Optimization of Production of Docosahexaenoic Acid (DHA) by *Thraustochytrium aureum* ATCC 34304

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By varying culture carbon source, lipid content in mycelium of *Thraustochytrium aureum* ATCC 34304 varied widely in the range 1-25% of biomass weight. Docosahexaenoic acid (DHA) content of mycelium lipid was higher (65-76%) when biomass lipid content was very low (1-2%) and lower (40-50%) when biomass contained a high lipid content (14-18%). DHA yields from glucose, starch and maltose were 270, 325 and 334 mg/L, respectively. DHA yield and content of biomass was optimal at an initial culture pH of 6.0. During the culture cycle of *T. aureum*, DHA content in lipids remained relatively constant with optimal DHA yield being observed after six days. Biomass, lipid content in biomass, DHA content in biomass and DHA yield were all optimal at a cultivation temperature of 28°C. However, the proportion of DHA in lipids declined with increase in temperature. Biomass, lipids in biomass and DHA yields were 13%, 42% and 47% higher, respectively, in light-exposed cultures as compared to dark cultures. A maximum yield of DHA of 511 mg/L was observed in light-exposed cultures containing 2.5% starch, where lipids accounted for over 20% of biomass dry weight.

KEY WORDS: DHA (docosahexaenoic acid), omega-3 fatty acids, *Thraustochytrium aureum*.

The beneficial effects on human health of the omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are widely accepted and have led to extensive nutritional and clinical studies on their effects in human physiology (1-4). DHA is produced from eicosapentaenoic acid by a desaturation and elongation mechanism and thus it shares a common biosynthetic pathway with omega-3 fatty acid precursors of eicosanoid compounds (3). DHA has perceived functions in the nervous, visual and reproductive systems (5-7), and in mammals and birds it is usually associated with phospholipids (8). The cerebral cortex, retina, testes and sperm of mammals, including humans, are particularly rich in DHA (9-11).

DHA is one of the most abundant components of the brain's structural lipids, and the lipid fraction of human mothers' milk contains thirty times the level of DHA observed in cows' milk lipid. DHA may be important in the development of brain tissue of babies. A deficiency of DHA in the human infant diet as a result of use of formula baby milk reduces DHA content in erythrocyte total lipid, phosphatidylcholine and phosphatidylethanolamine levels to half those found in normal breast-fed infants (8).

The current commercial source of DHA is fish and fish oils. Fish oil contains 7-14% of DHA in addition to EPA and other more saturated fatty acids. Because of the relatively low proportion of DHA in fish oil and the difficulties encountered in extraction and purification of omega-3 fatty acids (12-16), other sources of DHA and EPA are being sought among phytoplankton, seaweeds and fungi (17,18).

Among the lower fungi, individual species within the order Mucorales have been shown to produce substantial quantities of arachidonic acid, eicosapentaenoic acid and γ-linolenic acid (19-22). A survey of the literature (18) indicates that some lower fungi within the orders Saprolegniales and Entomorphorales, and especially the strain *Thraustochytrium aureum*, produce significant quantities of DHA (23). This non-filamentous pigmented marine species has been found to display light-stimulated growth (24,25).

We have previously shown that *T. aureum* ATCC 34304 can produce a DHA yield of up to 284 mg/L of culture broth, with around 50% of its lipid as DHA (unpublished data). The high proportion of DHA in total lipids of *T. aureum* and the relatively low levels of structurally related polyunsaturated fatty acids should simplify downstream processing methods for DHA recovery. Thus potential exists for the development and commercial exploitation of a fermentation process for production of DHA from *T. aureum*. In this paper some of the factors affecting growth and DHA production by *T. aureum* have been investigated and studies on optimization of DHA production have been carried out.

MATERIALS AND METHODS

Culture medium. The basal medium contained per liter: NaCl, 25 g; $MgSO_4 \cdot 7H_2O$, 5 g; KCl, 1 g; KH_2PO_4, 0.1 g; $CaCO_3$, 0.2 g; $(NH_4)_2 SO_4$, 0.2 g; sodium glutamate, 2 g; thiamine•HCl, 10 μg; $NaHCO_3$, 0.1 g; and vitamin B_{12}, 1 μg.

Culture maintenance. T. aureum ATCC 34304 was maintained on 3% agar slants containing the basal medium supplemented with 2% glucose and 0.2% yeast extract.

Culture conditions. An inoculum was prepared in 250-mL Erlenmeyer flasks containing 50 mL of the basal medium, supplemented with 2% glucose and 0.2% yeast extract. Cultures were grown at 25°C in light for 40 hr with orbital shaking at 300 rpm. Erlenmeyer flasks, 250 mL, containing 50 mL of the production medium, were inoculated with this 40-hr inoculum at a rate of 5% v/v and incubated on an orbital shaker at 300 rpm in light at 25°C for six days unless otherwise indicated. The orbital shakers were Lab-line incubator shakers, Model 3525, fitted with two 33-watt 24-inch fluorescent tubes in the lid at a distance of 14 inches for the shaker platform. Fermenter studies were carried out in a Chemap 2000 7.5-L *in situ* sterilizable fermenter with a working volume of 5 L, by using a 5% inoculum prepared in shake flasks. Fermenter settings were: temperature 25°C; aeration 2.5 L/min; and impeller speed, 300 rpm.

Biomass determinations. Dry weight of biomass was determined by vacuum filtration or centrifugation of fungal cell suspension, washing it with 1% NaCl and

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TABLE 1

Effect of Carbon Source on DHA Production by *T. aureum* ATCC 34304

Carbon source[a]	Biomass (g/L)	Lipids in biomass (% w/w)	DHA in Biomass (mg/g)	DHA in Lipids (% w/w)	DHA Yield (mg/L)
Fructose	1.2	0.9	6.1	64.5	7.2
Sucrose	1.1	1.7	12.3	74.5	13.5
Glucose	3.8	16.5	70.4	42.8	269.6
Starch	4.9	14.7	66.7	45.3	325.4
Lactose	1.1	1.7	11.3	65.2	11.9
Maltose	4.6	17.9	73.2	41.0	334.4
Linseed oil	5.5	25.2	12.1	4.8	67.0

[a]Each carbon source was added at 2% concentration in basal medium except linseed oil, which was used at 0.8% concentration.

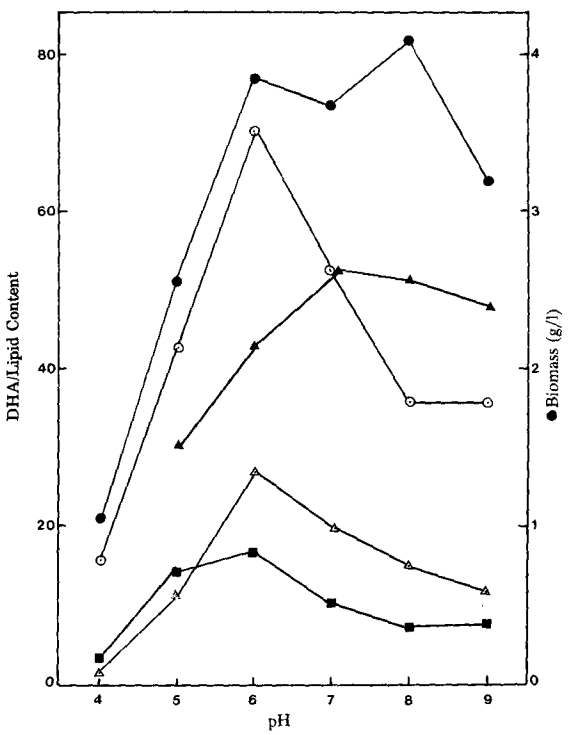

FIG. 1. Effect of initial pH on DHA production by *T. aureum* ATCC 34304. Culture medium—basal medium supplemented with 2% glucose. ○, DHA in biomass (mg/g); ■, lipids in biomass (% w/w); △, DHA yield (mg/100 mL); and ▲, DHA in lipids (% w/w).

distilled water and drying at 100°C for 12-16 hr.

Extraction and determination of lipids. The dried cells were weighed (20-40 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 (26). The extracted lipids were dried at 36°C under nitrogen atmosphere and methylated by using the method of Holub and Skeaff (27). The fatty acid methyl esters were then 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 (Shimadzu, Kyoto, Japan) was connected with a GC-14A data integrator. The GLC was fitted with a 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 with standard fatty acids supplied by Sigma Chemical Company (St. Louis, MO). Pentadecanoic acid (C15:0) was used as the internal standard. Data are the averages of three determinations.

RESULTS

The effect of carbon source on biomass, lipid and DHA production by *T. aureum* ATCC 34304 is summarized in Table 1. Highest biomass was produced when the organism was grown on linseed oil, while poor growth was observed on fructose, sucrose and lactose. Starch, glucose, maltose and linseed oil produced a high lipid content in biomass although, in the latter case, part of this lipid content could be due to association of linseed oil substrate with the recovered mycelium. Lipid content in biomass ranged from 1-25% and DHA content of lipid (excluding the linseed oil culture) ranged from 41-75%. In general, DHA content of lipid was higher (65-75%) when biomass lipid content was very low (1-2%) and lower (40-50%) when biomass contained a high lipid content (14-18%). Comparative DHA yields from glucose, starch and maltose were 270.0, 325.4 and 334.4 mg/L, respectively.

The effect of initial pH on DHA production in the basal medium containing 2% glucose is illustrated in Figure 1. DHA yield and content of biomass was optimal at an initial pH of 6.0.

Pattern of growth, lipid and DHA production by *T. aureum* ATCC 34304 with time is illustrated in Figure 2. Biomass and lipid content of biomass increased to a maximum at six days, after which biomass plateaus and lipid content declines. DHA content in lipids remains relatively constant at around 50% during the culture process. Optimal DHA yield is recovered after six days. The effect of varying the nitrogen source in the production medium is illustrated in Table 2. Lipid content of biomass and DHA yield was maximum in media containing 0.2% sodium glutamate. Effect of temperature on biomass, lipid and DHA production is illustrated in Figure 3. Biomass, lipid content of biomass, DHA content of biomass and DHA yield all increased with temperature and reached maximum values at 28°C. The proportion of DHA in lipids declined with increase in temperature. The fatty acid profiles observed at different temperatures are illustrated

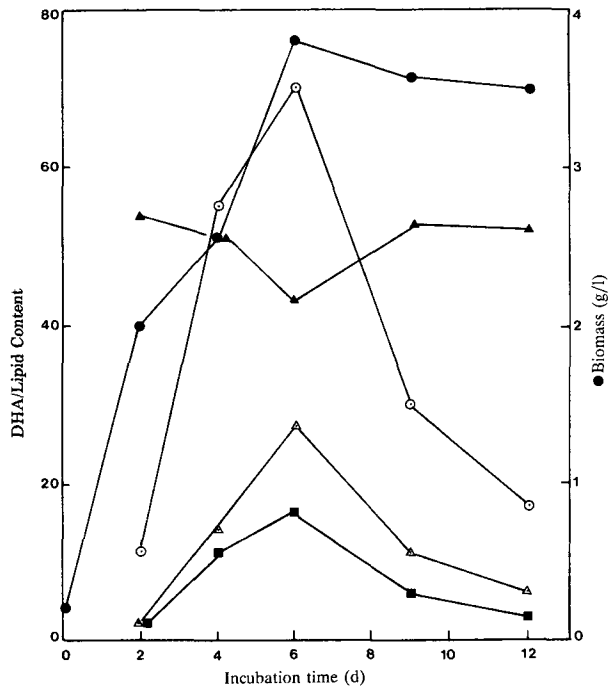

FIG. 2. Time course of growth, lipid and DHA production by *T. aureum* ATCC 34304 in shake flasks. Culture medium—basal medium supplemented with 2% glucose. Legends as in Figure 1.

in Table 3, indicating an increase in palmitic (16:0) and oleic (18:1) acids and concomitant decrease in DHA (22:6) and EPA (20:5) with increase in cultivation temperature.

An inoculum size of 5% was found to be optimum for production of DHA. The effect of inoculum age on subsequent DHA production is summarized in Table 4. While inoculum age had little affect on culture biomass, lipid content of biomass and DHA yield dramatically declined when 48-hr or 72-hr inocula were used instead of a 24-hr inoculum. Inoculum age did not significantly affect the proportion of DHA in lipids.

The effect of light on growth and DHA production by *T. aureum* ATCC 34304 is illustrated in Table 5. Biomass, lipids in biomass and DHA yields were 13%, 42% and 47% higher, respectively, in light-exposed cultures as compared to dark cultures.

The effect of starch or maltose concentration on growth, lipid and DHA production by *T. aureum* is presented in Table 6. A substantial increase in lipid content of biomass and yield of DHA was observed by increasing starch concentration to 2.5%. Lipids accounted for over 20% of biomass and DHA yield per liter of culture reached a maximum value of 511 mg/L.

The pattern of production of biomass, lipid and DHA in a laboratory fermenter is illustrated in Figure 4. Maximum DHA yield was 325 mg/L after 116 hr at 25°C by using the basal medium supplemented with 2% starch.

DISCUSSION

Mono-, di- and polysaccharides of glucose promoted good growth of *T. aureum* and produced a high lipid content in biomass. In contrast, *T. aureum* grew poorly on fructose, sucrose and lactose, and the lipid content of mycelia in these cultures was very low. This suggests that, where carbon substrate substantially limited mycelia growth, minimum conversion of carbohydrate to lipid occurred. Conversely, where substrates, present in excess, were shown to support good mycelial growth, substantial synthesis of lipid was promoted. Where lipid content was low (1–2%), DHA content in lipid was highest (64–75%). However, where high DHA yields were produced in cultures with high biomass lipid content, percentage of DHA in lipids was somewhat lower (40–45%).

Highest yield of DHA was observed when the initial culture pH was 6.0, where lipid and DHA contents in biomass were also optimal. At an initial pH of 6, DHA content in lipids was 42.8% and higner proportions of DHA in lipids were observed at more alkaline pH values. We have also found that an initial pH near 6 is optimal for production of EPA by *Mortierella elongata* NRRL 5513. An increase in unsaturated fatty acids with increasing culture pH has been reported in many fungi (28,29). Cantrell and Dowler (30) reported that the best pH for lipid production varies with fungal species but that lipid content is almost unchanged between 5.9–7.5. The latter comment is not in agreement with our findings for *T. aureum.*

When growth, lipid and DHA production by *T. aureum* was monitored with time, biomass and lipid content of biomass increased to a maximum after which biomass leveled out and lipid content declined. Wassef (31) noted that the amount of lipid produced by a given fungal species depended to a great extent on the developmental

TABLE 2

Effect of Nitrogen Source on DHA Production by *T. aureum* ATCC 34304

Nitrogen source[a]	Biomass (g/l)	Lipids in biomass (% w/w)	DHA in Biomass (mg/g)	DHA in Lipids (% w/w)	DHA Yield (mg/L)
Tryptone	3.5	10.5	44.6	42.5	156.0
Peptone	2.5	6.9	38.6	56.0	97.5
Malt extract	1.0	11.0	58.1	53.0	60.0
Yeast extract	5.0	10.5	44.5	46.9	247.7
Sodium glutamate	3.8	16.5	70.4	42.8	269.6

[a]The medium contained 0.2% of each nitrogen source and 2% glucose.

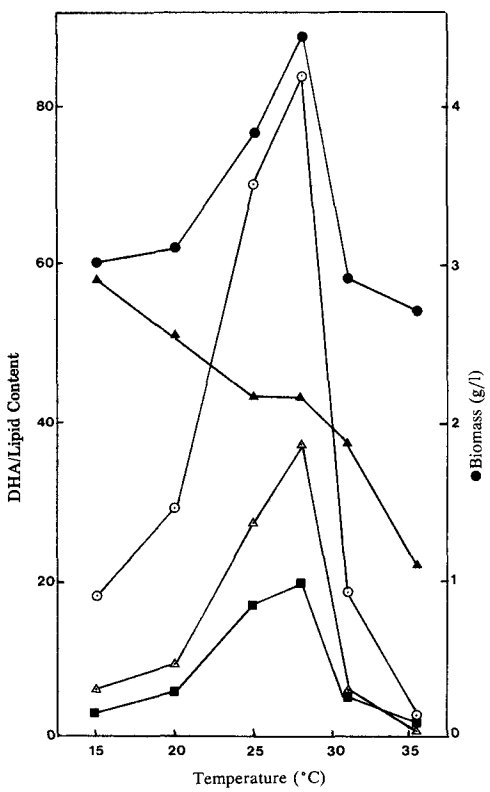

FIG. 3. Effect of temperatue on biomass, lipid and DHA production by *T. aureum* ATCC 34304. Culture medium—basal medium supplemented with 2% glucose. Legends as in Figure 1.

stage of growth. Fat formation in surface cultures of *Aspergillus nidulans* were found to accelerate at the later stages of growth, after which fat content decreased (32). A general biphasic pattern of lipid accumulation in oleaginous organisms during batch culture was described by Boulton and Ratledge (33). Lipid content of cells stays approximately constant when nutrients are in excess while after nutrient and especially nitrogen exhaustion, there is a continued build-up of lipid without a corresponding increase in biomass. This post-exponential lipid build-up was not observed with *T. aureum*. During post-exponential lipid depletion, DHA content in lipids remained relatively constant. In the case of EPA production by *M. elongata*, maximum biomass and lipid content of biomass were also observed at the same culture time (Bajpai *et al.*, unpublished results). However, in contrast to the above findings with DHA, as lipid content subsequently declined, EPA content of biomass and lipid continued to rise. Temperature optimum for DHA production by *T. aureum* was 28°C. As was observed with *T. aureum*, lower incubation temperatures generally result in an increase in unsaturation of fungal lipids (34,35). However, an increase in unsaturation has been observed in some fungi at higher incubation temperatures (36,37), while in others, little relationship has been found between fatty acid composition and culture temperature (38). In *Mucor* and *Rhizopus* species, reduction in temperature did result in production of more unsaturated lipids (31). Exposure of *T. aureum* cultures to light had a dramatic effect of increasing lipid content of biomass and DHA yields by 40–50% while biomass was increased by 13%. The ability of light to stimulate growth of *Thraustochytrium* species was first reported by Goldstein in 1963 (24). The

TABLE 3

Fatty Acid Profiles as a Function of Growth Temperature of *T. aureum* ATCC 34304

Fatty acids (% w/w)	Growth temperature					
	15°C	20°C	25°C	28°C	31°C	36°C
16:0	20.6	20.2	20.8	21.4	26.6	53.8
16:1	trace	4.4	0.9	0.8	3.6	12.0
18:0	trace	3.2	4.9	8.6	7.6	trace
18:1	6.7	7.5	18.7	14.5	16.3	11.9
18:2	trace	trace	3.7	3.4	4.0	trace
20:4	trace	2.9	2.3	1.0	trace	0.0
20:5	5.2	4.1	1.8	1.2	trace	0.0
22:6	57.7	51.1	42.8	43.1	37.0	22.3
Others (mainly 22:5)	9.8	6.6	4.1	6.0	4.9	0.0

TABLE 4

Effect of Inoculum Age on Production of DHA by *T. aureum* ATCC 34304

Inoculum age (h)	Biomass (g/L)	Lipids in biomass (% w/w)	DHA		
			in Biomass (mg/g)	in Lipids (% w/w)	Yield (mg/L)
24	3.9	18.9	91.8	48.6	357.1
48	3.9	5.6	29.2	52.1	114.2
72	3.9	4.2	22.6	53.8	88.1

TABLE 5

Effect of Light on Growth and DHA Production by *T. aureum* ATCC 34304

	Biomass (g/L)	Lipids in biomass (% w/w)	DHA in Biomass (mg/g)	DHA in Lipids (% w/w)	DHA Yield (mg/L)
Light	3.8	16.5	70.4	42.8	269.6
Dark	3.4	11.6	53.8	46.3	182.8

TABLE 6

Effect of Starch or Maltose Concentration on Production of DHA by *T. aureum* ATCC 34304

Starch concentration (% w/v)	Maltose concentration (% w/v)	Biomass (g/L)	Lipids in biomass (% w/w)	DHA in Biomass (mg/g)	DHA in Lipids (% w/w)	DHA Yield (mg/L)
2.0	—	4.9	14.7	73.8	50.1	360.1
2.5	—	4.9	20.3	103.8	51.0	510.5
3.0	—	5.0	20.2	101.1	50.1	506.5
—	2.0	4.6	16.8	82.0	48.9	374.7
—	2.5	4.8	15.8	75.4	47.8	358.2
—	3.0	4.8	17.6	84.9	48.3	410.1

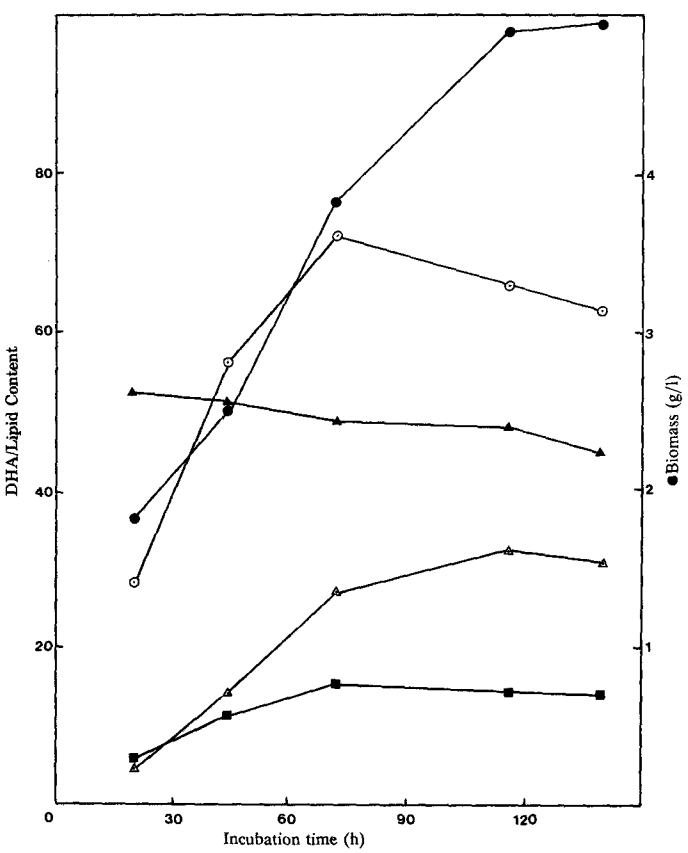

FIG. 4. Time course of growth, lipid and DHA production by *T. aureum* ATCC 34304 in a laboratory fermenter. Culture medium—basal medium supplemented with 2% starch. Legends as in Figure 1.

lower yield of DHA produced in the laboratory fermenter as compared to shake flasks most likely reflected its reduced light exposure.

In this study an optimum yield of 511 mg/L DHA was observed in shake flasks, with DHA constituting 10.4% of biomass and 51% of total lipids. Compared to other fungal fermentations, biomass yields observed in this study (5 g/L) are relatively low. By manipulating culture medium constituents and optimizing and controlling parameters such as aeration, pH and light in fermenters, potential exists to substantially increase fungal biomass production with a proportional increase in DHA yield. Future fermentation optimization studies would also investigate the use of nitrogen sources such as corn steep liquor or distiller's solubles, which are less expensive than yeast extract or glutamate.

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