

Production of Arachidonic Acid and Eicosapentaenoic Acid by *Mortierella alpina* CBS 528.72 on Date Waste

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SUMMARY

Arachidonic acid (ARA) and eicosapentaenoic acid (EPA) are important for human health and are produced by the fungus *Mortierella alpina*. Fermentation conditions may affect fatty acid profile of produced oil. Substrates in solid-state fermentation are usually renewable and low-cost agro-industrial waste. The aim of this research is to study simultaneous production of ARA and EPA by *M. alpina* CBS 528.72 in solid-state fermentation. Date waste and soybean meal served as carbon and nitrogen sources, respectively. Plackett-Burman design was used to evaluate the impact of eleven process variables on ARA and EPA production. Analysis of the results showed that the main variables with significant impact on production include seed age, and supplementation of linseed oil and nitrogen. These three variables were then studied in a central composite design for the optimization of production of both fatty acids. Finally, reconfirmation test was conducted to obtain experimental results from predicted optimum conditions. This report showed that simultaneous optimization of the production of ARA and EPA by *M. alpina* CBS 528.72 on date waste can be achieved by modification of process variables in solid-state fermentation.

Key words: arachidonic acid (ARA), date waste, experimental design, eicosapentaenoic acid (EPA), *Mortierella alpina*, solid-state fermentation

INTRODUCTION

Polyunsaturated fatty acids (PUFAs) such as arachidonic acid (ARA) and eicosapentaenoic acid (EPA) have significant pharmaceutical and nutritional value and great impact on human physiology due to their important roles as structural components of membrane phospholipids and eicosanoid precursors (1-3). Microorganisms, pig liver, adrenal glands and marine fish oil are all sources of PUFAs, e.g. ARA and EPA (4). However, there are some problems with marine resources due to limited fishing quotas and remote geographical locations and the necessity to remove cholesterol, undesirable odours and flavours (5). Some microalgae are suitable sources of EPA, but they require specific conditions for growth, so they are not economically suitable at all times. On the other hand, no plant produces PUFAs longer than C18, therefore genetic manipulation is necessary to achieve desirable PUFAs (6,7). Thus microorganisms are more preferable to animal-based sources for the production of PUFAs for use as food additives, dietary supplements or feedstock (6).

Production of microbial oil, known as single cell oil (SCO), by *Mortierella* sp. filamentous fungi, especially *M. alpina*, has been studied because they are not only a potential source of ARA but can also produce a wide range of PUFAs including ARA and EPA under different culture conditions. Also, similar to other moulds, they could use agricultural and food industrial wastes to manufacture products in solid-state fermentation in the absence or almost complete lack of free water (5). The biosynthesis of fatty acids in most organisms causes the formation of saturated fatty acids of palmitic and stearic acids. These fatty acids can then (by a series of sequential reactions using enzymes, mainly fatty acid elongase) metabolize to a wide range of PUFAs (6).

One of the main parameters that contribute to the production cost of PUFAs is that of the raw material (6). The most cost-effective substrates are the waste materials obtained

from food industry using solid-state fermentation (SSF) preferably when the product (oil) is intended for human consumption. The ratio of organic carbon to total nitrogen and available trace elements are important for microbial growth and SCO production in SSF (8,9). Date waste and soybean meal are rich in nutrients and could be used as carbon and nitrogen sources for SCO production.

Definite and appropriate designing of fermentation process experiments is important and necessary (10,11). Also, fungal growth in solid-state fermentation is simple and available (12). So far, most of the research on *M. alpina* has evaluated PUFA production, especially ARA, in submerged fermentation and few were done using SSF. In other studies, PUFA production was performed by culturing *M. alpina* in SSF on rice and wheat bran, peanut meal and sweet potato residue and their mixtures, press cake (sunflower, soybean, colza and olive), pearl barley, rice without bran, dehulled millet, cracked barley, peanut meal, brewer's spent grain, soybean hull, etc. (13).

The effect of different variables including temperature, time and carbon source concentration on ARA and EPA production is reported in the literature (13-15). However, a comprehensive study considering screening of possible influencing variables on the production of PUFA from date waste in solid-state fermentation has not been conducted. Also, possibility of simultaneous optimum production of these two fatty acids with health benefits has not been reported.

In this research, eleven process variables, namely particle size of the substrate, initial moisture content and pH of the substrate, carbon to nitrogen ratio of the substrate, supplementations of soybean oil and linseed oil, seed age, incubation temperature and time, heating pretreatment time and supplementation of nitrogen on the fourth day of SSF were investigated with Plackett-Burman design in producing ARA and EPA by *M. alpina* CBS 528.72 using date waste as carbon source and soybean meal as nitrogen source, and compared with results of other researchers.

MATERIALS AND METHODS

Microorganism and substrates

Mortierella alpina CBS 528.72 was obtained from the Westerdijk Fungal Biodiversity Institute (formerly Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands). All chemicals used in the experiments were obtained from Carlo Erba Reagents (Val de Reuil, France) unless specified differently. Pentadecaenoic acid used for the gas chromatography (GC) analyses was pur-

chased from Merck (Darmstadt, Germany). Malt agar was obtained from Liofilchem (Roseto degli Abruzzi, Italy).

The organism was maintained on malt agar slants, preserved at 4 °C and subcultured every 2 months (14). Date waste (byproduct of date palm syrup production) was obtained from Khorma Bone Jonoob Company (Bandar Abbas, Iran) and soybean meal from Behpak-Sobhan soy protein company (Behshahr, Iran), linseed oil and soybean oil were purchased from Behshahr Industrial Company (Tehran, Iran). The main chemical compositions of the solid substrates are listed in Table 1.

Mycelial suspension production

M. alpina CBS 528.72 was incubated at 23 °C on a medium containing (in g/L): glucose 10, yeast extract 5 and agar 20, with pH=(6.5±0.1), for 96 and 192 h based on Plackett-Burman experimental design (Table 2 and Table 3). Our previous research (data not shown) did not indicate the necessity of strict pH control. The pH was maintained in an interval of 12 h with 1 M NaOH or 1 M H₃PO₄ only up to the 5th day of fermentation. The mycelia were then homogenized with distilled water to produce a suspension of mycelium with an estimated mycelial fragment of (1.5-2)·10⁶ cells per mL (11,16).

Chemical analyses

Moisture content was adjusted by drying in a vacuum oven until obtaining a constant mass. The substrate pH was measured with a pH meter (MA235; Mettler Toledo, Greifensee, Switzerland), and then adjusted with 1 M NaOH or 1 M HCl (11,16). The percentage of organic carbon was estimated via wet oxidation method using potassium dichromate(VI) and titration with 0.5 M ammonium iron(II) sulfate (17). The percentage of total nitrogen was measured with an automated Kjeldahl apparatus (Kjeltec™ 8400; FOSS, Hilleroed, Denmark). The oil was extracted from dried substrate using *n*-hexane as solvent and ultrasound device (18). The Soxhlet method was used to measure oil content, while derivatization of fatty acids was done according to Jacobs *et al.* (19) and Metcalfe *et al.* (20). ARA and EPA content was determined by gas chromatograph (UNICAM 4600, Cambridge, UK) equipped with capillary BPX70 column (30 m×0.25 mm i.d., 0.25 mm film thickness; SGE, Melbourne, Australia) and flame ionization detector. Nitrogen was the carrier gas under the pressure of 20 kPa. The injector and detector temperatures were maintained at 250 and 300 °C, respectively. The injection volume

Table 1. The main compositions of different solid substrates used for production of ARA and EPA in solid-state fermentation

Substrate	w(organic carbon)/%	w(total nitrogen)/%	C/N	w(moisture)/%	w(oil)/%	w(PUFA)/(mg/g)			
						LA	ALA	ARA	EPA
Palm date waste	45.3±0.6	1.26±0.04	35.9±0.8	23.3±0.1	1.3±0.3	0.63±0.06	0.08±0.03	ND	ND
Soybean meal	43.95±0.05	8.0±0.2	5.5±0.2	6.60±0.04	1.4±0.2	2.4±0.2	0.30±0.02	ND	ND
Linseed oil	–	–	–	–	–	116.6±0.5	383.5±0.7	ND	ND
Soybean oil	–	–	–	–	–	384.0±0.6	45.1±0.6	ND	ND

Mean value±standard deviation, N=3; ARA=arachidonic acid, EPA=eicosapentaenoic acid, PUFAs=polyunsaturated fatty acids, LA=linoleic acid, ALA=α-linolenic acid, ND=not detected, --=not available

was 0.2 µL, with the split ratio of 50:1. Pentadecaenoic acid (15:0) was used as the internal standard.

Table 2. Plackett-Burman design for studying the impact of 11 variables on arachidonic acid (ARA) and eicosapentaenoic acid (EPA) production by *Mortierella alpina* in solid-state fermentation

Variable	Low level (-)	High level (+)
A: <i>d</i> (substrate particle)/mm	0.1-0.6	1.2-1.7
B: <i>w</i> (soybean oil)/% (by mass)	1	10
C: <i>w</i> (linseed oil)/% (by mass)	1	10
D: <i>w</i> (substrate initial moisture)/%	60-65	70-75
E: Initial pH of substrate	6	7
F: <i>t</i> (heating pretreatment)/min	20	60
G: Seed age/h	96	192
H: Substrate carbon to nitrogen ratio	17	21
J: Incubation temperature/°C (after 5 days)	12	20
K: <i>t</i> (incubation)/day	4	7
L: <i>w</i> (N)/% (by mass) on the fourth day	0	4

Solid-state fermentation

Based on the desired carbon to nitrogen ratio, a certain amount of date waste and soybean meal was mixed in flasks. Then other process variables, *e.g.* substrate particle size, moisture content, pH and the content of soybean oil and linseed oil were adjusted based on the experimental design (Table 2 and Table 3), and then the substrate was mixed. Heat pretreatment was carried out by autoclaving at 121 °C for 20 and 60 min. After adding 2 mL of mycelial suspension aged 96 and 192 h, the mixture was inoculated and incubated at 23 °C for 5 days. On the fourth day of incubation, nitrogen supplement containing potassium nitrate and yeast extract at the ratio 2:1 was added to some flasks according to the experimental design. On the

sixth day, the temperature was changed to 20 and 12 °C for another 4 and 7 days depending on the experimental design. The fermented substrate was dried in a freeze dryer and then ground in a lab mortar (11,13,14,16).

Statistical analysis

Statistical analysis of the obtained results from both Plackett-Burman design and central composite design (CCD) was performed using MINITAB statistical software v. 14 (21). The data were statistically evaluated using the analysis of variance (ANOVA). Three- and higher-order interactions were eliminated. All data are presented as the mean value±standard deviation of two independent experiments on different days. A *p*-value below 0.05 was considered statistically significant. Table 2 shows factors affecting the production of PUFAs using Plackett-Burman design at two levels. To do so, the Design-Expert v. 8 software (22) was also used (14,15). Range finding was based on literature review and previous research on solid-state production, which will be discussed later (3,8,13-15).

RESULTS AND DISCUSSION

Composition of solid substrates

Iran is ranked among the top countries in the world for production of dates as a result of which a large amount of this fruit is converted to agro-industrial wastes. Therefore, this rich source of sugar can be utilized as an available low-cost substrate for cell growth and production (23).

To enhance the growth of *Mortierella alpina* CBS 528.72 and single cell oil (SCO) production, analysis of substrate's main constituents is important (14). In this study, initial ratio of organic carbon to total nitrogen of date waste was calculated

Table 3. Plackett-Burman design in 12 runs for studying the impact of eleven process variables on arachidonic (ARA) and eicosapentaenoic acid (EPA) production (per g of dried fermented substrate) in solid-state fermentation

Run no.	Variable											<i>w</i> (ARA)/(mg/g)	<i>w</i> (EPA)/(mg/g)	<i>w</i> (oil)/%
	A	B	C	D	E	F	G	H	J	K	L			
1	+	+	-	+	+	+	-	-	-	+	-	(2.95±0.08) ^e	(0.07±0.02) ^b	17.5±0.8
2	-	+	+	-	+	+	+	-	-	-	+	(0.73±0.01) ^b	(0.04±0.01) ^a	23.0±1
3	+	-	+	+	-	+	+	+	-	-	-	(2.5±0.11) ^d	(0.06±0.02) ^b	15.99±0.02
4	-	+	-	+	+	-	+	+	+	-	-	(0.19±0.01) ^a	(0.04±0.01) ^a	15.0±0.4
5	-	-	+	-	+	+	-	+	+	+	-	(0.31±0.04) ^a	(0.04±0.01) ^a	14.7±1.1
6	-	-	-	+	-	+	+	-	+	+	+	(0.59±0.06) ^b	(0.02±0.01) ^a	6.5±1.2
7	+	-	-	-	+	-	+	+	-	+	+	(0.68±0.06) ^b	(0.06±0.01) ^b	7.4±0.7
8	+	+	-	-	-	+	-	+	+	-	+	(1.39±0.04) ^c	(0.01±0.01) ^a	17.7±0.9
9	+	+	+	-	-	-	+	-	+	+	-	(1.24±0.01) ^c	(0.06±0.01) ^b	23.3±0.9
10	-	+	+	+	-	-	-	+	-	+	+	(4.38±0.40) ^f	(0.87±0.09) ^c	25.5±0.5
11	+	-	+	+	+	-	-	-	+	-	+	(4.7±0.47) ^g	(1.86±0.08) ^d	18.8±1.0
12	-	-	-	-	-	-	-	-	-	-	-	(0.19±0.15) ^a	(0.01±0.01) ^a	5.9±1.1
Coeff. ARA*	+58	NSE#	+66	+88	NSE	NSE	-68	NSE	NSE	NSE	+42	-	-	
Coeff. EPA*	NSE	NSE	+23	+23	NSE	-22	-21	NSE	NSE	NSE	+22	-	-	

*Coefficients of factors influencing ARA and EPA production; A-L=variables the same as in Table 2, NSE=not significant effect. Values with the same letter in each column are not significantly different

to be 35.9 ± 0.8 (Table 1). Koike *et al.* (24) reported that carbon to nitrogen ratio of 7-15 was suitable for lipid production by *M. alpina*, which increased in range of 15-32. Generally, the best carbon to nitrogen ratio has been reported to be from 15:1 to 20:1 (13,24). Therefore, soybean meal was used as a nitrogen source and carbon to nitrogen ratio of the substrate was adjusted at two levels (17,23).

Soybean meal is suitable for producing some PUFAs like ARA and is economically justifiable (14). The oils extracted from date waste and soybean meal, soybean oil and linseed oil used as supplements did not contain ARA and EPA; therefore, production of the mentioned fatty acids could be related to the role of independent variables and their levels. In fact, date waste and soybean meal are also oil supplements which contain PUFAs including linoleic acid and α -linolenic acid (ALA), which stimulate the production of other PUFAs such as ARA and EPA in different species of *Mortierella*, e.g. *M. alpina* (25).

Influence of process parameters on ARA and EPA production

According to the results of Plackett-Burman design for ARA and EPA production (Table 3) based on the analysis of variance, the Pareto charts of variables (Fig. 1) were drawn. The coefficients of significant factors affecting each of them show that positive coefficients indicate direct relation between the variable and the considered response and negative coefficients indicate an inverse relationship between them (Table 3). Positive sign of the coefficient means that by increasing the level of a variable from low to high leads to increasing the dependent variable or response (in this study ARA and EPA production). Coefficient with a negative sign means that by changing the level of variable from low to high, the response decreases.

Particle size of the substrate

In this study, the coarse substrate particles have been shown to have a significant positive impact on the production of ARA compared with fine particles. However, the particle size was not an effective and significant factor in EPA production. Ghobadi *et al.* (18) showed that coarser substrate particles increase the production of ARA; thus, they introduced it as a significant effective factor.

In general, coarse substrate particles prevent aggregation of particles in aerobic fungi during fermentation and lead to more free air flow and better penetration into mycelium substrate, which improves the production of a product that requires further oxygen (26). Therefore, the size of substrate particles is important variable when SSF depends strongly on oxygen availability (18). Key point in SSF is that particle size of substrate has a critical and narrow range because it influences the mass and heat transfer as well as the surface to volume ratio of particle (which is important for availability and consumption of substrate). In fact, decreasing the particle size causes an increase in the surface to volume ratio, which

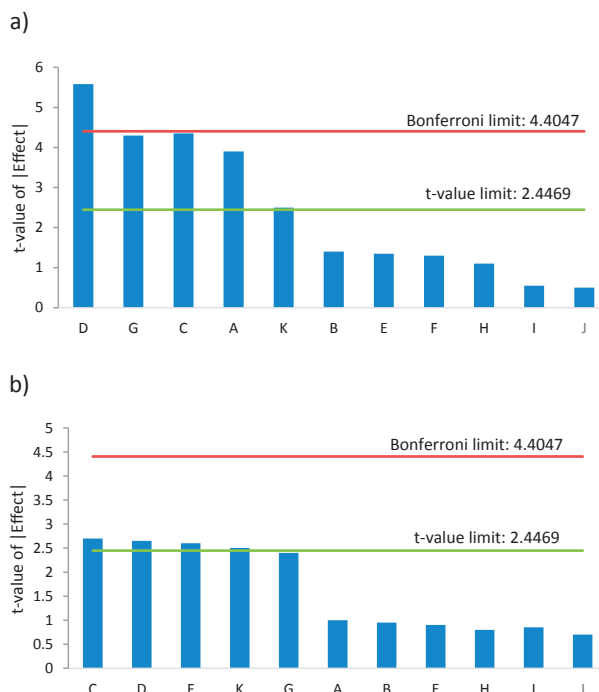


Fig. 1. Pareto chart of influence of process variables on: a) arachidonic acid (ARA) and b) eicosapentaenoic acid (EPA) production (A=particle size of substrate, B=soybean oil supplement, C=linseed oil supplement, D=substrate initial moisture content, E=initial pH of substrate, F=heating pretreatment time, G=seed age, H=carbon to nitrogen ratio, I=incubation temperature after 5 days, J=incubation time, K=nitrogen supplementation on the fourth day)

enables the contact between the particle and the enzymes produced by the microorganism. Further decrease of particle size leads to poor heat and mass transfer due to reduced inter-particle space. It has been shown that SSF with aeration increased the production of arachidonic acids, EPA, and total PUFAs (16). It can be interpreted from the results that both particle sizes used in this study were suitable for air transfer in culture and EPA production, while the fungus required further oxygen for production of ARA. PUFAs are produced through enzymatic unsaturation with oxygen; thus, adequate oxygenation is the key factor in achieving high PUFA content in mycelial cells (27).

Seed age

Seed age (the incubation period of mycelia prior to inoculation) is an effective and significantly negative factor in production of ARA and EPA by *M. alpina*. In other words, compared to the mycelia aged for 192 h, inoculation of young mycelia (aged for 96 h) increased the production of desired fatty acids. This was consistent with the research of Fakas *et al.* (28) who studied *M. isabellina* on solid culture medium of pear pomace and revealed the relationship between the unsaturation of fatty acids and mycelial age. They found that PUFAs were particularly high in young mycelia and their amount was reduced by ageing. PUFAs were better biosynthesized in fast-growing young mycelia, while their synthesis was significantly reduced in old mycelia.

Changes in fatty acid profiles with mycelium ageing are due to the changes in their biosynthesis process, particularly changes in the rate of fatty acid unsaturation.

Heating pretreatment time

The results showed a significantly inverse relationship between the duration of heating pretreatment of the substrate and EPA production, probably due to excessive hydrolysis or loss of some nutrients in the substrate and interference in the activity of unsaturated enzymes of *M. alpina*. Furthermore, no significant impact was observed on ARA production, probably because the changes in substrate did not affect the activity of the aforementioned enzymes. However, the solid substrate has to be treated to release the nutrients. Ghobadi *et al.* (18) hydrolyzed the substrate at 121 °C for 30 min. Therefore, in order to save energy, 20-30 min of autoclaving is recommended for this fungus (13).

Nitrogen supplementation

Adding nitrogen on the fourth day was one of the significant factors for producing ARA and EPA; in other words, adding 4 % (by mass) supplement to the substrate increased the amount of ARA and EPA. These results are in agreement with those of Jang and Yang (16), who supplemented solid substrates fermented by *M. alpina* with nitrogen sources, including yeast extract and nitrate, and realized that the produced PUFAs depended on the amount of nitrogen supplement. Supplementing with 3.75 to 7.5 % nitrogen source on the fourth growth day was suitable for producing ω -3 PUFAs, especially EPA (11). This finding is in agreement with the report of Ben-Amotz *et al.* (29) who indicated that high nitrogen concentrations could strengthen EPA production. Also Jang and Yang (16) found that culturing rice bran on solid substrate with nitrogen supplement resulted in better growth and adding nitrogen source on the fourth day in a column reactor increased EPA production.

Although lipid accumulation is affected by high ratio of carbon to nitrogen (30), further addition of nitrogen source (a combination of yeast extract and KNO_3) to the substrate on the fourth day of fermentation might enhance microbial growth and thus increase PUFA production (16).

Oil supplementation

Results of this research showed that the addition of linseed oil to the substrate was an effective and significant factor with a positive effect on the production of ARA and EPA by *M. alpina*, while the addition of soybean oil did not have a significant effect on the amount of produced ARA and EPA. In other words, incorporation of 10 % (by mass) linseed oil to the substrate increased the production of ARA and EPA. The results were consistent with reports by some researchers, where with the addition of 10 % (by mass) linseed oil to the solid substrate the production of PUFAs increased in most of the investigated species of *Mortierella* (11,16,19,31).

Results of Jang *et al.* (32) showed that cellular mass increased with the increase of concentration of soybean oil and became stable at 1 %. The amount of ARA and desaturation decreased with the increase of soybean concentration (31). Jang *et al.* (32) showed that the addition of 1 % soybean oil and 1 % linseed oil to rice bran increased the efficiency of total PUFAs, linoleic acid, ARA and EPA. This difference probably resulted from the difference in the type of substrate and conditions of fermentation (11,13).

Therefore, it could be concluded that adding 10 % (by mass) linseed oil caused greater increase of ARA and EPA than adding 10 % soybean oil. In other words, adding linseed oil had greater effect on desaturation of the oil produced by *M. alpina* than adding soybean oil to the substrate, which showed that, by culturing this fungus, linseed oil could be metabolized more effectively and that it had a positive effect on the production of PUFAs. Due to the rich content of oleic, linoleic and α -linolenic acids and use of precursors by *Mortierella* fungus, they could be desirable for producing PUFAs. The mentioned findings confirm that *M. alpina* is capable of using lipids added to the medium for producing PUFAs, which is done by desaturation and elongation of fatty acids (33).

Substrate initial moisture content

Initial moisture content of the substrate is very important for SSF (13). The results show that at the initial moisture of the substrate of 75 %, the production of ARA and EPA by *M. alpina* increased. Maximum and minimum moisture content in SSF depends on substrate characteristics, especially its water activity, because above a critical point the free water particles may appear, filling the inter-particle space between substrate particles and acting as a barrier for mass and heat transfer in SSF.

It had a significantly positive effect and was consistent with the results reported by other researchers on production of ARA in rice bran by *M. alpina*. Jang and Yang (16) found that optimum initial moisture for total PUFA, linoleic acid and ARA production by *M. alpina* in rice bran was about 57 %, while maximum initial moisture for the production of ALA and EPA was 64 %. Initial amount of moisture was reported to be about 70 to 75 % for producing ω -6 PUFAs and moisture of 60 to 65 % was suitable for producing ω -3 PUFAs (11,16). In contrast, in this work, 75 % moisture was shown to be suitable for EPA production. Moreover, in reports of Zhang and Hu (34) on soybean hull and Economou *et al.* (35) on sweet sorghum using *M. isabellina*, initial moisture of about 75 and 92 % respectively was reported as the reason for increased production of unicellular oil. This difference could be attributed to the discrepancy in substrate type and its moisture absorption because the amount of moisture is a function of absorbability of the substrate. Zdražil and Brunert (36) showed that low moisture decreased solubility of nutrients and substrate inflation. Also, high moisture caused loss of porosity, induced sticky substrate particles and decreased exchange of oxygen gas. Thus, very high and very low moisture are not suitable for metabolite production (11).

Incubation temperature

In this research, temperature had no significant effect on the production of ARA and EPA. However, other researchers have reported temperature as one of the functional factors in producing biomass, oil and fatty acids, especially by *M. alpina* (11,16,18). In other words, temperature results of this research did not imply inconsistency with results of other researchers because temperature might not be introduced as a significantly functional factor due to the selection of the studied temperature range and differences in other conditions of fermentation.

Nevertheless, in studies by other researchers (11,37), the temperature required for biomass production is usually reported higher than the temperature needed for production of PUFAs. *Mortierella* species such as *M. alpina* have shown the best growth at 23–25 °C and the largest amount of PUFA was produced at 16–20 °C. Also, some evidence indicates that the desaturation of ARA to EPA by n-3 desaturase is stimulated by growth of *Mortierella* at low temperature (6).

Incubation time

As with any other biotechnological fermentation, the optimum incubation time is important for metabolite production. *M. alpina* species produced the largest amount of PUFAs during 10 to 15 days. The important point was that excessive increase of fermentation time decreased economic conservation of the product produced by the microorganism. Considering this factor, optimum incubation time should be selected so that maximum efficiency is achieved during the minimum possible time (13,37).

Although a significantly negative effect of time on ARA production was reported Ghobadi *et al.* (18); in this report, it was not introduced among significant factors for production of ARA and EPA. This difference could be attributed to different species of fungus, types of substrate and selected time intervals. Probably, if different periods of time for their production were studied, it would have been reported as a significant factor because the two selected incubation times in this research were suitable for SCO production.

Fakas *et al.* (28) found that the amount of oil in pear pomace fermented by cultivation of *M. isabellina* fungus on the 9th day after insemination was about 12 % of the maximum value, which decreased after 12 days. Furthermore, Jang and Yang (16) reported that production of PUFAs by *M. alpina* on rice bran solid substrate for 12 days was stable and unchanged. The results showed a constant microbial growth in solid substrate during 12 days, and PUFA content in mycelium of fungus was accumulated without any decrease in efficiency.

Fakas *et al.* (28) studied PUFA production by *M. isabellina* in SSF of pear pomace and reported that production yield reached a maximum in young mycelia and then by further prolonging the fermentation time (up to 1 day), it decreased in old fungi. In another study, Jang and Yang (16) reported that production of PUFAs by *M. alpina* SSF was stable for 12 days of incubation without

a decrease in production yield. The reason for such difference between SSF reports may strongly depend on the applied microorganism and substrate. Carbon source in fruit waste is simpler than lignocellulose ligands of bran, so change in its content (and resulting changes in C/N ratio) may directly influence PUFA production. In some cases a slow and constant fungal growth and PUFA production were reported in SSF (11).

Carbon to nitrogen ratio of substrate

Some researchers have reported that carbon to nitrogen ratio of solid substrate is crucial for mould growth and lipid production (10,32). In this study, C/N ratio was not a significant factor for ARA and EPA production, probably due to the selected range of variables. In other words, both selected levels were in the optimum range for producing PUFAs. Other researchers (13,24) observed C/N ratio of the substrate in the range of 15–20, which is desirable for *M. alpina* growth and lipid production. C/N ratio of between 14.5 and 18.5 was suitable for EPA production and 19.8 and 21 was suitable for ARA and total PUFA production (9).

Also at different C/N ratio, the amount of other ingredients of date waste also varies. In fact, increasing the carbon source is a consequence of some other effective metal ions in date extract, as a complex medium, such as iron, potassium, magnesium, manganese, sodium, phosphorus and copper (data not shown). These metal ions can act as inhibitors of biomass and PUFA production directly or indirectly. Most growth inhibition compounds found in date extract are acids, phenol and quinone compounds (23).

Initial pH of substrate

The initial pH of the substrate is an important factor for fungal growth and has low impact on fatty acid type (13). In this study, selection of pH=6 and 7, which are the optimum values for production of PUFAs in SSF, was not recognized as a significant factor for ARA and EPA production. Jang *et al.* (9) also found that optimum pH for production of PUFAs in SSF by *M. alpina* is 6 to 7, and PUFA production decreased at a pH greater than 8 or lower than 5. Other researchers have also reported that slightly acidic to neutral pH, *i.e.* from 6 to 7, is optimal for production of ARA, EPA and PUFAs by *Mortierella* in SSF (5,16,38).

CCD design

The results for ARA and EPA production by *M. alpina* during SSF of date waste for 6 days according to CCD (Table 4) are presented in Fig. 2 and Fig. 3. In all trials, non-significant parameters of Placket-Burman design were fixed at the corresponding acceptable levels and CCD was designed based on three variables, linseed oil, seed age and mass fraction of nitrogen supplement, which were more significant in fermentation of both fatty acids. The variables were fixed at their most suitable levels (for ARA the fixed variables were D+G-C+A+L+ and for EPA C+D+F-G-L+, based on Table 2 coding).

Table 4. Central composite design for studying the impact of three selected variables and their levels (based on α : -1.34, -1, 0, +1 and +1.34) on arachidonic acid (ARA) and eicosapentaenoic acid (EPA) production by *Mortierella alpina* in solid-state fermentation

No	w(LSO)/%	Seed age/h	w(N)/%*	w(ARA)/(mg/g)	w(EPA)/(mg/g)
1	12	100	5.00	5.75 ^e	0.18 ^b
2	8	100	2.50	3.51 ^c	0.05 ^a
3	10	115	3.75	6.07 ^f	0.08 ^a
4	12	70	5.00	8.65 ⁱ	4.53 ^g
5	10	85	1.25	9.01 ^j	3.71 ^f
6	8	100	5.00	8.62 ⁱ	3.43 ^e
7	10	55	3.75	6.02 ^f	2.51 ^d
8	10	85	6.25	6.18 ^f	3.64 ^f
9	10	85	3.75	4.32 ^d	0.06 ^a
10	12	70	2.50	5.51 ^e	0.07 ^a
11	10	85	3.75	4.37 ^d	0.07 ^a
12	8	70	5.00	6.05 ^f	0.16 ^b
13	12	100	2.50	7.34 ^h	0.83 ^c
14	10	85	3.75	4.30 ^d	0.80 ^c
15	10	85	3.75	3.05 ^b	0.05 ^a
16	6	85	3.75	6.65 ^g	0.78 ^c
17	8	70	2.50	6.16 ^f	0.75 ^c
18	10	85	3.75	3.44 ^c	0.62 ^c
19	10	85	3.75	2.52 ^a	2.51 ^d
20	14	85	3.75	3.56 ^c	3.43 ^e

*Nitrogen supplementation on the fourth day, LSO=linseed oil supplement. Values with the same letter in each column are not significantly different

Fig. 2 and **Fig. 3** show the response surface plots of the ARA and EPA produced by *M. alpina* on date waste in SSF as a function of linseed oil, seed age and mass fraction of nitrogen supplement. According to the plots, by increasing the mass fraction of nitrogen source, production of ARA decreased, while linseed oil and seed age were at their optimum levels to improve production. CCD was focused on significant variables only.

According to the results obtained from data analysis, the highest produced mass fraction of ARA was 9.01 mg/g (trial number 5) when inoculation was conducted by young (85 h) seeds on date waste containing linseed oil 10 and nitrogen 1.25 % (by mass) ($p \leq 0.05$). The highest production of EPA of 4.53 mg/g (**Table 4**) was obtained in trial number 4, where date waste contained linseed oil 12 and nitrogen 5 % (by mass) when using young seeds (aged 70 h) ($p \leq 0.05$). It is worth mentioning that the obtained optimized levels of each CCD variable in fatty acid production are consistent with the results obtained using Plackett-Burman design. Between two examined levels of linseed oil in Plackett-Burman design (1 and 10), the higher was the better. Thus, in CCD, 5 levels were selected around 10 (6, 8, 10, 12 and 14) and again the highest was the best. In the case of seed age, between 2 levels (96 and 192) of Plackett-Burman design the lower was the better. Thus, in CCD, 5 levels were selected around 96 (55, 70, 85, 100 and 115) and again the lowest was the best (14). Also, Plackett-Burman design indicated that with supplementation of

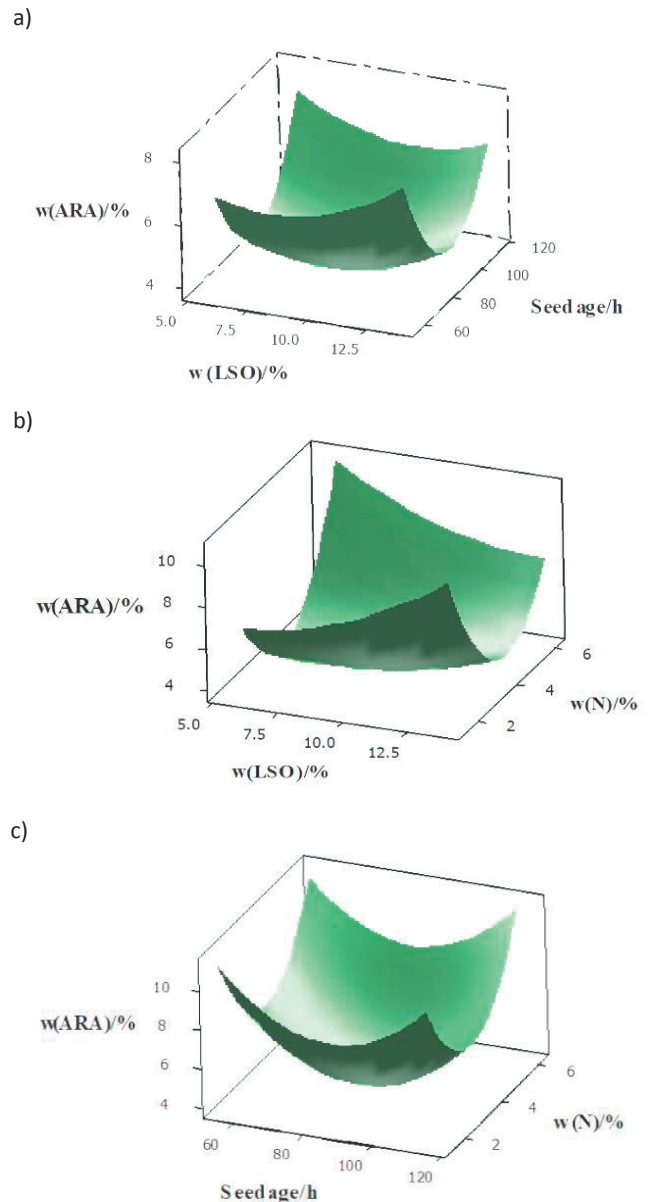


Fig. 2. Impact of main variables (seed age, nitrogen and linseed oil supplementation) and their interaction effects on arachidonic acid (ARA) production by *Mortierella alpina* in solid-state fermentation: a) ARA vs. seed age and linseed oil supplementation, b) ARA vs. nitrogen and linseed oil supplementation, and c) ARA vs. nitrogen supplementation and seed age

nitrogen (4 %) the production of both fatty acids increased, so CCD levels were adjusted around 4 (1.25, 2.50, 3.75, 5.00 and 6.25). ARA production was induced in the presence of 1.25 % nitrogen, but EPA production still increased with the increase of nitrogen to 6.25 %. Greater seed age led to low production of both fatty acids, as shown in **Fig. 2-Fig. 4**.

Fakas *et al.* (28) compared PUFA production by young and aged mycelia and showed that young are rich in polar lipids (containing almost 40 %, by mass, PUFAs), which decreased to less than 30 % by mass in aged mycelia, while neutral lipid

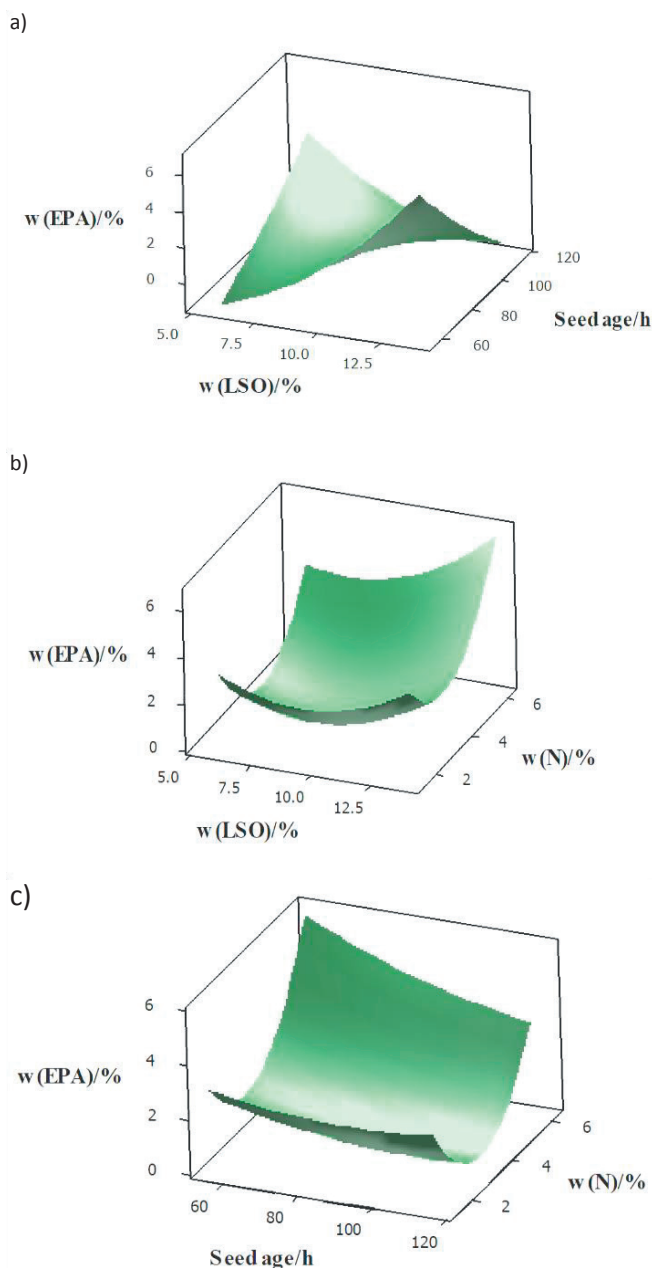


Fig. 3. Impact of main variables (seed age, nitrogen and linseed oil supplementation) and their interaction effects on eicosapentaenoic acid (EPA) production by *Mortierella alpina* in solid-state fermentation: a) EPA vs. seed age and linseed oil supplementation, b) EPA vs. nitrogen and linseed oil supplementation, and c) EPA vs. nitrogen supplementation and seed age

content increased in aged mycelia. They also report slight fluctuation of PUFA content in neutral lipids with age and finally indicate that PUFA biosynthesis is favoured in young, fast growing mycelia, while it decreases significantly in aged mycelia.

The coefficient of regression (R^2) for fatty acid production during 6 days of fermentation was 0.93. Analysis of variance of the stepwise regression showed a first order polynomial model that fits the data well. Also, in the residual plots a trend in the distribution of the residuals around the zero line

(means the goodness of fit) was not observed. The value of R^2 showed good agreement between experimental and predicted values. The F ratio for the lack-of-fit of the model for fatty acid production during 6 days of fermentation was not significant ($p>0.05$). The polynomial mathematical model is as follows:

$$Y(\text{fatty acid})=1.22+0.655w(\text{N})+0.346w(\text{linseed oil})+0.00440t(\text{mycelial incubation}) \quad /1/$$

Validation of data

Table 4 shows CCD used to study the impact of three selected variables and their levels (based on $\alpha=-1.34, -1, 0, +1$ and $+1.34$) on ARA and EPA production by *M. alpina* in solid-state fermentation. The results indicated that optimum conditions for ARA production may be achieved by supplementation of linseed oil and nitrogen source at 14.0 and 1.25 % (by mass) respectively and seed aged for 55 h.

The production of ARA and EPA under the optimized conditions (with the addition of optimum amounts of linseed oil and nitrogen at optimum seed age) was examined during 6 days of fermentation. The results showed that the yield of ARA and EPA after 6 days of fermentation reached (14.37 ± 0.06) and (11.35 ± 0.09) mg/g, respectively. It is worth mentioning that a control sample (date waste) was tested in order to validate the optimized conditions. The control sample was inoculated with a similar amount of fungi and almost 0.1 mg/g of ARA and EPA were produced after 6 days of fermentation.

The comparison of the obtained results with other reports showed that there is a variety of reports on PUFA production on different solid substrates. Total final lipid mass fraction reached 47.9 mg from 1 g of soybean hull, where fatty acid content consisted of 30.0 % of total lipids, and 80.4 % of total fatty acids were C16 and C18 (34). It can be concluded that 11.28 mg of each C16 and C18 are produced from 1 g of soybean hull. Also, in another report (18), arachidonic acid production by *M. alpina* CBS 754.68 in SSF reached 1.14 mg/g. *M. isabellina* produced the maximum GLA mass fraction of 2.9 mg per g of dry biomass in SSF (28). In SSF on rice bran *M. alpina* produced (in mg/g): total PUFAs 127, EPA 12, AA 6, arachidonic acid 6, ALA 5 and linoleic acid 117 after 12 days of incubation (11,16). When *Microsphaeropsis* sp. was cultured in pretreated cellulose containing wheat straw and wheat bran, SCO reached 74 mg/g and the SCO content of the dry fermented mass was 10.2 % (7). *M. alpina* and *M. basiparvispora* produced 6.4 and 5.8 mg of EPA, respectively, per g of sunflower press cake (19). Also in a semi-solid-state fermentation of *Mortierella isabellina* on sweet sorghum, oil production reached 11 g per 100 g of dry mass of substrate (35). To date, the maximum yield of PUFAs in SSF has been reported to be (103 ± 5.2) mg/g using *M. alpina* ATCC 32222 on rice bran, where ARA and EPA reached (33.35 ± 2.13) and (12.77 ± 1.61) , respectively (13).

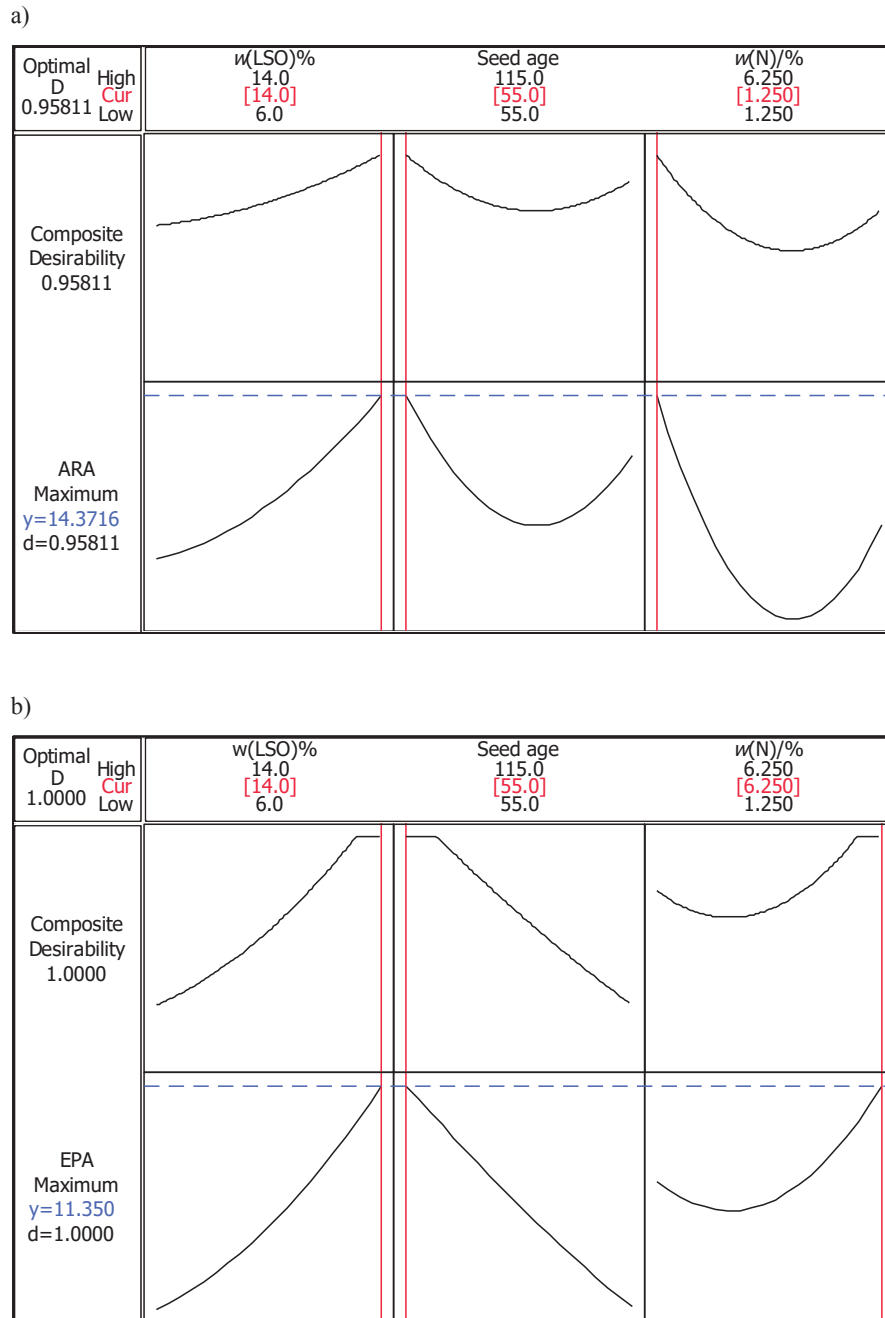


Fig. 4. Influence of optimum conditions and levels of main variables (seed age, nitrogen and linseed oil supplementation) on: a) arachidonic acid (ARA), and b) eicosapentaenoic acid (EPA) production by *Mortierella alpina* in solid-state fermentation

CONCLUSIONS

In this research, substrate initial moisture and the addition of linseed oil and nitrogen on the fourth day had a positive effect, while seed age had a negative effect on the production of arachidonic (ARA) and eicosapentaenoic acids (EPA). Particle size of the substrate had a significantly positive effect on ARA production by *Mortierella alpina*. Duration of heating pretreatment had a negative effect only on EPA production. In the selected range of variables, substrate initial moisture content of 70-75 %, addition of 10 % (by mass) of linseed oil and

4 % (by mass) nitrogen, particle size of the substrate of 1.2-1.7 mm and seed age of 96 h had high impact on the increase of ARA yield. Also, the addition of 10 % (by mass) linseed oil and 4 % (by mass) nitrogen supplement, seed age of 96 h, substrate initial moisture content of 70-75 % and 20 min of heating pretreatment had a high impact on the increased production of EPA. The highest values of produced ARA and EPA in Plackett-Burman design using *M. alpina* were 4.66 and 1.86 mg per g of dry fermented substrate, respectively (experiment 11). When conditions were optimized with response surface

methodology, ARA and EPA production reached (14.37 ± 0.06) and (11.35 ± 0.09) mg/g, respectively, after 6 days of fermentation.

However, for optimum production of EPA, supplementation of nitrogen source needs to be 6.25 % (by mass), while other conditions are similar. Therefore, it can be concluded that if the aim is simultaneous production of both fatty acids, a two-phase fermentation is required. All conditions are similar for both fatty acids, while a change in nitrogen supplementation from 1.25 to 6.25 leads to shift of production from ARA to EPA. It can be concluded that high levels of N supplementation supported the EPA production. This is most likely due to the role of components of this nitrogen source. It is obvious that developing the industry of converting date waste into worthy materials provides a great help for solving the problem of fatty acid deficiency.

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