Optimization of Arachidonic Acid Production by *Mortierella alpina* Wuji-H4 Isolate

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ABSTRACT: A fungal isolate Wuji-H4 with a dense-lobe rosette growth pattern on malt extract agar was identified as *Mortierella alpina* Peyronel. It was capable of producing 504 mg/L of arachidonic acid (AA) in the screening medium. Its AA content accounted for 42.4% of the total fatty acids. The AA yield was raised to 1,817 mg/L by a step-by-step approach, which uncovered that the preferred carbon source, nitrogen source, and temperature for fungal growth and lipid production were soluble starch, urea, and 24°C, respectively. Productivity was further optimized by exploiting the interactions between the constituents of the medium by the response surface method. A partial factorial design, followed by steepest ascent analysis, was carried out to locate the general vicinity of the optimal level of each nutrient. The response surface of AA production in this optimal region was then approximated with a full quadratic equation obtained from a three-factor/five-level central composite rotatable design. Maximum AA yield was predicted to occur in a medium that contained 99.7 g/L of soluble starch, 12.6 g/L of yeast extract, and 3.0 g/L of KH_2PO_4. Upon verification, the average experimental yield of AA (3,885 mg/L) was not significantly different from the predicted AA yield (3,940 mg/L), indicating that the response surface method had succeeded in exploiting the AA production potential of this new fungal isolate.

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KEY WORDS: Arachidonic acid, central composite rotatable design, content, *Mortierella alpina,* optimization, RSM, soluble starch, urea, Wuji-H4 isolate, yield.

Arachidonic acid (AA; 5,8,11,14-*cis*-eicosatetraenoic acid) is a C_{20} polyunsaturated fatty acid that is important as a natural constituent of biological membranes and a precursor of numerous eicosanoids, such as prostaglandins, thromboxanes, and leukotrienes that are presently the subjects of extensive nutritional and medical research owing to their unique biological activities (1–3). This dietarily and pharmacologically important fatty acid is currently isolated from porcine adrenal gland and liver as well as from sardines (4). However, these oils contain too little AA (0.2% w/w) to be practical sources for its industrial production. In addition, animal oils often contain other fatty acids with less desirable qualities. Hence, cultivation of microorganisms that are capable of producing larger amounts of AA has recently been proposed as an alternative method of production (5–7).

There are many marine algae and fungal species capable of producing AA, but only lower fungi of the Phycomycetes class, especially in the order of Mucorales, are rich in AA. Several species of the genus *Mortierella* have been suggested as promising AA producers (5). In shake-flask cultures, *M. elongata* 1S-5 produced 16.4–30.1% AA of the total fatty acid content, with a yield of 0.5–0.99 g/L (8); *Mortierella* sp. S-17 produced 0.96 g/L of AA at a carbon/nitrogen (C/N) ratio of 20 (9); *M. isabellina* 224 produced 505 mg/L of AA in the malt extract medium (10), while *M. alpina* [Centraalbureau voor Schimmelcultures (CBS)] 210.32 could produce 0.86–0.96 g/L of AA in 5–7 d (10,11). Totani and Oba (12) cultivated *M. alpina* IFO 8568 for 20 d and produced 2.11 g/L of AA in liquid medium. *Mortierella alpina* ATCC 16266 produced 2.1 g of AA per liter in media containing 10% glucose (13). Widespread interest in AA for nutritional and clinical uses continues to call for the search of particularly effective strains and optimal cultural conditions for its production.

We have recently found a new soil fungal isolate, Wuji-H4, with a high percentage of AA. This article reports, in addition to the morphological and physiological characteristics of this strain, the rapid optimization of its AA production through the response surface method (RSM), which is an efficient and powerful statistical inference method used to assess simultaneously the effects of several variables with only a fraction of the total number of the experiments otherwise required (14,15). It has been employed to solve multivariate problems and optimize several responses in many types of experimentation (16–18).

EXPERIMENTAL PROCEDURES

Screening for AA producers. More than 50 soil samples with various colors, moisture contents, and impurities were collected all over the island of Taiwan. To each 10 g of soil sample was added 5 mL of sterile water, and these were mixed vigorously. The slurry was filtered with cheesecloth to obtain

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an aliquot of 1–2 mL. Each milliliter of the aliquot was smeared onto a potato dextrose yeast (PDY) agar plate that contained 10 ppm of tetracycline to discourage bacterial growth. The plates were then incubated at 10°C for 2 wk, and visible colonies were collected and purified by the conventional streaking method. Overall, more than 200 pure fungal cultures, including Wuji-H4 isolate, were obtained and subjected to AA production test.

AA production test. Each pure isolate was grown on PDY agar plate at 20°C for 7 d. Thereafter, the mycelia were harvested, suspended in 5 mL of sterile water, and vortexed vigorously for 1 min in a 10 × 150 mm screw-cap test tube containing 30 glass beads 2–3 mm in diameter. Each 250-mL flask with 25 mL of screening medium was inoculated with 0.4 mL of the freshly prepared mycelium suspension. The optimized AA production medium for *M. alpina* CBS 210.32 (11) was used as screening medium and consisted of (in g/L): soluble starch, 100; yeast extract, 5; KH_2PO_4, 1.0; KNO_3, 2.1; $MgSO_4 \cdot 7H_2O$, 0.25. The culture was incubated at 20°C for 5 d in an orbital shaker at 200 rpm. After harvest, the dry mass, lipid content, and fatty acid composition of the mycelia were analyzed and compared.

Optimization of the culture conditions for AA production. The same soil isolate, Wuji-H4, was used throughout these experiments. Initially, the production of AA by this organism was evaluated in the screening medium (see above) with different carbon and simple nitrogen sources, replacing soluble starch and KNO_3, and at different temperatures. After fixing the carbon and nitrogen sources and the incubation temperature, a two-level 2^{5-1} partial factorial design, followed by the method of steepest ascent, was then carried out to find the general vicinity of the optimal concentrations of each medium component for AA production. The central composite rotatable design (14,15), consisting of a three-factor/five-level pattern with 17 design points (15 combinations with 2 more replications of the center point), was then conducted in this optimal vicinity to locate the true optimal medium composition for AA production. The Response Surface REGression (RSREG) procedure of the Statistical Analysis System (19) was used to fit the experimental yields of AA to the second-order polynomial equation. The cultivation conditions of these experiments were identical to those described above except that, in examining the effects of temperature and carbon and nitrogen sources, the medium was neutralized once with 5 N KOH 60 h after inoculation.

Biomass and lipid content determination. Fungal biomass was recovered from fermented broth by filtering through Whatman No. 2 filter paper (Maidstone, England). The mycelia on the filter paper were washed with 50 mL distilled water three times, weighed, and then homogenized with KG Ultra-Turrax-Antrieb T 25 (Janke & Kunkle GmbH & Co., Staufen, Germany) at 24,000 rpm for 2 min. About 3 g of the homogenized fungal mass was dried at 80°C for 24 h to calculate the mycelial dry weight (MDW). Another 3 g of the homogenized fungal mass was homogenized once more and then hydrolyzed and extracted according to the procedure of Moon and Hammond (20), except that the concentration of the alcoholic KOH solution was increased from 12 to 15% (wt/vol), and the volume of the second time hexane extraction was increased from 5 to 8 mL. The lipids extracted (in free fatty acid form) by the above procedure were flushed with nitrogen to remove hexane and weighed to determine the lipid content.

Fatty acid analysis. The dried fatty acids were methylated according to the H_2SO_4–CH_3OH method (21) and analyzed with a Shimadzu GC-14B gas–liquid chromatograph (Kyoto, Japan) fitted with an SP2330 capillary column (30 m × 0.25 mm i.d.; Supelco Co., Bellefonte, PA). The methylated fatty acids were identified and calibrated by using standard fatty acid methyl esters supplied by Sigma Chemical Co. (St. Louis, MO). The standard fatty acid methyl esters included 14:0, 16:0, 18:0, 18:1, 18:2, γ-18:3, 20:0, α-18:3, 20:4, 22:0, 20:5, and 22:6 and were eluted in that order. The mass spectrum of the AA (20:4) peak from the gas chromatographic (GC) outlet was measured with JEOL JMS-SX/SX 102A mass spectrometer in EI+ ion mode. The double-bond index (DBI, Δ/mol), or degree of lipid unsaturation, was calculated according to the method of Choi and coworkers (22), i.e., DBI = (1 × % monoene + 2 × % diene + 3 × % triene + ⋯)/% sum of all known fatty acids. The unrecognizable trace peaks in the gas chromatogram of each sample were neglected in this calculation.

RESULTS

Characteristics and identity of Wuji-H4 isolate and the authenticity of its AA peak. In more than 200 fungal isolates screened for their capability of producing AA, only 12 strains produced detectable amounts of AA in the GC spectra of their lipids. Wuji-H4, isolated from the roadside soil sample of Wuji, Taiwan, had a high content (42.4%) of AA in the lipid, while the AA content of lipids of the other 11 isolates were only in the range of 6.5–17.7%. Furthermore, Wuji-H4 isolate grew well in the AA screening medium and showed the highest AA yield of 504 mg/L. The authenticity of the AA peak in the GC spectrum of Wuji-H4 isolate was confirmed with mass spectrometry. The mass spectrum of this peak had a GC retention time of 16.64 min, a molecular ion peak at *m/z* 318 with a normalized intensity of 5.50, and intense fragment ion peaks at *m/z* 150, 119, 106, 105, 91, 79, and 67 with normalized intensities of 36.1, 33.0, 48.8, 45.8, 79.7, 100, and 65.6, respectively. Each of these peaks matched well to the corresponding peaks of the standard methyl arachidonate sample, which had a GC retention time of 16.51 min, a molecular ion peak at *m/z* 318 with a normalized intensity of 4.36 and intense fragment ion peaks at *m/z* 150, 119, 106, 105, 91, 79, and 67 with normalized intensities of 32.5, 33.4, 46.0, 46.4, 76.2, 100, and 63.4, respectively.

The Wuji-H4 isolate was identified by the CBS (Baarn, The Netherlands) as *M. alpina* Peyronel. Figure 1 shows the macro- and microscopic morphologies of this isolate. Colonies on malt extract agar (MEA) were white, grew fast

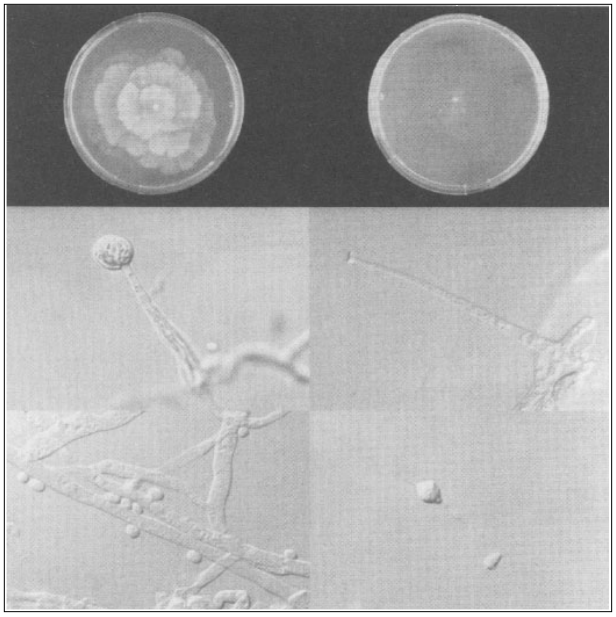

FIG. 1. The morphologies of Wuji-H4 isolate: top left and right show the respective colony appearance of this strain grown on MEA for 10 d and on SEA for 6 d at 25°C, middle left is a tapered sporangiophore arising from aerial mycelium with single sporangia (500×), middle right shows the collar at the top of the sporangiophore after fall-off of the sporangia (500×), bottom left presents the mycelium with scattering sporangiospores (500×), and bottom right shows the thick-wall spore-like swollen cells (500×) (photographed by CCRD, FIRDI, Taiwan).

with a rosette pattern of dense lobes, and attained a diameter of 85 mm in 10 d at 25°C. Most of the aerial mycelia were buried in the agar, some with a swollen end, and released oil-like droplets when broken. Differentiation of mycelia into a sporulation structure was not observed. Hyphae were heavy, and the younger ones were coenocyte without septum. Colonies on soil extract agar (SEA) were also white, grew moderately fast with scanty aerial mycelia with sporulation structure, and attained a diameter of 44–47 mm in 7 d at 25°C. Sporangiophores that arose from aerial mycelia were simple, not branched, no columellae but with apparent collar at the top, 55–110 μm tall, with a widened and often irregularly swollen base of 3.5–7 μm and tapering to 1.3–2 μm at the tip. Sporangia were nearly spherical, 10–12 μm in size, appeared singly on top of the sporangiophore, and could differentiate into numerous sporangiospores or fall off without separating into single spores. Sporangiospores were ellipsoidal, translucent, smooth, and 4–5 × 2.5–3 μm in size. All morphological and physiological characteristics of the Wuji-H4 isolate agreed well with the description of *M. alpina* Peyronel by Domsch *et al.* (23), except that this isolate did not sporulate readily in both MEA and SEA.

Effects of carbon source on AA production by Wuji-H4. The carbon sources listed in Figure 2, all at 10% (wt/vol), were tested for growth and production of lipid and AA by *M. alpina* Wuji-H4 isolate. Soluble starch produced the best results in terms of biomass yield (22.7 g/L), AA yield (1,061 mg/L), lipid content in biomass (12.5%), and AA content in lipid (41.6%). When the fungus was grown on easily assimilative substrates, such as glucose, sucrose and maltose, the pH dropped to 3.5–4.3, and reduced biomass and AA yields were observed as compared with soluble starch. The growth of this strain on lactose and glycerol resulted in poor biomass yields, lower AA yields, and higher final pH (7.8–8.3).

Effects of simple nitrogen source on AA production by Wuji-H4. The influence of replacing potassium nitrate in the screening medium with an equal weight of different nitrogen sources on the biomass, lipid, and AA production by Wuji-H4 isolate was compared, and the results are presented in Figure 3. Maximum yields of biomass (29.4 g/L) and AA (1,583 mg/L), DBI (2.5 Δ/mol) and AA content of lipid (46.8%) were observed with urea. Ammonium nitrate, ammonium sulfate, and ammonium chloride were inferior to potassium nitrate in terms of biomass growth and AA production. Higher final pH was observed in the media that contained urea (6.7) and potassium nitrate (5.9) than in other ammonium-based nitrogen sources.

Effects of temperature on AA production by Wuji-H4. The influence of temperature on biomass, lipid content of biomass, AA content of lipid, and AA yield of Wuji-H4 isolate was studied at 18, 24, 30, and 36°C in the screening medium enriched with 2 g/L of urea (Fig. 4). The culture was unable to grow at 36°C. Biomass production decreased and lipid content increased with increasing temperature from 18 to 30°C. The AA content of the lipid, and thus the degree of lipid unsaturation (DBI value), peaked at 24°C with respective values of 50% and 2.67 Δ/mol. Consequently, the highest AA yield of 1,817 g/L was obtained at 24°C, which was 30.7 and 66.7% higher than those of 18 and 30°C, respectively.

Optimizing AA yield of Wuji-H4 isolate by RSM. In aiming at a fast yield improvement of the Wuji-H4 isolate, the concentrations of the five components (soluble starch, yeast extract, urea, KH_2PO_4, and $MgSO_4 \cdot 7H_2O$) of the above medium were approached by the RSM at a fixed temperature of 24°C. Three basic steps were involved in our RSM study: (i) exploratory test—locating the factor region vicinal to the optimal response by running small-scale experiments (Tables 1 and 2); (ii) optimization—conducting a more extensive experiment in this new region that will permit the characterization of the response surface with higher-order models (Table 3); (iii) statistical and graphical analysis—using multiple regression techniques to compute the coefficients and the mathematical equation that relates the response with the factors, assessing the importance of the individual factors, the appropriateness of the equation, and the sensitivity of the response to each factor by statistical inference techniques (Table 4), and graphing the response surfaces or their contours to illustrate the changes in the response as a function of the various factors on the study (Fig. 5).

Table 1 shows the design and results of a 2^{5-1} fractional factorial experiment with the five medium components just mentioned. The lower and upper concentration levels of each component were normalized and coded to −1 and +1, respec-

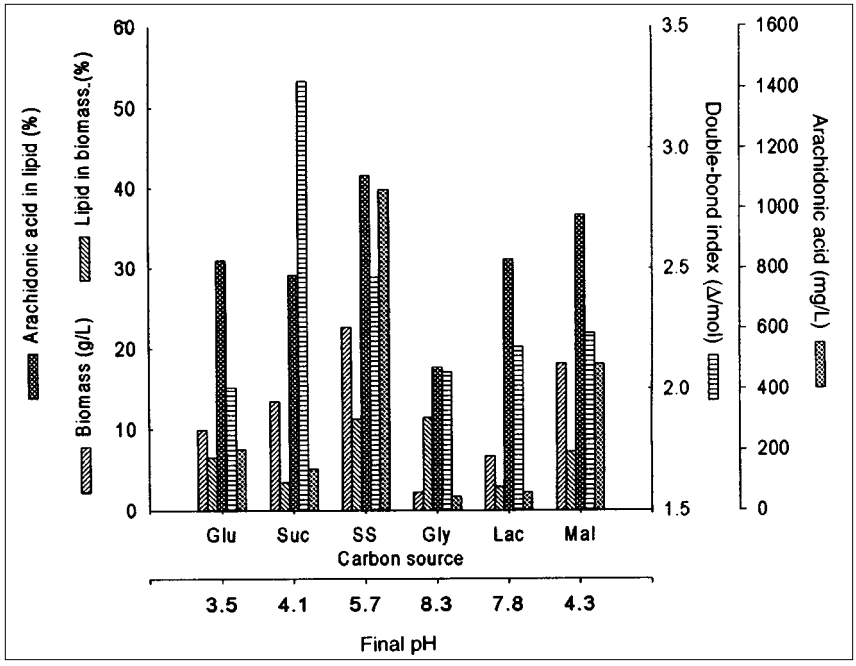

FIG. 2. Effects of different carbon sources on the growth and production of arachidonic acid by Wuji-H4 isolate (Glu: glucose; Suc: sucrose; SS: soluble starch; Gly: glycerol; Lac: lactose; Mal: maltose).

tively. The normalized factors with corresponding response of AA yield data in Table 1 were analyzed by the least-squares method, and the following first-order equation was obtained from the coded data:

$$\text{AA yield (mg/L)} = 1144 + 401X_1 - 180X_2 + 89X_3 + 73X_4 - 131X_5 \quad [1]$$

where X_1, X_2, X_3, X_4, and X_5 represented the coded concen-

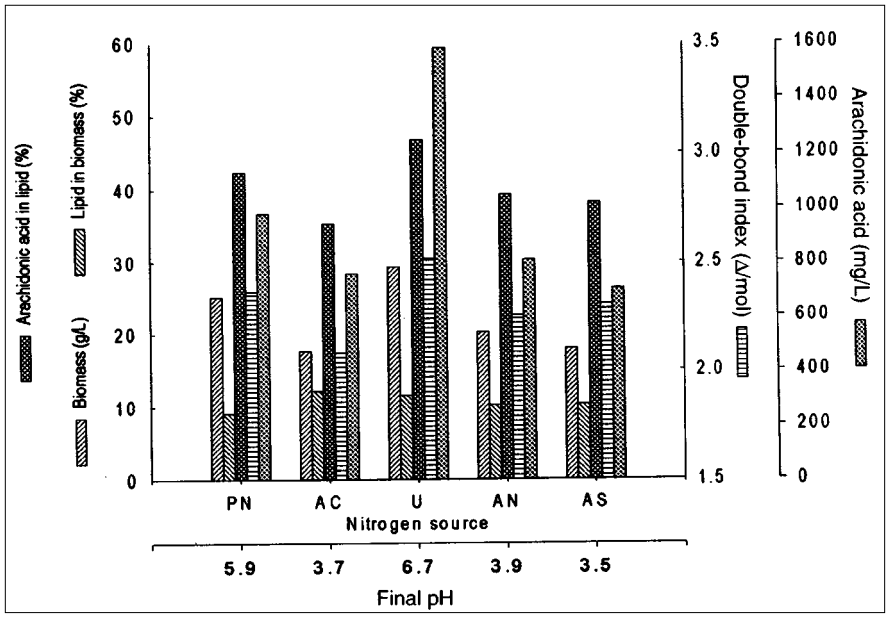

FIG. 3. Effects of different nitrogen sources on the growth and production of arachidonic acid by Wuji-H4 isolate (PN: potassium nitrate; AC: ammonium chloride; U: urea; AN: ammonium nitrate; AS: ammonium sulfate).

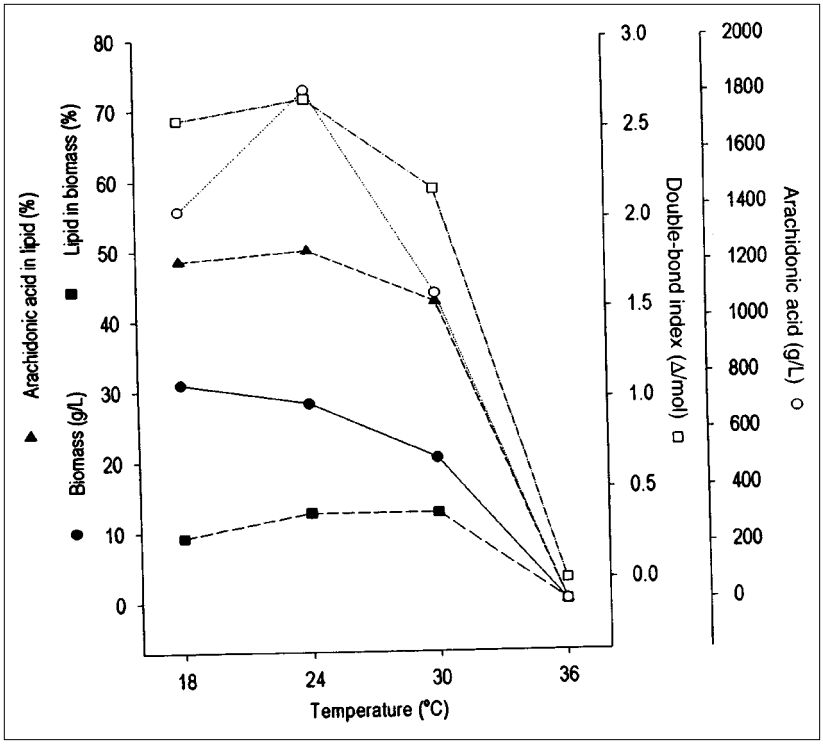

FIG. 4. Effects of incubation temperature on the growth and production of arachidonic acid by Wuji-H4 isolate.

tration of soluble starch, yeast extract, urea, KH_2PO_4, and $MgSO_4 \cdot 7H_2O$, respectively. Table 1 also indicates that the biomass and lipid content of Wuji-H4 isolate fluctuates significantly from 12.4 to 31.8 g/L and from 5.9 to 20.7%, respectively, with various medium compositions. However, the AA content was relatively constant and remained in the range of 38.3 to 46.3%.

Table 2 shows the step-by-step path of steepest ascent toward the optimal region of the response from the base point, which is the zero level (center) of the 2^{5-1} fractional factorial

TABLE 1
Results of the 2^{5-1} Fractional Factorial Design for the Production of Arachidonic Acid by Wuji-H4 Isolate[a]

	Factors					Biomass	Lipid content	AA	
Number	X_1	X_2	X_3	X_4	X_5	(g/L)	(%, dry wt)	Content (%)	Yield (mg/L)
1	−	−	−	−	+	12.4	6.9	37.8	392
2	+	−	−	−	−	20.7	20.7	46.3	1884
3	−	+	−	−	−	19.0	6.9	41.3	546
4	+	+	−	−	+	25.7	9.4	43.5	1053
5	−	−	+	−	−	22.0	14.2	38.3	1195
6	+	−	+	−	+	29.0	12.9	44.4	1661
7	−	+	+	−	+	23.5	6.0	44.1	620
8	+	+	+	−	−	31.1	9.5	41.4	1220
9	−	−	−	+	−	17.3	15.4	40.1	1065
10	+	−	−	+	+	23.9	14.0	42.6	1425
11	−	+	−	+	+	20.1	5.9	42.2	503
12	+	+	−	+	−	30.4	12.1	42.7	1571
13	−	−	+	+	+	22.0	9.8	43.7	939
14	+	−	+	+	−	28.5	15.9	44.8	2035
15	−	+	+	+	−	22.7	7.2	42.0	688
16	+	+	+	+	+	31.8	12.1	39.2	1510

[a]"−" and "+" represent the high and low levels (in g/L) of each factor (X), respectively: X_1 = yeast extract, 2.0 and 5.5; X_2 = urea, 1 and 5; X_3 = soluble starch, 60 and 100; X_4 = KH_2PO_4, 0.25 and 3.75; X_5 = $MgSO_4 \cdot 7H_2O$, 0.2 and 1.0.

TABLE 2
Results of Arachidonic Acid Production by Wuji-H4 Isolate Along the Path of Steepest Ascent of the 2^{5-1} Fractional Factorial Design[a]

		Factors					AA
		X_1	X_2	X_3	X_4	X_5	(mg/L)
i. Base point		3.75	3.0	80	2.00	0.60	
ii. Conc. range of unity level		1.75	2.0	20	1.75	0.40	
iii. Normalized coefficient		5.49	−2.47	1.22	1.00	−1.79	
iv. Relative conc. range (ii*iii)		9.61	−4.94	24.4	1.75	−0.72	
v. Proportion of iv (iv*0.164)		1.58	−0.81	4.0	0.29	−0.12	
vi. Step	0	3.75	3.00	80	2.00	0.60	1147
	1	5.33	2.19	84	2.29	0.48	1748
	2	6.91	1.38	88	2.58	0.36	2314
	3	8.49	0.57	92	2.87	0.24	2842
	4	10.07	0.00	96	3.16	0.12	3325
	5	11.65	0.00	100	3.45	0.00	3696
	6	13.23	0.00	104	3.74	0.00	3440
	7	14.81	0.00	108	4.03	0.00	3123

[a]X_1 = yeast extract; X_2 = urea; X_3 = soluble starch; X_4 = KH_2PO_4; X_5 = $MgSO_4 \cdot 7H_2O$.

design. The direction of each factor in the path was determined by the sign of each coefficient in Equation 1. The magnitude of each factor in each step of the path was derived from Equation 1 by (i) normalizing the coefficient of each factor by using the absolute value of the smallest coefficient as 1; (ii) obtaining the relative concentration range of each factor by multiplying the normalized factor with the respective concentration range of unity level in the original 2^{5-1} design; and (iii) multiplying the relative concentration range with a common portion (0.164) appropriate for all factors. As shown in Table 2, continuing increase in the AA yield to 3,576 mg/L was observed through the fifth step; however, both the sixth and seventh steps resulted in a decrease in the yield. These facts indicated that the inflection point (fifth step) was close to the point of maximum response of AA yield. At the inflection point, urea and magnesium sulfate were totally eliminated, and the concentration of the yeast extract was not bounded by the original 2^{5-1} fractional factorial design. Thus, the concentrations of the remaining three factors, which are soluble starch, yeast extract and KH_2PO_4, were further optimized around the inflection point by a three-factor/five-level central composite rotatable design (CCRD) as shown in Table 3. The five concentration levels of each factor were normalized and coded to −1.68, −1.0, 0, +1.0 and +1.68, respectively. The experiment numbers 1–8 in Table 3 included a 2^3 complete factorial design, numbers 9–14 were star points, and

TABLE 3
Results of the Central Composite Rotatable Design for the Production of Arachidonic Acid by Wuji-H4 Isolate[a]

	Factors			Biomass	Lipid content	AA	
Number	X_1	X_2	X_3	(g/L)	(%)	Content (%)	Yield (mg/L)
1	−1	−1	−1	26.7	21.7	48.5	2818
2	+1	−1	−1	31.3	13.3	42.1	1755
3	−1	+1	−1	29.9	23.1	50.3	3468
4	+1	+1	−1	36.3	20.3	43.8	3225
5	−1	−1	+1	28.2	18.0	49.4	2503
6	+1	−1	+1	33.9	15.2	49.3	2548
7	−1	+1	+1	27.4	14.7	37.6	1511
8	+1	+1	+1	36.5	18.2	37.2	2468
9	+1.68	0	0	37.4	12.5	39.1	1825
10	−1.68	0	0	23.2	17.1	37.5	1487
11	0	+1.68	0	35.2	20.8	48.9	3579
12	0	−1.68	0	27.8	13.3	38.8	1440
13	0	0	+1.68	31.4	19.4	41.8	2547
14	0	0	−1.68	29.7	22.9	39.4	2671
15	0	0	0	34.7	22.0	49.7	3795
16	0	0	0	33.5	23.9	46.9	3755
17	0	0	0	34.3	24.0	44.5	3658

[a]X_1 = soluable starch, 83.2–116.8 g/L; X_2 = yeast extract, 9.96–13.34 g/L; X_3 = KH_2PO_4, 2.61–4.29 g/L.

TABLE 4
Analysis of Variance of Fitting Arachidonic Acid Yield with a Quadratic Equation by Using Data Obtained from the Central Composite Rotatable Design

	Degrees of freedom	Sum of squares	R^2	F-ratio	Prob > F
Regression					
Linear	3	1893697	0.1737	2.997	0.1048
Quadratic	3	5219094	0.4787	8.260	0.0106
Crossproduct	3	2314444	0.2123	3.663	0.0714
Total regression	9	9427235	0.8648	4.973	0.0230
Residual					
Lack of fit	5	1464429		59.014	0.0167
Pure error	2	9926			
Total error	7	1474355			
Factor					
Starch	4	5797414		6.881	0.0142
Yeast extract	4	4633563		5.500	0.0253
KH_2PO_4	4	3476734		4.127	0.0498

the last three were the triplicates at the design center. Again, the biomass and lipid content scattered over a much wider range than the AA content of the lipid.

The data obtained from CCRD were fitted to a second-order polynomial with multiple regression, and the following equation was obtained from the noncoded data:

$$\text{AA yield (mg/L)} = -11{,}5760 + 852X_1 + 9442X_2 + 11{,}806X_3 - 6.51X_1^2 + 21.7X_1X_2 - 366X_2^2 + 57.7X_1X_3 - 798X_2X_3 - 1252X_3^2 \quad [2]$$

where X_1, X_2, and X_3 represent soluble starch, yeast extract, and KH_2PO_4, respectively. Analysis of variance of this fitting is presented in Table 4. It is evident that both second-order terms were more significant than the first-order (linear) term. For the total regression, the coefficient of determination was 0.86, and the *F* statistics were significant at 5%, indicating a good approximation of the data with the quadratic equation. Factor analysis showed that all three factors were significant at 5%, and soluble starch was the most influential factor. Furthermore, the canonical analysis predicts an optimal response of 3,940 mg/L for the AA yield in a medium that contains 99.7 g/L of soluble starch, 12.6 g/L of yeast extract, and 3.0 g/L of KH_2PO_4. The predicted optimal response was verified by conducting the experiments at these critical values. Five replicates at optimal condition resulted in an average AA yield of 3,885 mg/L with an average biomass, lipid content, and AA content of 32.5 g/L, 26.5%, and 45.1%, respectively. The predicted value and the experimental average were not significantly different at 5%.

The simplest way to characterize the response surface is to construct three-dimensional response surfaces or corresponding contour plots from the fitted model. Figure 5 shows the contour plots of the response of AA yield generated by holding one of the three factors at a time at its estimated optimal value. It is clear that the response of AA yield is a maximum at the concentration levels of soluble starch, yeast extract, and KH_2PO_4 mentioned above. Furthermore, the response is the least insensitive and most sensitive to changes in the concentrations of KH_2PO_4 and soluble starch, respectively. This is in agreement with the factor analysis shown in Table 4.

DISCUSSION

Most of the high producers of AA in the literature were strains of *M. alpina*, and all strains of *M. alpina* can grow rapidly with a variety of carbon sources, such as glucose, maltose, starch, glycerol, and oils and produce mycelia rich in AA (7,11–13,24–26). The Wuji-H4 isolate of this study provided further evidence for these statements. However, the choice of carbon source for AA production appeared to be species- or strain-dependent. Yamada *et al.* (8) reported that glucose was the most effective carbon source for AA production by *M. elongata.* Maltose, glycerol, *n*-hexadecane, and *n*-octadecane were also suitable for the production of AA. Bajpai *et al.* (13) reported that the highest amount of AA was produced by *M. alpina* grown in glycerol, but they preferred using glucose owing to the lower AA content of glycerol-grown culture. Shimnen *et al.* (24) reported that *M. alpina* 1*S*-4 gave almost the same mycelial yields and AA contents in media containing glucose, fructose, maltose, soluble starch, and corn starch. However, in this study, Wuji-H4 isolate produced significantly higher amounts of AA in soluble starch as a consequence of the highest biomass, lipid content, and AA content obtained in the medium containing this carbon source (Fig. 2).

In the present study, the pH of the media in Figures 2–4 was adjusted to neutral with 5 N KOH 60 h after inoculation. The final pH of the screening medium was about 3.6 without adjustment and was raised to 5.7–5.9 after single adjustment. This single adjustment of pH led to a twofold increase in AA production when the AA yield of soluble starch medium (1,061 mg/L) in Figure 2 was compared to that of the screening medium (504 mg/L).

Apart from the expensive yeast extract as a complex nitrogen source, the use of cheap and simple nitrogen sources for microbial lipid accumulation has also received attention from several workers. The results generally suggested that KNO_3 and $NaNO_3$ were better and, on the other hand, urea and NH_4Cl were inferior nitrogen sources for lipid accumulation in various cultures (27–29). However, the Wuji-H4 isolate of this study accumulated the highest level of lipid in the biomass growing in the medium containing 2.1 g/L of urea or NH_4Cl in addition to 5 g/L of yeast extract (Fig. 3). These dissimilar results may be explained by the C/N ratios of the media, because lipid accumulation is only favored by a fixed range of C/N ratios. Among the simple nitrogen sources, urea and KNO_3 have the highest (46.7%) and lowest (13.9%) nitrogen content, respectively. Yeast extract contains only 8.8% nitrogen (30). By calculating from these data, the initial C/N ratios in Figure 2 of this study ranged from 28.2 for urea to 54.6 for KNO_3, which were within the optimal range of 20 to

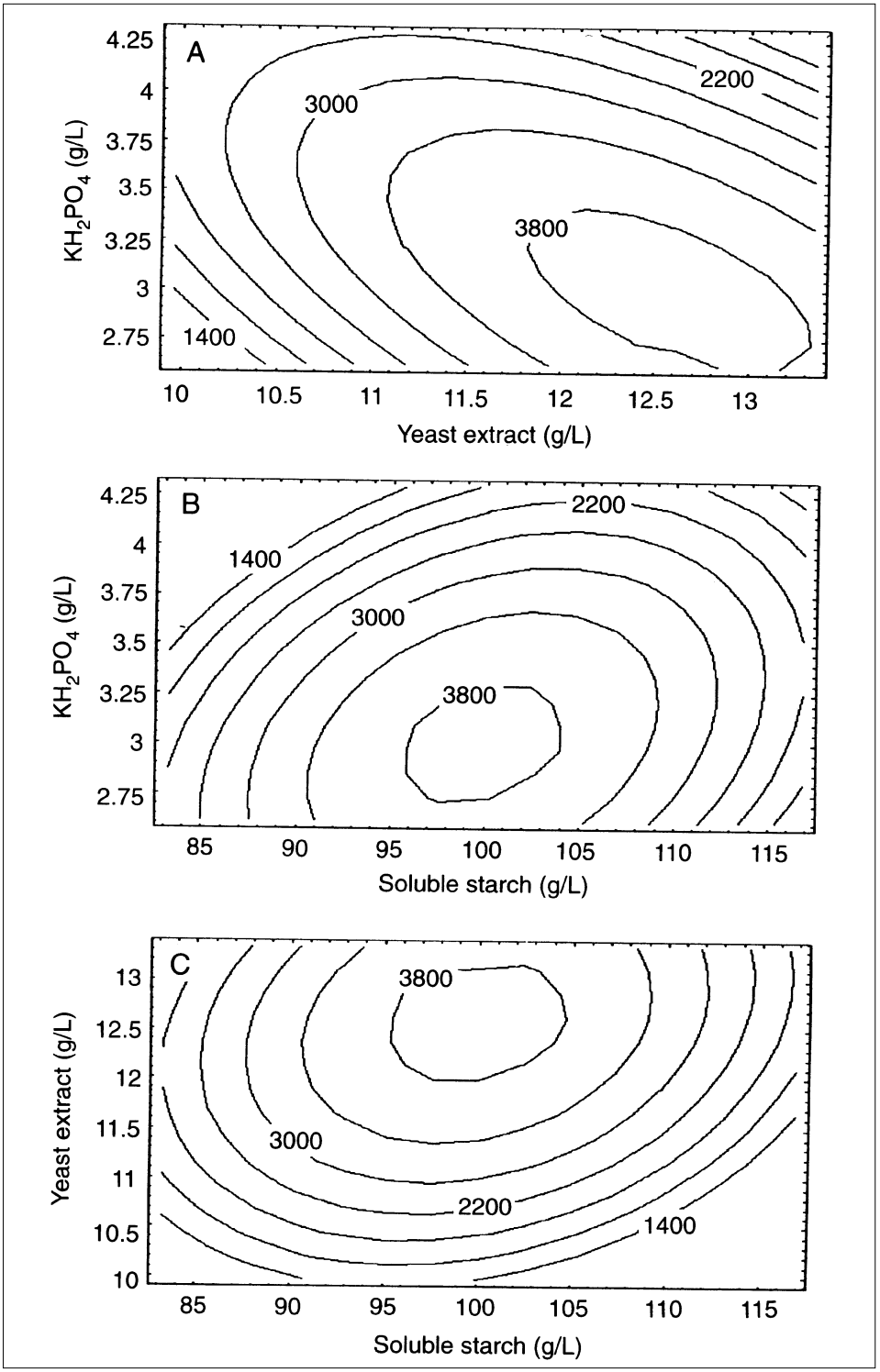

FIG. 5. Contour plots showing the arachidonic acid yield (in mg/L) of Wuji-H4 isolate with soluble starch, yeast extract, and KH_2PO_4. (A) Soluble starch kept at 99.7 g/L; (B) yeast extract kept at 12.6 g/L; (C) KH_2PO_4 kept at 3.0 g/L.

60 for lipid accumulation in most microbes (31). In other studies, media containing large amounts of urea, NH_4NO_3, or NH_4Cl as a single nitrogen source commonly had an initial C/N ratio too small to favor lipid accumulation as compared to that of $NaNO_3$ or KNO_3 on the same weight basis. The efficacy of urea or KNO_3 in shake-flask cultures may also be complicated by their capability of maintaining pH for optimal growth or lipid production (Fig. 3).

Temperature is an environmental factor reported to have a significant effect on the degree of unsaturation of constituent

fatty acids, i.e., when the growth temperature is lowered, the proportion of unsaturated fatty acids tends to increase (32,33) owing to increased membrane fluidity of the organisms that lack self-sustained temperature systems (34). Different maxima of AA content were reported in different strains of *M. alpina* at different temperatures. Shinmen *et al.* (24) reported that maximum values of 83–96 mg/g dry mycelia were obtained in strains 1*S*-4, 1-83, and CBS 210.32 at 28°C, whereas 98 mg/g dry mycelia was obtained in strain 20–17 at 22°C. Strain CBS 343.66 (35) and strain IFO 8568 (12) produced maximum AA contents of 32 and 62% of the lipid at 18 and 15°C, respectively. In this study, strain Wuji-H4 produced the most unsaturated lipid with a highest AA content of 50% at 24°C (Fig. 4). Despite these variations, the incubation temperatures that permitted optimal yields of AA for most species or strains of *M. alpina* were within ±4°C of the 24°C optimum of the Wuji-H4 isolate (8,12,13,24,35) as a result of the combined effects of rapid cell growth and high AA content at this temperature range.

All previously mentioned works related to AA production have attempted to optimize medium composition for AA production by using the "one-variable-at-a-time" technique. However, this technique does not take into account any joint effects of the variables on the response and may fail to locate the region of optimal response (14,15). In the present study, we adopted the RSM approach to locate the co-optimal levels of five individual medium components for the production of AA and to gain insight into the relative importance and the interactions of these components. A comparison of the coefficients of each factor in Equation 1, derived from the 2^{5-1} fractional factorial design (Table 1), suggested that an increase in the dosage of yeast extract (X_1), soluble starch (X_3), or KH_2PO_4 (X_4) of the medium would increase the AA yield. Contrarily, an increase in the dosage of urea (X_2) or magnesium sulfate (X_5) would exert a negative effect on AA production, and they were not required at higher concentrations of yeast extract (Table 2). These results suggested that the high yeast extract concentration alone had provided, in addition to the important growth factors, enough nitrogen, magnesium, and sulfur for the growth of Wuji-H4 isolate and its AA production. The beneficial effect of increasing KH_2PO_4 concentration on AA production was apparently due to the increased buffering capacity that promoted cell growth (data of final pH and biomass not shown). The pH of the medium was not adjusted during incubation in the RSM experiments because we found that this practice was cumbersome and could be omitted at high KH_2PO_4 concentration. The detrimental effect of high phosphate concentration on growth, commonly observed after autoclaving of glucose-based media, was not significant in our starch-based medium.

Yeast extract, having the largest coefficient of Equation 1, appeared to be the most profound factor to influence the AA yield in the path of steepest ascent to the vicinity of the optimal response. This result was different from the factor analysis of CCRD, which indicated that soluble starch was the most influential factor in the experiment (Table 4). The C/N ratio of the optimal medium (99.7 g/L soluble starch, 12.6 g/L yeast extract and 3.0 g/L KH_2PO_4) predicted from CCRD is 36.0. If we take the C/N ratio into account, the great effect of yeast extract in the path of steepest ascent may be simply due to a rapid shift of an unfavorable C/N ratio of 18.5 of the base point medium (step 0 of Table 2) to a more favorable region of C/N ratio after two or three steps. On the other hand, the C/N ratios of the 15 possible media combinations in the CCRD, ranging from 32.3 to 46.9, are more concentrated around the C/N ratio of the optimal medium. The lipid content data of the CCRD in Table 3 averaged higher and scattered in a smaller range than those of the 2^{5-1} fractional factorial design in Table 2 as a result of the centralizing C/N ratios around the optimum.

The AA yield of 3,885 mg/L in the 5-d culture of Wuji-H4 isolate, obtained with the critical concentrations of the three medium components (soluble starch, yeast extract, and KH_2PO_4) predicted by the quadratic equation, increased more than sevenfold over that of the initial screening medium. Li *et al.* (7) has obtained an AA yield of 5.3 g/L from a 7-d culture of *M. alpina* UW-1, both in shake flask and fermenter, but the yield was about 3.6 g/L after 5 d of incubation. The AA yields of *M. alpina* ATCC 32222 (3.7 g/L in 6 d) (26) and *M. alpina* 1*S*-4 (3.6 g/L in 7 d) (24) were the second highest reported in shake-flask and fermenter cultures, respectively. To our knowledge, on a 5-d basis, our AA yield is the highest ever reported. Furthermore, our optimal medium, consisting of only three components, is incomparably easier to prepare, whereas the medium of Li and coworkers (7) is complicated by the addition of canola oil as an additional carbon source and insoluble soy flour as a supplement to form dispersed growth. Both components will unquestionably affect the recovery of AA from fungal mycelia and increase production cost. Our results have clearly indicated that RSM has succeeded in the rapid exploitation of AA production potential of the new fungal isolate, Wuji-H4, by using a small number of observations. The success was achieved apparently through the finding of an appropriate combination of C/N ratio and buffering capacity of the medium, which favored large increases in lipid content and biomass, respectively. However, the lack of fit of the quadratic equation to the experimental data was significant (Table 4), indicating that no firm statements could be made on the response surfaces described by the quadratic equation without further experimentation. The optimal initial C/N ratio for the production of AA by *Mortierella* sp. *S*-17 was 20 (9) and that of *M. alpina* CBS210.32 was 54.8 of the initial screening medium of this study (11). These values are on the opposite sides of and quite different from the 36.0 of the Wuji-H4 isolate. Because different species/strains or methodologies were used in these studies, the real cause of such diverse results remains unknown and also warrants further investigations.

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REFERENCES

1. Goodnight, S.H., W.S. Harris, W.E. Conner, and D.R. Illingworth, Polyunsaturated Fatty Acid, Hyperlipidemia and Thrombosis, *Arteriosclerosis 2*:87–113 (1982).
2. Marx, J.L., The Leukotrienes in Allergy and Inflammation, *Science 215*:1380–1383 (1982).
3. Das, U.N., M.E. Begin, Y.S. Huang, and D.F. Horrobin, Polyunsaturated Fatty Acids Augment Free Radical Generation in Tumor Cells *in vitro*, *Biochem. Biophys. Res. Commun. 145*:15–24 (1987).
4. Ahern, T.J., Plant-Derived Catalysts and Precursors for Use in Prostaglandin Synthesis, *J. Am. Oil Chem. Soc. 61*:1754–1757 (1984).
5. Bajpai, P., and P.K. Bajpai, Arachidonic Acid Production by Microorganism, *Biotechnol. Appl. Biochem. 15*:1–10 (1992).
6. Sajbidor, J., M. Lamacka, J. Cista, and M. Certik, Microbial Production and Purification of Arachidonic Acid, *Biotechnol. Tech. 8*:561–564 (1994).
7. Li, Z.Y., Y. Lu, V.B. Yadwad, and O.P. Ward, Process for Production of Arachidonic Acid Concentrate by a Strain of *Mortierella alpina*, *Can. J. Chem. Eng. 73*:135–139 (1995).
8. Yamada, H., S. Shimizu, and Y. Shinmen, Production of Arachidonic Acid by *Mortierella elongata* 1*S*-5, *Agric. Biol. Chem. 51*:785–790 (1987).
9. Sajbidor, J., S. Dobronova, and M. Certik, Arachidonic Acid Production by *Mortierella* sp. S-17: Influence of C/N Ratio, *Biotechnol. Lett. 12*:455–456 (1990).
10. Buranova, L., T. Rezanka, and A. Jandera, Screening for Strains of the Genus *Mortierella*, Showing Elevated Production of Highly Unsaturated Fatty Acids, *Folia Microbiol. 35*:578–582 (1990).
11. Chang, C.C., and H.C. Chen, Production of Arachidonic Acid by *Mortierella alpina, J. Chinese Agric. Chem. Soc. 32*:395–405 (1994).
12. Totani, N., and K. Oba, A Simple Method for Production of Arachidonic Acid by *Mortierella alpina*, *Appl. Microbiol. Biotechnol. 28*:135–137 (1988).
13. Bajpai, P.K., P. Bajpai, and O.P. Ward, Arachidonic Acid Production by Fungi, *Appl. Environ. Microbiol. 57*:1255–1258 (1991).
14. Mason, R.L., R.F. Gunst, and J.L. Hess, *Statistical Design and Analysis of Experiments—with Applications to Engineering and Science*, John Wiley & Sons, New York, 1989.
15. Montgomery, D.C., *Design and Analysis of Experiments*, John Wiley & Sons, New York, 1991.
16. Chen, H.C., Response-Surface Methodology for Optimizing Citric Acid Fermentation by *Aspergillus foetidus*, *Process Biochem. 29*:399–405 (1994).
17. Houng, J.Y., K.C. Chen, and W.H. Hsu, Optimization of Cultivation Medium Composition for Isoamylase Production, *Appl. Microbiol. Biotechnol. 31*:61–64 (1989).
18. Ylimaki, G., Z.J. Hawrysh, R.T. Hardin, and A.B.R. Thomson, Response Surface Methodology in the Development of Rice Flour Yeast Breads: Sensory Evaluation, *J. Food Sci. 56*:751–759 (1991).
19. SAS, *SAS/STAT User's Guide, Version 6*, SAS Institute Inc., Cary, 1989.
20. Moon, N.J., and E.G. Hammond, Oil Production by Fermentation of Lactose and the Effect of Temperature on the Fatty Acid Composition, *J. Am. Oil Chem. Soc. 55*:683–688 (1978).
21. Christie, W.W., *Gas Chromatograph, and Lipids*, The Oily Press, Scotland, 1989.
22. Choi, S.Y., D.D.W. Ryu, and J.S. Rhee, Production of Microbial Lipid: Effects of Growth Rate and Oxygen on Lipid Synthesis and Fatty Acid Composition of *Rhodotorula gracilis*, *Biotechnol. Bioeng. 24*:1165–1172 (1982).
23. Domsch, K.H., W. Gams, and T.H. Anderson, *Compendium of Soil Fungi*, Academic Press, New York, 1980.
24. Shinmen, Y., S. Shimizu, K. Akimoto, H. Kawashima, and H. Yamada, Production of Arachidonic Acid by *Mortierella* Fungi: Selection of a Potent Producer and Optimization of Culture Conditions for Large-Scale Production, *Appl. Microbiol. Biotechnol. 31*:11–16 (1989).
25. Stredanska, S., D. Slugen, M. Stredansky, and J. Grego, Arachidonic Acid Production by *Mortierella alpina* Grown on Solid Substrates, *World J. Microbiol. Biotechnol. 9*:511–513 (1993).
26. Bajpai, P.K., P. Bajpai, and O.P. Ward, Production of Arachidonic Acid by *Mortierella alpina* ATCC 32222, *J. Ind. Microbiol. 8*:179–185 (1991).
27. Hansson, L., and M. Dostalek, Effect of Culture Conditions on Mycelial Growth and Production of γ-Linolenic Acid by the Fungus *Mortierella ramanniana, Appl. Microbiol. Biotechnol. 28*:240–246 (1988).
28. Yoon, S.H., J.W. Rhim, S.Y. Choi, W.D.D. Ryu, and J.S. Rhee, Effect of Carbon and Nitrogen Sources on Lipid Production of *Rhodotorula gracilis*, *J. Ferment. Technol. 60*:243–246 (1982).
29. Chesters, C.G.C., and J.F. Peberdy, Nutritional Factors in Relation to Growth and Fat Synthesis in *Mortierella vinacea, J. Gen. Microbiol. 41*:127–134 (1965).
30. Crueger, W., and A. Crueger, *Biotechnology: A Textbook of Industrial Microbiology*, Science Tech., Inc., Madison, 1984.
31. Moreton, R.S., Physiology of Lipid Accumulating Yeasts, in *Single Cell Oil*, edited by R.S. Moreton, Longman Scientific and Technical, Essex, England, 1988, pp. 1–32.
32. Sumner, J.L., E.D. Morgan, and H.C. Evans, The Effect of Growth Temperature on the Fatty Acid Composition of Fungi in the Order Mucorales, *Can. J. Microbiol. 15*:515–520 (1969).
33. Ferrante, G., Y. Ohno, and M. Kates, Influence of Temperature and Growth Phase on Desaturase Activity of the Mesophilic Yeast *Candida lipolytica, Can. J. Biochem. Cell Biol. 61*:171–177 (1983).
34. Fulco, A.J., Metabolic Alterations of Fatty Acids, *Annu. Rev. Biochem. 43*:215–241 (1974).
35. Lindberg, A.M., and G. Molin, Effect of Temperature and Glucose Supply on the Production of Polyunsaturated Fatty Acids by the Fungus *Mortierella alpina* CBS 343.66 in Fermentor Cultures, *Appl. Microbiol. Biotechnol. 39*:450–455 (1993).

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