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Arachidonic Acid Production by Fungi

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After preliminary screening, *Mortierella alpina* and *Mortierella elongata* were compared with respect to arachidonic acid content. *M. alpina* ATCC 16266 produced 2.1 g of arachidonic acid per liter in media containing 10% glucose while the highest percentage of arachidonic acid in lipid (43.3%) was observed at a glucose concentration of 2%. Arachidonic acid content in lipids increased to 66% during storage.

Arachidonic acid is a precursor of numerous eicosanoids and other compounds which are presently the subject of extensive medical research (6, 14, 19). Although arachidonic acid is presently isolated from animal adrenal gland and liver and from sardines, the yield is only 0.2% (wt/wt) (1). Arachidonic acid is also found in the cells of ciliated protozoa, amoebae, algae, and other microorganisms (3, 5, 8, 22) including *Mortierella* species (15, 17, 20, 21).

Media and culture conditions. GY medium contained the following (in grams per liter): glucose, 20; and yeast extract, 10. YM medium (20) consisted of the following (in grams per liter): glucose, 10; polypeptone, 5; yeast extract, 3; and malt extract, 3. HD medium (10) contained the following (per liter): glucose, 30.0 g; yeast extract, 5.0 g; KH_2PO_4, 2.4 g; KNO_3, 1.0 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeCl_3 \cdot 6H_2O$, 15 mg; $ZnSO_4 \cdot 7H_2O$, 7.5 mg; and $CuSO_4 \cdot 5H_2O$, 0.5 mg. Fungal strains were maintained on GY medium supplemented with 3% agar and were transferred every 2 months. Cultures were first grown on HD medium for 48 h at 25°C. This culture (5% [vol/vol]) was used to inoculate the production medium. Inoculum and production cultures were prepared in 250-ml Erlenmeyer flasks containing 50 ml of medium and shaken at 300 rpm on an orbital shaker.

Lipid analysis. Lipids were extracted from the dried fungal biomass by the method of Bligh and Dyer (4), and the extract was dried at 36°C and methylated (11). The fatty acid methyl esters, dissolved in *n*-hexane, were analyzed by gas chromatography. The degree of unsaturation in the lipid fraction was calculated by the method of Kates and Baxter (12).

Screening at 25°C. Although several fungal strains have been screened for lipid accumulation, only selected strains which have some potential for intracellular lipid production are reported here. *Mortierella alpina* ATCC 32221 did not produce arachidonic acid at 25°C (Table 1). *Conidiobolus* species exhibited low arachidonic acid content. Arachidonic acid content of the biomass of the remaining strains ranged from 2.2 to 8.4% (wt/wt). The lowest levels of biomass production and arachidonic acid yield occurred in cultures grown in YM medium for all strains. Of the three *Mortierella elongata* strains tested, NRRL 5513 produced the highest biomass and the highest arachidonic acid yield in GY and HD media. In GY medium, NRRL 5513 had a low total lipid content (11.5%) but arachidonic acid accounted for 33.9% of this lipid. In contrast, in NRRL 5513 grown in HD medium, the total lipid content was higher but the percentage of arachidonic acid in lipid was lower. Arachidonic acid yield from *M. elongata* NRRL 5513 grown in HD medium was 22% higher than the corresponding value for NRRL 5513 grown in GY medium.

M. alpina ATCC 16266 and ATCC 42430 were the best producers of arachidonic acid of this species. The highest arachidonic acid yields were observed in strains grown in HD medium, where the arachidonic acid content in lipid was 26.4 to 26.9%. In GY medium, arachidonic acid yield was lower, although it accounted for 41.6 to 43.3% of total lipid. Contents of arachidonic, oleic, linoleic, and linolenic acids in strain ATCC 16266 were 43.3, 13.4, 8.5, and 1.5% (wt/wt) of total lipid, respectively. Corresponding values for strain ATCC 36965 were 3.9, 25.2, 40.0, and 6.3% (wt/wt) total lipid.

Screening at 11°C. *Mortierella* strains were also grown at 11°C in HD medium for 10 days (Table 2). For *M. elongata*, biomass values were higher and arachidonic acid contents of biomass were lower when strains were grown at 11 than at 25°C. While the average arachidonic acid yields of the three strains grown at 11°C (0.39 g/liter) and those for the same strains grown at 25°C (0.38 g/liter) were similar, productivities (arachidonic acid produced per liter per day) were much lower for strains grown at 11 than at 25°C. Although biomass values observed for *M. alpina* ATCC 16266 and ATCC 42430 were higher when the strains were grown at 11°C, lipid and arachidonic acid contents of biomass were significantly lower, and, overall, higher yields were observed at a growth temperature of 25°C. Traces of arachidonic acid were synthesized by *M. alpina* ATCC 32221 at 11°C, although no such production was observed at 25°C.

M. alpina ATCC 16266, which manifested the highest yields of arachidonic acid, was selected for further studies. Although strains grown on GY medium produced a lower overall yield than those grown on HD medium, GY medium was selected for further investigation because strains grown on it produced a higher content of arachidonic acid in lipid (43%) than did strains grown on HD medium (27%).

Effect of initial pH. When the initial pH of the medium was varied, strain ATCC 16266 grew well in the pH range of 3.8 to 8.0 (Fig. 1). Lipid content of biomass, degree of lipid unsaturation, and arachidonic acid yield of ATCC 16266 were also highest when the initial pH was 6.0.

Effect of carbon source. When different compounds were tested as carbon source in GY medium, growth of ATCC 16266 was found to be very poor with lactose, starch, and sucrose as carbon source, moderate with maltose, fructose, and glucose, and very good with linseed oil and glycerol (Table 3). The arachidonic acid content in lipids was above

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TABLE 1. Comparison of fungal strains with respect to growth, lipid content, and arachidonic acid production in selected media[a]

Strain	Medium	Biomass (g/liter)	Lipids in biomass (% [wt/wt])	Arachidonic acid		
				In biomass (% [wt/wt])	In lipids (% [wt/wt])	Yield (g/liter of broth)
Mortierella elongata						
ATCC 16271	GY	7.6	21.5	3.2	15.0	0.25
	YM	6.6	21.3	3.3	15.5	0.22
	HD	11.1	27.0	2.6	9.5	0.29
ATCC 24129	GY	7.8	20.4	4.7	23.0	0.37
	YM	6.2	12.9	2.2	17.2	0.14
	HD	9.0	16.0	3.2	19.8	0.29
NRRL 5513	GY	12.7	11.5	3.9	33.9	0.49
	YM	8.7	10.4	2.3	22.2	0.19
	HD	13.7	22.2	4.2	19.0	0.58
Mortierella alpina						
ATCC 16266	GY	12.1	13.2	5.7	43.3	0.69
	YM	8.9	12.5	3.4	27.1	0.30
	HD	13.0	31.2	8.4	26.9	1.09
ATCC 42430	GY	11.7	10.0	4.2	41.6	0.49
	YM	8.6	11.4	4.8	42.4	0.41
	HD	12.4	23.0	6.1	26.4	0.75
ATCC 32221	GY	10.8	1.6	0.0	0.0	0.0
	YM	7.8	1.6	0.0	0.0	0.0
	HD	13.7	1.1	0.0	0.0	0.0
ATCC 36965	GY	10.5	3.3	0.1	3.9	0.01
Conidiobulus obscurus						
ATCC 36369	GY	2.9	1.0	0.1	10.0	0.0
ATCC 42977	GY	2.1	1.3	0.1	9.5	0.0

[a] Culture conditions: 25°C, 6 days. Data are the averages of three replicates.

40% (wt/wt) with starch, maltose, glucose, and fructose used as carbon source. Although glycerol utilization produced the highest yield of arachidonic acid, the arachidonic acid content in lipids was quite low. The highest arachidonic acid yields were produced on GY medium with glucose as the carbon source. The effect of glucose concentration on arachidonic acid production was also investigated (Fig. 2). Arachidonic acid contents in lipids, in biomass, and per liter of culture were at their maximum in medium with glucose concentrations of 20, 50, and 100 g/liters, respectively.

Effect of nitrogen source. Of various nitrogen sources added separately at a 1% concentration to the medium containing 10% glucose, yeast extract followed by peptone resulted in the highest arachidonic acid yields of 2.09 and 0.74 g/liter, respectively. Growth was very poor with other nitrogen sources. High percentages of arachidonic acid in

TABLE 2. Comparison of *Mortierella* strains with respect to growth, lipid content, and arachidonic acid production in HD medium at 11°C[a]

Strain	Biomass (g/liter)	Lipids in biomass (% [wt/wt])	Arachidonic acid		
			In biomass (% [wt/wt])	In lipids (% [wt/wt])	Yield (g/liter of broth)
M. alpina					
ATCC 16266	17.8	22.6	5.0	22.2	0.90
ATCC 32221	4.2	1.6	0.1	7.1	0.01
ATCC 42430	21.7	10.7	3.4	32.1	0.74
M. elongata					
ATCC 16271	15.8	27.7	2.5	9.0	0.39
ATCC 21429	13.0	21.1	2.7	12.8	0.35
NRRL 5513	14.6	23.3	3.0	13.0	0.44

[a] Data are the averages of three replicates.

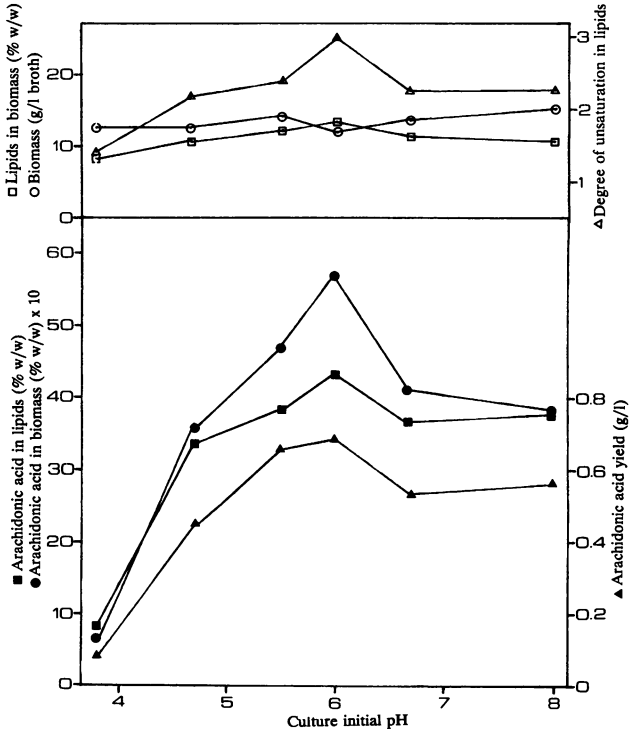

FIG. 1. Effect of initial culture pH on growth of strain and on arachidonic acid and lipid produced by *M. alpina* ATCC 16266.

TABLE 3. Effect of carbon source on growth, lipid content, and arachidonic acid production by *M. alpina* ATCC 16266[a]

Carbon source	Biomass (g/liter)	Lipids in biomass (% [wt/wt])	Degree of unsaturation in lipids	Arachidonic acid		
				In biomass (% [wt/wt])	In lipid (% [wt/wt])	Yield (g/liter of broth)
Fructose	12.8	12.2	2.43	4.9	40.1	0.63
Glucose	12.1	13.2	2.99	5.7	43.3	0.69
Glycerol	15.6	20.5	2.06	5.8	28.4	0.91
Lactose	4.0	5.1	2.23	1.7	33.6	0.07
Linseed oil	24.4	51.2	2.20	0.9	1.8	0.22
Maltose	13.5	11.3	2.45	4.8	42.2	0.64
Starch	4.6	5.0	2.48	2.0	40.3	0.09
Sucrose	3.8	4.4	2.10	1.2	27.9	0.05

[a] Culture conditions consisted of GY medium containing 2% (wt/vol) of each carbon source incubated at 25°C for 6 days. Data are the average of three replicates.

the lipid fraction were observed with urea (25.1%) and malt extract (23.8%) used as the nitrogen source.

Aging of mycelium. When the biomass, obtained with 5% glucose medium, was stored at 22°C for 1 week, the arachidonic acid content increased from 8.3 to 13.5% of dry biomass and from 25.3 to 41.3% of the lipid fraction. Similarly, arachidonic acid content in mycelium obtained with 2% glucose medium also increased from 5.7 to 8.7% dry weight and from 43.3 to 65.9% in the lipid fraction.

We have identified a strain of *M. alpina* (ATCC 16266) and media conditions which resulted in the production of 1.90 to 2.09 g of arachidonic acid per liter, representing 6.8 to 8.3% of biomass dry weight. The arachidonic acid yield observed is the highest so far reported in fungal shake-flask cultures (15, 21). Significantly higher arachidonic acid contents in biomass may be achieved by growth on agar plates (20). When recovered mycelium was stored for 7 days, arachidonic acid content increased to 65.9% of total lipid. A similar increase in arachidonic acid content to 67.4% was also reported by Shinmen et al. (17). With many microorganisms, a decrease in unsaturated fatty acid content occurs on aging (7). However, polyunsaturated fatty acids in *Ochromonas danica* (9) and *Phaeodactylum tricornutum* (2) increased with aging. Maximum yields of arachidonic acid in cultures of *M. elongata* were 0.96 to 0.99 g/liter (15, 21). In their fermentor studies, Shinmen et al. (18) reported a maximum arachidonic acid yield and percentage of arachidonic acid in lipid of 3.6 g/liter and 35% (wt/wt), respectively.

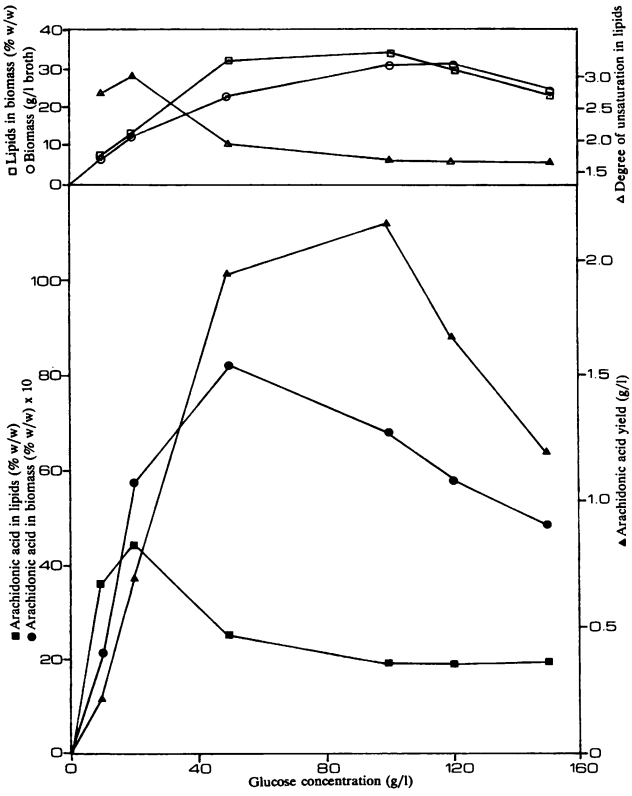

FIG. 2. Effect of glucose concentration on growth of strain and on arachidonic acid and lipid produced by *M. alpina* ATCC 16266.

Yields of arachidonic acid for *M. elongata* and *M. alpina* strains, cultured at 11°C, were similar to yields observed at 25°C in the same medium. In contrast, the production of eicosapentaenoic acid by several *Mortierella* species (18) and by *Chlorella minutissima* (16) is stimulated at reduced temperatures. Glucose was also reported to best support cell growth and arachidonic acid production by *M. elongata*, maximum arachidonic acid production being observed in media containing 100 g of glucose per liter (21). The accumulation of oleic and linoleic acids by the low-arachidonic-acid-producing strain, *M. alpina* ATCC 36965, suggests that the enzymatic desaturation and/or elongation reactions for conversion of linoleic to arachidonic acid (13) do not operate efficiently in this strain.

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REFERENCES

1. **Ahern, T. J.** 1984. Plant-derived catalysts and precursors for use in prostaglandin synthesis. J. Am. Oil. Chem. Soc. **61**:1754–1757.
2. **Arao, T., A. Kawaguchi, and M. Yamada.** 1987. Positional distribution of fatty acids in lipids of the marine diatom *Phaeodactylum tricornutum*. Phytochemistry **26**:2573–2576.
3. **Bergstrom, S., and H. Danielsson.** 1984. The enzymatic formation of prostaglandin E_2 from arachidonic acid, prostaglandins and related factors. Biochim. Biophys. Acta **90**:207–210.
4. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37**:911–917.
5. **Chu, F.-L. E., and J. L. Dupuy.** 1980. The fatty acid composition of three unicellular algal species used as food sources for larvae of the American oyster (*Crassostrea virginica*). Lipids **15**:356–364.

6. **Das, U. N., M. E. Begin, Y. S. Huang, and D. F. Horrobin.** 1987. Polyunsaturated fatty acids augment free radical generation in tumor cells *in vitro*. Biochem. Biophys. Res. Commun. **145:**15–24.
7. **Erwin, J.** 1973. Comparative biochemistry of fatty acids in eucaryotic microorganisms, p. 41–143. *In* J. A. Erwin (ed.), Lipids and biomembranes of eucaryotic microorganisms. Academic Press, Inc., New York.
8. **Erwin, J., and K. Bloch.** 1964. Biosynthesis of unsaturated fatty acids in microorganisms—structure and biosynthetic pathways are compared and related to physiological properties of the organisms. Science **143:**1006–1012.
9. **Gellerman, J. L., and K. Schlenk.** 1979. Methyl-directed desaturation of arachidonic acid to eicosapentaenoic acid in the fungus, *Saprolegnia parasitica*. Biochim. Biophys. Acta **573:** 23–30.
10. **Hansson, L., and M. Dostalek.** 1988. Effect of culture conditions on mycelial growth and production of γ-linolenic acid by the fungus *Mortierella ramanniana*. Appl. Microbiol. Biotechnol. **28:**240–246.
11. **Holub, B. J., and C. M. Skeaff.** 1987. Nutritional regulation of cellular phosphatidylinositol. Methods Enzymol. **141:**234–244.
12. **Kates, M., and R. M. Baxter.** 1962. Lipid composition of mesophilic and psychrophilic yeasts as influenced by environmental temperature. Can. J. Biochem. Physiol. **40:**1213–1227.
13. **Korn, E. D., C. I. Greenblatt, and A. M. Lees.** 1965. Synthesis of unsaturated fatty acids in the slime mold *Physarum polycephalum* and the zooflagellates *Leishmania tarentolae*, *Trypanosoma lewisi* and *Crithidia* sp.: a comparative study. J. Lipid Res. **6:**43–50.
14. **Marx, J. L.** 1982. The leukotrienes in allergy and inflammation. Science **215:**1380–1383.
15. **Sajbidor, J., S. Dobronova, and M. Certik.** 1990. Arachidonic acid production by *Mortierella* sp. S-17. Influence of C/N ratio. Biotechnol. Lett. **12:**455–456.
16. **Seto, A., H. L. Wang, and C. W. Hesseltine.** 1984. Culture conditions affect eicosapentaenoic acid content of *Chlorella minutissima*. J. Am. Oil. Chem. Soc. **61:**892–894.
17. **Shinmen, Y., S. Shimizu, K. Akimoto, H. Kawashima, and H. Yamada.** 1989. Production of arachidonic acid by *Mortierella* fungi: selection of a potent producer and optimisation of culture conditions for large scale production. Appl. Microbiol. Biotechnol. **31:**11–16.
18. **Shinmen, Y., H. Yamada, and S. Shimizu.** 1988. Microbial process for production of dihomo-γ-linolenic acid and eicosapentaenoic acid. European Patent Application 252716.
19. **Simopoulos, A. P.** 1989. Summary of the NATO advanced research workshop on dietary ω3 and ω6 fatty acids: biological effects and nutritional essentiality. J. Nutr. **119:**521–528.
20. **Totani, N., and K. Oba.** 1987. The filamentous fungus *Mortierella alpina*, high in arachidonic acid. Lipids **22:**1060–1062.
21. **Yamada, H., S. Shimizu, and Y. Shinmen.** 1987. Production of arachidonic acid by *Mortierella elongata* 1S-5. Agric. Biol. Chem. **51:**785–790.
22. **Yongmanitchai, W., and O. P. Ward.** 1989. Omega-3 fatty acids: alternative sources of production. Process Biochem. **24:**117–125.