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Lipid Characterization of *Mortierella alpina* Grown at Different NaCl Concentrations

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Effects of sodium chloride (NaCl) concentration on the lipid and fatty acid profiles of the polyunsaturated fatty acid (PUFA)-producing fungus, *Mortierella alpina* SC9, were investigated. The cells were cultivated in the medium with 4 different NaCl concentrations (0, 1, 2, 4%) for 6 days. The lipid and fatty acid profiles were analyzed by thin layer chromatography and gas chromatography. In the cultures with NaCl concentration up to 2%, PUFAs accounted for over 50% of the total fatty acids (TFAs) of the cells. Triacylglycerol (TAG) was the major lipid class, followed by monoacylglycerol (MAG) and diacylglycerol (DAG). TAG was found to contain the highest proportion of arachidonic acid (C20:4n-6, AA), suggesting that AA was mainly stored in the TAG. Comparing cultures at different NaCl concentrations indicated that TFA and TAG contents were higher in the cells grown at 2% NaCl. Similar results were found when 2% NaCl was added at day 3 of cultivation (late-log phase). In addition, the gene expression level of a TAG biosynthesis enzyme, diacylglycerol acyltransferase 2 (DGAT2), was also higher in the NaCl treated cells. This suggested that the increase of TFA and TAG contents might be related to the NaCl-stimulated DGAT2 expression.

KEYWORDS: Diacylglycerol acyltransferase 2; *Mortierella alpina*; polyunsaturated fatty acids; triacylglycerol

INTRODUCTION

The fungus *Mortierella alpina* has been widely used for polyunsaturated fatty acid (PUFA) production because of its oleaginous ability and unique fatty acid composition (*1*). Several strains of *M. alpina* are now being used for industrial arachidonic acid (C20:4n-6, AA) production in Europe, China, and possibly Japan (*1*). In addition to AA, the fungus produces several PUFAs such as linoleic acid (C18:2, LA), γ-linolenic acid (C18:3n-6, GLA), and dihomo-γ-linolenic acid (C20:3n-6, DGLA) (*1*). These PUFAs are beneficial to human health and are crucial for normal metabolic and physiological functions (*2*−*6*). Besides, the PUFAs are used in various foods and medical research. For instance, AA is shown to benefit infant brain and retinal development and thus has been included in the infant milk formulas (*2*−*4*). As a result, many studies have focused on PUFA production by *M. alpina* (*7*−*9*). Different cultivation optimizations for improving the AA production have been performed (*7, 8*), and cheaper carbon and nitrogen sources have been investigated, attempting to lower the PUFA production cost (*9*). Because of the unique fatty acid profile, *M. alpina* is also an important fungus for studying the PUFA biosynthesis pathway. Several genes of desaturases and elongase in the PUFA biosynthesis pathway have been cloned and transferred into other organisms (*10, 11*).

M. alpina is a fast-growing, filamentous, and saprophytic fungus. *M. alpina* might be isolated from many different geographical locations, such as the alpine area, tropical areas, and salt mashes (*12*−*14*). This implies that the fungus could adapt to various environmental conditions, and thus, several studies have been carried out to investigate the responses of *M. alpina* to different environmental conditions. These studies provided valuable information for improving PUFA production. For example, lowering cultivation temperature led to a higher degree of fatty acid unsaturation, whereas adjusting the pH of the medium value altered the proportion of AA in total fatty acids (TFAs) (*15, 16*). However, there is no study focusing on the effects of sodium chloride (NaCl) concentration on fatty acid and lipid class compositions of *M. alpina*. The effects of NaCl concentration on microorganisms including PUFA-producing species have been widely studied, and it was suggested that varying the NaCl concentration in the cultivation medium could affect the cellular fatty acid contents (*17*−*19*). In a study of the docosahexaenoic acid (DHA)-producing microalga *Crypthecodinium cohnii*, the strains *C. cohnii* ATCC30556 and RJH produced the highest fatty acid contents at 9 g/L NaCl, when compared with that at 0, 2, and 5 g/L (*17*). It was also reported that all the tested strains modified their fatty acid compositions in response to the change of NaCl concentration (*17*). In another PUFA-producing microorganism *Nitzschia laevis*, higher TFA and eicosapentaenoic acid (EPA) contents were obtained from the cells grown at a higher NaCl concentration (14 g/L) than that at a lower level (8 g/L) (*18*). In addition, the intracellular triacylglycerol (TAG) and lipid contents of *Dunaliella*

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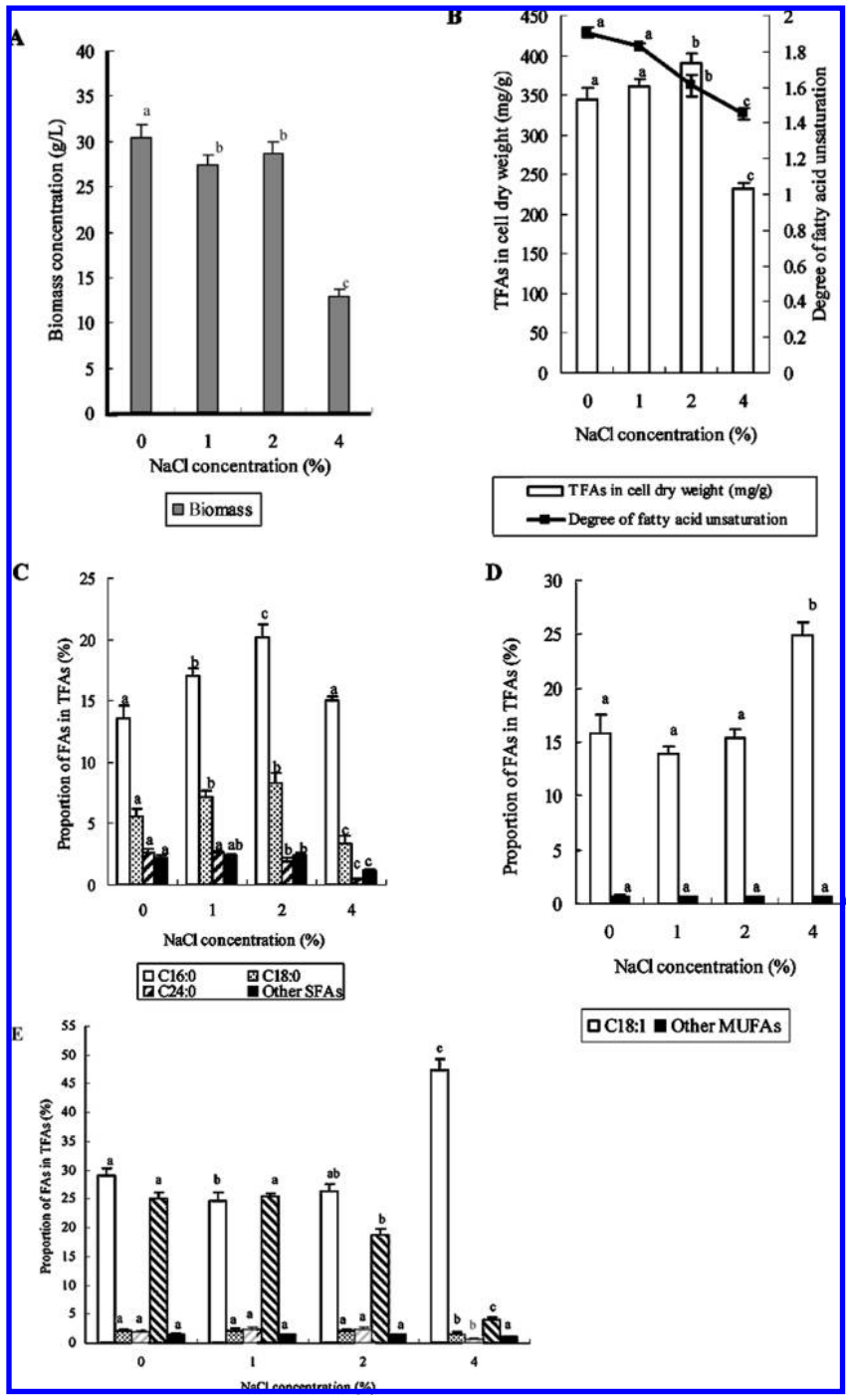

Figure 1. Growth characteristics and fatty acid profiles of *M. alpina* SC9 cultivated at different NaCl concentrations (0, 1, 2, 4%) for 6 days. Data are expressed as the mean ± SD of three replicates. Data marked with the same letters of each biomass concentration, TFA content, degree of fatty acid unsaturation, and fatty acid are not significantly different ($p < 0.05$). (**A**) Biomass concentration (g/L) of the cells. (**B**) Total fatty acid (TFA) content (mg/g of cell dry weight) and the degree of fatty acid unsaturation. The value was calculated according to Chen and Johns (*21*); ▽/mole = [1.0 (% monene) + 2.0 (% diene) + 3.0 (% triene) + 4.0 (% tetraene)]/100. (**C**) Fatty acid profile of saturated fatty acids (SFAs). Other SFAs included C14:0, C15:0, C20:0, and C22:0. (**D**) Fatty acid profile of monounsaturated fatty acids (MUFAs). Other MUFAs included C16:1, C20:1, and C24:1. (**E**) Fatty acid profile of polyunsaturated fatty acids (PUFAs). Other PUFAs included C18:3n-3, C20:2, and C20:3n-3. LA, linoleic acid; GLA, γ-linolenic acid; DGLA, dihomo-γ-linolenic acid; AA, arachidonic acid. FAs, fatty acids.

tertiolecta ATCC30929 increased with the addition of NaCl at the late-log phase of cultivation (*19*). As *M. alpina* could be isolated from saltmarshes with up to 4% NaCl (*14*), it is interesting to investigate the changes of intracellular TAG content and fatty acid composition in response to the change in NaCl concentration. If higher TFA and TAG contents of *M. alpina* could be obtained by adjusting the NaCl concentration in the cultivation medium, it will be beneficial to the oil production industry.

The aim of the present study was to characterize the lipid classes, fatty acid contents, and compositions of *M. alpina* grown at different NaCl concentrations. The effects of NaCl addition at the initial and the late-log phases of cultivation were investigated. In addition, the expression of a neutral lipid (TAG)

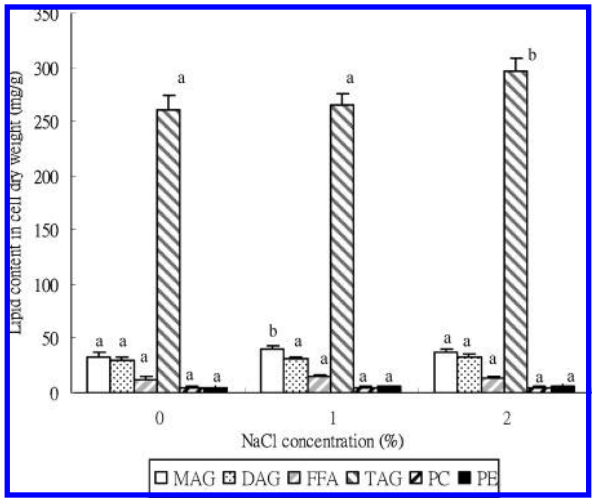

Figure 2. The contents (mg/g of cell dry weight) of different lipid classes of *M. alpina* SC9 after 6 days of cultivation at 3 different NaCl concentrations (0, 1, 2%). Data are expressed as the mean ± SD of three replicates. Data with the same letters of each lipid class are not significantly different ($p < 0.05$). MAG, monoacylglycerol; DAG, diacylglycerol; FFA, free fatty acid; TAG, triacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

biosynthesis enzyme gene, diacylglycerol acyltransferase 2 (DGAT2), was examined.

MATERIALS AND METHODS

Fungal Strain and Cultivation. *Mortierella alpina* SC9 was isolated and identified by molecular techniques as reported previously (*20*). For cultivation, an inoculum 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 containing 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 $CoCl_2.6H_2O$, and 0.13 mg of $CuSO_4$ (*15*) (with 0, 1, 2, or 4% of NaCl), were inoculated with 10% (v/v) of an exponentially growing inoculum and incubated at 23 °C with orbital shaking at 200 rpm in darkness for 6 days.

For studying the effects of NaCl addition on *M. alpina* SC9 at late-log phase during cultivation, the cells were grown without NaCl for 3 days (late-log phase) (*20*). The addition of 2% NaCl was then performed, and the cultures were grown for another 3 days.

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

Lipid Class Separation. Total lipids were extracted from 200 mg of the lyophilized biomass sample with a solvent mixture of chloroform−methanol−water (1:2: 0.8, v/v) (*21*). The extracted-lipids were then resuspended in chloroform with 80 mg/L butylated hydroxytoluene, and stored at −20 °C under nitrogen to prevent the samples from oxidation. The lipids were then separated by solid phase extraction with silica gel cartridge (sep-pak) into neutral lipids (NLs), and phospholipids (PLs) (*22*). The different lipid fractions were then concentrated under a stream of nitrogen and resuspended in 100 μL of chloroform. Lipid class separation and identification were carried out by thin-layer chromatography (TLC) using TLC plates (20 × 20 cm) coated with silica gel 60 (Merck, Darmstadt, Germany). NLs were eluted with a mixture of hexane−diethyl ether−acetic-water (70: 30: 1, v/v). PLs were eluted with chloroform−acetone−methanol−acetic acid−water (50:20:10:10:5, v/v) (*22*). Bands of different lipid classes were visualized by staining with 2,7-dichlorofluorescein (Sigma Chemical Co., St. Louis, MO) under UV light and identified by chromatography with authentic standards (Sigma Chemical Co., St. Louis, MO).

Fatty Acid Analysis. Either the lyophilized cells or the bands scraped from the TLC plates were used for fatty acid analysis. Fatty acid methyl esters (FAMEs) were prepared by trans-methylation with methanol−acetyl chloride and analyzed by an HP 6890 capillary gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a flame-ionization detector and an HP-INNOwax capillary column (30 m × 0.32 mm) (Agilent Technologies, Inc., Wilmington, DE) (*23*). Nitrogen was used as the carrier gas. Initial column temperature was set at 170 °C, which was subsequently raised to 230 at 1 °C/min and kept at 230 °C for 10 min. The injector was kept as 250 °C with an injection volume of 2 μL under splitless mode. The flame-ionization detector 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 (C17:0) as the internal standard (*20*).

RNA Extraction and Northern Blot Analysis. The harvested mycelia were ground with a mortar and pestle with liquid nitrogen. The ground powder was transferred into a 1.5 mL microfuge tube. The RNA was extracted with TRIzol reagent according to the manufacturer's instructions (Roche Applied Science, Indianapolis, IN). An equal amount of RNA (25 μg) for each sample was loaded into a 1% agarose gel. After electrophoresis, the RNA was transferred onto a nylon membrane (Amersham Biosciences, Piscataway, NJ). After drying, the RNA was UV-cross-linked for 2 min. The nylon membrane was hybridized in a hybridization buffer (50% formamide) containing the DIG-labeled probe (nonradioactive) at 50 °C overnight. The probe with the sequence of diacylglycerol acyltransferase 2 (DGAT2) gene with the accession number EA109470 in the NCBI database, was synthesized by the kit (Roche, Germany) following the manufacturer's instructions. The sequence of DGAT2 was amplified by using forward primer 5′TGAACACATCCTGCGGTCT and reverse primer 5′GCGGCATACTTGTCCTTGT in PCR. 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. After hybridization, the membrane was washed twice in 2 × SSC and 0.1% SDS at 50 °C for 15 min, and then washed twice in 0.5 × SSC and 0.1% SDS at 58 °C for 15 min. The signals were detected by adding the anti-DIG-AP conjugate (Roche). Chemiluminescent substrate CDP-Star (Roche) was added, and the membrane was exposed to an X-ray film. The signals were normalized by the 28S rRNA with the computer software ImageJ (*24*).

Statistical Analysis. Statistical analyses were performed by using the SPSS statistical package (SPSS, Inc. Chicago, IL). Analysis of variance (ANOVA) and least significant difference tests were performed. Difference at $p < 0.05$ was considered significant.

RESULTS AND DISSCUSION

Biomass Concentrations, TFA Contents, and Compositions of *M. alpina* SC9. *M. alpina* SC9 was cultivated in the medium with 4 different NaCl concentrations (0, 1, 2, 4%) for 6 days. As shown in **Figure 1A**, the biomass concentration was 30.51 g/L when *M. alpina* SC9 was grown in the medium without NaCl. The cells could grow at a higher NaCl concentration, but the biomass concentrations were decreased to 27.36 and 28.73 g/L at 1 and 2% NaCl, respectively. When the concentration of NaCl increased to 4% in the medium, the biomass concentration after 6 days of cultivation was significantly lower (12.90 g/L) ($p < 0.05$). However, the TFA contents of *M. alpina* were 345.29, 360.98, and 390.93 mg/g of cell dry weight at 0, 1, and 2% NaCl concentrations, respectively (**Figure 1B**). The TFA content of the cells at 2% NaCl concentration was significantly higher ($p < 0.05$). In contrast, the degree of fatty acid unsaturation (*25*) was the highest when the cells were cultivated without NaCl, whereas it was the lowest at 4% NaCl

Table 1. Lipid Class Fatty Acid Compositions of *M. alpina* SC9 Grown at Different NaCl Concentrations for 6 Days[a]

		proportion of fatty acid (%)														
		saturated fatty acids[c]					monounsaturated fatty acids[d]			polyunsaturated fatty acids[e]						
NaCl concentration (%)	lipid class[b]	C16:0	C18:0	C24:0	other SFAs	subtotal	C18:1 OA	other MUFAs	subtotal	C18:2 n-6 LA	C18:3 n-6 GLA	C20:3 n-6 DGLA	C20:4 AA	other PUFAs	subtotal	▽/mole[f]
0%	MAG	12.36 ± 0.98a	3.09 ± 0.54a	0.18 ± 0.19a	0.74 ± 0.01a	16.37 ± 0.88a	22.56 ± 0.78a	0.47 ± 0.11a	23.03 ± 0.76a	47.41 ± 1.84a	1.66 ± 0.04a	0.96 ± 0.14a	9.39 ± 1.66a	1.18 ± 0.05a	60.60 ± 1.25a	1.67 ± 0.07a
1%		12.87 ± 0.23ab	3.26 ± 0.14a	0.08 ± 0.13a	0.74 ± 0.04a	16.95 ± 0.45a	20.92 ± 0.69ab	0.15 ± 0.00b	21.07 ± 1.17b	43.42 ± 0.51b	1.81 ± 0.09b	1.52 ± 0.06b	14.13 ± 0.56b	1.10 ± 0.04ab	61.98 ± 2.15a	1.77 ± 0.02a
2%		13.87 ± 0.16b	3.64 ± 0.30a	0.08 ± 0.14a	0.66 ± 0.02b	18.25 ± 0.13b	19.90 ± 0.98b	0.17 ± 0.00b	20.07 ± 0.70b	43.18 ± 0.78b	2.26 ± 0.29b	1.81 ± 0.22b	13.36 ± 1.20b	1.07 ± 0.03b	61.68 ± 1.30a	1.75 ± 0.05a
0%	DAG	13.80 ± 1.62a	5.71 ± 0.85a	0.22 ± 0.07a	0.96 ± 0.06a	20.69 ± 1.11a	26.07 ± 1.07a	0.61 ± 0.23a	26.68 ± 0.99a	42.84 ± 2.64a	1.17 ± 0.17a	0.72 ± 0.17a	6.57 ± 1.47a	1.33 ± 0.06a	52.63 ± 2.11a	1.48 ± 0.10a
1%		14.32 ± 0.43a	5.33 ± 0.96a	0.12 ± 0.10a	0.72 ± 0.10b	20.49 ± 0.85a	23.45 ± 0.44b	0.40 ± 0.01a	23.85 ± 1.21b	45.22 ± 1.07a	1.44 ± 0.69a	0.62 ± 0.53a	7.66 ± 0.84a	0.72 ± 0.03b	55.66 ± 2.43a	1.53 ± 0.05a
2%		15.73 ± 0.04a	5.52 ± 0.25a	0.13 ± 0.14a	0.52 ± 0.02c	21.90 ± 0.28a	23.50 ± 1.47b	0.24 ± 0.01a	23.74 ± 1.31b	43.89 ± 0.73a	1.01 ± 0.21a	0.83 ± 0.11a	7.21 ± 1.57a	1.42 ± 0.04a	54.36 ± 1.98a	1.50 ± 0.04a
0%	FFA	16.07 ± 0.54a	12.38 ± 2.89a	2.03 ± 0.38a	3.07 ± 0.05a	33.55 ± 1.12a	20.94 ± 1.45a	0.67 ± 0.14a	21.61 ± 0.85a	33.32 ± 3.80a	1.00 ± 0.31ab	0.62 ± 0.47a	7.61 ± 3.63a	2.29 ± 0.04a	44.84 ± 2.64a	1.30 ± 0.15a
1%		19.31 ± 0.54b	18.11 ± 1.72b	2.82 ± 0.14a	4.77 ± 1.11b	45.01 ± 1.29b	18.10 ± 1.43a	0.59 ± 0.03a	18.69 ± 0.85b	25.22 ± 1.94b	0.48 ± 0.51a	1.21 ± 0.03a	8.81 ± 0.95a	0.58 ± 0.01b	36.3 ± 1.14b	1.11 ± 0.08a
2%		20.48 ± 0.72b	18.03 ± 0.56b	0.78 ± 0.70b	2.88 ± 0.11a	42.17 ± 1.05c	16.09 ± 1.90b	0.61 ± 0.03a	16.70 ± 1.82b	25.77 ± 2.25b	1.44 ± 0.16b	1.26 ± 0.51a	11.90 ± 2.71a	0.76 ± 0.00c	41.13 ± 2.20a	1.26 ± 0.09a
0%	TAG	12.98 ± 0.65a	5.40 ± 0.38a	3.28 ± 0.36a	2.46 ± 0.20a	24.12 ± 0.86a	13.82 ± 1.04a	0.65 ± 0.15a	14.47 ± 1.23a	25.19 ± 0.69a	2.28 ± 0.09a	2.24 ± 0.06a	30.29 ± 2.27a	1.41 ± 0.08a	61.41 ± 1.52a	2.03 ± 0.09a
1%		17.47 ± 1.65b	7.19 ± 0.57b	3.45 ± 0.23a	2.75 ± 0.05ab	30.86 ± 2.51b	11.66 ± 0.25b	0.77 ± 0.02a	12.43 ± 0.43b	19.52 ± 1.18b	2.31 ± 0.09a	2.72 ± 0.14b	30.59 ± 0.71a	1.57 ± 0.04b	56.71 ± 1.98b	1.93 ± 0.05a
2%		21.16 ± 0.12c	8.52 ± 0.57c	2.35 ± 0.25b	2.87 ± 0.19b	34.90 ± 2.17c	13.95 ± 0.46a	0.74 ± 0.02a	14.69 ± 0.65a	22.70 ± 0.79c	2.23 ± 0.08a	2.75 ± 0.11b	21.24 ± 0.40b	1.49 ± 0.04ab	50.41 ± 1.91c	1.64 ± 0.01b
0%	PC	34.73 ± 0.24a	7.69 ± 1.61a	1.58 ± 0.97a	3.97 ± 0.18a	47.97 ± 1.25a	7.09 ± 0.01a	0.68 ± 0.08a	7.77 ± 0.18a	27.56 ± 1.58a	3.53 ± 0.21a	1.88 ± 0.49a	7.77 ± 0.35a	3.52 ± 0.11a	44.26 ± 0.87a	1.19 ± 0.03a
1%		33.98 ± 0.09a	12.01 ± 1.45a	0.21 ± 0.16a	3.01 ± 0.13b	49.21 ± 1.84a	8.63 ± 3.96a	1.14 ± 0.02b	9.77 ± 0.21b	24.8 ± 3.63a	2.30 ± 1.35a	1.96 ± 0.85a	7.78 ± 1.54a	4.18 ± 0.15b	41.02 ± 0.97b	1.14 ± 0.02a
2%		33.47 ± 1.67a	20.40 ± 6.57b	0.91 ± 1.51a	3.32 ± 0.08c	58.10 ± 0.69b	10.66 ± 3.88a	0.99 ± 0.01c	11.65 ± 0.82c	16.09 ± 2.70b	1.65 ± 1.14a	1.92 ± 1.27a	8.30 ± 2.83a	2.29 ± 0.09c	30.25 ± 1.46c	0.94 ± 0.16b
0%	PE	19.31 ± 0.44a	8.95 ± 1.56a	1.44 ± 0.70a	2.92 ± 0.11a	32.62 ± 1.33a	13.51 ± 3.53a	2.11 ± 0.09a	15.62 ± 0.45a	21.40 ± 0.35a	5.43 ± 0.91a	2.19 ± 0.78a	21.80 ± 0.78a	0.94 ± 0.01a	51.76 ± 1.85a	1.71 ± 0.01a
1%		22.41 ± 0.91a	9.41 ± 1.82a	0.59 ± 0.20ab	0.37 ± 0.00b	32.78 ± 0.25a	15.84 ± 4.29ab	0.47 ± 0.01b	16.31 ± 0.66a	19.91 ± 0.44b	5.26 ± 0.20a	1.73 ± 0.44a	23.37 ± 2.48a	0.64 ± 0.01b	50.91 ± 0.41a	1.72 ± 0.08a
2%		24.42 ± 6.41a	10.05 ± 3.85a	0.22 ± 0.13b	1.96 ± 0.03c	36.65 ± 1.85b	20.07 ± 0.81b	1.25 ± 0.04c	21.32 ± 1.31b	17.58 ± 0.54c	5.47 ± 0.92a	1.58 ± 0.24a	16.17 ± 6.91a	1.23 ± 0.05c	42.03 ± 2.00b	1.45 ± 0.30a

[a] Data are expressed as the mean ± SD of three replicates. Data with the same letters in the same column of each lipid class are not significantly different ($p < 0.05$). [b] MAG, monoacylglycerol; DAG, diacylglycerol; FFA, free fatty acid; TAG, triacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine. [c] Other SFAs included C14:0, C15:0, C20:0, and C22:0. [d] OA, oleic acid; other MUFAs included C16:1, C20:1, and C24:1. [e] LA, linoleic acid; GLA, γ-linolenic acid; DGLA, dihomo-γ-linolenic acid; AA, arachidonic acid. Other PUFAs included C18:3n-3, C20:2, and C20:3n-3. [f] ▽/mole: the degree of fatty acid unsaturation. The value was calculated according to Chen and Johns (*21*); ▽/mole = [1.0 (% monene) + 2.0 (% diene) + 3.0 (% triene) + 4.0 (% tetraene)]/100.

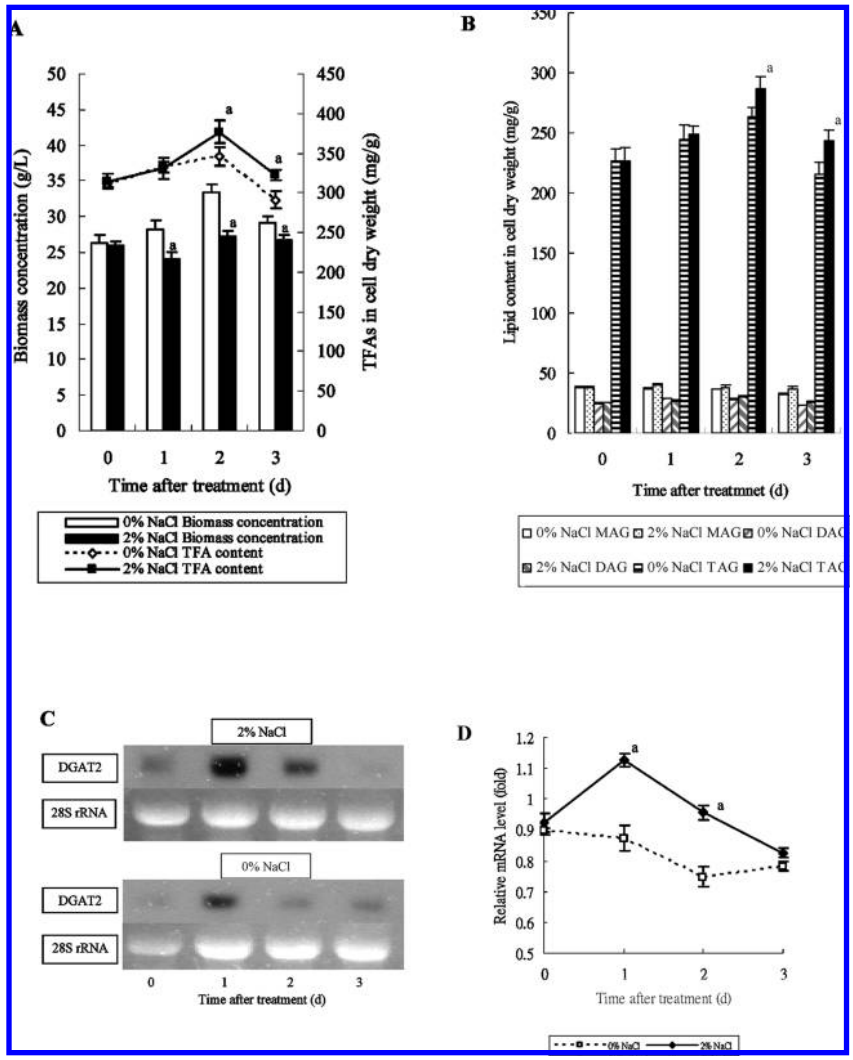

Figure 3. Effects of the addition of 2% NaCl at day 3 of cultivation on *M. alpina* SC9. Data are expressed as the mean ± SD of three replicates. Data marked the letter a are significantly different from that of the control ($p < 0.05$). (**A**) Biomass concentration (g/L) (bars) and total fatty acid (TFA) content (mg/g of cell dry weight) (lines). (**B**) Lipid class contents (mg/g of cell dry weight). MAG, monoacylglycerol; DAG, diacylglycerol; TAG, triacylglycerol. (**C**) Northern blot analysis of DGAT2 gene. (**D**) Relative mRNA level of DGAT2 gene. The level was normalized by the 28S rRNA in **C**.

($p < 0.05$), suggesting that *M. alpina* SC9 could regulate the fatty acid composition when facing the stress of NaCl. **Figure 1C−E** shows the profiles of different types of fatty acids, namely, saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and PUFAs. **Figure 1C** shows that when the cells were cultivated at 2% NaCl, the proportion of the predominant SFA, palmitic acid (C16:0), was 20.19%, which was higher ($p < 0.05$) than that cultivated without NaCl and at 1% NaCl (13.51% and 17.06%, respectively). As shown in **Figure 1D**, the predominant type of MUFAs was oleic acid (C18:1), and there was no significant difference between the cells grown at different NaCl concentrations (i.e., 0−2%). In contrast, more than 50% of the fatty acids belonged to PUFAs (**Figure 1E**) at all NaCl concentrations. This further suggested the potential of this fungus for PUFA production (*20*). AA and LA (C18:2) were the predominant PUFAs. The proportion of AA in the culture with 2% NaCl (18.74%) was lower than that at no NaCl (25.06%) or at 1% NaCl (25.48%) ($p < 0.05$). The AA proportion of the cells cultivated at 4% NaCl was the lowest, being only 4.01% ($p < 0.05$).

Regulating lipid contents and compositions in order to adapt to different environmental conditions have been reported in various organisms (*19, 26*). For instance, *Dunaliella* cells increased their intracellular accumulation of lipids when the initial NaCl concentration of cultivation increased (*19*). In *Catharanthus roseus*, the total lipid content increased significantly when salinity increased (*27*). When *Chromohalobacter israelensis* was cultivated at suboptimal salt concentrations, the degree of fatty acid unsaturation decreased (*28*). These findings were similar to the responses of *M. alpina* SC9 found in the present study. However, salt stress caused an increase in fatty acid unsaturation in halophilic *Hortaea werneckii* and halotolerant *Aureobasidum pullulans*, but a decrease in halophilic *Phaeotheca triangularis*, suggesting that there is no universal response to salt changes among all species and that the regulations could be more complex in eukaryotic cells because of the presence of intracellular membranes and organelles (*29*).

Lipid Classes and Their Corresponding Fatty Acid Compositions. The effects of NaCl (0, 1, 2%) on *M. alpina* SC9 lipid classes and their corresponding fatty acid profiles after 6 days of cultivation were investigated. The effects of 4% NaCl on the cells were not studied because the biomass concentration and TFA content obtained from the cells grown at that concentration were significantly lower ($p < 0.05$) than that of the cells at other NaCl concentrations (**Figure 1A** and **B**), which is undesirable for oil production. In **Figure 2**, the contents of different lipid classes are shown. The major lipid class

determined in *M. alpina* SC9 was TAG, followed by monoacylglycerol (MAG), diacylglycerol (DAG) and free fatty acid (FFA). The results were in accordance with studies on other oleaginous organisms such as *Schizochytrium mangrovei* and *Cunninghamella echinulata* that TAG was found to be the major lipid class (*30, 31*). These suggested the importance of TAG to the lipid accumulation in the oleaginous species. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were the only two phospholipids that could be detected in significant amounts. The contents of PC and PE (~5 mg/g of cell dry weight at all NaCl concentrations) were comparable to other studies on *M. alpina* (*32*). The contents of different lipid classes at various NaCl concentrations were not significantly changed, except that of TAG. The TAG content at 2% NaCl was the highest, reaching 296.55 mg/g ($p < 0.05$). In contrast, the TAG contents at 0 and 1% NaCl were 261.16 and 264.64 mg/g, respectively. This suggested that the increase of TFA content at 2% NaCl culture (**Figure 1B**) was contributed by the increased TAG content. In addition, the TAG yield (biomass concentration × TAG content) of the cells grown at 2% NaCl (8.52 g/L) was higher than that of the control cells (7.97 g/L). This suggested that the oil production using *M. alpina* SC9 was improved by adding 2% NaCl into the medium.

The fatty acid compositions of different lipid classes are shown in **Table 1**. When the cells were cultivated in cultures without NaCl, TAG was characterized by the highest AA proportion (30.29%). TAG was the dominant lipid class of the cells (**Figure 2**) suggesting that TAG was the major lipid class for AA storage in *M. alpina* SC9. This was in accordance with other oleaginous organisms that the major PUFAs are distributed mainly in TAG (*30, 31*). However, the fatty acid profiles of MAG and DAG were different from that of TAG that the proportions of AA were much lower, with only 9.39% and 6.57%, respectively. The major fatty acids found in MAG and DAG were C18:1 (22.56% in MAG and 26.07% in DAG) and C18:2 (47.41% in MAG and 42.84% in DAG).

Comparing the fatty acid profiles of TAG at different NaCl concentrations (**Table 1**) indicated that total PUFA proportions were 61.41, 56.71, and 50.41% at 0, 1, and 2% NaCl concentrations, respectively. However, the total PUFA proportions in MAG and DAG were not significantly influenced by the NaCl concentrations. Similar observations were given from a study on *C. echinulata*, where the amounts of the major PUFA (GLA) in MAG and DAG at different phases of cultivation were maintained constantly and changed slightly, respectively (*31*). Together with the insignificant changes of MAG and DAG contents as shown in **Figure 2**, the present study suggested that MAG and DAG were not the lipid classes for manipulating the PUFA contents in response to environmental changes. When comparing the PC fatty acid compositions, it was found that the total SFA proportion was higher when the cells were cultivated at 2% NaCl (58.10%) than that at 0 and 1% (47.97 and 49.21%, respectively). The total MUFA proportion in PC was also higher when the cells were cultivated at 2% NaCl (11.65%) than that at 0 and 1% (7.77 and 9.77%, respectively). In contrast, the total PUFA proportion in PC was the lowest when the cells were cultivated at 2% NaCl (30.25%). Similar trends were found when comparing the PE fatty acid compositions. It should be noted that in *C. roseus*, the proportions of the major PUFA (LA) in PC and PE increased with a higher NaCl concentration in the medium (*27*). This suggested that different organisms may have different mechanisms for regulating PUFA proportions in phospholipids. As phospholipids are highly specialized membrane components for regulating membrane fluidity in response to environmental changes (*29, 30*), the present study provided the information of how the phospholipid fatty acid compositions were regulated when *M. alpina* SC9 faced different NaCl concentrations.

Effects of 2% NaCl Treatment on *M. alpina* SC9. The present study suggested that the TFA and TAG contents of *M. alpina* SC9 were higher when the NaCl concentration of the cultivation medium was 2%. It has been reported that the addition of NaCl at late-log phase could increase the cellular content of TAG (*19*). In order to further confirm and elucidate the effects of NaCl on the TAG content of *M. alpina* SC9, the cells at late-log phase (day 3 of cultivation) (*20*) with no NaCl were subjected to a 2% NaCl treatment. **Figure 3A** shows that the addition of 2% NaCl into the medium would inhibit the growth of cells, as the 2% NaCl treated culture led to lower biomass concentrations than the control after 1 day of treatment. In contrast, after 2 days of treatment, the TFA content was higher in the cells at 2% NaCl than that with no NaCl. **Figure 3B** shows that the TAG content of 2% NaCl cultivated cells was higher than that of the control (no NaCl), further confirming that the increase of TFA content was contributed by the increased TAG content. This was in agreement with the study on *Dunaliella* cells in which the TAG content increased with the addition of NaCl at midlog or late-log phases of cultivation (*19*). As the biosynthesis of TAG requires DGAT2, which is an enzyme responsible for adding an acyl-chain to DAG to form TAG (*33*), the present study also characterized the effects of NaCl addition on this enzyme. Gene expression analysis was used to reveal the mRNA level of the DGAT2 gene. The changes of mRNA level of the DGAT2 gene after NaCl treatment are shown in **Figure 3C** and **D**. It was clearly found that after one day of the 2% NaCl addition, the DGAT2 expression level was higher than that of the control (0% NaCl). Once the DGAT2 gene was expressed at a higher level, more DGAT2 could be translated, and as a result, a higher amount of TAG would be synthesized in the cells (*33*). The increased lipid content may correspond to adaptive responses, such as glycerol production and cell volume changes (*19*). Although other mechanisms might also be involved in the increase of TAG content after the 2% NaCl treatment, the gene expression analysis strongly suggested that the increase of TAG content is related to the increased DGAT2 gene expression level.

In conclusion, the effects of NaCl concentration on lipid contents or classes of *M. alpina* SC9 were characterized. We had previously shown before that the fatty acid compositions of *M. alpina* SC9 were tightly regulated during different cultivation stages (*20*) and that this study showed that the whole cell fatty acid contents and compositions, the lipid class contents and fatty acid profiles, and the expression of DGAT2 gene were regulated when the cells were facing environmental changes. It was shown that the increase of TFA content at 2% NaCl was contributed by the increased TAG content, which might be related to the NaCl-stimulated DGAT2 expression. This study provided important information for understanding fatty acid metabolism of this oleaginous fungus and facilitated further optimization of PUFA production.

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