Production of 8,11-*cis*-Eicosadienoic Acid by a Δ5 and Δ12 Desaturase-Defective Mutant Derived from the Arachidonic Acid-Producing Fungus *Mortierella alpina* 1S-4

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ABSTRACT: The Δ5 and Δ12 desaturase (DS)-blocked mutants of an arachidonic acid-producing fungus, *Mortierella alpina* 1S-4, were obtained. These mutants accumulated 8,11-*cis*-eicosadienoic acid (20:2n-9). One of the mutants, M226-9, in which Δ5 and Δ12 DS are perfectly blocked, produced 1.68 mg of 20:2n-9 per mL of culture medium (101 mg/g dry mycelia) and no 5,8,11-*cis*-eicosatrienoic acid (20:3n-9) when grown in a medium containing 4% glucose and 1% yeast extract at 28°C for 2 d and then at 12°C for 12 d. The mycelial lipid comprised 77.4% triacylglycerol (TG) and 9.8% phosphatidylcholine (PC), among others. TG contained 69.0% of the total 20:2n-9, whose percentage in total TG fatty acids was 15.9%. The highest percentage (44.4%) of 20:2n-9 was found in PC. The addition of olive oil to the culture medium enhanced the production of 20:2n-9.

Paper no. J9301 in *JAOCS 76,* 1269–1274 (November 1999).

KEY WORDS: 20:2n-9, eicosadienoic acid, *Mortierella alpina.*

8,11-*cis*-Eicosadienoic acid (20:2n-9) is a polyunsaturated fatty acid (PUFA) of the n-9 series and the precursor of Mead acid (5,8,11-*cis*-eicosatrienoic acid, MA, or 20:3n-9) in the n-9 pathway (Fig. 1). PUFA of the n-9 series are known to exist in animals with essential fatty acid (EFA) deficiency (1,2). They have attracted great interest recently because they are synthesized *de novo* in animals, unlike n-6 and n-3 PUFA, and may function as natural analogs of n-6 and n-3 PUFA. Of the n-9 PUFA, MA has been studied in detail; it is found in human umbilical cords (3) and in the cartilage of young animals (4,5), fetuses, and infants (5), and is converted into leukotrienes of the 3-series (6,7). However, to our knowledge, there is no report to date of 20:2n-9 or its physiological role. One of the reasons for this may be its limited availability.

A very small amount of 20:2n-9 has been detected in the organs of EFA-deficient animals (2), but they cannot serve as practical or sufficient sources of 20:2n-9. In previous studies on MA production using a Δ12 desaturase (DS)-defective mutant (Mut48) (8) of an arachidonic acid (AA, or 20:4n-6)-producing fungus *Mortierella alpina* 1S-4 (9), we found that the mutant accumulated a small amount of 20:2n-9. However, its productivity was very low (approximately 4 mg/g dry mycelia or 0.2 g/L of culture medium and approximately 3% of total mycelial fatty acids).

We thought that accumulation of 20:2n-9 by the Δ12 DS-defective mutant could be increased if Δ5 DS in the mutant were blocked. A mutant defective in both Δ5 and Δ12 DS was

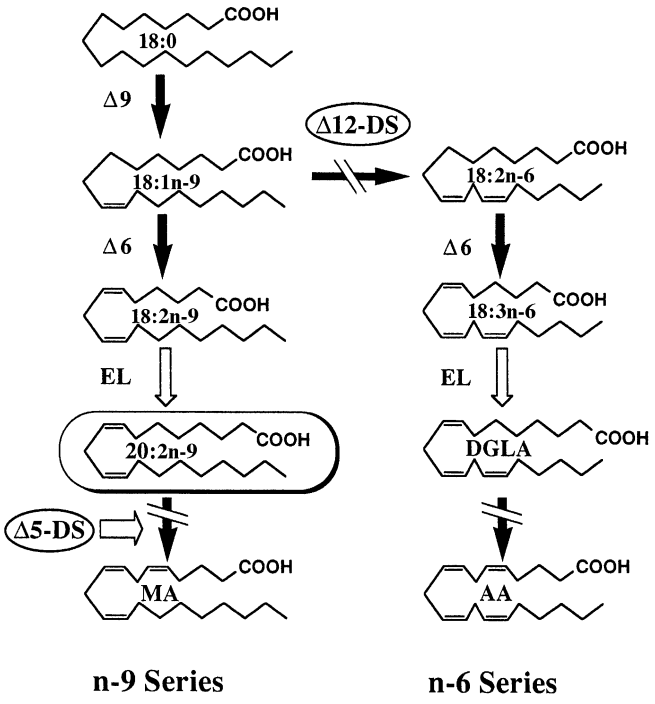

FIG. 1. Biosynthetic pathway for fatty acids in *Mortierella alpina* M226-9. Δ5 and Δ12 desaturases (DS) are blocked in M226-9. EL, elongase; DGLA, dihomo-γ-linolenic acid; AA, arachidonic acid; MA, Mead acid.

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obtained from the Δ12 DS-defective mutant, *M. alpina* Mut48. Here, the characterization of this mutant, the optimization of the culture conditions for 20:2n-9 production by the mutant, and the fatty acid profiles of the major lipid classes in the lipids produced are described.

MATERIALS AND METHODS

Chemicals. Oils (triacylglycerols) and oleate derivatives were obtained from Wako Pure Chemicals (Osaka, Japan), Nacalai Tesque (Kyoto, Japan), Nissan Chemicals (Tokyo, Japan) and Sigma (St. Louis, MO). All other chemicals used are commercially available and were described previously (10).

Mutagenesis and isolation of mutants. Mutagenesis and isolation of mutants were essentially performed according to the methods described previously (10). Spores of *M. alpina* Mut48 (8), which is a Δ12 DS-defective mutant derived from *M. alpina* 1S-4 (9), were treated with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG). The mycelial fatty acids of the colonies derived from 2000 MNNG-treated spores were analyzed by gas–liquid chromatography (GLC) as described below, and the strains with high contents of 20:2n-9 were stored on a potato-dextrose agar slant medium. One of the mutants, M226-9, in which Δ5 and Δ12 desaturation reactions are almost perfectly blocked, was selected as a potent producer of 20:2n-9.

Liquid culture. *Mortierella alpina* Mut48 and M226-9 were inoculated as a spore suspension into a 100-mL Erlenmeyer flask containing 20 mL of medium GY (2% glucose and 1% yeast extract, pH 6.0), followed by incubation with reciprocal shaking (120 strokes/min) at 28°C. For jar fermentation, M226-9 was precultured at 28°C for 2 d in 100 mL of the same medium, and the resultant culture was inoculated into 2 L of a medium composed of 4% glucose, 1% yeast extract, 2% olive oil, and 0.01% Adekanol (an antifoaming agent; Asahi Denka, Osaka, Japan), pH 6.0, in a 5-L jar fermentor (Mitsuwa, Osaka, Japan). Other conditions are given in each table and figure.

Analysis of fatty acids and lipids. Mycelia were harvested by suction filtration, washed with water, and then dried at 105°C for 2 h. The dried mycelia were directly treated with 10% methanolic HCl for transmethylation of mycelial fatty acids. The resultant fatty acid methyl esters were extracted with *n*-hexane and then analyzed by GLC, as described previously (11). A glass column (3 mm × 3 m) packed with Advance DS (Nishio Kogyo, Tokyo, Japan) was used. The GLC was operated at a constant column temperature of 195°C. Fungal lipids were extracted with a chloroform/methanol/water system according to Folch *et al*. (12). The lipids were separated into individual lipid classes by thin-layer chromatography (11), and the fatty acid composition of each lipid class was analyzed by GLC as described above. Lipid compositions

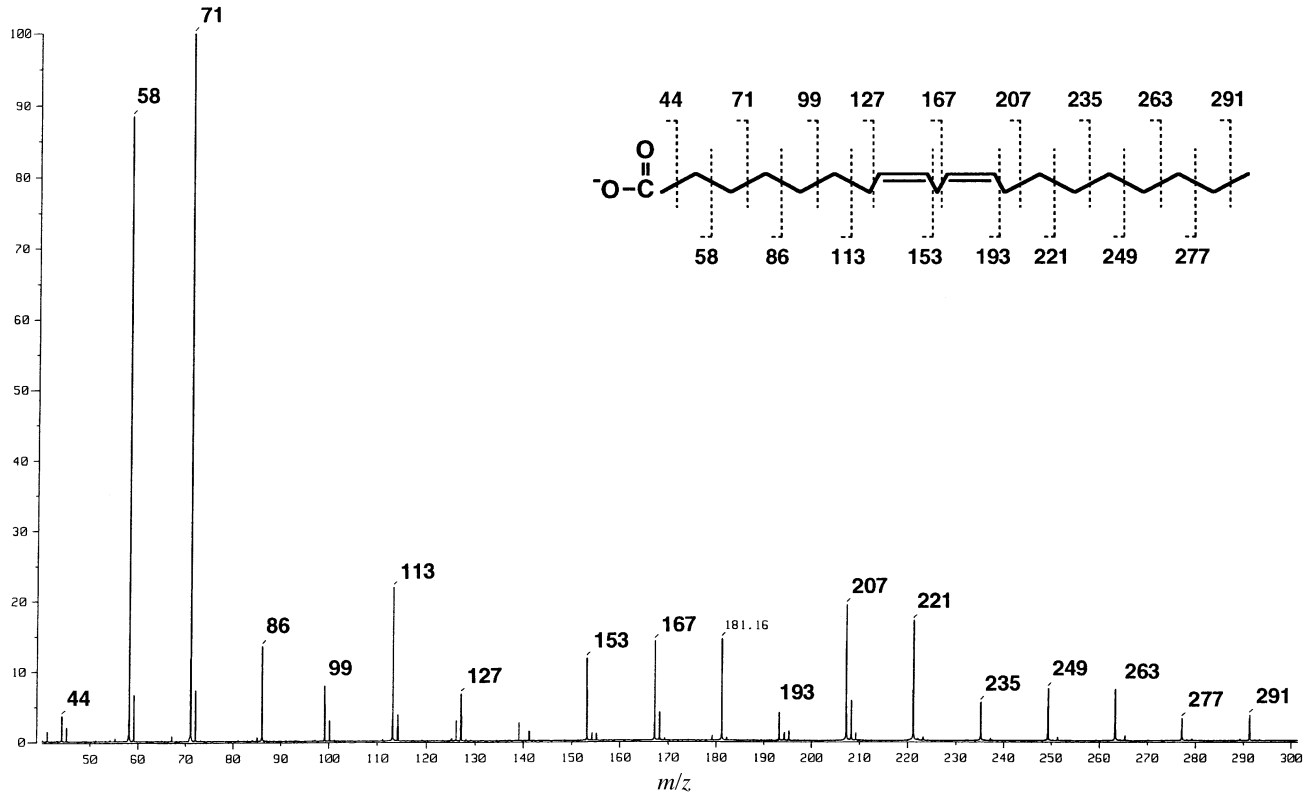

FIG. 2. Tandem (MS/MS) mass spectrum of free fatty acid 8,11-*cis*-eicosadienoic acid accompanied by chemical ionization (negative ion fast atom bombardment).

were calculated from the amounts of total fatty acids of individual lipid classes as recommended by Christie (13). Heptadecanoate was usually added as an internal standard before transmethylation. The compound 20:2n-9 was separated from the mixture of fatty acid methyl esters by reversed-phase high-performance liquid chromatography (HPLC) as described previously (9). The isolated fatty acid was saponified with KOH, and then its mass spectrum was determined.

Mass spectroscopy (MS). Negative ion fast atom bombardment (FAB) spectra were recorded on a JEOL HX110A/HX110A four-sector tandem mass spectrometer (Tokyo, Japan), equipped with an MS-ADS11 variable dispersion array detector, at 8 kV accelerating voltage. A negative ion FAB-MS/MS using helium to cause collision-induced dissociation (CID) in the collision cell, which was used at a pressure that reduced the intensity of precursor ions to 30–40%, was performed on the isolated free acid. Samples were mixed with a glycerol matrix on the FAB target.

Other methods. Fungal growth was measured by determining mycelial weight after drying at 105°C for 2 h. All values shown in figures and tables are means of three independent determinations. The differences between pairs of values were less than 5% of the means.

RESULTS

*Identification of 8,11-*cis*-eicosadienoic acid (20:2n-9)*. FAB–MS and –MS/MS were applied to identify the position of the double bond of the fatty acid. Its negative-ion FAB–MS spectrum showed a molecular-related ion at *m/z* 307 $(M - H)^-$; the CID MS/MS spectrum of $(M - H)^-$ involved prominent product ions as shown in Figure 2. The characteristic product ions due to α-cleavage of the double bond system were observed at *m/z* 127, 153, 167, and 193, respectively. These product ions indicated that the positions of the double bond are between C-8 and -9, and also C-11 and -12. Consequently, the isolated fatty acid produced by M226-9 was identified as 8,11-*cis*-eicosadienoic acid.

Time courses of changes in mycelial fatty acid contents. Time courses of the changes in the production of mycelial fatty acids in M226-9 are shown in Figure 3. As fungal growth increased, mycelial oleic acid (18:1n-9) accumulation increased, followed by mycelial 20:2n-9 accumulation. Production of 20:2n-9 increased markedly from the third to the ninth day, reaching 1.68 mg/mL of culture medium (101 mg/g dry mycelia) on the fourteenth day. As for fatty acid compositions, saturated fatty acids (palmitic and stearic acids) and monounsaturated fatty acid (18:1n-9) were accumulated in the early stage of cultivation, but their percentage in the total fatty acids decreased markedly from the second to the eighth day. In contrast, the percentage of 20:2n-9 in the total fatty acids increased markedly, from 3.4% on the second day to 20.0% on the seventh day.

Factors affecting 20:2n-9 production. (i) Growth temperature. Mycelial 20:2n-9 production was highest at 12°C (72.3 mg/g dry mycelia or 0.76 mg/mL of culture medium, Fig. 4A). The higher the growth temperature was, the lower the mycelial 20:2n-9 content and its ratio to total fatty acids were. The production of fungal mycelia did not change markedly with growth temperature, but the total fatty acid contents (data not shown) and mycelial 18:1n-9 contents decreased with increasing growth temperature.

(ii) Glucose concentration. Mycelial 20:2n-9 production increased markedly with increasing glucose concentration, reaching 74.5 mg/g of dry mycelia and 1.14 mg/mL of culture medium when glucose concentration was 4%, but the percentage of 20:2n-9 in the total fatty acids decreased gradually (Fig. 4B).

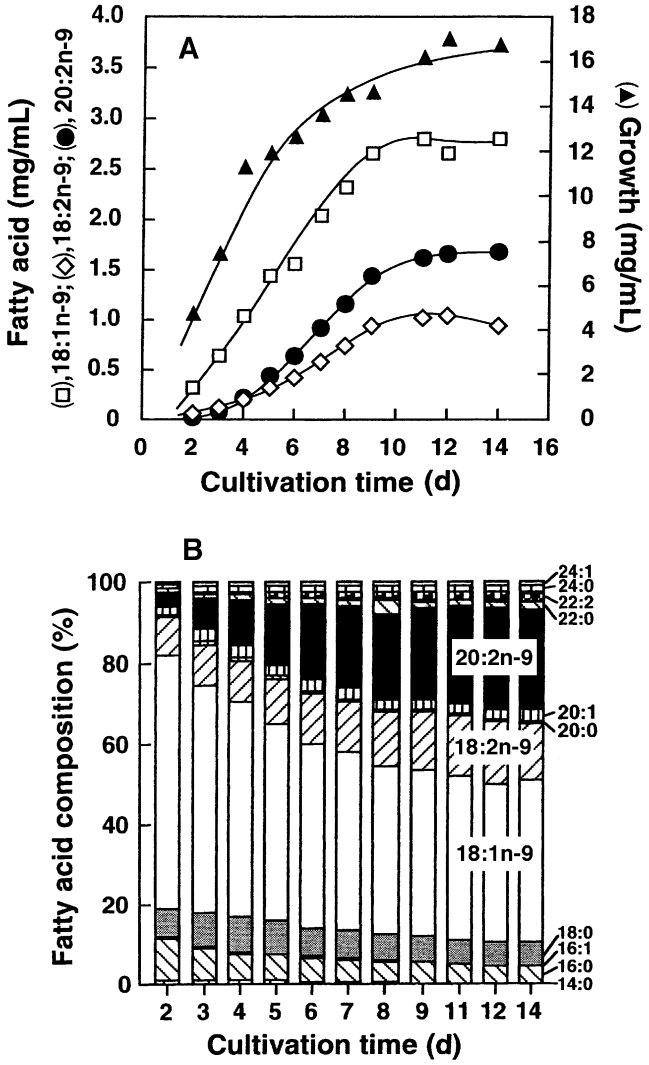

FIG. 3. Changes in the production of fatty acids in *M. alpina* M226-9. The fungus was cultivated in a medium containing 4% glucose and 1% yeast extract at 28°C for 2 d and then at 12°C for 12 d. (A) Fatty acid production and cell growth. (B) Fatty acid composition during cultivation. 14:0, myristic acid; 16:0, palmitic acid; 16:1, palmitoleic acid; 18:0, stearic acid; 18:1n-9, oleic acid; 18:2n-9, 6,9-octadecadienoic acid; 20:0, arachidic acid; 20:1, 11-eicosenoic acid; 20:2n-9, 8,11-eicosadienoic acid; 22:0, behenic acid; 22:2, docosadienoic acid; 24:0, lignoceric acid; 24:1, tetracosenoic acid. See Figure 1 for abbreviation.

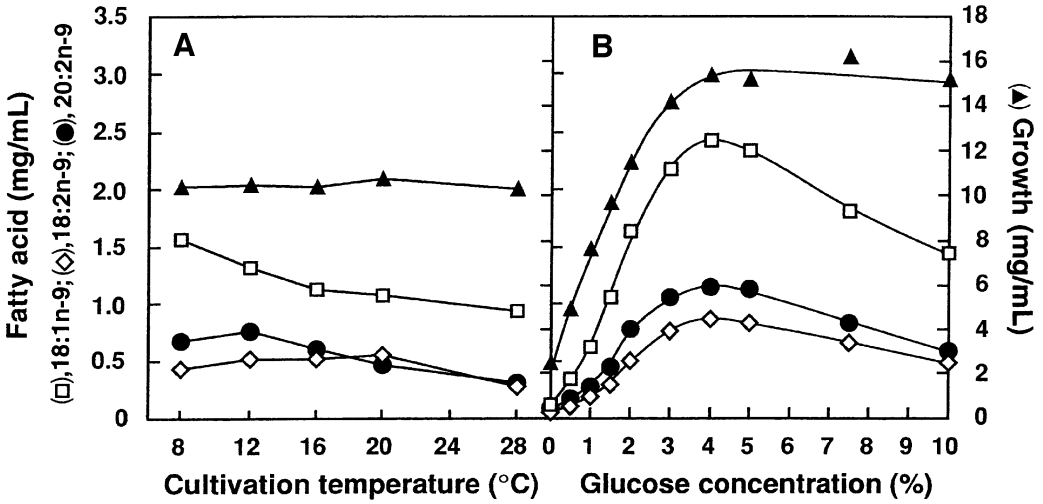

FIG. 4. Effects of (A) growth temperature and (B) glucose concentration on fatty acid production. (A) M226-9 was cultivated in a medium containing 2% glucose and 1% yeast extract at 28°C for 2 d and then at 8–28°C as indicated for 7 d. (B) M226-9 was cultivated in a medium containing 1% yeast extract and 0–10% glucose as indicated at 28°C for 2 d and then at 12°C for 7 d.

(iii) Oil addition. As oil addition was reported to support AA production (14), various oils and oleate derivatives were added to the culture medium. Supplements containing minimal concentrations of n-6 or n-3 fatty acids were used, because n-6 or n-3 fatty acids are thought to be desaturated and elongated prior to n-9 fatty acids (15). As shown in Figure 5, the additions of olive oil, high-oleic sunflower oil, peanut oil, camellia oil, palm oil, triolein, oleic acid, and ethyl oleate increased mycelial 20:2n-9 production. Production of 20:2n-9 with olive oil was about 1.6-fold higher than without it. Those oils had an abundance of 18:1n-9. Other oils that had the effect of increasing 20:2n-9 production were coconut oil, which had an abundance of medium-chain fatty acids, and jojoba bean oil, which had abundant 20:1n-9.

Production of 20:2n-9 in a bench-scale jar fermentor. On the basis of the culture conditions optimized above, bench-scale production of 20:2n-9 by strain M226-9 was performed using a 5-L jar fermentor. Fungal growth reached a plateau (24.4 g/L of culture medium) on the tenth day. Production of 20:2n-9 was low at first and increased markedly from the seventh to the thirteenth day, reaching 1.31 g/L of culture medium on the thirteenth day (corresponding to 51.0 mg/g dry mycelia and 7.6% of total fatty acids) (Fig. 6).

Compositions of major lipids and distribution of fatty acids in major lipid classes of M226-9. As shown in Table 1, the main lipid of M226-9 was triacylglycerol (TG), which constituted 77.4 mol% of the total lipids. Other major lipids were phosphatidylethanolamine (PE, 5.4 mol%) and phosphatidylcholine (PC, 9.8 mol%). About 69.0 mol% of the total 20:2n-9 produced was present in the TG fraction and the remainder mainly in the PC fraction (*ca*. 24.4 mol%). The highest proportion of 20:2n-9 (44.4 mol%) was found in PC. The proportion of 18:2n-9 was high in PC (29.9 mol%) and PE (34.2 mol%).

DISCUSSION

To date, several mutants have been obtained from an AA-producing fungus, *M. alpina* 1S-4. They are defective in one DS, i.e., Δ5 (16,17), Δ6 (18), or Δ12 DS (8). Another mutant defective in two DS, i.e., Δ5 DS and n-3 DS (19), was also obtained. In the present study, the second mutant defective in two DS has been obtained. This mutant (M226-9 strain) is defective in Δ5 DS and Δ12 DS and can accumulate a rare PUFA, 20:2n-9.

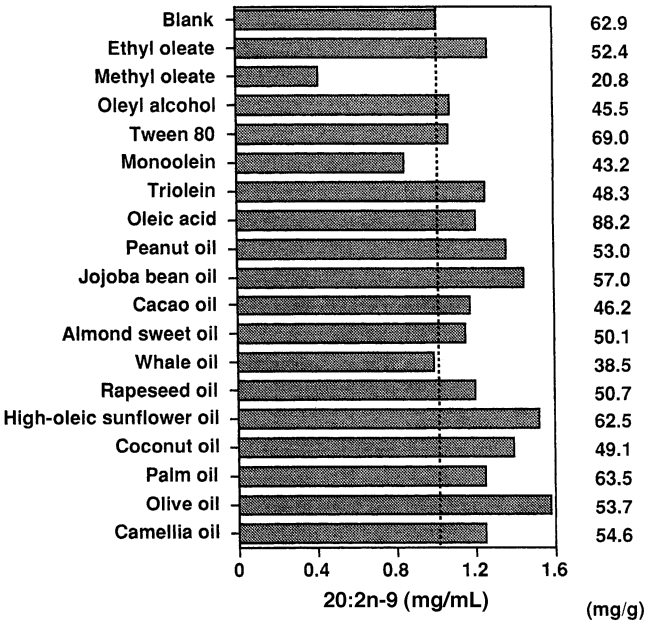

FIG. 5. Effect of oil addition on 20:2n-9 production. M226-9 was cultivated in a medium containing 2% glucose, 1% yeast extract, and each oil at 28°C for 2 d and then at 12°C for 7 d.

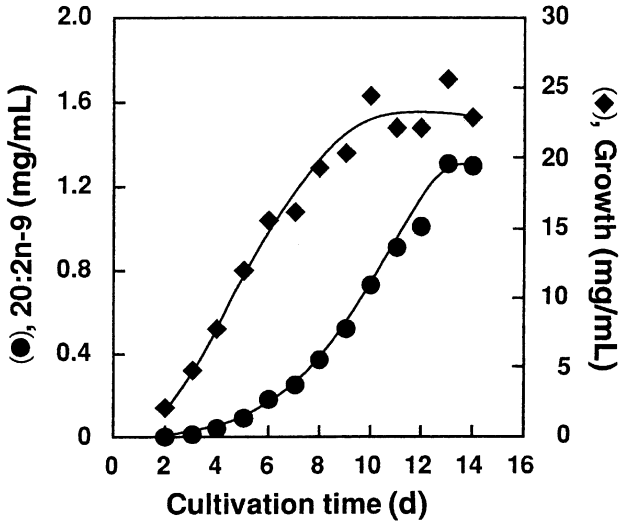

FIG. 6. Production of 20:2n-9 by jar fermentation. M226-9 was cultivated in 2 L of medium containing 4% glucose, 1% yeast extract, 2% olive oil, and 0.01% Adekanol in a 5-L jar fermentor at 28°C for 2 d and then at 12°C for 12 d with aeration at 1 vol/vol/min (= 2.0 L.min) and agitation at 300 rpm.

In Mut48 defective in Δ12 DS, 18:1n-9 is converted into MA through the n-9 pathway *via* 18:2n-9 and 20:2n-9; but in M226-9, 20:2n-9 is not converted into MA because of the Δ5 DS defect and is accumulated in the mycelia (Fig. 1). Production of 20:2n-9 was highest when M226-9 was cultivated at 12°C (Fig. 4A). This seems to be due to the activations of Δ6 DS and elongase because the proportions of saturated fatty acids and 18:1n-9 were higher at higher growth temperatures. At glucose concentrations higher than 4%, the production of 20:2n-9 and other fatty acids decreased gradually, but the mycelial mass did not change markedly (Fig. 4B). Fatty acid synthesis may have been suppressed to produce other cellular components.

The addition of 18:1n-9-abundant oils increased the production of 20:2n-9 (Fig. 5), suggesting that these oils were incorporated into the mycelia. Because of this, the mycelial 18:1n-9 content increased, and 18:1n-9 was likely to be converted into 20:2n-9 because of metabolic control. Under optimal culture conditions, 20:2n-9 production reached *ca.* 1.7 g/L of culture medium (*ca.* 100 mg/g dry mycelia) on the fourteenth day. Furthermore, bench-scale production of 20:2n-9 reached 1.3 g/L of culture medium (51.0 mg/g dry mycelia) (Fig. 6), suggesting that practical production of 20:2n-9 is possible.

The distribution of fatty acids in the major lipid class showed that the proportion of 20:2n-9 was high in PC and that the proportion of the other PUFA, 18:2n-9, was high in both PC and PE. It has been suggested (20) that membrane function is greatly dependent on membrane fluidity, which varies with the composition of the lipids and their fatty acid composition. The parent strain *M. alpina* 1S-4 and its various mutants, except M226-9, had large amounts of many different PUFA in their mycelia. However, there are only two PUFA, 20:2n-9 and 18:2n-9, in M226-9. Although it is uncertain if large amounts of PUFA are necessary for maintaining the membrane fluidity of M226-9, 20:2n-9 and 18:2n-9 seem to be incorporated selectively into major membrane lipids, PC and PE.

TABLE 1
Fatty Acid Compositions of the Major Lipids of *Mortierella alpina* M226-9[a]

	Fraction[b]						
	TG	FA	DG	PE	PC	PS	PA
Lipid composition (mol%)							
	77.4	3.3	0.8	5.4	9.8	2.6	0.7
Fatty acid composition (mol%)							
14:0	2.3	2.6	6.6	1.2	0.6	1.5	3.5
16:0	8.6	11.8	9.8	3.7	1.6	15.5	13.1
16:1	0.3	1.5	—[c]	—	—	—	—
18:0	4.2	6.5	11.4	1.2	0.5	2.0	6.4
18:1n-9	51.2	46.9	40.9	46.7	21.4	58.4	38.8
18:2n-9	8.2	9.1	7.0	34.2	29.9	13.0	19.7
20:0	0.4	2.5	—	—	—	—	—
20:1	3.5	2.3	3.8	4.0	1.4	—	—
20:2n-9	15.9	7.1	11.8	9.1	44.4	8.8	18.5
20:3n-9	—	—	—	—	—	—	—
22:0	1.0	1.6	2.4	—	—	—	—
22:1	0.3	0.7	—	—	—	—	—
22:2	2.3	1.4	1.9	—	—	—	—
24:0	1.1	2.5	4.3	—	0.3	0.7	—
24:1	0.8	3.5	—	—	—	—	—

[a]The fungus was cultivated in a medium containing 4% glucose and 1% yeast extract at 28°C for 2 d and then at 12°C for 7 d. The extracted lipids were separated into each fraction by thin-layer chromatography.
[b]TG, triacylglycerol; DG, diacylglycerol; FA, free fatty acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PA, phosphatidic acid.
[c]Undetectable.

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[Received July 1, 1999; accepted September 2, 1999]