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A Study of the Fatty Acid Metabolism of the Yeast *Pityrosporum ovale*

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The yeast *Pityrosporum ovale*, a skin saprophyte, will only grow if fatty acids of chain length greater than C_{10} are added to the culture medium. 9-Hydroxypalmitic acid is the major product of metabolism of even-carbon-number fatty acids; 9-hydroxystearic acid is also found. The optimum pH for this conversion is pH 4·5. The hydroxy fatty acids produced are found bound in a polar form in the aqueous phase of the culture medium. Growth of the organisms is facilitated by presentation of the substrate as a two-phase liquid system.

Pityrosporum ovale has been associated with several skin conditions including dandruff, seborrhea capitis and tinea versicola, though it is also found extensively on normal scalps. Several workers have been unable to demonstrate conclusively any pathogenic properties of the organism (Kile & Engman, 1938; Rocha, Silva, Lima & Goto, 1952).

The lipophilic nature of the organism was reported by Benham (1939, 1941), who found that lanolin or butterfat extracts facilitated growth on conventional media. Shifrine & Marr (1963) found that fatty acids were essential for growth. However, they concluded that oleic acid, which had previously been thought to be highly effective, was unable to support growth when pure.

The present paper reports the results of work undertaken to re-examine the mode of nutrition of the organism on the scalp, its substrate requirements and the metabolism of these substrates by the organism. The metabolism of several strains of the organism isolated from the heads of normal human subjects was compared with that of cultures obtained from national collections. The results of these studies are at some variance with those reported by other workers.

MATERIALS

Two cultures described as *Pityrosporum ovale* were obtained from the London School of Hygiene and Tropical Medicine. These were: L.S.H.T.M. 3073, which was isolated from dandruff in Calcutta in 1930, and L.S.H.T.M. 3074, which was isolated in 1930 from a case of tinea versicola; this was identified as *Malassezia furfur* at that time. Two other cultures were obtained from the Centraalbureau voor Schimmelcultures (Delft, The Netherlands); these were C.B.S. 1878 (Bizzozero) Castellani et Chalmers and C.B.S. 4162 (Bizzozero) Brumpt.

Twelve organisms were cultured from the heads of human volunteers; these conformed to the published morphological and cultural data available for *Pityrosporum* organisms (Lodder & Kreger-van Rij, 1952; Swift & Dunbar, 1965).

Mixed scalp-surface lipids were obtained from human volunteers by extraction of hair with ether–ethanol (9:1, v/v). Fatty acids were obtained from Sigma (London) Chemical Co. Ltd. (London, S.W. 6), Calbiochem Ltd. (London, W. 1) and Koch–Light Laboratories Ltd. (Colnbrook, Bucks.). Radioactive fatty acids were obtained from The Radiochemical Centre (Amersham, Bucks.). Their purity was established by gas–liquid chromatography; all were better than 95% pure.

METHODS

Cultivation. Routine cultivation and maintenance of strains of cells was undertaken on Littman's oxgall agar, containing 1% of peptone (Oxoid), 1% of glucose and 1·5% ox bile (Oxoid), sterilized by autoclaving. For experimental culture of *P. ovale*, peptone (2%)–glucose (1%) broth supplemented with lipid was found to be an excellent medium allowing differentiation between suitable and unsuitable lipid supplements. No growth occurred without lipid supplement. In each case the lipid supplement was introduced into the culture vessel in ether, dried under a stream of nitrogen with gentle warming and 25 ml. of aqueous medium added. After inoculation with 0·1 ml. of a suspension of washed cells (approx. 10^6) the culture was grown at 37° with constant agitation.

The lipid supplements used were prepared as described below.

Total scalp-surface lipids. Human scalps were washed with 500 ml. of ether–ethanol (9:1, v/v). The washings were discarded. One week later this procedure was repeated, hairdressing or washing being forbidden during this period. After filtration and concentration of the washings on a rotary evaporator, the residue was extracted with chloroform–methanol (2:1, v/v) and washed by the method of Folch, Lees & Sloane-Stanley (1957). Material contained in the lower chloroform-rich phase was termed total scalp-surface lipids.

Saponifiable and non-saponifiable fractions. Total scalp-surface lipid was hydrolysed for 1 hr. under reflux in 6% (w/v) KOH in ethanol–water (9:1, v/v). After addition of twice the volume of water, non-saponified material was removed by three extractions with equal volumes of hexane. N-HCl was added to adjust the pH to 2·0 and the mixture was re-extracted three times with equal volumes of ether.

Column fractionation of total scalp-surface lipids. Samples (1 g.) of total scalp-surface lipids dissolved in hexane were fractionated on a 100 g. silicic acid (Malinckrodt Chemical Works, St Louis, Mo., U.S.A.) column into four fractions eluted with 200 ml. of each of hexane, benzene, chloroform and methanol; these were termed fractions I–IV respectively. Thin-layer chromatography and fatty acid determinations were performed on all fractions.

Estimation of growth. Owing to the large quantities of lipid in the media used, straightforward aqueous centrifugal washing of cultures was found to be inadequate for the estimation of growth. Therefore in each case the entire culture including medium was extracted with ten times its volume of chloroform–methanol (1:1, v/v) acidified with 1% (v/v) of N-HCl and the defatted cells were then centrifuged down. They were dried in an oven at 80° for 24 hr. and growth was estimated as the weight of the sediment obtained.

Recovery of products. Lipid extracted from cells and medium after growth or incubation experiments in chloroform–methanol (2:1, v/v) containing 1% (v/v) of N-HCl was washed by the method of Folch *et al.* (1957); the chloroform layer was considered to contain the whole lipid extract. Total fatty acids were prepared as described above. Methyl esters of fatty acids were prepared by bubbling gaseous diazomethane in nitrogen through a solution of the fatty acids in ether–methanol (9:1, v/v) (Schlenk & Gellerman, 1960*a*,*b*) until the solution became yellow. After 10 min. the solvent was removed together with excess of diazomethane.

Thin-layer chromatography was used to separate the products of metabolism as follows: (1) whole lipid samples were separated on silica gel G (E. Merck A.-G., Darmstadt, Germany) by development in hexane–ether–formic acid (98%) (80:20:1, by vol.); (2) methyl esters of mono-, di- and tri-enoic acids and saturated fatty acids were separated on silica gel G containing 5% (w/v) of $AgNO_3$ (de Vries, 1962, 1963) in hexane–benzene (1:1, v/v); (3) monohydroxy fatty acid methyl esters were separated from others on silica gel G in ether–hexane (2:3, v/v) (Morris, 1964); (4) acetoxy methyl esters of monohydroxy fatty acids were separated on silica gel G in ether–hexane (1:3, v/v) (Morris, 1964). Components separated by these techniques were located by spraying the plates with 0·02% 2,7-dichlorofluorescein in ethanol and viewing under u.v. light, or by spraying with aq. 50% (v/v) H_2SO_4 and heating at 160° for 1 hr. Location of radioactive areas on thin-layer chromatograms was achieved by either scanning on a moving-bed gas-flow counter or by radioautography on Kodak 0800 Superspeed photographic plates.

Gas–liquid chromatography of methyl esters of fatty acids was performed on a Pye Argon gas chromatograph on columns of either 10% polyethylene glycol adipate or 10% Apiezon M on Gas-Chrom Z (Applied Science Laboratories Inc., State College, Pa., U.S.A.). Radiochemical gas–liquid chromatography was performed on similar columns by the technique of James & Hitchcock (1965).

Characterization of products. Fatty acids in ethanol solution were catalytically hydrogenated with platinum oxide under 1 atm. of hydrogen for 1 hr. with stirring.

Hydroxyl groups were reduced by refluxing the material dissolved in 1 ml. of hydrogen iodide and 0·5 ml. of propionic acid with 100 mg. of red phosphorus for 6 hr. (Yang & Stumpf, 1965).

Oxidation of hydroxy acids with chromium trioxide (15 mg.) in 0·5 ml. of acetic acid for 2 hr. at 80° yielded mono- and di-carboxylic acids, which were then separated as methyl esters by gas–liquid chromatography, allowing an assessment of the position of the hydroxyl groups (Yang & Stumpf, 1965).

Oxo groups were reduced to hydroxyl groups by treatment of ether–methanol (1:2, v/v) solutions of material with sodium borohydride at room temperature for 1 hr.

Acetoxy derivatives of hydroxy fatty acids were prepared by the rapid $HClO_4$-catalysed acetylation procedure of Fritz & Schenk (1959).

RESULTS

Identification of the lipid requirements for the growth of P. ovale

From experiments designed to measure the growth of *P. ovale* in media to which various lipid

Table 1. *Growth of* P. ovale *in 25 ml. of peptone–glucose broth with 25 mg. of various lipid supplements for 48 hr. at 37°*

Results (expressed as mg.) are the total weight of dry defatted cell residue recovered from the culture vessels. All values are means of triplicates; variation was not more than ±15%.

Lipid supplement	Growth (mg.)
None	1·2
Total scalp-surface lipid	18·0
Non-saponifiable fraction	2·7
Saponifiable fraction (as free fatty acids)	32·0
Hydrogenated saponifiable fraction	22·0
Column fraction I (largely hydrocarbons)	3·2
Column fraction II (triglycerides, cholesteryl esters and fatty acids)	23·2
Column fraction III (some fatty acids, mono- and di-glycerides, free sterols)	6·7
Column fraction IV (phospholipids)	4·9

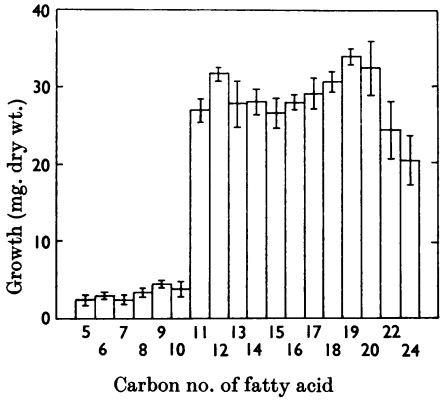

Fig. 1. Growth of *P. ovale* in 25 ml. of peptone–glucose broth with 25 mg. of individual fatty acids in ascending series of chain length. Sodium taurocholate (1%) was added to each flask. Growth is expressed as mg. of dry defatted cell residue after 72 hr. growth at 37°.

fractions had been added it was evident that the lipid found on the surface of the scalp can fulfil the lipid requirements of this organism (Table 1).

Saponifiable and non-saponifiable fractions. When the total scalp-surface lipid was separated chemically into saponifiable and non-saponifiable fractions it was conclusively evident that the greatest growth was associated with the presence of free fatty acid.

Saturated and unsaturated fractions. After hydrogenation the fatty acid fraction proved to be at least as active in promoting growth as the original scalp-surface lipid. This indicated that the requirement for fatty acids was not confined to those with preformed double bonds. The decrease in the yield of cells when grown on fully saturated fatty acids is discussed below.

Physically separated fractions. When the total scalp-surface lipid was separated on silicic acid columns the second fraction, which contained most of the free fatty acids, was found to be the most effective promoter of growth, though a smaller effect was observed with the fraction that contained fatty acid esters together with some free fatty acids. This confirmed the results of experiments with free fatty acids that had been isolated chemically.

Chain length of fatty acids. When individual saturated fatty acids of different chain lengths were used as supplements to media that would not otherwise support growth, the yields of cells increased markedly when the chain length exceeded 10 (Fig. 1). In the range 11–24 the actual chain length had little effect on the yield, nor did it matter whether the carbon number was odd or even.

Table 2. *Gas–liquid chromatography of the total fatty acid methyl esters recovered from cultures of* P. ovale *grown with* C_{11}–C_{24} *saturated fatty acids as supplements*

Each + sign represents approx. 20% of the total fatty acid recovered. Abbreviations: APM, Apiezon M; PEGA, polyethylene glycol adipate; R_v, retention volume relative to that of palmitate derivative (= 1·0).

Carbon no. of fatty acid given	...	...	11	12	14	15	16	17	18	19	20	22	24
Gas–liquid-chromatographic conditions	R_v												
10% APM at 200°, 40 ml./min.	0·73		+	+	+	+	+	+	+	+	+	+	+
	1·0		+	+	+		+	+	+	+	+	+	+
	1·27					+	+	+	+	+	+	+	+
	1·77		+	+	+	+	+	+	+	+	+	+	+
	2·51							+		+			
	3·64							+	+	+	+	+	
10% PEGA at 185°, 40 ml./min.	3·08		+										
	4·58			+									
	6·06					+							
	8·2			+	+	+	+	+	+	+	+		
	9·45		+										
	11·3					+		+		+			
	16·6				+								

Identification of lipid products of metabolism of P. ovale

When *P. ovale* was cultured on peptone–glucose broth for 72hr. with a small supplement of individual fatty acids in a series with 8–20 carbon atoms, the products of this metabolism were found to vary depending on the chain length of the substrate.

To identify these products, extracts of the entire culture plus cells were hydrolysed and the fatty acids methylated. Thin-layer chromatography of these methyl esters in ether–hexane (2:3, v/v) revealed two major spots regardless of chain length of the fatty acid supplement. The two spots were located at R_F 0·52 and 0·03 (under these conditions methyl 2-hydroxystearate had R_F 0·57).

Samples of the two major products were recovered for gas–liquid-chromatographic analysis by removal of the appropriate areas of the adsorbent from the plate and elution of this with ether. The ethereal solutions were subjected to gas–liquid-chromatographic analysis. Table 2 summarizes the gas–liquid-chromatographic data obtained when the fast-running component (R_F 0·52) produced from each fatty acid on which growth occurred was analysed. It will be seen that at 200° on a column of 10% Apiezon M on Celite a component with R_V (retention volume relative to that of palmitate derivative = 1·0) 1·77 was always found, though this was only the major component when fatty acids with an even carbon number of 16 or above were the substrate. Materials with R_V 0·73 and 3·64 were also observed in these cases.

When odd-carbon-number fatty acids were the substrate the major products appeared at R_V 0·73, 1·27 and 2·51, depending on the length of the carbon chain. Material collected from the gas–liquid chromatograph with R_V 1·77 on Apiezon M at 200° ran with R_V 7·61 on polyethylene glycol adipate at 185°. Acetylation of this material decreased its R_V on polyethylene glycol adipate to 5·57. On thin-layer chromatography with ether–hexane (1:3, v/v) its R_F was 0·47 (methyl 2-acetoxystearate and methyl 2-acetoxypalmitate both had R_F 0·53 under these conditions). Table 3 summarizes these results. It was suspected from these results that the fast-running materials located at R_F 0·52 on thin-layer chromatograms were monohydroxy fatty acids. Further evidence for this view is given below.

Analysis by gas–liquid chromatography of the material eluted from the slow-running component found on thin-layer chromatograms (R_F 0·03) from each fatty acid substrate showed the same pattern of peaks regardless of the chain length of the substrate. On Apiezon M at 230° (gas flow 100ml./min.) two peaks were always observed with R_V 95 and 166. Acetylation of this material decreased these retention volumes to 78 and 83 respectively, indicating the presence of hydroxyl groups. Treatment of these two substances with thionyl chloride followed by hydrolysis with ethanolic potassium hydroxide and catalytic hydrogenation yielded two peaks on gas–liquid chromatography that corresponded to those produced by methyl esters of saturated fatty acids with 22 and 26 carbon atoms respectively. We conclude that these compounds

Table 3. *Thin-layer-chromatographic and gas–liquid-chromatographic data on the methyl esters of fatty acids derived from cultures of* P. ovale *grown on peptone–glucose broth supplemented with fatty acids*

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; APM, Apiezon M; PEGA, polyethylene glycol adipate; R_V, retention volume relative to that of palmitate derivative (=1·0).

	Methyl esters			Acetoxy methyl esters	
	R_F	R_V		R_F	R_V
Growth condition	TLC in ether–hexane (2:3)	GLC on APM at 200°	GLC on PEGA at 185°	TLC in ether–hexane (1:3)	GLC on PEGA at 185°
Fast-running component					
Even series	0·52	0·73	7·61	0·47	5·57
		1·77			
		3·64			
Odd series	0·52	1·27			-
		2·51			
Slow running component		230			
Odd and even series	0·034	95			
		166			
2-Hydroxypalmitate	0·57	1·55	3·73	0·53	4·29
2-Hydroxystearate	0·57	3·55	6·70	0·53	8·04

are dihydroxy fatty acids with 22 and 26 carbon atoms respectively.

Biosynthesis of monohydroxy fatty acids. Cultures of the organism grown for 18hr. in Littman's oxgall broth were washed three times in peptone–glucose broth and incubated in fresh medium to which had been added [U-^{14}C]palmitic acid (100μg., 3μc) for periods ranging from 10min. to 48hr. The lipids were extracted and run on thin-layer chromatograms in hexane–ether–formic acid (98%) (80:20:1, by vol.). Radioautography revealed transient incorporation of label into the triglyceride and cholesteryl ester fraction together with a slower incorporation into material that did not move from the origin in this solvent.

Further samples of lipid extracted from these incubations were hydrolysed and the fatty acid methyl esters were chromatographed on thin-layer chromatograms in ether–hexane (2:3, v/v). In these cases a slow incorporation into material that ran with R_F 0·52 (fast-running component) was observed. This was the only incorporation of radioactivity observed on chromatograms and identification of this material was undertaken.

A sample was prepared by removal of adsorbent and elution with ether, and it was then divided into several portions. Radiochemical gas–liquid chromatography yielded one peak with R_V 1·77 on Apiezon M at 200° and R_V 7·6 on polyethylene glycol adipate at 185°. Catalytic hydrogenation did not vary the running position on thin-layer chromatography or gas–liquid chromatography on either column, indicating that the material was not unsaturated. Reduction with sodium borohydride produced no change in its migration characteristics on thin-layer chromatography or gas–liquid chromatography on either column, indicating the absence of oxo groups. After acetylation the material had R_V 5·57 on polyethylene glycol adipate at 185° and on thin-layer chromatography in ether–hexane (1:3, v/v) the radioactivity was localized at R_F 0·47 (methyl 2-acetoxystearate has R_F 0·53), indicating the presence of an esterifiable hydroxyl group. Reduction with red phosphorus and hydrogen iodide produced a saturated fatty acid that was identical with palmitic acid when run on gas–liquid chromatography, indicating that the original molecule contained 16 carbon atoms. Oxidation with chromium trioxide and subsequent chromatography of the methyl esters of the dicarboxylic acids yielded two major radioactive peaks, corresponding to the esters of azelaic acid and sebacic acid; other shorter-chain minor peaks were seen, but this was considered to be due to overoxidation. The hydroxyl group was thus located at C-9. The major radioactive product was therefore considered to be 9-hydroxypalmitic acid.

When [1-^{14}C]palmitic acid was added as a fatty acid supplement, good yields of a radioactive hydroxy acid were isolated; this proved to be composed entirely of 9-hydroxypalmitic acid. Oxidation of this material with potassium permanganate in acetic acid (Stumpf & James, 1963) and subsequent radiochemical gas–liquid chromatography of the fatty acid products indicated that all the radioactivity was located in the first carbon atom of the chain. It was concluded that no new synthesis of the hydroxy fatty acid had taken place, but that the organism had simply inserted a hydroxyl group into the palmitic acid chain.

From growth studies with stearic acid as the major fatty acid source, both 9-hydroxypalmitic acid and 9-hydroxystearic acid (R_V 3·64 on Apiezon M at 200°) were identified by the procedures described above. When the organism was incubated with [1-^{14}C]stearic acid, the radioactivity recovered in the hydroxy acid fraction was found to be located entirely in 9-hydroxystearic acid. Treatment with the potassium permanganate in acetic acid again indicated that all the radioactivity is located in the first carbon atom of the molecule, showing that a hydroxyl group is again inserted into the fatty acid carbon chain. A further conclusion from this observation is that, when 9-hydroxypalmitic acid is synthesized from stearic acid, the chain is shortened by removal of carbon atoms from the carboxyl end of the molecule before hydroxylation occurs. No new synthesis of the 9-hydroxypalmitic acid occurred.

Location of hydroxy acid

To ascertain whether the hydroxy fatty acids are intra- or extra-cellular, cultures grown in peptone–glucose broth with [U-^{14}C]palmitic acid were centrifuged and the culture medium and washed cells were extracted separately. Methyl esters of fatty acids were separated on thin-layer chromatograms and radioautographs prepared. These indicated that approx. 85% of the hydroxy acid was present in the medium.

When whole lipid was separated on thin-layer chromatograms in hexane – ether – formic acid (80:20:1, by vol.) and fractions were eluted from the adsorbent and hydrolysed, the fraction showing the largest quantity of hydroxy acid was found to be that remaining at the origin. This observation indicated that the hydroxy acids are bound in polar lipids that are present in the culture medium in a water-soluble form.

Effect of pH *and physical state of the substrate*

On incubation of pregrown cultures of washed organism in peptone–glucose broth buffered with phosphate to a range of pH values, the optimum conversion of [U-^{14}C]palmitate into hydroxy fatty

acid occurred at pH 4·5. This suggests that the organism prefers its substrate as free acid.

Growth was accelerated if the fatty acid substrate was incubated as a solution in hexane, paraffin oil or oleic acid. Evidence was obtained that oleic acid itself was only very slowly metabolized.

From these observations it is concluded that growth is facilitated by the presentation of the substrate at a liquid/liquid interface as opposed to a solid/liquid interface; this conclusion possibly explains the decrease in growth observed when hydrogenated scalp-surface fatty acids (solids at 37°) were used as substrate.

DISCUSSION

P. ovale occurs on the scalp in large numbers. Light- and electron-microscopic evidence of post-mortem and living scalp samples obtained by section, tape stripping or abrasion of the scalp indicates that the numbers of these organisms present on most scalps are in excess of previous estimates by several orders of magnitude. The lack of realization of this may stem from the unusual cultural requirements of the organism. Sampling techniques with standard media restrict the growth of *P. ovale* relative to other forms, and yield a picture that is not correct for this organism.

All of the 12 cultures identified as *P. ovale* by bacteriological and light- and electron-microscopic techniques behaved identically with respect to their utilization of lipid when compared with the strains obtained from the collections of type cultures.

The isolation medium used in this study was Littman's oxgall agar, which always yielded faster growth than peptone–glucose broth supplemented with lipid from whatever source. It had been hoped to discover the factors in which a lipid-supplemented peptone–glucose broth was deficient. A range of detergents, the most promising of which was sodium taurocholate, improved growth in this medium, though the rate of growth even with this medium was not as great as with Littman's oxgall agar. That taurocholate was not acting as an alternative carbon source was confirmed by the lack of growth of the organism in its presence without lipid supplement. The aqueous requirements for growth have not yet been fully investigated.

The specificity for fatty acid above C_{10} in chain length seems absolute. However, it is noteworthy that odd- or even-numbered carbon chains are accepted, the materials produced from the odd-carbon-number acids being different from those resulting from growth on even-carbon-number acids.

The examination of the normal fatty acid composition of *P. ovale* was hampered by difficulties encountered in attempting to remove unmetabolized substrate. Growth in a two-phase liquid system, such as palmitic acid dissolved in oleic acid over peptone–glucose broth, causes the organisms to distribute themselves largely in the lipid phase. Centrifugation at 3000*g* was insufficient to cause sedimentation of most of the organisms. Attempts to remove the lipid substrate with hexane followed by centrifugation again yielded distribution of organisms at the interface between the aqueous medium and solvent. In all experiments in which information was required on the normal fatty acid composition of the organisms, growth was continued until only minimal quantities of the original fatty substrate were detectable. Under these conditions only very small amounts of straight-chain saturated or monoenoic acids were observed.

Shifrine & Marr (1963) concluded that this organism is incapable of synthesizing long-chain fatty acids from C_2 units, and therefore exhibits a requirement for preformed fatty acid. The present conclusions agree with theirs except that in our experiments normal long-chain fatty acids were used for the synthesis of a range of monohydroxy and dihydroxy fatty acids. Oleic acid, which was a major product reported by Shifrine & Marr (1963), was never found to be synthesized by our organisms. Indeed, experiments were performed in which radioactively labelled oleic acid of high specific activity was fed to organisms or homogenates in the hope of indicating that biosynthesis of hydroxy acid proceeded via the monoene. In these cases very little radioactivity was found in the hydroxy acid produced. It is considered significant that the estimation techniques used by Shifrine & Marr (1963) involved oxidation of oleic acid to hydroxystearic acid followed by column fractionation. In this Laboratory, this technique led to the production of hydroxy acids that were not separated from 9-hydroxypalmitic acid by the solvents quoted.

The hydroxy acids produced by this organism from saturated precursors are found in a highly polar form in the aqueous medium after incubation. It is possible that glycolipids are being produced, as in *Torulopsis utilis* (Stanacev & Kates, 1963) and *Torulopsis magnoliae* (Tulloch, Spencer & Gorin, 1962).

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