5 g of yeast extract, and was made up with distilled water; it was pH 6.0. The fermentation medium for the production of ME, also pH 6.0, contained

(per liter) 100 g of glucose, 5 g of casamino acids, 0.1 g of KH_2PO_4 , 0.2 g of $MgSO_4 \cdot 7H_2O$, and 0.5 g yeast extract, and was made up with distilled water. Complete fermentation medium (100 ml) was placed in 500-ml Sakaguchi flasks and incubated aerobically at 26°C for one week on a reciprocal shaker (125 strokes/min) with 2% of a 2-day-old seed culture.

ME was isolated from the spent medium by removing the cells by centrifugation, then putting one liter of the supernatant, acidified to pH 4.5 with acetic acid, on a column of activated charcoal (8 × 50 cm). The column was washed with 6.0 l of distilled water and then eluted with 25% (v/v) ethanol, and fractions were checked for the presence of ME by thin-layer chromatography. The fractions containing the material were pooled and concentrated *in vacuo* below 50°C. The resulting concentrate (16.2 g) was further purified by chromatography on an activated charcoal column (5 × 55 cm) with 10% (v/v) ethanol as an eluent. The eluted material was concentrated *in vacuo* to yield 14.9 g of ME, which was pure by thin layer and high-performance liquid chromatographies. The resulting material was colorless and transparent, and contained 8.9~17.0% water as estimated by the Karl-Fisher method.

The structure of the isolate was characterized by ¹³C-NMR, FD-MS spectrometry, infrared (IR) spectrophotometry and other methods. NMR^{DMSO-d₆}_{TMS} δ_C at 60°C were: 101.3 (C-1' β -D-mannopyranoside), 78.4 (C-5'), 74.6 (C-3'), 73.3 (C-2), 72.1 (C-4, *O*-substituted C of erythritol), 71.6 (C-3), 71.5 (C-2'), 68.2 (C-4'), 64.2 (C-1), 62.4 (C-6') and 39.7 (methyl C of DMSO-d₆). Results from FD-MS gave the following *m/z* values: 285 (*M*+1)⁺, 569 (2*M*+1)⁺. Infrared spectrophotometry gave the following values for IR γ_{max} (NaCl) cm⁻¹: 3400 (OH), 2940 and 2840 (—CH₂—), 1075 (—O—), 1050 (primary OH), 898 (β -configuration of mannopyranoside). When the isolate was acid-hydrolyzed, high-performance liquid chromatography yielded two peaks which corresponded to mannose

FIG. 1. Production of ME by *Candida* sp. KSM-1529.

Experimental conditions are described in the text. Cell growth was monitored by measurement of the absorbance at 600 nm (*A*₆₀₀) of the medium at appropriate dilutions, made with chilled water.

and erythritol with a stoichiometry of almost 1:1. In addition, the isolate could be hydrolyzed by β -mannosidase from *Achatina fulica*, but not by α -mannoidase from jack beans. These results coincided with those obtained after saponification of MEL. The structure of 4-O- β -D-mannosyl-D-erythritol (ME), as derived by us, is probably the same as that reported by Boothroyd *et al.*⁶⁾ who isolated it from cultures of the fungus *Ustilago*, and that of ME synthesized chemically by Gorin *et al.*⁷⁾

As shown in Fig. 1, the greatest yield of ME was observed after 7 days of incubation (about 22 g/l), while the cell mass reached a plateau after 3 days. Mannose or fructose could replace glucose entirely, and glycerol, erythritol, mannitol, or galactose could partially replace glucose, to give a yield of ME of 7.0~18.1 g/l. However, other usable carbohydrates such as ribose, arabinose, rhamnose, sorbitol, and dulcitol did not support the accumulation of ME in the medium.

These results suggest indirectly that extracellular lipids with the surfactant-like structures are essential for the uptake of alkanes by microorganisms, and that the function of the lipids may be attributed to their emulsifying activities in the oil-water phase. In fact, microorganisms which grow on alkanes excrete surfactant-like materials, such as trehalose lipid,⁸⁾ peptide lipid,⁹⁾ and rhamnose lipid,¹⁰⁾ in addition to the sophorose lipid produced by *Torulopsis bombicola*.^{3,11)} Therefore, ME is probably synthesized by *Candida* sp. KSM-1529 as the direct precursor of MEL. ME is thought to be acylated, only when the organism is grown on alkanes or triglycerides, and then be

excreted into the extracellular environment to emulsify the insoluble substrates.

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