Production of 8,11,14,17-*cis*-Eicosatetraenoic Acid (20:4ω-3) by a Δ5 and Δ12 Desaturase-Defective Mutant of an Arachidonic Acid-Producing Fungus *Mortierella alpina* 1S-4

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ABSTRACT: A Δ5 and Δ12 desaturase-defective mutant of an arachidonic acid-producing fungus, *Mortierella alpina* 1S-4, produced 8,11,14,17-*cis*-eicosatetraenoic acid (20:4ω3) intracellularly when grown with linseed oil. Dihomo-γ-linolenic acid was the only C_{20} polyunsaturated fatty acid (4.9 wt% of total mycelial fatty acids) other than 20:4ω3. AA and 5,8,11,14,17-*cis*-eicosapentaenoic acid were not detected. The mycelial lipids consisted of 82.2% (by mol) triacylglycerol (TG), 7.1% diacylglycerol, 8.9% phospholipids (PL), and 1.9% free fatty acids. The percentage of 20:4ω3 was higher in PL (30.1%) than in TG (11.6%), and highest in phosphatidylcholine (38.9%). Under the optimal conditions with a 5-L jar fermenter, 20:4ω3 production amounted to 97.4 mg/g dry mycelia with a mycelial yield of 23 g/L on the twelfth day (corresponding to 2.24 g/L medium and 37.1% of total mycelial fatty acids).
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KEY WORDS: Δ5 Desaturase, Δ12 desaturase, eicosatetraenoic acid (20:4ω3), *Mortierella alpina*, mutant.

8,11,14,17-*cis*-Eicosatetraenoic acid (20:4ω3) is a C_{20} polyunsaturated fatty acid (PUFA) belonging to the n-3 group. It is a double bond-positional isomer of arachidonic acid (AA) and the precursor of 5,8,11,14,17-*cis*-eicosapentaenoic acid (EPA) in the n-3 pathway (Fig. 1). A small amount of 20:4ω3 is widely distributed in organisms having the n-3 pathway. There have been some reports that 20:4ω3 was converted to prostaglandins, such as AA and EPA, in ram seminal vesicles (1) and that the formed prostaglandin had an inhibitory effect on rabbit platelet aggregation (2). 20:4ω3 may act as a natural analog of AA or EPA, but little is known of its physiological role.

No practical source for collecting a large amount of 20:4ω3 has been reported. Even in fish oil, the only practical source known, the 20:4ω3 content is low (<5% of total fatty acids) and there are large amounts of other C_{20} PUFA, such as AA, EPA, and dihomo-γ-linolenic acid (DGLA), which are difficult to separate from 20:4ω3. During our studies on PUFA production involving AA-producing *Mortierella* fungi, we found 20:4ω3 was formed (*ca*. 0.5 mg/mL of culture medium) as a by-product (3). Large amounts of AA (2.44 mg/mL) and EPA (1.88 mg/mL) were produced in this case. Recently, we reported 20:4ω3 production by cultivating Δ5 desaturase-defective mutants of AA-producing *M. alpina* 1S-4 in a medium containing linseed oil (4). One of them, S14 strain, produced 1.65 mg of 20:4ω3 per mL of culture medium. The mycelial lipids contained no AA or EPA. The lipids, however, contained a large amount of DGLA and the productivity of 20:4ω3 was rather lower than those of AA (5), DGLA (6), and Mead acid (7), with *M. alpina* 1S-4 and its mutants.

Here we report 20:4ω3 production by a mutant designated as M226-9, which is defective in two desaturases, Δ5 and Δ12 desaturases (8). This mutant accumulated 6,9-*cis*-octadienoic acid (18:2ω9) and 8,11-*cis*-eicosadienoic acid (20:2ω9) when grown on glucose and yeast extract (Fig. 1). Since it cannot convert oleic acid to n-6 PUFA, it is expected that M226-9 produces less DGLA than S14, and that the ratio of 20:4ω3 to DGLA becomes larger. The conditions for bench-scale production with a 5-L jar fermenter and the distribution of fatty acids in the major lipid class of the resultant fungal lipids are described.

MATERIALS AND METHODS

Chemicals. Linseed oil, containing palmitic (8.8%, by weight), stearic (4.8%), oleic (11.8%), linoleic (16.3%), and α-linolenic (58.3%) acids, was purchased from Wako Pure Chemicals (Osaka, Japan). Linseed oil methyl ester was prepared by transmethylation as described below. All other reagents were commercially available and as described previously (6).

Microorganisms and cultivation. The fungi used were the *M. alpina* M226-9 strain (8), which is a Δ5 and Δ12 desaturase-defective mutant of *M. alpina* 1S-4, and the S14 strain (6),

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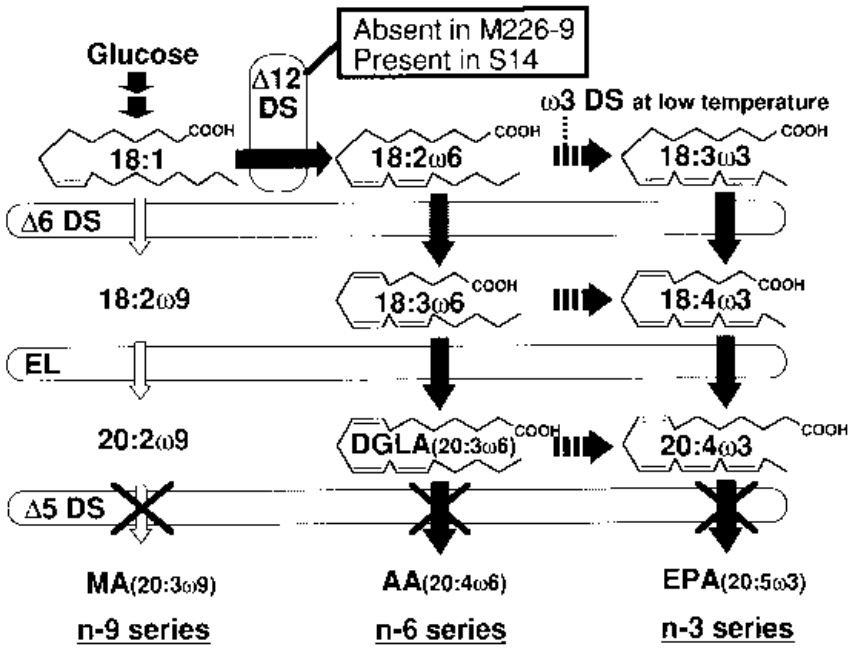

FIG. 1. Biosynthetic pathways for fatty acids in *Mortierella alpina* M226-9 and S14. Δ5 Desaturase is defective in both strains, and Δ12 desaturase is defective in only M226-9. ω3 Desaturase is active at low temperature. DS, desaturase; EL, elongase; DGLA, dihomo-γ-linolenic acid; MA, Mead acid; AA, arachidonic acid; EPA, eicosapentaenoic acid.

which is a Δ5 desaturase-defective mutant of 1S-4. The fungi were each inoculated as a spore suspension into 50-mL Erlenmeyer flasks containing 10 mL of medium GY (1% glucose and 1% yeast extract, pH 6.0) supplemented with linseed oil methyl ester, followed by incubation with reciprocal shaking (120 rpm), unless otherwise stated. For jar fermentation, the M226-9 strain was precultured at 28°C for 3 d in 200 mL of medium GY and the resultant culture was inoculated into 1.8 L of medium GY supplemented with 3.5% (vol/vol) linseed oil methyl ester and 0.01% Adekanol (Asahi Denka Industry, Tokyo, Japan) in a 5-L jar fermenter (Mitsuwa, Osaka, Japan). Other cultural conditions are given in each table and figure.

Fatty acid and lipid analyses. Mycelia were harvested by suction filtration, washed with water and ether, and then dried at 100°C for subsequent fatty acid analysis by gas–liquid chromatography (GLC) after transmethylation with methanolic HCl as described previously (9). Fungal lipids were extracted with a chloroform/methanol/water system (10). The lipids were separated into individual lipid fractions by thin-layer chromatography (9), and the fatty acid composition of each lipid fraction was analyzed by GLC as described above.

RESULTS

Comparison of the time courses of 20:4ω3 formation by M. alpina *M226-9 and S14.* M229-9 and S14 were grown in medium GY containing 2% (vol/vol) linseed oil methyl ester at 16°C for 11 d (Fig. 2). In both strains, mycelial fatty acids started to increase on the fifth day when the mycelial growth reached late-log phase. 20:4ω3 production by M226-9 reached 1.40 mg/mL of culture medium (13.5% of total fatty acids) on the eleventh day, with DGLA production amounting to only 0.51 mg/mL (4.9%). The other major fatty acids were 18:3ω3 (34.2%), 18:1 (16.1%), 18:2ω6 (11.6%), and 16:0 (5.9%), EPA not being detected. Under these conditions, n-9 fatty acids, such as 20:2ω9 and 18:2ω9, were not detected, although this strain formed n-9 fatty acids but no n-6 or n-3 ones when grown in a medium containing only glucose and yeast extract (8). On the other hand, 20:4ω3 production by S14 amounted to only 0.73 mg/mL (9.8% of total fatty acids), while DGLA production reached 0.78 mg/mL (10.4%). The ratios of 20:4ω3/DGLA were 2.75 (M226-9) and 0.94 (S14).

Factors affecting 20:4ω3 production by M. alpina *M226-9. (i) Growth temperature.* M226-9 was cultivated in medium GY containing 2% (vol/vol) linseed oil methyl ester at 28°C for 2 d and then at 10–28°C for 7 d (Fig. 3A). The dry mycelial weight was almost the same at 10–28°C (16.0–16.7 mg/mL of culture medium). 20:4ω3 production was high at 16 (1.24 mg/mL of culture medium) and 20°C (1.11 mg/mL). The percentages of 20:4ω3 among total fatty acids were also high at 16 (14.5%) and 20°C (13.1%) (data not shown). DGLA production remained almost the same (0.30–0.44 mg/mL), regardless of the growth temperature. The ratio of 20:4ω3/DGLA was high at 10°C (3.10) and decreased with increasing growth temperature (1.87 at 28°C).

(ii) Glucose concentration. The dry mycelial weight increased with increasing glucose concentration in the range of 0–2% (wt/vol) (Fig. 3b). 20:4ω3 production increased in the range of 0–1%, but remained unchanged in the range of 1–2% (1.07–1.10 mg/mL of culture medium). Therefore, the

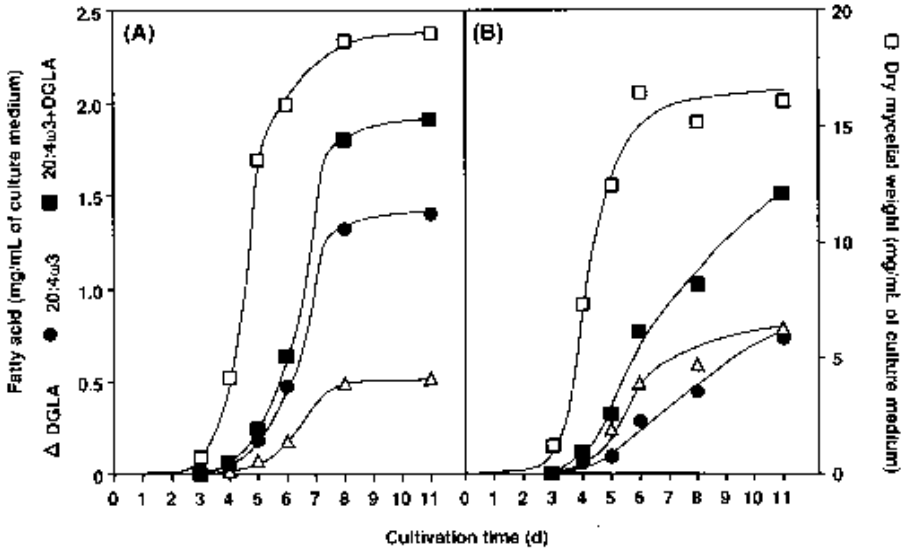

FIG. 2. Changes in the production of fatty acids by *M. alpina* M226-9 (A) and S14 (B). The fungi were cultivated in medium GY containing 2% (vol/vol) linseed oil methyl ester at 16°C for 11 d. For abbreviations see Figure 1.

20:4ω3 content was highest when the glucose concentration was 1% (59.5 mg/g dry mycelia). DGLA production decreased slightly with increasing glucose concentration. As for the fatty acid composition, the percentages of 20:4ω3 and DGLA were highest (12.7 and 5.3% of total fatty acids, respectively) when there was no glucose, and decreased to 8.0 and 2.6%, respectively, with increasing glucose concentration to 3% (data not shown). On the contrary, those of 18:0 and 18:1 were lowest (2.9 and 20.1%, respectively) when there was no glucose, and highest (5.2 and 23.8%, respectively) when the glucose concentration was 3% (data not shown).

(iii) Linseed oil methyl ester concentration. M226-9 was cultivated in medium GY containing 0–4% (vol/vol) linseed oil methyl ester (Fig. 3C). When there was no linseed oil methyl ester, 20:4ω3 and DGLA were not detected, 20:2ω9 being formed (0.20 mg/mL of culture medium) in place of them. When the linseed oil methyl ester level was 0.5–4%, no n-9 fatty acids were detected. As linseed oil methyl ester increased, 20:4ω3 production and dry mycelial weight increased. 20:4ω3 production amounted to 1.46 mg/mL (62.9 mg/g dry cells) when the linseed oil methyl ester level was 3%. DGLA production was high when the linseed oil methyl ester level was 2–4% (0.41–0.44 mg/mL). The ratio of 20:4ω3/DGLA increased with increasing linseed oil methyl ester.

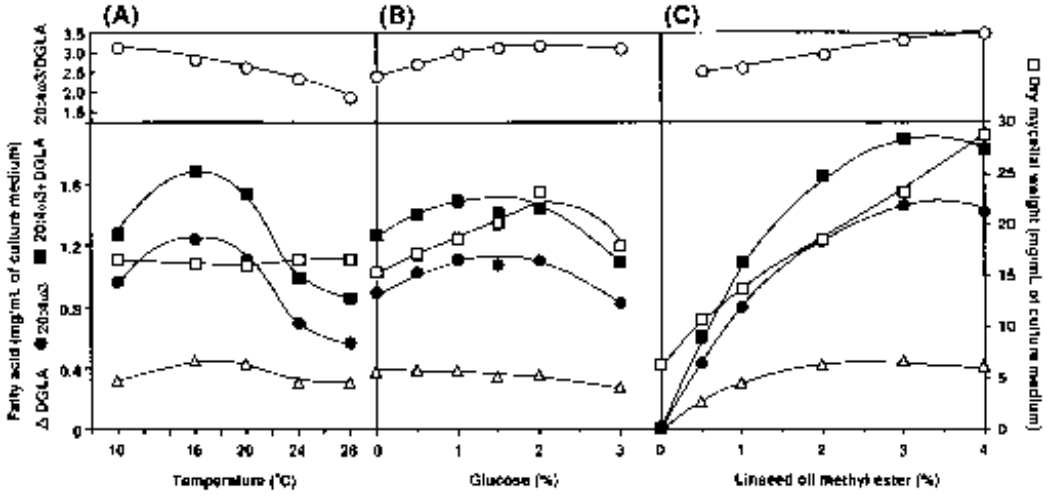

FIG. 3. Effects of growth temperature (A), glucose concentration (B), and linseed oil methyl ester concentration (C) on fatty acid production. (A) M226-9 was cultivated in medium GY containing 2% (vol/vol) linseed oil methyl ester at 28°C for 2 d and then at 10–28°C for 7 d. (B) M226-9 was cultivated in a medium comprising 1% yeast extract, 2% linseed oil methyl ester, and 0–3% glucose, as indicated, at 28°C for 2 d and then at 16°C for 7 d. (C) M226-9 was cultivated in medium GY containing 0–4% linseed oil methyl ester, as indicated, at 28°C for 2 d and then at 16°C for 7 d. For abbreviation see Figure 1.

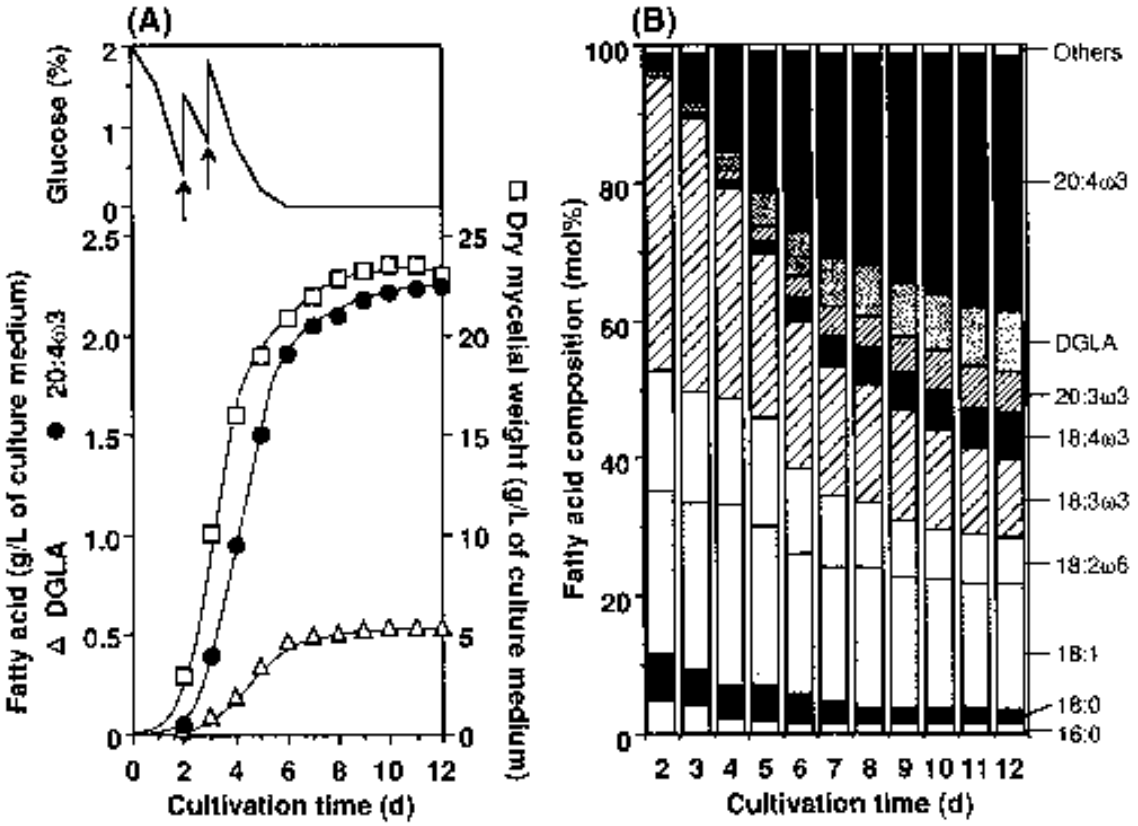

FIG. 4. 20:4ω3 production in jar fermentation. M226-9 was cultivated in 1.8 L of medium GY supplemented with 3.5% (vol/vol) linseed oil methyl ester and 0.01% Adekanol in a 5-L jar fermenter at 28°C for 2 d, and then at 18°C, with aeration at 1 vol/vol/min and agitation at 300 rpm. (A) Fatty acid production and cell growth. (B) Fatty acid composition during the cultivation.

Bench-scale production. Based on the culture conditions tested above, M226-9 was cultivated in a 5-L jar fermenter. Glucose was added on the 2nd and 3rd days and consumed completely within 6 d (Fig. 4A). Linseed oil methyl ester in the medium was also consumed within 3 d (data not shown). This resulted in rapid mycelial growth to about 23 g/L of culture medium. The increase in the dry mycelial weight was followed by an increase in 20:4ω3 production to 2.05 g/L of culture medium (93.2 mg/g dry mycelia) even on the seventh day, and 2.24 g/L (97.4 mg/g) on the twelfth day. DGLA production was much lower than 20:4ω3 production during the cultivation and reached 0.53 g/L on the twelfth day. At the early stage of the cultivation, the mycelial fatty acid composition was similar to that of the supplemented linseed oil methyl ester (Fig. 4B). Then, 18:3ω3 and 18:2ω6 that had been incorporated were rapidly converted to 20:4ω3 and DGLA, respectively, although 18:1

TABLE 1
Fatty Acid Compositions of the Major Lipids of *Mortierella alpina* M226-9 Grown with Linseed Oil Methyl Ester[a]

Fraction[b]	Lipid composition (mol%)	Fatty acid composition[c] (mol%)										
		16:0	18:0	18:1	18:2	18:3ω6 20:0	18:3ω3 20:1	18:4ω3	DGLA	20:3ω3	20:4ω3	24:0
TG	82.2	7.1	3.5	19.2	11.0	0.8	36.1	2.8	3.0	3.7	11.6	1.4
FA	1.9	16.6	13.4	45.2	8.9	—[d]	15.8	—	—	—	—	—
DG	7.1	7.2	3.3	24.2	14.1	0.4	44.5	1.6	0.8	1.0	2.7	—
PE	2.3	11.0	2.3	26.7	7.2	3.1	9.6	6.4	5.9	5.9	22.0	—
PC	3.9	12.9	1.3	8.9	6.0	1.5	5.8	13.2	7.4	4.3	38.9	—
PS	1.2	19.8	2.4	33.8	6.3	2.2	5.5	2.4	5.9	2.5	19.2	—
PA	1.5	17.5	2.5	19.0	5.8	1.3	7.6	8.9	5.2	3.8	28.3	—

[a]The fungus was cultivated in medium GY containing 2% linseed oil methyl ester at 28°C for 2 d and then at 16°C for 7 d. The extracted lipids were separated into fractions by thin-layer chromatography.
[b]TG, triacylglycerol; DG, diacylglycerol; FA, free fatty acids; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PA, phosphatidic acid; DGLA, dihomo-γ-linolenic acid.
[c]Data are means of two independent determinations. The first fatty acid of the paired ones was the major component.
[d]Undetectable.

changed little. The percentage of 20:4ω3 among the total fatty acids increased gradually to 37.1%, the ratio of 20:4ω3/DGLA being 4.22.

Lipid analysis. The extracted mycelial lipids were separated into individual lipid classes (Table 1). Of the total lipids 82.2% (by mol) comprised triacylglycerol (TG). As for 20:4ω3, 83.3% was distributed in TG. On comparing the fatty acid compositions of the classes, 20:4ω3 was one of the major fatty acids in phospholipids (PL), such as in phosphatidylcholine (PC) (38.9%), phosphatidic acid (PA) (28.3%), and phosphatidylethanolamine (PE) (22.0%), while it was only 11.6% in TG. High percentages of DGLA, 18:4ω3, and 18:3ω6 were also detected in PL, although 18:3ω3 and 18:2ω6 were abundant in TG and diacylglycerol (DG).

DISCUSSION

We have reported that Δ5 desaturase-defective mutants S14 and Mut44 produced 20:4ω3 when grown with linseed oil (4). A problem in using these mutants is that they accumulate large amounts of structurally related C_{20} PUFA, such as EPA, DGLA, and AA, which are difficult to separate from 20:4ω3. To study 20:4ω3 and to produce a 20:4ω3-containing oil for practical use, an oil with a high 20:4ω3 and a low EPA + DGLA + AA content is suitable. As shown in Table 2, Δ5 desaturase-defective mutant, S14, shows high 20:4ω3 productivity. The lipids produced by this mutant are free from both EPA and AA, but contain a large amount of DGLA. However, the ratio of 20:4ω3/(EPA + DGLA + AA) was in the range of 1–1.6. The present mutant, M226-9, also showed excellent 20:4ω3 productivity, especially on jar fermentation. The production of 20:4ω3 reached 2.24 g/L of culture medium, and the DGLA content drastically decreased, resulting in a 20:4ω3/ (EPA + DGLA + AA) ratio increase to 4.22.

Since M226-9 is defective in both Δ5 and Δ12 desaturases, it accumulates 20:2ω9, and cannot form any n-6 or n-3 PUFA when grown on glucose and yeast extract (8) (Fig. 1). M226-9 produced 20:4ω3 and DGLA, but did not form any n-9 PUFA when grown with linseed oil. A similar case was reported previously, i.e., a Δ12 desaturase-defective strain, producing Mead acid (20:3ω9), produces not 20:3ω9 but EPA and AA when grown with linseed oil (11). These probably resulted from the substrate specificity of Δ6 desaturase, i.e., Δ6 desaturase may act on 18:3ω3 and 18:2ω6 much more than on 18:1, and only n-3 and n-6 PUFA are formed. This is also presumed from the fact that rat liver Δ6 desaturase exhibits such a specificity (12). In the case of M226-9, glucose is converted to only saturated fatty acids and 18:1, and exogenous 18:3ω3 and 18:2ω6 in linseed oil are converted to 20:4ω3 and DGLA, respectively.

The effect of Δ12 desaturase defectiveness is shown in Figure 2. Although the production of 20:4ω3 and DGLA was almost the same (*ca.* 0.7 mg/mL of culture medium) in the case of S14, 20:4ω3 production (1.40 mg/mL) was 2.7 times higher than DGLA production (0.51 mg/mL) in the case of M226-9. DGLA production by M226-9 decreased, probably because 18:2ω6 was not synthesized from glucose and this led to increased 20:4ω3 production, since the n-3 and n-6 pathways share an enzyme system and compete with each other.

Figure 3a indicates that 20:4ω3 production was highest when M226-9 was grown at 28°C and then at 16°C. There are two biosynthetic pathways for 20:4ω3 (Fig. 1). One is the n-3 pathway, in which exogenous 18:3ω3 is converted to 20:4ω3 by Δ6 desaturase and elongase. The other is ω3 desaturation from n-6 to n-3 fatty acids, which is active only at low temperatures (13). In either case, the 20:4ω3 formed accumulates in M226-9 and

TABLE 2
Comparison of 20:4ω3 Productivities of *M. alpina* 1S-4, Mut44, S14, and M226-9

Strain [Mutant-type]	Culture conditions: Glucose (%)	Linseed oil methyl ester (%)	Temperature (°C)	Scale[a]	Culture period (d)	Mycelial mass (g/L)	20:4ω3 production (g/L)	% in total fatty acids: 20:4ω3	EPA	DGLA	AA	20:4ω3/(EPA + DGLA + AA)[b]	Reference
1S-4 [Wild strain]	2.0	3.0	12	F	16	28.2	0.50	3.2	12.0	3.0	15.7	0.10	3
Mut44 [Δ5 DS-defective]	1.0	2.0	28→16	F	9	15.2	0.47	5.5	0.6	8.8	6.3	0.35	4
S14 [Δ5 DS-defective]	1.0	4.0	28→16	F	9	24.8	1.65	11.6	—[c]	7.4	—	1.57	4
	2.0	4.0	28→16	J	12	20.7	1.60	26.0	—	13.0	—	2.00	
M226-9 [Δ5 and Δ12 DS-defective]	4.0	3.5	28→18	J	7	22.0	2.05	29.6	—	7.1	—	4.17	This work
					12	23.0	2.24	37.1	—	8.8	—	4.22	

[a]F, the fungus was grown in a 50-mL or 100-mL flask; J, the fungus was grown in a 5-L or 10-L jar fermenter.
[b]EPA, 5,8,11,14,17-*cis*-eicosapentaenoic acid; AA, arachidonic acid; DS, desaturase. For other abbreviation see Table 1.
[c]Undetectable.

is not further desaturated to EPA. At a higher temperature, 20:4ω3 production decreased, probably concomitant with decreasing activities of ω3 desaturase and other desaturases. At a lower temperature, ω3 desaturation is probably more prominent, which is the main reason for the high 20:4ω3/DGLA ratio at 10°C. But 10°C is thought to be too cold for the other reactions necessary for 20:4ω3 formation to proceed, and 20:4ω3 production was lower. Optimization of the glucose and linseed oil methyl ester concentrations is shown in Figures 3B and 3C. DGLA production did not increase with increasing glucose concentration. This is different from the case of S14, also indicating that glucose was not converted to DGLA by M226-9. With medium GY containing 3% linseed oil methyl ester, 20:4ω3 production reached 1.46 mg/mL of culture medium.

The productivity of 20:4ω3 in a jar fermenter was superior to that in a flask, which resulted from the increase in mycelial growth, mainly due to the addition of glucose. High mycelial growth is thought to lead to high production of total fatty acids and efficient conversion of 18:3ω3 to 20:4ω3.

The main lipid class was TG (82.2% of total lipids) (Table 1). Such a high proportion of TG is thought to be suitable for practical use. Among the n-3 fatty acids, percentages of 20:4ω3 and 18:4ω3 were higher in PL than in TG, and 18:3ω3 was lower in PL, suggesting that the fatty acids in PL are selectively incorporated. Similarly, percentages of DGLA and 18:3ω6 among n-6 fatty acids were higher in PL, suggesting that M226-9 adapts to a low growth temperature (16°C) by selectively incorporating highly desaturated fatty acids into PL. DGLA and 20:4ω3 were high in PL, and 18:3ω3 was high in TG; in these respects, M226-9 and S14 are similar (4). Although 20:4ω3 productivity by M226-9 was much greater than that by S14, the distributions of individual fatty acids among the main lipids were similar.

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