

Determination of Effective Variables on Arachidonic Acid Production by *Mortierella alpina* CBS 754.68 in Solid-State Fermentation using Plackett-Burman Screening Design

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Abstract—In the present study, the oleaginous fungus *Mortierella alpina* CBS 754.68 was screened for arachidonic acid production using inexpensive agricultural by-products as substrate. Four oilcakes were analysed to choose the best substrate among them. Sunflower oilcake was the most effective substrate for ARA production followed by soybean, colza and olive oilcakes. In the next step, seven variables including substrate particle size, moisture content, time, temperature, yeast extract supply, glucose supply and glutamate supply were surveyed and effective variables for ARA production were determined using a Plackett-Burman screening design. Analysis results showed that time (12 days), substrate particle size (1-1.4 mm) and temperature (20°C) were the most effective variables for the highest level of ARA production respectively.

Keywords—Arachidonic acid, *Mortierella alpina*, Solid-state fermentation, Plackett-Burman design

I. INTRODUCTION

ARACHIDONIC acid (ARA) is an omega-6 fatty acid 20:4(ω -6) that has some important roles in human, especially infants. Omega 6 is one of the two types of fats that are essential to stay healthy. The other type of nutritious fat is the Omega 3 fatty acids. It is the fat that the body uses to synthesize regulatory molecules such as prostaglandins (hormone like chemical messenger) and thromboxanes (involved in platelet aggregation and blood clotting). One of the major sources of polyunsaturated fatty acids are microorganisms, because oils and fats from animal sources have some problems like bad taste and odor and cholesterol supply [1], so PUFAs produced by microorganisms are more desirable to be used as food additives [2]. Submerged cultures of some microorganisms, including some *Mortierella* species like *M. alpina*, *M. isabellina* and *M. alliacea*, *Thamnidium elegans*, some *Cunninghamella* species like *C. elegans* and *C. echinulata*, *Mucor rouxii*, *Saccharomyces servisiae* and some other microorganisms were used to produce PUFAs. [3-10]. Among them, *Mortierella alpina* showed that is the best microorganism to produce some PUFAs like ARA. The submerged cultures of *Mortierella* are usually used for PUFA production with glucose or glycerol supplementation as the carbon source [11].

Many researches have been done using this microorganism to produce PUFAs like Arachidonic and Linolenic acid and EPA in submerged fermentation. Although it is easier to use submerged fermentation, a submerged culture has some deficiencies. It demands high energy and its wastes are more than a solid-state culture [1].

To be economically competitive, the production of PUFAs should be able to be performed at the rural level. Solid-state fermentation (SSF) can achieve this purpose, cause it reduces the cost of growing microorganisms, also it has high product yields and low wastewater output [1].

Some microorganisms have shown the ability of production of PUFAs in solid substrates. *Thamnidium elegans* can produce γ -Linolenic acid when it is grown on cereals [6]. Also, *Geotrichum candidum*, *Cunninghamella elegans*, *Mucor rouxii* and some other microorganisms can produce this fatty acid in solid state fermentation [7, 9, 12]. *Pythium ultimum* is able to produce ARA and EPA by growing on solid substrates [13].

Agricultural by-products are suitable media for PUFA production. Jang *et al.* (2000) established an experiment on some cereal brans and concluded that rice bran is the most suitable substrate for production of ARA by *M. alpina*. Production of other microbial lipids, like γ -Linolenic acid by *M. alpina* on cereals also has been surveyed [2]. Oilcakes are abundant inexpensive by-products of oil and fat factories which can be used for PUFA, especially ARA production.

In this study, four oilcakes, include sunflower, soybean, colza and olive oilcakes were investigated to choose the best substrate for ARA production, then effective variables for ARA production on the chosen substrate were determined using plackett-Burman screening design.

II. MATERIALS AND METHODS

A. Solid Substrate

Oilcakes were purchased from local oil factories. Initial ARA content of Sunflower, Soybean, colza and olive oilcakes were 0.00, 0.02, 0.014 and 0.00 mg/g of oil cake dry weight respectively and moisture content of them was 0.1, 0.1, 0.09 and 0.08 wet bases respectively.

B. Media and Culture Conditions

Mortierella alpina CBS 754.68 was purchased from the Centraalbureau Schimmelcultures (CBS, Netherlands). It was grown on slants and then plates of Malt agar at 20°C to obtain

suitable cultures ready for spore production. For obtaining spores, the fungus was grown for 17 days on Czapek-Dox agar plates at 28°C. Then, each plate has been washed with 3 milliliter of PFS solution and then was filtered to obtain pure spore suspension, free from mycelium. The pure spore suspension was mixed with 23% v/v of glycerol and was kept in 1.5 ml microtubes at -20°C freezer. Each microtube contained 1 ml of spore suspension including 10⁵ CFUs.

The first step of solid-state fermentation for selecting the best substrate was carried out in 500 ml Erlenmeyer. Each Erlenmeyer contained 10g of oilcake. Substrates were hydrolysed for 30 minutes in 121°C. This treat is necessary for the substrates and causes their nutrients to be released and available for consumption by fungus [11]. Moisture content was adjusted in a way that there was not any free water in each substrate, so it was 50%, 50%, 50% and 10% wet basis for sunflower, soybean, colza and olive oilcakes respectively. Each Erlenmeyer was inoculated with 1 ml of spore suspension and was kept in 20°C for 6 days, then in 12°C for 6 other days, according to Jang *et al.*, 2000. Decreasing the temperature after six days causes the higher levels of PUFAs, especially ARA production by *M. alpina* (Jang *et al.*, 2000). Finally, after this 12 day incubation, fatty acid analysis was carried out on the fermented oilcakes using gas chromatography to find the best substrate.

In the next step, experiments were carried out on the selected oilcake using a Plackett-Burman design at two levels, high (+) and low (-) for each variable. The design includes seven variables which carried out in 8 runs in a Plackett-Burman screening design. Factors studied include substrate particle size (A), moisture content (B), time (C), temperature (D), yeast extract supply (E), glucose supply (F) and glutamate supply (G). All the experiments were carried out triplicate in 500 ml Erlenmeyer contained 10g of substrate. Final data was the mean of the triplicate data. High and low levels of variables have been shown at Table I. Statistical analysis was carried out with Minitab 13 software.

TABLE I
EXPERIMENTAL VARIABLES AT DIFFERENT LEVELS USED FOR PLACKETT-BURMAN DESIGN.

Variables	Units	Experimental values	
		Lower level (-)	Higher level (+)
Substrate particle size	mm	0.2-0.6	1-1.4
Moisture content	%	50	70
time	day	2	6
temperature	°C	12	20
Yeast extract supply	g/l	0	10
Glucose supply	g/l	0	20
Glutamate supply	g/l	0	1

C. Lipid Extraction and Analysis

Produced lipid was extracted using n-hexane as the solvent. 3ml of n-hexane added to 1g of dried fermented substrate, then exposed to ultrasound waves in an ultrasonic bath for 3 hours to optimize the lipid extraction. N-hexane was then evaporated using nitrogen injection.

5 ml of 2% NaOH/methanol solution was added to the extracted lipids solution and then they were methylated by

2.175 ml of BF₃. Adding saturated NaCl and dissolving in n-hexane caused the separation of methylated fatty acids from aqueous layer [14]. PUFA content was determined by gas chromatography UNICAM 4600 (United Kingdom) which is equipped with capillary BPX70 column (30 m × 0.25 mm i.d., 0.25 mm film thickness; SGE, USA) and flame ionization detector. Nitrogen was the carrier gas under the pressure of 20 kPa. The injector and detector temperatures were maintained at 250°C and 300°C, respectively. The injection volume was 0.2 µL, with the split ratio of 50:1. Pentadecaenoic acid (15:0) was used as the internal standard.

III. RESULTS AND DISCUSSION

The first stage analysis showed that sunflower oilcake is the best substrate to produce ARA among other oilcakes, followed by soybean, colza and olive oilcakes (Table II). It might be because of fatty acid composition of sunflower oilcake that contains more than 90% of unsaturated fatty acids including oleic and linoleic acids. Substrates containing high levels of PUFAs, stimulate the ARA production by *M. alpina* [5].

TABLE II
ARACHIDONIC ACID CONTENT OF VARIOUS OILCAKES

Oilcake	Arachidonic acid content (mg/g of dry weight)
Sunflower	1.14
Soybean	0.53
Colza	0.38
Olive	0.066

IV. CONCLUSION

Oilcakes are suitable substrates to produce arachidonic acid by oleaginous fungi like *Mortierella alpina*. By growing of this fungus on various oilcakes, it was observed that sunflower oilcake is the best substrate to produce ARA. Time, substrate particle size and temperature are important variables that affected ARA production by *M. alpina*.

It is recommended that the study in high-volume solid-state reactors to examine whether the process is suitable for mass production of ARA using *M. alpina*. Also, there are many other variables that might affect ARA production by this microorganism, more researches on them might cause to achieve higher levels of ARA production by this fungus on solid substrates.

In the next step, the importance of the seven selected variables for ARA production including substrate particle size, moisture content, time, temperature, yeast extract supply, glucose supply and glutamate supply was investigated using Plackett-Burman design. The factors evidencing the *P*-values of less than 0.05 were considered to have significant effects on the response. Table III shows the ;

TABLE III
PLACKETT-BURMAN EXPERIMENTAL DESIGN AND RESULTS (MG OF PRODUCED
ARA/G OF DRIED SUBSTRATE WEIGHT)

Experiment	A	B	C	D	E	F	G	Y
1	+	-	+	-	-	+	+	3.93
2	+	+	-	-	+	-	+	2.81
3	+	+	-	+	-	+	-	3.11
4	-	+	+	+	-	-	+	3.36
5	+	-	+	+	+	-	-	4.18
6	-	+	+	-	+	+	-	2.65
7	-	-	-	+	+	+	+	2.43
8	-	-	-	-	-	-	-	1.64

Analysis results showed that there is significant positive effect for time, substrate particle size and temperature respectively. Figure 1 shows the Pareto chart of the effects for experimental variables. Other variables didn't show to have a significant effect on ARA production. 12 days incubation at 20°C causes to produce more amounts of ARA by *M. alpina*, while the best substrate particle size is in the limit of 1-1.4 mm, probably because of formation of hard stiff cakes that inhibits suitable aeration and growth of the fungus by using the lower substrate particle size. So such a SSF process is an appropriate inexpensive way to produce ARA which is suitable as dieteticum and probioteticum for human [15].

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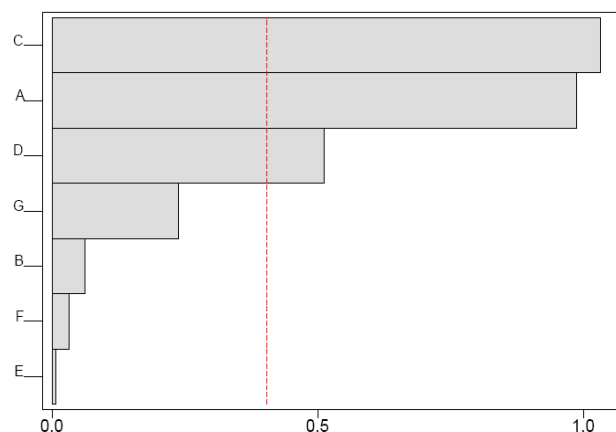


Fig. 1 Pareto chart of the effects for experimental variables. Alpha is 0.05

REFERENCES

- [1] H. D. Jang, S. S. Yang. Polyunsaturated fatty acids production with a solid-state column reactor. *Bioresource Technol.* 2008, 99: 6181-6189.
- [2] M. H. Cheng, T. H. Walker, G. J. Hulbert and D. R. Raman. Fungal production of eicosapentaenoic acid and arachidonic acids from industrial waste streams and crude soybean oil. *Bioresource Technol.* 1999, 67: 101-110.
- [3] S. Papanilolaou, M. Komaitis and G. Aggelis. Single cell oil (SCO) production by *Mortierella isabellina* grown on high-sugar content media. *Bioresource Technol.* 2004, 95: 287-291
- [4] T. Aki, Y. Nagahata, K. Ishihara, Y. Tanaka, T. Morinaha, K. Higashiyama, K. Akimoto, S. Fujikawa, S. Kawamoto, S. Shigeta, K. ono and O. Suzuki. Production of arachidonic acid by filamentous fungus, *Mortierella alliacea* strain YN-15. *J Amer Oil Chem Soc.* 2001, 78(6): 599-604
- [5] H. D. Jang, Y. Y. Lin and S. S. Yang. Polyunsaturated fatty acid production with *Mortierella alpina* by solid substrate fermentation. *Bot Bull Acad Sin.* 2000, 41: 41-48.
- [6] M. Certik, L. Slavikova and J. Sajbidor. Enhancement of nutritional value of cereals with γ -linolenic acid by fungal solid state fermentation. *Food Technol Biotechnol.* 2006, 44(1): 75-82
- [7] E. Conti, M. Stredansky, S. Stredanska and F. Zanetti. γ -Linolenic acid production by solid-state fermentation of *Mucorales* strains on cereals. *Bioresource Technol.* 2001, 76: 283-286.
- [8] H. Gema, A. Cavadia, D. Dimou, V. Tsagou, M. Komaitis and G. Aggelis. Production of γ -linolenic acid by *Cunninghamella echinulata* cultivated on glucose and orange peel. *Appl Microbiol Biotechnol.* 2002, 58: 303-307
- [9] P. Jangbua, K. Laoteng, P. Kitsubun, M. Nopparatana and A. Tongata. Gamma-linolenic acid production of *Mucor rouxii* by solid-state fermentation using agricultural by-products. *Lett Appl Microbiol.* 2009, 49(1): 91-97
- [10] M. Veen, C. lang. Production of lipid compounds in the yeast *Saccharomyces servisiae*. *Appl Microbiol Biotechnol.* 2004, 63: 635-646
- [11] M. Zhu, L.-J. Yu and Y.-X. Wu. An inexpensive medium for production of arachidonic acid by *Mortierella alpina*. *J Ind Microbiol Biotechnol.* 2003, 30: 75-79.
- [12] P. B. L. Fregolente, L. V. Fregolente, M. R. W. Maciel and P. O. Carvalho. Screening of microbial lipases and evaluation of their potential to produce glycerides with high gamma linolenic acid concentration. *Braz J Microbiol.* 2009, 40(4): 747-756
- [13] M. Stradansky, E. Conti and A. Salaris. Production of polyunsaturated fatty acids by *Pythium ultimum* in solid-state cultivation. *Enzyme Microb Technol.* 2000, 26: 304-307
- [14] L. C. Metcalf, A. A. Schmitz, and J. R. Pelka. Rapid preparation of methyl esters from lipid for gas chromatography analysis. *Anal Chem.* 1996, 38: 514-518.
- [15] S. Stredanska, D. Slugen, M. Stredansky and J. Grego. Arachidonic acid production by *Mortierella alpina* grown on solid substrates. *World J Microbiol Biotechnol.* 1993, 9: 511-513.