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ORIGINAL PAPER

Production of arachidonic acid and dihomo-γ-linolenic acid from glycerol by oil-producing filamentous fungi, *Mortierella* in the ARS culture collection

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Abstract The filamentous fungi of the genus *Mortierella* are known to produce arachidonic acid from glucose, and the species *alpina* is currently used in industrial production of arachidonic acid in Japan. In anticipation of a large excess of the co-product glycerol from the national biodiesel program, we are trying to find new uses for bioglycerin. We screened 12 *Mortierella* species: *M. alpina* NRRL 6302, *M. claussenii* NRRL 2760, *M. elongata* NRRL 5246, *M. epigama* NRRL 5512, *M. humilis* NRRL 6369, *M. hygrophila* NRRL 2591, *M. minutissima* NRRL 6462, *M. multidivaricata* NRRL 6456, *M. nantahalensis* NRRL 5216, *M. parvispora* NRRL 2941, *M. sepedonioides* NRRL 6425, and *M. zychae* NRRL 2592 for their production of arachidonic acid (AA) and dihomo-γ-linolenic acid (DGLA) from glycerol. With glucose as substrate all of the strains tested produced AA and DGLA. The total fatty acid content of 125 mg/g cell dry weight (CDW) and fatty acid composition for AA (19.63%) and DGLA (5.95%) in the mycelia of *M. alpina* grown on glucose were comparable with those reported by Takeno et al. (Appl Environ Microbiol 71:5124–5128, 2005). With glycerol as substrate all species tested grew on glycerol and produced AA and DGLA except *M. nantahalensis* NRRL 5216, which could not grow on glycerol. The amount of AA and DGLA produced were comparable with those obtained with glucose-grown mycelia. The top five AA producers (mg AA/CDW) from glycerol were in the following order: *M. parvispora* > *M. claussenii* > *M. alpina* > *M. zychae* > *M. minutissima.* The top five dry mycelia weights were: *M. zychae* > *M. epigama* > *M. hygrophila* > *M. humilis* > *M. minutissima.* The top five species for total fatty acids production (mg /g CDW) were: *M. claussenii* > *M. parvispora* > *M. minutissima* > *M. hygrophila* > *M. maltidivaricata*. We selected two species, *M. alpina* and *M. zychae* for further studies with glycerol substrate. Their optimum production conditions were determined. Time course studies showed that the maximum cell growth and AA production for both species were at 6 days of incubation. Therefore, glycerol can be considered for industrial use in the production of AA and DGLA.

Keywords Arachidonic acid · Dihomo-γ-linolenic acid · *Mortierella* · bioglycerin

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Introduction

The United States produces more than 18 billion pounds of soybean oil (SBO) annually with a yearly carryover of more than 300 million pounds. How to utilize this surplus oil effectively becomes a large economic issue in the agricultural community. SBO is a relatively cheap raw material at 2–25 cents per pound and is an attractive candidate for bioindustries. Our laboratories are trying to increase the usage of vegetable oils, focusing on physiologically active fatty acids, or bioactive fatty acids. Recently, production of biodiesel from vegetable oils has become a US national priority program. During the production of biodiesel, a huge amount of co-product glycerol will be produced. To find a new use for this co-product becomes equally important.

Polyunsaturated fatty acids (PUFAs) play important roles as structural components of membrane phospholipids

and as precursors of the eicosanoids of signaling molecules, including prostaglandins, thromboxanes, and leukotrienes [1, 4, 5, 10]. All mammals synthesize eicosanoids that are involved in inflammatory responses, reproductive function, immune responses, and the regulation of blood pressure [3]. The arachidonic acid (AA)-producing fungus, *Mortierella alpine* 1S-4, is used commercially to produce polyunsaturated fatty acids (PUFA) such as dihomo-γ-linolenic acid (DGLA) (n-6 PUFA, Δ8, Δ11, Δ14–20:3), AA (n-6 PUFA; Δ5, Δ8, Δ11, Δ14–20:4), and eicosapentaenoic acid (n-3 PUFA; Δ5, Δ8, Δ11, Δ14, Δ17–20:5) [9, 14]. Although these fatty acids are useful for researchers and consumers, their high cost and relative scarcity limit their application, and more efficient production strains are needed.

In this study, glycerol was tested as a potential substrate for the production of biologically active PUFAs such as AA. We screened all the filamentous fungi of the genus *Mortierella* in the ARS Culture Collection to compare their production of AA and DGLA from glucose and glycerol substrates. Judging from their AA production and yields of CDW, and total fatty acids from glycerol, we selected two strains, *M. alpina* and *M.zychae* for time course studies. We found that glycerol can substitute for glucose as a substrate for the production of these bioactive PUFAs.

Materials and methods

Strains, media, and growth conditions

Twelve *Mortierella* strains representing each species obtained from Dr. Kerry O'Donnell of the ARS Culture Collection, Peoria, IL were: *M. alpina* NRRL 6302, *M. claussenii* NRRL 2760, *M. elongata* NRRL 5246, *M. epigama* NRRL 5512, *M. humilis* NRRL 6369, *M. hygrophila* NRRL 2591, *M. minutissima* NRRL 6462, *M. multidivaricata* NRRL 6456, *M. nantahalensis* NRRL 5216, *M. parvispora* NRRL 2941, *M. sepedonioides* NRRL 6425, and *M. zychae* NRRL 2592. One loopful of mycelium from an agar slant was transferred into 25 mL glucose or glycerol growth medium (GY medium). GY medium contains either 2% (w/v) glucose or 2% glycerol, and 1% yeast extract at pH 6.0. Cultures were grown at 28 °C with reciprocal shaking at120 rpm for 6 days. The mycelia were harvested by suction filtration, washed with 50 mL distilled water and 50 mL ethyl ether, and dried at 120 °C for 3 h.

Chemicals

Arachidonic acid (purity 99%) was purchased from Sigma (St Louis, MO). Diazomethane was prepared from Diazald (Sigma, St Louis, MO). All other chemicals were reagent grade and used without further purification.

Products analyses

The dried cells were directly transmethylated with 10% methanolic HCl at 50 °C for 3 h, and the resultant fatty acid methyl esters were extracted with *n*-hexane, concentrated, and then analyzed by gas–liquid chromatography. Methyl ester derivatives were injected into an Agilent Technologies 6890N Network GC System equipped with a flame-ionization detector, a Supelco (Bellefonte, PA) SPB-1 capillary column (15 m × 0.32 mm inner diameter; 0.25 μm thickness), a 7683 series auto sample injector, and a Chem Station A.10.02 [1757]. The column temperature was kept at 200 °C isothermally. The injection and detector temperatures were 240 and 250 °C, respectively. The relative yield of products was calculated as the ratio of product peak area. GC/MS analyses were performed with a Hewlett-Packard Model 5890 gas chromatograph interfaced with a Model 5971 mass selective detector operating at 70 eV. The capillary column used was a Hewlett-Packard HP-5-MS cross-linked 5% phenyl methyl silicone, 30 m × 0.25 mm inner diameter, film thickness 0.25 μm. The carrier gas was helium and its flow late was 0.65 mL/min. The GC column was programmed from 190 °C for 3 min and then to 220 °C at a rate of 2 °C/min and then to 240 °C at 5 °C/min and then kept at 240 °C for 4 min. Experiments were conducted in triplicates, and the standard deviation was ±5%.

Identification of products

The products were identified by GC co-chromatography and GC/MS. GC co-chromatography of authentic AA and DGLA with the purified corresponding products showed one single peak. The GC/MS mass profile of the products matched well with the authentic AA, DGLA, and γ-linolenic acid.

Results and discussion

Screening of glucose-grown microbes for the production of AA

The cell dry weight, total fatty acids, fatty acids profiles, as well as mg AA produced per g CDW of the 12 glucose-grown *Mortierella* strains were analyzed. The results are shown in Table 1. All of the strains tested produced AA and DGLA. The total fatty acid 125 mg/g CDW and fatty acids composition for AA (19.63%) and DGLA (5.95%) in the mycelia of *M.* alpina grown on glucose were comparable with those reported by Takeno et al. [11, 14]. The top five AA producers (mg AA/g CDW) were in the following order: *M. alpine* (24.16) > *M. zychae* (14.65) > *M. hygrophila* (13.90) > *M. minutissima* (12.74) > *M. parvispora* (12.69). The top five CDW productions were: *M. multidivaricata*

Table 1 Fatty acid composition from *Mortierella* using glucose in the media

Mortierella species	Total fatty acids (mg/g) CDW	Fatty acid composition (%)							AA (mg/g CDW)
		16:0	18:0	18:1	18:2	18:3	20:3	20:4	
alpina NRRL 6302	125	15.00	7.90	29.01	9.07	–	5.95	19.63	24.16
claussenii NRRL 2760	51.28	12.25	4.07	24.42	16.85	–	6.66	14.90	7.64
elongata NRRL 5246	27.62	15.03	6.62	37.76	6.56	0.75	3.33	14.95	4.12
epigama NRRL 5512	80.24	5.44	2.09	18.36	5.52	–	6.15	11.54	9.26
humilis NRRL 6369	70.37	7.04	2.96	24.20	6.47	–	5.30	16.06	11.30
hygrophila NRRL 2591	85.1	12.80	–	24.58	14.04	–	5.84	16.34	13.90
minutissina NRRL 6424	81.73	8.36	3.86	24.21	11.77	–	3.83	15.59	12.74
multidivaricata NRRL 6456	55.1	4.23	1.92	33.10	5.47	0.66	4.65	11.76	6.48
nantahalensis NRRL 5216	43.47	10.15	3.74	23.92	11.07	–	8.14	14.91	6.41
parvispora NRRL 2941	86.66	4.56	1.21	14.48	9.51	–	3.53	14.65	12.69
sepedonioides NRRL 6425	80.21	17.49	4.11	29.16	11.61	3.41	3.06	14.16	11.35
zychae NRRL 2592	84.03	9.50	3.07	24.28	12.81	–	5.18	17.44	14.65

20:3 Dihomo-γ-linolenic acid

20:4 Arachidonic acid

CDW Cell dry weight

(0.49) > *M. parvispora* (0.30) > *M. humilis* (0.27) > *M. zychae* (0.23). And the top five strains for total fatty acids production (mg/g CDW) were: *M. alpina* (125) > *M. parvispora* (86.66) > *M. hygrophila* (85.10) > *M. zychae* (84.03) > *M. minutissima* (81.73) > *M. epigama* (80.24).

Screening of glycerol-grown microbes for the production of AA

In anticipation of a huge production of the co-product glycerol from the national biodiesel program, these 12 strains were grown on glycerol and the mycelia were analyzed for the production of AA and DGLA (Table 2). All the strains tested grew on glycerol and produced AA and DGLA except *M. nantahalensis* NRRL 5216, which could not grow on glycerol. The amount of AA and DGLA produced are comparable with those obtained with glucose-grown mycelia. The top five AA producers (mg AA/CDW) were in the following order: *M. parvispora* (18.16) > *M. claussenii* (15.93) > *M. alpina* (15.0) > *M. zychae* (14.21) > *M. minutissima* (13.04). The top five dry mycelia weights productions were: *M. zychae* (0.346) > *M. epigama*

Table 2 Fatty acid composition from *Mortierella* using glycerol as substrate

Mortierella species	Total fatty acids (mg/g) CDW	Fatty acid composition (%)							AA (mg/g) CDW
		16:0	18:0	18:1	18:2	18:3	20:3	20:4	
alpina NRRL 6302	82.19	9.52	4.18	19.33	13.24	–	7.34	18.25	15.00
claussenii NRRL 2760	101.69	11.97	5.84	26.13	18.02	–	6.59	15.70	15.93
elongata NRRL 5246	58.13	16.52	7.01	25.69	7.96	0.48	4.23	20.13	11.62
epigama NRRL 5512	80	10.63	3.43	16.41	6.45	–	7.03	12.09	9.66
humilis NRRL 6369	74.62	11.43	5.71	25.83	9.20	–	3.56	14.52	10.82
hygrophila NRRL 2591	86.02	10.48	3.61	19.41	8.75	–	4.18	14.49	12.43
minutissina NRRL 6424	94.86	12.39	3.50	21.32	10.86	0.49	4.06	13.75	13.04
multidivaricata NRRL 6456	82.96	6.84	3.09	35.55	6.96	–	3.68	12.27	10.17
nantahalensis NRRL 5216	–	–	–	–	–	–	–	–	
parvispora NRRL 2941	100.59	9.39	2.13	18.04	11.57	–	4.21	18.08	18.16
sepedonioides NRRL 6425	43.47	16.30	6.91	25.05	17.08	1.26	1.49	12.39	5.21
zychae NRRL 2592	78.030	10.38	3.90	23.25	7.77	0.44	5.90	18.24	14.21

20:3. Dihomo-γ-linolenic acid

20:4. Arachidonic acid

CDW Cell dry weight

(0.325) > *M. hygrophila* (0.279) > *M. humilis* (0.268) > *M. minutissima* (0.253). The top five strains for total fatty acids production (mg/g CDW) were: *M. claussenii* (101.69) > *M. parvispora* (100.59) > *M. minutissima* (94.86) > *M. hygrophila* (86.02) > *M. maltidivaricata* (82.96).

Judging from their AA production and yields of CDW, and total fatty acids from glycerol, we selected two strains, *M. alpina* and *M. Zychae* for further studies.

Effect of glycerol concentrations on the production of AA

Different concentrations of glycerol from 0.25 to 15% were used to study AA production by both *M. alpina* and *M. zychae*. The effect of glycerol concentrations on the production of AA by *M. alpina* is shown in Table 3. It appears that the fatty acid composition (%) was not influenced by the substrate concentration. However, substrate concentrations from 0.5 to 4% produced the highest cell dry weights. Higher amount of glycerol inhibit the growth of cells. On *M. zychae*, both the cell growth and AA (%) were higher at glycerol concentrations from 0.5 to 4% (Table 4). Glycerol concentrations greater than 10% slightly inhibited both the growth and the production of AA.

Effect of initial pH

The initial pHs of the media were adjusted with HCl to the pH values from 5 to 7.5. After 6 days of growth, the mycelia were collected and their arachidonic acid content analyzed. Results are shown in Tables 5 and 6. The effect of

Table 3 *Mortierella alpina* NRRL 6302 with different concentration of glycerol

Glycerol in media (%)	CDW (g)	Total fatty acids (mg/g) CDW	Fatty acid composition (%)						
			16:0	18:0	18:1	18:2	18:3	20:3	20:4
0.25	0.075	106.66	10.60	4.92	22.59	11.01	–	5.86	21.50
0.50	0.102	107.84	11.40	4.74	20.72	11.08	1.11	5.14	22.77
1	0.066	106.06	9.39	4.01	21.60	12.03	–	5.73	22.60
2	0.100	120.00	11.49	5.15	22.11	10.85	1.06	5.10	22.02
4	0.095	126.31	9.31	4.60	21.20	12.38	–	5.92	22.35
6	0.038	131.57	8.38	–	25.24	15.48	–	6.67	24.83
10	0.023	130.43	9.02	4.37	30.06	12.28	–	5.37	15.99
15	0.070	171.42	8.06	3.98	27.59	12.93	–	7.40	20.63

20:3 Dihomo-γ-linolenic acid
20:4 Arachidonic acid
CDW Cell dry weight

Table 4 *Mortierella zychae* NRRL 2592 with different concentrations of glycerol

Glycerol in media (%)	CDW (g)	Total fatty acids (mg/g) CDW	Fatty acid composition (%)						
			16:0	18:0	18:1	18:2	18:3	20:3	20:4
0.25	0.067	149.25	10.54	–	20.74	8.38	–	4.86	19.17
0.50	0.102	166.66	12.65	6.70	26.28	7.96	–	5.29	18.95
1	0.179	83.79	17.52	5.17	24.50	8.91	–	3.63	25.14
2	0.151	105.96	18.59	5.41	26.54	8.30	–	3.77	20.65
4	0.156	89.74	14.54	5.21	28.91	6.89	–	3.80	17.40
6	0.085	129.41	10.51	3.61	27.02	9.29	–	5.00	19.83
10	0.092	130.43	10.40	4.90	27.59	7.61	–	4.00	15.05
15	0.054	129.62	11.68	5.91	34.61	9.07	–	3.95	13.05

20:3 Dihomo-γ-linolenic acid
20:4 Arachidonic acid
CDW Cell dry weight

Table 5 *Mortierella alpina* NRRL 6302 grown on glycerol at different pH

Initial pH	CDW (g)	Total fatty acids (mg/g) CDW	Fatty acid composition (%)						
			16:0	18:0	18:1	18:2	18:3	20:3	20:4
5.5	0.080	112	10.02	4.40	21.55	11.46	–	7.66	18.33
6.0	0.099	101	11.23	4.97	22.87	12.03	–	6.01	21.05
6.5	0.080	112	11.17	4.58	23.98	11.90	–	5.64	18.73
7.0	0.080	81.00	8.87	5.29	24.46	7.94	–	5.44	18.38
7.5	0.080	75	11.17	4.35	25.15	10.92	–	5.64	20.33

20:3 Dihomo-γ-linolenic acid
20:4 Arachidonic acid
CDW Cell dry weight

Table 6 *Mortierella zychae* NRRL 2592 grown on glycerol at different pH

Initial pH	CDW (g)	Total fatty acids (mg/g) CDW	Fatty acid composition (%)						
			16:0	18:0	18:1	18:2	18:3	20:3	20:4
5.0	0.155	86.27	19.63	6.92	31.41	6.11	0.53	4.56	16.72
5.5	0.144	76.38	16.90	3.32	31.71	8.78	–	3.74	20.02
6.0	0.209	71.77	17.78	5.00	25.13	8.52	–	3.82	23.38
6.5	0.165	90.90	19.66	6.52	25.92	6.88	–	4.37	20.13
7.0	0.098	81.63	8.87	5.29	24.46	7.94	–	5.44	18.38
7.5	0.107	74.76	8.85	3.74	25.48	8.89	–	5.18	18.75

20:3 Dihomo-γ-linolenic acid
20:4 Arachidonic acid (AA)
CDW Cell dry weight

pH on the growth and mycelium percent AA content was not significant with *M. alpina* within the pH range tested. However, *M. zychae* showed an optimum for both cell growth and the production of AA at around pH 6.0. At pH higher than 7.0, *M. zychae* cells still produced AA; however, their mycelia weight were lower. In lower pH media, the cells grew and produced AA at a slightly reduced value.

Effect of rpm on the production of AA

The effect of shaking speeds on the production of AA were conducted from 80 to 200 rpm using both strain *M. alpina* and *M. zychae*. For strain *M. alpina*, the cell dry weight and the percent AA at 80, 120, 150 and 200 rpm were, CDW: 0.106, 0.136, 0.090, and 0.087 g for AA: 18.79, 19.63, 18.46 and 18.86%, respectively. It appears that 120 rpm is the best shaking speed for both the cell growth and the production of AA. For strain *M. zychae*, the values at 80, 120, 150, and 200 rpm were, CDW: 0.138, 0.175, 0.137, and 0.157 g and for AA: 16.25, 17.99, 14.16 and 15.36%, respectively. Again, the shaking speed of 120 rpm seems is the best for the cell growth and the production of AA for *strain M. zychae.*

Effect of temperature on the production of AA

The effect of temperature on the cell growth and the production of AA for both strains were conducted at temperatures between 15 and 35 °C. There was no cell growth for either strain at 35 °C. For *M. alpina*, the cell dry weight and AA in the percent fatty acid at 15, 20, 25, and 30 °C were, CDW: 0.086, 0.095, 0.115, and 0.092 g and for AA: 19.05, 20.65, 21.43, and 23.31%, respectively. For *M. zychae*, the values at 15, 20, 25, and 30 °C, were CDW: 0.175, 0.194, 0.237, and 0.226 g and for AA: 20.05, 22.81, 21.30 and 18.53%, respectively.

Time course of the production of AA on glycerol substrate

The production of AA over time was conducted with both strains, *M. alpina* and *M. zychae*. The mycelial growth starting from the second day, and the productions of AA are shown in Fig. 1. For *M. zychae*, AA production increases steadily up to 360 h, and reach at 34.53 mg/g dry cell weight. And for *M. alpina*, AA production increases up to 360 h, and reach at 28.68 mg/g dry cell weight. *M. alpina* grows more poorly and produces less AA than *M. zychae* with glycerol as asubstrate.

Shimizu's group studied the fatty acid metabolism in *M. alpina* 1S-4 through comparative analyses of fatty acid composition or the accumulation of derivative mutations [2–4]. They also cloned and sequenced the fatty acid desaturase and elongase genes and analyzed them functionally [6–8]. Wynn et al. [12, 13] identified the rate limiting step in AA production and the NADPH-producing step responsible for fatty acid synthesis through enzymatic analysis. We screened 12 *Mortirella* strains from our ARS Culture Collection for their production of AA and DGLA. They all produced these two important fatty acids from glucose as well as from glycerol. We identified the two best strains, *M. alpina* and *M. zychae* for future studies of their optimal production conditions. The DGLA productions in most cases were about one-fourth–one-third of AA. We also use these optimum production conditions to study their AA production time course from glycerol substrate. *M. alpina* grows more poorly and produces less AA than *M. zychae*

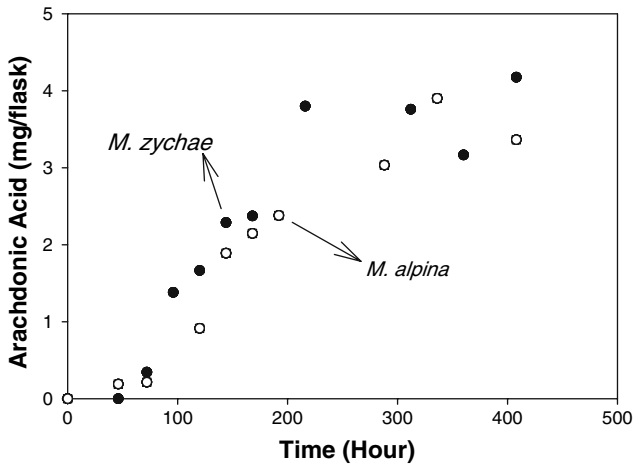

Fig. 1 Time course of AA Production from glycerol by *M. alpina* (*square*) and *M. zychae* (*circle*)

on glycerol. The yields obtained are as good as those with glucose as a substrate, indicating that glycerol can be used to substitute for glucose as an industrial scale production substrate.

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