Effect of Oxygen Levels on the Fatty Acids and Lipids of *Mucor rouxii*[1]

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ABSTRACT

The effect of aerobic and oxygen limiting (anaerobic) growth conditions upon the fatty acid and lipid composition of *Mucor rouxii* has been examined. The aerobic cells contained a range of fatty acids typical of phycomycetes, i.e. γ-linolenic acid, with an unsaturation index of 1.20, whereas the anerobic cells contained relatively high levels of shorter chained fatty acids and very low concentrations of unsaturated acids (unsaturation index = 0.025). The unsaturated compounds were monoolefinic tetra-, hexa-, and octadecenoic acids; and closer examination of their di-trimethylsilyl derivatives by gas chromatography and mass spectrometry showed that all three acids contained the double bond in the Δ^9 position. These results were consistent with a microaerobic biosynthetic pathway. In addition, there were major quantitative differences in the lipid composition of the two types of cells; and it was evident that the differences in growth environment markedly affected the cellular lipid and fatty acid compositions.

INTRODUCTION

It has been reported that *M. rouxii* (1-3) and other *Mucor* species (4) were capable of growth under both aerobic and anaerobic conditions. The aerobic cells were filamentous with considerable chlamydospore formation, whereas the anaerobic cells were yeast-like. This article describes the growth of a *M rouxii* isolate (5) aerobically (under helium) and shows that there are major differences in the lipids and fatty acid composition of the filamentous and yeast-like cells. Comparisons also are made between the lipid and fatty acid composition of the above cells and spores. The structures of the unsaturated fatty acids produced under anaerobic conditions also have been examined in detail to determine whether these compounds are produced microaerobically or via another pathway.

MATERIALS AND METHODS

Cultivation and Growth of *M. rouxii*

The isolation of *M. rouxii* (Calmette) Wehmer (HLX 1093) from sheep rumen contents has been described (5). Inocula of *M. rouxii* were prepared by adding sterile water (75 ml) to a culture maintained on defined medium to which agar (2%) had been added. The resulting spore suspension was decanted, and 3 ml (ca. 10^7 spores) used to inoculate 2 liters of medium contained in a 4 liter thick walled conical flask. The medium, described by Bartnicki-Garcia and Nickerson (6), was supplemented with Difco yeast extract (2%) and used for both aerobic and anaerobic growth. Aerobic growth was carried out without agitation at 25 C for 6 days. A stream of helium was bubbled through the medium used in the anaerobic experiments for 2 hr prior to inoculation (5). The flasks used in this study are shown (Fig. 1); opening the stopcock A permits evacuation and refilling of the flasks with helium (5). After refilling with helium, the clamp B is removed and a positive pressure of helium flushes both the flask and the deoxygenation trap which is

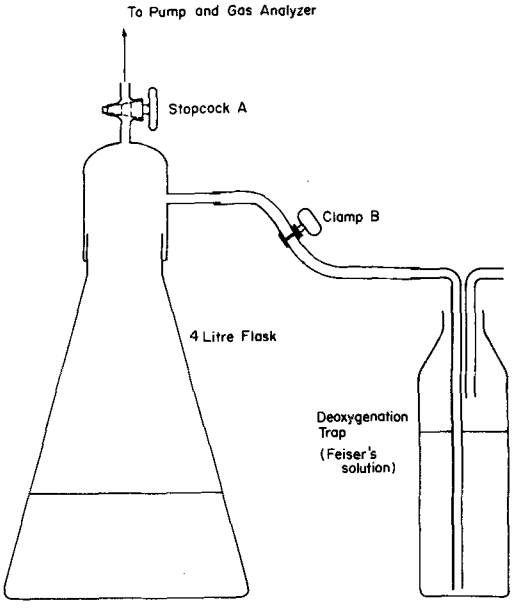

FIG. 1. Flasks used in the study.

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TABLE I

Fatty Acid Composition of Anaerobically Grown[a] *M. rouxii* at Specific Incubation Times

Incubation time (hr)	Mycelial[b] wt	Fatty acid composition									Unsaturation index
		8:0	10:0	12:0	14:0	14:1	16:0	16:1	18:0	18:1	
20	0.08	4.0	20.5	30.5	23.5	tr[c]	10.0	1.0	9.5	0.5	.015
30	0.26	6.5	48.0	21.0	13.1	tr	5.5	1.5	4.0	0.5	.020
40	0.49	4.0	34.0	26.5	21.2	tr	7.5	1.0	5.0	1.0	.020
50	0.57	tr	24.5	32.5	27.5	tr	8.0	2.0	2.0	1.5	.025
120	0.65	1.0	24.0	25.0	25.0	tr	14.0	1.0	8.0	1.5	.025

[a]Grown at 25 C.

[b]Expressed in terms of g/liter of media.

[c]tr = Trace.

filled with Fieser's solution (7). The excess gas pressure which develops during growth then can be released through the trap. Gas in the flasks was analyzed as previously described (5); and, in all cases, the anaerobic flasks were grown under an atmosphere containing less than 0.01% oxygen. The cells (both aerobic and anaerobic) were collected by filtration and then freeze-dried; spore suspensions were collected by centrifugation followed by freeze-drying.

Extraction of Fatty Acids and Lipids

The freeze-dried cells (or spores) were macerated with chloroform/methanol (2:1) (8) in a Waring blender and the mixture allowed to stand for 12 hr. A lipid extract was obtained by filtration to remove the cell debris. Fatty acids were obtained by concentrating the extract to dryness and saponifying the residue with 8% methanolic potassium hydroxide solution for 2 hr under reflux. The alkaline solution was diluted with water and the nonsaponifiable material removed by ether extraction; the aqueous layer was acidified with hydrochloric acid and extracted with ether to give the fatty acid fraction.

Lipid Analysis

The lipid fraction was purified by preparative thin layer chromatography (TLC) on Silica Gel $HF_{254+366}$ (Merck Darmstadt, Germany) using the solvent system, petroleum spirit/ether/methanol (90:30:15) as described by Sallee and Adams (9). The appropriate bands were visualized readily by long wave UV light and extracted with chloroform/methanol (2:1) to give the various lipid fractions.

Fatty Acid Analysis

The lipid and fatty acid fractions were transmethylated by refluxing for 90 min in benzene/methanol/sulfuric acid (20:10:1). The resultant fatty acid methyl esters then were purified by TLC (petroleum spirit/ether [97:3] as solvent system). Further purification of the fatty acid methyl esters according to degree of unsaturation was carried out using Silica Gel $HF_{254+366}$ plates impregnated with silver ni-

TABLE II

Fatty Acid Composition of Aerobically-Grown[a] *M. rouxii* at Specific Incubation Times

Incubation time (hr)	Mycelial[b] wt	Fatty acid composition									Unsaturation index
		8:0	10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	
24	0.01	tr[c]	5.00	11.4	18.4	21.4	17.9	3.0	20.9	1.40	0.40	0.43
48	0.20	0.60	13.2	22.2	26.5	15.9	6.9	2.6	8.50	1.60	1.00	0.22
72	0.31	0.40	12.0	19.6	24.0	16.8	6.4	4.0	9.50	3.20	4.00	0.33
96	0.38	2.70	12.5	14.2	16.9	12.9	4.50	8.50	17.6	4.80	5.50	0.48
120	0.56	0.70	8.10	12.1	17.0	12.5	4.70	5.90	20.7	7.20	11.0	0.73
144	0.58	0.15	6.30	10.6	15.6	11.9	4.30	7.00	22.8	8.60	12.1	0.81

[a]Grown at 25 C.

[b]Expressed in terms of grams/liter of media.

[c]tr = trace.

TABLE III

Fatty Acid and Lipid Composition of Spores of *M. rouxii* Grown at 25 C[a]

Lipid	Percent (by wt)	Fatty acid composition									Unsaturation index
		10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	
Triglycerides	22.0	4.5	8.5	12.0	34.5	6.5	10.5	15.0	5.5	2.0	0.39
Diglycerides	5.4	5.0	16.0	7.7	32.0	3.5	15.0	16.5	3.5	tr[b]	0.27
Polars	69.5	—	2.5	5.0	40.5	10.5	11.0	19.0	5.0	tr	0.40
Sterols	3.1										

[a]2-3 Week growth time.

[b]tr = trace.

trate (12% by wt) and eluted in benzene. The appropriate bands were visualized using long wave UV light.

The ester fractions were analyzed by gas liquid chromatography (GLC) on a stainless steel column (6 ft x 1/8 in.) packed with 8% HIEFF-1BP on Gas-Chrom Q (Applied Science) at a temperature of 140 C. The fatty acid methyl esters were identified by their relative retention times and by mass spectrometry. The unsaturated fatty acids were isolated by preparative GLC, converted into their di-trimethylsilyl derivatives, (10) and analyzed by mass spectrometry.

RESULTS AND DISCUSSION

The phycomycete, *M. rouxii* was capable of growth under both aerobic and anaerobic conditions and the differences in the cell growth environment were accompanied by changes in morphology. The aerobically grown cells were filamentous with considerable chlamydospore formation, whereas the anaerobic cells were yeast-like. The aerobic and anaerobic cells were harvested at various times from early to late log-phase growth and a summary of the mycelial wt and their fatty acid compositions is given in Tables I and II. The anaerobically grown cells readily adapted to growth at the low oxygen levels with a maximum growth rate occurring between 20-40 hr after inoculation. Similarly the growth rate of the aerobic cells was at a maximum between 24-48 hr after inoculation.

The fatty acid composition of the aerobic cells varied over the growth period, particularly with respect to the concentration of the 18:2 and 18:3 components. Their concentrations both increased with increasing age of the cells and not unexpectedly was accompanied by an increase in the unsaturation index of the fatty acids. The identity of the 18:3 component was shown to be γ-linolenic acid by comparative GLC and mass spectrometry with an authentic γ-linolenic acid standard. This acid commonly is found in phycomycetes, and Shaw (11) has proposed that γ-linolenic acid is a useful phylogenetic marker which distinguishes phycomycetes from the other orders of fungi which produce α-linolenic acid as the sole 18:3 species. The anaerobic cells contained only trace quantities of monoenoic acids (14:1, 16:1, and 18:1) and no dienoic or trienoic components and the unsaturation index was relatively constant over the entire growth period. There were

TABLE IV

Fatty Acid and Lipic Composition of *M. rouxii* (Aerobic)[a]

Lipid	Percent (by wt)	Fatty acid composition									Unsaturation index
		10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	
Triglycerides	22.6	8.0	19.0	16.5	9.0	6.0	4.0	14.0	7.5	16.5	0.84
Diglycerides	0.6	tr[c]	12.5	17.5	10.0	25.0	tr	25.0	10.0	tr	0.70
Free fatty acid	2.5	tr	2.0	9.5	20.5	7.0	7.5	28.0	10.5	13.5	0.96
Polars	60.5	4.0	3.0	8.0	13.5	10.0	1.0	22.0	14.5	25.0	1.36
Sterols	13.4										

[a]Grown at 25 C.

[b]Number of double bonds/mole fatty acid.

[c]tr = trace.

TABLE V

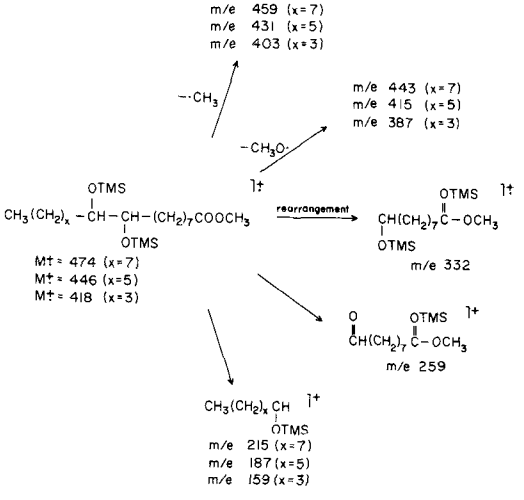
Fatty Acid Composition of Aerobically-Grown[a] *M. rouxii* at Specific Incubation Times

Lipid	Percent (by wt)	Fatty acid composition									Unsaturation index
		8:0	10:0	12:0	14:0	14:1	16:0	16:1	18:0	18:1	
Triglycerides	6.4	1.0	12.5	36.0	31.5	tr[a]	7.5	tr	11.0	0.5	.005
Diglycerides	1.8	—	2.0	26.0	29.0	tr	26.0	tr	13.5	2.5	.025
Polars	91.0	0.5	12.0	16.0	20.0	tr	19.5	0.5	27.0	2.0	.025
Sterols	0.8										

[a]tr = trace.

variations in the relative amounts of the short chained saturated fatty acids, i.e. 8:0, 10:0, 12:0, and 14:0, present in the anaerobic cells; but their overall concentration was 75-85% of the total fatty acids at 20, 30, 40, 50, and 120 hr after inoculation. In contrast, the aerobic cells contained only 32.6-62.3% short chain saturated acids. Similar results also have been observed for the fungus *Mucor genevensis* (4).

Since it is possible that the formation of the unsaturated compounds could occur microaerobically (via oxygen-dependent desaturases) or anaerobically (as in bacteria [12]), the position of the double bond in the above monounsaturated fatty acids was investigated. The olefinic esters were separated readily from the saturated acids by argentation TLC, and the R_f values indicated that the esters were *cis*-monoenes; further separation of the three components could be affected by preparative GLC. The monoenes were treated with $0_s\,0_4$/pyridine and the resulting diols converted into the ditrimethylsilyl (di-TMS) derivatives (10). The fragmentation pattern of all three di-TMS derivatives exhibited a common fragmentation pathway as shown in the equation; intense *m/e* 332 and 259 ions were observed as well as the expected $[M\text{-}CH_3O]^+$ species. In addition, an intense ion also was observed at *m/e* 215 for the 18:1 compound, at *m/e* 187 for the 16:1 ester, and at *m/e* 159 for the 14:1 ester. These results indicated that the structures of the olefinic fatty acids were 18:1ω9 (oleic acid), 16:1ω7 (palmitoleic acid), and 14:1ω5 (myristoleic acid); and these acids are typically produced by specific oxygen-dependent desaturation of the corresponding saturated fatty acid precursors. Thus, the samll quantities of unsaturated fatty acids were biosynthesized microaerobically, rather than by an anaerobic pathway.



The lipid and fatty acid compositions of the chloroform-methanol extracts of the aerobic and anaerobic cells and the spores are shown in Tables III-V. There were significant variations in the compositions of these extracts with relatively high levels of triglycerides (22.0 and 22.6%) and polar fatty acids (69.5 and 60.5%) in the spore and aerobic cell extracts. In contrast, the anaerobic cells contained only 6.4% triglyceride, with the polar fatty acid fraction being the major component (91.0%). The unsaturation indices for the aerobic cell lipids were higher than those observed for the spore lipid fractions, and this was primarily due to higher levels of the γ-linolenic acid. These results agreed with the data reported by Sumner and Morgan (13) for a number of mesophilic *Mucor* species. The sterol levels in all 3 extracts also varied considerably; the anaerobic cell extract contained only 0.8% sterols, whereas the aerobic cells and spores contained significantly larger quantities (13.8 and 3.1%) of extractable sterols.

Thus, the fatty and lipid composition of *M. rouxii* was markedly affected by the growth environment, particularly with respect to the concentrations of chloroform-methanol extractable lipids, as well as the relative amounts of short chained and unsaturated fatty acids present.

REFERENCES

1. Bartnicki-Garcia, S., and W.J. Nickerson, J. Bacteriol. 82:142 (1961).
2. Bartnicki-Garcia, S., Ibid. 96:1586 (1968).
3. Bartnicki-Garcia, S., and W.J. Nickerson, Biochim. Biophys. Acta 58:102 (1962).
4. Gordon, P.A., P.R. Stewart, and G.D. Clark-Walker, J. Bacteriol. 107:114 (1971).
5. Brewer, D., J.M. Duncan, S. Safe, and A. Taylor, Can. J. Microbiol. 18:1119 (1972).
6. Bartnicki-Garcia, S., and W.J. Nickerson, J. Bacteriol. 84:841 (1962).
7. Fieser, L.F., J. Amer. Chem. Soc. 46:2639 (1924).
8. Folch, J., M. Lees, and G.H. Sloane Stanley, J. Biol. Chem. 226:497 (1957).
9. Saliee, T.L., and G.M. Adams, J. Chromatogr. 51:545 (1970).
10. Capella, P., and C.M. Zorzut, Anal. Chem. 40:1458 (1968).
11. Shaw, R., Biochim. Biophys. Acta 98:230 (1965).
12. Kass, L.R., D.H.J. Brock, and K. Bloch, J. Biol. Chem. 242:4418 (1967).
13. Sumner, J.L., and E.D. Morgan, J. Gen. Microbiol. 59:215 (1969).

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