Technical

Production of Eicosapentaenoic Acid by *Mortierella* Fungi

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Mycelia of arachidonic acid-producing fungi belonging to the genus *Mortierella* were found to be rich sources of 5,8,11,14,17-*cis*-eicosapentaenoic acid (EPA). Production of EPA by these fungi was observed only when they were grown at low temperature (6–16 C). EPA comprised 5–20% of the total extractable mycelial fatty acids in most strains tested. No significant accumulation of EPA was observed on incubation at high temperature (20–28 C), at which the other major mycelial C-20 fatty acid, arachidonic acid, was still efficiently produced. In a study on the optimization of the culture conditions for EPA production by a selected fungi *M. alpina* 20–17, a medium containing glucose and yeast extract as major carbon and nitrogen sources, respectively, was found to be suitable. Periodic feeding of glucose during growth of the fungus and cultivation at high temperature (20 C) during the early growth phase followed by temperature shift to 12 C were found to be effective at increasing mycelial yield and reducing cultural period, respectively. Under the optimal culture conditions, the EPA production reached 0.49 mg/ml of culture broth (29 mg/g dry mycelia). This value accounted for 13.5% of the total fatty acids in the extracted lipids. Other major fatty acids in the lipids were palmitic acid (6.0%, by weight), stearic acid (5.3), oleic acid (6.2), linoleic acid (3.0), γ-linolenic acid (3.5) and arachidonic acid (60.0).

5,8,11,14,17-*cis*-Eicosapantaenoic acid (EPA) is a rare C-20 polyunsaturated fatty acid of potential pharmaceutical value. This fatty acid has been shown to be effective in preventing blood platelet aggregation (1). It has also been demonstrated to be useful for blood cholesterol reduction, thus reducing the risk of atherosclerosis (2). Several marine fish oil products have become available recently as lipid sources relatively rich in EPA. For practical purposes, however, these conventional sources are not satisfactory, due to their low EPA contents and the presence of other fatty acids with less desirable properties. A marine alga, *Chlorella minutissima* (3), a freshwater alga, *Monodus subterraneus* (4), a moss, *Lepotobryum pyriforme* (5), and *Euglena gracilis* (6), have been suggested as alternative sources of EPA. But, again, they are not so advantageous because of their poor growth rates and low lipid contents. To obtain more suitable sources for large-scale preparation of EPA, we have started screening lower classes of microorganisms, i.e., bacteria and fungi, as to the ability to accumulate lipids containing EPA. No attention has been paid so far to such microorganisms as sources of EPA.

In previous papers (7,8), we reported that several fungi accumulate large amounts of arachidonic acid when grown in usual media containing glucose as a major carbon source. We have now found that these arachidonic acid producers, especialy strains belonging to the genus *Mortierella* subgenus *Mortierella*, specifically accumulate EPA in their mycelia when grown at low temperature (9). In this work, the potential of various arachidonic acid-producing fungi as sources of EPA, and the environmental and nutritional conditions under which maximum EPA productivity can be obtained, were studied.

MATERIALS AND METHODS

Chemicals. Fatty acid methyl esters were purchased from Funakoshi Chemicals, Tokyo. All other reagents used in this work were of analytical grade and commercially available.

Microorganisms, media and cultivations. All fungal strains used were from our stock cultures (AKU Culture Collection, Faculty of Agriculture, Kyoto University). Medium GY contained 2% glucose and 1.0% yeast extract, pH 6.0. Medium YM was described previously (7). Each fungus was inoculated into 50-ml shaking flasks containing 10 ml of either medium GY or YM and then incubated at 12 C for seven days with reciprocal shaking (120 strokes/min), unless otherwise stated.

Extraction and determination of fatty acids. Fungal cells were harvested by suction filtration, washed with 50 ml of water and then dried at 100 C overnight. The dried cells were suspended in five ml of methylene chloride–10% methanolic HCl (1:1, v/v) for three hr at 50 C. As an internal standard, *n*-heptadecanoic acid (0.5 mg) usually was included in the methanolysis mixture. After extraction with 20 ml of *n*-hexane, followed by evaporation, the fatty acid methyl esters were dissolved in 0.05–0.1 ml of acetonitrile and then analyzed by gas liquid chromatography (GLC). The conditions for GLC were the same as those described previously (7) except for the following changes: glass column (3 mm × 2 m) packed with 5% Advans DS on 80/100 mesh Chromosorb W (Shimadzu, Kyoto); column temperature, 190 C, and injection port temperature, 240 C. Mycelial fatty acid composition values are given in weight percent.

Isolation of the EPA methyl ester from fungal mycelia. The mycelia from two separate cultures of *Mortierella alpina* 20–17, each of which was grown in 50 ml of medium GY in a 500-ml flask under the optimal conditions as described below, were collected by suction filtration and then washed with 200 ml of water. The procedures used for transmethylation and purifi-

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TABLE 1

Comparison of EPA Productivities and Changes in Mycelial Fatty Acid Composition in *Mortierella* Fungi Grown at 12 and 28 or 24 C[a]

Strain	Temperature (C)/ cultivation period (days)/medium	Productivity				Fatty acid composition (%)[b]								
		Mycelial mass (mg/ml of culture medium)	Total FA[c] (mg/g dry mycelia)	EPA content (mg/g dry mycelia)	EPA yield (μg/ml of culture medium)	16:0	18:0	18:1	18:2	18:3	20:3	20:4	20:5	others
M. hygrophila IFO 5941	12/7/GY[d]	5.5	94	9.8	54	16.3	3.8	30.7	8.9	11.3	2.5	13.6	10.4	2.5
	28/6/GY[d]	7.0	183	0	0	24.7	2.9	37.4	9.5	5.5	1.6	17.9	0	0.5
M. zychae CBS 652.68	12/10/GY	10.0	166	4.4	44	15.5	15.1	31.9	6.4	5.0	5.9	15.0	2.7	2.5
	28/7/GY	7.7	120	0	0	23.2	12.6	29.3	8.7	6.0	3.3	16.3	0	0.6
M. elongata CBS 121.71	12/10/GY	10.0	189	9.6	95	14.3	15.4	35.8	5.1	4.4	3.8	12.6	5.0	3.6
	28/7/GY	8.1	61	0	0	15.4	14.0	30.3	6.7	6.0	3.8	21.7	0	2.1
M. elongata 1S-5 AKU 3999	12/7/YM	5.8	76	6.0	35	15.5	4.2	31.6	8.9	7.6	2.9	15.5	7.9	5.9
	24/6/YM	6.9	166	0	0	14.5	8.0	34.0	7.6	6.0	2.6	23.6	0	3.7
M. parvispora 2S-13 AKU 3994	12/7/YM	3.0	77	8.4	25	8.8	2.2	24.5	15.3	13.8	2.4	14.4	10.9	7.7
	28/6/YM	8.0	201	0	0	7.8	9.0	56.3	5.3	6.9	1.9	9.8	0	0
M. schmuckeri NRRL 2761	12/7/GY	10.4	219	4.9	51	24.6	11.6	39.1	5.3	3.2	4.0	5.8	2.3	4.1
	28/5/GY	8.0	205	0	0	19.9	12.4	37.1	7.4	4.9	4.9	12.4	0	1.0
M. alpina 1S-4 AKU 3998	12/7/GY	3.5	59	8.1	28	8.7	2.2	16.8	14.4	10.1	3.0	28.4	13.9	2.9
	28/6/GY	9.5	318	0	0	17.9	5.9	11.3	9.8	4.1	3.3	47.7	0	0
M. alpina 2O-17 AKU 3996	12/7/GY	4.6	71	12.1	55	7.6	1.2	9.2	11.0	11.2	3.6	38.7	17.1	1.7
	28/6/GY	9.4	277	0	0	15.8	5.3	12.0	18.2	4.8	2.3	39.6	0	0.7
M. alpina CBS 250.53	12/10/GY	8.8	109	6.8	60	13.6	5.4	24.2	11.4	7.2	5.4	21.0	6.2	5.6
	28/7/GY	6.2	124	0	0	14.5	5.9	27.8	11.4	7.4	4.0	27.1	0	1.9
M. alpina 1-83 AKU 3995	12/7/GY	3.8	58	11.6	44	9.2	1.3	15.7	14.1	11.4	1.8	24.2	19.8	2.5
	28/6/GY	9.4	300	0	0	18.6	4.8	12.3	8.9	4.1	3.4	47.9	0	0
M. alpina CBS 219.35	12/10/GY	9.7	187	7.1	69	16.9	12.0	17.8	7.4	6.6	10.1	22.3	3.8	3.1
	28/7/GY	5.5	139	0	0	11.2	4.0	30.5	14.4	10.9	4.1	22.4	0	1.7

[a]Each strain was grown under conditions described in the text except for growth temperature and cultivation period as indicated.
[b]16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, γ-linolenic acid; 20:3, dihomo-γ-linolenic acid; 20:4, arachidonic acid; 20:5, EPA. α-Linolenic acid and other polyunsaturated fatty acids of the n-3 series were not detected.
[c]FA, fatty acid.
[d]Medium GY and medium YM contained 2% glucose and 1% yeast extract (pH 6.0), and 1% glucose, 0.5% peptone, 0.3% yeast extract and 0.3% malt extract (pH 5.0), respectively.

cation of EPA were essentially the same as described previously (7).

Other methods. Fungal growth was measured by determining the mycelial weight after drying at 100 C overnight. Glucose concentrations in media were measured with a commercially available kit (Blood Sugar-GOD-Perid-Test, Boehringer, Mannheim, Federal Republic of Germany) essentially according to the method of Werner et al. (10). Mass and ^1H NMR spectra were measured with a Hitachi M-80 and a Nicolet NT-360, respectively.

RESULTS

Screening of fungal strains capable of producing EPA. Two hundred and fifteen strains of Mucoraceae were assayed as to their EPA productivity at a low growth temperature (12 C), because several arachidonic acid-producing *Mortierella* strains had been found to accumulate detectable amounts of EPA in their mycelia only when grown at low temperature (9). Such strains were found in the genus *Mortierella* at a high frequency. All of them (105 strains) produced arachidonic acid as another major C-20 polyunsaturated fatty acid. Through this test, we selected the 11 strains listed in Table 1, the EPA productivities at 12 C of which were compared with those at 28 or 24 C. In every case, EPA accumulation was observed only at 12 C, regardless of the medium used (i.e., medium GY or medium YM). The data in Table 1 show that these EPA producers fall roughly into two groups as to their cellular fatty acid profiles. One group includes most strains other than *M. alpina*. They accumulated oleic acid as the most predominant cellular fatty acid (20-60%). *M. alpina* strains are unique in that arachidonic acid is one of the most predominant cellular fatty acids (20-50%). In particular, arachidonic acid comprised nearly 50% of the total cellular fatty acids in *M. alpina* 1S-4 and 1-83 at 28 C. On the other hand, there was not such a clear difference as to EPA contents between these two groups. We selected *M. hygrophila* and *M. alpina* 20-17 for the following experiments, because of their mycelial yields, lipid contents and EPA contents.

Effects of the growth temperature on the production of EPA. Both the strains grew well at low temperature (6-16 C), although the growth rates were somewhat lower than those at higher temperature (20-28 C). The mycelial yields obtained after five to seven days of cultivation in medium GY were comparable to

TABLE 2

Effect of the Growth Temperature on the Production of EPA[a]

Temperature (C)/ cultivation period (days)	Mycelial yield (mg/ml of culture medium)	Total FA[b] (mg/g dry mycelia)	Content of		Yield of		% in total FA[b]	
			EPA (mg/g dry mycelia)	Ara[b] (mg/g dry mycelia)	EPA (mg/ml of culture medium)	Ara[b] (mg/ml of culture medium)	EPA	Ara[b]
M. hygrophila IFO 5941								
6/11	7.7	148	15.3	11.1	0.12	0.09	10.3	7.5
12/6	9.7	163	14.6	16.3	0.13	0.16	6.5	8.1
16/6	8.7	220	6.9	26.8	0.06	0.23	3.1	12.2
20/6	7.7	167	6.2	31.6	0.04	0.24	3.7	18.9
28/6	7.4	133	0	75.6	0	0.17	0	17.6
M. alpina 20-17								
6/11	6.9	168	13.8	59.3	0.09	0.49	8.2	35.3
12/7	9.7	200	11.2	104.0	0.11	1.01	5.6	52.0
16/6	9.2	201	7.2	102.3	0.06	0.94	3.6	51.2
20/6	8.7	260	2.9	142.4	0.02	1.24	1.1	54.8
24/4	8.9	247	1.0	130.1	0.01	1.16	0.5	52.6
28/6	10.1	215	0	112.9	0	1.14	0	52.5

[a]Each strain was grown at various temperatures for 4 to 11 days, as indicated. Other conditions are given in the text.
[b]FA, fatty acid; Ara, arachidonic acid.

those on cultivation at higher temperature for four to six days. In both cases, the maximum EPA contents were obtained with mycelia grown at 6 C, as shown in Table 2. The maximum EPA production was, however, obtained at 12 C. Further elevation of the growth temperature brought about marked decreases in their EPA contents, although EPA accumulation was still observed up to 20 C. Conversely, their arachidonic acid contents increased with elevation of the growth temperature. The EPA content of *M. hygrophila* mycelia grown at 28 C was seven times higher than that at 6 C. It also should be noted that *M. alpina* is excellent in accumulating arachidonic acid. The arachidonic acid production reached 1.24 mg/ml of culture medium (142.4 mg/g dry mycelia) when cultivated at 20 C for six days. The arachidonic acid comprised more than 50% of the total mycelial fatty acid. A temperature shift from 28 to 12 C also induced EPA production. This occurred at any phase of growth, even when washed mycelia were allowed to stand at 12 C. When the temperature was shifted to 28 C after cultivation for four days at 12 C, no further increase in EPA accumulation was observed. More than 85% of the accumulated EPA during growth at 12 C remained unchanged on incubation of *M. hygrophila* for a further five days at 28 C. On the contrary, the EPA in *M. alpina* rapidly disappeared with the temperature shift (data not shown).

Other factors affecting EPA production. (i) Carbon sources. Various carbon compounds, including sugars, organic acids, alcohols, fatty acids, *n*-alkanes and oils, were tested as carbon sources in place of glucose in medium GY. Glycerol and maltose, and fructose were found to be effective carbon sources for *M. hygrophila* and *M. alpina*, respectively, producing almost the same mycelial yields and EPA contents as those with glucose. The EPA content of *M. hygrophila* grown with *n*-octadecane was double that with glucose, but the mycelial yield was poor (4.3 mg/ml). The optimum concentration of glucose at a fixed concentration of yeast extract (1%) was 2%, with which *M. hygrophila* and *M. alpina* accumulated 0.08 and 0.10 mg of EPA/ml of culture medium (11.1 and 11.3 mg/g dry mycelia), respectively. A further increase in the glucose concentration repressed the growth of both fungi.

TABLE 3

Effect of Glucose Feeding on the Production of EPA by *M. hygrophila* IFO 5941[a]

Cultivation period (days)	Day of feeding	Mycelial yield (mg/ml of culture medium)	Total FA[b] (mg/g dry mycelia)	EPA content (mg/g dry mycelia)	EPA yield (mg/ml of culture medium)
10		7.8	298	21.0	0.16
11	5,7,9	14.1	298	19.4	0.27
10	4,6,8	14.2	259	19.4	0.28
10	6,8	15.2	305	20.4	0.31

[a]*M. hygrophila* was grown in medium GY as indicated. Glucose was fed to maintain a level of 2% on the indicated days of cultivation. Compositions of medium GY and other conditions are given in the text.
[b]FA, fatty acid.

TABLE 4

Effect of a Temperature Shift and Glucose Feeding on the Production of EPA[a]

Cultivation period (days)	Glucose feeding	*M. hygrophila*				*M. alpina*			
		Mycelial yield (mg/ml of culture medium)	Total FA[b] (mg/g dry mycelia)	EPA content (mg/g dry mycelia)	EPA yield (mg/ml of culture medium)	Mycelial yield (mg/ml of culture medium)	Total FA[b] (mg/g dry mycelia)	EPA content (mg/g dry mycelia)	EPA yield (mg/ml of culture medium)
Exp. I (20 → 12 C)									
A 3	no	7.8	219	4.6	0.04	9.2	100	1.5	0.01
B 7	no	8.8	159	8.3	0.07	9.9	276	4.1	0.04
C 7	yes	8.0	388	17.8	0.14	6.7	96	29.1	0.20
D 7	yes	10.0	198	14.9	0.15	11.1	272	25.3	0.28
Exp. II (12 → 20 C)									
A 5	no	8.2	164	9.7	0.08	9.3	112	5.7	0.14
B 9	no	9.3	188	23.3	0.22	9.9	204	13.7	0.14
C 9	yes	7.1	610	40.9	0.29	7.1	308	25.0	0.18
D 9	yes	10.1	184	14.0	0.14	10.5	216	12.3	0.13

[a]In Experiment I, cultivation as performed in 4 flasks at the same time at 20 C. One of the flasks was analyzed on the 3rd day (A). The other three flasks were shifted to 12 C on the same day with (C and D) or without (B) glucose feeding to maintain the level of 2%; then, the cultivations were continued for a further 4 days. In D, yeast extract (0.2 mg/ml) also was added on the 3rd day. In Experiment II, the cultivations were started at 12 C. On the 5th day, one flask was analyzed (A). The other three flasks were shifted to 20 C with (C and D) or without (B) glucose feeding to maintain the level of 2%, and then the cultivations were continued for a further 4 days. In D, yeast extract (0.2 mg/ml) also was added on the 5th day. Other conditions are given in the text.
[b]FA, fatty acid.

(ii) Nitrogen sources. Various organic and inorganic compounds and natural nutrients were tested by adding each of them to medium GY to a final concentration of 0.5%, and by replacing the yeast extract in medium GY with 0.5% of each of them. Generally, the mycelial yields increased significantly on the addition of organic nitrogen sources such as tryptone, casamino acid, meat extract, etc., but the EPA contents of the two fungi decreased markedly. Only bactopeptone (Difco, Detroit, Michigan) was effective in increasing the mycelial yields without decreases in the EPA contents. In the replacement test, no nitrogen source superior to yeast extract was found, so far as tested. The optimum concentration of yeast extract at a fixed concentration of glucose (2%) was 1%.

(iii) Glucose feeding. Because the glucose in medium GY was consumed during the first four to five days of cultivation, and the presence of a high level of glucose at the initiation of the cultivation repressed the fungal growth, glucose was fed periodically to maintain a level of 2%. The data in Table 3 show that the mycelial mass increased about two-fold without a significant decrease in EPA content on feeding of glucose in every case tested. The maximum production of EPA (0.31 mg/ml, 20.4 mg/g dry mycelia) was attained on cultivation of *M. hygrophila* for 10 days with two successive feedings of glucose on the sixth and eighth days.

(iv) Combination of a temperature shift and glucose feeding. To reduce the growth period without any decrease in EPA production, each strain was grown under the conditions (with glucose feeding and a temperature shift) shown in Table 4. The maximum EPA production with *M. alpina* (0.28 mg/ml) was obtained when the fungus was grown at 20 C for three days and then at 12 C for a further four days with feeding of glucose and yeast extract. This value was about seven times higher than that obtained under the conditions without feeding. More than 11 days were required to obtain essentially the same value when the fungus was grown without a temperature shift (data not shown). In the case of *M. hygrophila*, the maximum EPA production (0.29 mg/ml) was obtained when it was grown at 12 C for the first five days and then for a further four days at 20 C with glucose feeding. However, feeding of yeast extract led to decreased production of EPA.

EPA production under optimum culture conditions. Based on the above results, *M. alpina* was cultivated in medium GY supplemented with 0.5% bactopeptone for 11 days at 12 C with two successive feedings of glucose (1% each) on the seventh and nineth days. The EPA production reached 0.49 mg/ml of culture medium (29 mg/g dry mycelia). This value accounted for 13.5% of the total extractable mycelial fatty acids. Other major fatty acids were palmitic acid (6.0%), stearic acid (5.3), oleic acid (6.2), linoleic acid (3.0), γ-linolenic acid (3.5) and arachidonic acid (60.0). Essentially the same results were obtained when the cultivation was carried out at 20 C for the first three days followed by cultivation for a further six days at 12 C with two successive glucose feedings (1% each; fifth and seventh days).

Isolation of the EPA. The EPA methyl ester (6.7 mg) was isolated from the lipids extracted from 15 g of wet mycelia of *M. alpina* grown under optimum culture conditions. The mass spectrum of the isolated methyl ester showed a molecular ion peak at m/z 316 (relative intensity, 7%) and intense fragment ion peaks at m/z 201, 180, 175, 173, 93, 91, 79 and 67 (relative intensity, 65, 100, 87, 52, 54, 74, 100 and 58%, respec-

tively). The ^1H NMR spectra in $CDCl_3$ with tetramethylsilane as an internal standard showed signals at 0.97 (*t*, 3H, CH_3), 1.72 (*m*, 2H, CH_2), 2.10 (*m*, 4H, CH_2), 2.33 (*t*, 2H, CH_2), 2.82 (*m*, 8H, CH_2), 3.67 (*s*, 3H, CH_3) and 5.38 ppm (*m*, 10H, C=C). These data corresponded well to those of authentic EPA methyl ester (Funakoshi Chemicals, Tokyo).

DISCUSSION

The results reported here show that fungal mycelia are rich sources of EPA. In particular, *M. alpina* 20-17 was found to accumulate about 0.5 mg of EPA/ml of culture medium (29 mg/g dry mycelia). There has been no report showing the possibility of fungal microorganisms being potential sources of it, before this work. These fungi are thought to be much more advantageous than the algal, moss and protozoal sources previously reported (3-6), because of their higher EPA contents, their higher growth rates in simple media and the simplicity of their manipulation. These features would make the use of these fungi as sources of EPA very promising.

All the EPA-producing strains found here accumulated polyunsaturated fatty acids of the n-6 series (i.e., γ-linolenic acid, dihomo-γ-linolenic acid and arachidonic acid) in their mycelia. However, no n-3 polyunsaturated fatty acid other than EPA was detected. This suggests that an n-6 PUFA may be a precursor of EPA. If this is the case, an enzyme(s) or enzyme system catalyzing the methyl-end directed desaturation (6,11) of one of these polyunsaturated fatty acids, probably arachidonic acid, may be formed or activated on cold adaptation. The resultant EPA may be necessary for maintaining a proper membrane fluidity in a low temperature environment. The mechanism underlying this cold-induced formation of EPA is now being studied (9).

For practical purposes, however, low temperature is disadvantageous because of low growth rate and high energy cost for cooling. The data in Table 4 and those obtained under optimal conditions suggest that combination of the temperature shift and glucose feeding during the growth of the fungi may be one of the effective ways of obtaining enough mycelia with high EPA content in a short cultivation period.

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