Production and Toxicity Assessment of Sophorosides from *Torulopsis bombicola*

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Production of sophorosides from *Torulopsis bombicola* cultivated on three different substrates (methyl oleate, olive oil, and rapeseed oil) was studied. The higher yield (60–70%) was obtained with olive oil, which also corresponds to the less expensive lipid source. Sophorosides did not show great antifungal and antibacterial activities except against *Candida parapsilosis* and *Staphylococcus aureus*. Other tested microorganisms were resistant to and sometimes stimulated by these compounds. Cytotoxicity of sophorosides on cultured human fibroblasts (human skin or MRC5 line) was almost the same as with cholesterol acetate but higher than with DL-α-lecithin. Moreover, on skin fibroblasts LC_{50} was near LC_{100} with sophorosides, indicating the possibility of acute toxicity in some cases.

INTRODUCTION

A number of microorganisms (bacteria, yeasts) are capable of emulsifying hydrocarbons during the substrate degradation process (Hisatsuka et al., 1971; Kretschmer et al., 1982; Inoue and Ito, 1982; Ito and Inoue, 1982). The production of extracellular glycosides by *Torulopsis* sp. was investigated by Gorin et al. (1961). When long-chain fatty acids are fermented by a yeast of this genus, the acids are hydroxylated at the penultimate or terminal carbon atom and the hydroxy acids produced are converted into sophorosides (Tulloch et al., 1962). Production of sophorosides from *Torulopsis bombicola* has been studied by using different carbon sources (Cooper and Paddock, 1984). However, the biological role of these extracellular glycolipids has been poorly investigated except for biosurfactant properties (Inoue and Ito, 1982), and they have been thought of as secondary metabolites (Bentley and Campbell, 1968) as a function of their production in the late exponential growth phase (Cooper and Paddock, 1984).

In this paper, we consider the production of sophorosides from *T. bombicola* cultivated on three different lipidic substrates, and because of their possible use as emulsifiant in food or pharmaceutical products, some biological properties (antifungal, antibacterial) of these compounds were investigated as well as their cytotoxicity.

MATERIALS AND METHODS

Microorganism. *T. bombicola* ATCC 22214 (Spencer et al., 1970) was obtained from the American Type Culture Collection (ATCC, Rockville, MD). The strain was maintained on honey (30%), yeast extract (0.25%), and agar (2.5%) medium at 4 °C and reactivated on the same medium before use.

Chemicals. Yeast extract and agar were purchased from Difco Laboratories (Detroit, MI), and olive and rapeseed oils came from a grocery. Methyl oleate was from Fluka (Buchs, Switzerland), cholesterol acetate and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma Chemical Co. (St. Louis, MO), synthetic DL-α-lecithin was from Nutritional Biochemicals Corp. (Cleveland, OH), and cell culture reagents were from Gibco (Grand Island, NY). Other products were purchased from Prolabo (Paris, France).

Culture Conditions. The medium (GYU) used for cultivation was adapted from the procedure of Cooper and Paddock (1984): glucose (10%), yeast extract (1%), and urea (0.1%), pH 4.0 (with 5 N H_2SO_4). Controlled cultivation was conducted in a 2-L Biolaffite fermentor using 1 L of working volume, under the following running conditions: temperature, 23 °C; agitation, 400 rpm; air flow, 1 L/min. Medium was sterilized by autoclaving at 120 °C for 20 min. To obtain sufficient inoculum, *T. bombicola* was first grown for 1 day at 23 °C in a 250-mL Erlenmeyer flask containing 50 mL of GYU medium and agitated on a rotary shaker (180 rpm). The cell suspension was standardized to 0.6 absorbance unit at 650 nm and inoculated into the fermentor. Lipidic substrate was added 2 days later (17 g) and every 4 successive days.

Extraction of Sophorosides. One day after the last lipidic substrate addition, the medium was decanted from the sludge of cells and heated to 70 °C. Most of the sophorosides were removed as a yellow-brown viscous syrup; the aqueous phase soluble sophorosides were extracted three times with equal volumes of ethyl acetate. The resulting brownish paste was washed three times with equal volumes of hexane to remove the remaining oil, and the insoluble residue was dried at 40° C under reduced pressure to yield a crude sophoroside mixture. The crude extract was analyzed by thin-layer chromatography according to the method of Cooper and Paddock (1984). The mobile phase was chloroform–methanol–water (65:15:2 v/v). The plates were Fisher Redi-Gel silica gel GF. Sophorosides were visualized by using a solution of α-naphthol.

Antifungal and Antibacterial Activities. Sophorosides obtained from fermentation with olive oil were used in this study. The pathogenic test organisms (Tables 1 and 2) included five bacteria, seven yeasts, seven dermatophytes, and five phytopathogens. Activity against yeasts and antibacterial properties were assessed by a microtitration method using 96-well microtitration culture plates as described before (Okeke et al., 1992). Yeasts and bacteria were first grown, respectively, on malt extract (1.5%) and agar (1.5%) (MEA) and yeast extract (0.25%), tryptone (0.5%), glucose (0.1%), and agar (1.5%), pH 7.2, media for 2–4 days at 37 °C. Microorganisms were suspended in the same filtered liquid media (OD_{600} = 1.5). Each well contained 200 μL of liquid medium, 10 μL of suspension of target microorganisms, and 10 μL of sophorosides or reference products (ketoconazole and kanamycin) (final concentrations from 0.1 to 5.0 g/L) solubilized in ethanol (final concentration did not exceed 5%). Controls included free medium and medium plus microorganisms with or without ethanol (10 μL). Incubation was run at 37 °C on a shaker (250 rpm). Each test was performed four times. Growth was evaluated after 24 and 48 h by recording OD_{600} with a multiwell scanning spectrophotometer (JBio, Paris, France). For sophorosides, the concentration inhibiting 50% of growth (IC_{50}) was calculated with the probit method. Results obtained with kanamycin and ketoconazole were expressed as minimal concentration giving 100% of inhibition [minimal bacteriostatic concentration (MBC)] and as minimal concentration giving 100% of cytotoxicity [minimal lethal concentration (MLC)]. Viability of microorganisms was assessed by pouring well suspensions onto appropriate solid media in Petri dishes.

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Table 1. Sensitivity of Seven Pathogenic Yeast Strains and Five Pathogenic Bacterial Strains to Sophorosides and Ketoconazole or Kanamycin in Shaken Liquid Medium

strain	IC_{50}[d] (g/L)	viability (0–5 g/L)	ref, ketoconazole (g/L) MFC[e]	MLC[f]
yeasts[a]				
C. albicans	0.62 < 1.02 < 1.69	+	0.20	0.30
Candida glabrata	0.19 < 0.29 < 0.43	+	nd[g]	
C. parapsilosis	0.08 < 0.18 < 0.40[h]	+	>0.30	>0.30
C. tropicalis 2R Ampho	0.28 < 0.53 < 1.00	+	0.15	>0.30
C. tropicalis	0.25 < 0.47 < 0.88	+	nd	
Cryptococcus neoformans 1	<0.1	+	0.05	0.10
Cryptococcus neoformans 2	2–3[h]	+	nd	

strain	IC_{50} (g/L)	viability (0–5 g/L)	ref, kanamycin (g/L) MBC[i]	MLC
bacteria				
E. coli	nd[h]	+	0.05	0.10
P. aeruginosa				
24 h	0.33 < 0.52 < 0.81	+	0.10	0.20
48 h	0.90 < 1.19 < 1.58	+		
S. aureus[b]				
24 h	<0.1	+	0.10	0.15
48 h	<0.1	+		
S. aureus[c]				
24 h	<0.1[j]	+	1.00	>1.00
48 h	<0.1[j]	+		
S. faecalis				
24 h	nd[h]	+	1.00	>1.00

[a] Results recorded after 48 h of incubation at 37 °C. [b] Penicillin resistant. [c] Penicillin sensitive. [d] IC_{50}, 50% inhibition concentration with 95% confidence boundaries. [e] MFC, minimal fungistatic concentration. [f] MLC, minimal lethal concentration. [g] Not determined. [h] Growth stimulated by sophorosides. [i] MBC, minimal bacteriostatic concentration. [j] Growth partly inhibited by ethanol. [k] Growth totally inhibited by ethanol.

Table 2. Sensitivity of Five Phytopathogens and Seven Dermatophytes to Sophorosides and Ketoconazole on Solid Malt Extract Medium[a]

strain	IC_{50}[b] (g/L)	ketoconazole (g/L) MFC[c]	MLC[d]
phytopathogens			
Drechslera sp.	0.59 < 0.78 < 1.03	nd[e]	
F. oxysporum	1.41 < 1.80 < 2.30	nd	
G. candidum	1.57 < 1.93 < 2.38	nd	
P. oryzae	0.97 < 1.70 < 2.98	nd	
V. lecanii	nd[f]	nd	
dermatophytes			
E. floccosum	1.92 < 2.58 < 3.47	0.005	0.01
M. canis	0.95 < 1.26 < 1.67	nd	
T. mentagrophytes 1	2.34 < 3.18 < 4.34	0.001	>0.30
T. mentagrophytes 2	1.50 < 2.00 < 2.65	0.100	>0.30
T. mentagrophytes 3	1.64 < 2.25 < 3.10	nd	
T. rubrum	1.13 < 1.55 < 2.12	nd	
T. tonsurans	2.44 < 3.52 < 5.08	nd	

[a] Results recorded after 10 days of incubation at 24 °C. [b] IC_{50}, 50% inhibition concentration with 95% confidence boundaries. [c] MFC, minimal fungistatic concentration. [d] MLC, minimal lethal concentration. [e] Not determined. [f] Absence of inhibition.

Results were recorded after 24 h at 37 °C; occurrence of colonies was researched.

The effect of sophorosides on the growth of some dermatophyte and phytopathogen strains was investigated on MEA medium. The assay was performed as described by Guiraud et al. (1992a). Seven flasks of MEA medium were autoclaved, and various amounts of sterile ethanolic stock solution of sophorosides (200 g/L) or ketoconazole were added. Final concentrations of the different compounds were 0.1, 0.25, 0.5, 0.75, 1.0, 2.5, and 5.0 g/L (final ethanol concentration did not exceed 2.5%). Media were poured into Petri dishes (90 mm), and strains were grown on the dishes for 10 days at 24 °C. Growth was evaluated by measuring the diameter of colonies at 4, 7, and 10 days. Morphological modifications and variations in pigmentation were observed. For each strain, control cultures were set up on MEA without products or ethanol and MEA without products but with ethanol (2.5%). IC_{50} values were calculated with values recorded at 10 days. Experiments were performed in triplicate.

Cytotoxicity Assessment. Cytotoxicity of sophorosides toward cultured fibroblasts was assessed with a modified colorimetric micromethod described by Mosmann (1983). Human diploid fibroblasts were obtained by biopsies of healthy skin from shaved forearms. MRC5 fibroblasts (ATCC CCL171) came from Flow Laboratories (Uxbridge, Great Britain). Culture conditions, preparation of 96-well microplates for assay, and reagents were performed as previously described by Richard et al. (1992). For the assay, the culture medium was withdrawn and replaced in each well by 100 μL of medium containing 0.001, 0.01, 0.05, 1, 5, 10, 30, 40, 50, 70, and 100 mg/L of sophorosides or 10, 30, 40, 50, and 70, mg/L of cholesterol acetate or lecithin, added as sterile ethanolic solutions (ethanol concentration did not exceed 1%); pH and osmolarity of all solutions were systematically checked. Microplates were incubated for 24 h at 37 °C under a water-saturated sterile atmosphere containing 6% CO_2 (Forma scientific incubator, Marietta). Then, the plates were rinsed twice with tyrode buffer (100 μL/well) (Guiraud et al., 1992b), and living cells were evaluated by their ability to transform the yellow tetrazolium salt MTT into blue-black formazan via the action of dehydrogenases in active mitochondria. The test was conducted as previously described by Richard et al. (1992) with replacement of DMSO by 100 μL of 2-propanol to dissolve formazan crystals in each well at the end of the assay, and then microplates were shaken for 4 h before results were recorded. Blank, positive control (100% survival) and evaluation of ethanol toxicity were simultaneously performed. Lethal concentrations 50% (LC_{50}) were calculated with the probit method.

RESULTS AND DISCUSSION

Production of Sophorosides. The production of biosurfactants by microorganisms has been mainly studied in bacteria (Hisatsuka et al., 1971; Zajic et al., 1977; Rosenberg et al., 1979; Kretschmer et al., 1982; Cirigliano and Carman, 1984). Among micromycetes, a yeast, *T. bombicola*, is the most efficient (Cooper and Paddock, 1984).

Sophoroside production (Table 3) varied greatly following the type of lipid added in the medium. Several previous works (Spencer et al., 1962; Tulloch et al., 1962; Tulloch and Spencer, 1968) have shown that the best yields

Table 3. Effect of Lipidic Substrate on Yield of Sophorosides from *T. bombicola*

lipidic substrate	pH	total crude sophorosides (g/L)	sophoroside % of lipidic substrate	lipidic substrate not fermented (g/L)
olive oil	2.34	48.0	70.6	0
	2.35	40.0	58.8	0
rapeseed oil	2.50	10.0	14.8	51.5
	2.54	7.0	10.3	66.7
	2.56	9.0	15.0	56.6
methyl oleate	2.43	53.1	78.1	0
	2.55	45.9	76.5	0

were given by esters of C_{18} fatty acids (saturated or unsaturated), so we used only substrates rich in C_{18} fatty acids. The highest yields were with methyl oleate (78.1 and 76.5%) followed by olive oil (70.6 and 58.8%). Rapeseed oil gave a very low yield (mean 13.4%), and growth with this oil was the only one that left a high amount of unused lipidic substrate (>50%). The more oleic acid the oil contained, the more sophorolipids were produced. Olive oil contains 83.5% of this acid, and the yield obtained was high, close to that observed with methyl oleate.

According to a commercial point of view, using olive oil is the less expensive production way (6 time less than with methyl oleate). In these conditions, the cost of production of sophorolipids ($2.75/kg) is not much different from the cost of synthetic surfactants (Cooper and Paddock, 1984).

Biosurfactant properties of sophorosides from *T. bombicola* have been studied by Cooper and Paddock (1984). These authors have shown that the glycolipids caused substantial lowering of surface tensions or interfacial tensions but were not able to stabilize either water–hydrocarbon or water–vegetable oil emulsions.

Toxicity of Sophorosides. Sophorosides could be of value to a number of industries, so it is important to know their toxicity, little information has been available to date.

A first series of tests dealt with microorganisms: bacteria, yeasts, and filamentous micromycetes (dermatophytes and phytopathogens). Some of them were sensitive to ethanol (the best solvent to dissolve sophorolipids); it was the case for *Streptococcus faecalis* (100% inhibition), *Staphylococcus aureus* (85% inhibition), and *Trichophyton mentagrophytes* 3 (20% inhibition). Ethanol was not toxic for the other strains; moreover, growth was sometimes stimulated, for example, in *Candida tropicalis* (+10%), *C. tropicalis* 2R Ampho (+15%), *Candida parapsilosis* (+8%), and *Escherichia coli* (+32%). Effect of sophorosides was evaluated by comparison with ethanol controls.

In all cases, also when ethanol is an inhibitor, it may be noticed that both bacteria and yeasts were inhibited but never killed by the glycolipids even at 5 g/L (Table 1). Results are given after 48 h of incubation for yeasts and both 24 and 48 h for bacteria. On the whole, yeasts were less sensitive to sophorosides than bacteria. Among bacteria, only the two strains of *Staphylococcus aureus* (one resistant and one sensitive to penicillin) were very sensitive to sophorosides when results were compared to those obtained with kanamycin in the same conditions. On the other hand, *E. coli* and *Pseudomonas aeruginosa* were highly resistant to the glycolipids. Among yeasts, *C. parapsilosis* was very sensitive when results were compared to those obtained with ketoconazole. *Candida albicans* was resistant (Table 1), in agreement with results reported by Ito et al. (1980), who found a strong inhibitory effect due to sophorosides when yeasts (especially *C. albicans*) were grown on media containing *n*-alkanes (C_{10}–C_{18}), while no influence was observed on the growth in media containing fatty acids, the corresponding alcohols, or glucose as a sole source of carbon.

Table 4. Cytotoxicity of Sophorosides, Cholesterol Acetate, and DL-α-Lecithin toward Normal Human Skin Fibroblasts and MRC5 Fibroblasts

product	LC_{50}[a] normal cells (mg/L)	LC_{50} MRC5 (mg/L)
sophorosides	28.4 < 31.0 < 33.9	38.6 < 41.3 < 44.2
cholesterol acetate	30.9 < 39.1 < 49.6	29.0 < 34.5 < 41.1
DL-α-lecithin	>70	>70

[a] LC_{50}, 50% lethal concentration with 95% confidence boundaries.

Moreover, a biphasic effect was observed with *C. parapsilosis*: growth decreased between 0.1 and 0.25 g/L, while it was stimulated between 0.5 and 1.5 g/L, and it was finally totally inhibited at 2 g/L (a precipitate occurred over 1 g/L). Similar observations were made for bacteria, e.g., *P. aeruginosa*. *E. coli* was highly stimulated by sophorosides at 24 h of incubation (increase of +149% was observed at 0.25 g/L), and then growth decreased slightly until 1.5 g/L (+6%) and rose again to reach +154% at 5 g/L; after 48 h of incubation, differences with control were reduced. On the whole, sophorosides were not very toxic toward bacteria and yeasts and MBC or MFC (minimal fungistatic concentration) measured with reference antibiotic or fungicide was often weaker than IC_{50} obtained with the glycolipids in the same conditions.

On the whole, filamentous micromycetes were more resistant than bacteria and yeasts (Table 2), but their growth rate was not increased. Five of them kept a regular-looking mycelium, even when growth was lower: *Epidermophyton floccosum, Fusarium oxysporum, Microsporum canis,* and *T. mentagrophytes* 1; and *Verticillium lecanii* was not inhibited by sophorosides whatever the concentration. The mycelium was more abundant with *Drechslera* sp. (0.75 g/L) and *T. mentagrophytes* 2 (0.5 g/L) and 3 (0.75 g/L), while it was scarce with *Geotrichum candidum* (0.75 g/L) and *Pyricularia oryzae* (0.25 g/L). Pigmentation was not changed in any case, which might show that metabolism of the strains was not much modified. For some strains, high concentrations acted on fructifications (inhibition, stimulation, deformation). For example, conidiogenesis was altered for *Drechslera* sp. (5 g/L), *P. oryzae* (1 g/L), *T. mentagrophytes* 3 (0.75 g/L), and *Trichophyton rubrum* (0.5 g/L). Deformations were observed for *P. oryzae* (0.25 g/L), *V. lecanii* (2.5 g/L), and *T. mentagrophytes* 3 (0.25 g/L). Conidiogenesis was stimulated for *Trichophyton tonsurans* as a function of the amount of sophorosides added. Dermatophytes resisted doses of sophorosides at least 10 times higher than the lowest doses of ketoconazole necessary to totally inhibit growth (Table 2).

Before the toxicity of sophorosides toward human fibroblasts was assessed, toxicity of ethanol was first evaluated. LC_{50} of ethanol was of 2.6% (95% confidence interval: 2.3–2.9%, probit method) in the culture medium for normal skin cells and 2.8% (95% confidence interval: 2.5–3.1%, probit method) for MRC5 cells. In both cases, 100% of survival was obtained with 1% which was the maximal level used in the experimentation. A second assay was conducted with culture media containing 10, 30, 40, 50, and 70 mg/L of sophorosides and cytotoxicity was compared with that of cholesterol acetate (natural product commonly used as surfactant) and DL-α-lecithin (commonly used synthetic surfactant). LC_{50} values were obtained precisely for sophorosides and cholesterol, while lecithin seemed to be the less toxic (LC_{50} over 70 mg/L in all cases). Sophorosides were a little more toxic than cholesterol for normal skin fibroblasts, while the contrary occurred for MRC5 cells (Table 4). It must be pointed

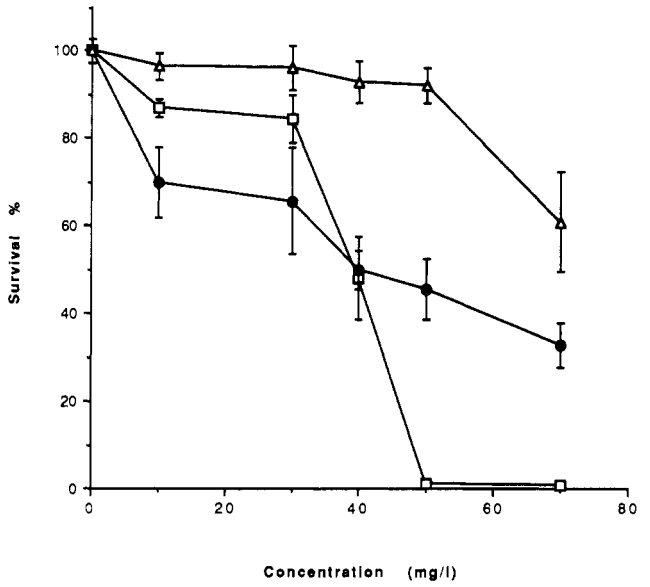

Figure 1. Survival of normal human skin fibroblasts grown in media containing sophorosides (□), cholesterol acetate (●), or DL-α-lecithin (△).

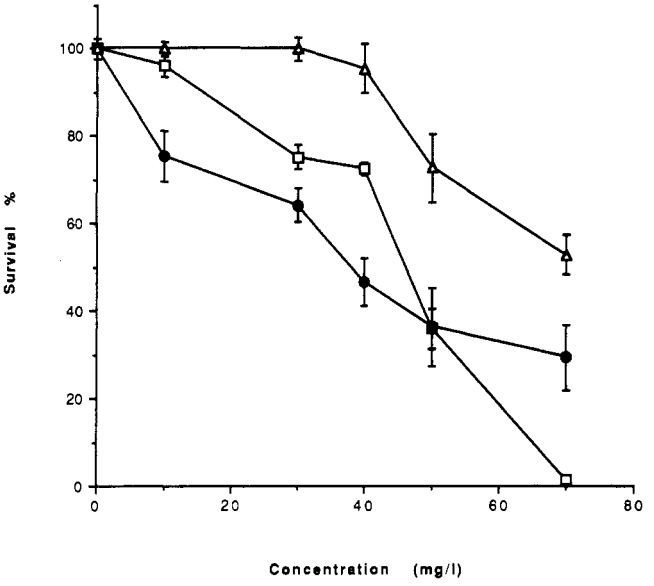

Figure 2. Survival of MRC5 fibroblasts grown in media containing sophorosides (□), cholesterol acetate (●) or DL-α-lecithin (△).

out that if LC_{50} values were close, the cytotoxicity curves of these two compounds were quite different. Sophorosides showed an acute toxicity expressed by an abrupt decrease of the curve over 40 mg/L, while this phenomenon was not observed with cholesterol (Figures 1 and 2). This was more obvious in the case of normal cells (Figure 1).

In conclusion, sophorosides from *T. bombicola* are slightly toxic for some bacteria and yeasts but are well tolerated by most yeasts, which confirms literature data (Ito et al., 1980), and by filamentous micromycetes, which was not reported until now. On cultured human fibroblasts (human skin or MRC5), toxicity of sophorosides was almost the same as with cholesterol acetate but higher than with DL-α-lecithin. On skin fibroblasts sophorosides showed LC_{50} values weakly higher than that with cholesterol acetate but not far from LC_{100} values. Owing to the possible use of sophorosides in food, cosmetology, or dermatology, further toxicity assays must be undertaken.

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