Enhancement of Arachidonic Acid Production by *Mortierella alpina* 1S-4

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ABSTRACT: The effect of mineral addition on arachidonic acid (AA) production by *Mortierella alpina* 1S-4 was evaluated. At first, the addition of minerals such as sodium, potassium, calcium, and magnesium was examined in flask cultures, and then the addition of phosphorus with the optimal amounts of the minerals was investigated in a 10-L jar-fermenter. As a result, 1.5% soy flour medium with the addition of 0.3% KH_2PO_4, 0.1% Na_2SO_4, 0.05% $CaCl_2 \cdot 2H_2O$, and 0.05% $MgCl_2 \cdot 6H_2O$ was found to enhance the AA yield 1.7-fold over that without mineral addition. When 1% yeast extract with the above mineral mixture was used, the AA yield was enhanced 1.35-fold over that without minerals. We also verified that an increase in the polar lipid content occurred in the case of only KH_2PO_4 addition, and that the above-mentioned increase in the AA yield was due to the minerals themselves, not a pH buffer effect. *JAOCS 75,* 1501–1505 (1998).

KEY WORDS: Arachidonic acid, arachidonic acid yield, lipid composition, mineral addition, morphology, *Mortierella alpina.*

Arachidonic acid (5,8,11,14-*cis*-eicosatetraenoic acid, AA) acts as a precursor for prostaglandins and leukotrienes, which have various regulatory effects and physiological activities. It is the most abundant C_{20} polyunsaturated fatty acid (PUFA) in humans, and plays important roles in infant nutrition (1,2). There are various sources of AA, including fungi (3), animal liver, tuna (4), and egg yolk. Fungal oil seems to be the most advantageous among these sources in the aspects of production cost and product handling, because of its high AA content and the structure of its triacylglycerol (TG).

In the previous paper (3), we reported the isolation of *Mortierella alpina* 1S-4 as a potent producer of AA and the fundamental culture conditions. In order to obtain a higher production yield, further optimization of the culture conditions is essential. Phosphorus, potassium, sulfur, calcium, sodium, and magnesium are major inorganic constituents of fungi (5); these minerals should be supplied sufficiently, and optimization of the additional concentration is important. Therefore, we attempted to enhance the AA productivity by optimization of the amounts of these minerals.

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MATERIALS AND METHODS

Microorganism and culture conditions. Mortierella alpina 1S-4 (3) was used throughout this study. A stock culture was stored on a Czapek medium (0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.001% $FeSO_4 \cdot 7H_2O$, 3% sucrose, and 2% agar) slant in a test tube at 5°C.

For the flask culture study, 2 mL medium containing 4% glucose and 1.5% soy flour, with or without the addition of minerals, pH 6.3, was prepared in a 10-mL Erlenmeyer flask, and then 0.1 mL of a spore suspension obtained from the stock culture was inoculated, followed by incubation for 7 d at 28°C at a reciprocal speed of 150 rpm.

For the jar-fermenter study, the inoculum was prepared in a 500-mL flask containing 100 mL medium including 1.8% glucose and 1% yeast extract, with shaking for 3 d at 28°C, and the main culture was carried out in a 10-L jar-fermenter (Able Corp., Tokyo, Japan) with a working volume of 5 L medium at 28°C, an inoculation rate of 2%, an agitation speed of 300 rpm, and an aeration rate of 5 L/min. The nitrogen and mineral sources in the culture medium are given in the legend to each figure. Glucose (1.8%) and soybean oil (0.2%) were used as the initial ingredients, and the pH was adjusted before inoculation and monitored during the culture by means of a pH electrode (DKK Corp., Tokyo, Japan). All jar-fermentations involved the fed-batch system: 0–0.9% glucose was fed at 1-d intervals to maintain the glucose concentration at 1–2%.

Analytical methods. Cell growth was evaluated as the dry cell weight (DCW) after suction filtration, washing with distilled water, and drying at 105°C for 2 h. Fatty acids in the total mycelial lipid were analyzed as follows: 20 mg dried cells was *trans*-methylated in methanolic HCl, and then fatty acid methyl esters were extracted and quantified by gas–liquid chromatography as described (6). The lipid composition of the mycelial oil extracted with *n*-hexane was examined with a thin-layer chromatography/flame-ionization detector (TLC/FID) analyzer (Iatroscan MK-5; Iatron, Tokyo, Japan) as previously reported (7). The values in the figures and ta-

bles are the means of at least three treatment replications, and data given in this paper are representative.

RESULTS

Optimization of mineral additions. The minerals listed in Figure 1 were examined as additive sources for AA production. When 0.05% Na_2SO_4, 0.025% $CaCl_2 \cdot 2H_2O$, and 0.025% $MgCl_2 \cdot 6H_2O$ were added, the AA yield was enhanced 1.3-, 1.6-, and 1.3-fold, respectively, over that without mineral addition. However, the addition of NaCl, KCl, or K_2SO_4 did not enhance the AA production. The addition of excess NaCl revealed the possibility of suppression of AA production. As far as DCW is concerned, there was little effect on it under all conditions examined (data not shown).

Based on the above results, the following mineral mixture was selected for the next experiment: 0.05% Na_2SO_4, 0.025% $CaCl_2 \cdot 2H_2O$, and 0.025% $MgCl_2 \cdot 6H_2O$. In addition to those of sodium, calcium, and magnesium, the effect of phosphorus was investigated in a 10-L jar-fermenter, as shown in Figure 2. The addition of only the above mixture and the same mixture with addition of 0.1% KH_2PO_4 did not enhance the AA production or cell growth. However, when the above mixture with 0.3% KH_2PO_4 was used, the AA yield and DCW after 8 d were enhanced 1.5- and 1.4-fold, respectively, over those obtained with the basal medium without minerals. Thus, the mixture of Na_2SO_4, $MgCl_2$, and $CaCl_2$ with 0.3% KH_2PO_4 was found to be essential for the increases in AA productivity and cell growth.

Furthermore, the effects of enrichment with Na_2SO_4, $MgCl_2$, and $CaCl_2$ were investigated with the medium containing 0.3% KH_2PO_4, as shown in Figure 3. As a result, the AA production and cell growth were found to be slightly enhanced or not affected. As observed on the culture broth, the liquid circulation rate was higher in the case of twofold mineral enrichment. The culture broth revealed the mixture of two types of morphology, filaments and small pellets (<2 mm in diameter), and the percentage of small pellets was higher in the enriched-mineral medium. The oxygen supply derived from the liquid circulation on agitation and aeration seemed to be sufficient under both conditions shown in Figure 3, although it was predicted that the effect of the liquid mixing conditions would be significant with an increase in the biomass concentration, especially for a large-scale production. Therefore, we selected the following mineral mixture composition as the optimal one for AA production: 0.3% KH_2PO_4, 0.1% Na_2SO_4, 0.05% $CaCl_2 \cdot 2H_2O$, and 0.05% $MgCl_2 \cdot 6H_2O$.

The effect of the nitrogen source in the basal medium was investigated, as shown in Figure 4. When the mineral mixture was not added, the AA yield and DCW were a little higher in the case of yeast extract than that of soy flour. On the contrary, when the soy flour medium with the mineral mixture was used, the AA yield and DCW became higher than those of yeast extract with the mineral mixture. The AA yields after 8 d with the media containing soy flour and yeast extract with the mineral mixture were enhanced 1.7- and 1.4-fold, respectively, over those without mineral addition. The AA content

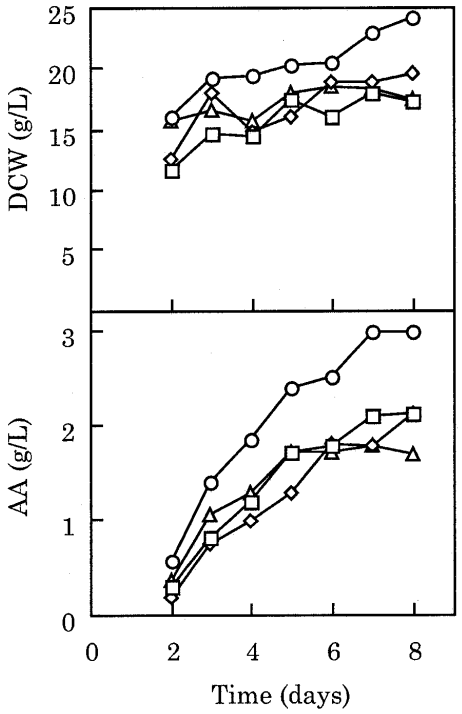

FIG. 2. Time course of AA production by *M. alpina* 1S-4 cultivated in a 10-L jar-fermenter for 8 d. Medium composed of 1.5% soy flour, 1.8% glucose, and 0.2% soybean oil, pH 6.0, with the addition of each of the indicated minerals, was used. Symbols: □ (KH_2PO_4, Na_2SO_4, $CaCl_2 \cdot 2H_2O$, $MgCl_2 \cdot 6H_2O$; 0, 0, 0, 0%); ◇ (0, 0.05, 0.025, 0.025%); △ (0.1, 0.05, 0.025, 0.025%); ○ (0.3, 0.05, 0.025, 0.025%). DCW, dry cell weight; AA, arachidonic acid. See Figure 1 for other abbreviation.

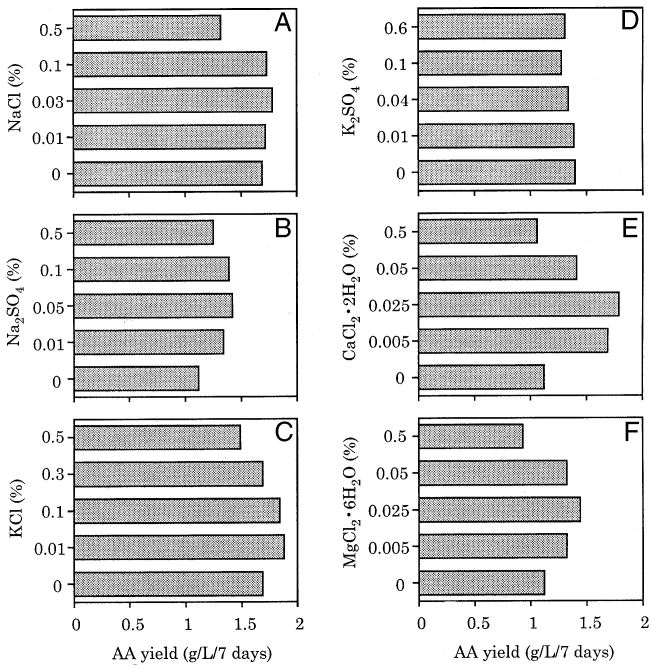

FIG. 1. Effects of mineral addition, (A) NaCl, (B) Na_2SO_4, (C) KCl, (D) K_2SO_4, (E) $CaCl_2 \cdot 2H_2O$, and (F) $MgCl_2 \cdot 6H_2O$, on arachidonic acid (AA) production by *Mortierella alpina* 1S-4 in flask cultures.

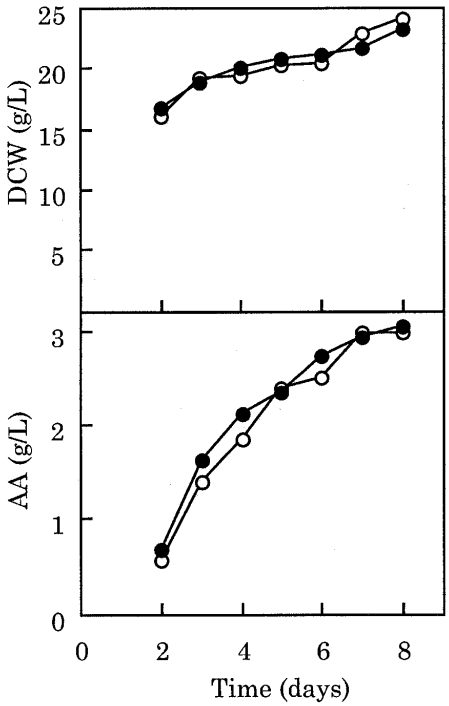

FIG. 3. Time course of AA production by *M. alpina* 1S-4 cultivated in a 10-L jar-fermenter for 8 d. Medium composed of 1.5% soy flour, 1.8% glucose, and 0.2% soybean oil, pH 6.0, with the addition of each of the indicated minerals, was used. Symbols: ● (KH_2PO_4, Na_2SO_4, $CaCl_2·2H_2O$, $MgCl_2·6H_2O$; 0.3, 0.1, 0.05, 0.05%); ○ (0.3, 0.05, 0.025, 0.025%). See Figures 1 and 2 for abbreviations.

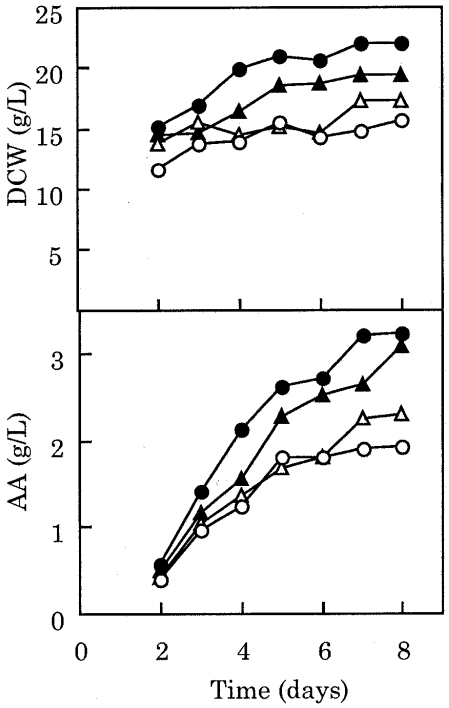

FIG. 4. Time course of AA production by *M. alpina* 1S-4 cultivated in a 10-L jar-fermenter for 8 d. Medium composed of 1.5% soy flour (circles) or 1% yeast extract (triangles), 1.8% glucose, and 0.2% soybean oil, pH 6.0, with the addition of each of the indicated minerals, was used. Symbols: ○ and △ (KH_2PO_4, Na_2SO_4, $CaCl_2·2H_2O$, $MgCl_2·6H_2O$; 0, 0, 0, 0%); ● and ▲ (0.3, 0.1, 0.05, 0.05%). See Figures 1 and 2 for abbreviations.

in the total fatty acid (tFA) was also enhanced only in the medium containing soy flour with mineral addition, as shown in Table 1, and the AA and tFA contents of mycelia also increased, from 12.4 (AA in DCW) and 36.8% (tFA in DCW) to 14.8 and 40.8%, respectively.

TABLE 1
Comparison of Fatty Acid Compositions[a]

Medium (%)				
KH_2PO_4	0	0.3	0	0.3
Na_2SO_4	0	0.1	0	0.1
$CaCl_2·2H_2O$	0	0.05	0	0.05
$MgCl_2·6H_2O$	0	0.05	0	0.05
Soy flour	1.5	1.5	0	0
Yeast extract	0	0	1.0	1.0
Fatty acid composition (%)[b]				
14:0	0.1	0.2	0.3	0.3
16:0	13.7	13.4	12.2	12.3
18:0	4.4	6.8	5.2	5.4
18:1	14.3	11.3	12.8	12.4
18:2	15.6	13.3	15.9	15.9
18:3	4.1	3.6	3.9	3.7
20:2	0.5	0.6	0.6	0.6
20:3	3.1	3.2	3.3	3.3
20:4	33.6	36.2	34.7	34.8
24:0	6.4	7.2	6.4	6.9
Others	4.2	4.2	4.7	4.4

[a]*Mortierella alpina* 1S-4 cultivated in a 10-L jar-fermenter for 8 d. Medium composed of 1.5% soy flour or 1% yeast extract, 1.8% glucose, and 0.2% soybean oil, pH 6.0, with the addition of minerals listed in table, was used.
[b]14:0, myristic acid; 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, γ-linolenic acid; 20:2, eicosadienoic acid; 20:3, dihomo-γ-linolenic acid; 20:4, arachidonic acid; 24:0, lignoceric acid.

Effect of mineral addition on the lipid composition. The effect of mineral addition on the lipid composition was also investigated, as shown in Table 2. Under the conditions of the basal medium without mineral addition, and with the mineral mixture of Na_2SO_4, $CaCl_2$, $MgCl_2$, and KH_2PO_4, over 98% of the extracted mycelial oil was composed of neutral lipid. However, when only KH_2PO_4 was added, the polar lipid content increased to 9.5% and the FA yield was the same as that of soy flour medium without minerals. The major components of neutral lipid and polar lipid were identified as TG and phospholipid (PL), respectively, in another experiment.

TABLE 2
Effects of Mineral Addition on the Lipid Composition of *Mortierella alpina* 1S-4 Cultivated in a 10-L Jar-Fermenter for 8 Days[a]

Medium (%)				
KH_2PO_4	0	0	0.2	0.3
Na_2SO_4	0	0	0	0.1
$CaCl_2·2H_2O$	0	0	0	0.05
$MgCl_2·6H_2O$	0	0	0	0.05
Soy flour	0	1.5	1.5	1.5
Yeast extract	1.0	0	0	0
Lipid composition (%)				
Neutral lipid	98.8	98.8	90.5	98.6
Polar lipid	1.2	1.2	9.5	1.4

[a]See Table 1 for culture conditions.

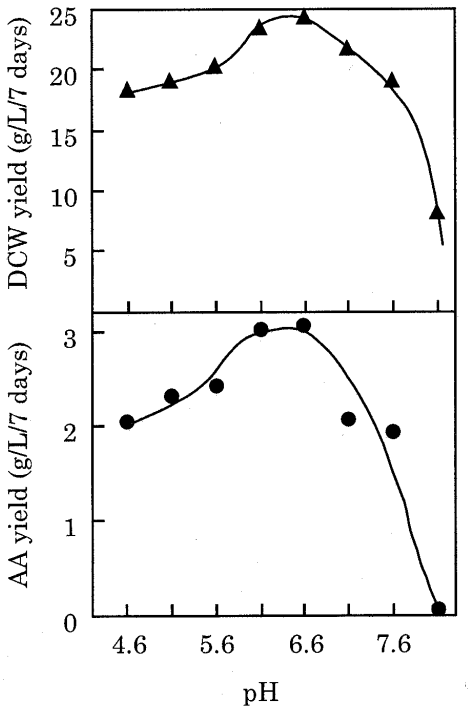

FIG. 5. Effects of pH on the AA yield and DCW of *M. alpina* 1S-4 cultivated in a 10-L jar-fermenter for 7 d. Medium composed of 1.5% soy flour, 1.8% glucose, 0.2% soybean oil, 0.3% KH_2PO_4, 0.1% Na_2SO_4, 0.05% $CaCl_2{\cdot}2H_2O$ and 0.05% $MgCl_2{\cdot}6H_2O$ was used. The pH was maintained constant at values (within ±0.15) of 4.6, 5.1, 5.6, 6.1, 6.6, 7.1, 7.6, and 8.1 throughout the cultivation by titration with NaOH and H_2SO_4. See Figures 1 and 2 for abbreviations.

Effect of pH on AA production. Minerals also have a buffer effect on pH. When the medium containing soy flour without the mineral mixture was used in the experiment in Figure 4, the pH decreased from 6.0 to 5.8 during the first 2 d and then gradually increased to 6.2. On the other hand, in the case of soy flour with mineral addition, the pH changed little during the first 2 d and then gradually increased to 6.5. In order to determine the effect of pH, AA production was investigated under a constant pH of 4.6, 5.1, 5.6, 6.1, 6.6, 7.1, 7.6, or 8.1 throughout the cultivation, as shown in Figure 5. As a result, the AA yield and DCW were found to be maximum at pH 6.1–6.6. If we compare this maximum yield to that at pH 5.6, the AA yield in 7 d was enhanced 1.26-fold, which was lower than the degree of enhancement with mineral addition. In the pH range of 5.1–6.6, the amounts of added NaOH and H_2SO_4 for initial adjustment and titration control were below 1 mM, which seemed to be negligible in comparison with the 0.1% (= 7 mM) Na_2SO_4 in the mineral mixture. At pH 4.6, the concentration of added NaOH and H_2SO_4 were each 2 mM. In the pH range of 7.1–8.1, added NaOH and H_2SO_4 amounted to about 10 mM and below 1 mM, respectively.

DISCUSSION

The addition of a mineral mixture comprising 0.1% Na_2SO_4, 0.05% $CaCl_2{\cdot}2H_2O$, 0.05% $MgCl_2{\cdot}6H_2O$, and 0.3% KH_2PO_4 enhanced the AA yield without a change in the lipid composition. It became clear that this effect was due to the minerals themselves, not a pH buffer effect. The enhancement degree of 1.5–1.7-fold was considered to be significant and would contribute to the reduction of manufacturing cost, because the mineral component constituted only 10% of the total cost of the medium with soy flour and glucose. To our knowledge, a mineral mixture with such a composition has not been reported prior to this study. Berkley (8) studied a medium with the addition of 0.01% $MgSO_4$ and mentioned that low magnesium was desirable for AA production. The addition of phosphorus for AA production has been studied by some groups (9–11); however, in all cases, sodium, magnesium, and calcium were not added. The addition of only 0.3% KH_2PO_4 did not enhance the AA production in another experiment involving a jar-fermenter (data not shown). Sajbidor *et al.* (12) used Czapek-mineral, in which calcium is not included. Hansson *et al.* (13) added phosphorus, sodium, calcium, and magnesium for γ-linolenic acid production, but the concentration of the added calcium was much lower than that in our medium.

The medium containing soy flour with the mineral mixture was better for AA production than that containing yeast extract. However, the contents of the above minerals in the basal nitrogen source were higher for soy flour than for yeast extract. Therefore, it was likely that the effect of mineral addition was due to the synergistic effect of the soy flour and minerals, not the replenishment of medium ingredients. But the mechanism underlying such an effect remains unclear.

As a result of the pH control experiment, the optimal pH range for AA production and cell growth was proved to be 6.1–6.6. The effect of pH on AA production was reported previously. Kyle (9) and Lindberg and Mollin (10) mentioned that AA productivity and cell growth were better under higher pH conditions and lower ones, respectively. Bajpai *et al.* (14) found the optimal initial pH range for AA production and cell growth was 6.0–6.7. However, there have been few studies with a constant pH throughout cultivation prior to this study. When the initial pH of the basal medium with the mineral mixture was 6.0, the pH was maintained in the range of 6.0–6.5 throughout the cultivation without control by titration with acid and/or alkali.

In addition to AA productivity, we verified that the addition of phosphorus affected the lipid composition. When only KH_2PO_4 was added, the PL content increased, but the tFA yield did not change. It has been known that PL is a precursor of TG, and, thus, PL and/or phosphate is considered to be the control factor of TG synthesis. Our result indicates a possibility that the TG synthesis from PL might be reduced. The mechanism underlying this change in lipid composition remains unclear, for one reason: fungal metabolism of PUFA production has not been fully explained (15). Therefore, it is predicted that further work of metabolic analysis will contribute to the FA production. As a practical conclusion, when TG is required as the end product, the addition of balanced minerals, i.e., phosphorus, sodium, calcium, and magnesium,

was found to be necessary so as not to cause a decrease in the product yield.

In terms of growth morphology, only KH_2PO_4 addition caused filamentous growth and increased viscosity. The addition of Na_2SO_4, $CaCl_2$, and $MgCl_2$ caused pellet growth and decreased viscosity. With addition of KH_2PO_4, Na_2SO_4, $CaCl_2$, and $MgCl_2$, the liquid circulation rate became higher in the case of enrichment with Na_2SO_4, $CaCl_2$, and $MgCl_2$. As observed on the mycelial growth, the growth rate was a little higher in the case of phosphate addition. This phenomenon agrees with the previous report (16) which mentioned that growth rate was higher in the case of filamentous growth. Further investigation of this phenomenon regarding morphology seems to be important to achieve a higher biomass concentration and scale-up.

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