Effects of Aging *Mortierella* Mycelium on Production of Arachidonic and Eicosapentaenoic Acids

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Arachidonic acid and eicosapentaenoic acid (EPA) are important intermediates of eicosanoid metabolism and are presently the subject of extensive nutritional and medical research. The effects of mycelial aging on production of these fatty acids were investigated as part of a research program directed toward examining the feasibility of economically producing these products by fungal fermentation.

Arachidonic acid content of *M. alpina* ATCC 32222 increased from 4.1–8.3% to 13–16% during aging while lipid content of mycelium increased from 14–18% to 33–45%. Maximum lipid content produced in biomass during storage declined as harvesting time was increased from 3 to 6 days while maximum arachidonic acid content in lipid increased. Maximum lipid and arachidonic acid was produced during aging at pH 8, whereas arachidonic acid content of lipids was highest in mycelium aged at pH 6. EPA content of *M. elongata* NRRL 5513 biomass increased during aging, reaching a maximum after 22–28 days. When the pH of the culture prior to harvesting was adjusted in the range of pH 4–9, pH values for development of maximum EPA in biomass and in lipids during storage were found to be 6 and 7, respectively. Temperature of aging had little effect on arachidonic acid or EPA content.

KEY WORDS: Arachidonic acid, eicosapentaenoic acid, *Mortierella alpina*, *Mortierella elongata*, mycelium aging.

Arachidonic acid and eicosapentaenoic (EPA) are important intermediates in eicosanoid metabolism. They are precursors of prostaglandins, leukotrienes and a large group of C20 and C22 compounds that are presently the subject of intrinsic nutritional and medical research (1–4). Supply of EPA from the current major commercial source, fish and fish oils, is unlikely to meet future requirements and alternative microbial sources are being sought (5). Arachidonic acid is presently isolated in low yields from animal adrenal gland and liver as well as from sardines (6). Lower fungi of the Phycomycetes class are a promising source of a variety of polyunsaturated fatty acids (7). These fungi, especially in the order Mucorales, are rich in gamma-linolenic acid, and the fermentation process for production of this fatty acid product has been commercialized (8–10). Many species of the genus *Mortierella* are rich in arachidonic acid and EPA, depending on the species and culture conditions (11–15). We have previously investigated factors affecting production of arachidonic acid and EPA by *Mortierella* strains producing high amounts of these acid components (16,17).

As microbial biomass ages, many microorganisms tend to store their energy source in the form of lipid, and this lipid is usually rich in saturated and monounsaturated fatty acids. With many microorganisms, a general decrease of unsaturated fatty acid as a function of time has been noted (18). However, in the case of a photosynthetic protist *Ochromonas danica* (19), a marine diatom, *Phaeodactylum tricornutum* (20), and some *Mortierella* species (12,15,17), the concentrations of polyunsaturated fatty acids increased significantly as the culture aged. However, factors that affect production of polyunsaturated fatty acids during aging of these species have not been examined. In this paper, we report aspects of this aging phenomenon with respect to production of arachidonic acid by *M. alpina* ATCC 32222 and EPA by *M. elongata* NRRL 5513.

MATERIALS AND METHODS

Chemicals. Standard fatty acids were purchased from Sigma Chemical Company, St. Louis, MO. Solvents and reagents were obtained from Aldrich Chemical Co., Milwaukee, WI, and British Drug House, Toronto, Canada. Linseed oil was supplied by Recochem Inc., Toronto, Canada. It contained (%, w/w): palmitic acid, 4.9; stearic acid, 3.0; oleic acid, 19.5; linoleic acid, 14.5; α-linolenic acid, 53.5; and other acids, 3.7.

Culture conditions. Mortierella strains were maintained on 3% agar containing 20 g/L glucose and 10 g/L yeast extract and were transferred every 2 mon. Culture medium for production of arachidonic acid by *M. alpina* contained (g/L): glucose, 50; yeast extract, 5; sodium nitrate, 3; K_2HPO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5 and $FeSO_4 \cdot 7H_2O$, 0.01. Medium for production of EPA by *M. elongata* NRRL 5513 contained (g/L): linseed oil, 20; KH_2PO_4, 2.4; KNO_3, 1; $CaCl_2 \cdot 2H_2O$, 0.1; $MgSO_4 \cdot 7H_2O$, 0.5; $FeCl_3 \cdot 6H_2O$, 0.015; $ZnSO_4 \cdot 7H_2O$, 0.0075; and $CuSO_4 \cdot 5H_2O$, 0.0005. Liquid cultures containing 50 mL of these media in 250-mL Erlenmeyer flasks were inoculated with mycelium from freshly grown agar cultures and incubated at 25°C with orbital shaking at 300 rpm. A 5% inoculum from these flasks was used to inoculate similar replicate Erlenmeyer flasks for production of arachidonic acid and EPA. *M. alpina* and *M. elongata* production cultures were incubated at 25°C and 15°C, respectively.

Mycelium aging. Mycelium was harvested by vacuum filtration, and approximately 5 g (wet weight) was stored in small petri dishes at different temperatures for aging.

Biomass determinations. Dry weight of biomass was determined by vacuum filtration or centrifugation of fungal cell suspension, washing it with 1% NaCl and distilled water and drying at 100°C for 12–16 hr.

Extraction and determination of lipids. The dried cells were weighed (20–40 mg) in Teflon-lined screw-cap test tubes of 10 mL capacity, and the lipids were extracted according to the procedure of Bligh and Dyer (21). The extracted lipids were dried at 36°C under nitrogen atmosphere and then methylated by the method of Holub and Skeaff (22). Then the fatty acid methyl esters were dissolved in 200 μL n-hexane, and a 1-μL sample was in-

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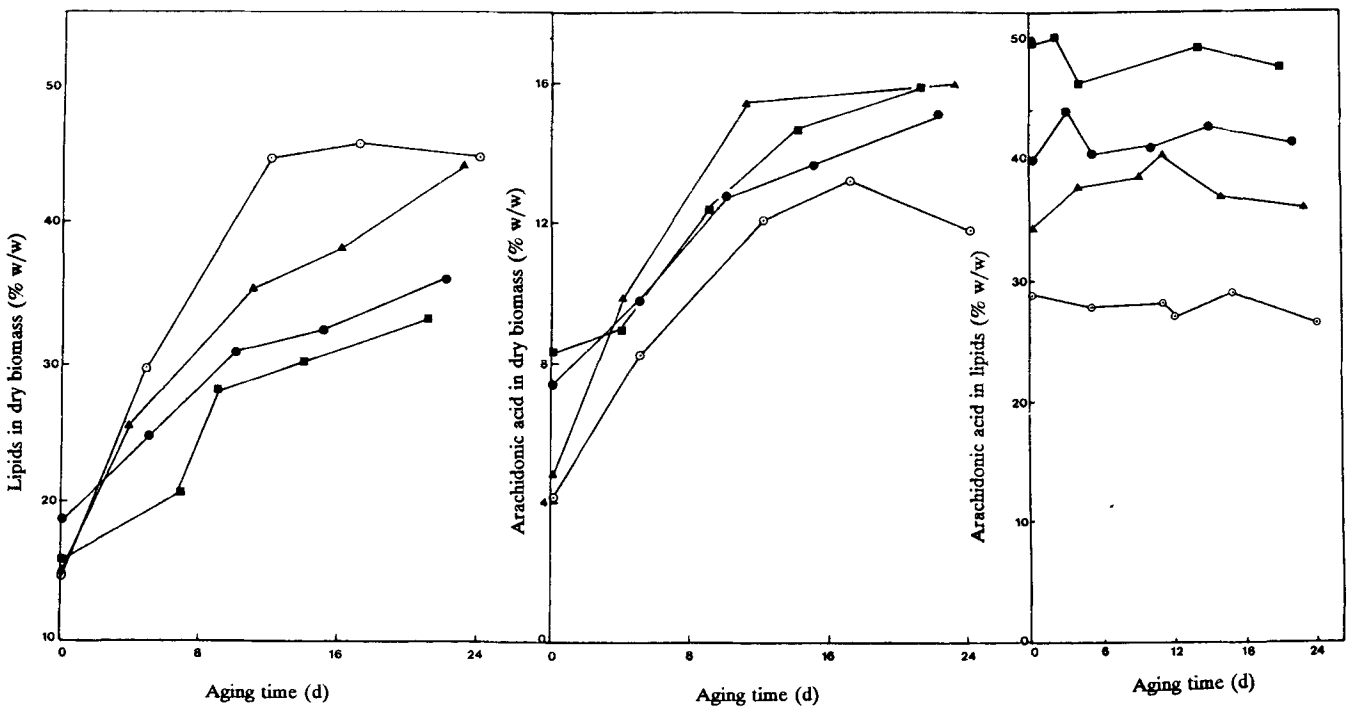

FIG. 1. Content of lipids and arachidonic acid in *M. alpina* biomass as a function of aging time for mycelium harvested after different periods of cultivation. Period of cultivation: ○, 3 days; ▲, 4 days; ●, 5 days; ■, 6 days. Cultivation time: 25°C; aging temperature: 25°C.

jected into a gas-liquid chromatograph (GLC) for analysis. The Shimadzu CR601 GLC (Kyoto, Japan) was connected with a GC-14A data integrator. The GLC was fitted with megabore column DB-225 (Chromatographic Specialties, Brockville, Ontario, Canada) and a flame ionization detector. Helium was used as the carrier gas. The fatty acid ester peaks were identified and calibrated by using standard fatty acids. Pentadecanoic acid (C15:0) was used as internal standard. Analytical data are the averages of three determinations.

RESULTS

Mycelium was harvested from *M. alpina* and *M. elongata* cultures at different times. In previous work (16,17), we have observed that *M. alpina* cultures reached a stationary phase of growth after 3 days at 25°C and that little variation in biomass was observed in these cultures between 3–6 days. Preliminary studies indicated that maximum EPA was produced by *M. elongata* at 15°C in linseed oil-containing media, and that maximum biomass was observed after approximately 7 days. Cultures of *M. alpina* used as a source of arachidonic acid and *M. elongata* as a source of EPA were harvested at intervals from 3–6 days and 7–10 days, respectively, and the effect of aging on the content of these acids was investigated.

Changes in lipid and arachidonic content of *M. alpina* mycelia during aging at 25°C are illustrated in Figure 1. Regardless of culture harvesting time, lipid content in biomass (dry weight basis) increased from 14–18% to 33–45%. Increase in lipid content of mycelium was greatest in cultures harvested after 3 days, and least in 6-day harvested cultures. Initial arachidonic acid content, as a percentage of dry biomass, ranged from 4.1–8.3% (w/w). General increases in arachidonic acid content were observed during the 24-day storage period at 25°C. Maximum arachidonic acid contents in biomass (dry basis) due to aging ranged from 13–16% (w/w). Plots of arachidonic acid content in lipids indicate that during storage the proportion of arachidonic acid in lipids remains relatively constant, ranging from 26–29% in 3-day harvested mycelium to 46–50% in 6-day harvested mycelium.

When the maximum content of lipid and arachidonic acid observed during storage is related to harvesting time, an interesting trend is observed (Fig. 2, Table 1). Maximum lipid content produced in biomass during storage declines as harvesting time is increased from 3–6 days while maximum arachidonic acid content in lipid increases. Maximum arachidonic acid content produced in biomass during storage was found to be relatively independent of harvesting time. The fatty acid spectra corresponding to the 3- and 6-day harvested and stored samples indicate that the 3-day material contains a greater percentage in lipid of palmitic (16:0), stearic (18:0) and oleic (18:1) acids and a lower percentage of arachidonic acid than the 6-day material.

By adjusting the culture pH in the range 4.0–9.0 prior to mycelia harvesting, the effect of pH on lipid and arachidonic acid development during a 6-day storage period at 25°C was investigated (Fig. 3, Table 2). Lipid and arachidonic acid content of biomass was maximum when mycelium was harvested from medium at pH 8, whereas the arachidonic acid content of lipids was highest in mycelia recovered from culture medium adjusted to pH 6. Fatty acid spectra of mycelia stored at pH 4,6 and 8 illustrate that the increased arachidonic acid content of

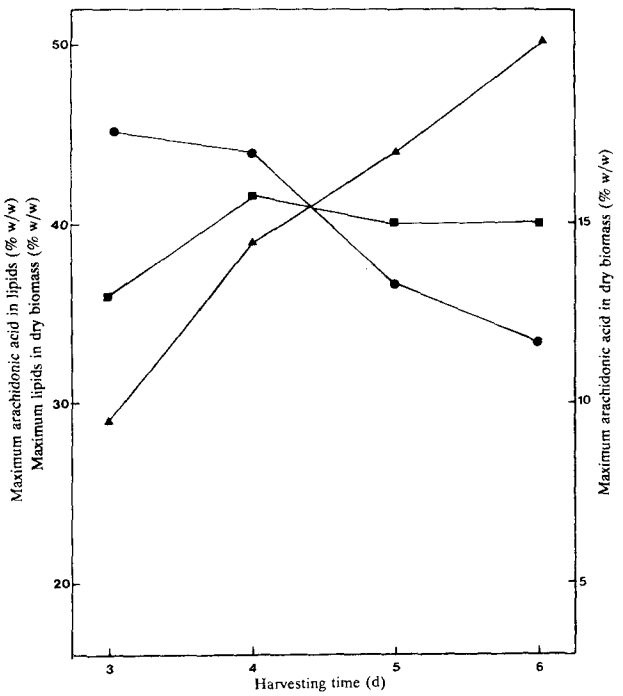

FIG. 2. Effect of culture harvesting time on maximum lipid and arachidonic acid content of *M. alpina* mycelium observed during aging: ▲, arachidonic acid in lipids; ■, arachidonic acid in biomass; ●, lipids in biomass. Cultivation temperature: 25°C; aging temperature: 25°C.

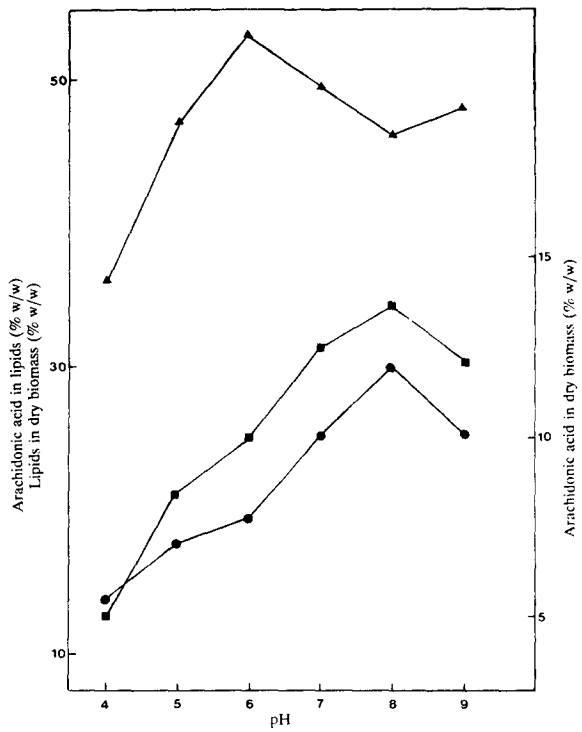

FIG. 3. Effect of adjusting culture pH prior to culture harvesting of lipid and arachidonic acid content of aged *M. alpina* mycelium: ▲, arachidonic acid in lipids; ■, arachidonic acid in biomass; ●, lipids in biomass. Cultivation temperature: 25°C; aging temperature: 25°C; aging time: 15 days.

TABLE 1

Fatty Acid Spectra (% of total fatty acids) of 3- and 6-day Harvested and Stored Samples

	16:0	16:1	18:0	18:1	18:2	18:3	20:3	20:4	20:5	Others
3 Days	12.2	0.2	17.5	14.3	6.1	0.7	5.9	28.9	0.0	14.1
6 Days	8.8	trace	10.2	10.5	4.7	trace	2.7	48.9	0.0	14.1

TABLE 2

Fatty Acid Spectra (% of total fatty acids) of Samples Stored at pH 4, 6 and 8

	16:0	16:1	18:0	18:1	18:2	18:3	20:3	20:4	20:5	Others
pH 4	13.0	trace	14.0	14.6	5.3	trace	2.1	36.0	0.0	15.0
pH 6	8.5	0.8	9.0	9.9	4.9	trace	2.4	51.3	0.0	13.1
pH 8	9.1	trace	10.5	11.3	5.1	trace	2.9	46.2	0.0	15.0

lipid occurs at pH 6 and 8 primarily at the expense of saturated palmitic, stearic and oleic acids. The effect of temperature on aging was investigated at 5, 15 and 25°C (Fig. 4). Overall, temperature appeared to have little effect on total lipid and arachidonic acid content.

The effect of aging time on lipid and EPA content was monitored in mycelia stored at 4°C over a 31-day period (Fig. 5, Table 3). Lipid content in mycelium harvested after 9 and 10 days increased to a maximum after 15–16 days of aging and then manifested a decline. Mycelium harvested after 7 days exhibited a dramatic increase in lipid content between 13 and 31 days of aging. EPA content of biomass and of total cell lipids also increased during aging. The fatty acid spectra of 9-day harvested mycelia before and after storage for 22 days indicate that EPA content in lipids increased 1.73-fold during storage, from 5.1% to 8.8% of total lipids.

The influence of harvesting time on the maximum content of lipids and EPA produced on subsequent aging is illustrated in Figure 6. Maximum lipid content decreased as the harvesting time was extended from 7 to 9 days. Only a small variation in maximum EPA content of aged mycelium as a function of harvesting time was observed. Maximum EPA content in lipid ranged from 7.0–8.4% (w/w) while maximum EPA content in biomass (dry basis) ranged from 28.8–32 mg/g. A limited investigation on the effect of temperature on EPA production during aging was carried out by incubation of the harvested mycelium at 4°C and 15°C (Table 4). Aging temperature manifested little overall effect on EPA contents in lipid and biomass. EPA-containing mycelium was also aged at 25°C. In this case there was essentially no increase in EPA during storage. The effect of adjusting the culture pH prior to harvesting on production of EPA during aging is illustrated in Table 5. The pH curves obtained are relatively flat over the range pH 4–9, with optima for EPA in biomass and in lipids observed at 6 and 7, respectively.

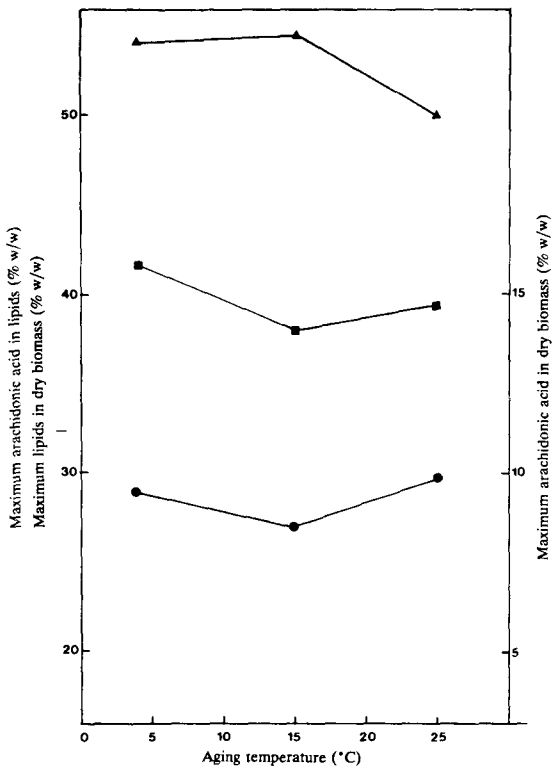

FIG. 4. Effect of storage temperature on maximum yields of lipids and arachidonic acid content observed in *M. alpina* mycelium during aging: ▲, arachidonic acid in lipids; ■, arachidonic acid in biomass; ●, lipids in biomass.

DISCUSSION

Extending the harvesting time prior to storage to increase the arachidonic acid or EPA content of lipid may facilitate recovery of these acids during downstream processing. Harvesting time had little effect on maximum arachidonic acid or EPA content of aged mycelium. Fatty acid spectra for *M. alpina* harvested after 3 and 6 days indicated a general increase in unsaturation level with increased culture time, and spectra presented for *M. elongata* before and after aging also indicated a shift to more polyunsaturated fatty acids during storage. Aging of harvested *M. alpina* for 6 days resulted in decomposition of the major mycelial fatty acids, palmitic, oleic and linoleic acid with arachidonic acid content of lipids concomitantly rising to nearly 70% of total fatty acids (12). Nevertheless, there is a general lack of data in the literature on changes in mycelial lipid composition following harvesting, with most reports describing effects of environmental conditions on fungal lipids during culture. Our results will, therefore, be discussed in the context of these reports.

The amount of lipid produced by a given species of fungus has been found to depend to a great extent on the developmental stage of growth and/or culture conditions (23). In the case of *A. nidulans*, fat formation in surface cultures at 25°C was found to accelerate at the later stages of growth after which fat content decreased (24). Boulton and Ratledge (25) described a general biphasic pattern of lipid accumulation in oleaginous organisms during batch culture. When nutrients are in excess, lipid content of cells stays approximately constant, while after nutrients and

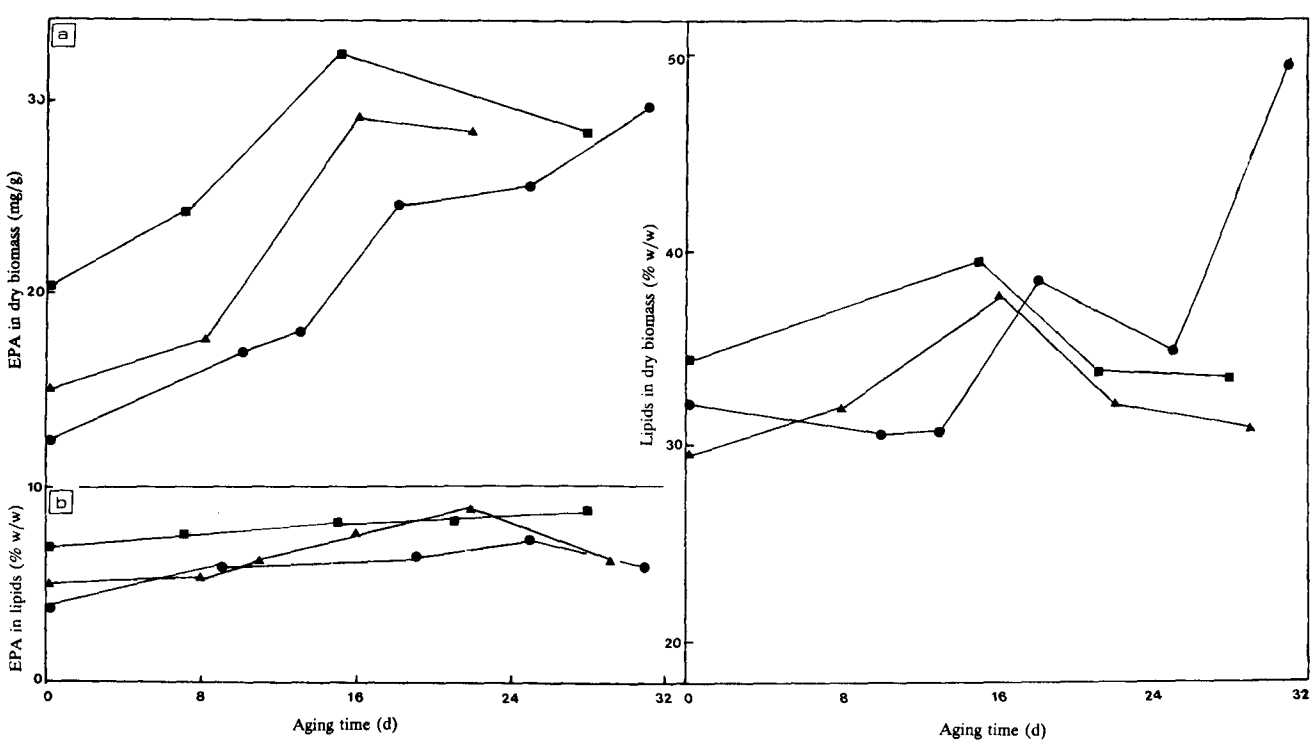

FIG. 5. Content of lipids and EPA as a function of aging time in *M. elongata* mycelium harvested after different periods of cultivation. Period of cultivation: ●, 7 days; ▲, 9 days; ■, 10 days. Cultivation temperature: 15°C; aging temperature: 4°C.

TABLE 3

Fatty Acid Spectra (% of total fatty acids) of 9-day Harvested Mycelia Before and After Storage for 22 days

	16:0	16:1	18:0	18:1	18:2	18:3	20:4	20:5	Others
Before	7.0	trace	3.3	21.8	13.6	34.8	5.3	5.1	9.2
After	6.6	0.4	2.6	18.2	12.3	30.7	8.3	8.8	12.2

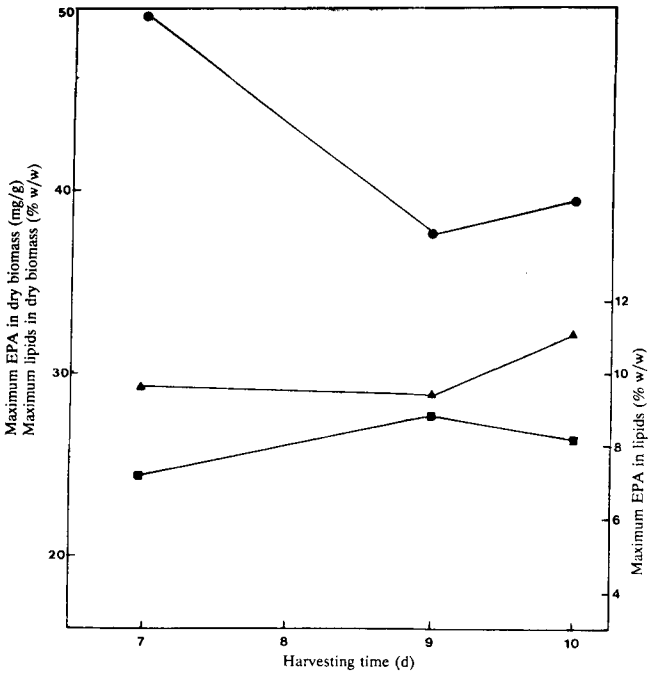

FIG. 6. Effect of culture harvesting time on maximum lipid and EPA content of *M. elongata* mycelium observed during aging: ▲, EPA in lipids; ■, EPA in biomass; ●, lipids in biomass. Cultivation temperature: 15°C; aging temperature, 4°C.

especially nitrogen are exhausted there is a continued build-up of lipid without a corresponding increase in biomass.

Maximum lipid and arachidonic acid was produced in *M. alpina* mycelia aged at pH 8, while maximum lipid and EPA was observed in *M. elongata* mycelium stored at pH 7 and 6, respectively. An increase in unsaturated fatty acids with increasing culture pH has been reported in many fungi (26).

It was noted that the temperature of aging had little effect on lipid, arachidonic acid (in the range 5–25°C) and EPA (in the range 4–15°C) contents of *M. alpina* and *M. elongata*. No increase in EPA content of *M. elongata* was observed during aging at 25°C. In cell culture studies, generally lower temperatures result in an increase in unsaturation (27,28). Lipids of *Mucor* and *Rhizopus* species were more unsaturated when fungi were grown at lower temperature (23).

Harvesting of mycelium from liquid culture media may have the effect of increasing oxygen supply to the stored mycelium since increased oxygen tension elevated unsaturated fatty acid content of fungi of the order Mucorales (29). The desaturase enzymes required for production of the unsaturated fatty acids require molecular oxygen as cofactor (27). The tendency for some fungi to produce polyunsaturated fatty acids at low temperatures may be due to the temperature-labile nature of desaturase enzymes (30). This may explain the observation that aging of *M. elongata* mycelium at 25°C did not increase EPA content.

The aging studies reported in this paper may have a practical application in the development of fungal systems for commercial production of arachidonic acid, EPA, and possibly DHA.

TABLE 4

Effect of Storage Temperature on Lipids and EPA Content Observed in *M. elongata* Mycelium During Aging[a]

Storage temperature (°C)	Component	Aging time (days) 0	7	8	15	21	28
4	EPA in dry biomass	24.0	20.2	—	32.0	28.0	28.0
15	(mg/g)	24.0	20.2	—	27.2	33.0	30.2
4	EPA in lipids	7.0	7.6	—	8.1	8.3	8.4
15	(% w/w)	7.0	6.7	—	7.4	—	8.1
4	Lipids in dry biomass	34.4	—	26.6	39.2	33.6	33.6
15	(% w/w)	34.4	—	30.0	36.6	33.0	30.2

[a]Cultivation time: 10 days; cultivation temperature: 15°C.

TABLE 5

Effect of Adjusting Culture pH Prior to Culture Harvesting on Lipid and EPA Content of Aged *M. elongata* Mycelium[a]

Component	Adjusted pH 4	5	6	7	8	9
EPA in dry biomass (mg/g)	26.6	31.2	34.8	31.6	30.4	28.2
EPA in lipids (% w/w)	8.8	10.0	9.0	10.9	9.6	8.1
Lipids in dry biomass (% w/w)	31.2	31.2	38.5	29.4	31.8	34.8

[a]Cultivation time: 10 days; storage time: 6 days; cultivation temperature: 15°C; storage temperature: 4°C.

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