International Journal of ChemTech Research
CODEN (USA): IJCRGG ISSN: 0974-4290
Vol.9, No.04 pp 547-554, 2016

Statistical optimization of polyunsaturated fatty acids production by *Mucor plumbeus* in submerged fermentation

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Abstract: In the present study, The production of polyunsaturated fatty acids (Linoleic, γ-linolenic and α-linolenic) was experimented in 15 fungal isolates. The results showed that only 6 isolates could produce linoleic, γ-linolenic and α-linolenic together namely, *Mucor plumbeus* (2 isolates), *Aspergillus peniciloides* (2 isolates), *Aspergillus niger, Penicillium funiculosum*. *Mucor plumbeus* (isolates No. 4 and 5) produced the highest amount of total PUFA reaching (5.38, 4.19 g/l) respectively proceeded by *Aspergillus sp* produced amount of PUFA vary between (1.21-1.62 g/l) whereas, *P.funiculosum* resulted 1.03 g/l of PUFA. The effect of environmental factors on the production of polyunsaturated fatty acids by *Mucor plumbeus* (isolate No. 4) was studied using the statistics test Response Surface Methodology (RSM). The optimum conditions for the production of polyunsaturated fatty acids by submerged culture were achieved using broth medium containing 60 g/l of glucose as a sole source of carbon and 7 g/l of yeast extract as a sole source of nitrogen. The initial pH was 7 during an incubation period which lasted 5 days at 20°C. Applying the optimum conditions obtained 10.24 g/l PUFA comparing with 5.38 g/l before applying them which indicate that the total PUFA increased about approximately 90%.

Keywords: polyunsaturated fatty acids; Response surface methodology (RSM); *Mucor plumbeus.*

Introduction

Polyunsaturated fatty acids (PUFA) have a unique biological activities[1,2] and clinical effects[3,4]. Nutritional studies have indicated their potential benefits to human health[5,6]. Marine fish is the traditional source of PUFA. However, marine resources are not sustainable due to limited fishing seasons, geographical locations and declining fish populations[7]. Moreover, the fish oil concentrates contain cholesterol and have unpleasant odour. Therefore, there is a need for alternative sources of PUFA[8].

The production of PUFA by microbial fermentation has been shown to be an ideal alternative owing to its amicability for the separation, purification, and industrialization[9]. Many microorganisms have an ability to store lipophilic substances with unusual compositions, including fungi, marine algae, diatoms, and some bacteria, are some potential sources[10,11]. Microorganisms have several advantages over plants such as high growth rate, simple cultural conditions, no requirement for sunlight and consistency of the product[12,13].

For efficient microbial PUFA production, it is critical to obtain a microbial strain with both high biomass and high PUFA content. Lipids from filamentous fungi have an unusual fatty acid composition, which is of commercial importance[12]. Recent research has focused mainly on maximizing PUFA yield by genetic manipulation as well as by medium optimization[14,15].

Several studies on fungi have shown that media variables affect their growth and lipid accumulation[13,16,17,18,19]. Studies of *Mortierella ramanniana var. ramanniana* indicated that PUFA production could be maximized by using a basal growth medium consisting of 5% dextrose and 1% yeast extract, supplemented with Mn^{2+} [20]. Another Study of *Mucor rouxii* showed that the optimum medium compositions were determined to be carbon source 30 g/L, nitrogen source 5 g/L, pH 5.5, temperature 30°C. Under the conditions, the biomass and Cellular lipid content concentration reached to the highest level[21]. In this study, We identified the optimum fermentation conditions from *Mucor plumbeus* as well as medium composition for maximal PUFA production.

Materials and Methods

Organisms

Fungus cultures isolated from Syrian soils were used in the present study. Soil samples were collected from different places in Damascus and its countryside and they were serially diluted in sterile saline solutions. A 100 µl aliquot of the final dilution was spread over potato dextrose agar (PDA) plates. The plates were incubated at room temperature for 8 days, then the isolates obtained were subcultured and purified on PDA plates for identification.

Media and cultivation conditions

The basal fermentation medium was used in this study included Glucose (30 g/l), yeast extract (5 g/l) and The initial pH of the fermentation medium was to 5.5 prior to autoclaving at 121°C for 15 min[22]. The fermentation medium in each flask was inoculated with a 4% (v/v) mycelium suspension of the seed culture.

Fatty acids methyl esters preparation

Four Reagents were used for this stage. Reagent 1 (45 g sodium hydroxide, 150 ml methanol, and 150 ml distilled water) for saponification, Reagent 2, (325 ml certified 6.0 N hydrochloric acid and 275 ml methyl alcohol) for methylation, Reagent 3 (200 ml hexane and 200 ml methyl tert-butyl ether) for extraction, Reagent 4, (10.8 g sodium hydroxide dissolved in 900 ml distilled water) for sample cleanup. 0.1 g of dry mycelium is placed in a clean tube. 3 ml of Reagent 1 is added to each tube containing biomass and heated in a boiling water bath for 5 minutes, at which time the tubes are vigorously vortexed for 5-10 seconds and returned to the water bath to complete the 30 minute heating. 4 ml of Reagent 2 is added for the cooled tubes. The tubes are briefly vortexed. Then, they are heated for 10 minutes at 80°C. 4 ml of Reagent 3 is added to the cooled tubes is followed gentle tumbling on a clinical rotator for about 10 minutes. The tubes are uncapped and the aqueous (lower) phase is pipetted out and discarded. Finally, About 7 ml of Reagent 4 is added to the organic phase remaining in the tubes, the tubes are tumbled for 5 minutes. The organic phase is pipetted into a GC vial for analysis[23].

Gas Chromotography settings

The gas chromatograph GC-17A (Shimadzu Co., Japan) was equipped with a fused silica capillary column (30 m×0.25 mm i.d.×0.25 m film thickness) and a flame ionization detector. The injector and detector temperatures were maintained at 250 and 260°C, respectively. The oven was programmed on 185°C for 35 minutes. The carrier gas, Helium, was used at a flow rate of 1.5 mL/min. The injection volume was 1 L with a split ratio of 60:1. Methyl esters of Linoleic, α-Linolenic, γ-Linolenic were used as standards for fatty acid identification and quantitation. Total fatty acids (production was calculated from the total peak areas of the chromatogram relative to the peak area of an internal standard[24].

Experimental design and data analysis

In order to select the significant variables for PUFA production, the independent variables of glucose concentration, yeast extract concentration, temperature, pH and fermentation time were considered and determine the optimum levels of the most significant independent variables for PUFA 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 2. All the variables were taken at a central coded value (considered as zero).

This design contains 54 treatments, experimental results of total PUFA production by a complete 5-factor, with 5 replications of the central point. The parameters of Eq. (1) were determined by multiple regression analysis, with the RSM method. The second-order polynomial regression equation, which shows the relationship between total PUFA concetration (Y) and 5 test variables in coded units, is represented by Eq:

$$Y= a + bX_1 + cX_2 + dX_3 + eX_4 + fX_5 + gX_1^2 + hX_2^2 + iX_3^2 + jX_4^2 + kX_5^2 + lX_1X_2 + mX_1X_3 + nX_1X_4 + oX_1X_5 + pX_2X_3 + qX_2X_4 + rX_2X_5 + sX_3X_4 + tX_3X_5 + uX_4X_5$$

Y: response
a: constant
b, c, d, e, f: linear coefficient
g, h, i, g, k: square coefficient
l, m, n, o, p, q, r, s, t, u: interaction coefficient

The effect of five independent variables namely, Temperature, (X_1),initial pH (X_2) and glucose concentration (X_3), Yeast extract concentration (X_4) and inocubation time (X_5) on total polyunsaturated fatty acids were studied during experimentation. The results of Linoleic, γ-Linolenic, α-Linolenic and total PUFA yields (g/l) were listed in table (3).

Results and Discussion

Fungal isolates content of polyunsaturated fatty acids

15 fungal isolates were studied in present research which were characterized morphologically by using microscope table (1). The results showed that only 6 isolates could produce linoleic, γ-linolenic, α-linolenic together in their cell composition. Isolate 4 (*Mucor plumbeus*) shows the highest content of total PUFA (5.38 g/l). Whereas, the rest of isolates produced amount of PUFA lower than it. Isolates 2 (*Mucor rasemusus*), 10 (*Penicillium verrucosam*), 12 (*Penicillium digitatum*) could not produce Linoleic acids but only produce GLA. α-linoleic acid (ω-3) was produced by 6 fungi and highest content was produced by *Mucor plumbeus* (3.09 g/l). The optimum culture conditions latter one was defined the in order to raise PUFA concentration to the highest rate.

Table 1: Fungai content of polyunsaturated fatty acids:

No.	Organisms	LA g/l	GLA g/l	ALA g/l	Total PUFA g/l
1	*Mucor rasemusus*	0.33	1.51	-	1.84
2	*Mucor rasemusus*	-	0.1	-	0.1
3	*Mucor plumbeus*	0.39	2.78	-	3.17
4	***Mucor plumbeus***	0.1	2.19	3.09	5.38
5	*Mucor plumbeus*	0.22	1.79	2.18	4.19
6	*Mucor plumbeus*	0.40	2.81	-	3.21
7	*Mucor plumbeus*	0.69	2.95	-	3.64
8	*Mucor plumbeus*	0.40	-	-	0.40
9	*Penicillium funiculosum*	0.06	0.35	0.62	1.03
10	*Penicillium verrucosam*	-	4.1	-	4.1
11	*Penicillium expansum*	0.26	-	2.1	2.36
12	*Penicillium digitatum*	-	5.40	-	5.40
13	*Aspergillus peniciloides*	0.06	0.1	1.36	1.52
14	*Aspergillus peniciloides*	0.06	0.48	0.67	1.21
15	*Aspergillus niger*	0.1	0.32	1.2	1.62

Optimization of the significant variables using RSM:

Table (3) showed the results of total PUFA produced from *Mucor plumbeus* isolate after optimizing medium conditions using the statical program (RSM) as in the adopted statistical design (table 2) that contains

temperature of fermentation medium, pH, glucose concentration, yeast extract concentration and fermentation period.

Table 2: Experimental variables at different levels used for the RSM approach:

Variabals	Symbol	Coded levels				
		-2	**-1**	**0**	**+1**	**+2**
Temperature (°C)	X_1	20	25	30	35	40
pH	X_2	4	5	6	7	8
Glucose (g/l)	X_3	20	40	60	80	100
Yeast extract (g/l)	X_4	0	2.5	5	7.5	10
Time (day)	X_5	5	6	7	8	9

Table 3: Experimental design and results of the central composite design for PUFA concentration:

Run no.	X_1	X_2	X_3	X_4	X_5	LA g/l	GLA g/l	ALA g/l	Total PUFA g/l
1	-1	-1	-1	-1	-1	0.03	1.70	2.39	4.13
2	+1	-1	-1	-1	-1	0.06	2.33	2.66	5.05
3	-1	+1	-1	-1	-1	0.01	1.03	1.33	2.37
4	+1	+1	-1	-1	-1	0.07	1.30	1.30	2.66
5	-1	-1	+1	-1	-1	0.04	1.91	2.88	4.83
6	+1	-1	+1	-1	-1	0.07	1.16	1.37	2.60
7	-1	+1	+1	-1	-1	0.06	2.50	3.27	5.83
8	+1	+1	+1	-1	-1	0.08	1.79	1.82	3.70
9	-1	-1	-1	+1	-1	0.15	3.19	4.53	7.87
10	+1	-1	-1	+1	-1	0.08	1.27	1.39	2.75
11	-1	+1	-1	+1	-1	0.25	2.68	3.68	6.61
12	+1	+1	-1	+1	-1	0.27	1.81	1.54	3.62
13	-1	-1	+1	+1	-1	0.22	2.99	3.59	6.79
14	+1	-1	+1	+1	-1	0.14	1.26	1.54	2.94
15	-1	+1	+1	+1	-1	0.35	2.77	4.03	7.15
16	+1	+1	+1	+1	-1	0.21	0.84	1.10	2.15
17	-1	-1	-1	-1	+1	0.04	1.60	1.78	3.42
18	+1	-1	-1	-1	+1	0.11	3.28	3.42	6.81
19	-1	+1	-1	-1	+1	0.06	2.02	2.91	4.99
20	+1	+1	-1	-1	+1	0.03	1.93	1.29	3.25
21	-1	-1	+1	-1	+1	0.03	1.61	1.59	3.23
22	+1	-1	+1	-1	+1	0.13	2.14	1.97	4.24
23	-1	+1	+1	-1	+1	0.05	1.67	1.83	3.56
24	+1	+1	+1	-1	+1	0.07	2.33	1.68	4.08
25	-1	-1	-1	+1	+1	0.13	1.47	2.06	3.66
26	+1	-1	-1	+1	+1	0.19	0.73	0.63	1.56
27	-1	+1	-1	+1	+1	0.29	1.38	3.05	4.73
28	+1	+1	-1	+1	+1	0.24	1.37	1.29	2.90
29	-1	-1	+1	+1	+1	0.14	1.97	2.28	4.39
30	+1	-1	+1	+1	+1	0.20	0.63	0.59	1.42
31	-1	+1	+1	+1	+1	0.27	3.67	3.59	7.54
32	-1	+1	+1	+1	+1	0.21	1.57	0.77	2.55
33	0	0	0	0	0	0.22	2.84	2.60	5.67
34	0	0	0	0	0	0.16	2.57	3.83	6.56

35	0	0	0	0	0	0.18	3.09	3.23	6.50
36	0	0	0	0	0	0.19	2.86	2.98	6.03
37	0	0	0	0	0	0.20	3.09	3.11	6.41
38	0	0	0	0	0	0.15	1.93	2.31	4.38
39	0	0	0	0	0	0.19	3.07	3.17	6.43
40	0	0	0	0	0	0.23	3.33	3.43	7.00
41	-2	0	0	0	0	0.08	2.45	3.51	6.04
42	+2	0	0	0	0	0.00	0.00	1.12	1.12
43	0	-2	0	0	0	0.18	2.34	2.13	4.65
44	0	+2	0	0	0	0.03	1.11	3.23	4.36
45	0	0	-2	0	0	0.29	1.69	3.51	5.49
46	0	0	+2	0	0	0.13	1.56	1.35	3.04
47	0	0	0	-2	0	0.00	0.01	0.01	0.03
48	0	0	0	+2	0	0.34	1.02	1.24	2.60
49	0	0	0	0	-2	0.20	2.91	2.19	5.31
50	0	0	0	0	+2	0.16	5.18	4.48	9.83
51	0	0	0	0	0	0.20	3.01	3.03	6.23
52	0	0	0	0	0	0.20	2.96	2.96	6.12
53	0	0	0	0	0	0.19	3.13	3.15	6.47
54	0	0	0	0	0	0.17	2.76	2.91	5.83

The results in table (3) clearly showed that there was a difference in total PUFA concentration in all experiments, and the experiment No (50) has the maximum value with total PUFA of 9.83 g/l when the temperature of fermentation, pH, concentration of the substrate (glucose), concentration of yeast extract, incubation period were 30°C, 6, 60 g/l, 5 g/l, 9 days respectively, while the minimal value was in the experiment No (47) that the total PUFA was 0.03 g/l when the temperature of fermentation, pH, concentration of the substrate (glucose), concentration of yeast extract, incubation period were 30°C, 6, 60 g/l, 0 g/l, 7 days respectively.

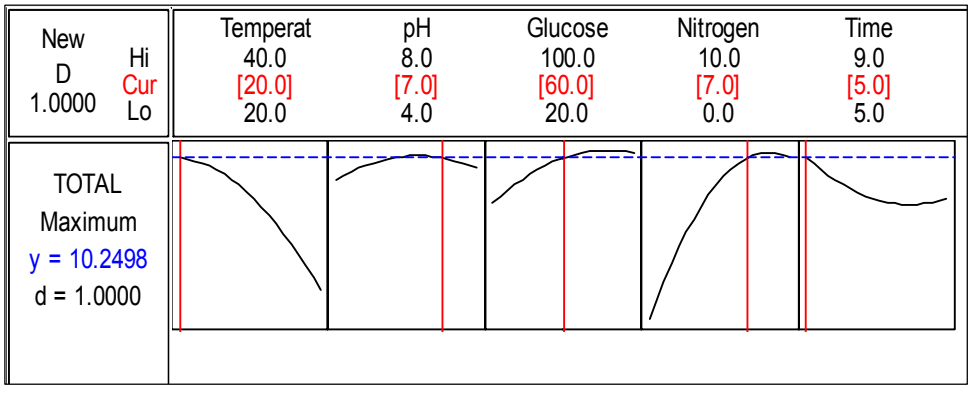

Figure 1: Optimum values of the culture parameters for total PUFA.

The optimum values of variables for production of PUFA were: temperature (20°C); pH 7; glucose concentration (60 g/l); yeast extract concentration (7 g/l) and incubation period (5 days), PUFA yield increased significantly up to 10.25 g/l under the optimum conditions (Figure. 1). Many researches defined the optimum culture conditions to yield the highest PUFA concentration From several fungi species and their results were almost agreed with the many studies21[,25,26,27]. Yang and Chern illustrated that the optimum values of nitrogen and carbon resources were 7.5 g/l and 30 g/l respectively and their results showed that the optimum inucubation period was 4 days to yield the highest concentration of g-linoleic acid and total PUFA from *Mucor rouxii*[21]. Li *et al* showed that 5 days was the optimum fermentation period to accumulate the maximum yields of g-linoleic acid and total PUFA produced from *Mucor recurvus*[25]. Ahmad *et al* demonstrated that *Mucor spp* produced the

maximum yields of g-linoleic acid and total PUFA when the medium pH was 6.5 [26]. Samadlouie *et al* concluded that the best incubation temperature was 21°C to create the maximum concentration of arachidonic acid and total PUFA *Mortierella alpine* CBS 754.68 because it was most suitable temperature for enzymes activity which synthetic PUFA[27].

The strain of *Mucor plumbeus* used in present study could be considered a good source for PUFA especially Linoleic, α-Linolenic and γ-linoleic acids. The yield of PUFA reached 10.24 g/l in the optimum conditions which is considered high value comparing with another organisms. For example, *Mucor recurvus* produced only 4.48 g/l[25]. In addition, total lipids yield was 8.2 g/l in *Mucor* spp[26].

Results of the statistical program RSM for total PUFA production

Table (4) showed the effect of the studied factors separately, the square of factors and the relationship between factors in total PUFA production, P-value o/f each factor, of fermentation temperature, yeast extract (nitrogen) concentration and fermentation period was less than 0.05 ($P<0.05$), therefore there was a significant linear effect of each of these three variables in PUFA production, while the P-value for each of pH, glucose concentration was more than 0.05 ($P>0.05$), while P-value for the effect of square factors of temperature, glucose concentration, yeast extract concentration and the fermentation period was less than 5%. The effect of interrelated factors on PUFA production was observed in table (4) where the correlation between temperature and yeast extract concentration, and between the fermentation period and yeast extract concentration were significant ($P<0.05$), while relationships between the other factors were not significant, and the $R^2 = 80.0\%$, for the regression equation of the five factors affected 80.0% change in PUFA production. The following equation was decuded depending on table (4):

$$Y= 1.510\ X_2 - 8.503\ X_5 - 0.023\ X_1^2 - 0.001\ X_3^2 - 0.183\ X_4^2 + 0.420\ X_5^2 - 0.072\ X_1X_4 - 0.169\ X_4X_5$$

Table 4: Estimated Regression Coefficients for Total PUFA:

Term	Coef	SE Coef	T-value	P-value
Constant	-5.737	20.7202	-0.277	0.784
Temperature	1.510	0.6162	2.45	0.020
pH	2.222	3.081	0.721	0.476
Glucose	0.126	0.1268	0.996	0.327
Nitrogen	4.471	0.9688	4.615	0.000
Time	-8.503	3.3115	-2.568	0.015
Temperature*Temperature	-0.023	0.0078	-2.947	0.006
pH*pH	-0.346	0.1959	-1.766	0.087
Glucose*Glucose	-0.001	0.0005	-2.072	0.046
Nitrogen*Nitrogen	-0.183	0.0313	-5.837	0.000
Time*Time	0.420	0.1959	2.146	0.040
Temperature*pH	-0.043	0.0401	-1.079	0.289
Temperature*Glucose	-0.003	0.002	-1.631	0.113
Temperature*Nitrogen	-0.072	0.016	-4.504	0.000
Temperature*Time	0.071	0.0401	1.778	0.085
pH*Glucose	0.016	0.01	1.597	0.120
pH*Nitrogen	0.121	0.0802	1.519	0.139
pH*Time	0.242	0.2004	1.207	0.236
Glucose*Nitrogen	0.001	0.004	0.287	0.776
Glucose*Time	-0.002	0.01	-0.193	0.848
Nitrogen*Time	-0.169	0.0802	-2.112	0.043

R-Sq = 80.0%

Conclusion

Mucor plumbeus was relatively able to produce high level of polyunsaturated fatty acids (Linoleic; α-Linolenic and γ-linoleic) and the optimum conditions for production of PUFA were achieved on broth medium

containing 60 g/l of glucose as a sole carbon source and 7 g/l of yeast extract as a sole nitrogen source with an initial pH of 7 during incubation period of 5 days at 20°C, these optimum conditions for total PUFA attained 10.24 g/l. There was a significant correlation among the temperature; yeast extract and the fermentation time in PUFA production at 5% confidence level and there was a correlation between the temperature and yeast extract, and between the fermentation time and yeast extract at 5% confidence level.

Acknowledgements

We are grateful to the Department of Food Science, and especial thanks to the technicians in this department.

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