Production of 5,8,11-*cis*-Eicosatrienoic Acid by a Δ12-Desaturase-Defective Mutant of *Mortierella alpina* 1S-4

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A mutant defective in Δ12-desaturase of an arachidonic-acid producing fungus, *Mortierella alpina* 1S-4, was shown to be a novel potent producer of Mead acid (5,8,11-*cis*-eicosatrienoic acid, 20:3ω9). The fungus produced several fatty acids of the n-9 family, *i.e.*, 6,9-*cis*-octadecadienoic acid (18:2ω9), 8,11-*cis*-eicosadienoic acid (20:2ω9) and 20:3ω9. Significantly high levels of these fatty acids were produced during growth at low temperatures (12–20°C). On submerged cultivation at 20°C for 10 days in a 5-L fermenter containing 2% glucose plus 1% yeast extract (pH 6.0), the production of 20:3ω9 reached *ca.* 0.8 g/L (56 mg/g dry mycelia), accounting for 15% (by wt) of the total mycelial fatty acids. The other major fatty acids were palmitic acid (6%), stearic acid (11%), oleic acid (45%), 18:2ω9 (12%) and 20:2ω9 (3%). Studies on the distribution of fatty acids among lipid classes showed that, irrespective of the growth temperature employed (12–28°C), *ca.* 70% (by mol) of 20:3ω9 was present in the triglyceride and the remainder in the phospholipid fraction, especially in phosphatidylcholine (PC). When the fungus was grown at 12°C, the proportion of 20:3ω9 in the PC fraction was *ca.* 55%.

KEY WORDS: **Eicosatrienoic acid, Δ12-desaturation, n-9 fatty acids, Mead acid, *Mortierella alpina*.**

Over 30 years ago Mead and Slaton (1) and Fulco and Mead (2) showed that the fatty acid that accumulated in the livers and other organs of essential fatty acid (EFA)-deficient rats had the 5,8,11-*cis*-eicosatrienoic acid (20:3ω9) structure. Since then, studies on 20:3ω9, sometimes called Mead acid, have indicated that it is a structurally important fatty acid found in the cellular membranes of EFA-deficient animals and that it has a limited metabolic pathway as compared to analogous EFAs. Early work by Struijk *et al.* (3), who compared the efficiencies of transformation of various fatty acids by cyclo-oxygenase from sheep seminal vesicle microsomes, demonstrated that no prostaglandin-like metabolite was detected when Mead acid was used as a substrate. Recently, Lagarde and co-workers (4) demonstrated that 20:3ω9 was converted, through the lipoxygenase pathway, to 12-hydroxy-5,8,10-eicosatrienoic acid, which was shown to have a biphasic effect on platelet aggregation.

Possibly due to the limited available sources and its high commercial price, Mead acid has not been as widely studied as analogous fatty acids, such as arachidonic acid (Ara) (5). Besides its presence in EFA-deficient organs, this fatty acid was found in umbilical cord vessels of neonates (6) and in the cartilage of young animals (7). However, none of these was promising as a practical source. Ghosh *et al.* (8) suggested a chemical method of preparation involving random reduction of Ara by hydrazine. This method not only requires Ara, which is expensive, but also gives a low yield of 20:3ω9.

In the previous paper (9) we reported the isolation of fatty acid desaturation-defective mutants from an Ara-producing fungus, *Mortierella alpina* 1S-4, one of which (Mut48) is unable to convert oleic acid (18:1) to linoleic acid (18:2). We also have detected several fatty acids of the n-9 family, such as 6,9-*cis*-octadecadienoic acid (18:2ω9), 8,11-*cis*-eicosadienoic acid (20:2ω9) and 20:3ω9 in its mycelia. The biosynthetic pathways for fatty acids in this mutant are shown in Figure 1. Since Mead acid can be converted to 3-series leukotrienes, which can be expected to show some profound biological activities, and because naturally available sources are rare, we attempted to optimize the conditions for the practical production of this fatty acid by this mutant. In the present paper, we report several factors affecting the Mead acid accumulation by this mutant, as well as its distribution among the major lipid classes.

EXPERIMENTAL PROCEDURES

Chemicals. All reagents used were commercially available and were as described previously (9).

Microorganism and cultivation. Mortierella alpina 1S-4 Mut48 (9) is a Δ12-desaturase-defective mutant derived from *M. alpina* 1S-4 (10,11). The fungus was inoculated as a spore suspension into a 10-mL Erlenmeyer flask containing 2 mL of medium GY (2% glucose plus 1% yeast extract, pH 6.0), and then the culture was incubated with reciprocal shaking (120 strokes/min) at 28°C for one week, unless stated otherwise.

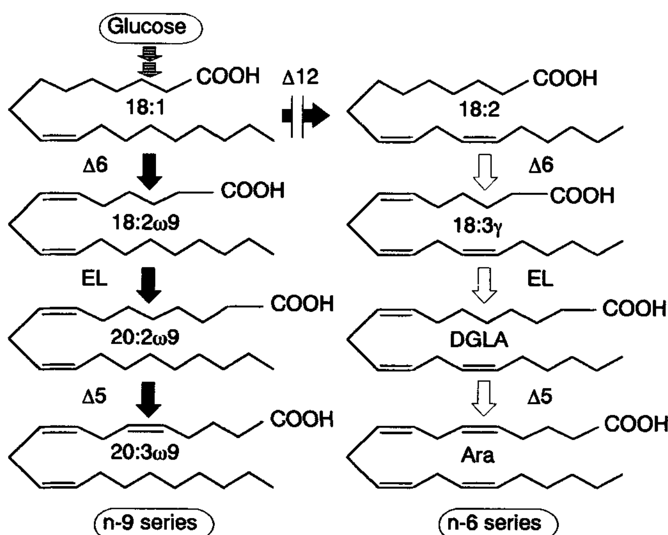

FIG. 1. Possible biosynthetic pathways for fatty acids in *M. alpina* 1S-4 Mut48. This fungus is defective in the desaturation of 18:1 to 18:2, and therefore no fatty acids of the n-6 family were found. The biosynthesis of 20:3ω9 would occur through the same sequential reactions as for Ara formation. Abbreviations: Δn, Desaturation at the Δn position; EL, elongation.

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Fatty acid and lipid analysis. Mycelial cells were harvested by suction filtration, washed with distilled water and then dried at 100°C overnight for subsequent fatty acid analysis by gas liquid chromatography (GLC) as described previously (12). Extraction and fractionation into lipid classes was carried out essentially by the same methods as described previously (9,12).

Isolation and characterization of fatty acids. 18:2ω9, 20:2ω9 and 20:3ω9 were separated from the mixture of fatty acid methyl esters by reverse-phase high-performance liquid chromatography on a Cosmosil column ($5C_{18}$-AR, 20 × 250 mm, Nacalai Tesque, Kyoto, Japan). The mobile phase was CH_3CN-H_2O (90:10, vol/vol) at a flow rate of 9 mL/min, and the effluent was monitored by means of ultraviolet detection (210 nm). The elution times of these fatty acids under the above conditions were 37, 64 and 46 min, respectively. The chemical structures of these acids were determined by proton nuclear magnetic resonance (NMR) and mass spectroscopy (MS). Mass spectra were recorded in the electron impact mode (70 eV) with a Hitachi M-80B (Tokyo, Japan), and ^{1}H-NMR with a Nicolet NT-360 (Madison, WI). The analytical data for the methyl ester derivatives, unless otherwise stated, were as follows.

18:2ω9, NMR δ ($CDCl_3$): 0.88 ppm (*t*, 3H), 1.26 (broad, 12H), 1.34 (*p*, 2H), 1.65 (*p*, 2H), 2.06 (*m*, 4H), 2.32 (*t*, 2H), 2.77 (*t*, 2H), 3.67 (*s*, 3H) and 5.35 (*m*, 4H). MS *m/z* (relative abundance, %): 294 (M^+, 16), 262 (5), 220 (6), 178 (5), 164 (7), 150 (17), 135 (17), 121 (22), 109 (33), 95 (66), 81 (95), 67 (100), 55 (76), 41 (93), 29 (40).

20:2ω9, NMR δ ($CDCl_3$: 0.88 ppm (*t*, 3H), 1.26 (broad, 12H), 1.34 (broad, 6H), 1.65 (*p*, 2H), 2.06 (*m*, 4H), 2.32 (*t*, 2H), 2.77 (*t*, 2H), 3.67 (*s*, 3H) and 5.35 (*m*, 4H). MS (picolinyl ester, see ref. 13 for preparation method) *m/z* (relative abundance, %): some of the useful fragment ions were 399 (M^+, 28%), 314 (6), 300 (8), 286 (3), 260 (5), 246 (6), 220 (3), 92 (100).

20:3ω9, NMR δ ($CDCl_3$): 0.88 ppm (*t*, 3H), 1.27 (broad, 12H), 1.71 (*p*, 2H), 2.10 (*m*, 4H), 2.32 (*t*, 2H), 2.80 (*t*, 4H), 3.67 (*s*, 3H) and 5.37 (*m*, 6H). MS *m/z* (relative abundance, %): 320 (M^+, 6%), 205 (7), 192 (9), 180 (15), 161 (16), 147 (10), 135 (15), 121 (14), 106 (47), 93 (68), 79 (91), 67 (100), 55 (43), 41 (83), 29 (30).

Other methods. Glucose concentrations were measured with a commercial kit (Blood Sugar-GOD-Pred-Test., Boehringer, Mannheim) according to the method of Werner *et al.* (14).

RESULTS

Accumulation of n-9 fatty acids in M. alpina *1S-4 Mut48.* Representative time courses of the changes in the mycelial contents of fatty acids are shown in Figure 2. The fungal growth usually reached a plateau after 3-d cultivation, and the total mycelial fatty acids reached the maximum of *ca.* 230 mg/g dry mycelia after five days. The amounts of 18:1 and saturated acids, *i.e.*, 16:0, 18:0, 20:0, 22:0 and 24:0, increased with growth, the maximal values being reached after three days and decreasing thereafter. On the other hand, 18:2ω9, 20:1 and 20:2ω9 reached their maximum levels after five days and then remained almost constant up to the tenth day of cultivation. 20:3ω9 increased markedly from three to five days, and continued increasing at a lower rate up to the tenth day of cultivaion, reaching *ca.* 22 mg/g dry mycelia.

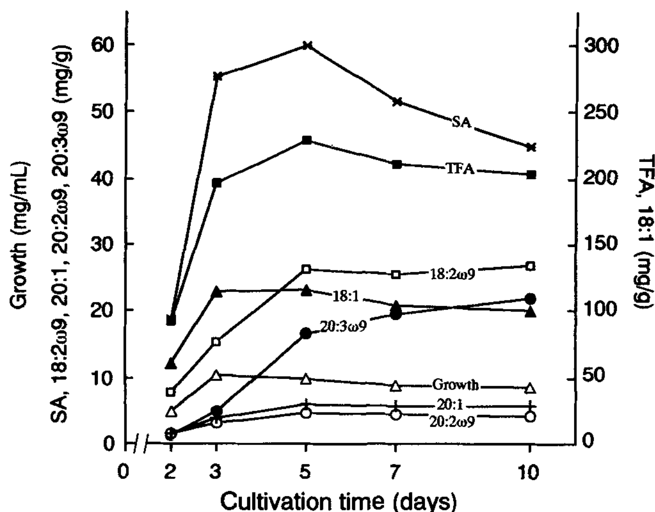

FIG. 2. Changes in the contents of fatty acids in *M. alpina* 1S-4 Mut48. The fungus was grown in medium GY at 28°C for the times indicated. SA, sum of saturated fatty acids; TFA, total fatty acids.

Factors affecting Mead acid production. Based on the previously determined conditions for the production of Ara by the wild-type strain (15), we investigated factors affecting the production of Mead acid as follows.

(i) *Glucose and yeast extract concentrations.* Like the wild-type strain, Mut48 grew well on a simple medium containing glucose and yeast extract as the basal constituents. As shown in Figure 3, the optimal glucose concentrations at two fixed yeast extract concentrations [0.5 and 1%, Fig. 3 (a) and (b)] were both 4%. Increasing the yeast extract concentration increased the mycelial mass, but decreased the production of 20:3ω9 markedly. The optimal concentration was 0.5%, as for the medium containing 2% glucose [Fig. 3 (c)].

(ii) *Growth temperature.* On cultivation at various temperatures, it was found that the range of 12–20°C was optimal for mycelial Mead acid accumulation. As shown in Figure 4, the production of 20:3ω9 increased significantly when the growth temperature was reduced from 28°C to either 12 or 20°C. The longer the period of cultivation, either at 12 or 20°C, the greater the yield of 20:3ω9. However, cultivation at both temperatures without preincubation at 28°C (0 days) resulted in a marked decrease in the amount of 20:3ω9, especially on growth at 12°C. Maximum production was obtained on cultivation at either 12 or 20°C for 6 days after 1-d preincubation at 28°C (1 d). The value was about double that obtained on cultivation at 28°C only (7 d).

(iii) *Aging effect.* It was previously found that the mycelial Ara in the wild-type strain increased when the latter was allowed to stand for a period of time (15). The same trend was observed in this study, but we found that the increase in mycelial 20:3ω9 was significantly promoted on aging at temperatures lower than 28°C. As shown in Figure 5, the rate of the increase in mycelial 20:3ω9 increased with decreasing temperature. The in-

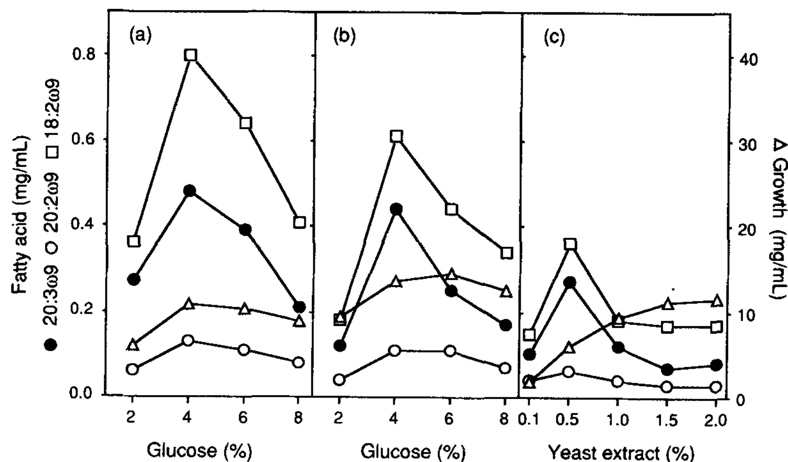

FIG. 3. Effects of the medium concentrations of glucose and yeast extract on Mead acid production. The fungus was grown at 28°C for 7 d in medium containing 0.5% yeast extract (a) or 1.0% yeast extract (b) and the indicated glucose concentrations. In (c), the glucose concentration was 2% and the yeast extract concentrations were as indicated.

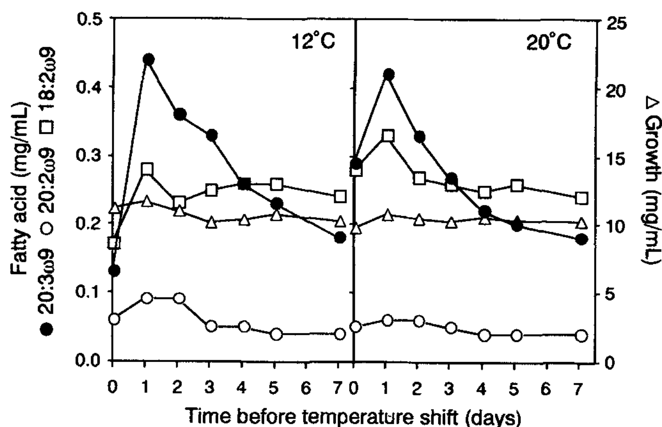

FIG. 4. Effect of a temperature shift on Mead acid production. The fungus was cultured in medium GY at 28°C for the times indicated, shifted to 12 or 20°C and then grown at the respective temperature. The total cultivation time before and after the temperature shift in all cases was seven days.

crease rates during the first 6 days on aging at 12, 20 and 28°C were calculated to be 2.6, 2.1 and 1.1 mg/g dry mycelia/day, respectively. On aging at 12°C for 6 days, mycelial 20:3ω9 increased to 230% of the level before aging, that is, from 12 to 27.7 mg/g dry mycelia.

(iv) *Oil addition.* Various oils were examined as to their effects on Mead acid production (Fig. 6). Except for shark, sardine, sesame and linseed oils, most oils, especially those of coconut, camellia and olive, enhanced the production of 20:3ω9. The addition of 2% (vol/vol culture broth) coconut oil increased the production of 20:3ω9 to about three times that without the oil supplementation.

(v) *Glucose feeding.* The fungus was grown under the conditions given in Table 1. Under condition I, glucose was added every two days to maintain the medium glucose level at 2%. Under condition II, the initial glucose concentration was 4% and no glucose feeding was performed during the cultivation. The glucose consumption rate under condition I was somewhat higher than under condition II—3.2 and 2.4 g (per 60 mL culture broth), respectively. The production of 20:3ω9, as well as its percentage based on total fatty acids, upon growth under the glucose feeding condition was the highest; the yield of Mead acid was about 1 mg per 1 mL of culture broth.

Bench scale production of Mead acid. According to the results as to factors affecting Mead acid production, the optimum conditions were determined as follows. Though addition of oils enhanced Mead acid production, it was usually accompanied by Ara contamination because of its formation from 18:2, which usually occurs in natural oils (Fig. 1). Thus, we considered cultivation under low temperature with glucose feeding as the optimum conditions for Mead acid production. Cultivation was performed as described in the legend to Figure 7. The production of Mead acid increased with cultivation time, reaching 0.83 g/L (56 mg/g dry mycelia) after 10 days [Fig. 7 (a)]. The percentage of Mead acid based on total fatty acids also increased gradually with cultivation time, at the expense of 18:1, and reached *ca.* 15% on the tenth day of cultivation [Fig. 7 (b)].

Distribution of fatty acids in major lipid classes. The fatty acid profiles of major lipid classes in the fungus grown at either 12, 20 or 28°C are given in Table 2. Irrespective of the growth temperature, about 73% (by mol) of the total lipids were triglycerides (TG), and the remainder were phospholipids which were composed of approximately 10% phosphatidylethanolamine (PE), 12% phosphatidylcholine (PC) and 5% phosphatidylserine (PS). As for Mead acid, about 70% (by mol) was found in TG and the remainder mainly in PC (*ca.* 25%). At any growth

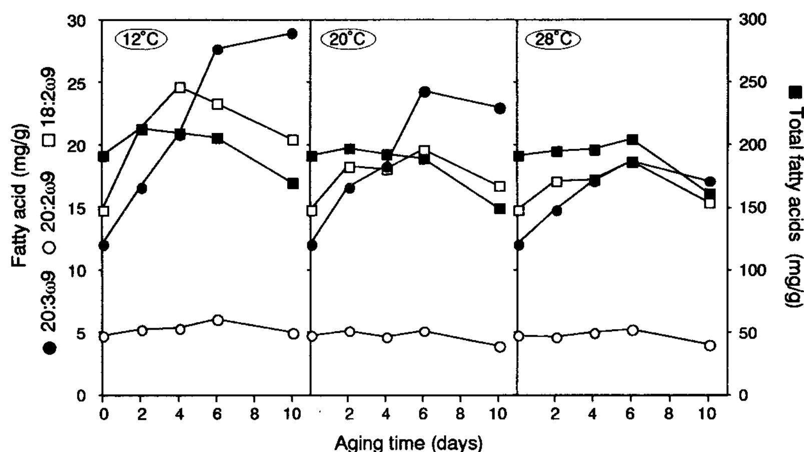

FIG. 5. Changes in the mycelial n-9 fatty acids on aging at various temperatures. Mycelium of Mut48 grown in medium GY at 28°C for 7 d was harvested, washed with distilled water and then divided into three portions of the same weight (*ca.* 10 g each). Each portion was allowed to stand at the temperature indicated in a siliconized Erlenmeyer flask, and samples were periodically removed for analysis of the fatty acid composition.

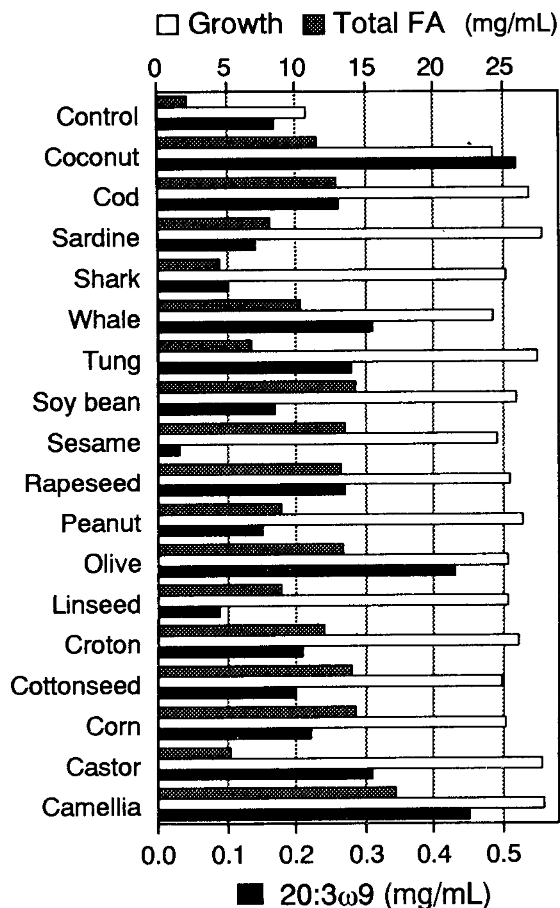

FIG. 6. Effects of various oils on Mead acid production. The fungus was cultured at 28°C for 7 d in medium GY supplemented with 2% (vol/vol culture broth) of one of the oils indicated.

temperature, the proportion of Mead acid was high in PC as compared with TG, PE or PS. For example, Mead acid accounted for 55.2% of the total fatty acids of PC for growth at 12°C, while the percentages in TG, PE and PS were 19, 14.8 and 15.2%, respectively

DISCUSSION

To our knowledge, the present paper is the first to describe the practical fermentative production of Mead acid, for which naturally available sources are presently rare. The Δ12-desaturase-defective mutant, Mut48, which was derived from an Ara-producing fungus, *M. alpina* 1S-4, is a novel and promising source of Mead acid. Under optimum culture conditions, the fungus produced *ca.* 0.8 g/L culture broth in 10 days of cultivation. This value is encouraging in that the production of Mead acid through animal experiments or chemical synthesis involves long and expensive procedures. Mead acid comprised *ca.* 15% of the mycelial total fatty acids, but it could be increased over a longer period of time. For example, we have found that Mead acid accounted for 33% on cultivation at 20°C for 2 wk (data not shown).

Studies on the distribution of fatty acids in the major lipid classes showed that the proportion of 20:3ω9 was significantly high in the PC fraction. It is known that membrane functions are greatly dependent on membrane fluidity, which varies with the compositional lipids and fatty acids composition. Only three polyunsaturated fatty acids, *i.e.*, 18:2ω9, 20:2ω9 and 20:3ω9, are present in this mutant. The occurrence of a significant amount of 20:3ω9 in the PC implies that 20:3ω9 and PC are important for the maintenance of membrane fluidity and, consequently, membrane functions.

The significance of Mead acid was previously reviewed by Lundberg (16), but the real biological activity of this fatty acid remains unclear. With a few exceptions (4,16,17), the biological activity of Mead acid has not been as widely studied as those of analogous fatty acids such as Ara, which could be due to the limited availability of sources, as well as its high commercial price. Like Ara, 20:3ω9 is converted *via* the 5-lipoxygenase pathway into 3-series leukotrienes, such as leukotriene B_3, which are expected

TABLE 1

Effect of Glucose Feeding on Mead Acid Production

	Total			Fatty acid composition (wt%)									
Condition[a]	Growth mg/mL	FA[b] mg/mL	20:3ω9 mg/mL	16:0	18:0	18:1	18:2ω9	20:0	20:1	20:2ω9	20:3ω9	22:0	24:0
I	21.5	7.45	0.97	9.8	6.8	48.5	11.8	0.7	2.7	3.0	13.2	1.5	2.0
II	16.2	4.20	0.42	11.6	6.2	52.1	10.9	0.6	2.7	2.5	10.2	1.2	2.0

[a]The fungus was cultured in a 300-mL Erlenmeyer flask containing 60 mL of medium GY (2% glucose plus 1% yeast extract, pH 6.0) (I) or the medium containing 4% glucose plus 1% yeast extract (pH 6.0) (II) at 28°C for one day, and then the cultivation temperature was decreased to 20°C. The fungus was grown at this temperature for nine days; for condition I, glucose was added as an 80% solution every two days to maintain the medium glucose level at 2%.
[b]FA, fatty acids.

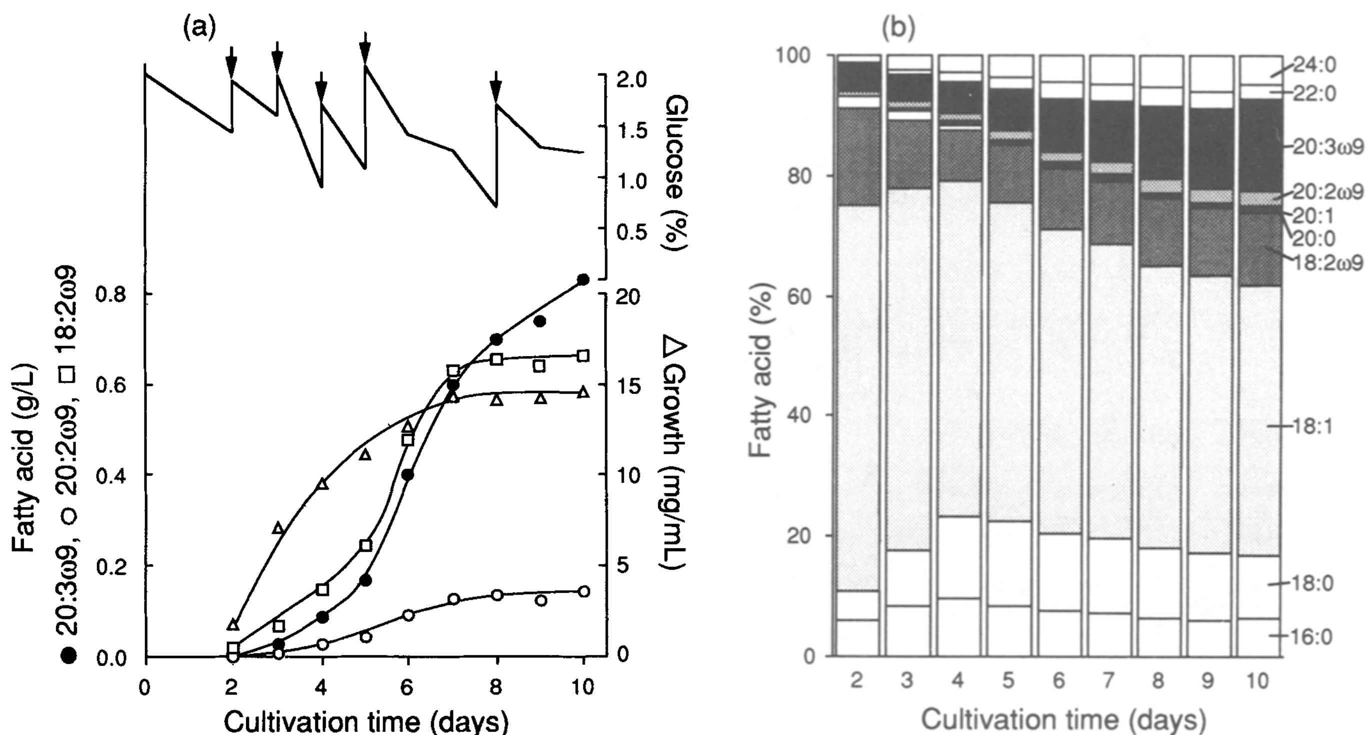

FIG. 7. Production of Mead acid under submerged culture conditions. The fungus was pre-cultured at 28°C for 3 d in 40 mL of medium GY and then the culture was inoculated into 2 L of medium GY containing 0.01% (vol/vol) Adekanol (Asahi Denka Industries, Tokyo, Japan), in a 5-L jar fermenter. Cultivation was performed at 20°C with aeration at 1 vol/vol/min and agitation at 300 rpm. Glucose was added at the times indicated by arrows (a). The changes in the fatty acid composition during growth are shown in (b).

to exhibit several unique biological activities. Recently, a proper balance of n-3/n-6 polyunsaturated fatty acids in the diet has been considered necessary for human health. However, no attention has been paid to the n-9 fatty acids, possibly because of their low amounts in most cells as compared with those of n-3 or n-6 fatty acids. It should be realized that in some tissues, such as cartilage, they occur in relatively high concentrations (7). In recent reports it was demonstrated that EFA-deficiency suppresses normal inflammation reactions (18) and that tissues from animals deficient in EFA are not rejected by normal heterologous recipients on organ transplantation (19), but it was unclear whether 20:3ω9 itself and/or the simultaneous decrease in Ara are responsible for these phenomena. Determination of the significance of Mead acid must await future nutritional studies on the effects of dietary n-9 fatty acid-containing oils, and the oil reported here, which is produced by the Δ12-desaturase-defective mutant of *M. alpina* 1S-4, would be useful for such studies.

TABLE 2

Fatty Acid Profiles of Major Lipid Classes in *M. alpina* 1S-4 Mut48 Grown at Various Temperatures[a]

Lipids	Lipid comp. mol%	Fatty acid composition (mol%)									
		16:0	18:0	18:1	18:2ω9	20:0	20:1	20:2ω9	20:3ω9	22:0	24:0
28°C											
TG[b]	76	15.3	6.6	50.6	8.8	0.5	2.2	2.1	7.3	1.9	4.7
PE[b]	9	8.4	3.4	61.3	15.9	*c*	3.2	0.7	7.1	—	—
PC[b]	11	8.0	2.3	40.2	16.4	—	1.6	1.2	30.3	—	—
PS[b]	4	19.4	4.7	61.7	7.9	—	0.6	0.2	5.4	—	—
20°C											
TG	72	8.6	6.7	52.2	11.3	0.6	3.1	3.3	10.7	1.4	2.2
PE	11	5.2	2.9	52.5	25.0	0.2	3.5	0.8	9.9	—	—
PC	13	3.5	1.4	29.8	27.2	0.3	1.4	1.4	35.0	—	—
PS	5	14.9	3.5	58.4	12.7	—	0.8	0.5	9.3	—	—
12°C											
TG	73	4.7	5.4	50.1	9.2	0.4	4.4	3.7	19.0	1.3	1.8
PE	10	3.8	1.9	49.0	24.2	0.2	5.2	1.0	14.8	—	—
PC	12	2.8	0.8	20.6	18.5	0.1	1.5	0.4	55.2	—	—
PS	5	12.3	2.2	56.6	11.6	—	1.2	0.9	15.2	—	—

[a]The fungus was grown at either 28 or 20°C for 7 d, or it was grown at 28°C for 2 d and then at 12°C for a further 8 d (12°C).
[b]TG, triglycerides; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine.
[c]Undetectable.

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