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SOPHOROLIPIDS FROM *TORULOPSIS BOMBICOLA* AS MICROBIAL SURFACTANTS IN ALKANE FERMENTATIONS

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ABSTRACT

The possible role of sophorolipids from *Torulopsis bombicola* was investigated in alkane fermentation. Sophorolipids and related model compounds specifically stimulated the growth of strains of *Torulopsis* yeasts on insoluble alkanes and may act as a specific growth factor. There may be more than one way for a yeast to be stimulated to incorporate alkanes for growth.

INTRODUCTION

In alkane fermentation by yeasts, production of extracellular glycolipids has often been observed (Stodola *et al.*, 1967; Iguchi *et al.*, 1969), but the biological role of these extracellular lipids has not yet been investigated and they have been thought of as secondary metabolites (Bentley & Campbell, 1968). At the same time in biochemical engineering, another characteristic is that the main test organisms in alkane fermentation for SCP production are *Candida* yeasts (Erickson & Nakahara, 1975). Suggestions have been made that too much attention has focused on these organisms and that other organisms should presumably be examined.

Sophorolipids produced by *Torulopsis* sp. were found by Gorin *et al.*(1961). Tulloch *et al.*, (1968) studied fractions of the glycolipid mixture from *T. apicola*(later classified as *T. bombicola* by Spencer *et al.*,1970), produced during fermentation of octadecane. They were composed mainly of 17-L-[(2'-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]octadecanoic acid 6',6"-diacetate, its 1,4"-lactone, and the 6"-monoacetate of the lactone. Recently, a strain of *T. bombicola*, capable of producing a large quantity of sophorolipid, was isolated in this laboratory. This strain grew poorly on insoluble alkanes in a simple semi-synthetic medium, but very well in an enriched complex medium. This study was initiated to clarify the mechanism by which *Torulopsis* yeasts incorporate the alkanes. The sophorolipid was suggested to be required for the growth of the yeast strains on alkanes as a specific biosurfactant by an unidentified mechanism.

EXPERIMENTAL

Organisms: *T. bombicola* ATCC 22214 (as used by Gorin *et al.*, 1961 and Tulloch *et al.*, 1968) and *T. bombicola* KSM-36 were used extensively. The latter strain was isolated from a cabbage leaf in this laboratory. This yeast excretes the same principal fractions of sophorolipid mixture as those produced by *T. bombicola* ATCC 22214, at concentrations reaching 120-130 gr/l, when grown on a glucose medium enriched with plant oils such as safflower oil, instead of octadecane (unpublished data). Other yeasts used for this experiment are described in the text.

Media and cultivations: The following simple YNB medium were used. The YNB medium consisted of the following components (gr./l): Yeast Nitrogen Base, 7.5; yeast extract, 2.0; carbon source, suitable amount; and distilled water (pH 5.6). One hundred-milliliter portions of the growth medium were placed in 500-ml Sakaguchi flasks, or 10-ml portions in test tubes (23 x 200 mm). For inocula from YM slants, the cell suspension was standardized to 0.5 - 0.6 absorbance unit at 650 nm (A_{650}). Flasks or test tubes of the growth medium were incubated aerobically at 30 C on a reciprocal shaker or a test tube rotary shaker with 0.2% of the standardized cell suspension.

Growth measurement: Cell growth was monitored by measuring A_{650}, using appropriate dilution with chilled saline. To remove insoluble carbon sources which interfered with A_{650} readings, the cultured media were cooled to 4°C to solidify the carbon sources on the medium surfaces, and then extracted gently with chilled ethyl acetate. Measurement of growth in terms of cell mass was also carried out. Cells from a known volume of cultured media were centrifuged at 20,000 rpm and washed twice with chilled saline; the cell paste was dried at 80°C for several days and weighed. A_{650} reading of 1.0 corresponded to approximately 520 and 500 mg dry cell/l for *T. bombicola* KSM-36 and *T. bombicola* ATCC 22214, respectively.

Fermentation of sophorolipids: To obtain a crude sophorolipid mixture, *T. bombicola* KSM-36 was used. The growth conditions and fermentation medium were those used previously (Ito *et al.*, 1980). The sophorolipids were isolated by extraction with ethyl acetate. The resulting brownish paste was washed with hexane to remove the remaining safflower oil, and the washed material was dried to yield crude sophorolipid mixture (thereafter designated as SL) (32g. from one liter of fermentation medium).

Preparation of related model compounds: 17-L- (2'-0- β-D-glucopyranosyl-β-D-glucopyranosyl)oxy octadecenoic acid (SL-acid type) was prepared by alkaline hydrolysis of SL and C_1-C_{10} SL esters (SL-methyl to decyl esters) were then prepared by conventional methods. α-SL-ethyl ester (1'-α-glucoside), hydrogenated SL-ethyl ester, and SL-methyl ester heptaacetate were synthesized according to Tulloch *et al.*, (1968).

Chemicals: Yeast Nitrogen Base and yeast extract were from Difco and Daigo Eiyo (Japan), respectively. Forty-three nonionic surfactants including 14 polyoxethylene (POE) alkylethers (hydrophile-lipophile balance values with 7.5-19.5), 5 POE nonylphenylethers (9.8-18.9), 3POE alkylesters (13.4-13.7), 8 POE-sorbitol oleylesters (3.6-13.8), 3 Tweens (11.5-15.6), 3 Spans (4.3-6.7), and 7 sugar esters (3-16) were of Kao Soap (Japan) production. Alkanes and other chemicals were purchased from Wako Pure Chemicals (Japan).

RESULTS

Stimulation of growth on alkanes by sophorolipids: Using *T. bombicola* KSM-36 and *T. bombicola* ATCC 22214, growth responses on a number of carbon sources were examined in YNB medium. Despite having the ability to grow on fatty acids, fatty alcohols, glucose, and glycerol, these strains were found to grow poorly on C_8 - C_{22} alkanes. Especially, the C_8 - C_9 and C_{14} - C_{22} alkanes were hardly attacked, whereas the C_{10} - C_{13} alkanes supported growth (Table 1). When some organic nutrient, such as peptone and corn steep liquor, was added, both strains grew very well on the C_{10} - C_{20} alkanes, accompanied by extracellular production of sophrolipids. Since the glycolipid mixture had surfactant-like structure, it was both interesting and important whether the glycolipids act as a growth stimulant for *Torulopsis* on alkanes. In the presence of SL-methyl ester, growth of *T. bombicola* on C_{10} - C_{20} alkanes was accelerated uniformly. Growth was consistently higher than in the absence of SL-methyl ester, but growth on C_8, C_9, and C_{22} was not observed under the conditions used. As shown in Table 2, SL and related model compounds such as SL-methyl ester, SL-ethyl ester, and SL-acid type, stimulated the growth of *T. bombicola* KSM-36 and *T. bombicola* ATCC 22214 on hexadecane. SL-methyl ester heptaacetate was inert. SL-methyl and SL-ethyl esters gave strong enhancing effect, but final cell yields with the synthetic SL-alkyl esters were almost the same as that with SL. Hydrogenated SL-ethyl ester and its stereoisomer, α-SL-ethyl ester, also gave the same levels of growth, showing that strict stereospecificity is not required for the growth stimulating activity.

TABLE 1. Growth of T. bombicola KSM-36 on Alkanes having C_8 to C_{22}.

n-Alkane (2%)	Growth (gr. dry cells/l)*					
	No addition			SL-methyl ester added (0.02%)		
	Incubation time (days)					
	4	7	10	4	7	10
C_8	0.0	0.0	0.0	0.0	0.0	0.0
C_9	0.0	0.0	0.0	0.0	0.0	0.0
C_{10}	1.03	3.00	3.52	1.70	3.88	4.13
C_{11}	1.45	3.56	4.55	1.88	6.30	8.06
C_{12}	3.38	6.87	8.26	4.16	9.83	10.40
C_{13}	0.36	2.17	6.16	3.64	6.84	8.26
C_{14}	0.0	0.0	1.00	4.00	10.26	10.79
C_{15}	0.0	0.0	0.53	1.39	9.96	10.45
C_{16}	0.0	0.0	1.32	4.16	10.30	10.44
C_{18}	0.0	0.0	0.77	1.96	5.38	7.34
C_{20}	0.0	0.0	0.0	0.52	0.77	0.96
C_{22}	0.0	0.0	0.0	0.0	0.0	0.0

*T. bombicola ATCC 22214 gave similar growth responses.

TABLE 2. Effect of Sophorolipid and its Related Model Compounds on the Growth of T. bombicola with Hexadecane.

Supplementation (mM)	Growth (gr. dry cells/l/5 days)	
	KSM-36	ATCC 22214
No addition (0.40)	0.33	0.35
SL (0.40)	2.96	2.75
SL-Acid type (0.40)	1.45	1.30
SL-Methyl ester (0.40)	4.42	4.05
SL-Ethyl ester (0.40)	4.25	3.92
SL-Ethyl ester (0.62)	3.60	3.88
Hydrogenated SL-ethyl ester (0.62)	3.60	3.59
~~d~~-SL-Ethyl ester (0.62)	3.44	3.18
Sl-Methyl ester hepta-acetate (0.62)	0.25	0.38

A number of exogenous additives (e.g., vitamins, trace metals, purine and pyrimidine bases, amino acids, or DL-carnitine added individually up to 3 mM each) did not serve as an alternate growth stimulator for either strain of *T. bombicola* in YNB medium (data not shown). Nor did any of the nonionic surfactants tested (see EXPERIMENTAL), added at 0.02%.

Effect of alkyl chain length of the model compounds: As shown in Fig. 1, SL-acid type and short chain C_1 - C_5 SL esters stimulated the growth of *T. bombicola* KSM-36 on hexadecane, whereas longer chain C_6 - C_{10} SL esters did and easily solubilized in YNB medium while the inert SL-alkyl esters were practically water-insoluble. These results may indicate that hydrophilic character of SL-alkyl ester plays an important role for the uptake of alkanes by *T. bombicola*.

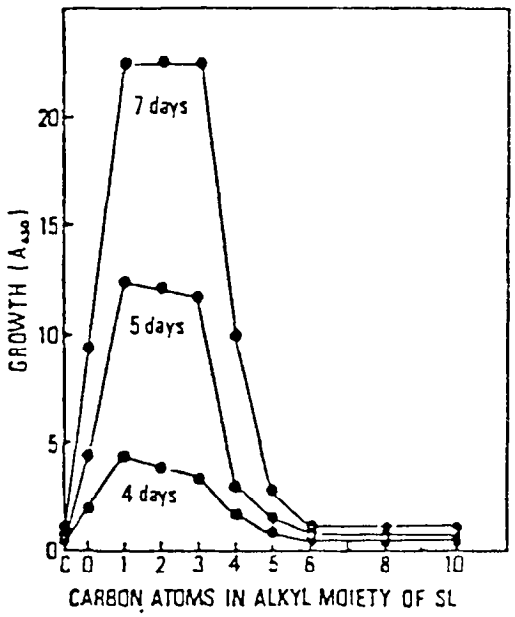

Fig. 1. Time course of growth of T. bombicola KSM-36 on hexadecane as a function of the alkyl chain length of SL. Each SL-alkyl ester was added at 0.02%. C: cultured without SL.

Surface activities of sophorolipids: To demonstrate surface activities of SL-alkyl esters used for this experiment, an aqueous solution of each glycolipid, in varying concentration, was placed in contact with an equal volume of hexadecane in test tubes and shaken vertically. Emulsion type was determined by conductivity, visual observation, and/or dye solubility. All emulsions in the hexadecane-water system were of oil in water type and not stable at 30 C. Especially, the emulsions with SL-acid type and C_1 - C_5 SL esters were extremely unstable.

Critical micelle concentration and surface tension were 3.2×10^{-2}% and 34.8 dynes/cm for SL-acid type; 4.5×10^{-3} and 38.0 for SL-methyl ester; 2.0×10^{-3} and 40.0 for SL-ethyl ester; 2.9×10^{-3} and 43.5 for hydrogenated SL-ethyl ester; 2.9×10^{-3} and 42.0 for -SL-ethyl ester: 1.4×10^{-3} and 39.5 for SL-propyl ester;[3] 7.6×10^{-4} and 37.5 for SL-butyl ester; 4.6×10^{-4} and 36.5 for SL-amyl ester; 3.2×10^{-4} and 36.5 for SL-hexyl ester; 2.8×10^{-4} and 35.8 for SL-octyl ester; and 5.3×10^{-4} and 38.5 for SL-decyl ester.

Effect of sophorolipids on the growth of various yeasts: With hexadecane as sole carbon source, the growth responses to SL and SL-ethyl ester were examined for various alkane-utilizing yeasts. Growth stimulation by SL and/or SL-ethyl ester was almost exclusively seen with *Torulopsis* yeasts such as *T. bombicola*, *T. apicola*, *T. gropengiesseri*, *T. magnoliae*, and *T. candida*. In contrast, the growth of most strains of typical alkane-utilizing yeasts such as *Candida*, *Pichia*, *Debaryomyces*, *Endomyces*, *Saccharomycopsis*, and *Lodderomyces* was inhibited considerably or not affected (data not shown).

DISCUSSION

In order to understand the availability and utilizability of alkane droplets by yeasts, three different hypotheses have been presented: (1) by direct contact of the cells with large oil drops (Einsele *et al.*, 1973); (2) contact with the fine oil droplets (accommodated oil) (Aiba *et al.*, 1969; Moo-Young *et al.*, 1971); uptake of the dissolved alkanes (Gomma *et al.*, 1973). This divergence may be due to the use of different yeast strains or under different culture conditions. Despite the extensive study of alkane-utilizing yeasts for SCP production, *Candida* yeasts have been the most intensively studied organisms in the field of biochemical engineering. Protrusions (slime-like outgrowths) (Osumi *et al.*, 1975), to which alkanes presumably adhere, were observed electron microscopically on the surface of alkane-grown *C. tropicalis*. Käppeli *et al.*, (1977, 1978) demonstrated that alkane microdroplets adhered nonenzymatically to the cell wall of alkane-grown *C. tropicalis* and that a polysaccharide-fatty acid complex isolated from the cell wall was responsible for this affinity. In these cases, the hydrohobicity or strong affinity to alkanes of cell surface may be one important factor for facilitating the transport of the substrates to the site of alkane oxidation inside the cells.

In the present study, it is shown that sophorolipid may act as a specific growth stimulant for *T. bombicola* on insoluble alkanes. The authors have shown that the observed growth rates of typical alkane-utilizing *Candida* yeasts on alkanes decrease with addition of SL (1980). In addition, *in vivo* alkane oxidation in *T. bombicola* (Heinz *et al.*, 1969) is presumed to be analogous to that in *Candida* yeasts (Coon *et al.*, 1973; Azoulay *et al.*, 1976). Therefore, it seems very important to study the nature of nonionic C_1 - C_5 SL esters in order to reduce confusions and to explore their stimulative effect on the uptake of alkanes by *Torulopsis* yeasts. SL-ethyl ester and its stereoisomeric derivatives give the same levels of growth, suggesting that sophorolipid is involved in a nonenzymatic process. Synthetic surfactants which improve growth rates have been found by Tanaka & Fukui (1971), Wang and Ochoa (1972), and Whitworth *et al.*, (1973). Such surfactants frequently act to emulsify alkanes, decrease the interfacial tension, and increase the interfacial area. However, a number of synthetic nonionic surfactants (see EXPERIMENTAL) were unable to replace the SL-alkyl esters, which themselves have no emulsifying activity for alkanes. In addition, the cell wall of *T. bombicola* had no binding affinity to hexadecane during growth on the alkane in the presence of the glycolipids (unpublished data).

Although available evidence is not sufficient to explain the function of sophorolipid, these results suggest that the basic structure of the glycolipid is plausibly a specific growth stimulant of *Torulopsis* yeasts on alkanes, and that mechanism for uptake of the substrates in the alkane fermentation by the yeasts remains unidentified.

A broth of a strain of *Candida* containing peptide-lipid has been reported to stimulate slightly the growth of the yeast on alkanes (Iguchi *et al.*, 1969), but it is not clear that the lipid itself functioned as the growth stimulant. *Torulopsis* yeasts seem to be the first such yeasts so far shown to require a specific biosurfactant for growth on alkanes.

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