Effects of Mineral Addition on the Growth Morphology of and Arachidonic Acid Production by *Mortierella alpina* 1S-4

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ABSTRACT: The culture conditions for arachidonic acid (AA) production by *Mortierella alpina* 1S-4 were investigated by means of a morphological study with the aims of obtaining a high AA yield and scale-up. In a 50-L jar fermentor study, a medium containing 3.1% soy flour and 1.8% glucose with 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 be optimum. The AA yield reached 9.8 g/L/7 d, and the major morphology was small pellets (1–2 mm). However, in the case of the only addition of KH_2PO_4, the major morphology was filaments. The apparent viscosity increased to 2240 cp, thereby requiring a high agitation speed to maintain adequate oxygen tension, which caused mycelial damage due to shear stress and therefore a decrease in the AA yield. When a medium with Na_2SO_4, $CaCl_2$, and $MgCl_2$ was used, the major morphology was large pellets (2–3 mm), and mass transfer limitation through the pellet wall caused a decrease in the AA yield. Based on these results, a scale-up study was carried out under the optimal conditions described above. An AA yield of 10.9 g/L/8 d was obtained in a 10-kL industrial fermentor, and the major morphology was small pellets.
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KEY WORDS: Arachidonic acid, arachidonic acid yield, mineral addition, morphology, *Mortierella alpina*, scale-up, viscosity.

Arachidonic acid (5,8,11,14-*cis*-eicosapentaenoic acid, AA) has various regulatory effects and physiological functions in humans, and plays important roles in infant nutrition (1,2). The importance of AA has become more obvious recently, and there have been some reports on AA production by *Mortierella* fungi (3–7) and safety evaluation of these fungal products (8,9). In the previous study (10), we reported the effectiveness of mineral addition to the basal medium; it was found to enhance the AA yield and to change the growth morphology.

In general, sufficient mass transfer and oxygen supply are required to obtain high productivity in an aerobic fermentation. In the case of a fungal fermentation, the medium composition not only influences the productivity, but, as a side effect, may also induce a change in growth morphology (11). The growth morphology has a strong effect on the physical properties of the fermentation broth, which causes numerous problems in large fermentors with respect to gas dispersion, mass and heat transfer, and homogenization. Thus the fungal morphology is often considered to be one of the key parameters in industrial fermentation. For the production of fungal metabolic products, the desired morphology varies from one product to another (12). Therefore, it is important to find the optimal morphology for production and to study the influence of minerals on the morphology.

In spite of this obvious need, there have been few reports of morphological studies on AA production by *Mortierella* fungi. For commercial production, a high biomass concentration is essential because it is an intracellular product. Besides, AA production requires adequate oxygen, because AA is formed through enzymatic desaturation which comprises oxygenation (13). Thus an adequate oxygen supply by means of agitation, aeration, and morphological control is a key factor in obtaining a higher AA content in the cells. Therefore, we examined the effects of mineral addition on the morphology, AA productivity and cell growth, and then attempted to achieve scale-up to an industrial fermentor by controlling the optimal morphology.

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 slant of 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 jar fermentor study, an 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 then the main culture was carried out in a 50-L jar fermentor (Mitsuwa Bio Systems Co., Ltd., Osaka, Japan) with a working volume of 25 L under such conditions as 26°C, an inoculation rate of 0.5%, an aeration rate of 25 L/min, and a headspace pressure of 200 kPa. The agitation speed was

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changed during the culture to maintain the dissolved oxygen (DO) concentration at 10–15 ppm. The DO concentration was monitored with a DO electrode (Ingold, Switzerland). A basal medium (pH 6.0) containing 3.1% soy flour, 1.8% glucose, and 0.1% soybean oil, was used. Mineral sources added to the medium are indicated in the figure legends in this paper. All jar fermentations were performed with the fed-batch system: 0–3.2% glucose was fed at 1-d intervals to maintain the glucose concentration at 1–2.5%.

For the industrial fermentor study, an inoculum was prepared in a 50-L jar fermentor containing a 30-L medium including 1% yeast extract, 1.8% glucose, and 0.1% soybean oil, followed by cultivation for 2 d at 28°C, aeration rate of 30 L/min, agitation speed of 200 rpm, and headspace pressure of 150 kPa. The main culture was carried out in a 10-kL fermentor (Kansai Chemical Engineering Co., Hyogo, Japan), with a working volume of 6 kL; in a medium containing 3.1% soy flour, 1.8% glucose, 0.1% soybean oil, 0.3% KH_2PO_4, 0.1% Na_2SO_4, 0.05% $CaCl_2 \cdot 2H_2O$ and 0.05% $MgCl_2 \cdot 6H_2O$, pH 6.0; under such conditions as 26°C, inoculation rate of 0.5%, aeration rate of 360 m^3/h, and headspace pressure of 200 kPa. Changes in the agitation speed for DO control and glucose feeding were performed as described.

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 lipids were analyzed as follows: 20 mg dried cells was *trans*-methylated in methanolic HCl, and then the fatty acid methyl esters were extracted and quantified by gas–liquid chromatography as described (14).

For morphological evaluation, the whole culture broth was passed through sieves of 1 mm, 2 mm, and 4 mm aperture, and then the DCW and fatty acid content of each cell fraction were determined as explained above. The apparent viscosity of the whole culture broth was measured with a rotary viscometer (Model DV-II; Brookfield Engineering Lab. Inc., MA).

The mycelial damage due to mechanical stress was evaluated as the leakage of intracellular nucleotides as follows: filtrated medium was diluted with distilled water, and then the absorbance at 260 nm (A260) was measured with a spectrophotometer (Model UV-160; Shimadzu, Kyoto, Japan) as reported previously (15).

RESULTS

50-L Jar fermentor study. In the previous study involving a 10-L jar fermentor (10), the addition of KH_2PO_4, Na_2SO_4, $CaCl_2$, and $MgCl_2$ to the basal medium containing 1.5% soy flour was found to enhance AA productivity. However, the addition of only KH_2PO_4 did not enhance productivity, and caused filamentous growth. The addition of Na_2SO_4, $CaCl_2$, and $MgCl_2$ did not enhance it either, and caused pellet growth. In order to obtain a higher AA yield, a higher biomass concentration is needed, and for this purpose, the nitrogen source has to be increased. Therefore, the effect of mineral addition, as shown in the legend to Figure 1, was examined under the conditions of an enriched nitrogen source of 3.1% soy flour in a 50-L jar fermentor.

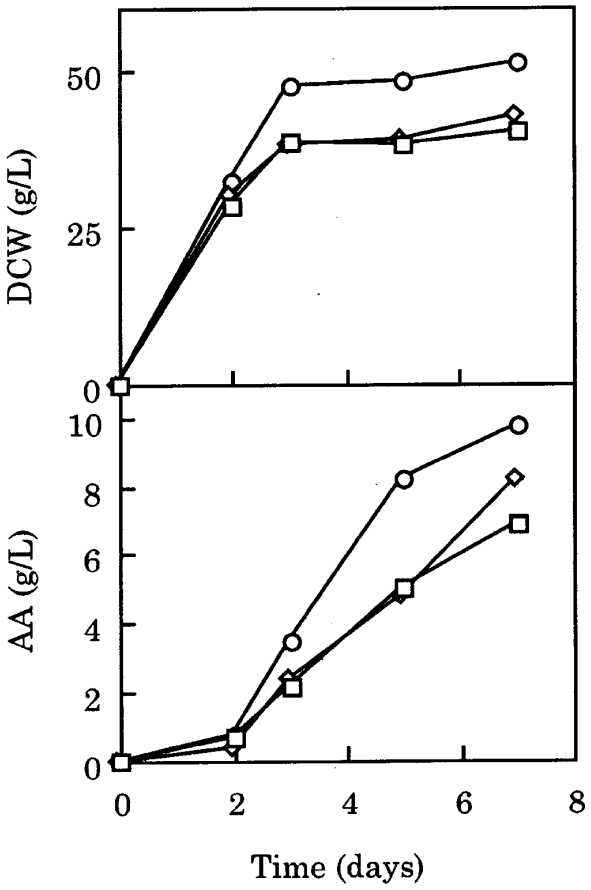

FIG. 1. Time course of arachidonic acid (AA) production by *Mortierella alpina* 1S-4 cultivated in a 50-L jar fermentor for 7 d. Condition A, □ (KH_2PO_4, Na_2SO_4, $CaCl_2 \cdot 2H_2O$, $MgCl_2 \cdot 6H_2O$; 0.3, 0, 0, 0%). Condition B, ◇ (0, 0.1, 0.05, 0.05%). Condition C, ○ (0.3, 0.1, 0.05, 0.05%). DCW, dry cell weight.

After 7 d under the condition of the addition of KH_2PO_4, Na_2SO_4, $CaCl_2$, and $MgCl_2$, the AA yield and dry cell weight (DCW) reached the highest values, 9.8 g/L and 51.1 g/L, respectively, among the conditions shown in Figure 1. The major morphology was small pellets (1–2 mm in diameter); the agitation speed was controlled within 100–400 rpm to maintain DO; the apparent viscosity reached 550 cp; and the amount of leaked nucleotides per cell (A260/DCW) was 0.323, as shown in Table 1.

However, when KH_2PO_4 was omitted from the mineral mixture, the AA yield and DCW were lower. The major morphology was large pellets (2–4 mm); agitation speed was controlled within 100–300 rpm; and apparent viscosity and A260/DCW were the lowest among the three conditions.

When only KH_2PO_4 was added, the AA yield and DCW were the lowest among the three conditions. The major morphology was filaments (0–1 mm); agitation speed was controlled within 100–500 rpm; and apparent viscosity and A260/DCW were the highest among the three conditions.

TABLE 1
Effects of Mineral Addition on Morphology, Apparent Viscosity, and Nucleotide Leakage.[a]

Conditions[b]	DCW of each fraction in whole broth (%)			Apparent viscosity (cp)	A260/DCW ([abs.]/[g/L])
	0–1 mm	1–2 mm	2–4 mm		
A	60.8	39.2	0	2240	0.415
B	12.4	40.8	46.8	30	0.280
C	47.3	52.7	0	550	0.323

[a]Culture broth on the 7th d of a 50-L jar fermentation was used for analysis.
[b]Condition A, (KH_2PO_4, Na_2SO_4, $CaCl_2 \cdot 2H_2O$, $MgCl_2 \cdot 6H_2O$; 0.3, 0, 0%). Condition B, (0, 0.1, 0.05, 0.05%). Condition C, (0.3, 0.1, 0.05, 0.05%). DCW, dry cell weight.

In an attempt to determine the relationship between morphology and AA productivity, the AA content per DCW of each cell fraction separated through sieves was determined. The AA content of the 1–2 mm fraction was found to be the highest under all three conditions, as shown in Figure 2.

10-kL Industrial fermentor study. Based on the results of the 50-L fermentor study, scale-up to a 10-kL fermentor was carried out. A medium composed of a basal medium with KH_2PO_4, Na_2SO_4, $CaCl_2$ and $MgCl_4$ was used, and the time course shown in Figure 3 was obtained. The AA, total fatty acid (TFA), and DCW values after 8 d were 10.9 g/L, 24.2 g/L, and 50.6 g/L, respectively, and the AA content of TFA increased to 45.0% after 8 d of cultivation, as shown in Table 2. The major morphology was small pellets (1–2 mm in diameter); apparent viscosity of the whole broth on the 8th d was 115 cp; and agitation speed was controlled within 27–94 rpm to maintain the DO concentration during the culture.

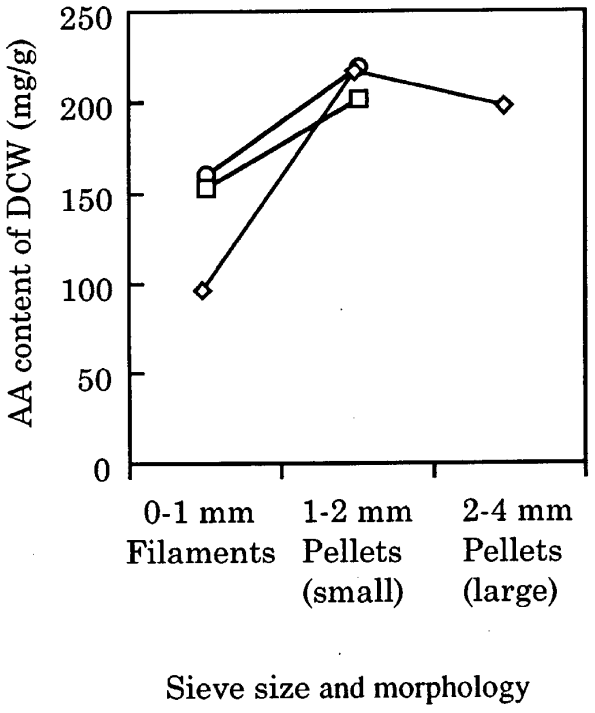

FIG. 2. Relationship between the AA content and morphology. Culture broth on the 7th d of a 50-L jar-fermentation study was used for analysis. The symbols and abbreviations are the same as in Figure 1.

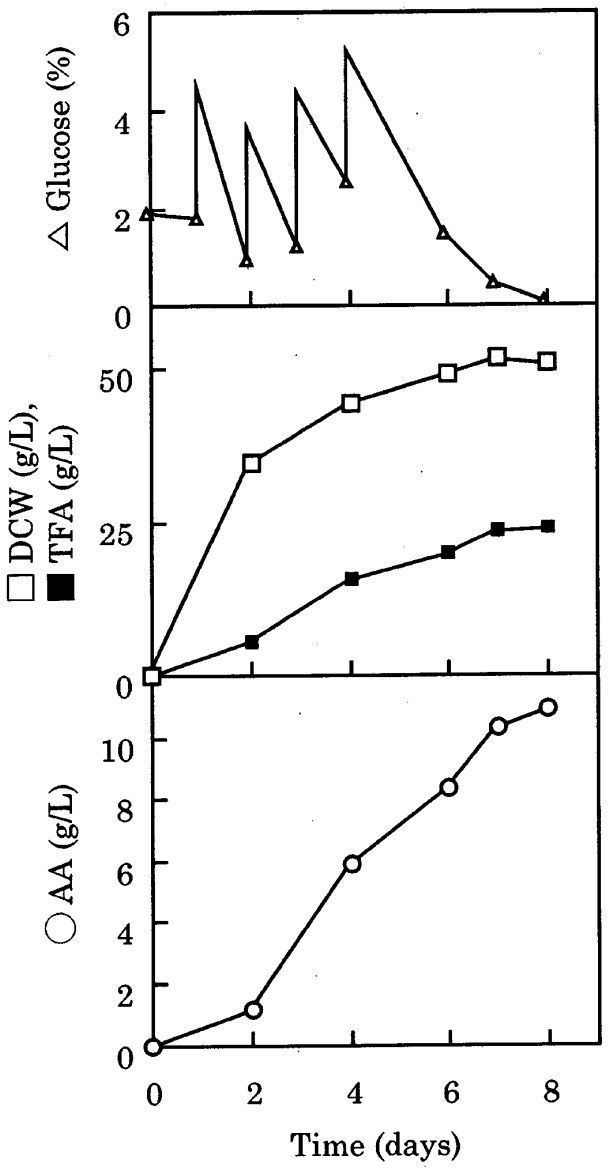

FIG. 3. Time course of AA production by *M. alpina* 1S-4 cultivated in a 10-kL industrial fermentor for 8 d. AA (○), DCW (□), total fatty acid (■), and glucose (△) are shown. See Figure 1 for abbreviations.

DISCUSSION

The effects of minerals such as KH_2PO_4, Na_2SO_4, $CaCl_2$, and $MgCl_2$ on the growth morphology and AA production became clear in this study. The culture broth revealed various morphologies, and AA productivity differed among these morphological forms, even though the whole culture broth was well mixed. Prior to this study, there have been few reports on the product distribution as a function of morphology

TABLE 2
Fatty Acid Composition Obtained in a 10 kL Industrial Fermentor (after 8 d of cultivation)

Fatty acid composition (%)[a]										
14:0	16:0	18:0	18:1	18:2	18:3	20:2	20:3	20:4	24:0	Others
0.4	12.8	7.8	6.9	10.1	2.5	0.3	3.8	45.0	7.7	2.7

[a]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.

in a well mixed broth, because many fungal metabolites are extracellular products and their measurement is difficult. Based on the resultsof our testing, we obtained a high AA yield in an industrial fermentor by controlling the morphology and maintaining an adequate DO concentration with a low agitation speed due to the low viscosity.

The 0–1 mm, 1–2 mm, and 2–4 mm broth fractions contained filaments, small pellets and large pellets, respectively, and the DCW of each fraction of whole broth was quantified as a percentage. We found that the apparent viscosity became higher with increasing percentages of the filament fraction (0–1 mm). Sieve separation was considered to be a useful means of morphological quantification.

Under the viscous condition resulting from the use of only KH_2PO_4, a higher agitation speed of up to 500 rpm was needed to maintain an adequate DO concentration, and this increased the shear stress, which in turn decreased the AA productivity. The mycelial damage due to high shear stress was evaluated as the increase in the leakage of intracellular nucleotides.

On the contrary, only Na_2SO_4, $CaCl_2$ and $MgCl_2$ addition caused pellet growth, an increase in pellet diameter, and a reduction in viscosity. Large pellets are subject to mass transfer limitation, which produces nutrient and oxygen gradients through the pellet wall (16), suggesting that the decrease in AA productivity was due to this phenomenon. In the case of small pellet formation, it was likely that the area inside the pellets was not susceptible to oxygen limitation and, also, was not exposed to shear stress. An adequate DO could be maintained at a lower agitation speed than that for filamentous growth because of the low viscosity. Therefore, under the condition of KH_2PO_4, Na_2SO_4, $CaCl_2$, and $MgCl_2$ addition, the AA yield was the highest.

For industrial production, increasing the mixing and thereby the oxygen transfer rate by lowering the broth viscosity is valuable, and mass transfer limitations through the pellet wall should be prevented (17). Therefore, morphological control seemed to be the key factor in achieving successful scale-up, and mineral addition was found to be a suitable means of achieving it. Based on the results of these investigations on morphology, we could reproduce almost the same AA yield in a 10-kL fermentor as that in a 50-L jar fermentor.

The mechanism underlying this morphological regulation remains unclear. However, it is likely that there are two factors—biochemical and physicochemical—that are responsible for the change in morphology. Under the condition of KH_2PO_4 addition, the initial growth rate was a little higher than that of no addition. This phenomenon was estimated from the glucose consumption rate and on observation of mycelia (data not shown). The higher growth rate seemed to induce the formation of more elongated hyphae, which were liable to be cut and dispersed on agitation, and then the larger amount of mycelial fragments caused the filamentous growth. This phenomenon agrees with another report (12), which stated that the growth rate was low in the case of pellet formation and that it was high in the case of filaments. On the other hand, the addition of Na_2SO_4, $CaCl_2$, and $MgCl_2$ induced pellet growth. The ionic strength seemed to affect pellet formation. Braun and Vecht-Lifshitz (18) stated that calcium ion and polycations induced mycelial aggregation and pellet formation. In order to obtain the optimal morphology, we must control these two opposite forces, favoring dispersion and aggregation. In this investigation, this was made possible by means of modification of the mineral composition.

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