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Suitability of lipid materials for culture of *Malassezia* as evaluated from its cellular fatty acid composition

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***Malassezia* is a facultative or obligatory lipophilic yeast. We devised new lipid-supplemented media suitable for the culture of *Malassezia*. *Malassezia furfur* and *M. pachydermatis* grew well on both solid and liquid media supplemented with creaming powder preparations which are commercially available at moderate prices. Striking differences were found between the cellular fatty acid compositions of *M. furfur* grown on media supplemented with creaming powder and that grown on media with conventional olive oil. *Malassezia furfur* grown on media with olive oil had nearly the same fatty acid composition as olive oil, with C18 : 1 amounting to 80%, while that grown on media supplemented with creaming powder had C16, C18 : 1 and C18 : 2 as the principal components. The use of these supplementary lipids appeared not to inhibit the normal synthesis of fatty acid in *M. furfur*. For the culture of *M. pachydermatis*, media supplemented with creaming powder were also found more suitable than lipid-free media. The media devised are considered excellent, because they appear to provide a more natural growth environment for *Malassezia*.**

Key Words——cellular fatty acid; creaming powder; *Malassezia furfur*; *Malassezia pachydermatis*; medium.

Malassezia species, facultative or obligatory lipophilic microorganisms, are common residents on homeothermal animal skin. Known strains of *Malassezia* can be classified into three species: *Malassezia furfur* (Robin) Billon 1889, *M. pachydermatis* (Weidman) Dodge 1935 and *M. sympodialis* Simmons & Gueho (Simmons and Gueho, 1990). *Malassezia furfur*, a common resident on human skin, can not only be isolated from normal skin but is also involved in the pathogenesis of superficial mycosis. *Malassezia pachydermatis* has been isolated from various animals and can be distinguished from the two other species by its active growth even on a lipid-free medium. *Malassezia sympodialis* has been described as a new species, but its pathogenic role remains unclear.

Olive oil has been routinely used for the culture of *M. furfur*. When used alone, however, it does not mix homogeneously with a basal medium. To increase its affinity for other ingredients, it is often supplemented with Tween (Michael Dorn and Roehnert, 1977; Takahashi et al., 1981; Ushijima et al., 1981; Jan Faergemann, 1985; Uchida and Yamaguchi, 1991), sodium taurocholate, gall powder (Takahashi et al., 1981; Ushijima et al., 1981) or monoglycerol (Jan Faergemann, 1985; Uchida and Yamaguchi, 1991).

The use of olive oil gives rise to problems: its removal from harvested cells and its disturbance of drug resistance tests (Uchida and Yamaguchi, 1991). Though *M. furfur* can grow well on glucose blood agar without olive oil, procurement of animal blood is difficult at present. Sodium taurocholate and gall powder are almost identical in composition but these are too expensive for routine use as additives.

We, therefore, used creaming powder preparations that are commercially available at moderate prices. Creaming powder is suitable as a lipid additive because it easily mixes with a basal medium. This study was designed to compare the cellular fatty acid compositions of cultures grown on solid and liquid media containing lipid additives such as creaming powder and to evaluate the suitability of lipid-supplemented media for the culture of *Malassezia*.

Materials and Methods

Microorganisms *Malassezia furfur* (IFO 0656, TIMM 1847, TIMM 2718) and *M. pachydermatis* (IFO 10064, TIMM 1983, TIMM 1984) were mainly used, together with MP 001, MP 002 and MP 003 isolated from the field.

Compositions of media Strains were precultured on YM agar (Difco) containing 0.5% milk fat creaming powder (DC) (Creap; Morinaga Milk Industry).

Solid media for experimental cultivation were prepared using YM agar to which (I) DC, (II) vegetable fat creaming powder (VC) (Creaming Powder; Morinaga Milk Industry), (III) powdered baby milk (BM) (Infant Formula F&P-f; Meiji Milk Product) or (IV) fresh cream (FC) (Sujahta; Nagoya Seiraku) was added to the concentration of

3% (w/v). In addition, potato dextrose agar (PDA) (Nissui) supplemented with 3% DC, VC or BM was also used.

Liquid media for experimental cultivation were prepared using a YM liquid medium supplemented with (V) DC or (VI) VC at concentration of 0.5% or 1% in 500-ml Erlenmeyer flasks.

Cultivation and harvesting To examine the effects on cellular fatty acid of the different lipids added to the solid media, strains grown for 4 d at 37°C on the precultivation medium were inoculated into the (I)–(IV) media and incubated statically at 37°C for 96 h. Cultured cells were harvested with a spatula, washed three times with physiological saline and once with distilled water, then freeze-dried. In the case of cells cultured with olive oil (Kozakai Seikagaku), the oil was poured over the YM agar plate to form a thin layer over the whole surface. Cells cultured on this medium were washed with *n*-hexane and acetone to remove adhering oil.

To examine the effects on cellular fatty acid of the different lipids added to the liquid medium, cells grown for 4 d at 37°C on the precultivation medium were inoculated into the (V) and (VI) media and were incubated at 37°C for 96 h with constant shaking. Cultured cells were separated by centrifugation at 10,000 rpm for 10 min. The cells were washed three times with physiological saline and once with distilled water, then freeze-dried.

Preparation of samples for gas chromatographic analysis Ten mg of the lyophilized cells was mixed with 1.5 ml of 5% methanol hydrochloride (Kokusan Chemical Works) in screw-top test tubes. The yeast cells were heated on a hot dry bath at 100°C for 3 h, then methyl esters were extracted with three 4-ml portions of *n*-hexane. The *n*-hexane extract was washed with distilled water, dehydrated with anhydrous sodium sulfate (Hikotaro Shudzui), concentrated in a vacuum evaporator and dried in a stream of nitrogen gas. The methyl esters obtained were dissolved in a small amount of acetonitrile and analyzed by use of a gas chromatograph (Hitachi, Model 163) equipped with a flame ion detector (FID) using a deactivated 20% DEGS, Uniport B (GL Science) – packed glass column (2 mm ID × 2 m). Analysis was carried out isothermally at 190°C with nitrogen gas as a carrier. Fatty acid components were identified by comparing their ECL with authentic standards. Lipids were extracted from the medium supplements with chloroform : methanol (1 : 2), and methylation was carried out by the above method.

Determination of protein Protein concentrations were determined by the method of Folin-Lowry (Morris, 1948).

Determination of sugar Sugar concentrations were determined by the anthrone method (Johanson, 1953).

Results

Compositions of supplementary lipids The major components of the supplementary lipids are shown in Table 1. The fat contents of DC, VC, FC and BM were all approximately 30%. FC differed in having a low non-fat portion and a high content of water. Olive oil was composed almost totally of fat.

Table 1. Principal components of supplementary lipids (%)[a].

Material[b]	Fat	Non-fat	(Sugar	Protein)
DC	28.0	69.5	(60.1	7.7)
VC	35.0	61.8	(53.5	5.8)
BM	25.0	72.2	(57.8	12.2)
FC	27.5	7.9	(3.0	4.4)
Ov	99.9	—		

a) Quoted from component tables on respective product labels.
b) DC: milk fat creaming powder, VC: vegetable fat creaming powder, BM: powdered baby milk, FC: fresh cream, Ov: olive oil.

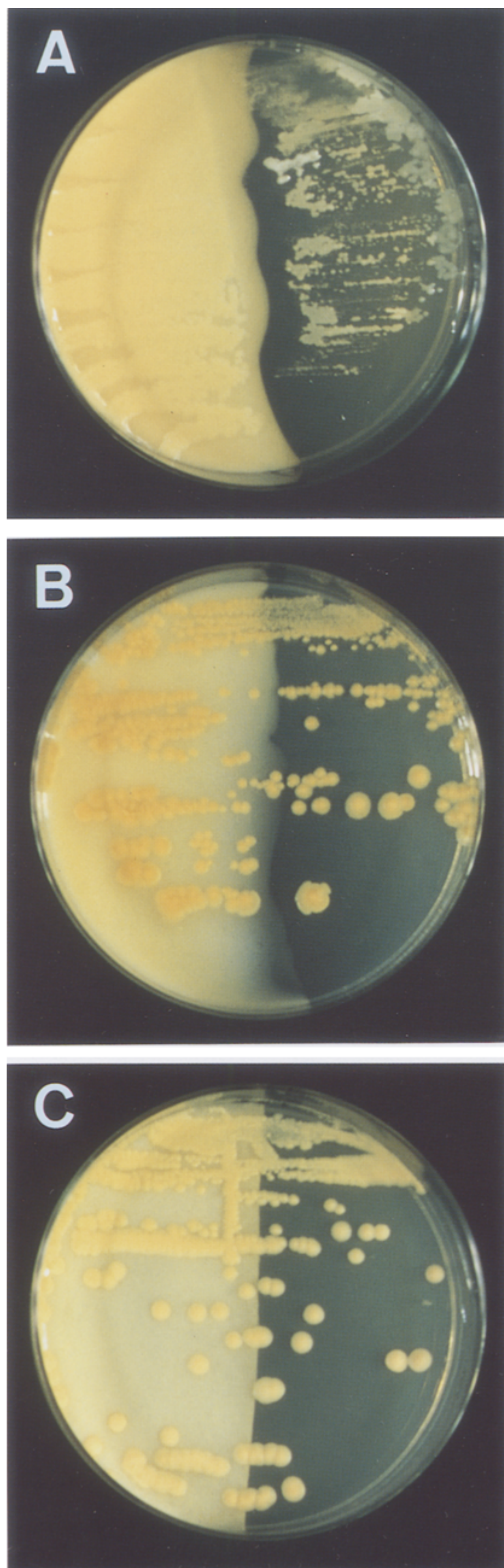
The major fatty acid components of these lipids are shown in Table 2.

DC is a creaming powder preparation made of milk fat, to which it has a similar fatty acid composition, with relatively high contents of C16 and C18:1. VC is a creaming powder preparation made of vegetable fat, mainly palm seed oil. It is characterized by the abundance (48%) of C12 as the principal component, with other components consisting mostly of saturated fatty acids. BM is made of milk fat mixed with soybean oil and has a higher ratio of C18:2 than the other supplementary lipids. In addition to C18:2, the major components of BM were C16 and C18:1. FC is made of milk fat, as is DC, and has nearly the same fatty acid composition as milk fat. In olive oil, C18:1 amounted to 80%.

Growth in solid media supplemented with lipids To compare the growth under the same culture condition of cells cultured on a solid medium supplemented with the lipids and on a solid medium supplemented with olive oil, *M. furfur* was streaked onto a plate containing, on one half, YM agar spread with olive oil and, on the other half, PDA containing 3% DC (Fig. 1A). The yeast clearly showed more active growth on the side of the plate containing DC (shown at left). When harvesting the yeast grown on the medium with olive oil, it was necessary to wash off olive oil adhering to the cells, while the yeast grown on the DC-supplemented medium could be harvested and cleaned more easily.

Malassezia pachydermatis was similarly cultured using two plates. On one plate, YM agar was applied to the right side and PDA containing 3% DC to the left (Fig. 1B). On the other plate, YM agar was applied to the right side and PDA containing 3% VC to the left (Fig. 1C). In both cases, the yeast growth on the side to which creaming powder had been added was equally as active as on the lipid-free side.

Cellular fatty acid composition of *M. pachydermatis* The cellular fatty acid compositions of *M. pachydermatis* grown on solid media supplemented with the lipids are shown in Table 3. The yeast grown on lipid-free PDA or YM agar as control had C16, C18:1 and C18:2 as its principal components. On PDA or YM agar containing 3% DC, the principal components were likewise C16, C18:1 and C18:2. On PDA or YM agar containing 3% VC, the major fatty acid components were C12, C16,



C18:1 and C18:2, this being the only lipid from which C12 was detected. On PDA or YM agar containing 3% BM, the distribution of cellular fatty acid components was similar to that in culture with DC, with principal components consisiting of C16 and equal abundances of C18:1 and C18:2. Thus in culture with the supplementary lipids, both basal media yielded substantially the same fatty acid compositions.

Cellular fatty acid composition of *M. furfur* The cellular fatty acid compositions of *M. furfur* grown on solid media supplemented with the lipids are shown in Table 4.

In the yeast grown on media containing DC, VC, BM and FC, the content of C18:1 was highest. Culture in the presence of DC resulted in similar cellular fatty acid compositions regardless of whether PDA or YM was used as the basal medium. The content of C18:1 was highest, followed by C16. The content of C18:2 was smaller, and this constituted the major difference from *M. pachydermatis*. The yeast grown on media with VC was characterized by a lower percentage of C16 and higher percentages of C12, and higher percentage C18:2 compared to those grown in the presence of DC. The growth with BM showed a different pattern of fatty acid composition, with a large increase in C18:2 and a large decrease in C16.

The yeast grown on the olive oil media required washing with hexane and acetone to remove olive oil before preparation of samples for cellular fatty acid analysis. The cellular fatty acid compositions in the washed samples were similar to the fatty acid composition of olive oil itself.

Concentrations of cellular protein and sugar in *M. furfur* The concentrations of protein and sugar of dried *M. furfur* cells are shown in Table 5. When grown on media with VC, BM or DC, the yeast consisted almost totally of protein and sugar. When grown on media with olive oil, cellular protein and sugar decreased to less than approximately 30% in total.

Effects of lipids added to liquid medium on cellular fatty acid To examine the possibility that cultivation in liquid medium supplemented with lipid may entail difficulty in harvesting cells because of unutilized lipid remaining in the medium, cellular fatty acid composition was compared between cells washed with organic solvent and those washed by a routine procedure after culture on YM broth supplemented with DC or VC.

When *M. furfur* was grown on a liquid medium supplemented with 1% DC or VC, the cellular fatty acid compositions of cells washed with organic solvent and those washed by a routine procedure were similar (Table 6).

Fig. 1. A. *Malassezia furfur* was streaked onto a plate containing, at right, YM agar spread with olive oil and, at left, PDA containing 3% DC, and incubated at 37°C for 3 d.
B. *Malassezia pachydermatis* was streaked onto a plate containing YM agar at right and PDA containing 3% DC at left and incubated at 37°C for 3 d.
C. *Malassezia pachydermatis* was streaked onto a plate containing YM agar at right and PDA containing 3% VC at left and incubated at 37°C for 3 d.

Table 2. Principal fatty acid components of supplementary lipids (%).

Material	Fatty acids (%)[a]							
	C10	C12	C14	C16	C16:1	C18	C18:1	C18:2
DC	3	4	13	36	2	11	27	1
VC	6	48	18	12		14	1	
BM	1	4	4	23	1	9	37	20
FC	3	5	12	21	1	7	39	4
Ov				11	1	4	80	5
Milk fat	3	3	9	27	1	10	35	3
Palm seed oil	7	54	17	10		7	1	1
Soybean oil				9		4	28	54

a) C10: capric acid, C12: lauric acid, C14: myristic acid, C16: palmitic acid, C16:1: palmitoleic acid, C18: stearic acid, C18:1: oleic acid, C18:2: linoleic acid.

Table 3. Principal cellular fatty acid components of *M. pachydermatis* IFO 10064.

Medium	Fatty acids (%)							
	C12	C14	C16	C16:1	C18	C18:1	C18:2	C18:3
PDA[a]		1	16		5	40	37	
YM[b]		1	17		3	31	47	
DC+PDA		3	18	1	5	44	28	
DC+YM		4	19	1	4	42	29	
VC+PDA	9	8	18	1	3	35	26	
VC+YM	13	9	18	1	4	29	25	
BM+PDA		2	16	1	6	35	36	
BM+YM		2	17	1	6	35	37	2

a) Potate dextrose agar.
b) YM agar.

Table 4. Principal cellular fatty acid components of *M. furfur* IFO 0656.

Medium	Fatty acids (%)								
	C12	C14	C14:1	C16	C16:1	C18	C18:1	C18:2	C18:3
DC+PDA	1	7	1	21	4	7	52	6	1
DC+YM	1	5	1	21	3	9	50	5	5
VC+YM	5			11	8	10	41	18	
BM+YM		1		6	3	5	48	35	1
FC+YM	1	6	1	26	3	12	41	7	1
Ov+YM				7	3	3	79	8	
Ov+PDA				8	2	3	81	6	

When *M. pachydermatis* was grown on a liquid medium supplemented with 1% DC, however, the cellular fatty acid composition differed between cells washed with organic solvent and those washed by a routine procedure; the contents of C16, C18 and C18:1 differed by approximately 10%. In the presence of 0.5% DC, on the other hand, the cellular fatty acid compositions of cells washed by the two methods were similar. When *M. pachydermatis* was grown on a liquid medium supplemented with 0.5% or 1% VC, the growth was poor.

Table 5. Cellular protein and sugar in *M. furfur* (mg/g dried cells).

Medium	Protein	Sugar
VC+YM	780	160
BM+YM	635	150
DC+YM	620	140
FC+YM	510	185
Ov+YM	315	40
Ov+PDA	220	42

Table 6. Cellular fatty acid composition of *Malassezia* strains grown in liquid medium supplemented with DC or VC.

Concentration of lipids	Strain[a]	Fatty acids (%)							
		C12	C14	C14:1	C16	C16:1	C18	C18:1	C18:2
1% DC	M.f	1	2		17	3	9	56	7
1% DC	M.f-w[b]		1		16	4	9	57	8
1% VC	M.f	1	2		9	2	12	51	15
1% VC	M.f-w[b]	2	3		12	2	14	51	15
1% DC	M.p	2	9	2	41	2	12	27	2
1% DC	M.p-w[b]	2	11	2	30	4	5	37	5
0.5% DC	M.p		1		15	5	1	58	17
0.5% DC	M.p-w[b]		1		16	3	3	55	18

a) M.f: *M. furfur* IFO 0656; M.p: *M. pachydermatis* IFO 10064.
b) w: Washed with organic solvent.

Comparison of cellular fatty acid compositions of yeast grown on liquid and solid media Comparison of the cellular fatty acid composition of *M. pachydermatis* grown with 1% DC on liquid and solid media (Table 7) revealed a higher content of C16 and a lower content of C18:2 on liquid medium than on solid medium. With 0.5% DC, a higher content of C18:1 and a lower content of C18:2 were found on liquid medium than on solid medium.

Malassezia furfur grown with 1% DC had similar cellular fatty acid profiles whether grown on a liquid medium or a solid medium (Table 8), with C16 and C18:1 as its principal components. When this yeast was grown on a liquid or solid medium with 1% VC, however, the cellular fatty acid profiles differed.

Discussion

Ready-made creaming powders, such as those used in this experiment, are commercially available in two types: animal fat preparations and vegetable fat preparations. Powdered baby milk is stable in composition and is easy to obtain, although it is more expensive. DC, VC and BM mixed very well with the basal media and were thus convenient for use as additives. FC, a liquid preparation of

Table 7. Cellular fatty acid composition of *M. pachydermatis* IFO 10064 grown in solid and liquid media supplemented with DC.

Medium	Fatty acids (%)							
	C12	C14	C14:1	C16	C16:1	C18	C18:1	C18:2
1%-L[a]	2	11	2	30	4	5	37	5
1%-S[b]		4		19	1	4	42	29
0.5%-L[c]		1		16	3	3	55	18
0.5%-S[d]		3		15	1	7	39	35

a) Cultivated in liquid medium supplemented with 1% DC.
b) Cultivated in solid medium supplemented with 1% DC.
c) Cultivated in liquid medium supplemented with 0.5% DC.
d) Cultivated in solid medium supplemented with 0.5% DC.

Table 8. Cellular fatty acid composition of *M. furfur* IFO 0656 grown in solid and liquid media supplemented with 1% DC or 1% VC.

Medium	Fatty acids (%)							
	C12	C14	C14:1	C16	C16:1	C18	C18:1	C18:2
DC-L[a]		1		16	4	9	57	8
DC-S[b]	1	5	1	21	3	9	50	5
VC-L[a]	2	3		12	2	14	51	15
VC-S[b]	6	6		10	13	8	38	19

a) L: Cultivated in liquid medium.
b) S: Cultivated in solid medium.

animal fat, does not stand long-term preservation, but it was superior to olive oil in that it mixed better with the basal media than olive oil. Olive oil consists almost completely of fat. When used singly, it did not mix with the basal media. In cultivation on the solid media spread with olive oil, independent colonies were not formed, because colonies were carried away by the drift of oil.

In drug resistance tests of *Malassezia*, some drugs can be remarkably affected by the antagonistic action of lipids (Uchida and Yamaguchi, 1991). The choice of a medium is difficult, and this has been an obstacle in drug resistance tests. However, the supplementary lipids suggested by us mixed homogeneously with the basal media and were found suitable for use in drug resistance tests. In cultivation on solid media containing creaming powder, it was confirmed that all strains of *M. furfur* and *M. pachydermatis* achieved active growth. These media were found highly useful, in that drug resistance tests can be performed under the same culture conditions for the two species having different lipid requirements.

In *M. pachydermatis*, no large differences were found in its cellular fatty acid composition regardless of whether it was grown on solid media supplemented with DC or BM or on lipid-free media (Table 3). This finding suggests that since *M. pachydermatis* can grow well without lipid, the differences in fatty acid composition of lipid between DC and BM had no direct effect on the cellular fatty acid composition of this yeast. When VC was used, on the other hand, substantial amounts of C12 were contained in the cellular fatty acid composition of *M. pachydermatis*. It seemed that VC exerted a significant effect, but it is not known whether C12 was stored or utilized. However, it was considered that the use of these supplementary lipids on solid media did not inhibit the normal synthesis of fatty acid in *M. pachydermatis*, and the media with these lipids were more suitable for the subculture of *M. pachydermatis* than the lipid-free media, particularly for the isolation of wild strains from the field.

The cellular fatty acid compositions of *M. furfur* grown on media supplemented with olive oil were predominated by C18:1. This reflected the fatty acid composition of olive oil itself. In this experiment, the supplementary lipids produced no significant disparities in the ratios of cellular fatty acid components. When *M. furfur* was cultured in the presence of olive oil, lipid was its major constituent, with very small amounts of protein and sugar, compared to culture with the other lipids. This result suggests that cells cultured with olive oil incorporated surplus oil, which was only accumulated in cells and was not utilized for the biosynthesis of fatty acid. It is presumed that since *M. furfur* is isolated from mammals such as human, its normal cellular fatty acid composition would be similar to that obtained by culture with animal fat. The lipid-supplemented media proposed by us are considered excellent, because these would provide a more natural growth environment for *M. furfur*.

Culture using liquid media supplemented with creaming powder was carried out because mass culture is needed for experiments such as extraction of somatic enzymes. *Malassezia furfur* exhibited good growth on all media, but *M. pachydermatis* showed poor growth on some media. The problem with cultivation on a liquid medium is the removal of oil remaining in the medium, which adheres to cells. This problem was solved by reducing the concentrarion of creaming powder. In culture of *M. furfur*, pure cells could be obtained by normal washing when DC or VC was used at concentration of 1%, and in culture of *M. pachydermatis* when DC was used at concentration of 0.5%. With higher concentrations, it was necessary to wash cells with distilled water several more times or to wash with organic solvent.

After culture on a liquid medium, the cellular fatty acid compositions of *M. pachydermatis* grown with 1% DC differed from that grown with 0.5% DC. With 0.5% DC, culture on a liquid medium resulted in good growth, but with 1% DC the growth was poor.

In culture of *M. pachydermatis* on liquid medium, low concentration of lipid was considered sufficient for the growth, and high concentration seemed to have an adverse effect on the growth. In culture on solid media, whether DC was used at 1% or 0.5%, good growth was achieved with similar ratios of principal components, C16, C18:1 and C18:2.

Malassezia furfur achieved good growth on both liquid and solid media supplemented with DC or VC. Supplementation with DC or VC was suitable for the culture of *M. furfur* as evaluated from its cellular fatty acid composition.

When *M. furfur* or *M. pachydermatis* was grown on liquid and solid media, the cellular fatty acid profiles were often dissimilar, even if the same supplementary lipid was used at the same concentration. This may be because the physical conditions of the culture influence the area of contact between the yeast and the lipid substrate.

We consider that the new media with creaming powder preparations proposed here are more suitable than the conventional lipid materials used for *Malassezia*. Creaming powder preparations are available at moderate prices, the yeasts grown on these media can be harvested and cleaned more easily, and drug resistance tests can be performed under the same conditions. In addition, these media were suitable from the standpoint of the cellular fatty acid compositions of these yeasts. The usefulness of creaming powder as an additive was thus supported.

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