Production of Eicosapentaenoic Acid by *Saprolegnia* sp. 28YTF-1

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ABSTRACT: *Saprolegnia* sp. 28YTF-1, isolated from a freshwater sample, is a potent producer of 5,8,11,14,17-*cis*-eicosapentaenoic acid (EPA). The fungus used various kinds of carbon sources, such as starch, dextrin, sucrose, glucose, and olive oil for growth, and olive oil was the best carbon source for EPA production. The EPA content reached 17 mg/g dry mycelium (0.25 mg/L) when the fungus was grown in a medium that contained 2.5% olive oil and 0.5% yeast extract, at pH 6.0 and 28°C for 6 d with shaking. Accompanying production of arachidonic acid (AA; 3.2 mg/g dry mycelia, EPA/AA = 5.1) and other ω6 polyunsaturated fatty acids was low. Both EPA content and EPA/AA ratio increased in parallel by lowering growth temperature. Triglyceride was the major mycelial lipid (*ca.* 84%), but EPA comprised only 2.2% of the total fatty acids of this lipid. About 40% of the EPA produced was found in polar lipids, such as phosphatidylethanolamine (EPA content, 28.2%), phosphatidylcholine (13.6%), and phosphatidylserine (21.2%).
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KEY WORDS: Arachidonic acid, eicosapentaenoic acid, *Saprolegnia* sp.

5,8,11,14,17-*cis*-Eicosapentaenoic acid (EPA), a rare C-20 polyunsaturated fatty acid (PUFA) of potential pharmaceutical value, has attracted much interest because of its unique biological activities (1–5). This fatty acid has been shown to be effective in the prevention and curing of thrombosis, arteriosclerosis, and other blood-circulatory diseases. Some marine fishes also require EPA as an essential fatty acid for growth. It is usually one of the major cellular fatty acids in these fishes.

Several marine fish oil products have become available recently as lipid sources that are 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. As alternative sources of EPA, marine microalgae *Chlorella minutissima* (6) and *Euglena glacilis* (7) and a marine bacterium (8) have been suggested, but again, their oil productivity and the EPA level in the oil or lipid are not satisfactory. Several fungi belonging to genera such as *Mortierella* (9,10), *Saprolegnia* (11), and *Pythium* (12,13) have been reported to produce relatively large amounts of oil with EPA. Therefore, these fungi are also candidates as practical sources of EPA. The lipids produced by these fungi contain large amounts of arachidonic acid (AA). The mycelial EPA/AA ratio in these fungi is usually 0.7–3.3 (11,14).

In previous papers (9,10,15,16), we reported that the AA-producing fungus *Mortierella alpina* and taxonomically related fungi accumulate EPA when grown at low temperature or in a medium that contains linseed oil. But AA content was also relatively high, and the EPA/AA ratio was low in these fungi. From a nutritional point of view, an EPA-containing lipid with a low AA level is preferable in some cases, to eliminate the diverse biological activities of AA. We have now found that two freshwater fungal strains, identified as *Saprolegnia* spp., produce large amounts of an EPA-containing lipid with a low AA level. The present paper reports the isolation of a high EPA/AA ratio strain and the culture conditions for the EPA production by an isolate, *Saprolegnia* sp. 28YTF-1.

MATERIALS AND METHODS

Chemicals. Olive oil was purchased from Nakalai Tesque (Kyoto, Japan). Sardine oil was purchased from Yamakei Industries (Osaka, Japan) and converted to methyl esters with methanolic sulfuric acid (17). The methyl esters were composed of 16:0 (14 wt%), 16:1 (10%), 18:0 (3%), 18:1ω9 (29%), 18:2ω6 (8%), 18:3ω3 (4%), 20:4ω6 (3%), 20:4ω3 (9%), and 22:6ω3 (11%). All authentic fatty acids were obtained from Sigma (St. Louis, MO). All other reagents were of analytical grade.

Microorganisms. Saprolegnia sp. 28YTF-1 and 28GTF were isolated from freshwater samples, obtained in Yamanashi prefecture, by the conventional streaking method on 2% agar plates of PYM medium [1% glucose, 1% Polypeptone (Daigo Eiyo, Japan), 0.5% malt extract, 0.5% yeast extract, and 0.5% methyl esters of sardine oil, pH 6.0]. All other strains were from our culture collection (AKU culture collection, Faculty of Agriculture; Kyoto University, Kyoto, Japan).

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Media and cultivation. Each strain was grown in PYM or OY medium (1% olive oil and 0.5% yeast extract, pH 6.0). In the screening experiments, each strain was inoculated into 4 mL of OY medium in a 20-mL Erlenmeyer flask and then incubated with reciprocal shaking (120 strokes/min) at 28°C for one week.

Optimization of the culture conditions for EPA production. These experiments were carried out with the same isolate, *Saprolegnia* sp. 28YTF-1, throughout. The compositions of the liquid media and the cultivation conditions for EPA production are described earlier or given in the legends of the respective figures.

Lipid analysis. The fungal mycelia were harvested by suction filtration and then treated twice with chloroform–methanol–water according to the procedure of Bligh and Dyer (18). The resultant lipid extract was evaporated to dryness under reduced pressure at 35°C and then used as the sample for transmethylation. For analysis of the fatty acid composition of the triglyceride or phospholipid fraction, the extracted lipid was separated on a silica gel thin-layer plate (Kieselgel 60, 200 × 200 × 0.25 mm; E. Merck, Germany). Solvent systems of hexane–diethyl ether-formic acid (8:2:0.2, vol/vol/vol) and chloroform–methanol–water (65:25:4, vol/vol/vol) were used for triglycerides and phospholipids, respectively. The gel corresponding to the bands of triglycerides and phospholipids, stained with 0.01% primuline in 80% acetone, was scraped off. The lipids were then transmethylated usually by treatment with 10% methanolic HCl. As an internal standard, *n*-pentadecanoic acid (0.2 mg) was usually included in the methanolysis mixture. Fatty acid methyl esters were extracted with 4 mL of *n*-hexane, and then the extracts were concentrated in a centrifugal evaporator at 40°C.

The fatty acid methyl esters were dissolved in acetonitrile and then analyzed by gas–liquid chromatography (GLC). The analytical conditions were as follows: Apparatus, Shimadzu GC-14B (Kyoto, Japan), equipped with a flame-ionization detector (FID) and a split injector; column, Rascot Silar-5CP (50 m × 0.25 mm i.d. (Nihon Chromato Works, Tokyo, Japan); column temperature, 215°C; injection port temperature, 250°C; helium as carrier gas (inlet pressure, 200 kPa), N_2 as make-up gas (60 mL/min); air and H_2, 60 kPa; and integrator, Shimadzu C-R6A.

RESULTS

Isolation of microorganisms that produce a high EPA/AA ratio. The intercellular production of EPA was examined for about 1500 isolates from soil, freshwater, and sea water samples, and 54 isolates showed EPA production. All of them coproduced AA, and the cellular EPA content and EPA/AA ratio were 0.5–6.5 mg/g dry mycelium and 0.1–1.5, respectively, on growth in OY medium, except for two isolates, 28YTF-1 and 28GTF. The EPA contents of these strains were more than 10 mg/g dry mycelium, and AA was less than 0.3 mg/g dry mycelium (EPA/AA > 5) (Table 1).

Identification of the isolates. Both isolates, 28YTF-1 and 28GTF, grew fast on YPGS agar medium (0.8% yeast extract, 2% glucose, 0.2% K_2HPO_4, 0.1% $MgSO_4 \cdot 7H_2O$ and 1.5% agar), and the colonies attained a diameter of 60–80 mm in 5 d at 25°C with little aerial mycelium. The hyphae were hyaline and profusely branched. They were aseptate and 20–40 μm in diameter. The zoosporangia were produced at the tips of the hyphae. They were cylindrical, and 180–200 μm long and 30–40 μm in diameter. The zoospores were globose and about 10 μm in diameter. The production of cylindrical zoosporangia at the hyphal tips may place the present isolates in the genus *Saprolegnia* of the family Saprolegniaceae. More detailed studies of the taxonomic characteristics of the present strains are now being done to decide their taxonomic positions at the specific rank.

Comparison of EPA productivities of the isolates and other Saprolegnia *strains.* Because these two isolates were identi-

TABLE 1
Eicosapentaenoic Acid and Arachidonic Acid Production by the Isolated Filamentous Fungi and Several *Saprolegnia* Fungi[a]

Strain[a]	Growth (mg/mL)	Fatty acid Yield (mg/L) AA[b]	Fatty acid Yield (mg/L) EPA[b]	Fatty acid Content (mg/g) AA	Fatty acid Content (mg/g) EPA	EPA/AA ratio
Isolate 28YTF-1	8.5	22.6	123.8	2.7	14.6	5.5
Isolate 28GTF	7.9	19.7	104.4	2.5	13.2	5.5
Saprolegnia delica CBS 345.62	6.9	12.9	12.7	2.4	2.4	1.0
Sparolegnia decline CBS 536.67	6.5	22.6	15.6	3.5	2.4	0.7
Saprolegnia ferax CBS 534.67	8.9	26.1	30.2	2.9	3.4	1.2
Saprolegnia hypogyna CBS 869.72	6.7	60.8	40.6	9.1	6.1	0.7
Saprolegnia lapponica CBS 284.38	8.3	20.4	29.3	2.5	3.5	1.4
Saprolegnia litralis CBS 535.67	8.8	77.6	31.0	8.8	3.5	0.4
Saprolegnia parasitica CBS 540.67	7.4	50.9	36.6	7.2	5.2	0.7
Saprolegnia turfosa CBS 313.81	6.2	74.1	22.2	11.9	3.6	0.3

[a]All strains were grown in medium OY at 28°C for 7 d under the conditions given in the Materials and Methods section. All values are means of three determinations, and the standard deviation is less than 7%.
[b]Abbreviations: AA, arachidonic acid; EPA, eicosapentaenoic acid.

fied as *Saprolegnia* spp., EPA productivities of these isolates were compared with those of other *Saprolegnia* strains from our stock culture. Neither the EPA content nor the EPA/AA ratio in any of the stock cultures tested was satisfactory (Table 1).

Culture conditions for the production of EPA by Saprolegnia *sp. 28YTF-1. (i) Effect of carbon sources.* Various kinds of carbon sources, such as glucose, fructose, soluble starch, dextrin, and potato flour, were utilized as carbon sources for growth. High mycelial growth with high EPA content was obtained when the fungus was grown with some natural oils, such as olive oil, sesame oil, or cocoa butter. The best EPA production (116 mg/L, 13.5 mg/g dry mycelia) was attained in a medium that contained 1% olive oil and 0.5% yeast extract (pH 6.0).

(ii) Effect of culture time. The mycelial yield in OY medium reached the maximum after 5 d and then decreased gradually. The amount of EPA and AA increased markedly in 3 to 5 d of cultivation, and reached 200 mg/L culture broth (20.4 mg/g dry mycelium) after 6 d. The EPA/AA ratio (*ca.* 5.5) did not vary significantly during cultivation (data not shown).

(iii) Effect of growth temperature. EPA and AA production and the EPA/AA ratio in OY medium were investigated at various growth temperatures. EPA production increased slightly and AA production decreased with lowering of the growth temperature. The maximum EPA/AA ratio (10.9) and EPA production (176.4 mg/L) were attained at 6°C (Table 2).

(iv) Effects of additional carbon and nitrogen sources. The carbon compounds listed in Figure 1 were tested as additional carbon sources in the basal medium (1.25% olive oil and 0.5% yeast extract, pH 6.0) for EPA production. As shown in Figure 1, when olive oil was added, both growth (12.4 g/L) and the mycelial EPA content (19 mg/g dry mycelia) increased. The values were 1.5 and 1.3 times higher, respectively, than those without the addition. As a result, 1.8 times higher production of EPA per liter medium (230 mg/L) was attained with only a small decrease in the EPA/AA ratio. Similarly, methyl stearate was effective in increasing the mycelial

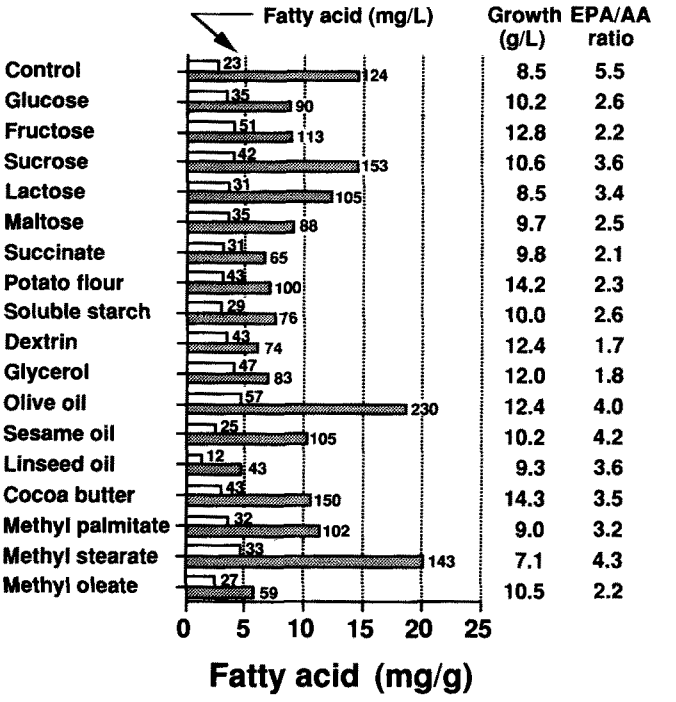

FIG. 1. Effect of added carbon sources on the production of eicosapentaenoic acid (EPA) (meshed bars) and arachidonic acid (AA) (open bars) by *Saprolegnia* sp. 28YTF-1. The fungus was cultivated in a medium composed of 1.25% olive oil, 0.5% yeast extract, and 1.25% of each additional carbon source, as shown, for 5 d under the conditions given in the Materials and Methods section. All values are means of three determinations, and the standard deviation is less than 7%.

TABLE 2
Effect of the Growth Temperature on Eicosapentaenoic Acid and Arachidonic Acid Production by *Saprolegnia* sp. 28YTF-1[a]

Cultivation temperature (°C)[b]	Growth (mg/mL)	Fatty acid Yield (mg/L) AA	Fatty acid Yield (mg/L) EPA	Fatty acid Content (mg/g) AA	Fatty acid Content (mg/g) EPA	EPA/AA ratio
6	7.7	16.1	176.4	2.1	23.1	10.9
12	7.9	21.3	157.1	2.7	20.1	7.4
20	7.9	28.0	153.2	3.6	19.4	5.5
28	7.6	27.3	152.6	3.6	20.2	5.6

[a]*Saprolegnia* sp. 28YTF-1 was cultured in 2 mL of OY medium in a 10-mL Erlenmeyer flask with reciprocal shaking. All values are means of three determinations, and the standard deviation is less than 7%. See Table 1 for abbreviations.
[b]Cultivation was performed at 28°C for 2 d, followed by a shift to the indicated temperature and then growth at that temperature. The total cultivation time—that is, before and after the temperature shift—in all cases was 7 d.

content of EPA (20 mg/L). In this case, mycelial growth was low (7.1 g/L), and EPA production reached 150 mg/L. In most cases, the addition of carbohydrates brought about decreased production of EPA, accompanied by a low EPA/AA ratio. The addition of a nitrogen source to the basal medium generally resulted in decreased production of EPA, but mycelial growth was rapid and dense (data not shown). As shown in Figure 2, maximum production of EPA (250 mg/L; EPA/AA = 5.1) was observed at a concentration between 2.5 to 3.7% olive oil.

Distribution of fatty acids among major mycelial lipids. Upon cultivation at 28°C, the major fungal lipids were triglycerides (TG), phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) (Table 3a). In the neutral lipid fraction [TG and diglycerides (DG)], oleic acid was the abundant fatty acid, and the fatty acid compositions of these fractions were similar to that of olive oil. By contrast, fatty acids with more than three double bonds, such as AA and EPA, were mainly distributed in the polar lipid fraction. About 40% of the EPA was found in the phospholipid fractions (PC, PE, and PS). A similar distribution was also found for AA (Table 3b).

DISCUSSION

EPA is accumulated in mycelia of *Saprolegnia* fungi grown at room temperature (24–30°C), and AA is proposed to be

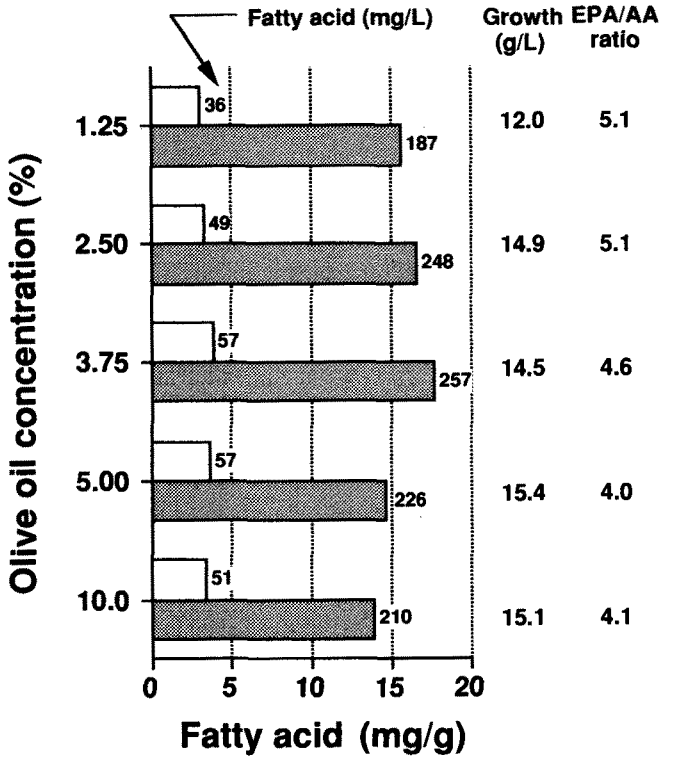

FIG. 2. Effect of olive oil concentration on the production of EPA (meshed bars) and AA (open bars) by *Saprolegnia* sp. 28YTF-1. The fungus was grown at 28°C for 5 d in a medium containing 0.5% yeast extract and the indicated concentrations of olive oil. All values are means of three determinations, and the standard deviation is less than 7%. See Figure 1 for abbreviations.

converted to EPA through the ω3 (Δ17) desaturation reaction (11). Several studies on the EPA accumulation in *Saprolegnia* fungi have been reported (14), but all the strains used in those studies were unsatisfactory as to content of EPA and EPA/AA ratio. In the present study, we show that *Saprolegnia* sp. 28YTF-1 and 28GTF can efficiently utilize some natural oils as carbon sources for growth and as substrates for PUFA synthesis. It is also shown that both EPA productivities and EPA/AA ratios in these strains are considerably higher than those in other *Saprolegnia* fungi (Table 1).

When *Saprolegnia* sp. 28YTF-1 was grown in the medium with olive oil as a carbon source, the resultant mycelial fatty acid compositions of the neutral lipid fractions were similar to that of olive oil. This result strongly suggests that olive oil was directly incorporated into the mycelium and then used as both carbon and energy source for growth. Only a small part of these fatty acids in the oil was utilized as substrate for PUFA biosynthesis. That about 50% of the PUFA were present in the polar lipids, whose content in the total lipids comprised only 10%, and the fatty acid compositions of these polar lipids were quite different from those of neutral lipids support the above assumption. Consequently, it appears that the desaturation reactions leading to EPA in this fungus utilize fatty acids incorporated into phospholipids.

Lowering the growth temperature increased the EPA content and the EPA/AA ratio in this fungus. The same phenomenon was observed in *Mortierella* fungi, in which EPA is newly produced from AA below 20°C through the ω3 (Δ17) desaturation reaction (19). Such temperature-dependent de-

TABLE 3
Fatty Acid Compositions of the Major Lipids (a) and Distribution of Abundant Fatty Acids among Major Lipids (b) in *Saprolegnia* sp. 28YTF-1 on Growth at 28°C

(a)

Fraction[a]	Lipid[b] comp. (%)	Fatty acid composition (%)									
		14:0	16:0	16:1	18:0	18:1	18:2ω6	18:3ω6	20:3ω6	AA	EPA
TG	84.2	1.0	9.8	1.3	2.0	74.8	5.1	0.4	trace[c]	0.7	2.2
DG	5.7	0.5	6.0	0.9	2.0	77.4	5.5	0.6	trace	1.1	4.1
FA	3.2	0.7	19.9	1.9	10.8	58.6	2.5	trace	trace	0.4	4.1
PE	2.6	2.2	11.3	0.9	1.2	32.9	8.2	1.0	trace	7.9	28.2
PC	3.3	1.0	15.3	2.4	0.8	43.8	13.6	1.8	trace	2.9	13.6
PS	1.0	1.6	30.0	1.1	1.5	26.0	11.2	trace	trace	6.7	21.2
Oilve oil[c]		trace	9.9	—[d]	trace	83.7	6.4	—	—	—	—

(b)

Fatty acid	Fatty acid comp. (%)	Fatty acid distribution (%)					
		TG	DG	FA	PE	PC	PS
Total fatty acid	100	84.2	5.7	3.2	2.6	3.3	1.0
18:1	71.9	87.7	6.1	2.6	1.2	2.0	0.4
18:2	5.5	78.7	5.7	1.5	3.9	8.3	2.0
AA	1.0	53.8	6.3	3.5	20.3	9.5	6.5
EPA	3.5	52.4	6.7	0.4	21.3	13.1	6.0

[a]Abbreviations: TG, triglyceride; DG, diglyceride; FA, free fatty acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; 14:0, myristic acid; 16:0, palmitic acid; 16:1, palmitoleic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2ω6, linoleic acid; 18:3ω6, γ-linolenic acid; 20:3ω6, linolenic acid; see Table 1 for other abbreviations. All values are means of three determinations, and the standard deviation is less than 7%.
[b]Lipid composition. The indicated percentages of fatty acids were distributed in these fractions. Such minor lipids as sterols, sterol esters, and monoglyceride were not included in the calculation.
[c]Fatty acid composition of the olive oil used as the carbon source for growth.
[d]—, Undetectable.

saturation may be beneficial to the fungi for maintaining proper membrane fluidity during cold-adaptation.

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