Production of Arachidonic Acid by Filamentous Fungus, *Mortierella alliacea* Strain YN-15

Tsunehiro Aki[a,*], Yumiko Nagahata[a,1], Katsuyuki Ishihara[a], Yoshio Tanaka[a], Tsutomu Morinaga[a,2], Kenichi Higashiyama[b], Kengo Akimoto[b], Shigeaki Fujikawa[b], Seiji Kawamoto[a], Seiko Shigeta[a], Kazuhisa Ono[a], and Osamu Suzuki[a]

[a]Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, Higashi-Hiroshima 739-8527, and [b]Institute for Fundamental Research, Suntory Ltd., Shimamoto, Mishima, Osaka 618-0001, Japan

ABSTRACT: A filamentous fungus producing significant levels of arachidonic acid (AA, C20:4n-6) was isolated from a freshwater pond sample and assigned to the species *Mortierella alliacea*. This strain, YN-15, accumulated AA mainly in the form of triglyceride in its mycelia. An optimized culture in 25 L of medium containing 12% glucose and 3% yeast extract yielded 46.1 g/L dry cell weight, 19.5 g/L total fatty acid, and 7.1 g/L AA by 7-d cultivation in a 50-L jar fermenter. Assimilation of soluble starch by YN-15 was notably enhanced by the addition of oleic acid, soybean oil, ammonium sulfate, or potassium phosphate to a starch-based medium. Using starch as a main carbon source in the pre-pilot scale cultivation improved the production of AA by up to 5.0 g/L. *Mortierella alliacea* strain YN-15 is therefore a promising fungal isolate for industrial production of AA and other polyunsaturated fatty acids.

Paper no. J9821 in *JAOCS 78,* 599–604 (June 2001).

KEY WORDS: Arachidonic acid, microbial lipid production, *Mortierella alliacea,* polyunsaturated fatty acid, single-cell oil.

Arachidonic acid (AA, C20:4n-6) is an essential component of structural lipids in the cellular membranes of mammals. Because dietary AA does not have to be desaturated and elongated before tissue incorporation, and because it is a poor substrate for β-oxidation, AA can be rapidly and preferentially enriched in membrane phospholipids (1,2). AA is released from membrane lipids by phospholipases and metabolized into a large group of eicosanoids (prostaglandin group 2) including prostaglandin, thromboxane, leukotriene, and prostacyclin, which are important for intercellular signaling at various stages of growth and development (3). Eicosapentaenoic acid (EPA, C20:5n-3), on the other hand, is a precursor of the other family of eicosanoids (prostaglandin group 3). Because EPA itself and the eicosanoids derived from it exhibit an antagonizing effect against the eicosanoids stemming from AA (4), provision of an adequate balance of their precursors, n-6 and n-3 essential fatty acids, in the daily diet is important for physical homeostasis (5). In addition, an imbalance of dietary intake of n-6 and n-3 fatty acids may be a predisposing factor for carcinogenesis, impaired learning ability, moodiness, and drug sensitivity (6–8). Preterm infants who received a diet rich in docosahexaenoic acid (DHA, C22:6n-3) showed improved visual acuity (9), but AA levels in their sera decreased and their growth was reduced significantly (10). Accordingly, recognition is developing that AA must be complemented with n-3 polyunsaturated fatty acids (PUFA) in appropriate ratio in semisynthetic milk and nutritional supplements (5).

In investigations of sources of PUFA, microbes producing lipids rich in PUFA have been isolated and evaluated (11). A series of fungi belonging to the genus *Mortierella* has been extensively studied for their AA productivity through optimizing cultivation conditions (12,13). In fact, the production of AA and other PUFA by *Mortierella* species is considerably higher than by other characterized microbes (13). This seems to depend largely on their ability to grow as well as a high capacity to accumulate lipids. Under industrial conditions one would like to use a microbe that ferments cheap raw materials, e.g., starch. We report the isolation of a new fungal strain satisfying these criteria.

MATERIALS AND METHODS

Reagents and media. Fatty acids and fatty acid methyl ester standards were purchased from Sigma (St. Louis, MO). Yeast extract and 10% methanolic hydrochloride were obtained from Difco (Detroit, MI) and Tokyo Kasei (Tokyo, Japan), respectively. Polypeptone, corn steep liquor, and soybean meal were obtained from Wako (Osaka, Japan).

Screening of fungi producing PUFA. Grains of rice were put on a Petri dish with a small amount of distilled water and autoclaved. A freshwater pond sample (2–3 mL) was poured onto the plate and incubated for 2 d at 28 or 15°C. Mycelia that appeared around the rice grains were picked up and streaked onto a GY-agar plate composed of 2% glucose, 1% yeast extract, and 2% agar. The plates were incubated for 7 d, and mycelia were restreaked onto a fresh GY plate. After further incubation, a fungal colony was scraped and dried at 105°C followed by fatty acid analysis as described below. Cultures were considered pure when there was no significant difference in fatty acid compositions from colonies before and after purification.

Culture conditions. Using a 200-mL baffled flask with a working volume of 50 mL (medium composition depended on parameters being tested), we then prepared a culture that was placed on a rotary shaker at 160 rpm for 7 d at 28°C under a limited aerobic condition. Cells were harvested on filter paper, and their weight was determined gravimetrically. To prepare 25 L of the medium for a pre-pilot scale fermentation, we separately autoclaved an 11-L carbon source solution and a 14-L

*To whom correspondence should be addressed at Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, 1-4-1 Kagamiyama, Higashi-Hiroshima 739-8527, Japan. E-mail: aki@hiroshima-u.ac.jp

[1]Present address: Sagami Chemical Research Center, Sagamihara, Kanagawa 229-0012, Japan.

[2]Present address: Faculty of Biological Resources, Hiroshima Prefectural University, 562 Shichizuka, Shobara, Hiroshima 727-0023, Japan.

mixture of a nitrogen source and additives (see Fig. 3), with pH adjusted at 7.0, and then combined them in a 50-L stirred tank fermenter. Various carbon and nitrogen sources were employed as independent variables. The fermenter was inoculated with 100 mL of seed culture. During a batch cultivation of the inoculum at 28°C, aeration and agitation rates were manually controlled between 12.5 and 25 L/min and 100 and 500 rpm, respectively, so that dissolved oxygen was maintained at 50 ppm under a headspace pressure of 200 kPa.

Lipid analysis. We extracted total lipids from harvested wet mycelia with a chloroform/methanol (2:1, vol/vol) mixture in the presence of glass beads as previously described (14). Total lipids (100 mg) were fractionated into neutral and polar lipids by elution on a silicic acid column (Unisil, Clarkson, PA), initially with chloroform and then with methanol. After evaporation of the eluates, the amount of each lipid fraction was determined gravimetrically. The neutral lipids were resolved by thin-layer chromatography (TLC) on a precoated silica gel plate (Kieselgel 60, 200 × 200 × 0.25 mm; Merck, Darmstadt, Germany) in a one-dimensional, double-development system, first with benzene/diethyl ether/ethanol/28% ammonia water (50:40:2:0.5, by vol) and next with *n*-hexane/diethyl ether (94:6, vol/vol). The plates were sprayed with 10% sulfuric acid followed by heating at 130°C for 15 min. Neutral lipids were quantitatively analyzed using a densitometer (AE-6910; ATTO, Tokyo, Japan).

The polar lipids were fractionated on a silica gel fluorescent TLC plate (Kieselgel 60 F_{254}; Merck), developed with chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5, by vol). Phospholipids were visualized under an ultraviolet lamp and extracted with a mixture of chloroform/methanol (2:1, vol/vol).

Fatty acids that had been separated on the TLC plates were methyl-esterified by treatment with 10% methanolic hydrochloride at 60°C for 3 h. Authentic eicosanoic acid was added quantitatively as an internal standard. The fatty acid methyl esters were analyzed on a gas–liquid chromatograph (GC-17A; Shimadzu, Kyoto, Japan) equipped with a split injector, a TC-70 capillary column (GL Science, Tokyo, Japan), and a flame-ionization detector with temperature programming (holding at 190°C for 10 min followed by temperature increasing to 200°C at 1°C/min). Assignment of fatty acids in their methyl-esterified form was conducted by GC–mass spectrometry (GC mate; JEOL, Tokyo, Japan).

Analysis of carbohydrate contents. Total and reducing sugars in culture supernatants were measured by the phenol-sulfuric acid method (15) and the Somogyi-Nelson procedure (16), respectively, with glucose as a standard.

RESULTS

Isolation of arachidonic acid-producing fungus, M. alliacea. To obtain starch-utilizing microbes, we used rice grains as an isolation probe. Of about 1000 fungal isolates from a freshwater sample obtained in Hiroshima, Japan, one grew comparatively fast on an agar plate and produced a significant amount of arachidonic acid (AA; 64% of total fatty acid) in its cells. When this microbe, designated as YN-15, was cultivated in a GY liquid medium on a small scale, the yield of dried whole cells was about 1.3 g per 50 mL. Analysis of the fatty acid composition of the cellular total lipid (8.6 g/L) showed that AA constituted 28.7% of the total fatty acid (Table 1, line 1).

From morphological analysis of YN-15 we concluded that the strain belonged to the species *M. alliacea*, on account of the presence of chlamydospore and expanded gemmae with short extending mycelia, a characteristic shape of sporangia, and the absence of septa in the mycelia (Fig. 1; Ref. 17). We are unaware of the existence of any other literature describing *M. alliacea* as an AA producer. The related AA-producing species, *M. alpina* and *M. hygrophila*, are known to accumulate EPA when grown below 20°C (18). The YN-15 strain also produced EPA in its mycelia as well as other n-3 fatty acids at 15°C (data not shown). The addition of sesamin, a lignan compound found in sesame oil, to the YN-15 culture resulted in the mycelial accumulation of dihomo-γ-linolenic acid (DGLA, C20:3n-6; 15% of total fatty acid) while the AA content was substantially reduced, as was reported previously in a study of *M. alpina* (19).

Lipid and fatty acid compositions of M. alliacea *YN-15.* The total extractable lipid in the mycelia of YN-15 was fractionated into neutral and polar lipids by column chromatography and then into lipid classes by TLC. The neutral lipid, which was a dominant component (7.24 g/L; 84% of total

TABLE 1
Lipid and Fatty Acid Compositions of *Mortierella alliacea* YN-15[a]

Lipid classes	Lipid (g/L)	Principal fatty acids (%)							
		C16:0	C18:0	C18:1n-9	C18:2n-6	C18:3n-6	C20:3n-6	C20:4n-6	C24:0
Total lipid	8.57	21.1	7.3	16.5	9.8	3.4	5.8	28.7	4.9
Neutral lipids	7.24	22.1	7.6	16.0	9.2	2.7	6.3	29.3	5.2
Triacylglycerol	3.77	21.5	7.5	14.2	8.8	2.6	8.0	34.5	2.6
Free fatty acid	1.64	22.6	8.3	19.5	10.2	3.3	2.0	23.2	2.2
Sterol ester	0.92	23.5	6.7	16.8	9.2	2.3	7.0	19.1	5.1
Other neutral lipids	0.91	ND	ND	ND	ND	ND	ND	ND	ND
Polar lipids	1.32	15.9	5.4	19.5	12.7	6.9	3.0	25.1	3.2
Phosphatidylethanolamine	0.40	12.8	6.8	20.0	8.7	5.8	4.2	30.5	3.9
Phosphatidylcholine	0.37	20.7	4.5	21.9	15.3	7.7	1.8	19.8	2.0
Phosphatidylglycerol	0.21	13.2	4.5	14.2	15.9	7.7	2.7	24.3	3.1
Other polar lipids	0.34	ND	ND	ND	ND	ND	ND	ND	ND

[a]Strain YN-15 was cultivated in a medium containing 8% glucose and 3.2% yeast extract, pH 6.0, with shaking at 28°C for 7 d. C16:0, palmitic; C18:0, stearic; C18:1n-9, oleic; C18:2n-6, linoleic; C18:3n-6, γ-linolenic; C20:3n-6, dihomo-γ-linolenic; C20:4n-6, arachidonic; and C24:0, lignocerid acids. Minor fatty acid components are not shown. ND, not determined.

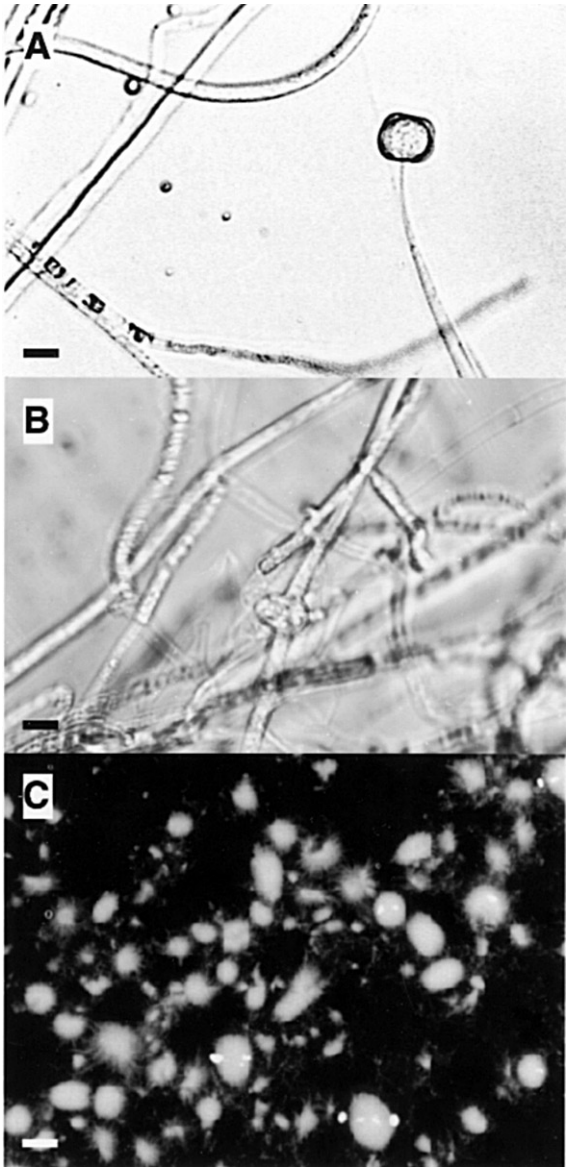

FIG. 1. Photomicrographs of sporangium (A), mycelia containing oil drops (B), and mycelial pellets (C) of *Mortierella alliacea* strain YN-15. Horizontal bars indicate 5 μm (A and B) and 1 mm (C).

lipid), consisted mainly of triacylglycerol (52% of neutral lipid), free fatty acid (23%), and sterol ester (13%) (Table 1). The polar lipid (1.32 g/L; 16% of total lipid) contained phosphatidylethanolamine (30% of polar lipid), phosphatidylcholine (28%), and phosphatidylglycerol (16%). With regard to the fatty acid composition of the lipid fractions, the content of AA was the highest (34.5%) in the triacylglycerol fraction. Although this fraction contained γ-linolenic acid (GLA, C18:3n-6) and DGLA, neither exceeded 10%.

Productivity of lipids rich in AA by M. alliacea *YN-15.* We evaluated the YN-15 strain's productivity of AA by cultivating it in liquid media containing different carbon and nitrogen sources. As summarized in Table 2, optimal growth and production of total intracellular fatty acids and AA were achieved by using a combination of glucose and yeast extract. When their contents in the medium were 8 and 2.4%, respectively, AA production was 3.9 g/L (44.6% in total fatty acid). Use of soluble starch and soybean meal gave excellent cell growth and AA productivity. Although we also tested the utility of inorganic compounds, e.g., ammonium acetate and ammonium sulfate, as a sole nitrogen source, a lower biomass yield was obtained (data not shown). Thus, we selected glucose and soluble starch as major carbon sources, and yeast extract and soybean meal as nitrogen sources for further study.

We examined the growth, total fatty acid content, and AA production of YN-15 at higher concentrations of medium components (Fig. 2). The optimal concentrations for AA production in each combination of carbon and nitrogen sources were obtained at (A) 15% glucose and 3% yeast extract, (B) 10% glucose and 3% soybean meal, (C) 12% soluble starch and 2.4% yeast extract, and (D) 12% soluble starch and 3% soybean meal. In most cases, 12% of the carbon source and a carbon/nitrogen ratio of 4 to 5 led to production of the highest content of AA (35–45%) in total fatty acid.

Figure 3 shows the effect of adding oils or organic/inorganic compounds, at 0.2% to the starch-based medium, on AA production by YN-15. In comparison to a medium with no additive (1.82 g AA/L), the addition of oleic acid, soybean oil, ammonium sulfate, or dipotassium phosphate increased yields of total fatty acid and AA about 1.5-fold. Moreover, a simultaneous addition of soybean oil and dipotassium phosphate to the basal medium boosted AA productivity up to 4 g/L but a further supplement with ammonium sulfate counteracted this effect (data not shown). Since we obtained similar results for the effect of additives on the glucose-based medium, we used 0.2% each of dipotassium phosphate and soybean oil for large scale cultivations.

In using the optimized conditions, batch cultures at prepilot plant scale (25 L) were carried out in 50-L jar fermenters. The dissolved oxygen concentration was kept at 50 ppm during the cultivation, which affected the maintenance of macroscopic mycelial morphology of smooth pellets (20). The highest productivity of AA was obtained with a medium consisting of glucose and soybean meal (Fig. 4). On day 6, the fermentation resulted in a biomass of 46.1 g/L, total fatty acid yield of 19.5 g/L, and AA yield of 7.1 g/L, i.e., 1.2 g of AA/L/d; 36.4% of total fatty acid as AA. The yields of biomass and AA vs. glucose consumed (102 g/L as total sugar) was 45.2 and 7.0%, respectively. In using media comprising either glucose plus yeast extract or soluble starch plus soybean meal, AA productivity was about 5 g/L. In the case of starch medium, a low level of residual reducing sugar in the culture broth was observed throughout the cultivation.

DISCUSSION

A number of strains in the genus *Mortierella*, e.g., *M. alpina* and *M. elongata*, have been studied as AA producers (11–13). Although this is the first report focusing on the species *M. alliacea*, the YN-15 strain has some properties similar to those of *M. alpina* strain 1S-4 (13,18,19) with respect to lipid and fatty acid production. The n-3 fatty acids, mainly EPA, accumulated in mycelia of the YN-15 when cultivated at 15°C.

TABLE 2
Effect of Different Carbon and Nitrogen Sources on Arachidonic Acid Production by *Mortierella alliacea* YN-15[a]

Carbon source	Nitrogen source	Dry cell weight (g/L)	Total fatty acid (g/L)	Arachidonic acid (% in total fatty acid)	Arachidonic acid (g/L)
Experiment 1					
Glucose	Yeast extract	25.0	4.50	43.3	1.95
Maltose	Yeast extract	18.4	2.96	21.6	0.64
Saccharose	Yeast extract	5.0	0.12	34.6	0.04
Lactose	Yeast extract	4.2	0.08	35.4	0.03
Soluble starch	Yeast extract	23.5	3.68	43.2	1.59
Soluble starch	Soybean meal	41.7	6.80	37.4	2.54
Experiment 2					
Glucose	Yeast extract	33.0	8.81	44.6	3.93
Glucose	Soybean meal	32.5	9.92	37.5	3.72
Glucose	Corn steep liquor	27.2	6.33	28.1	1.78
Glucose	Polypeptone	24.8	3.76	35.4	1.33
Glucose	Tryptone	21.5	3.15	23.5	0.74
Glucose	Malt extract	6.5	2.51	34.7	0.87

[a]Strain YN-15 was cultivated with shaking at 28°C in media containing 8% of each carbon source and 1.6% yeast extract or soybean meal, pH 6.0, for 7 d (experiment 1) or 8% glucose and 2.4% of each nitrogen source, pH 6.0, for 7 d (experiment 2).

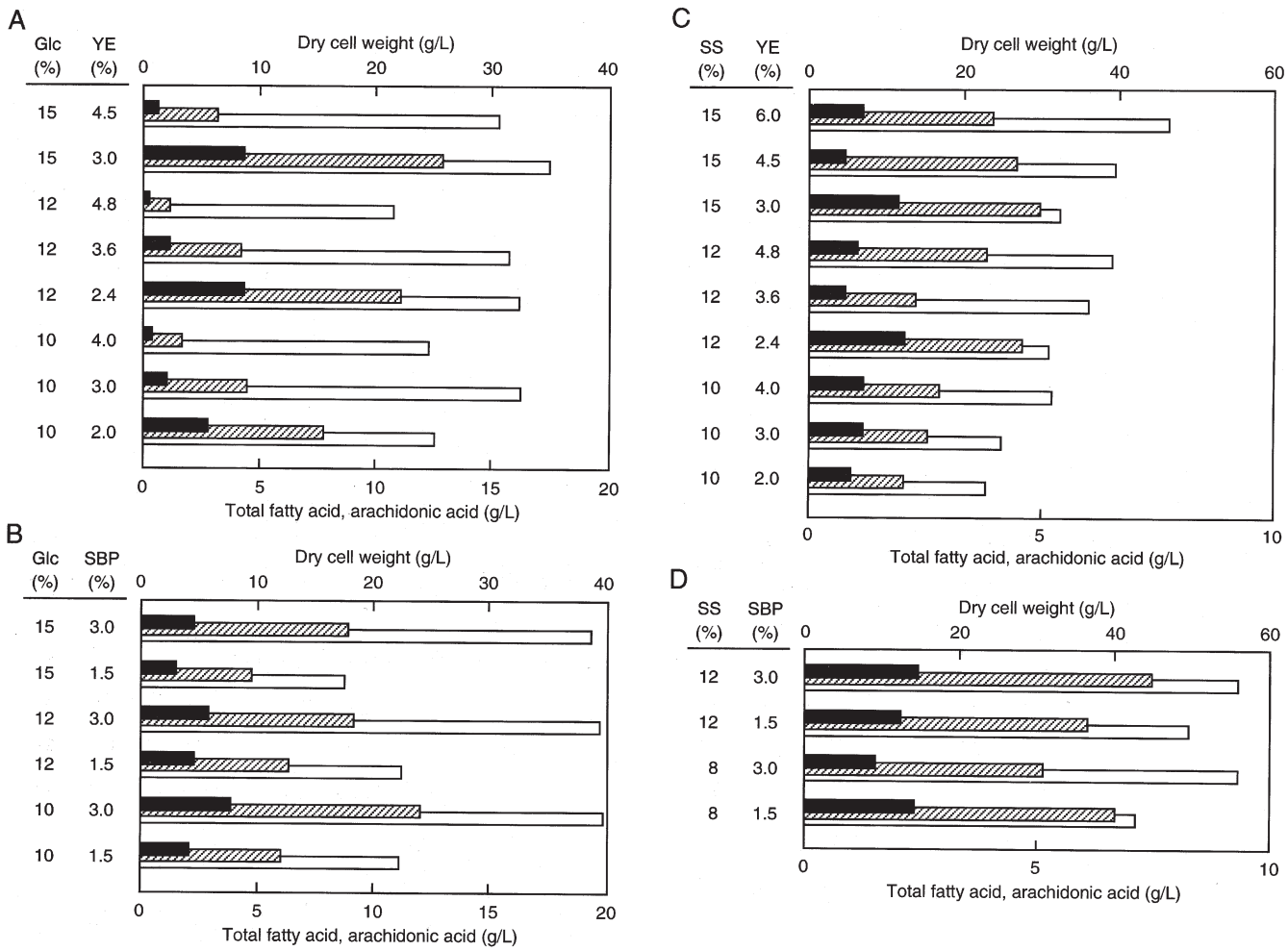

FIG. 2. Effects of media carbon and nitrogen sources and their concentrations on lipid content and arachidonic acid (AA) production in *Mortierella alliacea* YN-15. The strain was cultivated at 28°C for 7 d in a medium consisting of (A) glucose (Glc) and yeast extract (YE); (B) Glc and soybean meal (SBP); (C) soluble starch (SS) and YE; or (D) SS and SBP, at the indicated concentrations, pH 7.0. Dry cell weight (open bars), yields of total fatty acid (hatched bars), and AA (solid bars) were measured as described in the text.

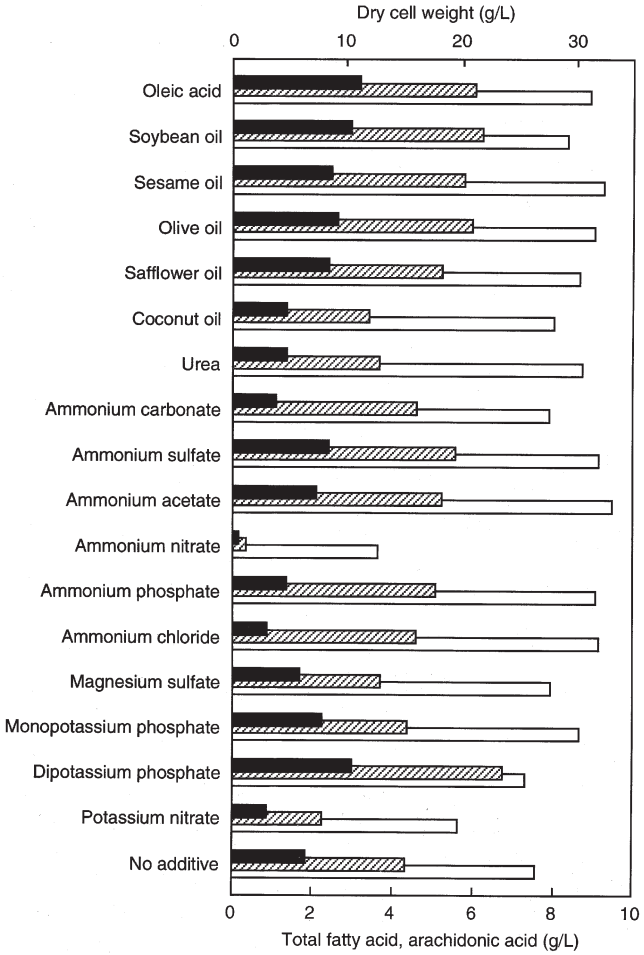

FIG. 3. Influence of oil and salt additives on production of AA by *M. alliacea* YN-15. The YN-15 strain was cultivated in media containing 12% soluble starch, 2.4% yeast extract, and 0.2% of indicated material, pH 6.0, in a 200-mL baffled flask at 28°C for 7 d. For key and abbreviations see Figure 2.

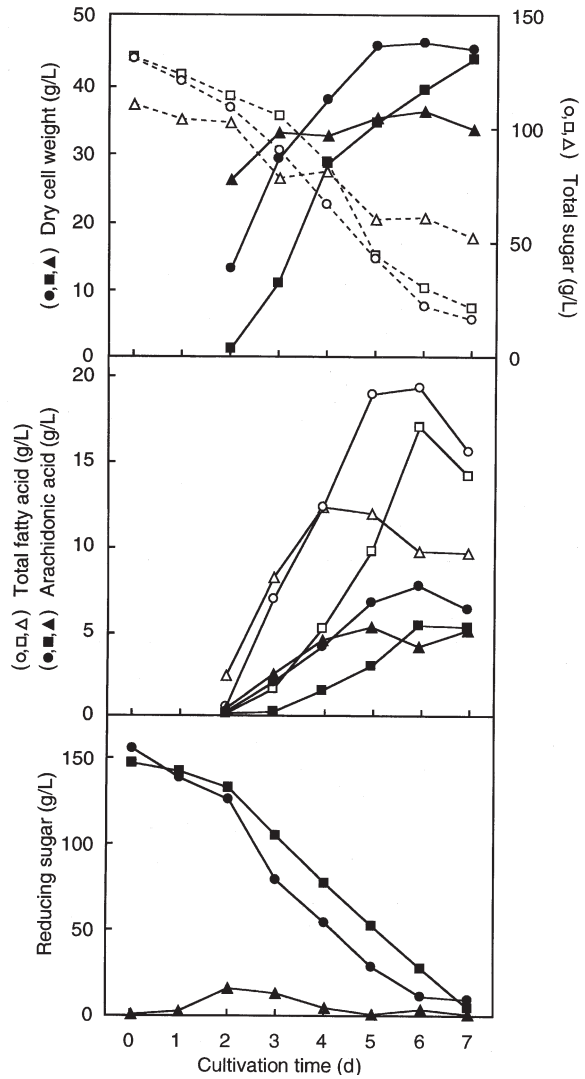

FIG. 4. Pre-pilot scale fermentation of AA by *M. alliacea* YN-15. Cultivations were conducted at 28°C in 25-L batches of media containing 12% carbon source, 3% nitrogen source, 0.2% dipotassium phosphate, and 0.2% soybean oil, pH 7.0. Detailed conditions are described in the text. (●,○) glucose and soybean meal; (■,□) glucose and yeast extract; (▲,△) soluble starch and soybean meal.

This alteration of fatty acid profile is considered a consequence of thermal induction of ω-3 desaturase that converts n-6 fatty acids into n-3 acids (18). Whether the desaturase induction is at the gene expression level is open to discussion, since the fungal ω-3 desaturase has yet to be identified.

The cultivation of YN-15 in the presense of sesamin or sesame oil resulted in a reduction of AA content and an accumulation of DGLA, as in the 1S-4 strain (data not shown). The effect of sesamin and related lignan compounds was examined in an *in vitro* desaturase assay using cell extracts from 1S-4, and these lignan compounds appeared to inhibit the desaturation of DGLA at the Δ5 position noncompetitively (19). Besides, the enhancement of AA productivity by the addition of phosphate salts and oils rich in oleic acid, such as soybean oil (Fig. 3), is also a property common to *M. alliacea* and *M. alpina* (18). The role of phosphate salts may be not only in their buffering effect but also in their control of growth morphology (21), as discussed below.

Mortierella alliacea YN-15 thus shares some characteristics with other *Mortierella* species with respect to the basic properties of lipid and fatty acid biosynthesis. However, the YN-15 strain possesses at least two advantages favorable for use in industrial fermentation. One of them is its adaptability to highly concentrated media, which leads to a potential merit of reducing the feeding processes in large-scale cultivation. In this report, in which a batch culture of YN-15 was studied at the pre-pilot scale (25 L medium in a 50-L tank), a maximal AA production of 7.1 g/L/6-d was obtained when glucose (12%) and soybean meal (3%) were used as main medium components (Fig. 4). The AA productivity (1.18 g/L/d) was comparable to or even higher than those previously reported. As for batch cultivation, Totani *et al.* (12) described a pilot-scale cultivation of *M. alpina* in a medium containing 10% potato dextrose, where the fermentation yield at 16 d was 0.69 g AA/L/d. Optimization of AA production with response surface methodology by Chen *et al.* (22) succeeded in yielding 0.78 g AA/L/d in a 10% starch medium, but the experi-

ment has yet to be conducted on a large scale. Nevertheless, we used the higher concentration of glucose (12%) in our study, and up to 85% of this was consumed by YN-15 (Fig. 4). Furthermore, since we have observed high levels of cell growth and AA production in 15% glucose media (Fig. 2), the fermentation may be further improved by the use of even more concentrated media. In this regard, it might be interesting to study the effect of medium osmolarity on lipid production.

The other advantage of YN-15 is its excellent starch assimilation, which was expected since this strain was isolated using rice grains as carbon source. The use of starch instead of glucose would avoid or reduce the effects of catabolite repression and osmotic stress, especially at high concentrations. Our prepilot scale cultivation in starch-based medium produced an AA yield of 5 g/L on day 5 (Fig. 4). A productivity of 1 g/L/d is even higher than those in previous batch culture studies (12,22). However, after day 6, the accumulated fatty acids seemed to be utilized as an energy source to maintain cell functions, for the AA content decreased. The problem was that about 40% of the total sugar (as starch) remained to be assimilated at the end of the cultivation. This should be solved by allowing YN-15 to employ starch as a sole carbon source. That the liberation of reducing sugar was insignificant over the second half of the cultivation period indicated saccharification of the starch was a limiting step. Furthermore, the activity of amylolytic enzyme(s) was extremely low, and therefore the culture accumulated branched polysaccharides that are not susceptible to saccharogenic enzymes like glucoamylase and glucosidases. Indeed, we detected the activity of exo-type enzymes that act on starch as well as oligosaccharides but not on branched sugar, but we could not detect any end-type enzymes such as amylase (Tanaka, Y., and T. Aki, unpublished data). Studies on this and other amylolytic enzymes from YN-15 are ongoing. Moreover, use of cheaper raw starch could be desirable from an industrial viewpoint, although we used soluble starch for the present study.

The strain YN-15 has another potentially useful feature in that the mycelia tend to form packed pellets with a diameter of ~2 mm, rather than unstable filaments (Fig. 1C). Higashiyama *et al.* (23) studied the relation of mycelial morphology to AA production in *M. alpina* 1S-4. Pellets of the 1S-4 mycelia were partially shaved off during cultivation, and the filamentous fraction generated contained less lipid. Morphology is also regulated by mineral addition (21) and dissolved oxygen (20). We assume intertwined mycelia in the "viable pellets" are able to capture the air bubbles. This could be profitable for cultivation in high concentration media where dissolved oxygen is limited.

REFERENCES

1. Leyton, J., P.J. Drury, and M.A. Crawford, *In Vivo* Incorporation of Labeled Fatty Acids in Rat Liver Lipids After Oral Administration, *Lipids 22*:553–558 (1987).
2. Banerjee, N., and M.D. Rosenthal, High-Affinity Incorporation of 20-Carbon Polyunsaturated Fatty Acids by Human Skin Fibroblasts, *Biochim. Biophys. Acta 835*:533–541 (1985).
3. Kragballe, K., and J.J. Voorhees, Arachidonic Acid and Leukotrienes in Dermatology, *J. Invest. Dermatol. 81*:293–296 (1983).
4. Swann, P.G., D.L. Venton, and G.C. Le Breton, Eicosapentaenoic Acid and Docosahexaenoic Acid Are Antagonists at the Thromboxane A2/Prostaglandin H2 Receptor in Human Platelets, *FEBS Lett. 243*:244–246 (1989).
5. Carlson, S.E., The Role of PUFA in Infant Nutrition, *INFORM 6*:940–946 (1995).
6. Watanabe, S., H. Hayashi, K. Onozaki, and H. Okuyama, Effect of Dietary Alpha-Linolenate/Linoleate Balance on Lipopolysaccharide-Induced Tumor Necrosis Factor Production in Mouse Macrophages, *Life Sci. 48*:2013–2020 (1991).
7. Yamamoto, N., M. Saitoh, A. Moriuchi, M. Nomura, and H. Okuyama, Effect of Dietary Alpha-Linolenate/Linoleate Balance on Brain Lipid Compositions and Learning Ability of Rats, *J. Lipid Res. 28*:144–151 (1987).
8. Nakashima, Y., S. Yuasa, Y. Hukamizu, H. Okuyama, T. Ohhara, T. Kameyama, and T. Nabeshima, Effect of a High Linoleate and a High Alpha-Linolenate Diet on General Behavior and Drug Sensitivity in Mice, *Ibid. 34*:239–247 (1993).
9. Carlson, S.E., A.J. Ford, S.H. Werkman, J.M. Peeples, and W.W. Koo, Visual Acuity and Fatty Acid Status of Term Infants Fed Human Milk and Formulas With and Without Docosahexaenoate and Arachidonate from Egg Yolk Lecithin, *Pediatr. Res. 39*:882–888 (1996).
10. Carlson, S.E., R.J. Cooke, S.H. Werkman, and E.A. Tolley, First Year Growth of Preterm Infants Fed Standard Compared to Marine Oil n-3 Supplemented Formula, *Lipids 27*:901–907 (1992).
11. Radwan, S.S., Sources of C_{20}-Polyunsaturated Fatty Acids for Biotechnological Use, *Appl. Microbiol. Biotechnol. 35*:421–430 (1991).
12. Totani, N., K. Someya, and K. Oba, Industrial Production of Arachidonic Acid by *Mortierella*, in *Industrial Applications of Single Cell Oils*, edited by D.J. Kyle and C. Ratledge, American Oil Chemists' Society, Champaign, 1992, pp. 52–60.
13. Certik, M., and S. Shimizu, Biosynthesis and Regulation of Microbial Polyunsaturated Fatty Acid Production, *J. Biosci. Bioeng. 87*:1–14 (1999).
14. Aki, T., Y. Matsumoto, T. Morinaga, S. Kawamoto, S. Shigeta, K. Ono, and O. Suzuki, Lipid Composition of Newly Isolated Polyunsaturated Fatty Acid Producing Fungus, *Achlya* sp. ma-2801, *J. Ferment. Bioeng. 86*:504–507 (1998).
15. Dubois, M., K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith, Colorimetric Method for Determination of Sugars and Related Substances, *Anal. Chem. 28*:350–356 (1956).
16. Somogyi, M., Notes on Sugar Determination, *J. Biol. Chem. 195*:19–23 (1952).
17. Linnemann, G., *Mucorales: Eine Beschreibung Aller Gattungen und Arten Dieser Pilzgruppe*, edited by H. Zycha and R. Siepmann, J. Cramer, Lehre, Germany, 1969, pp. 187–197.
18. Shimizu, S., H. Kawashima, Y. Shinmen, K. Akimoto, and H. Yamada, Production of Eicosapentaenoic Acid by *Mortierella* Fungi, *J. Am. Oil Chem. Soc. 65*:1455–1459 (1988).
19. Shimizu, S., K. Akimoto, Y. Shinmen, H. Kawashima, M. Sugano, and H. Yamada, Sesamin Is a Potent and Specific Inhibitor of Delta 5 Desaturase in Polyunsaturated Fatty Acid Biosynthesis, *Lipids 26*:512–516 (1991).
20. Higashiyama, K., K. Murakami, H. Tsujimura, N. Matsumoto, and S. Fujikawa, Effects of Dissolved Oxygen on the Morphology of an Arachidonic Acid Production by *Mortierella alpina* 1S-4, *Biotechnol. Bioeng. 63*:442–448 (1999).
21. Higashiyama, K., T. Yaguchi, K. Akimoto, S. Fujikawa, and S. Shimizu, Effects of Mineral Addition on the Growth Morphology of and Arachidonic Acid Production by *Mortierella alpina* 1S-4, *J. Am. Oil Chem. Soc. 75*:1815–1819 (1998).
22. Chen, H.C., C.C. Chang, and C.X. Chen, Optimization of Arachidonic Acid Production by *Mortierella alpina* Wuji-H4 Isolate, *Ibid. 74*:569–578 (1997).
23. Higashiyama, K., S. Fujikawa, E.Y. Park, and M. Okabe, Image Analysis of Morphological Change During Arachidonic Acid Production by *Mortierella alpina* 1S-4, *J. Biosci. Bioeng. 87*:489–494 (1999).

[Received November 28, 2000; accepted March 5, 2001]