

Hydrolysis of Fatty Acid Esters by *Malassezia furfur*: Different Utilization Depending on Alcohol Moiety

PETER MAYSER¹, DAGMAR FÜHRER², REINHOLD SCHMIDT² and KARL GRÜNDER¹

Departments of ¹Dermatology and Andrology and ²Internal Medicine, Justus Liebig University, Giessen, Germany

The lipophilic yeast *Malassezia furfur* belongs to the resident skin flora but has been implicated in various skin diseases. While topical oily preparations may support its growth, their formulation may be altered by yeast-dependent enzymatic degradation. Different synthetic fatty acid (mono-)esters used as refatting agents were mixed with 10^4 , 10^5 and 5×10^5 yeasts/ μ l, respectively, and incubated at 35°C for a maximum of 48 h on selective agar for pathogenic fungi (Merck). The amount and pattern of generated free acids were evaluated by densitometric and gas chromatographic analysis, while yeast density was determined in a Neubauer chamber. Depending on the inoculum, yeast-dependent hydrolysis occurred immediately and was best effected in ethyl esters, followed by isopropyl esters, whereas hydrolysis of decyl oleate was only limited. Of the fatty acids released, unsaturated fatty acids were more stimulative to growth than saturated fatty acids; no toxic effects were observed.

In conclusion, yeast-dependent hydrolysis of these synthetic fatty acid (mono-)esters was critically dependent on alcohol moiety, while growth promotion was best effected by unsaturated fatty acids. These results may help to improve the compatibility of topical formulations, especially in seborrheic areas. **Key words:** refatting agents; yeast-dependent hydrolysis; growth-promoting effects.

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P. Mayser, Department of Dermatology, Justus Liebig University Giessen, Gaffkystr. 14, D-35385 Giessen, Germany.

Malassezia furfur is a dimorphous lipophilic yeast which belongs to the resident microflora normally found on human skin. Under these saprophytic conditions, the yeast phase predominates, while yeast-to-mycelium transformation has been associated with *Pityriasis versicolor* (1). Depending on its micromorphology, the yeast phase has been given a variety of names, e.g. *Pityrosporum ovale/orbiculare*, while the term *M. furfur* has been used to describe the mycelial form. Today, *M. furfur* is the name formally accepted for both phases of growth (2).

Yeast phase *M. furfur* has been implicated in a range of other skin diseases, including *Malassezia* folliculitis (3), dandruff (4), seborrheic dermatitis (5), psoriasis (6) and atopic eczema (7).

Although factors favouring skin affections are not exactly known, endogenous or exogenous modification of cutaneous lipids may play a role as a growth promoter leading to parasitic evolution. Previous studies have shown that topical application of lipid-containing formulations, such as suntan cream or oil bath, can support growth and thereby exacerbate *Malassezia*-related skin diseases (8–11). On the other hand, its ability to generate free fatty acids by utilizing several triglycerides suggests that this yeast may contribute to the formation of comedones in the sebaceous follicles of man (12). Therefore, it is of interest in which way and to what extent *M. furfur* might utilize cosmetically important lipids.

In this study, different fatty acid monoesters used as refatting agents and spreading promoters in cosmetic-pharmaceutical preparations were tested for yeast-dependent hydrolysis and growth-supporting effects.

MATERIAL AND METHODS

Materials

Organisms: Strains 9207, 9208, 9209 and 92010 of *M. furfur*, isolated in our laboratory from the sternal region of healthy volunteers, were used. All yeasts fulfilled the criteria mentioned elsewhere (2).

Fatty acid monoesters: Table I shows a list of the oils tested. Esters were either bought (Sigma Chemical Company, St. Louis, USA) or kindly provided by the manufacturers.

Methods

Culturing: Cultures were first grown aerobically on selective agar for pathogenic fungi (Merck), overlaid with a thin layer of olive oil at 35°C. After 8–10 days, cells were harvested and suspended in the pure ester. By determining yeast density in a Neubauer chamber (dilution media xylene), suspensions were prepared to a density of 10^4 , 10^5 and 5×10^5 cells/ μ l, respectively. Selective agar for pathogenic fungi (Merck) was poured out on small glass Petri dishes (3 cm in diameter) and overlaid with 100 μ l of each suspension. After incubation at 35°C in the dark (0, 1, 3, 7, 24 and 48 h), the suspension was taken up with 2 ml chloroform

Table I. Oils tested

Ester	Manufacturer	Trade name	Purity*	Batch-No.
Decyl oleate	Henkel, Düsseldorf, FRG	Cetiol V		10063056
Ethyl linoleate	Induchem, Dübendorf, CH	Safester A-75	75%	206069
Ethyl oleate	Unichema Iberica	Estol 3659 ETO		
Isopropyl linoleate	Van Dyk, Belleville, USA	Ceraphyl IPL		1142
Isopropyl myristate	Sigma, St. Louis, USA	M 8136	98%	62HO421
Isopropyl oleate	Unichema Bebington, UK	Priolube 1406		MPSO 1933
Isopropyl palmitate	Henkel, Düsseldorf, FRG	Rilanit IPP		1272101

* as far as available.

Table II. Generation time of *Malassezia* strain 92010 in different esters

Ester	t(h) [10000 cells/ μ l]	t(h) [100000 cells/ μ l]	t(h) [500000 cells/ μ l]
Decyl oleate	No growth	No growth	35.8
Isopropyl palmitate	21.8	41.4	96
Isopropyl myristate	16.8	29.1	41.4
Isopropyl oleate	13.3	27.3	30.1
Isopropyl linoleate	9.5	12.9	17.1
Ethyl oleate	8.6	10.9	11.3*
Ethyl linoleate	5.1	5.6*	8.9*

Total volume 100 μ l; incubation 48 h and * 24 h, respectively.

and transferred to gas-dense tubes. In parallel, cell density was determined by a hemacytometer (dilution with xylene), and cell morphology was carefully observed by light microscopy. In most cases, cells were counted after 48 h; in case of high growth rate, determination was required after 24 h.

Thin-layer chromatography (TLC) and densitometry: TLC was performed to determine the amount of free fatty acid (FFA) generated by *Malassezia*. Ten μ l of each sample taken up with CHCl_3 were applied to HPTLC 60 plates (Merck) by means of a Camag Linomat IV and fractionated by TLC (elution media hexane:diethylether:formic acid 80:20:2 v/v/v). After development, each plate was dipped in a solution of Primulin (Sigma, Deisenhofen, Germany) (stock 0.1% in methyl alcohol; final solution in acetone 1:10 v/v), according to Wright (13). Results were quantified densitometrically with a Camag TLC scanner 2 using Camag software (Camag, Muttenz, Switzerland) and parallel processing of authentic fatty acid standards. Absolute values were obtained by comparison with the corresponding fatty acid standard curves. Amounts of FFA present at the time $t=0$ (primarily oleic acid due to the inocula) were subtracted from each measured value.

Gas chromatographic analysis (GC): GC was performed to determine the pattern of generated fatty acids. Respective bands on HPTLC plates were scraped off, eluted with $\text{CHCl}_3/\text{MeOH}$ (2:1 v/v) and dried under N_2 . FFAs were spiked with heptadecanoic acid as internal standard and

converted to methyl esters by mixing with ethereal diazomethane according to Pace-Asciak (14). The ethereal layer was dried, redissolved in chloroform and transferred to the gas chromatograph. GC was performed on a Chrompack gas chromatograph CP 9000, using a CP-88 fused silica capillary column (50 M \times 0.25 MM; Chrompack AG, Germany). The program used an initial oven temperature of 160°C for 2 min. The temperature was then raised at a rate of 4°C min⁻¹ to 220°C, where it was held for 3 min. The injector and the detector were maintained at 250°C and 300°C, respectively. The fatty acid methyl esters were detected by use of a flame ionization detector, and peak area integration was performed.

RESULTS

Growth-promoting effects

Table II shows the generation time of *M. furfur* in the esters at inocula of 10^4 , 10^5 and 5×10^5 cells/ μ l. The total volume was 100 μ l, the incubation time 48 and 24 h. In the latter case, the number of cells could not be determined after longer periods of incubation ($> 6 \times 10^6/\mu$ l).

Growth promotion can be graduated in the sequence ethyl ester > isopropyl ester > decyl ester, the latter requiring a

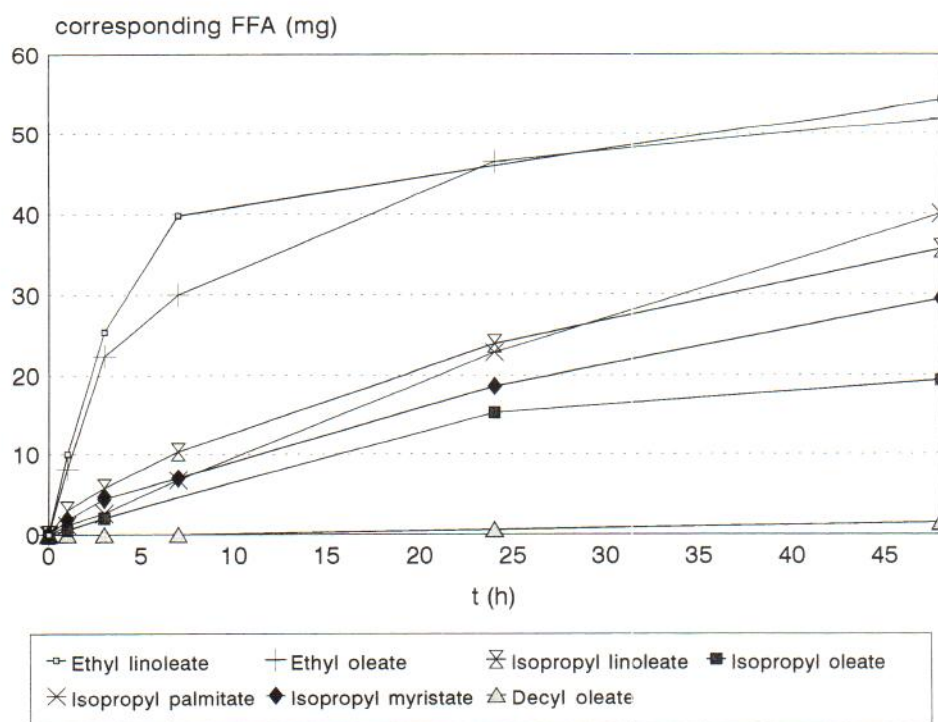
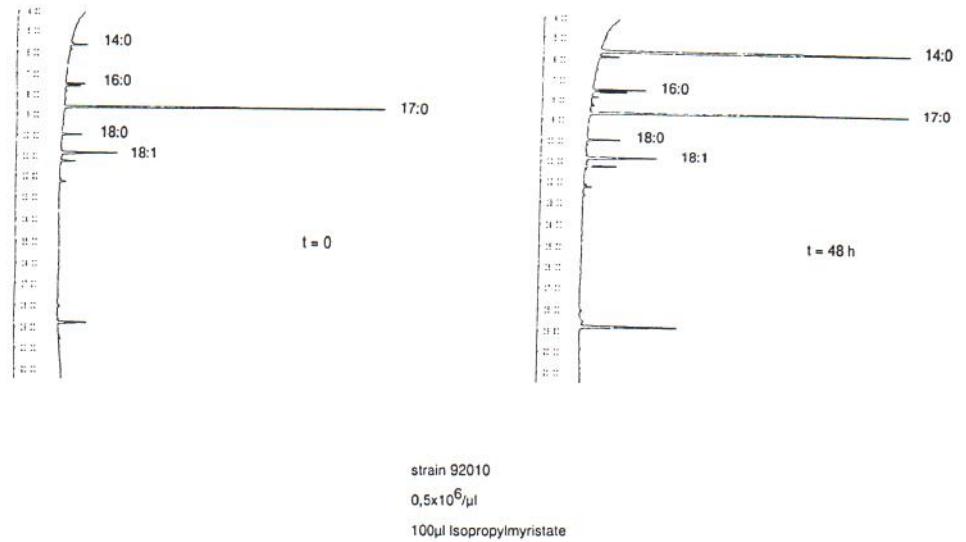


Fig. 1. Time-dependent hydrolysis of different fatty acid monoesters by *Malassezia furfur* strain 92010; 5×10^5 cells/ μ l; total incubation volume 100 μ l.

Fig. 2. Gas chromatographic pattern of strain 92010 ($5 \times 10^5/\mu\text{l}$) before and after incubation in isopropyl myristate (Sigma). Oleic acid peak due to inoculum.



minimum inoculum for growth to be initiated. These results were also obtained with other *Malassezia* strains and higher inocula. Of the fatty acids released, unsaturated fatty acids were more stimulative to growth than saturated fatty acids. In addition, depending on incubation time and inoculum, the consistency of the medium changed from liquid to solid in the esters of saturated fatty acids (isopropyl palmitate, -myristate) due to the higher melting point of generated FFA. This effect was accompanied by a dramatic increase in the generation time (Table II).

Even at high concentrations of FFA, no toxic effects were observed. Formation of mycelial elements was not detected.

Generation of free fatty acids

Fig. 1 shows the formation of FFA depending on fatty acid ester and incubation time at an inoculum of 5×10^5 cells/ μl . With the

exception of decyl oleate, 33.5–37.5 mg generated FFA corresponds to 50% hydrolysis. In general, hydrolysis occurred most rapidly in ethyl esters, followed by isopropyl esters, whereas the decyl ester required higher inocula and longer incubation time to be hydrolysed. For example, an inoculum of 1.5×10^6 cells/ μl yielded only 34% hydrolysis of this ester after 48 h. Among the isopropyl esters, isopropyl oleate is an exception in that it was only slightly hydrolyzed at low inocula. In all esters, maximum hydrolytic activity was achieved at shorter incubation times with increasing inocula. For example, at an inoculum of 500.000 cells/ μl , ethyl esters showed more than 10% hydrolysis after only 1 h. However, high inocula and long incubation time resulted in excessive cell growth, so that maximum values of generated FFA were limited by yeast-dependent consumption (Fig. 1).

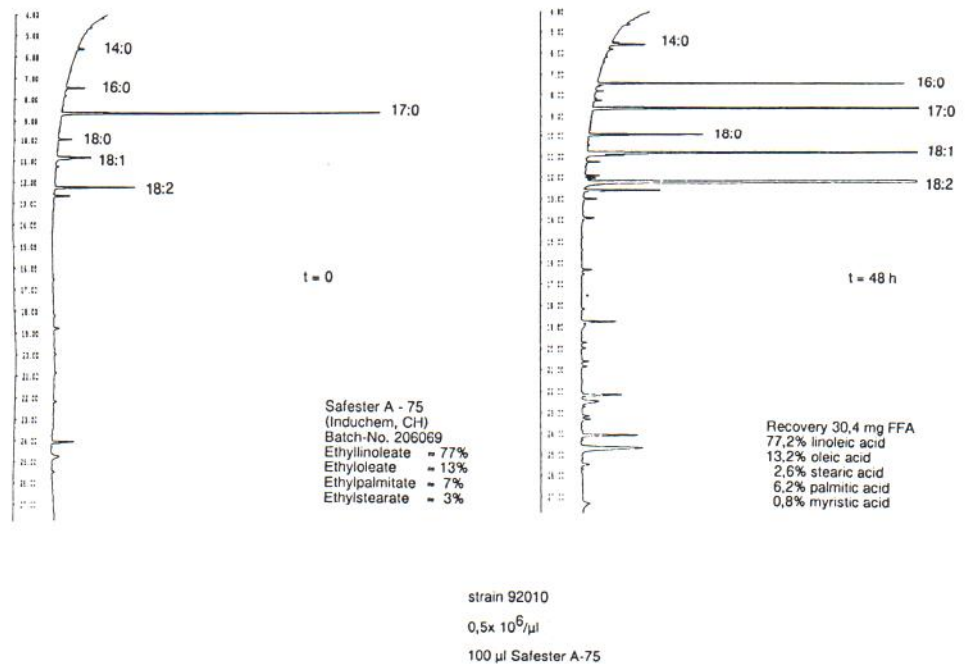


Fig. 3. Gas chromatographic pattern of strain 92010 ($5 \times 10^5/\mu\text{l}$) before and after incubation in Safester-A-75. Patterns of free fatty acids correspond to relative amounts of esters.

Interstrain variability

Variances between the individual strains (9207, 9208 and 9209) were determined for isopropyl myristate over an incubation period of 48 h at inocula of $10^6/\mu\text{l}$. With increasing time of incubation, higher differences among the individual strains were observed.

Spectrum of fatty acids released

The gas chromatographic spectrum of the generated FFA showed primary release of FFA corresponding to the ester used. It was problematical that most of the esters were not sufficiently pure to exclude degradation or transformation. Fig. 2 shows the fatty acid spectrum obtained by incubation of isopropyl myristate, which is obtainable with a 97% degree of purity. The result is almost exclusively myristic acid, because the low oleic acid peak is due to the culture medium. Fig. 3 shows the GC analysis of Safester-A75 (batch no. 206069). According to the manufacturer, this preparation is a combination of 77% ethylene oleate, 13% ethyl oleate, 7% ethyl palmitate and 3% ethyl stearate. After an incubation time of 48 h ($100 \mu\text{l}/0.5 \times 10^6$ cells), 30.4 mg FFA can be demonstrated in the GC, consisting of 77.2% free linoleic acid, 13.2% oleic acid, 6.2% palmitic acid, 2.6% stearic acid and 0.8% myristic acid. Despite extensive growth and an incubation time of 48 h, the resulting fatty acid pattern qualitatively corresponds to the combination of esters used. According to these data, transformation, chain elongation or degradation can be excluded, although degradation products of less than 10 carbon atoms cannot be demonstrated by this method.

DISCUSSION

Using an in vitro test system without emulsifiers, the present study investigated the interaction between the skin-resident lipophilic yeast *M. furfur* and liquid lipids in a model comparable to the skin surface. It yielded information about the growth-promoting potential of these substances, which may be of pathophysiological significance. In addition, yeast-dependent changes in their cosmetic-pharmaceutical properties were revealed, especially the generation of FFA.

In the case of synthetic lipids (15), i.e. different fatty acid esters on the basis of univalent alcohols, the model showed that the degree of hydrolysis was determined by alcohol moiety, while growth promotion was best effected by unsaturated fatty acids generated during incubation. Therefore, we cannot confirm the growth-inhibiting effects of high levels of unsaturated fatty acids described by Nazarro-Porro et al. (16). Growth stimulation and the formation of high amounts of FFA on the skin, especially in seborrheic areas, may be of pathophysiological importance – and undesired in external therapy. Correlations between the activity of the disease and the number of skin-resident yeasts have been suggested for *Malassezia* folliculitis, seborrheic eczema and dandruff (3–5). FFA may be irritating on the skin surface, and some fatty acids, such as palmitic or myristic acid, are thought to have comedogenic properties and to play an important role in the pathogenesis of acne (18). According to investigations by Marples et al. (19), *M.*

furfur normally contributes only to a minor extent and at a low density of *Propionibacterium acnes* to the generation of FFA on the skin. Based on our findings, increased amounts might be achieved by topical application of therapeutical or cosmetic substances that are potentially utilized by this lipophilic yeast, especially in seborrheic areas. Under the conditions investigated, FFAs are generated rapidly and in large amounts, depending on the inoculum and the esters tested. This is surprising, since synthetic lipids were investigated. It appears that the hydrolytic enzyme activity of this yeast, which might have been induced by the triglyceride-containing medium olive oil, has only a minor substrate specificity. Under this assumption, other synthetic topical lipids could also be hydrolyzed immediately without requiring enzyme induction. So far, investigations on lipase of *M. furfur* have not included substrate specificity (20, 21). A factor that is difficult to assess for the clinical significance of our results is the density of *M. furfur* on normal skin.

As a part of the resident skin flora, *M. furfur* has been shown at various body sites, with a predominance of the scalp and the upper trunk (92–100% incidence in adults) (4, 22).

Quantitative data on yeast density are rare. Leeming et al. (23) found the highest population density on the upper trunk (1.1×10^4 CFU/cm² on the chest; 8.9×10^3 on the back) and head (4.8×10^3 forehead, 1×10^4 on the cheek). Regarding the ostium of the sebaceous follicle, only qualitative data are available. With 13% of the follicle colonized, *M. furfur* showed the highest value of all microorganisms demonstrated, the average reaching 10^2 cells, mainly located in the follicular orifice (24).

Because the actual concentrations were difficult to estimate, tests were performed over a relatively broad range (10^4 bis $5 \times 10^5/\mu\text{l}$). It is possible that the conditions in the follicular orifice correspond to those used in our tests. In addition, yeast density, short generation periods (in our model less than 6 h depending on the substrate) and potential differences among the various strains might be responsible for interindividual variations, which would explain the different tolerance of topicals or the possible induction of diseases associated with *M. furfur*. However, regarding the univalent fatty acid alcohols tested here, these topical influences can be minimized by the use of esters from long-chain univalent alcohols and short-chain fatty acids (decyl oleate and [not shown] hexyl laurate). In cooperation with galenists, these preliminary investigations might be expanded to chemically different substances. On the other hand, should hydrolysis of a topical ester be desired for cosmetic or pharmaceutical reasons, this could be optimized by suitable combinations. For example, local deficiency of linoleic acid, which is discussed in the pathogenesis of acne, might be favourably influenced, particularly at the follicular orifice, by topical application of ethyl linoleate because of the high density of *M. furfur* at this site (25). In this connection it is interesting that yeast-dependent degradation or transformation of the generated FFA was not demonstrated, even after 48 h of incubation (Figs. 2, 3).

The in vitro model presented here provides information about interactions between the resident lipophilic yeast *M. furfur* and topical lipids or lipid-like substances. Supplemented by investigations in vivo, the results may help to improve the compatibility of topicals, especially in seborrheic areas.

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