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Production of Arachidonic Acid by *Mortierella* Fungi

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Abstract The growing interest in the application of arachidonic acid (ARA) in various fields of health and dietary requirements has elicited much attention on the industrial production of ARA-containing oil by the cultivation of *Mortierella* fungi. For the industrial production of ARA, various studies, such as isolation of a high-potential strain and optimization of culture conditions, have been conducted. Studies including the investigation of morphology are important because ARA is accumulated in the mycelia, and thus cultivation with high biomass concentration is essential for obtaining a high ARA yield. Combining the results derived from various studies, a high ARA yield was attained in an industrial fermentor. These ARA production techniques are applicable to the production of other polyunsaturated fatty acids (PUFAs), and will contribute to the improvement of fermentation technology especially in the field of fungal cultivation.

Keywords: arachidonic acid, *Mortierella*, morphology, industrial production

INTRODUCTION

Production of Microbial Lipid

It has been known that microorganisms have the potential to accumulate lipid intracellularly since more than a hundred years ago. Lipid content is usually less than approximately 10% of dry biomass. However, some microorganisms, the so-called oleaginous microorganisms, have the potential to accumulate lipid in their bodies equivalent to about 50% of dry biomass. A study on the application of microbial lipid using baker's yeast was carried out by Nageli and Loew in 1878, but it was not until some decades later that the microbial lipid began to attract attention [1]. Trials on the commercial production of microbial oil were performed by Lindner's group in Germany during World War I in order to alleviate food shortage [2]. Since then, to develop new edible oil sources to replace vegetable and animal oils, various types of research with the aim of oil production by oleaginous microorganisms have been carried out. However, microbial oils have been commercialized few because of the high production cost as compared with the low cost of conventional edible oil extracted from natural resources.

Recently, various physiological functions have been found in lipids that originate in unique natural sources such as rare plants, fish and microbial cells. With the research on the physiological functions of lipids, the production of microbial lipids has become attractive again. Among the known microbial lipids, polyunsaturated fatty acids (PUFAs) have attracted great interest because of their valuable functions. The first trial of PUFA production with the target being γ-linolenic acid (C18:3 ω6) was pioneered in the UK [1] and Japan [3] using *Mucor* fungi. Thereafter, various PUFAs have been studied with the aim of realizing effective production. For example, arachidonic acid (ARA, C20:4 ω6), dihomo-γ-linolenic acid (C20:3 ω6) and mead acid (C20:3 ω9) are produced by *Mortierella* fungi [4], and docosahexaenoic (DHA, C22:6 ω3) and eicosapentaenoic acids (C20:5 ω3) are produced by microalgae and bacteria [4].

Importance of Arachidonic Acid

Arachidonic acid (ARA; 5,8,11,14-*cis*-eicosatetraenoic acid) is a major constituent and plays the role of maintaining membrane fluidity in biological cells. In animals, ARA content is about 10% in vital organs such as blood and liver. ARA has also attracted attention as a precursor of prostaglandins, thromboxane, prostacyclin, and leucotrienes, which have potent and various physiological actions including uterine muscle contraction, relaxation, vasodilatation, and antihypertensive action [5].

Various physiological functions of ARA have been reported, *e.g.*, protection of gastric mucosa [6,7], treatment of skin psoriasis [8], reduction of fatty liver [9], killing of tumor cells [10], and improvement of lipid

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metabolism of cirrhotic patients [11]. Lately, a new function of ARA was reported, namely, arachidonylethanolamide, also called anandamide [12], and *sn*-2 arachidonylglycerol [13] may function as a natural ligand for the cannabinoid receptor, which is expressed in areas of the central nervous system that contribute to the control of memory, cognition, movement and pain perception. This kind of role has attracted attention as a novel function of PUFAs.

In addition to the above-mentioned biological activities, the nutritional aspect of ARA has also been highlighted along with other PUFAs. ARA has been extensively investigated particularly as a substance essential for the growth of infants. Carlson *et al.* [14,15] suggested that ARA could improve the first year of growth of preterm infants. Lanting *et al.* [16] monitored the growth of infants who had been fed breast milk or formula milk based on their behaviors until the age of 9, and found that the incidence of encephalopathy in the infants fed formula milk was about twice as high as that in those fed breast milk. This is surmised to have been due to the lack of PUFAs, such as DHA and ARA, in formula milk while these PUFAs are present in breast milk and may play an important role in development of the brain. O'Connor *et al.* [17] carried out a clinical trial on premature infants taking formula milk with or without ARA and DHA supplementation, and found that the benefits of supplementing formulas for premature infants were observed in the indexes of visual acuity, motor development and communicative development. Birch *et al.* [18] performed a clinical trial on term infants. They fed three types of formulas, such as that with ARA and DHA supplement, that with only DHA supplement, and that without supplement. Mental development index was the highest in the group of infants fed formula containing ARA and DHA supplement. Furthermore, there have been various reports that PUFA may play a role in brain development, and it is recommended that the composition of infant formula be as close as possible to breast milk, the ideal nutrition for infants.

From the above-mentioned background, it has strongly been desired to develop oil containing ARA abundantly, safely, and economically as a usable ingredient of food.

MICROORGANISMS FOR ARACHIDONIC ACID PRODUCTION

ARA exists widely in the animal kingdom, and has been isolated from lipids extracted from the adrenal gland and the liver of animals. However, because such organs contain ARA in small amounts, isolation from these organs is insufficient to meet the demand for ARA. Methods to produce ARA by cultivation of various microorganisms capable of producing ARA have been proposed, as shown in Table 1. Among them, those belonging to the genus *Mortierella* have been studied by many groups, and particularly, those belonging to species *alpina* show a high potential for ARA production. ARA is synthesized through many enzymatic steps of fatty acid synthesis, elongation of fatty acid and desaturation of fatty acid, as shown in Fig. 1. The genus *Mortierella* has two subgenus, *Mortierella* and *Micromucor* [35]. Only those belonging to the subgenus *Mortierella* can synthesize C20 PUFAs, such as dihomo-γ-linolenic acid (C20:3 ω6) and ARA. On the other hand, those belonging to the subgenus *Micromucor* can synthesize C18 PUFA, but not C20 PUFAs. The fatty acid composition of 50 *Mortierella* subgenus isolates was analyzed by Amano *et al.* [35]. They found that ARA composition of *alpina* was higher than those of any

Table 1. Previous reports on arachidonic acid production

Microorganism	ARA yield/cultivation period	Scale	Ref.
Submerged culture			
Mortierella alpina 1S-4	13 g/L/10d	10-kL fermentor	[19]
M. alpina ATCC 32222	11 g/L/11d	250-mL flask	[20]
M. alpina ATCC 32221	11 g/L/16d	500-L fermentor	[21]
M. alpina UW-1	5.5 g/L/6d	20-L fermentor	[22]
M. alpina LPM 301	4.5 g/L/8d	30-L fermentor	[23]
M. alpina ATCC 42430	4.1 g/L/6d	20-L fermentor	[24]
M. alpina Wuji-H4	3.9 g/L/5d	250-mL flask	[25]
M. alpina DSA-12	3.3 g/L/6d	500-L fermentor	[26]
M. alpina CBS 343.66	1.0 g/L/8d	5-L fermentor	[27]
Mortierella alliacea YN-15	7.1 g/L/6d	50-L fermentor	[28]
Mortierella schmuckeri S12	2.3 g/L/3d	14-L fermentor	[29]
Mortierella sp. S-17	0.96 g/L/7d	1-L flask	[30]
Mortierella elongata SC-208	0.49 g/L/5d	250-mL flask	[31]
Pythium irregulae ATCC 10951	3.1 g/L/8d	250-mL flask	[32]
Solid-state culture			
M. alpina IFO 8568	13 g/kg-medium/20d		[33]
M. alpina CCF 185	36 g/kg-medium/21d	300-mL flask	[34]

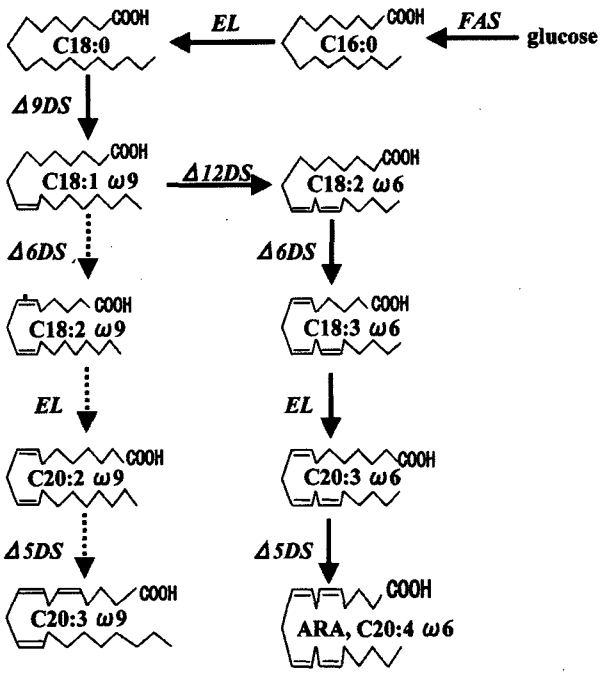

Fig. 1. Biosynthetic pathway of polyunsaturated fatty acids in *Mortierella alpina*. Abbreviations: *FAS*, fatty acid synthesis; *EL*, elongation; *DS*, desaturation.

other species.

In terms of cultivation methods, there are two types: the submerged culture and the solid-state culture. In order to industrialize PUFA production, the submerged culture seems to be the better choice. For one, the scaling up of cultivation and biomass recovery is easier, and ARA yield reported in the solid-state culture is less than that in the submerged culture. Moreover, in the case of solid-state culture, the cultivation period is more than 20 days because of the low growth rate, as shown in Table 1.

In terms of industrial production, the improvement of ARA yield and the reduction of cultivation time have an impact on the production cost. In terms of ARA yield, high yields of over 10 g/L have been reported in some studies [19-21], suggesting that *M. alpina* has a high potential for ARA production. For the reduction of the cultivation time, ARA productivity has to be evaluated. *M. alpina* 1S-4 [19] and *M. alliacea* YN-15 [28] have ARA productivities higher than 1 g/L per day. In order to commercialize the ARA-containing oil that originated from microorganisms, in addition to the cost evaluation mentioned above, safety evaluations have to be carried out. Lately, with the increase in the interest in the PUFAs produced by microorganisms, various studies on the safety of the product have been conducted. There have been various reports indicating the safety of *M. alpina* and its products [36-39].

EFFECTS OF CULTURE CONDITIONS ON ARACHIDONIC ACID PRODUCTION AND MYCELIAL MORPHOLOGY

In general, sufficient mass transfer and oxygen supply are required to obtain high productivity in aerobic fermentation. In the case of fungal fermentation, the medium composition not only influences the productivity, but also may induce a change in mycelial morphology as a side effect [40]. The morphology has a strong effect on the physical properties of the fermentation broth, and causes numerous problems in large fermentors with respect to gas dispersion, mass and heat transfer, and homogenization [41]. Thus, mycelial 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: for example, pellet growth is preferable for citric acid production by *Aspergillus niger* [42], itaconic acid production by *Aspergillus terreus* [43], and pravastatin precursor production by *Penicillium citrinum* [44]; and filamentous growth is preferable for penicillin production by *Penicillium chrysogenum* [45], fumaric acid production by *Rhizopus arrhizus* [46], and enzyme production by *Aspergillus niger* [47,48]. Therefore, it is important to find the optimal morphology for the production of the target metabolite.

For the commercial production of ARA, a high biomass concentration is required for high productivity because ARA is an intracellular product. Besides, ARA production requires adequate oxygen, because PUFAs are formed by enzymatic desaturation that comprises oxygenation [1]. Thus, adequate oxygen supply by means of agitation, aeration, and morphological control is the key factor for obtaining a higher ARA content in the cells. Therefore, we review the effects of culture conditions on the morphology and ARA productivity in this article.

Effects of Carbon and Nitrogen Sources

In cultures of *Mortierella* for ARA production, glucose is most frequently used as the carbon source [19-34]. Shinmen *et al.* [49] and Aki *et al.* [28] investigated various carbon sources for ARA production using strains of *M. alpina* and *M. alliacea*, respectively, and found that glucose was suitable for ARA production. In the case of *M. alliacea*, starch was also usable as expected, because this strain was isolated using rice grain. Totani *et al.* [21] reported the effect of glucose concentration on ARA production. They pointed out that more than 20% glucose inhibited the growth of *M. alpina* and high glucose concentration induced the formation of filamentous morphology. This influence of glucose concentration was also observed by Lindberg and Molin [27]. Various studies have also indicated that vegetable oil addition was beneficial to enhance ARA yield [28,49].

As for the nitrogen source, yeast extract (YE) and soybean meal (SM) are widely used for the culture of *Mortierella*. Aki *et al.* [28] investigated various nitrogen

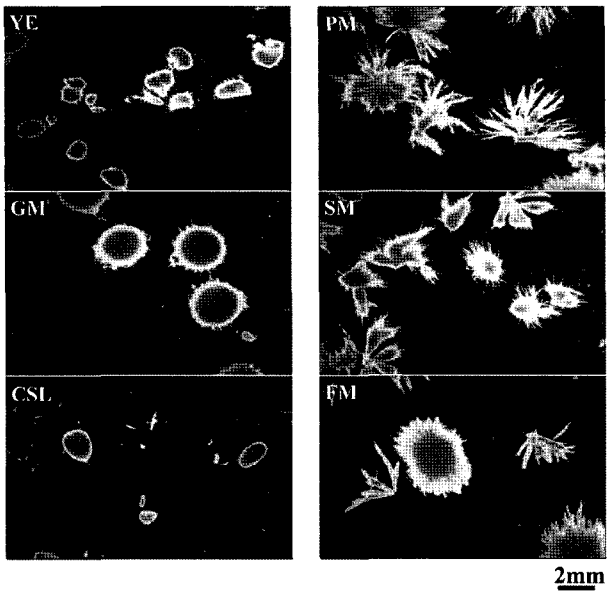

Fig. 2. Morphology of *Mortierella alpina* in cultures grown using yeast extract (YE), gluten meal (GM), corn steep liquor (CSL), Pharmamedia (PM), soybean meal (SM), and fish meal (FM) as nitrogen source. Images were captured after cultivation and were magnified 7×.

sources, and concluded that YE and SM were suitable. In the case of Higashiyama *et al.* [50], the medium comprising YE was better than that comprising SM. However, when some minerals were added to the basal medium, the medium comprising SM was better than that comprising YE. Park *et al.* [51] investigated various nitrogen sources, such as YE, SM, corn steep liquor (CSL), Pharmamedia (PM), fish meal (FM), and gluten meal (GM), and their effects on ARA production and mycelial morphology. They found that smooth pellets (or circular pellets) were obtained when YE, GM or CSL was used, and fluffy pellets (or feather-like pellets, radial morphology) were obtained when SM, PM or FM was used, as shown in Fig. 2. As a result of investigation of the relationship between morphological type and ARA productivity, the fluffy pellet morphology was suggested to be more suitable for ARA production than the smooth pellet morphology. Totani *et al.* [21] used peptone and wheat bran in the culture of *M. alpina*, and obtained a high ARA yield of 11 g/L. Only a small amount of biomass was obtained in the medium comprising ammonium nitrate, sodium nitrate, ammonium acetate, or ammonium sulfate as the nitrogen source [28,52], suggesting that *Mortierella* fungi hardly assimilate inorganic nitrogen source, and require amino acid or protein for obtaining a certain amount of biomass.

Koike *et al.* [53] reported the effect of carbon to nitrogen ratio (C/N) on ARA production and mycelial morphology in the culture of *M. alpina*. Cultivation was carried out at various C/N ratios under the constant condition of C(glucose)+N(SM) = 50 g/L. As a result, in the presence of excess N source, biomass yield depended on the N concentration, and in the presence of excess C source, the total fatty acid (tFA) yield increased with C concentration whereas the ARA/tFA was constant. In terms of ARA production, the optimum C/N ratio was found to be in the range of 15 to 20. As for the morphology [54], at C/N ratios below 20, the morphological size was constant. However, at C/N ratios above 20, the morphological size increased proportionaly to the C/N ratio. This phenomenon seems to contradict the result of Totani *et al.* [21] that high glucose concentration induced the formation of filamentous morphology. The reason for these contradictory results is presumed to be as follows. The difference in physical stress between the cultures might be large, because the studies of Park *et al.* [54] and Totani *et al.* [21] were carried out using a shake flask and a jar fermentor, respectively. Therefore, the morphology was dependent on the synergistic effects of glucose concentration and physical stress.

Effects of Mineral Addition

In order to obtain a higher product yield, optimization of the trace element composition in the medium is also important besides the fundamental medium composition, as mentioned in the above subsection. Phosphorus, potassium, sulfur, calcium, sodium, iron, and magnesium are the major inorganic constituents of fungi [55]; these minerals should be supplied sufficiently, and optimization of the additional concentration is important. Therefore, various groups have attempted to enhance ARA productivity by optimization of the amounts of minerals added.

Totani *et al.* [56] investigated the mineral requirements in the culture of *M. alpina*. They found that phosphorus, potassium, iron and manganese are essential for cell growth, and iron and manganese play significant roles in lipid synthesis. A negative effect due to the addition of only iron or only manganese was also observed. Sajbidor *et al.* [57] studied the influence of calcium, magnesium, manganese and iron on ARA production in the culture of *Mortierella* sp. They found that a low concentration of manganese was beneficial for ARA production, but a high concentration of that repressed lipid accumulation. An inhibitory effect of iron was also observed. The importance of manganese addition is common to these two reports [56,57]. Kyle [24] found that the addition of iron, zinc, and copper enhanced ARA yield in the culture of *M. alpina*, and that phosphorus supplement to that medium was a much better choice. One reason for these phenomena is that acetyl-CoA carboxylase, which catalyzes the conversion of acetyl-CoA into malonyl-CoA, requires bivalent metal ions as the cofactor [58]. Minerals added may act as a cofactor of this enzyme system that catalyzes the initial step of fatty acid synthesis.

Although various studies on the influence of minerals have been carried out, as shown in the above-mentioned examples, there has been little investigation on the influence of minerals on the morphology. Ionic

Table 2. Effect of mineral addition on morphology, apparent viscosity, and nucleotide leakage

	DCW of each fraction in whole culture broth (%)			Apparent viscosity[c] (cp)	A_{260}/DCW[d] ([abs.]/[g/L])	ARA yield[e] (g/L)
	0-1 mm[b]	1-2 mm[b]	2-4 mm[b]			
A[a]	60.8	39.2	0	2240	0.415	6.8
B[a]	12.4	40.8	46.8	30	0.280	8.3
C[a]	47.3	52.7	0	550	0.323	9.8

Abbreviations: DCW, dry cell weight; A260, absorbance at 260 nm; ARA, arachidonic acid.
[a] Conditions for mineral addition: 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%).
[b] Sieve aperture size used for separation of culture broth.
[c] Measured by a B-type viscometer.
[d] Nucleotide leakage for evaluation of mycelial damage due to mechanical stress.
[e] ARA yield obtained after cultivation for seven days in a 50-L fermentor.

strength is reported to have an effect on mycelial morphology [59]. The reason is that the cell wall of microorganisms is mostly negatively charged, and tends to separate or disperse due to electric repulsion. The relationships between electric charge of cell wall and cell aggregation/dispersion level have been observed in cultures of *Rhizopus* [60] and brewing yeast [61]. Mycelial dispersion may be suppressed by an increase in ionic strength, suggesting that pellet formation is consequently induced. Therefore, it is important to investigate the effect of mineral composition on mycelial morphology. Higashiyama *et al.* [62] studied the effects of minerals on both mycelial morphology and ARA production in the culture of *M. alpina,* and the results are listed in Table 2. In a 50-L jar fermentor study, the medium containing soy flour and glucose, together with KH_2PO_4, Na_2SO_4, $CaCl_2$ and $MgCl_2$, was found to be the optimum. The ARA yield reached 9.8 g/L after seven days of cultivation, with small pellets being the major morphology (diameter 1-2 mm). However, in the case of the addition of only 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; however, this caused mycelial damage due to shear stress, resulting in a decrease in the ARA yield. When a medium containing Na_2SO_4, $CaCl_2$, and $MgCl_2$ was used, the major morphology was large pellets (diameter 2-4 mm), which limited mass transfer through the pellet wall, also causing a decrease in the ARA yield. In an attempt to determine the relationship between morphology and ARA productivity, the ARA content in the dry cells of each cell fraction separated by sieving was measured. ARA content in the 1-2 mm cell fraction was found to be the highest under all three conditions, as shown in Fig. 3. In conclusion, optimization of mineral composition has to be carried out in two aspects: physiological or nutritional aspect and morphological aspect.

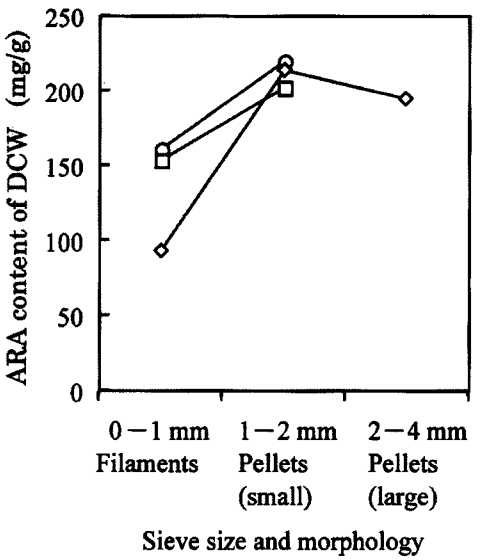

Fig. 3. Relationship between ARA content and mycelial morphology in the culture of *Mortierella alpina*. Culture broth on the 7th day of a 50-L fermentation study was used for analysis. Culture condition A, □; condition B, ◇; condition C, ○. Conditions A-C are indicated in Table 2.

Effects of DO Concentration

PUFAs are formed by elongation and desaturation from a carbon source in the mycelia. The desaturation involves an aerobic reaction by oxygenation [1]; therefore, dissolved oxygen (DO) seems to be an important factor for PUFA production. Various studies have indicated the importance of oxygen in the desaturation of fatty acids [63,64].

The effect of 2-3 ppm DO on ARA production by *Entomophthora exitalis* has been studied [65]. There have been some reports of attempts to monitor DO concentration and control the agitation rate to prevent DO limitation in cultures for γ-linolenic acid production [66-69] and ARA production [21]. In general, fungi are physically weak; therefore, the agitation rate has to be controlled within a certain range. An improvement of γ-linolenic acid productivity by changing the impeller design has been reported [70]; however, it may not be practical, especially on an industrial scale. It is possible

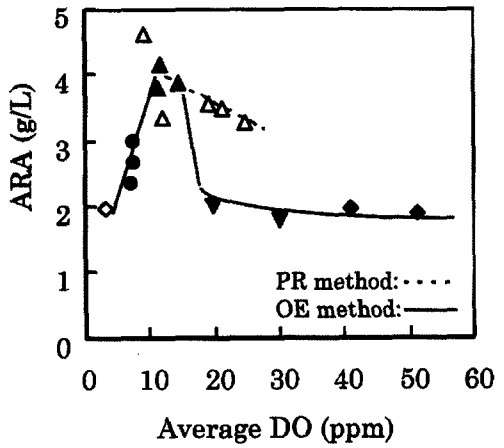

Fig. 4. Effects of average DO concentration on ARA yield in the culture of *Mortierella alpina*. Symbols: ● (conditions for [i]: headspace pressure [kPa], oxygen concentration of inlet gas [%], aeration rate [vvm], 150, 21, 1); ◇ ([ii]: 150, 21, 0.05-1); △ ([iii]: (180, 21, 1), (225, 21, 1), (300, 21, 1), (340, 21, 1), (380, 21, 1)); ▲ ([iv]: (150, 25, 1), (150, 27, 1), (150, 29, 1)); ▼ ([v]: (150, 37, 1), (150, 55, 1)); ◆ ([vi]: (150, 73, 1), (150, 90, 1)). Conditions [iii] and [iv]-[vi] are performed by the PR and OE methods, respectively.

to increase DO by low-temperature fermentation, but this technique is limited because of the effect of temperature on the fatty acid composition [27,71].

The above studies on PUFA production were all conducted under normal atmospheric pressure and in the presence of atmospheric air containing 21% oxygen. Higashiyama *et al.* [72] reported the effect of DO concentration above the atmospheric equilibrium level on ARA production and mycelial morphology in the culture of *M. alpina*. Cultivations were carried out under constant DO at various levels in the range of 3 to 50 ppm using a 50-L fermentor. To maintain a DO concentration above 7 ppm, either the oxygen-enrichment method (OE method; supplying oxygen-enriched gas with 25-90% oxygen concentration) or the pressurization method (PR method; pressurization of headspace in the range of 180 to 380 kPa) was used. As a result, the optimum DO concentration range was found to be 10-15 ppm, as shown in Fig. 4. In this optimum DO concentration range, the ARA yield was enhanced by about 1.6-fold compared to that obtained at 7 ppm DO, and there was no difference in ARA productivity between the OE and PR methods. Beyond this DO range, the difference in ARA productivity and morphology between the PR and OE methods became significant. When the DO concentration was maintained at 20-50 ppm using the OE method, the morphology changed from filaments to pellets, and the ARA yield decreased drastically because of stress due to the limited mass transfer through the pellet wall. When the DO concentration was maintained at 15-20 ppm using the PR method, the morphology did not change, and the ARA yield decreased only slightly. This phenomenon suggested that the decrease in productivity and the morphological change were caused by not only DO concentration but also oxygen concentration in the supplied gas.

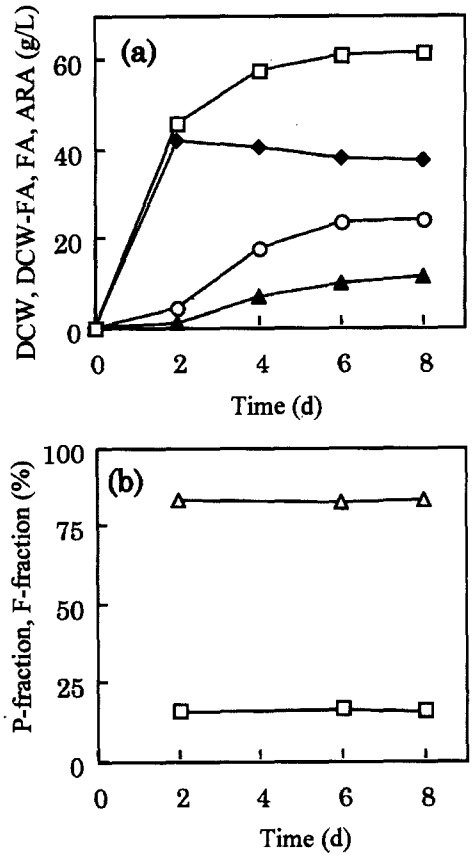

Fig. 5. Time course of ARA production by *Mortierella alpina* 1S-4 in a 10-kL fermentor. (a) dry cell weight (DCW, □), total fatty acid (FA, ○), arachidonic acid (ARA, ▲), and fat-free biomass (DCW-FA, ◆); (b) percentage of fat-free biomass in pellet fraction (P-fraction, △), and filament fraction (F-fraction, □).

INDUSTRIAL PRODUCTION OF ARACHIDONIC ACID

The scale-up of the cultivation process has been conventionally based on such factors as the oxygen transfer coefficient, DO concentration, power consumption for agitation, and impeller tip speed. In spite of the investigations of scale-up strategies, a complete scale-up strategy has not been established, and as Humphrey mentioned, scale-up is still an art, not a science [73]. Hiruta *et al.* [74] studied the agitation conditions for scale-up from a 30-L fermentor to a 600-L fermentor in the culture of *Mortierella ramanniana* for γ-linolenic acid production. They demonstrated that production and morphology were successfully scaled up from a 30-L fermentor to a 10-kL fermentor based on the impeller tip

speed. Tsujimura *et al.* [75] reported the application of fluid velocity distribution analysis to the scale-up of peroxidase production by the filamentous fungus *Arthromyces ramosus*. They also succeeded in applying this method to the scale-up of ARA production by *M. alpina* 1S-4 from a 50-L fermentor to a 10-kL fermentor [76]. As Fig. 5 shows, a high ARA yield of 11 g/L and a DCW of 61.6 g/L were obtained after cultivation for eight days in a 10-kL fermentor [77]. During the first two days, fat-free biomass (DCW-FA) increased, but from the 2nd to the 8th day, it was almost constant, indicating that the growth phase and the oil accumu-lation phase were distinct from each other. They also evaluated the morphology by the sieve separation method. The percentage of the pellet fraction (>0.5 mm of sieve aperture) was considerably higher than that of the filament fraction (0-0.5 mm of sieve aperture). The high percentage of pellet formation facilitated the mixing of the cultivation broth, and consequently, a high-yield production was attained in an industrial-scale fermentor.

CONCLUSION AND FUTURE PERSPECTIVES

In order to achieve high-yield ARA production in an industrial scale, various techniques are required, *i.e.*, isolation of a strain that has high potential for PUFA synthesis, optimization of culture medium, control of mycelial morphology to an appropriate form, and investigation of the scale-up methods. By combining these techniques, a high ARA yield was attained in an industrial fermentor, as shown in Fig. 5 [77].

The increasing demand for biologically important PUFAs has led to the search for their sources. By modifying the ARA production process of *M. alpina*, various PUFAs can be produced. This involves two techniques, one is the mutation technique and the other is the desaturase blocking technique. As examples of the latter, lignan compounds from sesame seed oil [78,79] or curcumin from turmeric [80] were reported to inhibit Δ5 desaturase. By adding these desaturase inhibitors to the culture medium of the ARA-producing fungus *M. alpina*, dihomo-γ-linolenic acid (DGLA, C20:3 ω6) was produced. The former technique, fungal mutation, resulting in the suppression or activation of specific desaturase, is beneficial not only for the production of tailor-made PUFAs but also for studying PUFA biosynthesis [81]. For example, the Δ5 desaturase-defective mutant, *M. alpina* S14, was used for DGLA production. Using this mutant, a DGLA yield of 7 g/L was achieved in a 10-kL fermentor after cultivation for 12 days [82]. As for other PUFAs, mead acid (C20:3 ω9) production by a Δ12 desturase-defective mutant [83], 8,11-*cis*-eicosadienoic acid (C20:2 ω9) production [84] and 8,11,14,17-*cis*-eicosatetraenoic acid (C20:4 ω3) production [85] by Δ5 and Δ12 desaturase-defective mutants of *M. alpina* have been reported.

The control of mycelial morphology is the key technique for improving PUFA yield by high-concentration cultivation. As mentioned in the last section, various studies on morphology have been carried out. In spite of the importance of morphology and the number of studies published, the mechanism of morphology formation has not been completely clarified. Analysis of morphology change is beneficial for improving the fermentation technology, especially for the filamentous fungi. In this respect, the image analysis technique has become a useful tool with the recent development of computer technology [86]. Various investigations have applied this technique for morphological classification, as well as for kinetic and physiological studies [87]. The image analysis technique was applied to ARA production, and effects of culture conditions on mycelial morphology were quantified [51,53,54]. In another study [77], sieve separation was used in order to analyze morphological sizes ranging from a few micrometers (size of filaments) to a few millimeters (size of pellets). In general, culture broth consists of filaments and pellets; therefore, the image analysis technique combined with sieve separation is a useful tool for the precise analysis of morphological change.

Pellet morphology allows easier mixing and better

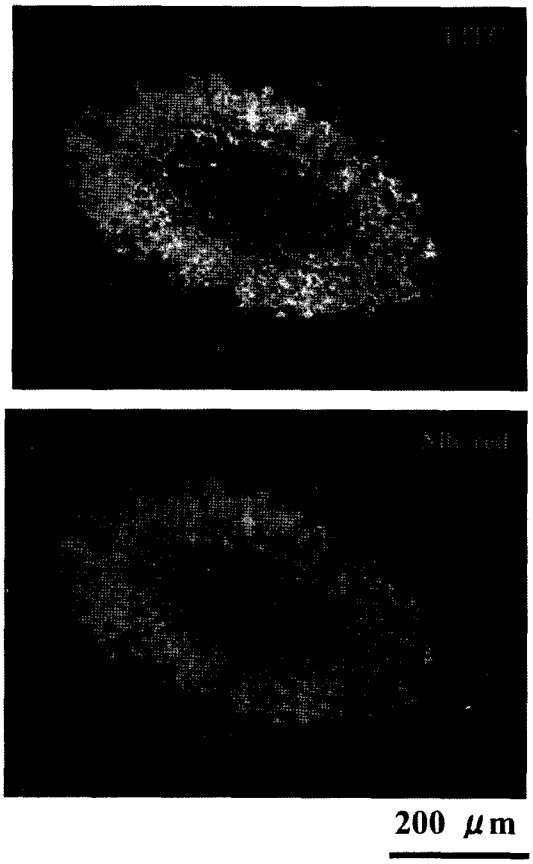

Fig. 6. Pellet cross section of *Mortierella alpina* stained with FITC and Nile red. Green and red colors indicate fluorescence of FITC and Nile red, respectively.

mass transfer to the culture broth. However, growth may be restricted due to substrate limitation in the region of the dense pellet core when the pellet radius exceeds a critical value. From this point of view, various studies to analyze the pellet intrastructure have been reported. Park *et al.* [88] investigated the distributions of viability and mycelial density in the pellets of *Streptomyces fradiae* by labeling with fluorescein isothiocyanate (FITC). Wittler *et al.* [89] used a microprobe to measure oxygen transfer into the pellets of *Penicillium chrysogenum*. However, there have been few investigations of the product distribution inside the pellets. This is probably because almost all of the products produced by fungal cultivation are extracellular. Using the characteristic that PUFAs are accumulated inside the cell, Hamanaka *et al.* [90] analyzed the product distribution in the pellets of *M. alpina*. In order to distinguish mycelia and lipid bodies inside the cell, FITC and Nile red [91] were used to stain mycelia and lipid bodies, respectively. After labeling with FITC and Nile red, the mycelial pellet intrastructure was visualized by fluorescein microscopy, and it was found that a cavity or a low-density region of mycelia and lipid existed at the center of the pellet, as shown in Fig. 6. The application of this visualization technique opens the possibility of analyzing the intrastructure of fungal pellets and performing new types of fungal biological studies.

In industrial production, quality control and process diagnosis must be conducted accurately. In the case of PUFA production by fungal cultivation, PUFA-enriched oil accumulated in the mycelia is usually analyzed by conventional time-consuming methods, such as chromatography or weighing after extraction with organic solvent. If an oil-detectable sensor were developed, it would not only contribute to the improvement of the control of the cultivation process, but also provide valuable information to further our biochemical understanding of oil production. Dielectric analysis is a non-invasive technique that can characterize the structure and passive electrical properties of viable biological cells [92]. The merits of dielectric analysis are summarized into four points: (i) it is easy to perform and less time-consuming; (ii) it is applicable to high cell concentrations; (iii) it enables direct measurement of cell concentration; and (iv) it detects only viable cells [93]. Based on these merits, it has been used for monitoring cell growth in fungal cultivation [94,95]. During the cultivation of oleaginous microorganisms, cytoplasmic conductivity is predicted to decrease with the accumulation of oil in the cell. From this point of view, Higashiyama *et al.* [96] attempted to estimate the oil content in the mycelia of *M. alpina* during cultivation by dielectric analysis. As a result, good correlation between cytoplasmic conductivity and oil content in the mycelia was obtained. This result indicates that dielectric analysis enables us to estimate the oil content in the mycelia and also provides a useful tool for monitoring cell growth and for controlling the cultivation process for PUFA production.

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