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Production of high yields of arachidonic acid in a fed-batch system by *Mortierella alpina* ATCC 32222

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Abstract Of six strains of *Mortierella* tested, *Mortierella alpina* ATCC 32222 produced the highest yields of arachidonic acid. Supplementation of soy flour (1% w/v) and vegetable oils (1% v/v) significantly increased the biomass, lipid content and arachidonic acid level. Replacement of $NaNO_3$ with corn steep liquor (1% w/v) also improved arachidonic acid production. A fed-batch culture system at 25 °C, producing a high biomass (52.4 g/l) and arachidonic acid content (9.1 g/l) in 8 days, was developed. A fed-batch system at low temperature (15 °C) gave even higher arachidonic acid levels (11.1 g/l) in 11 days.

Introduction

Arachidonic acid (5,8,11,14-*cis*-eicosatetraenoic acid, 20:4, AA), a biogenic precursor of prostaglandin and leukotrienes, has been a subject of intensive medical research (Marx 1982; Das et al. 1987; Simopoulos 1989). It is the most abundant C_{20} (ω-6 class) polyunsaturated fatty acid (PUFA) in humans and has an important role in the structure and function of biological membranes (Yamada et al. 1989). Although, AA can be extracted from porcine liver, adrenal glands and fish oil, these sources contain very little AA, therefore alternative sources are being sought.

AA has been found to be present in the cells of some protozoa, algae and fungi (Yongmanitchai and Ward 1989; Shinmen et al. 1989; Gandhi and Weete 1991). Lower fungi of the class Phycomycetes, especially in the order Mucorales, are a promising source of a variety of PUFA (Yongmanitchai and Ward 1989). Species of *Mortierella* produce substantial quantities of AA under different culture conditions (Lindberg and Molin 1993; Ward 1995), and we have found different strains of *M. alpina* to be efficient producers of the acid (Bajpai et al. 1991a,b; Li et al. 1995). We report here conditions for producing high biomass density and titer of AA in a fed-batch system using *M. alpina* ATCC 32222.

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Materials and methods

Microorganisms

Different strains of *Mortierella alpina* and *Mortierella elongata* were obtained from the American Type Culture Collection (ATCC, Rockville, USA). Cultures were maintained on 3% agar slants containing 2% glucose and 1% yeast extract and subcultured every 2 months.

Culture conditions

The basal medium contained (per liter): 50 g glucose, 5 g yeast extract, 3 g $NaNO_3$, 1 g KH_2PO_4, 0.5 g $MgSO_4$, 0.5 g KCl, 1.45 mg $FeCl_3$, 0.01 mg $CuSO_4$, 4.3 mg $MnCl_2 \cdot 4H_2O$, 0.13 mg $CoCl_2 \cdot 6H_2O$, and 0.3 mg $ZnCl_2$. The pH of the medium was adjusted to 6.0 before autoclaving. Inocula were prepared in 250-ml flasks containing 50 ml medium. The culture was grown at 25 °C for 48 h with shaking at 200 rpm. Conical flasks containing 50 ml production medium were inoculated with this 48-h-grown inoculum at a rate of 5% v/v and incubated on an orbital shaker (New Brunswick, N.J., USA) at 200 rpm. The values given for each experiment are means of two to three treatment replications. Some experiments were done at least twice, and data given in this paper are representative.

Analytical methods

The dry weight of biomass was determined by centrifugation of the fungal cell suspension, washing with distilled water and drying at 100 °C for 12–16 h. The lipids were extracted following the method of Bligh and Dyer (1959). The methyl esters, prepared as described by Holub and Skeaff (1987), were analyzed by gas chromatograph (Shimadzu GC-14A, Kyoto, Japan) as described earlier (Singh et al. 1996). The fatty acids were identified and quantified using methyl esters of quantitative standard fatty acids supplied by Sigma (St. Louis, USA). Pentadecanoic acid (15:0) was used as internal standard (nomenclature used for fatty acids: myristic 14:0, palmitic 16:0, stearic 18:0, oleic 18:1, linoleic 18:2, linolenic 18:3, arachidonic 20:4, and eicosapentaenoic 20:5).

Results

Four strains of *M. alpina* and two strains of *M. elongata* were tested for growth, total lipid production and their AA acid yields in the basal medium (Table 1). Except for *M. alpina* ATCC 42430, the *M. alpina* strains were better producers of AA than *M. elongata* strains. Although *M. elongata* ATCC 32325 possesses a high lipid content in its biomass (19.1% w/w), AA comprised only 2.3% w/w of this biomass and 12% w/w of the total lipid. *M. alpina* ATCC 42430 produced the least amount of lipid in biomass (5% w/w), with 16:0 and 18:1 as major fatty acids. Although *M. alpina* ATCC 16266 and *M. alpina* ATCC 32222 exhibited similar biomass contents and AA yields, *M. alpina* ATCC 32222 contained a lower AA content in lipid (30.7% w/w) as compared to that of *M. alpina* ATCC 16266 (46.3% w/w).

All the strains of *M. alpina* and *M. elongata* exhibited pellet formation during their growth in the basal medium. However, incorporation of soy flour (1% w/v) into the growth medium prevented pelleted growth, and all the strains exhibited dispersed filamentous growth with about a twofold increase in biomass and AA contents. Since *M. alpina* ATCC 32222 produced maximum biomass (24.6 g/l), and AA yield (3.1 g/l), this strain was used in further experiments in the basal medium containing 1% w/v soy flour.

A typical profile of growth and AA production by *M. alpina* ATCC 32222 is illustrated in Fig. 1. The biomass content increased rapidly during the first 3 days and reached a plateau after 5 days. The lipid content in biomass increased in parallel with the biomass and reached a maximum level (23.9% w/w) after 6 days. A maximum AA yields (3.1 g/l) was observed after 6 days of incubation. The profile of fatty acids as a function of incubation time indicates that the AA content continued to increase for 7 days and thereafter remained more or less constant (Fig. 2). A concomitant decrease in 14:0,

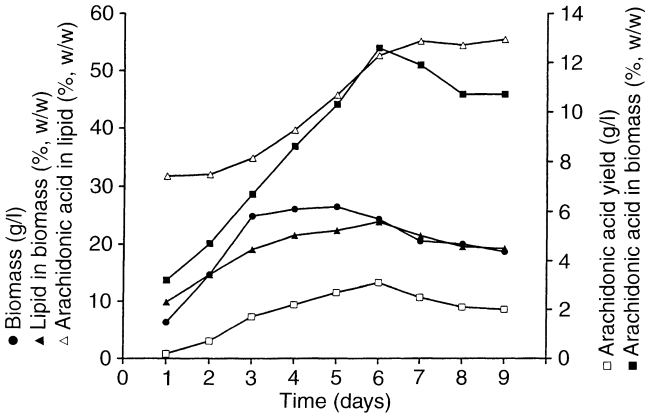

Fig. 1 Time course of arachidonic acid production by *Mortierella alpina* ATCC 32222. The culture was grown in the basal medium containing 1% w/v soy flour at pH 6.0 and 25 °C on a rotary shaker (200 rpm)

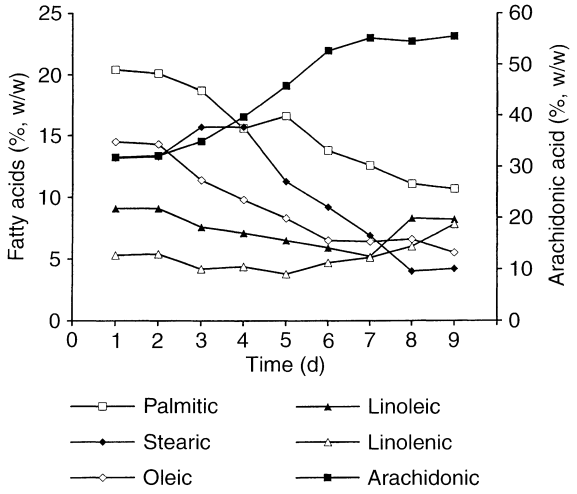

Fig. 2 Relative amounts of fatty acids produced by *M. alpina* ATCC 32222

Table 1 Production of biomass and lipids by different strains of *Mortierella*. All the strains were grown in basal medium containing 50 g/l glucose at pH 6.0 and 25 °C for 6 days with orbital shaking at 200 rpm. Soy flour (1% w/v) supplemented (+) and non-supplemented (−) cultures. *Others* includes 14:0 (0.4%–3.8% w/w); 18:0 (3.4%–14.0% w/w); 18:2 (1.2%–18.7% w/w); 18:3 (3.1%–9.4% w/w); 20:5 (0%–0.7% w/w). *AA* arachidonic acid

Strain/ATCC no.	Soy flour addition	Biomass (g/l)	Lipid in biomass (% w/w)	AA in biomass (% w/w)	AA yield (g/l)	Predominant fatty acids (% w/w)			
						16:0	18:1	20:4	Others[2]
M. alpina 16266	−	13.1	15.0	6.9	0.9	8.5	10.9	46.3	34.3
	+	23.7	23.4	11.5	2.7	9.6	11.1	49.1	30.2
M. alpina 32221	−	9.7	16.2	3.1	0.2	12.2	45.5	19.2	23.1
	+	17.3	20.0	6.2	1.1	20.5	14.0	30.9	34.6
M. alpina 32222	−	13.7	20.3	6.3	0.9	22.3	20.3	30.7	26.7
	+	24.6	23.8	12.5	3.1	13.8	6.5	52.7	27.0
M. alpina 42430	−	10.3	5.0	0.9	0.1	19.9	39.0	17.5	23.6
	+	21.2	9.5	2.9	0.6	19.2	13.7	30.8	36.3
M. elongata 32325	−	9.7	19.1	2.3	0.2	20.7	36.1	12.0	31.2
	+	16.9	20.9	5.2	0.9	25.3	20.4	25.1	29.2
M. elongata 42661	−	7.9	14.7	1.9	0.2	20.8	39.7	12.8	26.7
	+	13.5	17.9	3.9	0.5	16.3	36.1	21.7	25.9

16:0 and 18:1 acids was observed with the increase in incubation time.

Addition of vegetable oil (1%, v/v) to the medium stimulated biomass and lipid production by *M. alpina* ATCC 32222 (Table 2). Corn- and canola-oil-supplemented cultures exhibited the maximum AA yield (4.7 g/l), which was about 50% higher than the AA yield (3.1 g/l) of the culture without any supplementation. In general, there was a slight decrease in AA content in lipid accompanied by an increase in 18:1 and 18:2 acids of all the oil-supplemented cultures.

Replacement of $NaNO_3$ with corn steep liquor significantly improved the AA yield of *M. alpina* ATCC 32222 (Table 3). Corn steep liquor at the 1% w/v level was most effective and produced the maximum AA yield (4.9 g/l). Although a higher concentration of corn steep liquor (1.5% w/v) produced a higher yield of biomass (33.5 g/l), the lipid content (19.8% w/w) and AA yield (3.7 g/l) were significantly reduced.

In an attempt to increase the AA yield of *M. alpina* ATCC 32222 further, a fed-batch system was employed in shake flasks with basal medium supplemented with 1% w/v soy flour, 1% w/v corn steep liquor and 1% v/v corn oil at 25 °C. Additional glucose (20 g/l) was supplied each day to the medium after 3 days of fermentation. The results presented in Fig. 3a indicate a dramatic

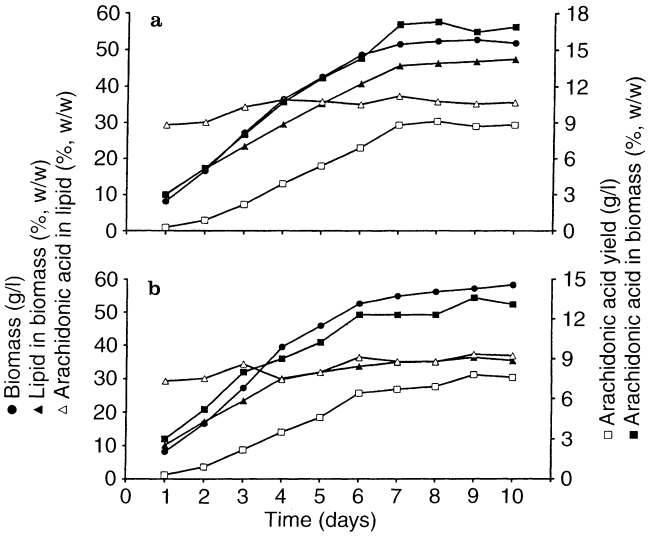

Fig. 3a, b Effect of glucose and nutrient addition on arachidonic acid production by *M. alpina* ATCC 32222. The fermentation was initiated as a batch culture with basal medium containing 50 g/l glucose, 1% w/v soy flour, 1% w/v corn steep liquor and 1% v/v corn oil. After 3 days, additional glucose and nutrients (at 40% of the initial amounts) were supplied at 1-day intervals. **a** Addition of glucose (20 g/l), **b** addition of glucose and nutrients. In **b**, supplemented medium contained 20 g/l glucose, 2 g/l yeast extract, 0.4 g/l KH_2PO_4, 0.2 g/l $MgSO_4$, 0.2 g/l KCl, and 4 g/l corn steep liquor

Table 2 Effect of vegetable oil supplementation on the production of biomass and lipids by *Mortierella alpina* ATCC 32222. The culture was grown in basal medium containing 50 g/l glucose and 1% w/v soy flour at pH 6.0 and 25 °C for 6 days with orbital shaking at 200 rpm. *Others* includes 14:0 (0.3%–0.4% w/w); 18:0 (5.8%–9.2% w/w); 18:3 (3.3%–4.7% w/w)

Parameters	Vegetable oil (1% v/v)				
	None	Corn	Soy	Peanut	Canola
Biomass (g/l)	24.4	29.7	29.9	29.3	30.5
Arachidonic acid					
In biomass (% w/w)	12.6	15.8	15.0	12.9	15.5
Yield (g/l)	3.1	4.7	4.5	3.8	4.7
Predominant fatty acids (% w/w)					
16:0	13.8	9.9	10.5	8.7	10.8
18:1	6.5	11.5	16.4	8.7	11.8
18:2	5.9	18.8	12.6	18.0	19.2
20:4	52.7	47.7	46.1	47.9	43.7
Others	21.1	12.1	14.4	16.7	14.5

Table 3 Effect of corn steep liquor supplementation on the production of biomass and lipids by *M. alpina* ATCC 32222. The culture was grown in basal medium containing 50 g/l glucose and 1% w/v soy flour at pH 6.0 and 25 °C for 6 days with orbital shaking at 200 rpm. $NaNO_3$ was replaced with corn steep liquor. *Others* includes 14:0 (0.4%–0.5% w/w); 18:0 (5.9%–6.5% w/w); 18:3 (3.8%–4.7% w/w)

Parameters	Corn steep liquor			
	0	5 g/l	10 g/l	15 g/l
Biomass (g/l)	24.4	27.9	30.9	33.5
Arachidonic acid				
In biomass (% w/w)	12.6	14.1	15.9	11.1
Yield (g/l)	3.1	3.9	4.9	3.7
Predominant fatty acids (% w/w)				
16:0	13.8	10.5	11.2	10.7
18:0	9.2	7.7	8.2	7.9
18:1	6.5	7.6	9.1	8.2
20:4	52.7	61.3	58.5	56.2
Others	17.8	13.9	13.0	17.0

increase in biomass, lipid and AA contents. High biomass (52.4 g/l) and AA yield (9.1 g/l) were achieved in 8 days. In a similar experiment (Fig. 3b), glucose (20 g/l) and other medium components (40% of the initial concentration) were supplied to the cultures. Although a higher biomass level (58.3 g/l) was obtained, the AA yield (7.8 g/l) was much lower.

A fed-batch system was also implemented in the same medium but at lower temperature. Thus *M. alpina* ATCC 32222 was grown initially for 3 days to maximize growth, which has a temperature optimum at 25 °C, thereafter additional glucose (20 g/l) was supplied each day and cultures were incubated at 15 °C until the end of the fermentation period. Although a lower growth rate was observed at low temperature, the cellular lipid and AA contents significantly increased (Fig. 4). This procedure gave a maximum lipid (46.7% w/w) and AA yield (11.1 g/l) after 11 days of fermentation. With the increase in fermentation time, an increase in the level of AA and a decrease in 16:0, 18:0 and 18:1 acids was observed (Fig. 5).

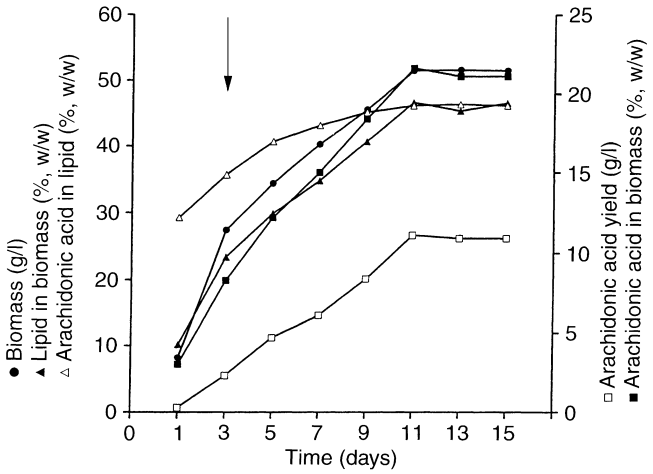

Fig. 4 Effect of glucose addition and temperature shift on arachidonic acid production by *M. alpina* ATCC 32222. The fermentation was initiated as batch culture at 25 °C with medium containing 50 g/l glucose, 1% w/v soy flour, 1% w/v corn steep liquor and 1% v/v corn oil. After 3 days, additional glucose (20 g/l) was supplied at 1-day intervals and cultures were further incubated at 15 °C. *Arrow* the temperature shift

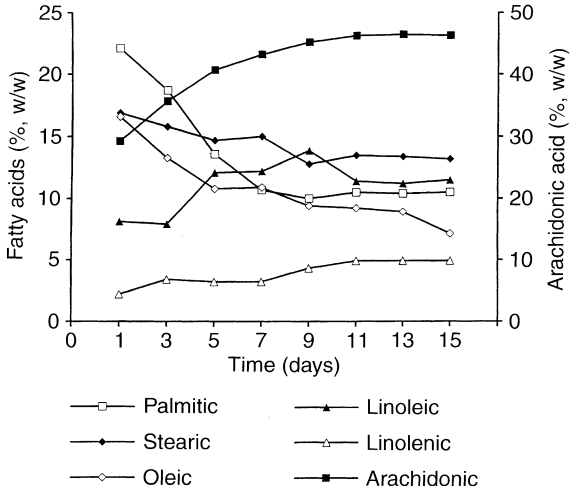

Fig. 5 Effect of glucose addition and temperature shift on relative amounts of fatty acid produced by *M. alpina* ATCC 32222

Discussion

Culture conditions for *M. alpina* ATCC 32222 were developed in a fed-batch system producing a high arachidonic acid yield (11.1 g/l) in 11 days. The AA content of the mycelia was affected by the fermentation conditions and medium components. This strain is advantageous in that it contains no PUFA other than AA in high concentration, making it a promising source of AA for commercial exploitation.

Product formation in fungal fermentation may be influenced by growth morphology (Byrne and Ward 1989). All the strains of *M. alpina* and *M. elongata* used in the present investigation exhibited pellet formation during their growth in basal medium. Since pellet formation reduces the growth rate and causes an undesirable lag phase in cultures, dispersed rather than pelleted growth has been preferred for the production of biomass and AA by *M. alpina* UW-1 (Li et al. 1995). The use of relatively insoluble medium constituents, such as soy flour (1%, w/v), appeared to counteract pellet formation in *Mortierella* (Li et al. 1995).

The amount of lipid produced by a given microbial species depends to a great extent on the development stage of growth. In *M. alpina* ATCC 32222, biomass accumulated rapidly during first 3 days and thereafter remained more or less constant. However, a considerable turnover in fungal lipids was noticed in this strain after the stationary phase was reached. Generally post-exponential build-up of lipid has been noticed in oleaginous microorganisms after nutrient, and especially nitrogen, exhaustion (Ratledge 1989).

In *Mortierella* species, 18:2 is desaturated to form 18:3, which is then elongated, forming 20:3, which is desaturated to form AA (Shinmen et al. 1989). Addition of vegetable oil (18:1 and 18:2 as major fatty acids) to the basal medium significantly enhanced the AA yield of *M. alpina* ATCC 32222. Some of the fatty acid supplement is possibly utilized as a precursor for arachidonic acid synthesis (Shinmen et al. 1989). Supplementation of the culture medium with natural oil has also been found to increase the accumulation of AA by *Echinsporangium transversalis* NRRL 3116 (Kengo et al. 1989), *M. alpina* IS-4 (Shinmen et al. 1989) and *M. alpina* UW-1 (Li et al. 1995).

In a fed-batch system, where glucose was fed to the fermentation medium at regular intervals, high biomass and AA yield (9.1 g/l) were observed. Under conditions of carbon and energy limitation, accumulated lipids are used as carbon and energy supply by fungi (Weete 1980).

High carbon and low nitrogen levels are known to support good lipid accumulation (Ratledge 1989). Our results for batch feeding indicate that similar or greater improvements might be realized by continuous culture as long as carbon and energy are in excess.

A fed-batch system with glucose feeding and temperature shifting produced the highest AA yield (11.1 g/l). The beneficial effects of temperature shifting have been noticed in cultures producing polyunsaturated fatty acids such as eicosapentaenoic acid (Shimizu et al. 1988), AA (Lindberg and Molin 1993) and docosahexaenoic acid (Singh et al. 1996). The degree of unsaturation in the fatty acid composition is known to be influenced by temperature, i.e. when the growth temperature is lowered, the proportion of unsaturated fatty acids to saturated fatty acids tends to increase as a result of increased membrane fluidity as an adaptation to the cold environment (Suutari and Laasko 1994).

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