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Statistical optimization of arachidonic acid production by *Mortierella alpina* CBS 754.68 in submerged fermentation

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Abstract

Arachidonic acid is an essential fatty acid in human nutrition. In the present study, production of arachidonic acid by *Mortierella alpina* CBS 754.68 was evaluated in submerged fermentation. The fermentation variables were selected in accordance with the Plackett-Burman (PB) design and further optimized via response surface methodology (RSM). Five significant variables, namely glucose, yeast extract, temperature, agitation rate, and fermentation time were selected for the optimization studies. The statistical model was constructed via central composite design (CCD). Following the optimization step arachidonic acid production increased by approximately 660.5%, when compared to the screening step. The results indicate that carrying out the fermentation under the conditions of glucose at 50 g/l; yeast extract at 14 g/l; temperature of 22°C; agitation rate of 180 rpm, and fermentation time of 8 days will increase the arachidonic acid production up to 3 g/l. Results show that the optimization of culture conditions could greatly increase arachidonic acid production by *Mortierella alpina* CBS 754.68.

Keywords: Arachidonic acid; *Mortierella alpina*; Plackett-Burman (PB) design; Response surface methodology (RSM)

INTRODUCTION

There has been increasing interest in the microbial production of lipid containing polyunsaturated fatty acids (PUFA) in the past decade (Ratledge, 1993, 1982). Arachidonic acid (ARA, 5, 8, 11, 14-*cis* eicosatetraenoic acid), a long chain polyunsaturated fatty acid, is an essential fatty acid in human nutrition, and a biogenetic precursor of the biologically active prostaglandins, and leukotrienes (Gill and Valivety, 1997). As a component of mature human milk, ARA is necessary for the neurological and neurophysiological development of both term and preterm infants (Brick *et al*., 2000; Bougle *et al*., 1999). Many expert organizations, including FAO and WHO, have recommended that ARA should be supplied as a supplement in the infant formula (FAO/WHO Expert Committee, 1994). This dietary and pharmacologically important fatty acid is currently isolated from porcine adrenal gland and liver as well as from sardines (Sakuradani and Shimizu, 2009; Certik and Shimizu, 1999). However, these oils contain too little ARA (0.2% w/w) to be practical sources for its industrial production. In addition, animal oils often contain other fatty acids with fewer desirable qualities. Hence, cultivation of microorganisms that are capable of producing larger amounts of ARA has been proposed as an alternative method of production (Ward and Singh, 2005; Yuan *et al*., 2002; Chen *et al*., 1997; Singh and Ward, 1997). ARA is present in the cells of some eukaryotic microorganisms. Lower fungi of the Phycomycetes group are a promising source of a variety of PUFAs (Radwan, 1991). Many species of the genus *Mortierella* are known to be rich in ARA under suitable culture conditions (Sakuradani and Shimizu, 2009; Shinmen *et al*., 1989; Totani and Oba, 1988).

The traditional one-at-a-time optimization strategy is relatively simple, and the individual effects of medi-

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um factors can be graphically depicted without statistical analysis. Unfortunately, it frequently fails to locate the region of optimum response in such procedures. Thus, fractional and/or full factorial designs provide an efficient approach to optimization. A combination of factors generating a certain optimum response can be identified through factorial design and response surface methodology (RSM) (Box *et al*., 1978). RSM is a useful experimental design to study the effect of several factors influencing the responses by varying them simultaneously and carrying out a limited number of experiments (Haaland, 1990). The principal objective of this study was to screen the significant variables by the Plackett-Burman design, and further to optimize the levels of the screened variables for ARA production by employing *Mortierella alpina* CBS 754.68 in submerged fermentation using RSM.

MATERIALS AND METHODS

Microorganism, inoculum preparation and culture conditions: *Mortierella alpina* CBS 754.68 was purchased from Centraalbureau Schimmelcultures (CBS, Netherlands). The culture was maintained on malt agar and subcultured every two months and stored at 3 ± 1°C. *M. alpina* CBS 754.68 was initially grown on sterile malt agar in Petri dishes at 22°C for 7 days, and then transferred to 100 ml of seed culture medium, which contained (g/l): glucose 30, and yeast extract 7. The seed culture was incubated at 30ºC for 2 days in a shaker at a rotation speed of 130 rpm. The contents of the seed culture flask were then mixed well by a miller to produce a mycelium suspension. All the fermentation experiments were performed in 500 ml Erlenmeyer flasks containing 100 ml of basal fermentation medium according to the experimental design procedure (Table 1).

Table 1. Experimental variables at different levels used for the Plackett-Burman design.

Variables	Unit	Experimental values	
		Low level (-1)	High level (+1)
Glucose	g/l	20	100
Lactose	g/l	20	100
Yeast extract	g/l	5	20
Peptone	g/l	5	20
Temperature	°C	15	30
Fermentation time	day	4	7
Agitation rate	rpm	150	250

Furthermore, the basal fermentation medium included the following salt components (g/l): $K_2HPO_4.3H_2O$ 4.0; $CaCl_2.2H_2O$ 0.1 and $MgSO_4.7H_2O$ 0.5. The initial pH of the fermentation medium was adjusted to 6.5 prior to autoclaving at 121°C for 15 min (Zhu *et al*., 2003). The fermentation medium in each flask was inoculated with a 5% (v/v) mycelium suspension of the seed culture. The value given for each experiment is the mean of two replications.

Lipid extraction: Fungal mycelia were harvested by suction filtration, washed with 50 ml of distilled water, dried at 105°C for 2 h and weighed to obtain the dry cell weight (DCW) (Koike *et al*., 2001). The dried mycelia were ground into a fine powder for subsequent extraction by organic solvent. The lipid content of the grounded powder was extracted with 2 ml of n-hexane by an ultrasonicator for 3 h and separated by centrifugation at 2000 rpm (Jang *et al*., 2005; Folsch *et al*., 1957).

Fatty acid analysis: The extracted lipid was dissolved in 5 ml of 2% NaOH/MeOH solution and methylated by 2.175 ml of BF_3. The methylated fatty acids were separated from the aqueous layer by adding saturated NaCl and dissolved in n-hexane (Metcalf *et al*., 1996). PUFA content was determined by a gas chromatography UNICAM 4600 (United Kingdom) equipped with a capillary BPX70 column (30 m × 0.25 mm i.d., 0.25 mm film thickness; SGE, USA) and flame ionization detector (UNICAM 4600, UK). Nitrogen was used as the carrier gas under the pressure of 20 kPa. The injector and detector temperatures were maintained at 250°C and 300°C, respectively. The oven was maintained or 6 min at 160°C for 6 min, then increased to 180°C at 20°C/min, maintained for 9 min at 180°C, increased further to 190°C at 20°C/min and finally maintained for 25 min at 190°C. The injection volume was 0.2 µl, with the split ratio of 50:1. The fatty acids were identified and quantified using methyl esters of the quantitative standard fatty acids supplied by Sigma (St. Louis, USA). Pentadecanoic acid (15:0) was used as the internal standard.

Experimental design and data analysis: In order to select the significant variables for ARA production, the independent variables of carbon sources (glucose and lactose), nitrogen sources (yeast extract and peptone), temperature, agitation rate, and fermentation time

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were considered and screened via the Plackett-Burman (PB) design. A total of seven variables were included for screening and each independent variable was tested at two levels, high level (+1) and low level (-1). The variables and their experimental matrix are listed in Tables 1 and 2, respectively. The main effect of each variable on ARA production was estimated as the difference between the two averages of measurements made at the higher and lower levels. The significance of each variable was determined via Student's *t*-test. The *P*-value, as the most common means of assessing significance, was also evaluated for each variable. The *P*-value is the probability that the magnitude of a parameter estimate is due to random process variability. Only confidence levels above 90% were accepted in the present study. Therefore, it is possible to rank the variables with respect to their effects on the ARA concentration.

The next step was to determine the optimum levels of the most significant independent variables for ARA production. Therefore, the response surface methodology (RSM) was adopted using a central composite design (CCD). The significant variables were assessed at five coded levels (-2, -1, 0, +1, +2), as is shown in Table 3. All the variables were taken at a central coded value (considered as zero). The experimental design and the results of CCD are listed in Table 4. By using this design, the experimental data were fitted according to equation (1), as a second-order polynomial equation including the linear and cross effects of the variables:

$$Y = \beta_0 + \sum_{j=1}^{k}\beta_j X_j + \sum_{j=1}^{k}\beta_{jj} X_j^2 + \sum\sum_{i\langle j}\beta_{ij} X_i X_j \quad (1)$$

Where, Y is the predicted response, X_i and X_j are independent variables, β_0 is the offset term, β_i is the ith linear coefficient, β_{ii} is the ith quadratic coefficient, and β_{ij} is the ijth interaction coefficient.

The statistical software package, SAS 9.1 (SAS institute, Cary, NC), was used for the regression analysis of the experimental data, to make the RSM design and also to plot the contour graphs. The statistical significance of the model equation and its terms was evaluated via the Fisher's test. The quality of fit of the second-order polynomial model equation was expressed via the coefficient of determination (R^2) and the adjusted R^2. The fitted polynomial equation was then expressed in the form of contour plots in order to illustrate the relationship between the responses and the experimental levels of variables in this study.

Table 2. Experimental design and results of the Plackett-Burman design for ARA concentration.

Run no.	Experimental values							ARA concentration (mg/l)
	Glucose	Lactose	Yeast extract	Peptone	Temperature	Agitation rate	Fermentation time	
1	+1	-1	-1	+1	+1	-1	+1	29.1±3.5
2	+1	+1	-1	-1	+1	+1	-1	0
3	+1	+1	+1	-1	-1	-1	+1	42.0±4.0
4	-1	+1	+1	+1	+1	-1	-1	32.5±1.0
5	+1	-1	+1	+1	-1	+1	-1	23.8±2.5
6	-1	+1	-1	+1	-1	+1	+1	62.3±5.4
7	-1	-1	+1	-1	+1	+1	+1	37.3±3.2
8	-1	-1	-1	-1	-1	-1	-1	81.8±0.9

Table 3. Experimental variables at different levels used for the RSM approach.

Variables	Unit	Symbol	Coded levels				
			-2	-1	0	+1	+2
Glucose	g/l	X_1	20	35	50	65	80
Yeast extract	g/l	X_2	10	12	14	16	18
Temperature	°C	X_3	10	13	16	19	22
Agitation rate	rpm	X_4	75	100	125	150	175
Fermentation time	day	X_5	6	7	8	9	10

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Table 4. Experimental design and results of the central composite design for of ARA concentration.

Run no.	Variables					ARA concentration (mg/l)
	X_1	X_2	X_3	X_4	X_5	
1	-1	-1	-1	-1	+1	113.7±13.6
2	-1	-1	-1	+1	-1	53.0±2.2
3	-1	-1	+1	-1	-1	58.2±3.0
4	-1	-1	+1	+1	+1	230.2±15.1
5	-1	+1	-1	-1	-1	21.0±3.0
6	-1	+1	-1	+1	+1	47.1±4.1
7	-1	+1	+1	-1	+1	21.0±1.4
8	-1	+1	+1	+1	-1	95.6±5.5
9	+1	-1	-1	-1	-1	73.6±2.9
10	+1	-1	-1	+1	+1	55.5±4.8
11	+1	-1	+1	-1	+1	57.0±2.2
12	+1	-1	+1	+1	-1	137.7±7.6
13	+1	+1	-1	-1	+1	53.3±1.1
14	+1	+1	-1	+1	-1	48.9±4.6
15	+1	+1	+1	-1	-1	49.5±1.3
16	+1	+1	+1	+1	+1	216.4±5.1
17	-2	0	0	0	0	80.3±3.2
18	+2	0	0	0	0	49.2±4.4
19	0	-2	0	0	0	131.8±4.7
20	0	+2	0	0	0	29.3±4.7
21	0	0	-2	0	0	29.9±3.3
22	0	0	+2	0	0	59.2±1.1
23	0	0	0	-2	0	45.1±1.3
24	0	0	0	+2	0	622.5±21.0
25	0	0	0	0	-2	92.2±1.3
26	0	0	0	0	+2	78.6±3.2
27	0	0	0	0	0	87.2±4.64
28	0	0	0	0	0	113.2±3.6
29	0	0	0	0	0	92.5±2.5
30	0	0	0	0	0	97.0±3.6

Table 5. Estimated effect, regression coefficient and corresponding *t* and *p* values for ARA concentration in the Plackett-Burman design.

Variables	Effect	Coefficient	Standard error	*t*-value	*p*-value
Glucose	-29.76	-14.88	2.142	-6.96	0.000[b]
Lactose	-8.82	-4.41	2.142	-2.06	0.074[c]
Yeast extract	-9.43	-4.72	2.142	-2.20	0.059[c]
Peptone	-3.34	-1.67	2.142	-0.78	0.458[a]
Temperature	-27.76	-13.88	2.142	-6.48	0.000[b]
Agitation rate	-15.52	-7.76	2.142	-3.62	0.007[b]
Fermentation time	+8.16	+4.08	2.142	+1.90	0.093[a]

[a]Non-significant at P < 0.05, [b]Significant at P < 0.01, [c]Significant at P < 0.1

RESULTS

Screening of the significant variables using the PB design: The results of the PB design for ARA concentration in the screening step are shown in Table 2. Table 5 shows the main effects, the associated *t*-values and the significant levels (*p*-value) of the variables on the response in the screening step. The variables showing the *p*-values of less than 0.1 were considered to have a significant effect on the response and were then selected for the

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Table 6. Analysis of variance for the regression model of ARA concentration by RSM.

Source	DF	SS	MS	*F*-value	*p*-value
Model	20	0.0916	0.0045	25.13	0.0001
Linear	5	0.0495	0.0099	54.34	0.0001
Quadratic	5	0.0142	0.0028	15.58	0.0001
Cross product	10	0.0278	0.0027	15.29	0.0001

R^2 =0.9280; adj- R^2 =0.8911; DF, degree of freedom; SS, sum of squares; MS, mean square.

next step. Therefore, the variables of glucose, yeast extract, temperature, agitation rate, and fermentation time were selected for further optimization in RSM (Table 4).

Optimization of the significant variables using RSM: The design matrix and the corresponding results of RSM experiments to determine the effects of five independent variables are shown in Table 4. The data obtained from CCD were fitted to a second-order polynomial with multiple regressions and the following equation (2) was obtained from the coded data:

$$Y=0.101701 - 0.00369X_1 + 0.019733X_2 - 0.01199X_3 - 0.02188X_4 - 0.00256X_5 + 0.006003X_{11} + 0.008517X_{22} + 0.013555X_{33} - 0.01479X_{12} + 0.007146X_{14} - 0.01285X_{24} - 0.01836X_{34} + 0.007988X_{35} \quad (2)$$

Where, Y is ARA concentration; X_1, glucose concentration; X_2, yeast extract concentration; X_3, temperature; X_4, agitation rate and X_5, fermentation time. The analysis of variance (ANOVA) of this fitting is presented in Table 6. The regression equation obtained from the ANOVA showed that the multiple correlation coefficient (R^2) was 0.9280 (the value >0.75 indicates a good fitness of the model). This is an estimate of the fraction of overall variation in the data accounted by the model and thus the model is capable of explaining 92.80% of the variation in response. It ensures a satisfactory adjustment of the quadratic model to the experimental data. The 'adjusted R^2' was found to be 0.8911 indicating that the model is good. Also, the 'Model *F*-value' of 25.13 implies that the model is significant.

In order to determine the optimal levels of each variable for maximum ARA production, the contour plots were constructed by plotting the response against each of the two independent variables, while maintaining the other variables at their fixed (zero) levels.

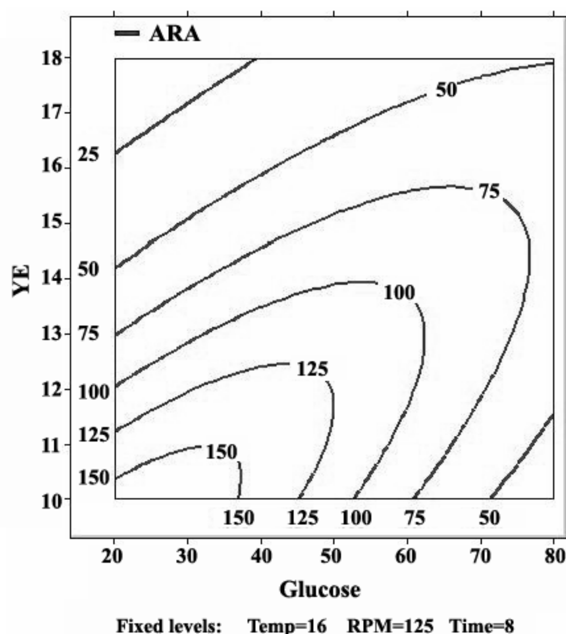

Figure 1. Contour plot showing the effects of glucose and yeast extract (YE) on ARA concentration (mg/l) based on the central composite experimental result.

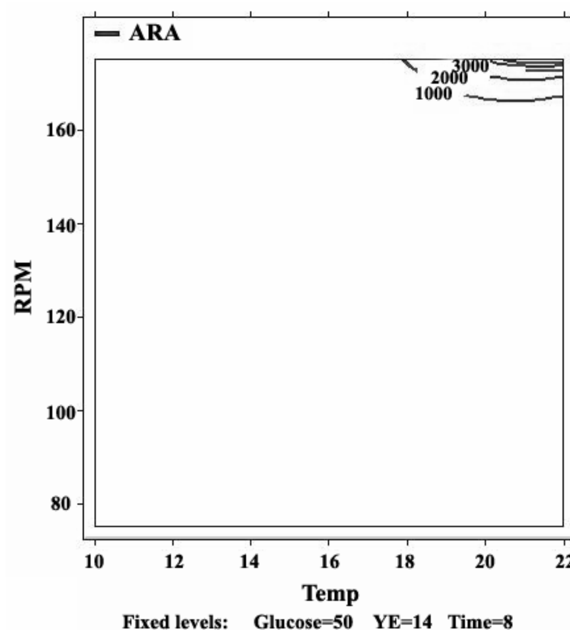

Figure 2. Contour plot showing the effects of temperature (TEMP) and agitation rate (RPM) on ARA concentration (mg/l) based on the central composite experimental result.

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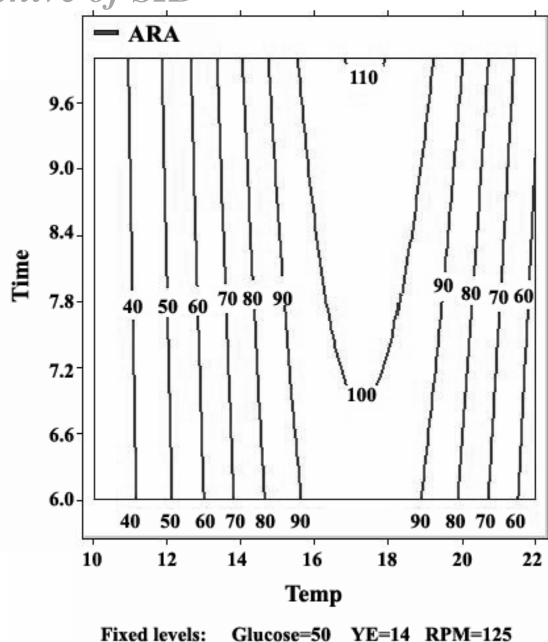

Figure 3. Contour plot showing the effects of temperature (TEMP) and fermentation time (TIME) on ARA concentration (mg/l) based on the central composite experimental result.

Figure 1 shows the contour plot of the effects of glucose and yeast extract on ARA concentration. Figure 2 shows the contour plot of the effects of temperature and agitation rate on ARA concentration. The effects of temperature and fermentation time on ARA concentration are shown in Figure 3.

DISCUSSION

According to Table 2, maximum level of ARA produced in the screening step was 81.8 mg/l. Also, Table 5 shows that glucose and temperature have a high probability value ($p<0.01$) and were determined to be the most significant variables. Moreover, the other variables except peptone were significant at $p<0.1$. Therefore, glucose and yeast extract were chosen among carbon and nitrogen sources for the optimization step, respectively.

Glucose was selected as the most suitable carbon source for fungal lipid production, which is in agreement with the findings of other researchers (Yuan *et al*., 2002; Shinmen *et al*., 1989; Totani and Oba, 1988). The rapid and efficient conversion of glucose into lipid is explained by an assumption that glucose is metabolized exclusively via the glycolysis pathway to pyruvate. The low utilization of the tested disaccharide (i. e. lactose) could be elucidated by the insufficient activity of invertase, namely β-galactosidase (Ahmed *et al*., 2006; Stredanska and Sajbidor, 1993). Yeast extract showed a more significant effect than peptone on ARA concentration. Vitamins and other growth factors present in the yeast extract probably led to a positive effect on lipid synthesis and ARA production (Stredanska and Sajbidor, 1993).

The other variables including temperature, agitation rate, and fermentation time along with glucose and yeast extract were selected for further investigation in the next step. The fermentation time exerted a positive effect while the other variables had a negative effect on ARA concentration (Table 5). Therefore, the range and central point for glucose, yeast extract, temperature and agitation rate were chosen at their corresponding low levels in the PB design. However, the range and central point for fermentation time were chosen at its corresponding high level in the PB design (Table 3).

Maximum ARA levels produced in the optimization step was 622.5 mg/l (run number 24) which in comparison to the screening step had increased by 6.6 times (Table 4). Regarding the estimated *p*-value from the ANOVA results (Table 6), the effects of culture conditions on ARA concentration were considered to be significant. The values of R^2 showed a good agreement between the experimental and predicted data for the regression. The statistical value of *F*-test probability showed that the model was accurate in describing the experimental data.

Results of this study show that glucose and yeast extract concentrations below 4% and 1%, respectively, can increase ARA concentration (Fig. 1). Similar observations have also been reported by Koike *et al*. (2001) and Shinmen *et al*. (1989).

According to Figure 2, the increase of both temperature and agitation rate up to 22°C and 180 rpm and further, respectively, can increase ARA concentration up to 3000 mg/l. This Figure shows the great importance of choosing suitable levels of temperature and agitation rate on ARA production. Moreover, the results of run numbers 23 and 24 of the central composite design for ARA concentration (Table 4) emphasize the great importance of increasing agitation rate up to 175 rpm.

Figure 3 shows that ARA production is less sensitive to the changes in fermentation time within the defined levels. Fatty acids are primary metabolites and their production increases by the increase in cell growth and biomass production (Singh and Ward,

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1997). It has been shown that a long period of time has a negative effect on biomass production due to cell lysis. Therefore, timing should be kept in a reasonable way, to guarantee sufficient biomass production and lower fermentation costs as much as possible (Yuan *et al*., 2002; Totani and Oba, 1988). The optimal condition was calculated using the SAS software (numerical method) as 50 g/l, 14 g/l, 22°C, 180 rpm and 8 days for glucose, yeast extract, temperature, agitation rate and fermentation time, respectively. The model predicted a maximum response of ARA concentration under the mentioned condition.

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References

Ahmed SU, Singh SK, Pandey A, Kanjilal S, Prasad RN (2006). Effect of various parameters on the production of γ-linolenic acid in submerged fermentation. *Food Technol Biotechnol*. 44: 283-287.

Bougle D, Denise P, Uimard F, Nouvelet A, Penniello MJ, Guillois B (1999). Early neurological and neurophysiological development of the preterm infant and polyunsaturated fatty acids supply. *Clin Neurophysiol*. 110: 1363-1370.

Box GEP, Hunter SW, Hunter JS (1978). Statistic for experiments an introduction to design, data analysis and model building. John Wiley, New York.

Brick EE, Garfield S, Hoffman DR, Uauy R, Birch DG (2000). A randomized controlled trial of early dietary supply of long chain polyunsaturated fatty acids and mental development in term infants. *Develop Med Child Neurol*. 42: 174-181.

Certik M, Shimizu S (1999). Biosynthesis and regulation of microbial polyunsaturated fatty acid production. *J Biosci Bioen*. 87: 1-4.

Chen HC, Chang CC, Chen CX (1997). Optimization of arachidonic acid production by *Mortierella alpina* Wuji-H4 isolate. *JAOCS*. 74*:* 569-578.

FAO/WHO Expert Committee (1994). The role of essential fatty acids in neural development: implications for prenatal nutrition. *Amer J Clin Nutr*. 57: 703-710.

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

Gill I, Valivety R (1997). Polyunsaturated fatty acids, Part l: Occurrence, biological activities and application. *Trends Biotechnol*. 15: 401-409.

Haaland PD (1990). *Experimental design in biotechnology*. Elsevier Science, New York.

Jang HD, Lin YY, Yang SS (2005). Effect of culture media and conditions on polyunsaturated fatty acids production by *Mortierella alpina*. *Bioresource Technol*. 96: 1633-1644.

Koike Y, Cai HJ, Higashiyama K, Fujikawa S, Park E (2001). Effect of consumed carbon to nitrogen on mycelial morphology and arachidonic acid production in cultures of *Mortierella alpina*. *J Biosci Bioeng*. 91: 382-389.

Metcalf LC, Schmitz AA, Pelka JR (1996). Rapid preparation of methyl esters from lipid for gas chromatography analysis. *Anal Chem*. 38: 514-518.

Radwan SS (1991). Sources of C20-polyunsaturated fatty acids for biotechnological use. *Appl Microbiol Biotechnol*. 35*:* 421-430.

Ratledge C (1982). Single cell oil. *Enzyme Microbial Technol*. 4: 58-60.

Ratledge C (1993). Single cell oil-Have they a biotechnological future? *Trends Biotechnol*. 11: 278-284.

Sakuradani E, Shimizu S (2009). Single cell oil production by *Mortierella alpina*. *J Biotechnol*. 144: 31-36.

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

Singh A, Ward OP (1997). Production of high yields of arachidonic acid in a fed-batch system by *Mortierella alpina* ATCC 32222. *Appl Microbiol Biotechnol*. 48: 1-5.

Stredanska S, Sajbidor J (1993). Influence of carbon and nitrogen sources on the lipid accumulation and arachidonic acid production by *Mortierella alpina*. *Acta Biotechnol*. 13: 185-191.

Totani N, Oba K (1988). A simple method for production of arachidonic acid by *Mortierella alpina*. *Appl Microbiol Biotechnol*. 28: 135-137.

Yuan C, Wang J, Shang Y, Gong G, Yao J, Yu Z (2002). Production of arachidonic acid by *Mortierella alpina* I_{49}-N_{18}. *Food Technol Biotechnol*. 40: 311-315.

Ward OP, Singh A (2005). Omegra-3/6 fatty acids: alternative sources of production. *Process Biochem*. 40: 3627-3652.

Zhu M, Yu LJ, Wu YX (2003). An inexpensive medium for production of arachidonic acid by *Mortierella alpina*. *J Ind Microbiol Biotechnol*. 30: 75-79.

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