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Production of arachidonic acid by *Mortierella alpina* ATCC 32222

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SUMMARY

When *Mortierella alpina* ATCC 32222 was incubated in a glucose salts medium at 25 °C the biomass (17.5 g/l) contained 9.62% arachidonic acid which amounted to 54% (w/w) of total biomass lipids. When the glucose concentration in the medium was varied from 0 to 150 g/l, the percentage of arachidonic acid in biomass and in lipids was highest at a glucose concentration of 30 g/l, but highest yield of arachidonic acid per litre of culture broth was observed at a glucose concentration of 100 g/l. While production of biomass reached a plateau of 17 g/l after a 3-day incubation at 25 °C, the percentage of arachidonic acid in lipids and biomass increased dramatically from 3 to 6 days with a concurrent arachidonic acid yield increase from 0.89 to 1.63 g/l. Optimum initial culture pH for arachidonic acid production was in the range 6.0–6.7. By increasing the concentration of the glucose salts medium three-fold, yields of biomass and arachidonic acid were increased to 35.8 g/l and 3.73 g/l, respectively.

INTRODUCTION

Arachidonic acid (ARA; 5,8,11,14-*cis*-eicosatetraenoic acid) is a precursor of prostaglandins, leukotrienes and a large group of C_{20} compounds which are presently the subject of intrinsic medical research [10,14,18,26]. The current preparative source of ARA is from animal liver and adrenal glands and from sardines. However, ARA content per unit dry weight is only 0.2% so alternative sources are being sought. ARA is also found in the cells of ciliated protozoa, amoebae, algae and other micro-organisms [1,4,9,12].

Lower fungi of the Phycomycetes class are a promising source of a variety of polyunsaturated fatty acids [31]. These fungi, especially in the order Mucorales, are usually rich in γ-linolenic acid and *Mortierella ramanniana* and *Mortierella vinacea* produced 390 and 230 mg/l of this acid respectively [15]. A fermentation process for production of γ-linolenic acid has been commercialised [27,28]. Many species of the genus *Mortierella* have been found rich in arachidonic acid (ARA) or eicosapentaenoic acid (EPA), depending on species and culture conditions. *M. alpina* accumulates EPA at 12 °C in the absence of α-linolenic acid precursor but not in the temperature range 20–28 °C, which is optimal for growth [3,23]. At the higher temperatures, *M. alpina* contains a high content of ARA. In contrast, *M. elongata* NRRL 5513 synthesised both ARA and EPA at 25 °C without the precursor α-linolenic acid, which is present in linseed oil [3].

We have found *M. alpina* ATCC 32222 to be an efficient producer of ARA and factors affecting production of ARA and other fatty acids are described in this report.

MATERIALS AND METHODS

Chemicals

Standard fatty acids were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A. Solvents (HPLC grade) and regents (Analar or ACS reagent grade) were procured from Aldrich Chemical Co. Inc., Milwaukee, WI, and BDH Chemicals, Toronto, Ontario. Sugars and other media nutrients were obtained from Difco, Detroit, MI.

Culture conditions

Three different media, with or without modification, were used to investigate ARA production. The GY medium contained (per litre): glucose, 20 g and yeast extract, 10 g. The YM medium [30] consisted of (per litre): glucose, 10 g; polypeptone, 5 g; yeast extract, 3 g; and malt extract, 3 g. HD medium was prepared according to the procedure of Hansson and Dostalek [15] which contained (per litre): glucose, 30.0 g; yeast extract, 5.0 g; KH_2PO_4, 2.4 g; KNO_3, 1.0 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeCl_3 \cdot 6H_2O$, 15 mg; $ZnSO_4 \cdot 7H_2O$, 7.5 mg; and $CuSO_4 \cdot 5H_2O$, 0.5 mg.

M. alpina ATCC 32222 was first grown in Petri dishes

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having GY medium with 3% agar for 72 h at 25 °C. The inoculum was grown in HD medium in Erlenmeyer flasks by incubation for 48 h at 25 °C with orbital shaking at 300 rpm. Then it was inoculated (5% v/v) into 50 ml medium in 250-ml Erlenmeyer flasks and incubated at 25 °C and 11 °C for 6 and 10 days respectively, with orbital shaking at 300 rpm. Yields of arachidonic acid were optimum after these incubation times.

Culture strains were maintained on 3% agar slants containing the GY medium at 4 °C and were transferred every 2 months.

Biomass determinations

Mycelium was harvested from cultures by vacuum filtration or centrifugation of fungal cell suspension. Dry weight of biomass was determined by washing the harvested mycelium with 1% NaCl and distilled water and drying at 100 °C for 12–16 h.

Extraction and determination of lipids

The dried cells were weighed (25–50 mg) in teflon-lined screw cap test tubes of 10 ml capacity and the lipids were extracted according to the procedure of Bligh and Dyer [5]. The extracted lipids were dried at 36 °C under nitrogen atmosphere and then methylated using the method of Holub and Skeaff [17]. Then the fatty acid methyl esters were dissolved in 200 μl *n*-hexane and a 1-μl sample was injected into a gas-liquid chromatograph (GLC) for analysis. The Shimadzu CR601 GLC was connected with GC-14A data integrator. The GLC was fitted with megabore column DB-225 (Chromatographic Specialities, Brockville, Ontario) and a flame ionization detector. Helium was used as the carrier gas. The fatty acid ester peaks were identified and calibrated using standard fatty acids supplied by Sigma Chemical Company, St. Louis, MO. Pentadecanoic acid (C15 : 0) was used as internal standard.

RESULTS

Production of biomass and lipids by *M. alpina* ATCC 32222 was first investigated in three media by incubation at 25 °C for 6 days. The results are presented in Table 1. The HD medium promoted the highest biomass production and this biomass contained the highest content of lipids. In addition, ARA constituted 54.11% (w/w) of total lipids in the biomass recovered from the HD medium amounting to a yield of 1.68 g ARA per litre of culture. Cells of *M. alpina* contain ARA but no eicosapentaenoic acid (EPA) when incubated at 25 °C.

M. alpina ATCC 32222 was also cultivated in the HD medium at 11 °C for 10 days. The data on biomass production and yield and distribution of lipids are presented in Table 2. While the percentage of lipids in biomass increased by incubation at the lower temperature, ARA yield and content of total fatty acids was reduced by 17%

TABLE 1

Effect of medium on arachidonic acid production by *M. alpina* ATCC 32222 at 25 °C

Parameter	Medium		
	GY	YM	HD
Biomass (g/l)	9.27	6.39	17.50
Lipids in biomass (% w/w)	10.37	13.16	17.78
ARA			
(a) in biomass (% w/w)	5.13	2.97	9.62
(b) in lipids (% w/w)	49.48	22.54	54.11
(c) yield (g/l)	0.48	0.19	1.68
Fatty acids (% w/w)			
16 : 0	8.45	13.55	9.01
16 : 1	Trace	Trace	Trace
18 : 0	3.06	8.48	6.77
18 : 1	11.51	28.63	8.31
18 : 2	7.92	7.01	7.30
18 : 3	3.26	8.87	Trace
20 : 3	2.53	2.54	2.48
20 : 4	49.48	22.54	54.11
20 : 5	0.00	0.00	0.00
Others	13.79	8.38	12.02

Data are the average of three replicates.
Incubation time 6 days.

TABLE 2

Arachidonic acid production in HD medium by *M. alpina* ATCC 32222 at 11 °C

Biomass (g/l)	15.15
Lipids in biomass (% w/w)	22.25
ARA	
(a) in biomass (% w/w)	9.22
(b) in lipids (% w/w)	41.45
(c) yield (g/l)	1.40
Fatty acids (% w/w)	
16 : 0	11.92
16 : 1	Trace
18 : 0	9.28
18 : 1	19.17
18 : 2	4.13
18 : 3	0.62
20 : 3	Trace
20 : 4	41.45
20 : 5	1.90
Others	11.53

Data are the average of three replicates.

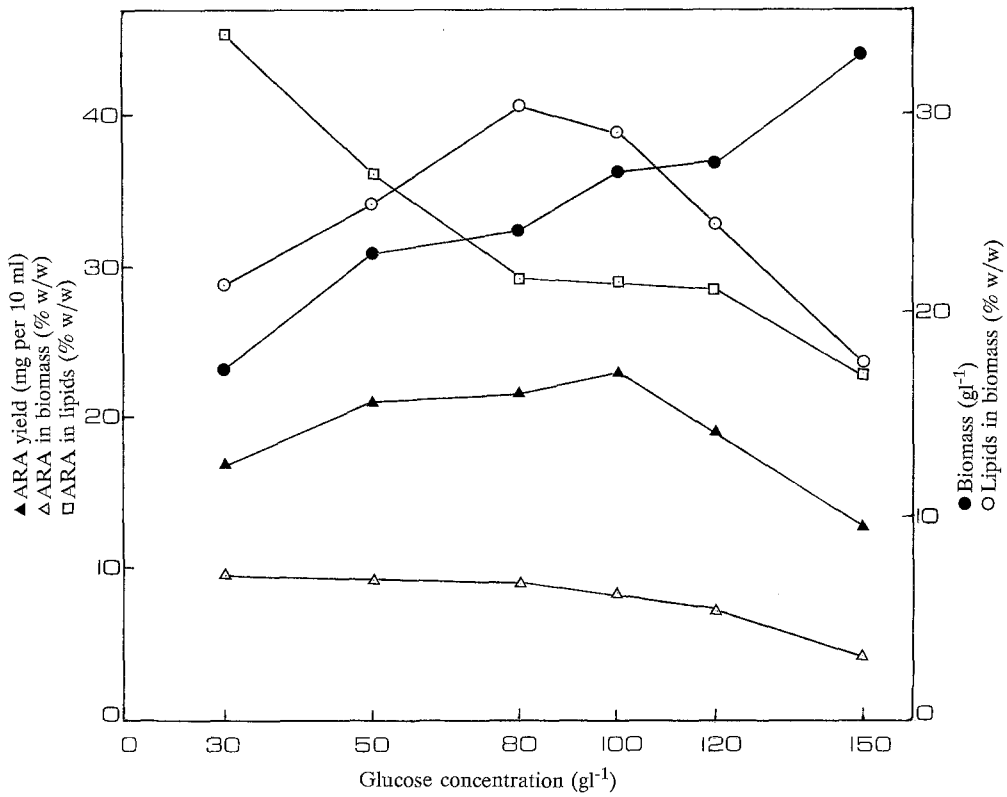

Fig. 1. Effect of glucose concentration on growth and arachidonic acid production by *Mortierella alpina* ATCC 32222. Incubation conditions: HD medium, 25 °C, 6 days.

and 24%, respectively, when compared with the higher temperature incubation.

The initial glucose concentration in the HD medium was varied in the range 30–150 g/l in order to investigate the effect of glucose content in the medium on production of biomass and lipids. The results are presented in Fig. 1. Biomass concentration increased with increasing glucose concentration to a maximum of 33.23 g/l biomass in the medium containing 150 g/l glucose. Lipid content in biomass was optimal (30.19%, w/w) when the medium

TABLE 3

Effect of initial pH on growth and ARA production by *M. alpina* ATCC 32222

pH		Biomass (g/l)	Lipids in biomass (% w/w)	ARA		
Initial	Final			In biomass (% w/w)	In lipids (% w/w)	Yield (g/l)
3.8	6.5	17.10	9.39	2.49	26.47	0.425
4.7	6.7	17.58	7.04	2.63	37.40	0.463
5.5	7.0	17.58	9.92	4.23	42.67	0.744
6.0	7.2	17.50	17.78	9.62	54.11	1.684
6.7	7.6	16.90	18.07	9.43	52.18	1.593
8.0	7.7	16.50	14.59	7.83	53.70	1.292
9.0	7.8	15.93	15.94	7.08	44.43	1.128

Data are the average of three replicates.
Incubation conditions: HD medium, 25 °C, 6 days.

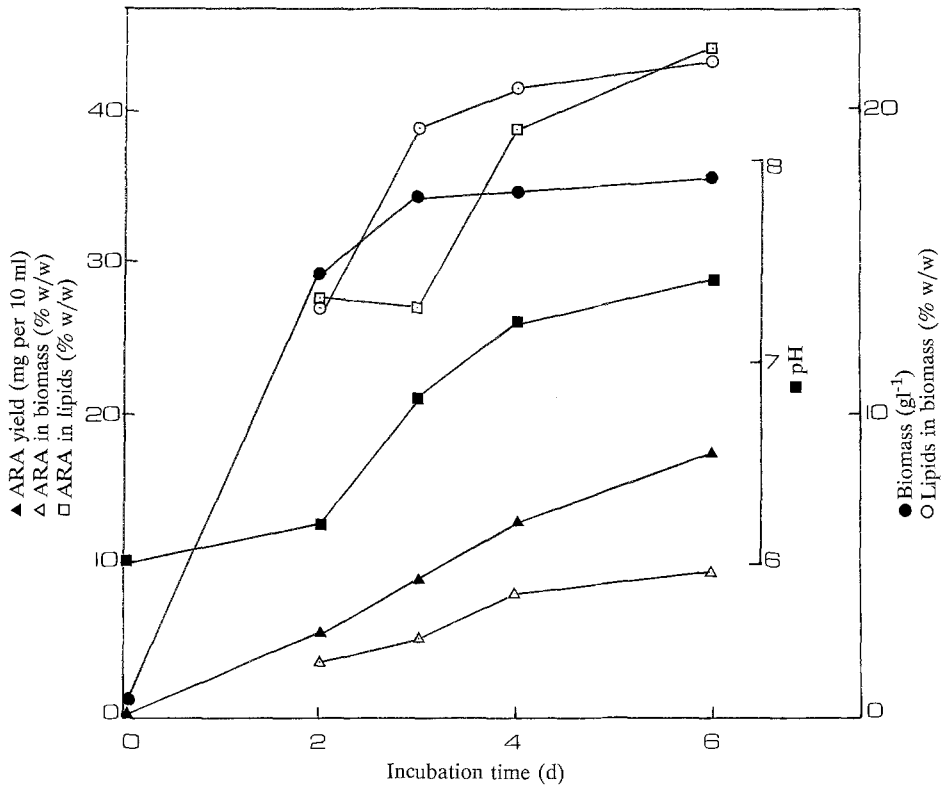

Fig. 2. Pattern of growth and arachidonic acid production by *Mortierella alpina* ATCC 32222 with time. Incubation conditions: HD medium, 25 °C, 6 days.

contained 80 g/l glucose. ARA content, both as a percentage of biomass and percentage of total lipids, was highest at a glucose concentration of 30 g/l and declined with increasing glucose concentration in the medium. Optimal yield of ARA, 2.27 g/l, was observed at a glucose concentration of 100 g/l. However, yields observed in the range 30–120 g/l were greater or equal to 75% of the optimum value.

The pattern of production of ARA with time by *M. alpina* ATCC 32222 in the HD medium at 25 °C is presented in Fig. 2. Although biomass and lipid production reaches a plateau after 3 days, a dramatic increase in ARA content as a percentage of biomass, as a percentage of the lipids and in yield per litre of culture broth is observed during the stationary phase of growth.

The effect of initial pH (after autoclaving) on ARA production by *M. alpina* ATCC 32222 is presented in Table 3. Initial pH had a relatively small effect on biomass production. The optimum initial pH range for production of lipids in biomass and ARA was found to be 6.0–6.7.

The effect of substituting the complex nitrogen source (5 g/l) in the HD medium on growth and ARA production by *M. alpina* ATCC 32222, incubated for 6 days at 25 °C, was investigated. Yeast extract resulted in better ARA production than peptone, tryptone or a 50:50 combination of yeast extract and peptone.

The effect of carbon source in the HD medium on fungal growth, lipid and ARA production is presented in Table 4. Fungal growth was poor in medium containing xylose or sucrose. With other carbon sources, biomass production ranged from 16.85 g/l for dextrin to 28.21 g/l for linseed oil. ARA content of biomass was very low and content of lipids in the medium was very low when linseed oil was the carbon source. ARA content of biomass produced with fructose, maltose, glycerol and glucose ranged from 9.6 to 10.75% (w/w) with ARA accounting for 44.7–54.1% of total fatty acids. Yields of ARA per litre of culture broth containing these carbon sources ranged from 1.63 to 1.96 g/l.

The effect of doubling and tripling the components of the HD medium on production of biomass and ARA were investigated (Table 5). Triple-strength medium resulted in a doubling of the yield of biomass produced and a 2.2-fold increase in yield of ARA which amounted to 3.73 g ARA per litre of culture broth. The lipid content in the biomass of the triple-strength medium was 63% higher than its content in biomass from the single-strength medium, although ARA content in biomass was similar in both

TABLE 4

Effect of carbon source on arachidonic acid production by *Mortierella alpina* ATCC 32222

Carbon source	Biomass (g/l)	Lipids in biomass (% w/w)	ARA		
			In biomass (% w/w)	In lipids (% w/w)	Yield (g/l)
Glycerol	18.21	24.03	10.75	44.73	1.958
Glucose	17.50	17.78	9.62	54.11	1.684
Fructose	17.54	21.10	10.46	49.57	1.835
Xylose	2.30	5.23	2.32	44.38	0.053
Maltose	16.96	19.35	9.60	49.61	1.628
Sucrose	6.20	7.34	2.01	27.33	0.124
Dextrin	16.85	11.26	5.38	47.76	0.906
Starch	18.10	12.81	6.66	52.00	1.205
Olive oil*	20.10	45.43	8.09	17.82	1.627
Linseed oil*	28.21	30.88	1.60	5.17	0.451

Data are the average of three replicates.
Incubation conditions: HD medium, 25 °C, 6 days.
* These oils do not contain arachidonic acid.

media. As a result, ARA content in lipids of the triple-strength medium was 35.9% compared to an ARA content in lipids of 54.1% in the single-strength medium. Stearic acid (18 : 0) content of the triple-strength medium was 19.92% compared to 6.77% in single-strength medium.

TABLE 5

Effect of increasing the strength of the HD medium on biomass growth and lipid production

Parameter	HD	2HD	3HD
Biomass (g/l)	17.50	27.00	35.80
Lipids in biomass (% w/w)	17.78	25.76	28.98
ARA			
(a) in biomass (% w/w)	9.62	10.02	10.41
(b) in lipids (% w/w)	54.11	38.90	35.92
(c) yield (g/l)	1.68	2.71	3.73
Fatty acids (% w/w)			
16 : 0	9.01	10.00	10.13
16 : 1	0.00	0.00	0.00
18 : 0	6.77	16.32	19.92
18 : 1	8.31	10.74	10.90
18 : 2	7.30	7.57	7.78
18 : 3	Trace	Trace	Trace
20 : 3	2.48	4.38	3.93
20 : 4	54.11	38.90	35.92
20 : 5	0.00	0.00	0.00
Others	12.02	12.09	11.42

Data are the average of three replicates.
Incubation conditions: 25 °C, 6 days.

DISCUSSION

We have described culture conditions which result in production of 54% (w/w) of the lipid content of *M. alpina* ATCC 32222 as ARA. In addition, we have observed that, by increasing glucose or whole medium concentration, overall yields of ARA per litre of culture are significantly increased although ARA content in lipids is somewhat reduced. We have demonstrated production of ARA in shake flask cultures to a yield of 3.73 g/l with ARA constituting 10.41% of biomass. Using the same incubation time and temperature, maximum ARA production by *M. alpina* ATCC 16266 was in the range 1.9–2.1 g/l in a medium containing 10 g/l yeast extract, 100 g/l glucose and no salt supplement [3].

It was noted that while biomass production of *M. alpina* ATCC 32222 reached a plateau after 3 days, ARA content increased substantially between 3 and 6 days. The photosynthetic protist *Ochromonas danica* [31] and the marine diatom, *Phaeodactylum tricornutum* [2], were found to increase their content of poly-unsaturated fatty acids significantly as cultures grew older. In contrast, with many microorganisms a general decrease in unsaturated fatty acid content occurs on ageing [11]. It has previously been noted that ageing of *Mortierella* mycelium following harvesting also results in an increased production of poly-unsaturated fatty acids [3]. The increased synthesis of ARA in the stationary phase of cell culture and the continued production of ARA during mycelium ageing are likely to be one and the same process. The observation that ARA production increases with

glucose concentration up to 100 g/l glucose is consistent with findings for other *Mortierella* strains [3,30]. Glucose is the most commonly used sugar for fungal fat production and is efficiently converted into lipids by a number of fungi [29]. Indeed a high carbon : nitrogen ratio was found to be best for fungal growth and lipid production by *Mortierella vinacea* and *Mortierella ramanniana* [8,15]. This may be explained by the general observation that lipid accumulation is often triggered by depletion of nitrogen. In contrast, EPA production by *M. elongata* was highest when glucose concentration in the medium was low [30].

Poly-unsaturated fatty acids are synthesized in eukaryotes from saturated or mono-unsaturated precursors via metabolic processes involving both chain elongation and desaturation [7]. In addition to fatty acid synthases (FAS) type I and type II, which produce long-chain fatty acids from acetate in prokaryotes and eukaryotes, a third enzyme, FAS type III, is associated with microsomal membranes of eukaryotes [6,20]. This appears to function primarily as an elongation system for medium and long-chain fatty acids produced by cytosolic type I enzyme or originating from external sources [20,21]. No FAS type III has been characterized to date. While little is known about the enzymes involved in poly-enoic fatty acid biosynthesis, the mechanism appears to be similar to that of oxidative mono-enoic fatty acid formation [22]. Oleoyl-CoA or oleoylphosphatidylcholine are used as substrates by particulate enzymes requiring molecular oxygen and NAD(P)H [11,13,16,19]. Shimizu et al. [24] have proposed a pathway for production of arachidonic acid by *Mortierella* species. Linoleic acid (18 : 2) is desaturated to form γ-linolenic acid (18 : 3) which is then elongated, forming dihomo-γ-linolenic acid (20 : 3) which is desaturated to form arachidonic acid. The low or trace levels of γ-linolenic acid and dihomo-γ-linolenic acid observed in our study suggest that the conversion of linoleic acid to arachidonic acid is very rapid and efficient. It was noted that at the lower incubation temperature some EPA was produced whereas at 25 °C no EPA was detected. Shimizu et al. [23] have suggested that EPA production may be due to activation of enzyme(s) which convert arachidonic acid to EPA, probably by methyl-end directed desaturation.

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