Appl Microbiol Biotechnol (2004) 66: 243–248
DOI 10.1007/s00253-004-1682-7

Eiji Sakuradani · Yuriko Hirano · Nozomu Kamada · Masutoshi Nojiri · Jun Ogawa · Sakayu Shimizu

Improvement of arachidonic acid production by mutants with lower n-3 desaturation activity derived from *Mortierella alpina* 1S-4

Received: 5 March 2004 / Revised: 24 May 2004 / Accepted: 31 May 2004 / Published online: 5 August 2004

Abstract Five mutants were obtained, Y11, Y135, Y164, Y180 and Y61, capable of accumulating higher amounts of arachidonic acid (AA) than *Mortierella alpina* 1S-4, an industrial strain for the production of AA-rich triacylglycerol (TG). This is thought to be due to low or no activity of n-3 desaturation with conversion of AA to eicosapentaenoic acid, which functions at a cultural temperature below 20°C. In small-scale cultivation under optimum conditions, Y11 and Y61 respectively accumulated 4.97 mg/ml and 4.11 mg/ml of AA, using a high concentration of glucose at 20°C, compared with 3.74 mg/ml for *M. alpina* 1S-4. In a 5-l jar fermentor, the AA content in Y11 and Y61 kept increasing during cultivation, with consumption of the glucose in the medium; and this reached 1.48 mg/ml and 1.77 mg/ml (118 mg/g, 120 mg/g of dry mycelia) at day 10, respectively, compared with 0.95 mg/ml (86 mg/g of dry mycelia) for *M. alpina* 1S-4. From the results of lipid analysis, the TG contents of Y11 and Y61 in the major lipids were significantly higher than that of *M. alpina* 1S-4; and the AA percentages in TG of Y11 and Y61 were also higher. Both Y11 and Y61 are potential producers of TG rich in AA.

Introduction

Arachidonic acid (5,8,11,14-*cis*-eicosatetraenoic acid; AA), as a precursor for prostaglandins, leukotrienes and thromboxanes, has various regulatory effects and physiological functions in humans; and it plays important roles in infant nutrition (Carlson et al. 1993; Gill and Valivety 1997). Thus, AA is one of the essential C_{20} polyunsaturated fatty acids (PUFAs) for humans. Although *Mortierella* fungi (Shinmen et al. 1989), animal liver, fish oil and egg yolk are well known as sources of AA, the fungal triacylglycerol (TG) seems to be the most prominent source among them in terms of high AA content, low production cost and ease of handling.

In a previous paper (Yamada et al. 1987), we reported that *M. alpina* 1S-4 isolated from soil shows the highest productivity among the *Mortierella* species tested. Furthermore, mineral addition in the culture medium influences the growth morphology and enhances the AA production by *M. alpina* 1S-4 (Higashiyama et al. 1998). AA yield is enhanced 1.7-fold over that without minerals on cultivation in a 10-l jar fermentor. *M. alpina* 1S-4 is now used as a practical strain for the large-scale production of TG rich in AA. Some PUFAs such as dihomo-γ-linolenic acid (8,11,14-*cis*-eicosatrienoic acid; DGLA) and Mead acid (5,8,11-*cis*-eicosatrienoic acid) are also produced by mutants derived from *M. alpina* 1S-4 (Jareonkitmongkol et al. 1993; Sakuradani et al. 2002). The optimal temperature for AA production by *M. alpina* 1S-4 ranges from 16°C to 28°C. The fungus accumulates n-3 PUFAs through n-3 desaturation activated by temperatures below 20°C, when part of the AA synthesized through the n-6 pathway is further converted to 5,8,11,14,17-*cis*-eicosapentaenoic acid (EPA; Shimizu et al. 1988). Thus, the AA production of this fungus decreases at low cultural temperature.

We assumed that a mutant with no n-3 desaturation activity is not capable of producing EPA and results in a higher production of AA. In order to accomplish a high production of AA, screening of mutants derived from *M. alpina* 1S-4 was carried out. As a result, we isolated some mutants with low or no n-3 desaturation activity and found each kind of mutant produced higher amounts of AA when compared with the parental strain. This paper describes the isolation of the mutants and evaluation of their AA productivities under various cultivation conditions.

E. Sakuradani · Y. Hirano · N. Kamada · M. Nojiri · J. Ogawa · S. Shimizu (✉)
Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University,
Kitashirakawa-oiwakecho, Sakyo-ku,
Kyoto, 606-8502, Japan
e-mail: sim@kais.kyoto-u.ac.jp
Tel.: +81-75-7536113
Fax: +81-75-7536128

Materials and methods

Chemicals

Adekanol, a polyether-type antifoaming agent, was purchased from Asahi Denka Industry (Tokyo, Japan). All other chemicals were commercially available and obtained as described by Jareonkitmongkol et al. (1992a).

Mutagenesis and isolation of mutants

M. alpina 1S-4, the wild strain (Shinmen et al. 1989; Yamada et al. 1987), was used for mutagenesis. Mutagenesis and isolation of mutants were essentially performed according to the methods described by Jareonkitmongkol et al. (1992a). Spores of *M. alpina* 1S-4 were treated with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) as a mutagen. On the first screening, each strain was cultivated on solid medium containing 1% glucose and 0.5% yeast extract, pH 6.0, at 28°C for 2 days and then at 12°C for 2 days. The mycelial fatty acid profiles of 3,000 colonies derived from MNNG-treated spores were analyzed by gas–liquid chromatography (GLC) as described below. Five mutants with high AA contents were selected: Y11, Y61, Y135, Y164 and Y180.

Liquid culture

M. alpina 1S-4 and its mutants were each inoculated as a spore suspension into a 20-ml Erlenmeyer flask containing 4 ml of medium containing glucose and yeast extract, followed by incubation with reciprocal shaking (120 strokes/min), unless stated otherwise. For lipid analysis, fungal strains were cultivated in a 300-ml Erlenmeyer flask containing 50 ml of medium including 2% glucose and 1% yeast extract, with shaking, for 7 days at 28°C or for 5 days at 20°C after preincubation for 2 days at 28°C. For bench-scale production, fungal strains were each precultured in a 300-ml Erlenmeyer flask containing 50 ml of medium including 2% glucose and 1% yeast extract, with shaking, for 2 days at 28°C; and the following main culture was carried out in a 5-l jar-fermentor (Able Corporation, Tokyo, Japan) with a working volume of 2.5 l of medium including 2% glucose, 1% soy flour, 0.05% Adekanol, 0.3% KH_2PO_4, 0.1% Na_2SO_4, 0.05% $CaCl_2{\cdot}2H_2O$, 0.05% $MgCl_2{\cdot}6H_2O$, pH 6.0, with a 2% inoculum, an agitation speed of 300 rpm and an aeration rate of 1 l/min. During cultivation in jar-fermentor, the glucose concentration was maintained at 1–3% by glucose-feeding. For other conditions for jar-fermentations, see Fig. 4.

Fatty acid and lipid analyses

Mycelial growth was evaluated as the dry mycelial weight 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 of dried mycelia were directly transmethylated in methanolic HCl and the resultant fatty acid methyl esters were extracted with *n*-hexane and quantified by GLC as described by Shimizu et al. (1991). Mycelial lipids were extracted with the chloroform/methanol/water system described by Folch et al. (1957). Neutral and polar lipids were separated by thin-layer chromatography (Shimizu et al. 1991) and the fatty acid composition of each lipid class was analyzed by GLC, as described above.

Other methods

All the values in the figures are means of three independent determinations, unless stated otherwise. The differences among the values were less than 5% of the means.

Results

Isolation of mutants for AA production

Through the analysis of the fatty acid composition of mycelia obtained from 3,000 MNNG-treated spores of *M. alpina* 1S-4, five mutants were selected (Y11, Y135, Y164, Y180, Y61) which had higher AA content and/or lower EPA content when grown at 12°C. The percentage of EPA in the total fatty acids in Y135, Y164 and Y180 was 0.32, 0.71 and 0.55%, respectively, in contrast to 2.70% in *M. alpina* 1S-4. Y11 showed no accumulation of EPA at all. These four mutants seemed to have low or no n-3 desaturation activity. Only Y61 was different from the other mutants in fatty acid composition: in spite of having the same percentage of EPA as the parent, it had a higher percentage of AA in the total fatty acids. These mutants were used for further evaluation. Although other mutants were obtained with slightly higher AA production in the first experiment, most of them could not show this character in the second. Furthermore, mutants with a low growth rate or producing odd-chain fatty acids, such as 15:0 and 17:0, were obtained with a frequency of about 10 per 3,000 samples, which might obstruct AA production.

Factors affecting AA production

Growth temperature

M. alpina 1S-4 and five mutants were cultivated at 28°C and then the growth temperature was shifted to 12, 16, 20, 24 or 28°C to determine the optimal temperature for AA production (Fig. 1). All strains grew well at all tested temperatures. Although there were no morphological differences among all mutants and *M. alpina* 1S-4 cultivated on solid media, the morphological form of Y164 was the “filamentous” type in submerged culture

and those of other mutants were "pellet" types (for a definition of each term, see Park et al. 1999). All strains produced their highest amount of AA in culture broth at 16°C or 20°C (respectively, *M. alpina* 1S-4, Y11, Y135, Y180 gave 1.07, 2.21, 1.65, 1.65 mg/ml at 16°C, whereas Y61, Y164 gave 1.79, 1.99 mg/ml at 20°C). These values of all five mutants were 1.5- to 2.1-fold higher than that of *M. alpina* 1S-4. In each of the above cultural conditions, the EPA contents of *M. alpina* 1S-4, Y135, Y164, Y180 and Y61 were 0.03, 0.01, 0.01, 0.05 and 0.01 mg/ml, respectively, whereas only Y11 could not produce EPA during cultivation.

Glucose concentration in medium

All strains were cultivated in medium containing 0–10% glucose and 1% yeast extract at 28°C for 2 days and then at 20°C for 7 days (Fig. 2). Growth of *M. alpina* 1S-4, Y135 and Y180 increased in proportion to the glucose concentration, up to 4%, and decreased under conditions above 4%. In contrast, Y11, Y61 and Y164 could grow well at high glucose concentration (5–10%). The maximum amounts of AA production of *M. alpina* 1S-4, Y135 and Y180 reached 2.69, 3.61 and 3.86 mg/ml of culture broth with 4% glucose, respectively. Those of Y61 and Y164 reached 4.20 mg/ml and 3.46 mg/ml at 5%, respectively, and that of Y11 was 4.05 mg/ml at 7.5%. Thus, all the mutants were shown to accumulate amounts of AA at each optimal glucose concentration 1.3- to 1.6-fold higher than that for *M. alpina* 1S-4.

AA productivity under individual optimum condition in small-scale cultivation

All strains were cultivated in each optimum condition, referring to growth temperature and glucose concentration, as shown in Fig. 3. The growth of all strains increased with consumption of the added glucose in the medium and then, with the exception of Y11, growth declined with exhaustion of the glucose. Because of the high initial concentration of glucose (8%), the growth of Y11 linearly increased until day 14. The maximum AA production of *M. alpina* 1S-4, Y11, Y61, Y135, Y164 and Y180 reached 3.74 mg/ml (AA content in total mycelial fatty acids was 57.5% at day 14), 4.97 mg/ml (51.3% at day 14), 4.11 mg/ml (58.3% at day 16), 3.51 mg/ml (60.0% at day 16), 4.19 mg/ml (50.6% at day 14) and 3.93 mg/ml (59.3% at day 16), respectively. These results indicate that greater values in both AA production and mycelial growth can be obtained with Y11, Y61 and Y164, on cultivation with a high initial glucose concentration in the medium.

Bench-scale production

Considering the AA productivities in small-scale cultivation, Y11 and Y61 were selected for bench-scale production, using a 5-l jar fermentor (Fig. 4). The medium used contained soy flour as a nitrogen source, as reported for the large-scale production of PUFA (Higashiyama et al. 1998). During cultivation, 50 g of glucose was fed to each jar fermentor at days 2 and 4 to maintain a 1–3% glucose concentration. The final glucose concentrations in the media at day 10 were 2.0, 1.4 and 1.5% in the jar

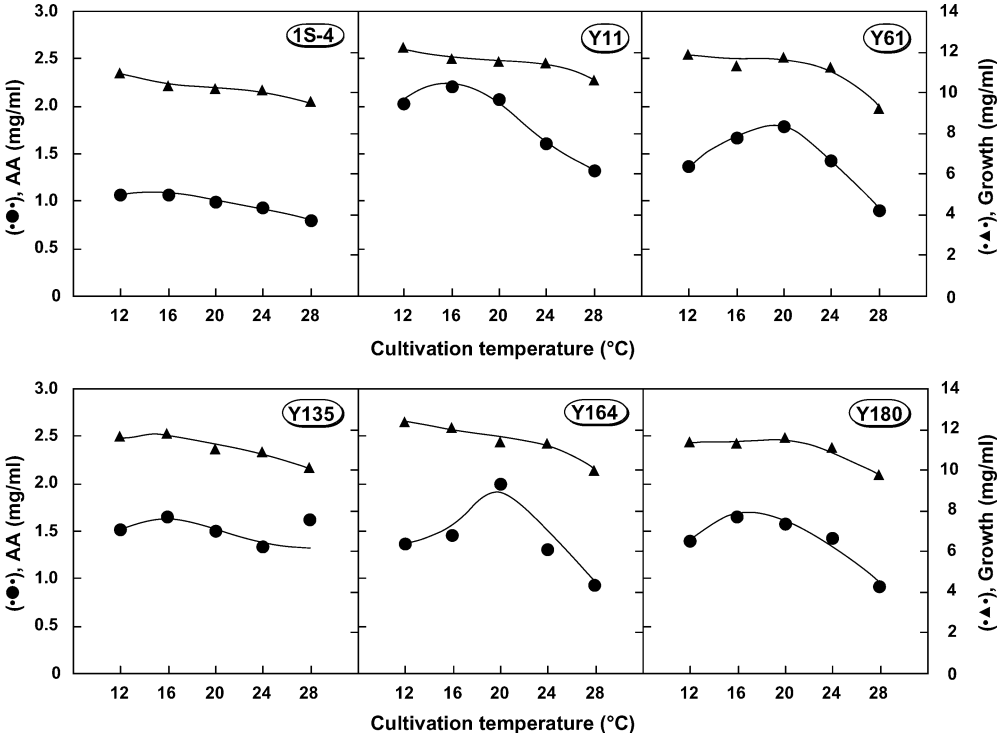

Fig. 1 Effects of growth temperature on AA production by *M. alpina* 1S-4, Y11, Y61, Y135, Y164 and Y180. Each stain was cultivated in a medium containing 2% glucose and 1% yeast extract at 28°C for 2 days and then at the indicated temperature (12–28°C) for 7 days

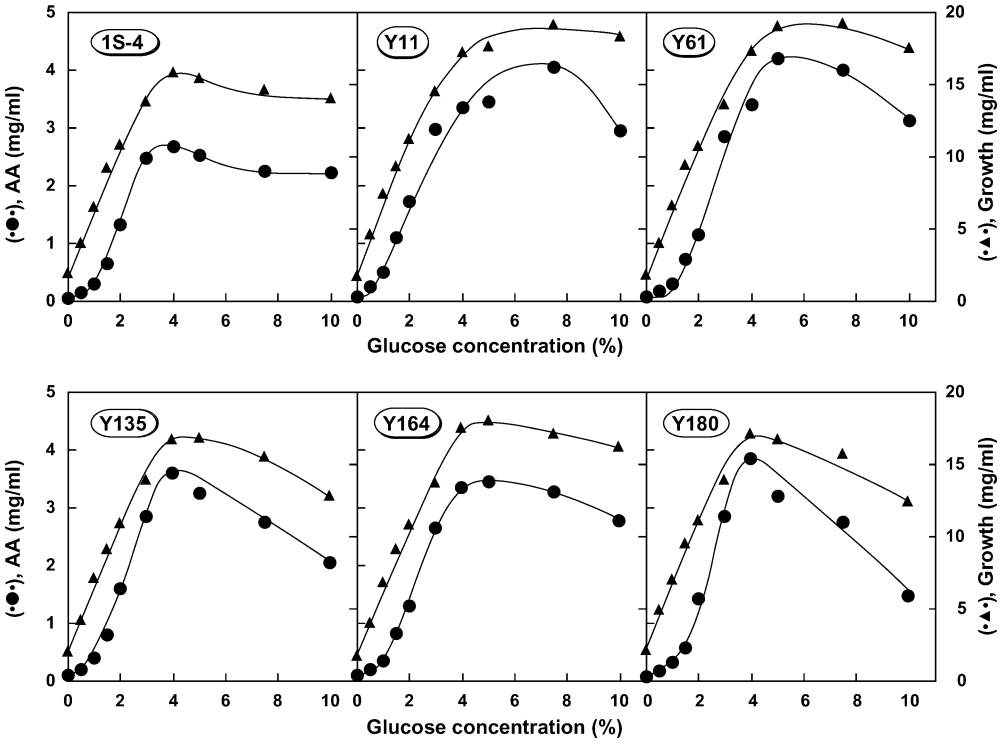

Fig. 2 Effects of glucose concentration in the medium on AA production by *M. alpina* 1S-4, Y11, Y61, Y135, Y164 and Y180. Each strain was cultivated in a medium containing 1% yeast extract and 0, 0.5, 1, 1.5, 2, 3, 4, 5, 7.5 or 10% glucose at 28°C for 2 days and then at 20°C for 7 days

fermentors of *M. alpina* 1S-4, Y11 and Y61, respectively. Growth of *M. alpina* 1S-4 and Y61 reached 12.9 mg/ml and 15.4 mg/ml at days 4 and 5, respectively, and then decreased gradually. Although the growth of Y11 was slow at the early stage, it reached 14.5 mg/ml at day 8. The AA production of *M. alpina* 1S-4 reached 0.95 mg/ml (86 mg/g of dry mycelia) at day 6 and then decreased gradually, whereas those of Y11 and Y61 kept increasing during cultivation and reached 1.48 mg/ml and 1.77 mg/ml (118 mg/g, 120 mg/g of dry mycelia) at day 10, respectively. The maximum amounts of AA in Y11 and Y61 were 1.56- and 1.86-fold greater than that in *M. alpina* 1S-4, respectively. The AA contents were 28.7, 35.0 and 38.8% of total fatty acids in *M. alpina* 1S-4, Y11 and Y61 at day 10, respectively. The percent AA in *M. alpina* 1S-4 at day 6, when AA production reached a maximum, was only 24.0%.

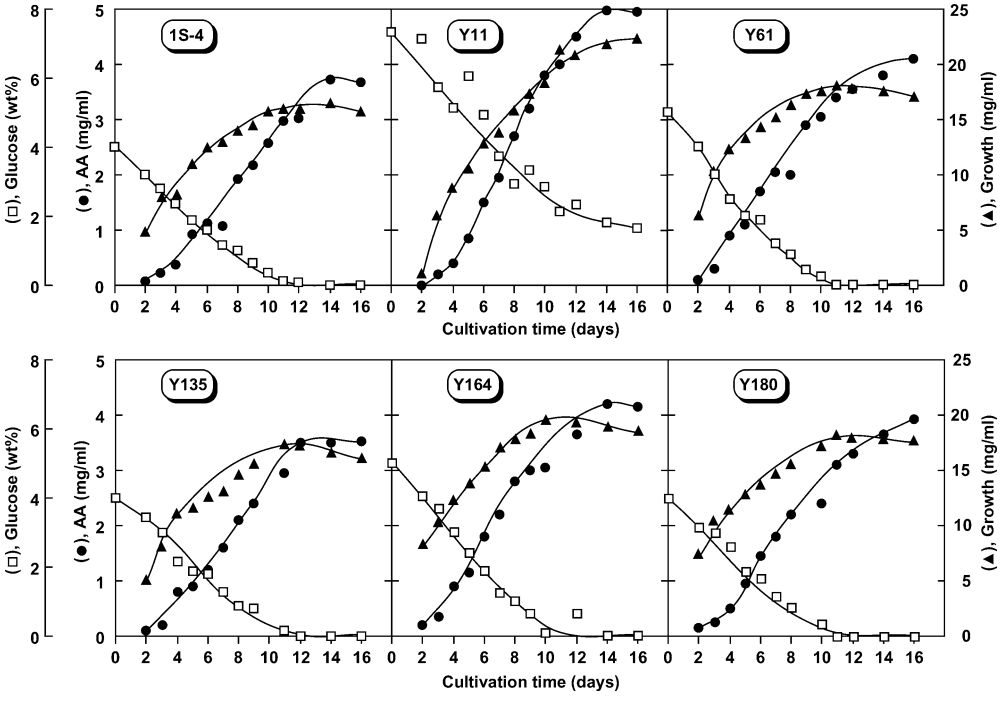

Fig. 3 Time-courses of AA content, growth and glucose consumption. Each fungus was cultivated in medium containing 4.0, 5.0 or 7.5% glucose and 1% yeast extract at 28°C for 2 days and then at 16°C or 20°C for 14 days. The medium for cultivation of *M. alpina* 1S-4, Y135 and Y180 contained 4% glucose; that for Y61 and Y164, 5%; and that for Y11, 7.5%. The cultivation temperature for Y61 and Y164 was 20°C and for others 16°C

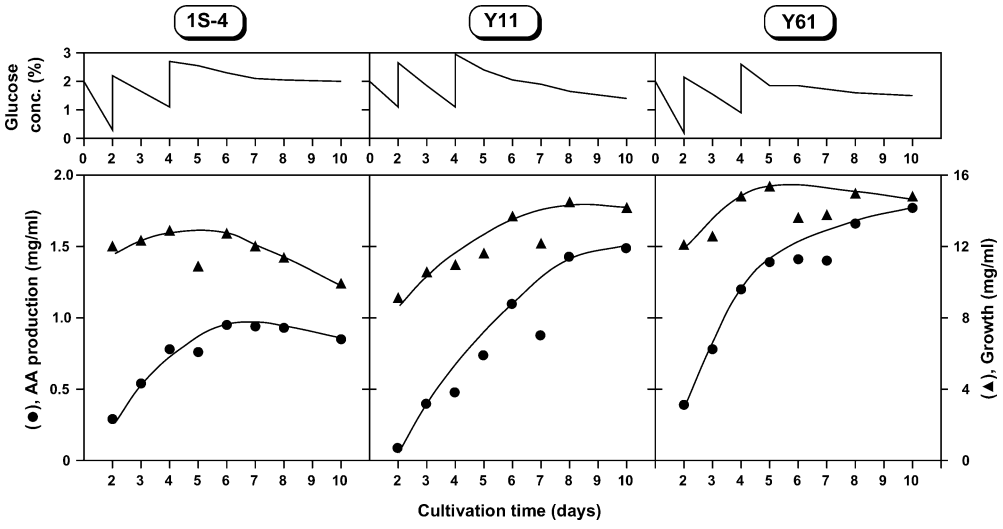

Fig. 4 Production of AA in a 5-l jar fermentor. Each fungus was cultivated in 2.5 l of medium (2% glucose, 1% soy flour, 0.01% Adekanol 0.3% KH_2PO_4, 0.1% Na_2SO_4, 0.05% $CaCl_2 \cdot 2H_2O$, 0.05% $MgCl_2 \cdot 6H_2O$, pH 6.0) at 28°C for 4 days and then at 20°C for 6 days, with aeration at 1 l/min and agitation at 300 rpm

AA and EPA contents of major lipids

The lipid analysis revealed that the contents of TG in Y11 [90.2 (85.4)%] and Y61 [89.2 (84.6)%] cultivated at 28°C (20°C) were higher than that of *M. alpina* 1S-4 [82.5 (76.8)%]. As shown in Fig. 5, the AA contents of major lipids in Y11 and Y61 were higher than that in *M. alpina* 1S-4. Especially, the AA contents of TG and polar lipid in Y11 cultivated at 20°C were significantly higher, with no accumulation of EPA.

Discussion

Previous reports described some kinds of desaturation activity-defective mutants (Jareonkitmongkol et al. 1993; Sakuradani et al. 2002) and some of them have been used for the large-scale production of PUFAs. GLC chromatograms of the fatty acid methyl esters prepared from those mutants, which greatly differed from that of the wild strain, were very specific. In the search for higher AA-accumulating mutants, as described in the present paper, we carefully focused on the level of AA in total fatty acids in the GLC chromatograms rather than their apparent profiles. One criterion was to select mutants with a lower content of EPA, even if they were cultivated at low temperature. This was based on the assumption that AA content as a final product of n-6 PUFAs would increase in strains with n-3 desaturation-deficiency, transforming n-6 to n-3 PUFAs.

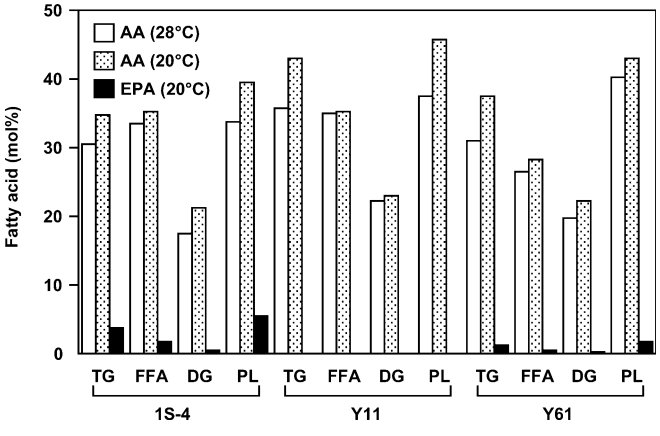

Fig. 5 AA and EPA contents in major lipid fractions. The cultural condition is described in the Materials and methods. Fatty acid content is represented as mol% total fatty acids. *DG* Diacylglycerol, *FFA* Free fatty acid, *PL* polar lipid

Four of the mutants obtained in this study seemed to have a lowered n-3 desaturation activity. Particularly, Y11 accumulated no EPA at 12°C, owing to apparently complete deficiency in n-3 desaturation. Thus far, a n-3 desaturation-defective mutant, K1, had been obtained from a Δ5 desaturation-defective mutant, Mut44 (Jareonkitmongkol et al. 1992b, 1994). K1 accumulated DGLA as a major PUFA instead of AA. The percentage of DGLA in total fatty acids in K1 at cultivation temperature of 12°C was higher than that in Mut44, although the growth rate of K1 was much smaller.

All mutants grew well at 16°C or 20°C to the same extent as *M. alpina* 1S-4, whereas they were greatly influenced by glucose concentration in an individual manner. Y11 and Y61 were capable of growing well, even in medium containing >5.0% glucose. They were assumed to have an improved glucose-utilizing ability or a relaxed glucose repression to some extent. When all strains were cultivated in the respective optimum conditions, Y11, Y61 and Y164 showed higher AA production, because of the higher initial glucose concentration in the medium. However, *M. alpina* 1S-4 could reach the same level of AA production as Y11 or Y61, even when cultivated in medium containing 5.0% or 7.5% glucose. As to fatty acid compositions, the low AA content (51.3%) in Y11 was probably due to immature cells growing with the remaining glucose in the medium. The evaluation of the AA productivities of Y11 and Y61 in 5-l jar fermentors also clearly demonstrated that these mutants show essentially reproducible results (1.56- and 1.86-fold greater AA production, respectively, than that of the

parent). However, the AA production of Y164 was very poor compared with others (data not shown). This was probably because of its "filamentous" morphological shape, which has been shown to have lower AA productivity (Higashiyama et al. 1999) in liquid culture. Thus, Y164 seemed not to be suitable for practical purposes. Another practical advantageous feature of Y11 and Y61 on cultivation at 20°C was their higher TG content (85.4–84.6%) in total mycelial lipids than that (76.8%) of the parental *M. alpina* 1S-4. In conclusion, unique features of Y11 and Y61, such as an improved ability of glucose utilization, allowing active growth in the late phase of cultivation, and a higher cellular content of TG, as demonstrated here should be useful for the practical production of fungal TG rich in AA.

Acknowledgements This work was supported in part by a Grant-in-Aid for Scientific Research (No. 15658024 to S.S.) from the Ministry of Education, Science, Sports, and Culture, Japan, by a grant (to S.S.) from the COE for Microbial Process Development Pioneering Future Production Systems (COE Program of the Ministry of Education, Science, Sports, and Culture, Japan) and by the Project of the New Energy and Industrial Technology Development Organization (NEDO), Japan.

References

Carlson SE, Werkman SH, Peeples JM, Cooke RJ, Tolley EA (1993) Arachidonic acid status correlates with first year growth in preterm infants. Proc Natl Acad Sci USA 90:1073–1077

Folch J, Lees M, Sloane-Stanley GH (1957) A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226:497–509

Gill I, Valivety R (1997) Polyunsaturated fatty acids, part 1: occurrence, biological activities and application. Trends Biotechnol 15:401–409

Higashiyama K, Yaguchi T, Akimoto K, Fujikawa S, Shimizu S (1998) Enhancement of arachidonic acid production by *Mortierella alpina* 1S-4. J Am Oil Chem Soc 75:1501–1505

Higashiyama K, Fujikawa S, Park EY, Okabe M (1999) Image analysis of morphological change during arachidonic acid production by *Mortierella alpina* 1S-4. J Biosci Bioeng 87:489–494

Jareonkitmongkol S, Shimizu S, Yamada H (1992a) Fatty acid desaturation-defective mutants of an arachidonic-acid-producing fungus, *Mortierella alpina* 1S-4. J Gen Microbiol 138:997–1002

Jareonkitmongkol S, Kawashima H, Shirasaka N, Shimizu S, Yamada H (1992b) Production of dihomo-γ-linolenic acid by a Δ5-desaturase-defective mutant of *Mortierella alpina* 1S-4. Appl Environ Microbiol 58:2196–2200

Jareonkitmongkol S, Sakuradani E, Shimizu S (1993) A novel Δ5-desaturase-defective mutant of *Mortierella alpina* 1S-4 and its dihomo-γ-linolenic acid productivity. Appl Environ Microbiol 59:4300–4304

Jareonkitmongkol S, Sakuradani E, Shimizu S (1994) Isolation and characterization of an ω3-desaturation-defective mutant of an arachidonic acid-producing fungus, *Mortierella alpina* 1S-4. Arch Microbiol 161:316–319

Park EY, Koike Y, Higashiyama K, Fujikawa S, Okabe M (1999) Effect of nitrogen source on mycelial morphology and arachidonic acid production in cultures of *Mortierella alpina*. J Biosci Bioeng 88:61–67

Sakuradani E, Kamada N, Hirano Y, Nishihara M, Kawashima H, Akimoto K, Higashiyama K, Ogawa J, Shimizu S (2002) Production of 5,8,11-eicosatrienoic acid by a Δ5 and Δ6 desaturation activity-enhanced mutant derived from a Δ12 desaturation activity-defective mutant of *Mortierella alpina* 1S-4. Appl Microbiol Biotechnol 60:281–287

Shimizu S, Kawashima H, Shinmen Y, Akimoto K, Yamada H (1988) Production of eicosapentaenoic acid by *Mortierella* fungi. J Am Oil Chem Soc 65:1455–1459

Shimizu S, Jareonkitmongkol S, Kawashima H, Akimoto K, Yamada H (1991) Production of a novel ω1-eicosapentaenoic acid by *Mortierella alpina* 1S-4 grown on 1-hexadecene. Arch Microbiol 156:163–166

Shinmen Y, Shimizu S, Akimoto K, Kawashima H, Yamada H (1989) Production of arachidonic acid by *Mortierella* fungi. Appl Microbiol Biotechnol 31:11–16

Yamada H, Shimizu S, Shinmen Y (1987) Production of arachidonic acid by *Mortierella elongata* 1S-5. Agric Biol Chem 51:785–790