JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Polyunsaturated Fatty Acids (PUFAs) Content of the Fungus *Mortierella alpina* Isolated from Soil

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Twenty-five isolates of *Mortierella* spcies were prepared, which can be used for the production of polyunsaturated fatty acids (PUFAs)-rich oil for nutritional supplements. The fatty acid contents were determined after heterotrophic fermentation. The content of total fatty acids (TFAs) in the cell dry weight of all isolates including two commercially purchased *Mortierella alpina* strains ranged from 207.51 to 370.11 mg/g, whereas PUFAs were the dominant fatty acid type. The highest PUFA-containing strain, *M. alpina* SC9, was identified and confirmed as a new strain of *M. alpina* through comparison analysis of the sequences of internal transcribed spacers 1 and 2 (ITS1 and ITS2) and the 5.8S rDNA region. During a 7-day fermentation, the PUFAs content of *M. alpina* SC9 varied between 189.83 and 240.00 mg/g, with a remarkable correlation between the oleic acid (C18:1, OA) and arachidonic acid (C20:4n-6, AA) contents and between the linoleic acid (C18:2n-6, LA) and AA contents, suggesting the PUFA content in the fungus is tightly regulated. This study provides a framework of isolation, identification, and characterization of an important PUFA-producing species, *M. alpina*.

KEYWORDS: ***Mortierella alpina*; polyunsaturated fatty acids (PUFAs); soil fungus; heterotrophic**

INTRODUCTION

Polyunsaturated fatty acids (PUFAs) are increasingly in demand due to the beneficial effects on human health. Extensive studies have shown that PUFAs have two main functions: (1) regulating membrane fluidity and membrane proteins and (2) serving as metabolite precursors, such as prostaglandins and leukotrienes (*1*). For example, PUFAs such as linoleic acid (C18:2, LA) are essential fatty acids for humans and, together with other n-6 PUFAs, are crucial for normal metabolic and physiological functions (*2*). It was also suggested that oral supplementation with PUFAs (especially arachidonic acid, C20:4n-6, AA) could prevent alloxan-induced diabetes mellitus and protect various tissues from oxidative stress (*3*). Besides, AA and docosahexaenoic acid (C22:6n-3, DHA) have been shown to be beneficial to infants' brain and retinal development (*4*) and thus have been recommended to be included in infant milk formulas (*5*). Other n-3 and n-6 PUFAs such as eicosapentaenoic acid (C20:5n-3, EPA), γ-linolenic acid (C18:3n-6, GLA), and dihomo-γ-linolenic acid (C20:3n-6, DGLA) are also used in many foods and medical research (*2*, *5*, *6*). As PUFAs have their unique structures and functions, deficiencies of PUFAs would cause various abnormalities in humans (*1*).

The worldwide production of different PUFAs is increasing yearly (*7*). Sources of a majority of n-6 PUFAs are from seeds of some plants, such as evening primrose (*Oenothera biennis*) and borage seeds (*Borago officinalis*), which are relatively rich in GLA (*1*). However, due to a lack of enzymatic system, PUFAs with >18 carbons, such as AA, are not produced in plants naturally (*1*). Some other conventional sources, such as porcine liver, adrenal gland, and fish oil, are not desirable due to their own limitations (*1*). Therefore, the production of PUFAs has been switching from conventional sources to microbial sources. Several studies have shown that *Mortierella* species, members of the family Mortierellaceae, order Mortierellales, class Zygomycetes, are the most promising organisms for n-6 PUFA production, including those PUFAs with 20 carbons, for example, AA. *Mortierella alpina* is one of the most important industrial species for PUFA production (*8*).

Arachidonic acid is one of the main PUFAs produced from *M. alpina*. As one of the oleaginous fungi, however, *M. alpina* can also produce other PUFAs in significant amounts, such as LA, GLA, DGLA (*9*), EPA, and DHA, which might be induced by lowering the temperature (*10*) or adding exogenous oils (*11*, *12*), suggesting this fungus is a potential producer of many commercially important PUFAs, both n-3 and n-6. In addition, as the fungus could produce AA and DHA, oil products with good n-3 and n-6 ratios may be developed as new desired food products.

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10.1021/jf0700071 CCC: $37.00
Published on Web 04/18/2007

The aim of this study was to investigate the PUFA contents of *Mortierella* species isolated from soil, which would explore the potential of new strains for PUFA production. Besides, the fungus identity with the highest PUFA content was further confirmed, showing the usefulness of molecular biology in conjunction with conventional morphological analysis. Moreover, changes of fatty acid compositions during different phases of cultivation were investigated, which would add to our understanding of how the fungus modifies fatty acids during different growth stages. Together with the growth characteristics of the fungus, this study would provide information of regulations of fatty acid compositions during fermentation and would facilitate further cultivation optimizations.

MATERIALS AND METHODS

Fungus Isolation and Identification. Two *M. alpina* strains, ATCC 16266 and ATCC 42430, were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). All other fungi were isolated from soil of different locations, including Hong Kong, France, China, and Canada. The soil direct isolation method (*13*) was used. Cooled and molten potato dextrose agar (PDA) (8−10 mL) was poured into a Petri dish with 0.005 g of soil. The plates were incubated at 4 °C and in darkness (*14*). After colonies had formed, the edges of colonies were transferred to new plates repeatedly until pure fungal colonies were obtained. For identification, the isolated fungi were cultivated on 2% agar (bacto agar, 20 g/L) (Becton, Dickinson and Co., Sparks, MD). Mycelium was mounted on a glass slide, and sporangiophores were observed under a microscope. The morphologies of the isolates were compared to those of ATCC 16266 and ATCC 42430 and descriptions in the literature (*15*, *16*). Purified and identified *Mortierella* species isolates were maintained on PDA slants at 4 °C and subcultured once every 3 months.

Heterotrophic Growth. A precultured broth was prepared in 250-mL Erlenmeyer flasks, each containing 50 mL of the preculture medium consisting of (per liter) 20 g of glucose and 10 g of yeast extract for 2 days with orbital shaking at 200 rpm in the dark. Erlenmeyer flasks of 250 mL, each with 50 mL of fermentation medium consisting of (per liter) 50 g of glucose, 10 g of soy flour, 10 g of corn steep liquor, 10 mL of corn oil, 5 g of yeast extract, 3 g of $NaNO_3$, 1 g of KH_2PO_4, 0.5 g of $MgSO_4$, 0.5 g of KCl, 4.3 mg of $MnCl_2$, 1.45 mg of $FeCl_3$, 0.3 mg of $ZnCl_2$, 0.13 mg of $CoCl_2 \cdot 6H_2O$, and 0.13 mg of $CuSO_4$ (*5*), were inoculated with 10% (v/v) of an exponentially growing inoculum and incubated at 23 °C with orbital shaking at 200 rpm in darkness.

Determination of Cell Dry Weight. The fungal cells from the fermentation broth were harvested by filtration and washed with distilled water twice. Cell dry weight was determined by lyophilizing to obtain a constant weight.

Determination of Glucose Concentration. Residual glucose concentration was determined according to the 3,5-dinitrosalicylic acid method (*17*).

Analysis of Fatty Acid Contents. Cells were harvested and lyophilized for fatty acids analysis. Fatty acid methyl esters (FAMEs) were prepared by transmethylation with methanol/acetyl chloride and analyzed by using a HP 6890 capillary gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a flame ionization detector (FID) and a HP-INNOwax capillary column (30 m × 0.32 mm) (Agilent Technologies, Inc., Wilmington, DE) (*18*). Nitrogen was used as carrier gas. Initial column temperature was set at 170 °C, which was subsequently raised to 230 °C at 1 °C/min. The injector was kept as 250 °C with an injection volume of 2 μL under splitless mode. The FID temperature was set at 270 °C. FAMEs were identified by chromatographic comparison with authentic standards (Sigma Chemical Co., St. Louis, MO). The quantities of individual FAMEs were estimated from the peak areas on the chromatogram using heptadecanoic acid as the internal standard.

Genomic DNA Extraction, Sequencing, and Data Analysis. The total genomic DNA extraction was performed according to the method of Lee and Taylor (*19*). In brief, the mycelium was harvested from a 7-day-grown PDA plate, ground with a mortar and pestle with liquid nitrogen, and transferred into a 1.5-mL microfuge tube. Lysis buffer [50 mM Tris-HCl (pH 7.2), 50 mM ethylenediaminetetraacetic acid (EDTA), 1% β-mercaptoethanol, 3% sodium dodecyl sulfate (SDS)] was added, vortexed, and incubated at 65 °C for 1 h. An equal volume of phenol/chloroform/isoamyl alcohol [25:24:1 (v/v)] (Sigma Chemical Co.) was mixed with the solution and centrifuged at 10000*g* for 15 min. The upper phase was transferred to a new microfuge tube, and 3 M sodium acetate (NaOAc) and isopropanol were used to precipitate the DNA. The DNA pellet after centrifugation of 10000*g* for 2 min was washed with 70% ethanol. The washed pellet was dried and resuspended in Tris−EDTA (TE) buffer (10 mM Tris-HCl, 0.1 mM EDTA).

A DNA segment containing the 3′ end of 18S rDNA, internal transcribed spacer 1 (ITS1), 5.8S rDNA, internal transcribed spacer 2 (ITS2), and the 5′ end of 28S rDNA was amplified by using forward primer ITS5 (5′GGAAGTAAAAGTCGTAACAAGG) and reverse primer ITS4 (5′TCCCCGCTTATTGATATGC) (*20*). PCR was performed in a total volume of 50 μL, with 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.5 μM of each of the primers ITS4 and ITS5, 0.2 mM dNTP mixture, 20 ng of genomic DNA, and 1.5 units of *Taq* DNA polymerase. The PCR conditions were as follows: initial denaturation at 94 °C for 5 min followed by 30 cycles of 94 °C for 45 s, 57 °C for 45 s, and 72 °C for 1 min and a final extension at 72 °C for 5 min. The amplified products were separated on 1% agarose gel and detected by staining with ethidium bromide. All PCR products amplified from strains *M. alpina* ATCC16266, *M. alpina* ATCC 42430, and *M. alpina* SC9 genomic DNA showed only one band. The PCR products were purified by GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Piscataway, NJ) following the instructions of the manufacturer. The PCR products were then sequenced in both directions using an automatic ABI3100 sequencer. The sequences of *M. alpina* ATCC 16266, *M. alpina* ATCC42430, and *M. alpina* SC9 were submitted to GenBank, with accession numbers EF202180, EF202181, and EF202182, respectively.

Other sequences used in this study were obtained from GenBank. The sequences were aligned by using the CLUSTAL X program (*21*), and the alignment was corrected manually. An identities matrix was calculated by the program CLUSTAL X. A neighbor-joining (NJ) tree was constructed with *Mucor plumbeus* as the outgroup. The bootstrap values were obtained from 1000 replications of NJ analyses.

RESULTS AND DISCUSSION

Isolation and Identification of *Mortierella* Species from Soil. Twenty-five isolates of *Mortierella* species were isolated from soil. After 7 days of cultivation on 2% agar, mycelium was mounted on a glass slide and sporangiophores were observed under microscope. The morphologies of the sporangiophores of all isolated and identified *Mortierella* species isolates were matched with that of *M. alpina* ATCC 16266, *M. alpina* ATCC 42430, and keys describing *M. alpina* (*15*, *16*). All of the sporangiophores were nonbranch. The lengths of the sporangiophores were between 20 and 120 μm, with an irregular and wide swollen base. Although all of the isolate morphologies matched the descriptions of *M. alpina*, they were assigned as *Mortierella* species at this stage.

Growth Characteristics, Fatty Acid Contents, and Compositions of 27 Isolates of *Mortierella* Species after 6 Days of Cultivation. The growth characteristics of all the isolates of *Mortierella* species including two commercially available strains, *M. alpina* ATCC 16266 and *M. alpina* ATCC 42430, in submerged culture are shown in **Table 1**. All of the isolates could grow with glucose and corn oil as carbon sources heterotrophically. It was in accordance with that *M. alpina* could grow in different carbon sources, including glucose (*9*). After a 6-day fermentation, biomass concentration varied from 24.96 g/L (*Mortierella* sp. FC2) to 36.2 g/L (*Mortierella* sp. HK1). High biomass concentration implied that the production of PUFAs would be more effective and at a lower cost (*8*). As for

Table 1. Biomass Concentration and Total Fatty Acid Contents of 25 Isolates of *Mortierella* Species and 2 *M. alpina* Strains after 6 Days of Cultivation[a]

fungal isolate	source	biomass concn (g/L)	TFAs[b] in cell dry wt (mg/g)
ATCC 16266	ATCC	31.13 ± 0.21	332.05 ± 10.06
ATCC 42430	ATCC	33.53 ± 0.48	252.52 ± 3.60
HK1	Hong Kong	36.20 ± 0.61	370.14 ± 9.56
HK2	Hong Kong	30.47 ± 1.01	236.59 ± 4.03
HK3	Hong Kong	29.76 ± 0.40	207.53 ± 13.80
HK4	Hong Kong	33.02 ± 0.56	273.12 ± 4.74
FC1	France	28.66 ± 0.77	231.81 ± 24.90
FC2	France	24.96 ± 1.29	275.45 ± 23.72
FC3	France	28.69 ± 1.40	268.79 ± 7.74
FC4	France	29.63 ± 2.74	225.27 ± 27.33
FN1	France	26.64 ± 0.88	256.62 ± 11.52
FN2	France	30.83 ± 1.28	290.29 ± 10.53
SC1	China	30.59 ± 1.00	283.21 ± 6.21
SC2	China	30.10 ± 1.13	284.79 ± 23.61
SC3	China	29.73 ± 1.39	313.23 ± 21.85
SC4	China	28.96 ± 1.59	337.16 ± 16.21
SC5	China	29.31 ± 1.29	260.81 ± 23.38
SC6	China	28.36 ± 1.41	277.62 ± 3.49
SC7	China	30.03 ± 0.97	314.27 ± 16.11
SC8	China	30.41 ± 0.65	304.10 ± 15.53
SC9	China	33.60 ± 1.49	353.52 ± 14.13
SC10	China	28.56 ± 0.53	305.90 ± 13.60
SC11	China	34.52 ± 0.73	340.71 ± 13.84
SC12	China	27.30 ± 1.13	276.04 ± 5.46
VA1	Canada	30.94 ± 0.92	306.92 ± 17.78
VA2	Canada	31.04 ± 0.81	257.97 ± 17.63
VA3	Canada	28.62 ± 1.52	273.37 ± 2.29

[a] Data are expressed as mean ± SD of three replicates. [b] Total fatty acids content in cell dry weight.

total fatty acids (TFAs) content (as mg/g of cell dry weight), the highest TFAs content (370.11 mg/g) was attained from *Mortierella* species HK1 and the lowest (207.53 mg/g) from *Mortierella* species HK3. It seems that there was no correlation between the TFAs content and the geographical locations of the *Mortierella* species isolates in this study, but as some isolates could accumulate >300 mg/g of cell dry weight (i.e., 30% of cell dry weight) as fatty acids in nonoptimized batch culture, this study showed the potential of *Mortierella* species as natural oil producers.

The contents of different fatty acids of all 25 isolates of *Mortierella* species and the two ATCC *M. alpina* strains are shown (**Table 2**). These included total saturated fatty acids (SFAs), total monounsaturated fatty acids (MUFAs), and total polyunsaturated fatty acids (PUFAs). The total SFAs content (as mg/g of cell dry weight) of all isolates varied from 42.41 mg/g (*Mortierella* sp. FC4) to 85.33 mg/g (*M. alpina* ATCC 16266), whereas that of total MUFAs varied from 24.18 mg/g (*Mortierella* sp. HK3) to 71.19 mg/g (*M. alpina* ATCC 16266). In contrast, the dominant category of fatty acids was found to be PUFAs, which ranged from 116.97 mg/g (*Mortierella* sp. FC4) to 229.60 mg/g (*Mortierella* sp. SC9 and *Mortierella* sp. SC11). OA (C18:1) was the major MUFA in all of the isolates, and the content of the total MUFAs was not as high as that of the total PUFAs, implying MUFAs were not used for oil storage in the genus. Despite this, both MUFAs and PUFAs could decrease total plasma cholesterol and low-density lipoprotein cholesterol significantly (*23*). The major fatty acids of PUFAs identified in all isolates were LA, GLA, DGLA, and AA. All of the major PUFAs stored in the isolates are important fatty acids in nutraceutical and pharmaceutical uses (*2*, *3*, *5*, *6*), especially AA, which is the most valuable n-6 PUFA (*5*). The AA contents (as mg/g of cell dry weight) were different in all isolates, ranging from 13.33 mg/g (*M. alpina* ATCC 42430) to 109.29 mg/g (*Mortierella* sp. FC2). The variations of AA content among different species were common within the genus *Mortierella* (*14*), but the reason for that remained unknown. In this study, the geographical locations are not related to the AA content of *Mortierella* species. Moreover, no EPA was detected in any of the isolates, which was suitable as a supplement in infant formula (*5*). Among all of the species, *Mortierella* sp. SC9, the highest PUFA-containing (64.94% of TFAs are PUFAs) strain, with significant contents of AA (97.90 mg/g of cell dry weight, higher than that of *Mortierella* sp. SC11), was chosen for the following experiment.

Molecular Sequencing Analysis of *M. alpina* SC9. The main use of PUFA-rich product is as a nutritional supplement in food products, and the safety of sources should be guaranteed. It has been suggested that *M. alpina* has extremely low pathogenic potential toward warm-blooded animals, as this fungus cannot survive at 37 °C (*24*), and the PUFA-rich oil produced from *M. alpina* could be used for nutritional products for human and infant consumption (*25*, *26*). All of the isolates in this study were assigned as *Mortierella* species through morphological comparison. However, before the isolate can be used in the nutraceutical and pharmaceutical industries, the identity of the selected isolate should be confirmed. In this study, *Mortierella* species SC9 (the highest PUFA-containing strain) was further confirmed as a new strain of *M. alpina* through molecular phylogenetic analysis. Sequences of ITS1, 5.8S rDNA, and ITS2 of *M. alpina* ATCC16266, *M. alpina* ATCC 42430, and *Mortierella* species SC9 were identified in this study (**Table 3**). The sequences were aligned with two other *M. alpina* strains (*M. alpina* CBS528.72 and *M. alpina* AY310443), three *Mortierella* species, and *Mucor plumbeus*. The *Mortierella* species were chosen for comparison in this study because they are the closest relatives with *M. alpina* (*27*, *28*). The percentages of identities are given in **Table 4**. It is shown that the percentages of identities between those *M. alpina* were >94% (including *Mortierella* sp. SC9), whereas that between *M. alpina* and other *Mortierella* species were all <90%. The identity between *Mortierella* species SC9 and *M. alpina* AY310443 was 99% (only three base pairs were different, data not shown), strongly suggesting that the *Mortierella* species SC9 is a new strain of *M. alpina*. In addition, an NJ tree was constructed using *Mucor plumbeus* as the outgroup (**Figure 1**). It is clearly shown that *Mortierella* species SC9 was claded together with other *M. alpina* strains with a high bootstrap value. Therefore, *Mortierella* species SC9 was assigned as *M. alpina* SC9 and was confirmed as a new strain of *M. alpina*. This sequence-based identification in this study would be useful for other researchers verifying the identities of different strains of *M. alpina*.

Growth Characteristics of *M. alpina* SC9 during 7 Days of Cultivation. Growth characteristics of *M. alpina* SC9 during 7 days of submerged cultivation are shown in **Figure 2a**. The cells were at their log phase from days 0 to 3, with biomass concentration increasing from 0.75 to 33.10 g/L. During that time, glucose concentration decreased from 50 to 9.6 g/L, implying that glucose was consumed rapidly by *M. alpina* SC9. Similar findings were observed in Zhu et al. (*29*), and it was suggested that *M. alpina* could grow more rapidly in the early days of cultivation when the initial glucose concentration was low (50 g/L) (*29*). The cells were at their late log phase from days 3 to 4, during which period the biomass concentration increased slightly further to 34.29 g/L. Afterward, the culture was in stationary phase from days 4 to 6, followed by death

Table 2. Fatty Acid Contents of 25 Isolates of *Mortierella* Species and 2 *M. alpina* Strains after 6 Days of Cultivation[a]

	fatty acid content (mg/g)																					
	saturated fatty acids								monounsaturated fatty acids					polyunsaturated fatty acids								
fungus	14:0	15:0	16:0	18:0	20:0	22:0	24:0	subtotal	16:1	18:1 OA[b]	20:1	24:1	subtotal	18:2 LA	18:3 n-3	18:3 n-6 GLA	20:2	20:3 n-3	20:3 n-6 DGLA	20:4 AA	subtotal	▽/mol[c]
ATCC 16266	0.93 ± 0.03	0.30 ± 0.03	49.01 ± 0.36	25.77 ± 0.89	2.32 ± 0.03	3.98 ± 0.08	3.02 ± 0.06	**85.33 ± 3.35**	0.43 ± 0.03	68.87 ± 2.60	1.76 ± 0.22	0.13 ± 0.00	**71.19 ± 1.60**	119.71 ± 2.91	1.49 ± 0.06	8.67 ± 0.33	1.86 ± 0.08	3.62 ± 3.77	5.88 ± 0.06	34.30 ± 0.80	**175.53 ± 8.43**	1.54 ± 0.03
ATCC 42430	1.44 ± 0.28	0.23 ± 0.03	33.13 ± 1.75	10.08 ± 0.33	0.78 ± 0.03	0.66 ± 0.03	0.76 ± 0.03	**47.08 ± 2.19**	0.56 ± 0.03	59.35 ± 0.47	0.71 ± 0.11	0.10 ± 0.03	**60.72 ± 3.54**	122.10 ± 1.55	2.27 ± 0.06	4.82 ± 0.19	0.40 ± 0.03	0.08 ± 0.00	1.72 ± 0.17	13.33 ± 0.66	**144.72 ± 2.15**	1.53 ± 0.01
HK1	1.18 ± 0.08	0.19 ± 0.08	41.90 ± 1.50	22.17 ± 0.94	2.11 ± 0.06	2.89 ± 0.19	4.07 ± 0.25	**74.51 ± 2.98**	0.48 ± 0.03	67.95 ± 3.66	0.74 ± 0.08	0.26 ± 0.03	**69.43 ± 3.23**	130.94 ± 2.66	1.70 ± 0.06	9.73 ± 0.80	0.85 ± 0.03	0.26 ± 0.00	6.44 ± 0.25	76.28 ± 0.58	**226.20 ± 10.33**	1.87 ± 0.02
HK2	0.43 ± 0.06	0.24 ± 0.03	27.82 ± 1.25	16.49 ± 1.72	1.58 ± 0.08	2.89 ± 0.33	5.30 ± 0.30	**54.75 ± 2.19**	0.26 ± 0.06	27.37 ± 3.74	0.50 ± 0.00	0.17 ± 0.03	**28.30 ± 3.54**	63.01 ± 5.57	0.83 ± 0.25	10.01 ± 0.28	1.02 ± 0.08	0.47 ± 0.06	4.75 ± 0.17	73.45 ± 5.84	**153.54 ± 2.15**	2.11 ± 0.04
HK3	0.33 ± 0.03	0.39 ± 0.06	27.79 ± 2.74	13.55 ± 0.53	1.18 ± 0.11	2.12 ± 0.11	3.67 ± 0.08	**49.03 ± 4.85**	0.25 ± 0.03	23.22 ± 0.53	0.48 ± 0.03	0.23 ± 0.06	**24.18 ± 1.91**	55.18 ± 1.75	0.73 ± 0.08	9.05 ± 0.61	1.25 ± 0.08	0.54 ± 0.03	4.61 ± 0.28	62.96 ± 2.96	**134.32 ± 7.04**	2.09 ± 0.04
HK4	0.41 ± 0.08	0.25 ± 0.03	31.22 ± 1.25	18.54 ± 1.33	1.67 ± 0.19	3.22 ± 0.08	4.81 ± 0.33	**60.12 ± 3.61**	0.25 ± 0.03	34.52 ± 1.41	0.60 ± 0.03	0.19 ± 0.08	**35.56 ± 0.69**	81.03 ± 2.99	1.12 ± 0.06	9.56 ± 1.16	1.26 ± 0.19	0.57 ± 0.06	5.30 ± 0.42	78.60 ± 3.02	**177.44 ± 3.74**	2.07 ± 0.04
FC1	1.32 ± 0.19	0.51 ± 0.06	33.08 ± 1.08	13.10 ± 0.58	1.04 ± 0.06	1.21 ± 0.03	1.51 ± 0.06	**51.77 ± 6.98**	0.28 ± 0.03	48.56 ± 2.96	1.02 ± 0.03	0.19 ± 0.03	**50.05 ± 3.83**	80.24 ± 1.99	1.02 ± 0.06	7.49 ± 0.64	0.39 ± 0.30	0.83 ± 0.50	1.92 ± 0.08	38.10 ± 1.27	**129.99 ± 14.44**	1.71 ± 0.02
FC2	0.83 ± 0.06	0.36 ± 0.08	24.32 ± 1.22	16.09 ± 0.66	1.79 ± 0.28	2.81 ± 0.25	4.38 ± 0.50	**50.58 ± 3.56**	0.30 ± 0.06	33.14 ± 2.30	1.07 ± 0.06	0.94 ± 1.11	**35.45 ± 6.42**	54.37 ± 2.66	0.63 ± 0.11	12.01 ± 0.19	2.40 ± 0.06	1.02 ± 0.11	9.70 ± 0.69	109.29 ± 3.91	**189.42 ± 13.79**	2.38 ± 0.04
FC3	1.83 ± 0.06	0.30 ± 0.03	43.52 ± 2.74	20.37 ± 0.94	1.96 ± 0.08	3.09 ± 0.72	2.07 ± 0.89	**73.14 ± 3.99**	0.35 ± 0.06	55.34 ± 2.08	1.77 ± 0.50	0.19 ± 0.00	**57.65 ± 3.12**	79.21 ± 0.64	1.13 ± 0.19	9.68 ± 0.03	1.21 ± 0.58	0.46 ± 0.30	8.22 ± 1.47	38.09 ± 4.85	**138.00 ± 1.16**	1.60 ± 0.05
FC4	1.91 ± 0.14	0.56 ± 0.06	26.85 ± 1.80	10.54 ± 0.25	0.88 ± 0.19	0.50 ± 0.06	1.17 ± 0.11	**42.41 ± 5.91**	0.25 ± 0.22	64.54 ± 2.91	0.90 ± 0.14	0.20 ± 0.03	**65.89 ± 7.11**	80.26 ± 3.68	1.13 ± 0.03	9.44 ± 0.66	0.29 ± 0.08	0.14 ± 0.06	4.96 ± 0.30	20.75 ± 1.97	**116.97 ± 14.74**	1.58 ± 0.02
FN1	0.80 ± 0.11	0.28 ± 0.00	31.33 ± 1.16	14.14 ± 0.33	1.15 ± 0.14	1.49 ± 0.33	2.05 ± 0.28	**51.24 ± 2.49**	0.36 ± 0.03	56.69 ± 4.16	1.10 ± 0.14	0.23 ± 0.03	**58.38 ± 6.21**	80.20 ± 4.43	1.13 ± 0.25	10.34 ± 1.16	0.87 ± 0.36	0.28 ± 0.14	5.83 ± 1.47	48.35 ± 4.74	**147.00 ± 3.40**	1.82 ± 0.05
FN2	1.71 ± 0.17	0.29 ± 0.06	42.12 ± 1.44	18.32 ± 1.25	1.65 ± 0.22	1.97 ± 0.33	2.44 ± 0.22	**68.50 ± 2.60**	0.35 ± 0.03	62.73 ± 1.19	0.61 ± 0.06	0.20 ± 0.03	**63.89 ± 2.18**	112.80 ± 5.60	1.39 ± 0.33	5.11 ± 0.30	0.52 ± 0.08	0.17 ± 0.03	3.89 ± 0.47	34.02 ± 3.05	**157.90 ± 8.39**	1.58 ± 0.02
SC1	0.68 ± 0.00	0.42 ± 0.03	40.87 ± 1.80	16.29 ± 0.89	1.39 ± 0.17	2.83 ± 0.06	6.20 ± 0.36	**68.68 ± 3.40**	0.40 ± 0.03	38.86 ± 2.24	0.85 ± 0.08	0.37 ± 0.00	**40.48 ± 3.25**	77.47 ± 3.21	1.16 ± 0.17	8.07 ± 0.50	1.73 ± 0.14	0.59 ± 0.06	6.15 ± 0.11	78.88 ± 5.37	**174.05 ± 1.93**	1.99 ± 0.08
SC2	0.85 ± 0.17	0.40 ± 0.14	42.80 ± 1.36	17.83 ± 0.25	1.59 ± 0.08	3.36 ± 0.58	5.58 ± 0.58	**72.41 ± 5.83**	0.37 ± 0.08	38.36 ± 3.74	0.80 ± 0.06	0.34 ± 0.00	**39.87 ± 4.05**	71.76 ± 9.78	1.03 ± 0.25	8.34 ± 1.08	1.77 ± 0.22	0.68 ± 0.25	7.06 ± 0.33	81.87 ± 6.43	**172.51 ± 15.86**	1.99 ± 0.04
SC3	0.63 ± 0.08	0.47 ± 0.11	44.04 ± 3.49	18.45 ± 2.38	1.79 ± 0.19	3.26 ± 0.06	8.14 ± 0.91	**76.78 ± 9.07**	0.38 ± 0.06	36.24 ± 2.35	0.88 ± 0.08	0.28 ± 0.06	**37.78 ± 4.35**	79.99 ± 9.58	0.94 ± 0.42	9.24 ± 0.55	1.94 ± 0.25	0.85 ± 0.14	6.95 ± 0.53	98.76 ± 4.46	**198.67 ± 14.51**	2.08 ± 0.03
SC4	1.25 ± 0.03	0.40 ± 0.06	50.30 ± 1.25	20.53 ± 0.58	1.69 ± 0.08	3.00 ± 0.11	5.46 ± 0.36	**82.63 ± 5.73**	0.44 ± 0.03	56.23 ± 2.24	1.05 ± 0.11	0.34 ± 0.03	**58.06 ± 3.83**	105.12 ± 4.16	1.31 ± 0.11	8.73 ± 0.89	1.58 ± 0.19	0.67 ± 0.14	5.97 ± 0.89	73.09 ± 4.65	**196.47 ± 8.37**	1.82 ± 0.04
SC5	0.63 ± 0.06	0.44 ± 0.08	38.76 ± 2.66	16.43 ± 0.89	1.51 ± 0.08	3.13 ± 0.44	6.34 ± 0.53	**67.24 ± 3.39**	0.37 ± 0.03	26.06 ± 2.58	0.81 ± 0.06	0.39 ± 0.06	**27.63 ± 4.72**	60.88 ± 6.15	0.83 ± 0.19	7.64 ± 0.33	1.59 ± 0.03	0.63 ± 0.08	6.94 ± 0.25	87.43 ± 5.48	**165.94 ± 15.36**	2.11 ± 0.03
SC6	0.69 ± 0.03	0.42 ± 0.06	42.50 ± 2.16	18.63 ± 1.02	1.72 ± 0.06	3.64 ± 0.42	7.27 ± 0.94	**74.87 ± 4.46**	0.33 ± 0.03	30.01 ± 5.01	0.72 ± 0.03	0.36 ± 0.06	**31.42 ± 5.33**	60.60 ± 4.54	0.86 ± 0.22	8.63 ± 0.14	1.80 ± 0.08	0.81 ± 0.11	7.33 ± 0.33	91.30 ± 5.40	**171.33 ± 1.59**	2.07 ± 0.03
SC7	0.79 ± 0.14	0.41 ± 0.06	45.38 ± 2.41	21.31 ± 1.05	1.76 ± 0.25	4.37 ± 0.39	7.17 ± 0.39	**81.19 ± 4.52**	0.25 ± 0.14	38.31 ± 1.52	0.82 ± 0.06	0.44 ± 0.03	**39.82 ± 3.70**	74.48 ± 4.43	1.19 ± 0.58	8.58 ± 1.77	1.82 ± 0.30	0.79 ± 0.08	8.33 ± 0.28	98.08 ± 2.66	**193.27 ± 10.63**	2.04 ± 0.04
SC8	0.61 ± 0.11	0.40 ± 0.03	40.96 ± 1.16	19.31 ± 0.80	1.58 ± 0.25	3.16 ± 0.17	6.60 ± 0.22	**72.62 ± 0.99**	0.33 ± 0.03	38.28 ± 3.13	0.82 ± 0.06	0.46 ± 0.08	**39.89 ± 5.28**	74.74 ± 3.10	1.28 ± 0.33	9.09 ± 0.47	1.82 ± 0.08	0.76 ± 0.11	5.93 ± 0.11	97.97 ± 4.27	**191.59 ± 9.65**	2.09 ± 0.03
SC9	0.57 ± 0.03	0.35 ± 0.03	43.59 ± 0.33	15.70 ± 0.33	1.59 ± 0.06	3.11 ± 0.17	6.79 ± 0.22	**71.70 ± 3.01**	0.35 ± 0.00	50.67 ± 3.35	0.92 ± 0.00	0.28 ± 0.03	**52.22 ± 4.84**	113.07 ± 4.57	1.45 ± 0.11	8.10 ± 0.58	1.84 ± 0.19	0.81 ± 0.11	6.43 ± 0.75	97.90 ± 5.18	**229.60 ± 9.23**	2.05 ± 0.04
SC10	0.52 ± 0.08	0.37 ± 0.06	39.64 ± 1.75	15.75 ± 1.36	1.41 ± 0.06	2.88 ± 0.17	6.24 ± 0.03	**66.81 ± 3.18**	0.24 ± 0.03	42.61 ± 1.47	0.89 ± 0.08	0.24 ± 0.03	**43.98 ± 3.53**	87.84 ± 0.94	1.16 ± 0.06	7.59 ± 0.44	1.84 ± 0.08	0.80 ± 0.03	5.99 ± 0.19	89.89 ± 1.77	**195.11 ± 7.16**	2.06 ± 0.02
SC11	0.51 ± 0.03	0.24 ± 0.00	34.54 ± 1.05	12.23 ± 1.47	1.60 ± 0.08	3.07 ± 0.22	6.10 ± 0.33	**58.29 ± 4.16**	0.34 ± 0.00	51.18 ± 1.50	0.99 ± 0.03	0.31 ± 0.06	**52.82 ± 3.99**	119.73 ± 3.57	1.40 ± 0.06	8.89 ± 0.47	2.59 ± 0.03	1.12 ± 0.11	6.71 ± 0.50	89.16 ± 1.83	**229.60 ± 8.45**	2.08 ± 0.02
SC12	0.63 ± 0.08	0.50 ± 0.06	30.94 ± 0.61	15.04 ± 1.02	1.60 ± 0.25	3.97 ± 0.08	7.51 ± 0.39	**60.19 ± 2.41**	0.28 ± 0.03	30.36 ± 2.69	0.86 ± 0.08	0.47 ± 0.06	**31.97 ± 3.11**	57.69 ± 3.07	0.83 ± 0.42	8.09 ± 0.25	1.66 ± 0.33	0.86 ± 0.19	7.87 ± 0.80	106.88 ± 4.13	**183.88 ± 1.87**	2.29 ± 0.03
VA1	0.71 ± 0.19	0.55 ± 0.19	43.22 ± 0.80	9.52 ± 0.86	1.04 ± 0.08	0.71 ± 0.08	1.84 ± 0.17	**57.59 ± 3.05**	0.52 ± 0.06	63.23 ± 3.71	1.10 ± 0.17	0.21 ± 0.06	**65.06 ± 6.10**	126.28 ± 3.91	2.03 ± 0.66	7.12 ± 1.00	0.71 ± 0.55	1.17 ± 0.83	2.15 ± 0.17	44.81 ± 4.29	**184.27 ± 10.59**	1.75 ± 0.04
VA2	0.67 ± 0.11	0.52 ± 0.03	36.22 ± 1.58	7.58 ± 0.25	0.88 ± 0.06	0.70 ± 0.08	1.52 ± 0.11	**48.09 ± 4.15**	0.39 ± 0.22	52.18 ± 3.32	0.88 ± 0.11	0.18 ± 0.06	**53.63 ± 5.56**	100.73 ± 5.32	1.29 ± 0.22	6.81 ± 0.47	0.70 ± 0.14	1.16 ± 0.36	1.86 ± 0.19	43.70 ± 6.56	**156.25 ± 8.89**	1.80 ± 0.05
VA3	0.66 ± 0.17	0.52 ± 0.11	39.28 ± 2.71	9.95 ± 0.97	1.18 ± 0.08	1.34 ± 0.08	3.36 ± 0.39	**56.29 ± 3.67**	0.52 ± 0.14	39.53 ± 1.91	1.37 ± 0.22	0.19 ± 0.06	**41.61 ± 4.60**	74.49 ± 8.81	0.90 ± 0.11	7.79 ± 0.86	1.07 ± 0.08	2.76 ± 0.06	2.79 ± 0.22	85.67 ± 4.24	**175.47 ± 17.59**	2.11 ± 0.03

[a] Data are expressed as mean ± SD of three replicates. [b] OA, oleic acid; LA, linoleic acid; GLA, γ-linolenic acid; DGLA.dihomo-γ-linolenic acid; AA, arachidonic acid. [c] ▽/mol, degree of fatty acid unsaturation. The value was calculated according to the method of Chen and Johns (*22*); ▽/mol = [1.0 (% monene) + 2.0 (% diene) + 3.0 (% triene) + 4.0 (% tetraene)]/100.

Table 3. Fungal Species Used for *Mortierella* Species SC9 Identification and Corresponding Accession Numbers of the Sequences of ITS1, 5.8S rDNA, and ITS2

fungus	accession no.	ref
M. alpina ATCC 16266	EF202180	this study
M. alpina ATCC 42430	EF202181	this study
Mortierella species SC9	EF202182	this study
M. alpina CBS 528.72	AJ271629	*27*
M. alpina AY310443	AY310443	*28*
M. humili	AJ878778	*28*
M. gamsi	AJ878508	*28*
M. macrocystis	AJ878781	*28*
Mucor plumbeus	AJ878776	*28*

Table 4. Percentage of Identities of Nine Fungi Examined in This Study Based on Sequences of ITS1, 5.8S rDNA, and ITS2[a]

	1	2	3	4	5	6	7	8	9
1. *M. alpina* ATCC 16266		96	94	95	94	81	81	77	56
2. *M. alpina* ATCC 42430			97	98	97	82	82	77	57
3. *Mortierella* species SC9				97	99	81	81	75	56
4. *M. alpina* CBS528.72					97	80	79	74	59
5. *M. alpina* AY310443						81	80	75	56
6. *M. humili*							84	80	59
7. *M. gamsi*								88	60
8. *M. macrocystis*									59
9. *Mucor plumbeus*									

[a] Identities were calculated by the program CLUSTAL X (*21*).

phase until the end of cultivation (day 7). TFA content (as mg/g of cell dry weight) decreased from 414.50 to 341.11 mg/g from days 1 to 4. The high initial TFA content (day 1, 414.50 mg/g) of *M. alpina* SC9 might be due to absorption of exogenous corn oil. From days 4 to 6, the TFA content increased from 341.11 to 353.56 mg/g. This might be due to the exhaustion of nitrogen sources (*30*) or a low concentration of glucose (<10 g/L) (*29*). A sharp decrease of TFA content (from 353.56 to 297.87 mg/g) was found from days 6 to 7, suggesting that when the cells were at the death phase, the stored TFAs were used to maintain cellular functions (*31*).

Changes of Different Categories of Fatty Acids (FA) of *M. alpina* SC9 during 7 Days of Fermentation. Besides general growth characteristics, the contents of different fatty acids were recorded (**Figure 2b**). As shown in the figure, PUFAs was the main type of FA in the fungus during the whole period of cultivation, and the trend of changes was similar to that of TFAs (**Figure 2a**). In contrast, the content of MUFAs decreased from 114.86 to 45.25 mg/g from days 1 to 7, further showing MUFAs are not the desirable type of FA for storage in this fungus. SFA content increased from days 1 to 4 from 59.65 to 87.09 mg/g, but decreased to 62.79 mg/g at day 7, implying the importance of SFAs during the log phase of this fungus. In addition, the degree of fatty acid unsaturation gradually increased from 1.47 (day 1) to 2.05 (day 6), which was in good agreement with other studies (*32*) or other organisms (*33*).

Changes of Fatty Acid Content of *M. alpina* SC9 during 7 Days of Fermentation. The changes of fatty acid content of *M. alpina* SC9 in 7-day batch cultivation are shown in **Figure 2c**. From days 1 to 7, the contents of OA (C18:1) and LA (C18:2) in cell dry weight decreased from 113.24 to 43.79 mg/g and from 230.54 to 91.45 mg/g, respectively. In contrast, the content of AA in cell dry weight increased from 3.40 to 97.9 mg/g from days 1 to 6. High contents of OA and LA further implicated that the corn oil (of which 25% is OA and 58% is LA) (*2*) was absorbed by *M. alpina* SC9 at the beginning of cultivation. In addition, the correlation coefficients between contents of C18:1 and AA and that of C18:2 and AA were found to be −0.9444 and −0.9373, respectively, suggesting there were enzymatic conversions of OA and LA to AA in the fungus *M. alpina* SC9 during fermentation. In the *M. alpina* PUFA enzymatic system, OA is first converted to LA by delta-12 desaturation, which is then converted to GLA (C18:3, n-6) by Δ-6 desaturation. Two carbon atoms are then added to the GLA by Δ-6 specific elongation to form DGLA (C20:3, n-6) which is finally converted to AA by Δ-5 desaturation (*10*). The contents of GLA and DGLA in cell dry weight increased from days 1 to 4 (from 2.03 to 7.61 mg/g and from 0.50 to 6.24 mg/g, respectively). The contents of C16:0 and C18:0 in cell dry weight increased from 48.62 and 8.25 mg/g (day 1) to 55.53 and 20.16 mg/g (day 4), respectively. The content of C16:0 in cell dry weight then decreased to 37.98 mg/g and that of C18:0 decreased to 12.99 mg/g at day 7. Together with **Figure 2b**, it is suggested that for PUFA-rich oil production, late stationary phase is preferred for cell harvesting as the content of the undesirable SFAs is at the least amount, whereas the degree of fatty acid unsaturation and the percentage of PUFAs in TFAs (64.94% of TFAs) are the highest. All major PUFAs (LA, GLA, DGLA, and AA) are valuable fatty acids and are beneficial to human health, suggesting the PUFA-rich oil produced by *M. alpina* SC9 would have high potential in food and medical industries. Together with the degree of fatty acid unsaturation changes, these findings suggested that the fatty acid compositions were tightly regulated in the fungus during different phases of cultivation.

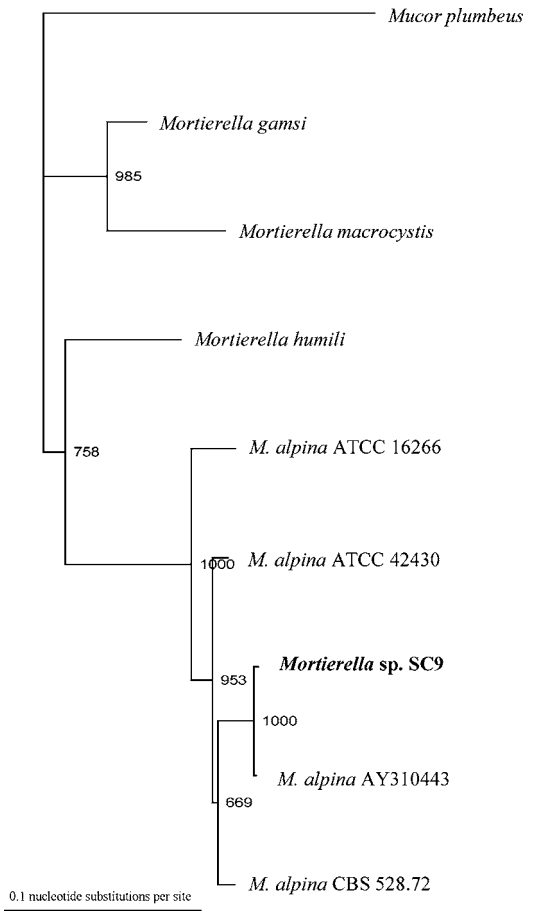

Figure 1. Phylogenetic relationship of the nine fungi based on ITS1, 5.8S rDNA, and ITS2 regions of DNA sequences by neighbor-joining analysis. Bootstrap values were obtained with 1000 replications and are shown at the nodes. The tree was rooted with *Mucor plumbeus* as outgroup.

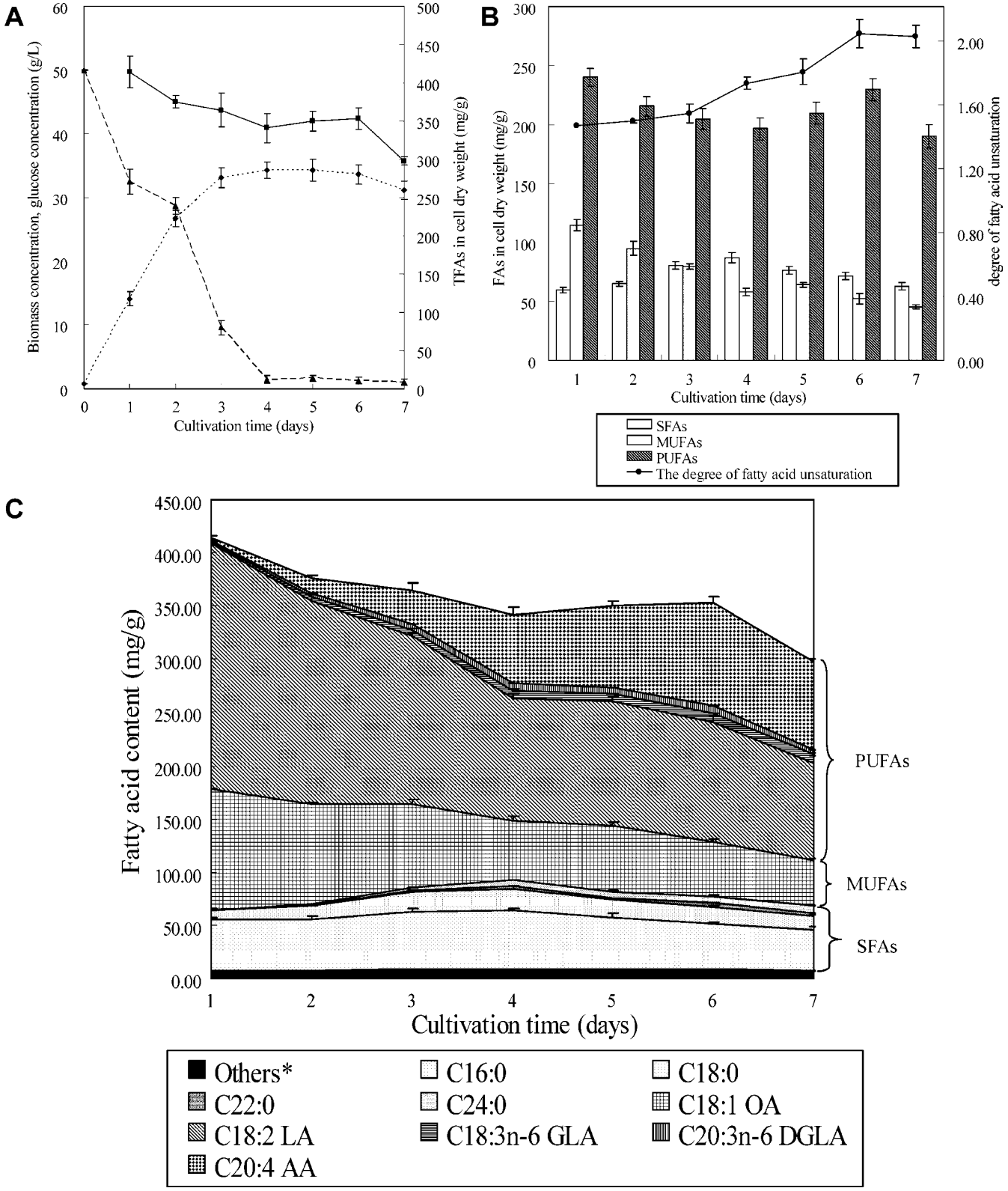

Figure 2. (**a**) Growth characteristics of *Mortierella alpina* SC9 during 7 days of cultivation: ◆, biomass concentration; ■, total fatty acids (TFAs) in cell dry weight; ▲, glucose concentration. Data are expressed as mean ± SD of three replicates. (**b**) Three categories of fatty acids content (mg/g of cell dry weight) and the degree of fatty acid unsaturation of *Mortierella alpina* SC9 during 7 days of cultivation. SFAs, total saturated fatty acids content in cell dry weight; MUFAs, total monounsaturated fatty acids in cell dry weight; PUFAs, total polyunsaturated fatty acids content in cell dry weight. SFAs include C14:0, C15:0, C16:0, C18:0, C20:0, C22:0, and C24:0. MUFAs include C16:1, C18:1, C20:1, and C24:1. PUFAs include C18:2 (linoleic acid, LA), C18:3n-6 (γ-linolenic acid, GLA), C18:3n-3, C20:2, C20:3n-6 (dihomo-γ-linolenic acid, DGLA), C20:3n-3, and C20:4 (arachidonic acid, AA). The degree of fatty acid unsaturation was calculated according to the method of Chen and Johns (*22*): [1.0 (% monene) + 2.0 (% diene) + 3.0 (% triene) + 4.0 (% tetraene)]/100. Data are expressed as mean ± SD of three replicates. (**c**) Fatty acid content (mg/g of cell dry weight) of *Mortierella alpina* SC9 during 7 days of cultivation. Data are expressed as mean ± SD of three replicates. *Others include C14:0, C15:0, C16:1, C18:3n-3, C20:0, C20:1, C20:2, C20:3n-3, and C24:1.

In conclusion, 25 isolates of *Mortierella* sp. were isolated from soil and were shown to be potential PUFA (including AA) producers. The highest PUFA-containing strain in this study was *M. alpina* SC9, which was further confirmed as a new strain of *M. alpina* through molecular sequencing comparison. PUFA proportion and the degree of fatty acid unsaturation of *M. alpina* SC9 were the highest at stationary phase (day 6), whereas fatty acid contents continuously changed during cultivation. Information obtained from this study would contribute to the improvement of PUFA production and add to our understanding of fatty acid metabolism in the fungus *M. alpina*.

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Received for review January 2, 2007. Revised manuscript received March 15, 2007. Accepted March 16, 2007. This research was supported by the RGC (the Research Grants Council of Hong Kong), the CRCG (The University of Hong Kong Committee on Research and Conference Grants), and the Outstanding Young Researcher and Outstanding Research Student Supervisor Awards of The University of Hong Kong.

JF0700071