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Isolation and identification of native lower fungi for polyunsaturated fatty acid (PUFA) production in Thailand, and the effect of carbon and nitrogen sources on growth and production

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This research focuses on isolation and identification of polyunsaturated fatty acid (PUFA) producing fungi from natural sources of Thailand, followed by experimental designs for carbon and nitrogen utilizations of the potential strains. The results show that 8 and 16 isolates of lower fungi from freshwater and wet-fallen leaves, respectively, could produce PUFAs. Among these isolates, the NR06 that was isolated from wet-fallen leaves at Tad Ta Phu waterfall, Nakhon Ratchasrima province. They showed the highest level productions of both biomass (15.49 ± 0.24 $g \cdot l^{-1}$) and total fatty acids ($16.44\pm0.30\%$). The major essential fatty acid composition was found to be arachidonic acid (ARA; C20:4n6) ($32.24\pm0.35\%$). The minors were linoleic acid (C18:2n6) ($8.26\pm0.59\%$) and γ-linoleic acid (GLA; C18:3n6) ($5.48\pm0.08\%$). Besides the morphological characterization, taxonomic identification by the 636 bp-ITS region sequencing and phylogenetic analysis were performed. It was demonstrated that the fungal isolate NR06 was classified in the closest species of *Mortierella elongata* with 99% similarity (GenBank accession no. KF181625). Statistically based experimental designs (Design Expert 7.1 software) were applied to the optimization of biomass and fatty acid production in the NR06 culture. Using the glucose as a carbon source, there was a significant effect on both biomass and ARA production (p-values < 0.0001), whereas using yeast extract as a nitrogen source affected only ARA production (p-value $= 0.02$). It was suggested that the NR06 isolate could be further optimized for an improvement as a potential ARA producing strain.

Key words: Polyunsaturated fatty acids (PUFAs), arachidonic acid (ARA), lower fungi, *Mortierella elongata.*

INTRODUCTION

Polyunsaturated fatty acids (PUFAs) are long-chain fatty acids containing more than one double bond in their backbone. Particularly, long chain PUFAs that are classified as ω-3 and ω-6 essential fatty acids (EFA), play important roles in both biomedical and nutraceutical area (Certik and Shimizu, 1999). Their biological functions are conducted via two principles. Firstly, as a part of lipid subunits in membrane of cells and organelles, PUFAs

modulate membrane dynamics, fluidity, flexibility and selective permeability. They also regulate membrane-associates processes, including membranes transport system, intercellular communication, interactions with extracellular components, cell-stimulus responses and control of some gene expressions (Gill and Valivety, 1997). Secondly, as the precursors of eicosanoids (prosta-glandins, prostacyclins, thromboxanes and leukotrienes), these metabolites are essential for maintaining and regulating many diverse metabolic processes. These processes reduce inflammation and pain, control cholesterol, triglyceride and lipoprotein levels in plasma, affect platelets composition and functions, and especially regulate cognitive and visual development of fetuses and infants (Ward and Singh, 2005). As a whole, PUFAs have an impact on various cellular biochemical activities, and they are implicated in both physiological and pathological conditions including carcinogenesis and cardiovascular diseases. Since the essential fatty acids could not be synthesized *de novo* in mammals as lack of the key enzyme desaturases, the principal polyunsaturated fatty acids, that is, arachidonic acid (C20:4; ARA), eicosa-pentaenoic acid (C20:5; EPA) and docosahexaenoic acid (C22:6; DHA) are nowadays extensively used as dietary supplements for health and infant formulas.

The major source of PUFAs is marine fish. Their global stocks are gradually limited, resulting in inadequate production in a near future. Besides, the despicable tastes and odors of fish oil, shows that some fish especially salmon, sardine, tuna and hake, are often contaminated with heavy metals (cadmium, lead and mercury) and organic pollutants (dioxins, dioxin-like compounds and furans) that are toxic to human (Domingo et al., 2007). Related to health risks derived from the environment contaminant found in fish, a remarkable promotion of fish consumption as a source of omega-3 PUFAs has been reviewed scientifically for adverse and beneficial effects (Domingo, 2007). Thus, several assorted sources containing various types of PUFAs, that is, microalgae, bacteria, yeast, fungi and transgenic plants have been alternatively proposed.

The green alga, *Parietochloris incina*, was reported to be a potent producer of ARA. Since more than 90% of the ARA was deposited onto storage triglyceride molecules, EFA bioavailability is important in further studies (Bigogno et al., 2002). While natural and transgenic plants are the main alternative sources of PUFAs for human consumption, oleaginous microorganisms, parti-cularly fungi also gain prominence as potential sources. In comparison with the plants, besides the main advantages of less competition for land to grow and the independence to seasonal variations

variations, fatty acid profiles from fungi are naturally high in EFAs. They can also be induced to synthesize higher yields of targeted fatty acids by controlling environmental and nutritional conditions (Certik and Shimizu, 1999; Dyal and Narine, 2005). Since lipogenesis mechanism in certain strains was well studied, various desaturase and elongase mutants can be manipulated. Also, it is able to incorporate and transform exogenous fatty acids (Dong and Walker, 2008; Shimizu and Sakuradani, 2009). More-over, high growth rates on wide substrates would provide utilizing cheap materials including local agricultural products and industrial by-products or wastes (Fakas et al., 2008; Gema et al., 2002). Production of fungal storage lipids as the single cell oils (SCO) have been obviously targeted to Zygomycetes especially of the genera *Cunningghamella*, *Mucor*, *Mortierella*, *Rhizopus* and *Zygorhynchus* (Yongmanitchai and Ward, 1989; Gill and Valively, 1997; Gema et al., 2002; Shimizu and Sakuradani, 2009). However, lipid accumulation was observed when the organisms were under stress conditions, for example, a high carbon to nitrogen ratio in the growth medium (Murphy, 1991). Besides, some fungal strains have been indicated as promising sources of certain long chain fatty acids, the ability of their fermentation to compete economically with traditional sources of EFA is limited by relatively low productivities and excessively long fermen-tation times (Barclay and Zeller, 1996; Leman, 1997). So far, many researches have been focusing on finding potential species those produce high specific EFA with relatively inexpensive cost and effective fermentation systems for industrial scales.

The aim of this study was to isolate the native lower fungi producing PUFAs from Thailand. To the best of our knowledge, this is the first survey for the isolation of oleaginous fungi from a freshwater and wet-fallen leaves from various natural sources, for which no previous data are available. The potential isolates for PUFAs production would be further characterized and investigated. Taxonomic identification was carried out through molecular methods. The effect of carbon and nitrogen sources on growth and ARA production was also evaluated.

MATERIALS AND METHODS

Isolation of lower fungi

Freshwater from rivers, waterfalls and reservoirs, and wet-fallen leaves around there were samples for isolation of PUFA producing fungi. The samples were collected into sterile bottles, from 5 provinces of Thailand, that is, Lopburi, Rayong, Chonburi, Nokorn

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Abbreviations: PUFAs, Polyunsaturated fatty acids; **ARA,** arachidonic acid.

Ratchasima, Chachoengsao and Trang. For the freshwater samples, mixed vegetable seeds (basil seeds, hairy basil seeds, cucumber seeds and pumpkin seeds) were put into the vials containing 5 ml of distilled water, followed by autoclaving at 121°C for 15 min. The freshwater samples were added to the vials and incubated at 25°C for 24 h. When fungal mycelia appeared around the seeds, freshwater samples were poured out. To suppress bacterial growth, the fungal seeds were washed three times with 50 ml of the antibiotic mixture solution (0.25 mg·l^{-1} of penicillin and 0.25 mg·l^{-1} streptomycin). The fungal seeds were further cultivated on potato dextrose agar (PDA) at 25°C for three to five days. For the wet-fallen leaves samples, they were cut into 0.5 x 0.5 cm pieces and washed three times with 50 ml of the antibiotic mixture solution. Then they were transferred to glucose peptone yeast extract (GPY) agar (0.1% yeast extract, 0.1% peptone, 1.0% glucose, 1.6% sea salt and 1.5% agar) and incubated at ambient temperature for 24 h. The morphology of all appeared fungi from both PDA and GPY plates was characterized. The fungal mycelia from different plates were mounted on slides for general staining with lactophenol cotton blue and oleagineous staining with Sudan Black B to observe sporangiophores and fat globules under a compound microscopy, respectively. The lower fungi (no septate fungi) with fat globules were selected to cultivate on PDA for more two to three times. Isolated pure cultures were maintained on PDA slants, stored at 4°C, and subcultured every two weeks.

Cultivation and harvest

After three to five days of growth on PDA, pure mycelia with agar were cut into 1.5x1.5 cm pieces and minced. They were transferred to 50 ml of potato dextrose broth (PDB) and cultivated on rotary shaker at 200 rpm and 25°C for five days. Growth fungi were taken and analyzed for polyunsaturated fatty acids. Culture broth was filtered by Whatman No.1 filter paper and washed three times with distilled water. The supernatant was determined for glucose residual by high-performance liquid chromatography (HPLC: HPLC model Waters 2690) equipped with a SHODEX SH-1011 COL analytical column and a refractive index detector. The mobile phase was 5 mM sulfuric acid and nanopure water with a flow rate of 0.6 ml·min^{-1}, and the column temperature was 60°C. The fungal cells were freeze dried for three days, followed by determination of biomass and polyunsaturated fatty acid content.

Extraction and fatty acid analysis

The fifty milligrams of fungal cells were added to the extraction tube containing 100 µl of internal standard (1% (w/v) heptadecanoic acid (C17:0)), and 2.0 ml of 5% (v/v) H_2SO_4 in methanol. The solution was mixed well and incubated in water bath at 90°C for 1 h. After cooling at room temperature, 1 ml of hexane and 1 ml of distilled water were added. The solution was centrifuged at 1,000 rpm for 5 min. The supernatant was analyzed for PUFA composition with gas chromatography (GC-Shimadzu), equipped with Omega Wax^{TM} 250 in a fused silica (Rtx1 fused silica) capillary column (0.25 mm ID x 30 m) and a flame ionization detector (FID). Nitrogen and hydrogen were used as carrier gases at a flow rate of 1.22 ml·min^{-1}. The column and detector temperatures were 150 and 260°C, respectively. An injection was performed in split mode 100:0 at 250°C. Lipid and fatty acid compositions were determined and paralleled with the chromatograms of standard polyunsaturated fatty acids (fatty acid methyl esters (FAME) chemical standards, Supelco, U.S.A.), i.e., palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2n6), ɣ-linolenic acid (GLA, C18:3n6), eicosenoic acid (C20:1n9), dihomo-linolenic acid (DGLA, C20:3n6), arachidonic acid (ARA, C20:4n6) and eicosapentaenoic acid (EPA, C20:5n3).

DNA sequencing of nuclear ribosomal internal transcribed space (ITS) region and phylogenetic analysis

DNA extraction was modified from O'Donnell et al. (1997) method. The fine powder of fungal mycelia of 50-100 mg was added to 1.5 ml-microcentrifuge tube. Lysis buffer (1 M EDTA 50 ml and 50 mM Tris HCl 10 ml in 1.16 g·l^{-1} of NaCl), 700 µl, was added and mixed well. The solution was incubated in heat block at 65°C for 1 h. It was centrifuged at 12,000 rpm, 25°C, for 20 min, and transferred to a new microcentrifuge tube. Equal volume of phenol : chloroform : isoamylalcohol (24:25:1) was added and turned up side down slowly for 20 times. The mixture was kept at -20°C for overnight. After that, the mixture was centrifuged at 12,000 rpm, 4°C, for 10 min. The DNA pellets were washed with 75% ethanol and air dried for overnight. Finally, DNA was resuspended in 20-40 fold of elution buffer NE (Macherey-Nagel GmbH & Co. KG, Germany). PCR amplification in ITS region was performed by using a pair of both primers ITS5 (5′ GGA AGT AAA AGT CGT AAC AAG G 3′) and ITS4 (5′ TTC TCC GCT TAT TGA TAT GC 3′) was used in 50 µl PCR reactions, which contained 35.2 µl of 10x buffers, 5.0 µl of 25 mM MgCl$_2$, 1.5 µl of 1 mM dNTP, 4.0 µl of forward and reverse primers (10 µM), 1.5 µl of 20-100 ng DNA sample, 1.8 µl of ddH$_2$O and Taq DNA polymerase (1 unit/20 µl) (Fermentas, Canada). The thermal cycling for PCR protocol was initially denatured at 94°C for 2 min. Then, the 2-35 cycles were performed at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min. The final extension step was managed at 72°C for 10 min, were incubated at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, and final extension step was managed at 72°C for 10 min. After analysis with 1.5% (w/v) agarose gel electrophoresis, the PCR products were purified exactly following the manufacturer's instruction of Nucleo spin extract II (Macherey-Nagel GmbH & Co. KG, Germany). The PCR products of the NR06 isolate were further directly sequenced by Marcrogen (Korea). Each sequence was checked for ambiguous bases and assembled using Bio edit V.7.0.5 (Hall, 1999). The consensus sequences for each DNA region were multiple aligned by Cluster W 1.6 along with other sequence obtained from the GenBank database. The phylogenetic analysis were performed using PAUP*4.0b 10 and similarity test of ITS region.

Effect of carbon and nitrogen sources on growth and PUFA production

This study was performed in flask scale for carbon and nitrogen assimilation. The appropriated carbon and nitrogen sources will promote growth and polyunsaturated fatty acid production. The carbon sources in this experiment included cellulose, dextrose, starch, glycerol, glucose, sucrose and xylose, in a concentration of 10 g·l^{-1} with yeast extract 5 g·l^{-1}, MgSO$_4$·7H$_2$O 1.0 g·l^{-1} and K$_2$HPO$_4$ 1.0 g·l^{-1}. The nitrogen sources used in this study were corn meal, corn steep solid, meat extract, NH$_4$NO$_3$, peptone, soytone, yeast extract, in a concentration of 5.0 g·l^{-1} with glucose 10.0 g·l^{-1}, MgSO$_4$·7H$_2$O 1.0 g·l^{-1} and KH$_2$PO$_4$ 1.0 g·l^{-1}. The samples were taken every 3 and 5 days of cultivation times. All samples were determined in triplicate for dried cell weight and polyunsaturated fatty acid production. The experimental design using statistical program (Design expert 7.1 software) was employed to analyze the analysis of variance (ANOVA) of the quadratic regression models. It was used to screen factors affecting the production of biomass and ARA by the NR06 isolate. The variable evaluations are listed in Table 4. These included seven nutrients (glucose, glycerol, sucrose, meat extract, soytone, peptone and yeast extract) and four dummies (unassigned variables). This statistic program consists of seven variables with 30 runs (N) with D1 to D4 being the dummy variables employed to evaluate the standard errors (SE) of the experiments (Saelao et al., 2011).

Statistical analyses were applied to identify the variables that had significant effects on the responses (biomass, ARA production and total fatty acid (TFA)). The effect by variable ($E_{(Xi)}$) on a response was determined by substrate on the average response of the lower level (Ri^-) from that of the high level (Ri^+) using the following standard equation:

$$E(X_i) = 2\frac{[\sum R_i^+ - \sum R_i^-]}{N} \tag{1}$$

Where, N is total number of experiments or runs (N=30). The effects of the dummy variables were used to calculate SE as follows:

$$SE = \sqrt{\frac{\sum (Ed)^2}{N}} \tag{2}$$

Where, Ed is the effect of each dummy variable and n is the number of dummy variables (n = 4).

$$Y = \beta_0 + \sum_{i=1}^{k}\beta_i x_i + \sum_{i=1}^{k}\beta_{ii} x_i^2 + \sum_{i=1}^{k}\beta_{ij} x_i x_j \tag{3}$$

Where, Y is the predicted response, β_0 is the model constant, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the interaction coefficient.

RESULTS

A total of 10 freshwater samples and 75 wet-fallen leaves samples from different natural sources, that is, Bang Pakong River (Chachoengsao Province), Lopburi River (Lopburi Province), Sikao River (Trang Province), Bang Phra Reservoir (Chonburi Province), Khao Cha Mao (Rayong Province), Wang Champi Waterfall and Tad Ta Phu Waterfall (Nakorn Rachasima Province) were used for determination of fungal growth and selected for pure colonies on PDA plates. Fatty acid producing strains of lower fungi were found to be 8 and 16 isolates from freshwaters and wet-fallen leaves samples, respectively (Table 1). The results show that the biomass concentration varied from 1.20-15.49 g·l^{-1} for all isolates. The lowest biomass production was found in the CC04 culture and the highest biomass producer was the NR06 culture. Similarity, total lipid content (percent) production varied from 2.11±0.12 to 16.44±0.30%, with the same cultures of the lowest and the highest. Thus, the NR06 isolate was considered as a potent culture for the maximum production of biomass (15.49±0.24 g·l^{-1}) and total lipid (16.44±0.30%) among all 24 isolates, and it was selected for further characterization and identification.

The NR06 isolate was cultivated from wet-fallen leaves near Tad Ta Phu waterfall, Nakorn Ratchasima Province. Morphological characteristics of the NR06 isolate are shown in Figure 1. After cultivation on PDA for 7-10 days, the culture was occurred as fluffed up white cottony mycelia in Petri dish (Figure 1A). Non-septated mycelia were classified as lower fungus, with many lipid globules

inside those stained by Sudan black B, and their swollen sporangiophores were observed (Figure 1B, C and D). Molecular phylogeny characterization of the NR06 isolate was carried out for taxonomic identification using *ITS* gene sequence. The PCR product obtained 636 bp-ITS gene which was further used to perform BLAST. The lower fungus isolate NR06 was identified to the closest species as *Mortierella elongata* with 99% similarity, with GenBank accession no. KF181625.

The fatty acid composition of all fatty acid producing strains was analyzed by GC (Table 2). Total fatty acid contents of fungal isolates were C16-C20 fatty acids, i.e. palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2n6), ɣ-linolenic acid (GLA, C18:3n6), eicosenoic acid (C20:1n9), dihomo-linolenic acid (DGLA, C20:3n6), arachidonic acid (ARA, C20:4n6) and eicosapentaenoic acid (EPA, C20:5n3). Although palmitic, stearic and oleic acids were commonly found in all isolates, polyunsaturated fatty acid profiles among the isolates were remarkably different. Ten (10) isolates from a total of 24 fungal isolates could produce essential unsaturated fatty acids. The NR06 isolate which showed the highest biomass and fatty acid content, could produce significantly high level of essential long chain unsaturated fatty acids ($p \le 0.05$), especially arachidonic acid (ARA; C20:4n6) (32.24±0.35 %). Other unsaturated fatty acids, i.e., linoleic acid (C18:2n6), ɣ-linoleic acid (GLA; C18:3n6) and oleic acid (C18:1) were also found in lipid profiles of the NR06 culture, with the levels of 8.26±0.59, 5.48±0.08 and 23.48±0.22%, respecttively (Figure 2).

The effect of various carbon sources, i.e., cellulose, dextrose, glycerol, glucose, starch, sucrose and xylose, on growth and polyunsaturated fatty acid production by the lower fungal NR06 isolate are demonstrated in Table 3. The results indicate that glycerol, dextrose and glucose gave relatively high biomass with the values of 8.37±0.72, 7.93±0.67 and 7.87±0.72 g·l^{-1}, the ARA productions of 22.51±1.9, 22.07±1.22 and 21.00±0.01% (w/w, ARA by total fatty acid) and the total fatty acids of 12.61±0.12, 10.83±0.56, 11.41±1.58% (w/w, total fatty acid by biomass), respectively. Thus, glycerol, dextrose and glucose were chosen for further studies as carbon source variables. Various nitrogen sources, i.e., corn meal, corn steep solid, meat extract, peptone, soy tone and yeast extract, except NH_4NO_3, were found to support growth in a range of 6.10±0.10 to 7.10±0.26 g·l^{-1}. However, since ARA productions from media containing meat extract, peptone, soytone and yeast extract were 23.62±2.46, 23.91±9.86, 32.36±4.12 and 27.03±6.41% (w/w, ARA by total fatty acid), respectively, these nitrogen sources were selected for the variables.

All selected carbon and nitrogen sources were evaluated to be the important factors for statistical program, Design expert 7.1 software, USA. The variable factors of substrate combination between carbon and nitrogen sources were performed in Table 4. After 30 trials, the results showed

Table 1. Biomass and total fatty acid contents of fungal isolates from various natural sources.

Natural sources	Samples	Fungal isolates	DW (g·l^{-1})	TFA (%)
Bang Pakong river, Chachoengsao province	Leaves	CC01	4.78±0.16^i	3.65±0.34^{ghi}
		CC02	1.59±0.19^{no}	3.32±0.13^{ghijk}
		CC03	1.49±0.23^o	2.22±0.15^{lm}
		CC04	1.20±0.08^o	2.11±0.12^m
Lopburi river, Lopburi province	Freshwater	LB01	11.19±0.23^c	10.38±0.22^c
		LB02	10.33±0.08^d	2.50±0.21^{km}
		LB03	10.93±0.13^c	4.83±0.11^f
		LB04	4.98±0.19^i	4.02±0.13^{fgh}
Sikao river, Trang province	Freshwater	TR03	4.85±0.07^i	4.74±0.36^f
Bang Phra reservoir, Chonburi province	Leaves	CB01	7.25±0.13^g	3.05±0.23^{ijkl}
		CB02	8.23±0.25^f	3.92±0.47^{fghi}
		CB04	12.12±0.24^b	2.99±0.05^{ijklm}
		CB05	6.31±0.08^h	2.67±0.16^{jklm}
		CB06	3.09±0.17^j	4.68±0.04^f
	Freshwater	CB07	6.11±0.18^h	3.54±0.17^{ghij}
		CB08	2.60±0.08^k	6.61±0.24^e
Khao Cha Mao waterfall, Rayong province	Leaves	RY01	7.43±0.16^g	10.39±0.94^c
		RY02	7.52±0.42^g	8.45±1.41^d
Wang Champi waterfall, Nakorn Ratchasima province	Leaves	NR01	2.33±0.11^{kl}	9.61±0.07^c
		NR02	1.89±0.15^{mn}	13.86±0.11^b
		NR03	2.08±0.18^{lm}	14.11±0.24^b
		NR04	9.34±0.16^e	4.10±0.25^{fg}
	Freshwater	NR05	1.20±0.07^o	3.12±0.18^{hijkl}
Tad Ta Phu waterfall, Nakorn Ratchasima province	Leaves	NR06	15.49±0.24^a	16.44±0.3^a

Data expressed as means±SD of three replicates. Different superscript letters are significantly different ($p \leq 0.05$). *DW, weight of dry biomass; TFA, total fatty acid.

wide variation of responses for biomass, total fatty acid content and ARA production. The amounts of biomass production varied from 7.06 to 26.46 g·l^{-1}. The obtained total fatty acids were in a wide range of 5.06 to 56.3% (w/w) with ARA productions varying from 12.06 to 56.3% (w/w) (Table 5). Variables of selected carbon sources (glucose, sucrose and glycerol) and selected nitrogen sources (meat extract and yeast extract) had a significant effect on growth with confidence levels >95.0%. The statistical experimental design revealed that biomass and ARA production were significantly affected by growth of cell cultures of the NR06 isolate with coefficients of determination (R^2) of 0.9956 and p-values <0.05.

The combination of carbon and nitrogen sources using the experimental design displayed the best results from the mixtures of substrates as in the equations. The ANOVA of the quadratic regression models for growth and ARA production indicated the 'Prob>F' <0.0015 and 0.0011, respectively. The models implicit that both statistical designs maintain the significance of experimental data. The statistically experimental design coefficients estimated by regression analysis for each factor are also shown in Table 6. The significance of each coefficient was determined by p-values. The smaller the p-values, the higher the significance of the corresponding coefficients. The results showed that glucose as the carbon source affected both biomass and ARA production (p-values of <0.0001), while yeast extract as the nitrogen source affected only ARA production ($p = 0.02$). The mathematical model, incorporating the different interactions of low and high levels of varied factors, is proposed with effect on growth (Equation 4) and ARA production (Equation 5).

$Y_{ARA (\% of TFA)}$ = -16.0 A+0.63 B+1.42 C-2.57D-1.47 E+0.11 F -2.49G+1.62 H+0.92 J-0.045 K+0.086 L-0.082A B -1.59 A C+1.92A D1.10A E-0.46A+1.52 AG -0.26A H-0.63A J-0.021AK-0.40 A L-0.66BC (4)
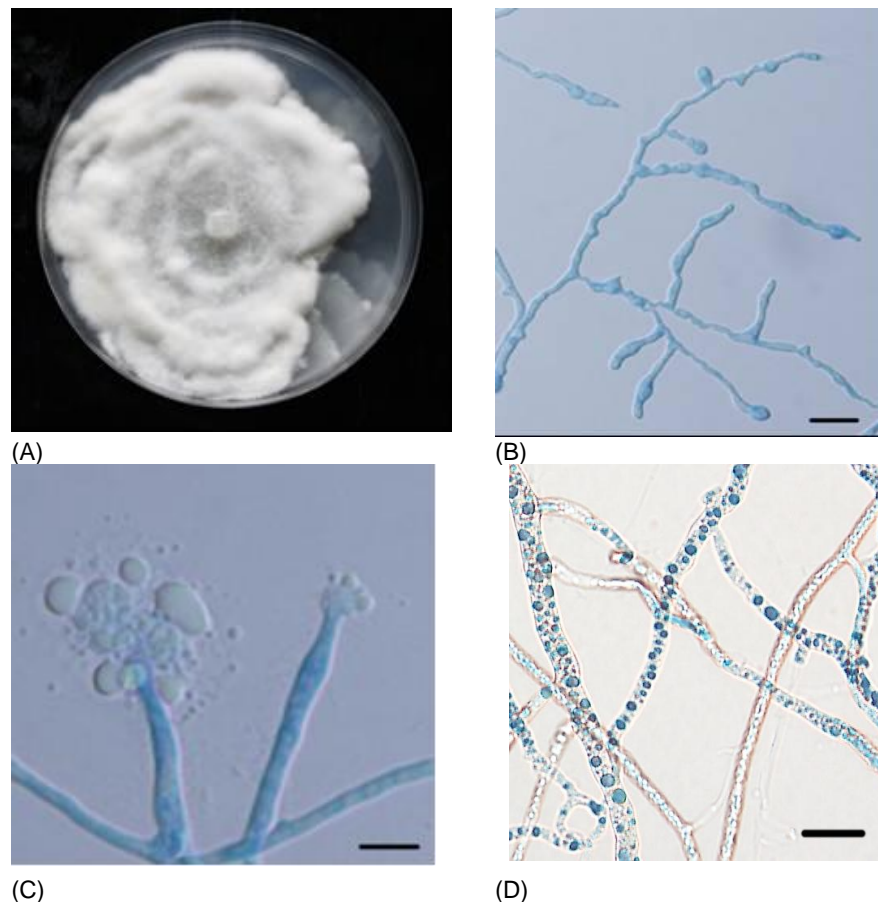
Figure 1. Morphological characteristics of the NR06 isolate. (A) Growth on PDA plate (B) The spores, 20x. (C) The sporangiophores, 40x. (D) The mycelia with many lipid globules, 40x. Horizontal bars indicate 1 μm (B, C and D).

$$Y_{(biomass\ g \cdot L\text{-}1)} = +3.99A\text{-}0.45\ B+0.56\ C+0.25\ D\text{-}0.28E+0.39\ F+0.33G\ +0.39\ H\text{-}0.82\ J\text{-}0.17\ K+0.28L\text{-}0.43A\ B+0.86\ A\ C+0.049AD+0.012A\ E\text{-}0.30\ A\ F+0.85\ AG+1.17A\ H\text{-}0.057\ AJ\text{-}0.40AK\text{-}0.64AL\text{-}1.48BC \qquad (5)$$

DISCUSSION

A number of the lower fungi, especially *Mucor* spp., *Mortierella* spp., *Rhizopus* spp. and *Cunninghamella* spp., have been exploited for the production of PUFA as they have the ability to synthesize intracellular lipids from carbohydrates and store them in the mycelium as triacylglycerols. However, the fungal economic yield is still limited. Thus, attempts have been continued to search for either the best new producer or the same strains with improved cultivation conditions for specific fatty acid productions. Since the degree of unsaturated fatty acids increases with decreased temperature, the production of EPA and ARA is stimulated by lower temperatures (Hansson and Dostálek, 1988). Botha et al. (1999) developed an isolation procedure for ARA

producing species at low temperature (5°C). Chen et al. (1997) screened for low temperature (10°C) ARA producer fungal strains and obtained the Wuji-H4 isolate with high content of ARA (42.4%) in lipids. Shimizu et al. (1988) investigated the effect of temperature on the fatty acid composition of *Mortierella* fungi. It was observed that the optimum temperature for ARA production in *Mortierella alpina* was 28°C while that of EPA production was stimulated at lower temperatures. In the present study, screening for the production PUFA was practically performed at 25°C. Fatty acid producing strains of native lower fungi could be isolated from wet-fallen leaves (16 isolates), more than those from freshwater (8 isolates). Aki et al. (2001) demonstrated a filamentous fungus isolated from freshwater pond samples, assigned to the species *Mortierella alliacea* YN-15 that accumulated high level of ARA in its mycelia. More native lower fungi, *Mortierella* sp. (Botha et al., 1999) and *Mucor rouxii* (Mamatha et al., 2010) were isolated from soil samples. Kamlangdee and Fan (2003) isolated DHA (docosahexaenoic acid, 22:6n3) producing strains of *Schizochytrium* sp. from fallen, senescent leaves from mangrove tree

Table 2. Fatty acid composition of 24 fungal isolates.

Isolates	Fatty acid composition (percentage of total fatty acid)									
	C16:0 (Palmitic acid)	C16:1 (Palmitoleoic acid)	C18:0 (Stearic acid)	C18:1 (Oleic acid)	C18:2n6 (Linoleic acid)	C18:3n6 (GLA*)	C20:1n9 (Eicosenoic acid)	C20:3n6 (DGLA*)	C20:4n6 (ARA*)	C20:5n3 (EPA*)
CC01	27.48 ± 0.52^c	1.56 ± 0.28^e	2.92 ± 0.15^{ijk}	30.10 ± 0.49^i	14.23 ± 0.30^j	ND	ND	ND	7.52 ± 0.37^c	12.08 ± 0.13^g
CC02	29.74 ± 0.16^b	1.30 ± 0.08^f	2.86 ± 0.23^{ijk}	35.10 ± 0.20^f	12.60 ± 0.34^k	ND	ND	ND	ND	9.91 ± 0.16^i
CC03	31.83 ± 0.25^a	2.41 ± 0.23^d	3.29 ± 0.38^{hij}	30.28 ± 0.18^i	14.25 ± 0.02^j	ND	ND	ND	6.76 ± 0.51^d	11.45 ± 0.34^h
CC04	22.32 ± 0.28^{ef}	1.59 ± 0.49^e	2.00 ± 0.05^{kl}	31.16 ± 0.29^h	19.30 ± 0.49^g	ND	ND	ND	10.19 ± 0.09^b	13.97 ± 0.07^d
LB01	17.36 ± 0.15^{ik}	0.34 ± 0.16^{hi}	11.07 ± 0.25^{bc}	32.21 ± 0.24^g	36.39 ± 0.28^e	ND	0.59 ± 0.25^b	ND	ND	ND
LB02	18.55 ± 0.30^i	0.59 ± 0.34^{hi}	3.07 ± 0.25^{ij}	16.36 ± 0.16^n	57.82 ± 0.28^b	ND	ND	ND	ND	ND
LB03	18.77 ± 0.45^i	0.84 ± 0.16^{fgh}	2.03 ± 0.17^{kl}	11.25 ± 0.41^p	63.54 ± 0.63^a	ND	ND	ND	ND	ND
LB04	12.65 ± 0.44^n	34.27 ± 0.44^a	4.52 ± 0.42^g	41.62 ± 0.58^c	ND	ND	ND	ND	ND	ND
TR03	22.11 ± 0.22^f	1.07 ± 0.25^{efg}	3.82 ± 0.23^{ghi}	23.58 ± 0.18^l	49.26 ± 0.01^c	ND	ND	ND	ND	ND
CB01	21.16 ± 0.28^g	ND	1.43 ± 0.52^l	10.81 ± 0.30^{pq}	19.54 ± 0.87^g	0.97 ± 0.08^{hi}	ND	ND	7.45 ± 0.31^i	17.03 ± 0.20^a
CB02	18.17 ± 0.60^{ij}	ND	1.09 ± 0.09^l	10.27 ± 0.07^f	18.25 ± 0.42^h	1.11 ± 0.20^{hi}	ND	ND	6.20 ± 0.07^c	12.61 ± 0.40^f
CB04	23.18 ± 0.28^d	ND	1.19 ± 0.05^l	12.97 ± 0.11^o	23.34 ± 0.17^f	22.52 ± 0.52^c	ND	ND	1.79 ± 0.19^h	2.37 ± 0.21^k
CB05	16.67 ± 0.15^j	ND	3.83 ± 0.17^l	28.88 ± 0.30^j	10.98 ± 0.18^l	33.74 ± 0.21^a	ND	ND		
CB06	17.52 ± 0.33^j	ND	2.75 ± 0.28^{ghi}	10.67 ± 0.16^{pq}	16.05 ± 0.23^i	1.03 ± 0.06^{hi}	ND	ND	6.32 ± 0.16^e	16.17 ± 0.31^b
CB07	18.14 ± 0.12^{ij}	ND	1.12 ± 0.04^l	11.45 ± 0.14^p	16.24 ± 0.16^i	0.63 ± 0.10^j	ND	ND	5.78 ± 0.19^f	13.45 ± 0.30^e
CB08	10.90 ± 0.18^o	ND	6.53 ± 0.32^f	45.08 ± 0.22^b	13.12 ± 0.23^k	0.39 ± 0.17^j	ND	ND	4.40 ± 0.28^g	8.44 ± 0.17^j
RY01	20.62 ± 0.52^{gh}	5.89 ± 0.89^b	12.03 ± 1.39^a	7.47 ± 1.04^s	22.82 ± 0.72^f	20.17 ± 0.45^d	ND	ND	ND	ND
RY02	20.12 ± 0.72^h	6.06 ± 0.66^b	4.05 ± 0.69^{gh}	21.50 ± 0.88^m	23.46 ± 0.62^f	23.93 ± 2.02^b	ND	ND	ND	ND
NR01	20.29 ± 0.20^h	4.10 ± 0.18^c	8.57 ± 0.40^{de}	48.11 ± 0.34^a	8.88 ± 0.10^m	6.67 ± 0.31^e	ND	ND	ND	ND
NR02	22.45 ± 0.13^{def}	ND	11.84 ± 0.26^{ab}	37.34 ± 0.39^d	5.10 ± 0.15^n	2.10 ± 0.11^{gh}	0.77 ± 0.06^a	ND	15.68 ± 0.42^i	0.56 ± 0.20^l
NR03	23.04 ± 0.22^{de}	ND	10.54 ± 0.58^c	36.08 ± 0.14^e	6.07 ± 0.3^n	3.16 ± 0.19^g	0.60 ± 0.20^b	2.53 ± 0.24^b	15.18 ± 0.28^i	0.54 ± 0.25^l
NR04	13.97 ± 0.49^m	0.21 ± 0.02^{hi}	5.66 ± 0.33^f	26.11 ± 0.37^k	8.96 ± 0.71^m	2.28 ± 1.20^g	ND	19.92 ± 0.57^a	ND	15.01 ± 0.32^c
NR05	19.98 ± 0.21^h	ND	7.85 ± 0.19^e	25.58 ± 0.38^k	46.59 ± 0.48^d	ND	ND	ND	ND	ND
NR06	15.09 ± 0.74^i	ND	9.38 ± 0.26^d	23.07 ± 0.22^l	8.26 ± 0.59^m	5.48 ± 0.08^f	ND	ND	32.34 ± 0.35^a	ND

Data expressed as means±SD of three replicates. Different superscript letters are significantly different ($p \leq 0.05$). ND = Not detectable. GLA, γ-linolenic acid; DGLA, dihomo-linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid.

(*Kandelia candel*). Since the NR06 was isolated from wet-fallen leaves at the waterfall, unfortunately there was no record on a type of the collected leaves. Thus, interestingly, it was suggested to study further with an original tree of the leaves.

The NR06 isolate, which was subsequently identified as *M. elongata*, produced the highest level of total fatty acids contents, which mainly contained ARA (more than 30%). Similar results were also found in *M. alpina* and *M. alliacea* (Shinmen et al., 1989). As the PUFAs are intracellular products, high biomass concentration is required for commercially high productivity. Since the effects of several environmental factors, that is, temperature, pH, the chemical composition
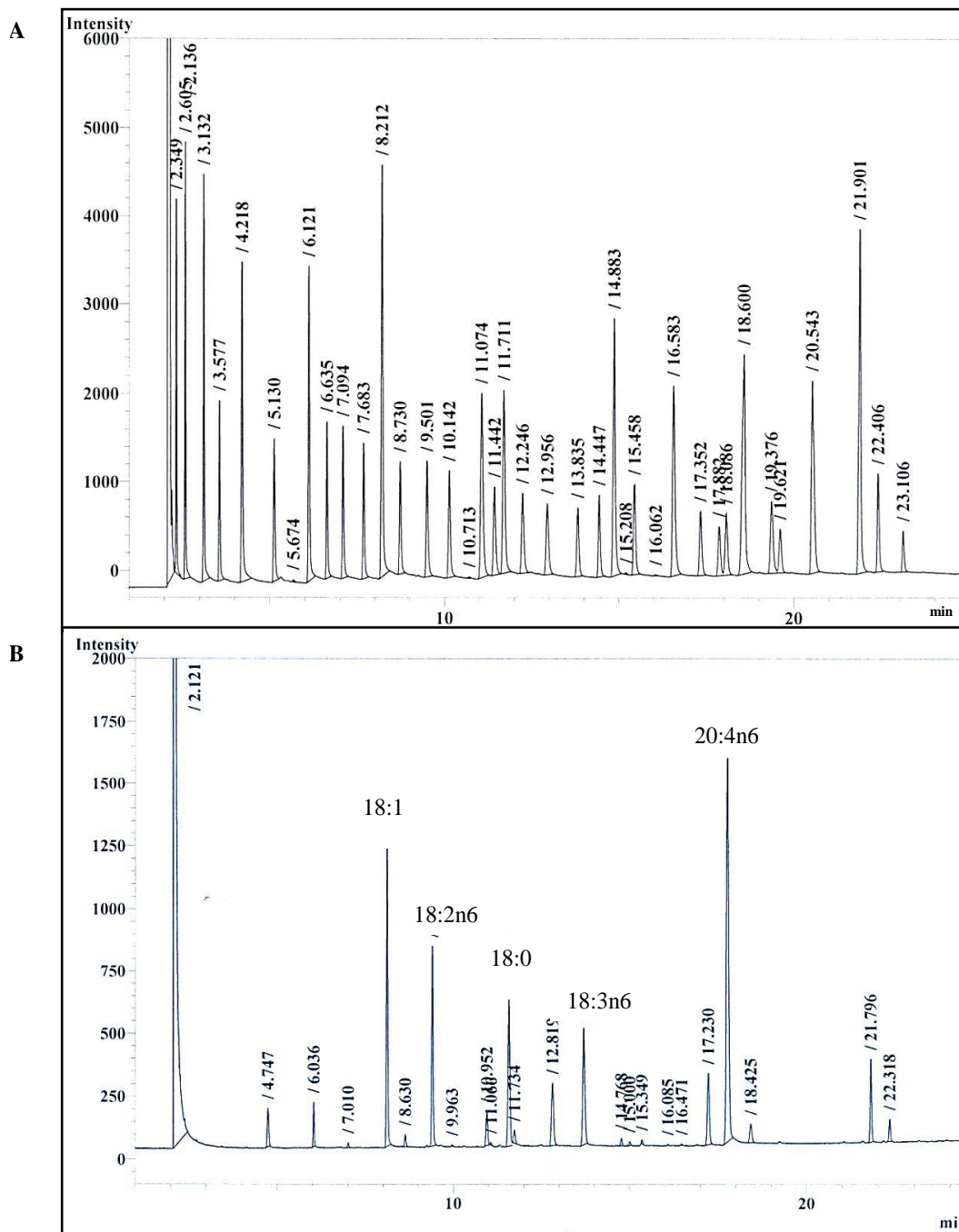
Figure 2. Chromatogram of fatty acid methyl esters (FAME) chemical standards (Supelco, U.S.A.) (A) and chromatogram of the NR06 isolate (B).

of the nutrient media and culture age, have been studied extensively, it was shown that the fungal strains could utilize a wide variety of carbon sources. In the cultures of *Mortierella* for ARA production, glucose is the most frequently used carbon source. Aki et al. (2001) investigated various carbon sources for the strains of *M. alpina* and *M. alliacea*, and found that glucose was suitable for ARA production. In the case of *M. alliacea*,

which was isolated from rice grains, usage of starch as a carbon source was also practical. Totani et al. (2002) reported the effect of glucose concentration on ARA production. They pointed out that more than 20% glucose inhibited the growth of *M. alpina* and high glucose concentration also induced the formation of filamentous morphology. Various studies have also demonstrated that vegetable oil addition was beneficial to enhance ARA

Table 3. Growth, fatty acid content and productions of ARA and EPA of the NR06 isolate in the media containing various carbon and nitrogen sources.

Media	Biomass (g·l^{-1})	Total Fatty acid (%w/w)	PUFA in TFA (%W/W)	
			ARA	EPA
Carbon sources (1%)				
Cellulose	5.13±0.83	4.83±0.15	27.24±5.04	0.66±0.06
Dextrose	7.93±0.67	10.83±0.56	22.07±1.22	0.37±0.06
Glycerol	8.37±0.72	12.61±0.12	22.51±1.91	0.29±0.24
Glucose	7.87±0.72	11.41±1.58	21.00±0.01	0.45±0.00
Starch	7.13±0.72	7.89±2.01	20.07±1.26	0.55±0.11
Sucrose	6.00±0.46	4.45±1.10	21.77±3.05	1.09±0.54
xylose	3.83±0.12	5.48±0.13	27.42±1.98	0.79±0.34
Nitrogen sources (0.5%)				
Corn meal	7.03±0.91	14.15±0.03	9.27±0.04	0.10±0.01
Corn steep solid	7.03±0.40	21.62±1.07	11.45±1.75	0.24±0.01
Meat extract	7.10±0.26	10.52±0.40	23.62±2.46	0.41±0.00
NH_4NO_3	4.90±0.89	8.17±1.27	12.2±1.44	ND
Peptone	6.43±0.35	10.34±6.41	23.91±9.86	0.41±0.21
Soytone	6.10±0.10	6.15±0.71	32.36±4.12	0.41±0.01
Yeast extract	6.77±0.59	7.55±1.21	27.03±6.41	0.57±0.09

Data expressed as means±SD of three replicates. ND = Not detectable. ARA, arachidonic acid; EPA, eicosapentaenoic acid; PUFA, polyunsaturated fatty acid; TFA, total fatty acid.

Table 4. Selected variables were chosen for studies of biomass, total fatty acid and ARA productions by cell cultures of the NR06 isolate, using the statistical program, Design Expert 7.1 software.

Code	Variables	Low level (+) (g·l^{-1})	High level (-) (g·l^{-1})
A_1	Glucose	10	60
B_2	Glycerol	10	60
C_3	Sucrose	10	60
D_4	Meat extract	5	15
E_5	Soytone	5	15
F_6	Peptone	5	15
G_7	Yeast extract	5	15
H	Dummy 1 (-)	-	-
I	Dummy 2 (-)	-	-
J	Dummy 3 (-)	-	-
K	Dummy 4 (-)	-	-

yield (Dong and Walker, 2008).

The ratio of carbon and nitrogen (C/N) in the growth media is considered as the most important factor to achieve biomass and lipid accumulation (Fakas et al., 2008). Complex organic nitrogen sources such as yeast extract and corn steep liquor are generally practicable for growth. Yokochi et al. (1998) investigated several alternative carbon and nitrogen sources for the algal culture of

Schizochytium limacinum SR 21. It was found that glucose, fructose, glycerol and oleic acid led to high levels of cell growth over 10 g·l^{-1} and DHA production over 0.5 g·l^{-1}, while saccharose, lactose, maltose, starch and linseed oil decreased levels of DHA production. Among various nitrogen sources tested, corn steep liquor or yeast extract had the highest DHA production levels (over 0.5 g·l^{-1}). The fungal strain *M. alpine*, could produce

Table 5. Statistical program, Design Expert 7.1 software, design matrix to evaluate variables affecting biomass, total fatty acid and ARA production by the NR06 isolate.

Run	A	B	C	D	E	F	G	H	I	J	K	ARA production (%, w/w)	TFA production (%, w/w)	Biomass production (g·l^{-1})
1	+	+	-	+	+	+	-	-	-	+	-	14.37	18.80	14.70
2	-	+	+	-	+	+	+	-	-	-	+	46.12	9.31	8.40
3	+	-	+	+	-	+	+	+	-	-	-	14.58	17.51	26.46
4	-	+	-	+	+	-	+	+	+	-	-	36.56	7.57	7.26
5	C	C	+	-	+	+	-	+	+	+	-	59.28	6.35	9.20
6	C	C	C	+	-	+	+	-	+	+	+	37.27	8.50	10.16
7	+	C	C	-	+	-	+	+	-	+	+	14.08	21.34	17.50
8	+	+	C	-	-	+	-	+	+	-	+	19.22	16.34	16.44
9	+	+	+	-	-	-	+	-	+	+	-	14.06	18.13	14.94
10	+	+	+	+	-	-	-	+	-	+	+	52.25	9.46	9.14
11	+	+	+	+	+	-	-	-	+	-	+	13.70	19.53	17.82
12	-	-	-	-	-	-	-	-	-	-	-	49.10	7.83	8.64
13	C	C	C	C	C	C	C	-	-	-	-	27.09	14.29	9.92
14	C	C	C	C	C	C	C	-	-	-	-	16.90	16.53	10.70
15	C	C	C	C	C	C	C	-	-	-	-	20.41	8.82	11.78
16	-	-	+	-	-	-	+	+	+	-	+	56.31	5.76	7.52
17	+	-	-	+	-	-	-	+	+	+	-	16.75	19.18	14.02
18	-	+	-	-	+	-	-	+	+	+	+	50.79	12.06	10.56
19	+	-	+	-	-	+	-	-	+	+	+	14.38	18.26	17.34
20	+	+	-	+	-	-	+	-	-	-	+	13.40	19.98	16.64
21	+	+	+	-	+	-	-	+	-	-	-	17.59	17.65	16.86
22	-	+	+	+	-	+	-	-	+	-	-	51.86	13.19	7.06
23	-	-	+	+	+	-	+	-	-	+	-	34.32	9.14	9.06
24	-	-	-	+	+	+	-	+	-	-	+	40.92	6.49	8.90
25	+	-	-	-	+	+	+	-	+	-	-	12.06	23.07	13.48
26	-	+	-	-	-	+	+	+	-	+	-	48.73	9.11	9.62
27	+	+	+	+	+	+	+	+	+	+	+	13.47	13.17	16.38
28	C	C	C	C	C	C	C	0	0	0	0	23.49	15.29	9.10
29	C	C	C	C	C	C	C	0	0	0	0	22.97	15.71	9.38
30	C	C	C	C	C	C	C	0	0	0	0	25.68	16.19	10.20

The four variables (D1-D4) are designed as "dummy variables". +, high level; -, low level; ARA, arachidonic acid; TFA, total fatty acid; C, central values.

ARA 5.3 g·l^{-1}, where glucose is a carbon source and yeast extract a nitrogen source (Jang et al., 2005). Zhu et al. (2003) described a method for ARA production by *M. alpina* on glucose/defatted soybean meal and sodium nitrate with the yield of 1.87 g·l^{-1} ARA (17.3% of total lipids) from 31.2 g biomass in 7 days. Lan et al. (2002) showed that glutamate enhanced ARA production, while other PUFAs were retained. For 7 days of growth, the yields of biomass and ARA were 25 and 1.4 g·l^{-1}, respectively. Eroshin et al. (1996) developed a medium containing aspirin to select ARA producing strain of *Mortierella* sp., and obtained three isolates producing up to 40% of ARA in total lipid content. Production of ARA in photoautotrophic algae, such as *Porphyridium cruentum* and *Parietochloris incise*, appeared to be optimal under conditions of slow growth in nitrogen free or nitrogen-starved conditions (Ward and Singh, 2005). Kyle (1994) described a method for production of ARA by the heterotrophic alga, *Pythium insidiosum*. Relatively high rate of growth (15 g in 50 h) were achieved in glucose-yeast extract medium. Biomass oil content was 5-6% (0.75-0.9 g·l^{-1}, of which 30-35% was ARA). However, ARA yields and productivities were only 0.3 and 0.15 g·l^{-1}·h^{-1}, respectively.

ARA productivity of the NR06 strain was evaluated by cultivation in liquid media containing different carbon sources and nitrogen sources. Optimization of growth and production of total intracellular fatty acids including ARA was accomplished by a combination of glucose and yeast extract, which was evaluated by the Design

Table 6. Regression coefficients of the variables in response to biomass, total fatty acid and ARA productions.

Variable	Biomass production						ARA production					
	Coefficient estimate	SE	SS	df	F value	p value (Prob > F)	Coefficient estimate	SE	SS	df	F value	p value (Prob >F)
Model	10.18	0.32	543.43	1	37.76	0.0015	30.86	0.74	6908.04	1	24	0.0011
A	3.99	0.16	372	1	620.78	<0.0001	-16.05	0.74	6145.22	1	469.60	<0.0001
B	-0.45	0.16	4.78	1	7.97	0.0476	0.63	0.74	9.41	1	0.72	0.4351
C	0.56	0.16	7.39	1	12.32	0.0247	1.42	0.74	48.08	1	3.67	0.1134
D	0.26	0.35	0.34	1	0.57	0.4910	-2.57	0.74	157.51	1	12.04	0.0179
E	-0.29	0.35	0.43	1	0.72	0.4437	-1.47	0.74	51.42	1	3.93	0.1043
F	0.41	0.39	0.67	1	1.12	0.3493	0.11	0.74	0.32	1	0.024	0.8827
G	0.32	0.35	0.5	1	0.83	0.4135	-2.49	0.74	148.26	1	11.33	0.02
H	0.37	0.39	0.54	1	0.91	0.3953	1.62	0.74	62.65	1	4.79	0.0803
I	-0.84	0.39	2.8	1	4.67	0.0968	0.92	0.74	20.19	1	1.54	0.2693
J	-0.19	0.39	0.14	1	0.24	0.6521	-0.045	0.74	0.048	1	3.68^{E-003}	0.9540
K	0.3	0.39	036	1	0.59	0.4840	0.086	0.74	0.18	1	0.013	0.9123

The four variables (H, I, J and K) are designed as ''dummy variables''. A, glucose; B, glycerol; C, sucrose; D, meat extract; E, soytone; F, peptone; G, yeast extract; SE, standard error; SS, sum of square; df, degree of freedom; ARA, arachidonic acid.

expert 7.1 software. Moreover, to enhance the production of polyunsaturated fatty acids, the combination of carbon and nitrogen sources using the statistically experimental design revealed the best results obtained from the mixtures of substrates as in the equations. These results agree with those of Ghobadi et al. (2011) who used the Plackett-Burman screening design providing the effect of combination of substrates for enhancement of biomass and ARA yield of *M. alpina* CBS754.68. Saelao et al. (2011) also optimized biomass and ARA production by a novel marine gliding bacterium, *Aureispira maritima* using response surface methodology. They reported that tryptone and culture temperature affected biomass, whereas pH and agitation rate had a significant effect on ARA production. Subsequent statistical optimization of these four factors was verified to increase both biomass and ARA yield.

The isolated native NR06 fungal strain from the present study was classified as *M. elongata*, with the high biomass of 15.49±0.24 g·l^{-1}, total fatty acids of 16.44±0.30% and the major essential fatty acid of arachidonic acid (ARA; C20:4n6) (32.24±0.35%). It has been recommended that further optimizations are required for improvement to be a potential ARA producing strain.

Conflict of interests

The authors did not declare any conflict of interest.

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