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Production of high yields of docosahexaenoic acid by *Thraustochytrium roseum* ATCC 28210

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Culture conditions for growth and docosahexaenoic acid (DHA) production by *Thraustochytrium roseum* ATCC 28210 were investigated with a view to increasing DHA titers. A medium was formulated (Medium 6) which produced a biomass and DHA content of 10.4 g L^{-1} and 1011 mg L^{-1}, respectively, in a 5-day incubation. A fed-batch culture system was also developed which achieved biomass and DHA titers of 17.1 g L^{-1} and 2000 mg L^{-1}, respectively, in 12 days.

Keywords: *Thraustochytrium roseum*; fed-batch; lipid; docosahexaenoic acid

Introduction

Long-chain polyunsaturated fatty acids (PUFAs) are important dietary constituents. Their beneficial effects on human health are widely accepted and hence led to extensive nutritional and clinical studies on their effects on human physiology [8,9,13]. These fatty acids have been utilised in the prevention and treatment of heart disease and high blood pressure, in the inflammatory area (for example, treatment of asthma, arthritis and psoriasis) and for some cancer treatments [32]. PUFAs are found naturally in low levels in porcine liver as arachidonic acid, and in fish oils as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA, C22:6 ω3). DHA is synthesized from EPA by a desaturation and elongation mechanism and shares a common biosynthetic pathway with ω-3 fatty acid precursors of ecosanoid compounds [7]. DHA makes up about 60% of the structural lipid in the grey matter of the brain and is most active in neural tissues. It appears to be essential for the normal growth and functional development of the brain [11]. In some severe neurological disorders, including Alzheimer's disease, DHA levels are depleted.

The largest commercial source of DHA is fish oil, which contains 7–14% DHA. The main barriers to substantial utilization of fish oils as a source of ω-3 fatty acids relate to the undesirable fishy flavour of such products, the oxidative instability of fish oils and difficulties in producing concentrates of the individual ω-3 fatty acids [32]. Microorganisms offer an alternative and unlimited source of PUFAs. Production of PUFAs by lower fungi (such as species of *Mortierella* and *Thraustochytrium*) and some heterotrophic microalgae is typically in the form of triglycerides within the cell [27,32]. The diversity of species can facilitate the selection of microbial strains producing a large proportion of their lipid material as a single predominant fatty acid. Alternative sources are being sought amongst phytoplankton, algae, fungi and bacteria [5,20,34,35]. Some fungal species belonging to the genus *Thraustochytrium* produce significant quantities of DHA [2–4]. A high proportion of DHA in the total lipids of *Thraustochytrium* spp and relatively lower levels of structurally related PUFAs would simplify downstream processing of DHA [32,35].

A large number of variables affect microbial production of fatty acids, including medium composition [14,17,28,31], nutrient starvation [25], fatty acid precursor addition [26], pH, temperature [15,24,30], light intensity [10] and oxygen supply [12]. We have described culture conditions for the production of DHA by *T. aureum* [2,3] and *T. roseum* [21] producing maximum DHA yields of 0.51 g L^{-1} and 0.85 g L^{-1}, respectively. We report here the effects of medium components and carbon source supply on DHA production by *T. roseum* ATCC 28210 and conditions producing very high titers of DHA.

Materials and methods

Microorganisms

T. roseum 28210 was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The culture was maintained on 3% agar slants containing Medium No. 1 (Table 1) supplemented with yeast extract (2 g L^{-1}) and subcultured every 2 months.

Inocula were prepared in 250-ml Erlenmeyer flasks containing 50 ml medium and were grown at 25°C for 48 h with orbital shaking at 200 rpm. Erlenmeyer flasks (250 ml) containing 50 ml of production medium were inoculated with this 48-h inoculum at a rate of 5% v/v and incubated on an orbital shaker at 200 rpm.

Analytical methods

Dry weight of biomass was determined by centrifuging a known volume of fungal cell suspension, washing the cells with distilled water and drying them at 100°C for 12–16 h. Total carbohydrate content was determined by the method of McReady *et al* [22]. For extraction and determination of lipids, dried cells were weighed in 10-ml Teflon-lined screw cap tubes and the lipids were extracted using chloroform/methanol (1 : 2, v/v) following the method of Bligh and Dyer [6]. The extracted lipids were dried at 36°C

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Table 1 Effect of medium composition on docosahexaenoic acid production by *Thraustochytrium roseum* ATCC 28210

	Medium No.					
	1	2	3	4	5	6
Components (per litre)						
Starch (g)	25.0	25.0	25.0	25.0	25.0	25.0
$(NH_4)_2SO_4$ (g)	0.2	0.2	0.2	0.2	0.2	0.2
Na-glutamate (g)	2.0	2.0	2.0	2.0	2.0	2.0
Yeast extract (g)	–	2.0	–	–	–	2.0
KH_2PO_4 (g)	0.1	0.1	0.2	0.1	0.1	0.2
NaCl (g)	25.0	25.0	25.0	25.0	10.0	10.0
KCl (g)	1.0	1.0	1.0	1.0	1.0	1.0
$CaCO_3$ (g)	0.2	0.2	0.2	0.2	0.2	0.2
$NaHCO_3$ (g)	0.1	0.1	0.1	0.1	0.1	0.1
$MgSO_4$ (g)	5.0	5.0	5.0	5.0	5.0	5.0
$FeCl_3 \cdot 6H_2O$ (mg)	–	–	–	2.9	–	2.9
$CuSO_4 \cdot 5H_2O$ (mg)	–	–	–	0.02	–	0.02
$MnCl_2 \cdot 4H_2O$ (mg)	–	–	–	8.6	–	8.6
$CoCl_2 \cdot 6H_2O$ (mg)	–	–	–	0.26	–	0.26
$ZnCl_2$ (mg)	–	–	–	0.6	–	0.6
Thiamine (μg)	10.0	10.0	10.0	10.0	10.0	10.0
Vitamin B12 (μg)	1.0	1.0	1.0	1.0	1.0	1.0
Analytical parameters						
Final pH	7.8	8.1	7.1	7.9	7.8	–
Biomass (g L^{-1})	6.1	8.6	7.2	8.0	6.4	–
Lipid in biomass (%)	16.5	18.0	17.9	18.7	15.8	–
DHA						
in biomass (mg g^{-1})	86.9	104.2	104.1	90.3	87.6	–
in lipid (%, w/w)	52.7	58.1	58.2	48.3	55.5	–
yield (mg L^{-1})	528.0	892.0	752.0	724.0	557.0	–

Medium No. 1 is from Ref [2]. Media numbers 2–6 were modified from Medium No. 1; pH of the media was adjusted to 6.0 before autoclaving. Cultures were incubated at 25°C for 5 days on a rotary shaker (200 rpm).

under nitrogen and methylated with 6% H_2SO_4 in methanol using the method of Holub and Skeaff [19]. The methyl esters were finally dissolved in hexane and analyzed by gas chromatography. The Shimadzu GC-14A (Kyoto, Japan) was connected with a CR601 Chromatopac data integrator. The GC was fitted with a fused silica column (DB-17, Chromatography Specialities, Brockville, ONT, Canada) and a flame ionization detector with helium as the carrier gas. The fatty acid methyl ester peaks were identified and quantified using standard fatty acids supplied by Sigma (St Louis, MO, USA). Pentadecanoic acid was used as an internal standard.

Results and discussion

Growth and DHA production of *T. roseum* was studied in media 1–5 (Table 1). Medium No. 1 was used earlier in studies on the production of DHA by *T. aureum* [3] and *T. roseum* [21]. This medium produced a biomass and DHA yield of 6.1 g L^{-1} and 528 mg L^{-1}, respectively, with *T. roseum* ATCC 28210 in a 5-day incubation. Media 2–5 are modifications of Medium 1. Supplementation with yeast extract (Medium No. 2) resulted in an increased biomass content (8.6 g L^{-1}) and DHA yield (892 g L^{-1}). Medium No. 3 contained an increased phosphate concentration to minimize pH variation and improved DHA yield by about 40%. Supplementation of Medium 1 with trace metals improved growth of *T. roseum* (Medium No. 4). Several metal ions promote synthesis of lipids and fatty acids in microorganisms [1,27,33,35]. While a deficiency of manganese decreased the PUFA content of *Mortierella* sp S-17 [29], more lipid and PUFAs were accumulated by *M. ramanniana* when the medium was supplemented with copper and zinc [18]. Acetyl CoA carboxylase catalyses the initial step of fatty acid synthesis and requires bivalent metal ions (Mg^{2+} or Co^{2+}) as cofactors [16,23]. When concentrations of trace elements were increased above the values shown in Medium No. 4, a reduction in DHA content was observed (data not shown).

No significant differences in DHA yields were observed with this marine organism when the sodium chloride content of Medium No. 1 (25 g L^{-1}) was reduced to 10 g L^{-1} (Medium No. 5).

The time course of biomass and DHA production was studied in a medium incorporating yeast extract, the trace metal supplement, the lower sodium chloride level and the higher phosphate concentration from the above studies (Medium No. 6). About 84% of total carbohydrates were utilized by *T. roseum* within 4 days of incubation. Biomass content increased with cultivation time and reached the maximum (10.4 g L^{-1}) after 5 days (Figure 1). At this time, 95% of the substrate had been consumed by *T. roseum*, giving a maximum value of lipid content in biomass of 19.6%, w/w. DHA content in biomass and overall DHA yield also increased with the cultivation time, showing a positive correlation with biomass yield and reaching maximum values of 102.2 mg g^{-1} and 1061 mg L^{-1}, respectively, after 5 days. DHA content in the lipid remained more or less constant (50–54%) for up to 5 days and decreased slightly during post-exponential lipid depletion. The relationship between the fatty acid profile and incubation time is provided in Table 2. There was no significant change in the amount of total saturated or total unsaturated fatty acids. An increase in oleic and linoleic acids was observed with a concomitant decrease in DHA after 6 days.

Attempts were made to increase biomass and DHA concentrations further in cultures incubated under the conditions described above in Medium No. 6 by supplying additional doses of carbohydrate (starch or glucose) equivalent to 10 g L^{-1}, after both 4 and 6 days in a fed-batch

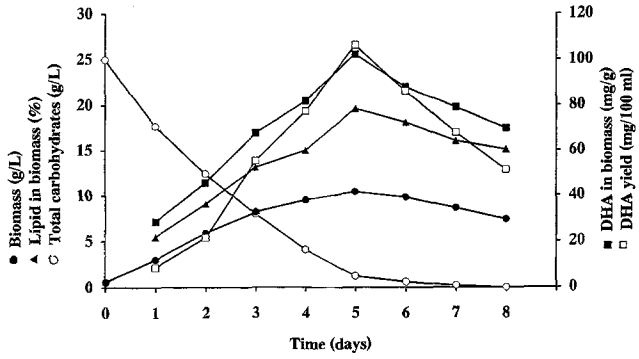

Figure 1 Biomass and DHA production by *Thraustochytrium roseum* ATCC 28210. The culture was incubated in Medium No. 6 at 25°C for 5 days on a rotary shaker (200 rpm). ●, Biomass (g L^{-1}); ▲, Lipid in biomass (%); ○, total carbohydrates (g L^{-1}); ■, DHA in biomass (mg g^{-1}); □, DHA yield (mg per 100 ml).

Table 2 Time course of fatty acid synthesis by *Thraustochytrium roseum* ATCC 28210

Time (days)	Fatty acids (%, w/w)								
	14:0	16:0	18:0	18:1	18:2	18:3	20:4	22:6	others
1	2.1	14.8	4.8	11.6	3.9	0	0.4	52.4	10.0
2	4.5	10.1	4.5	13.8	3.2	0	0.5	50.1	13.3
3	4.2	11.6	4.2	14.5	3.6	0.2	1.8	51.9	8.0
4	3.6	8.5	5.1	16.3	3.0	0.4	2.5	54.6	6.0
5	3.2	7.8	4.2	16.0	2.2	1.5	3.0	52.3	9.8
6	2.9	9.3	3.9	18.0	2.8	1.6	3.5	48.6	9.4
7	2.0	9.6	3.3	19.8	3.5	1.6	3.0	49.6	7.6
8	1.9	9.8	3.3	22.2	4.1	1.7	3.1	46.3	7.6

system. The results are presented in Figure 2. Additional starch dramatically increased biomass, lipid and DHA contents. Maximum biomass (12.1 g L^{-1}), lipid in biomass (23.7%) and DHA in biomass (118 mg g^{-1}) were produced after a fermentation of 8 days. This procedure gave the highest DHA yield (1433 mg L^{-1}) achieved to date. Supply of additional glucose resulted in a DHA yield of 1130 mg L^{-1} after 8 days.

The fed-batch system was extended in further experiments in shake flasks containing Medium No. 6 (Figure 3). In each case, 10 g L^{-1} aliquots of starch were added to the culture after 4, 6, 8, 10, and 12 days. In the case of (b), 0.8 g L^{-1} of yeast extract was also added at each addition point. In the case of (c), sodium glutamate, 0.8 g L^{-1}; $(NH_4)_2SO_4$, 0.08 g L^{-1}; KH_2PO_4, 0.08 g L^{-1} and $MgSO_4$, 2 g L^{-1} were added at each addition point. Maximum biomass and DHA production were observed in fed-batch media, system (c), after 12 days. Biomass and DHA yields were 17.1 g L^{-1} and 2.0 g L^{-1}, respectively. The percentage of DHA in total lipids was 48.8%.

Thus conditions were established in a fed-batch culture system which produced high biomass density and very high titers of DHA.

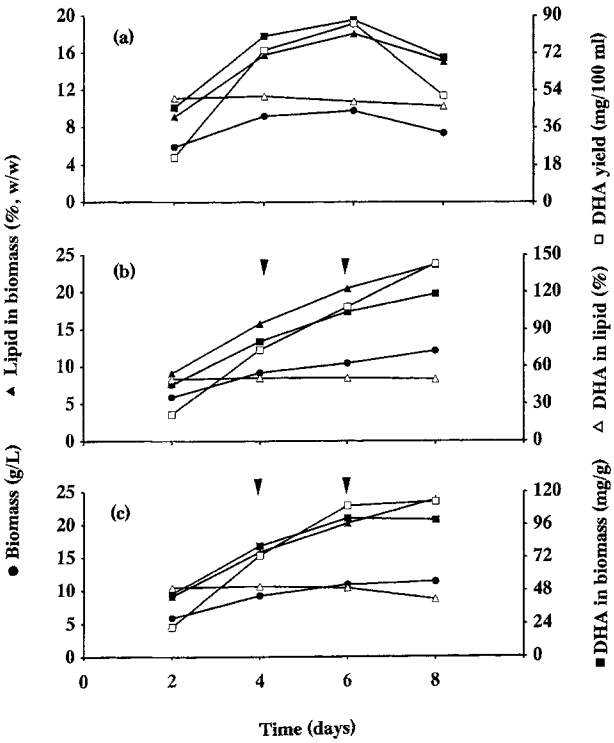

Figure 2 Effect of carbon source supply on DHA production by *Thraustochytrium roseum* ATCC 28210. The culture was incubated in Medium No. 6 containing 25 g L^{-1} starch at 25°C on a rotary shaker (200 rpm). Additional carbon source was supplied after 4 and 6 days of fermentation: (a) no addition; (b) starch (10 g L^{-1}); and (c) glucose (10 g L^{-1}) addition. Arrowheads indicate the times of carbon source addition. ●, Biomass (g L^{-1}); ▲, Lipid in biomass (%, w/w); ■, DHA in biomass (mg g^{-1}); △, DHA in lipid (%); □ DHA yield (mg per 100 ml).

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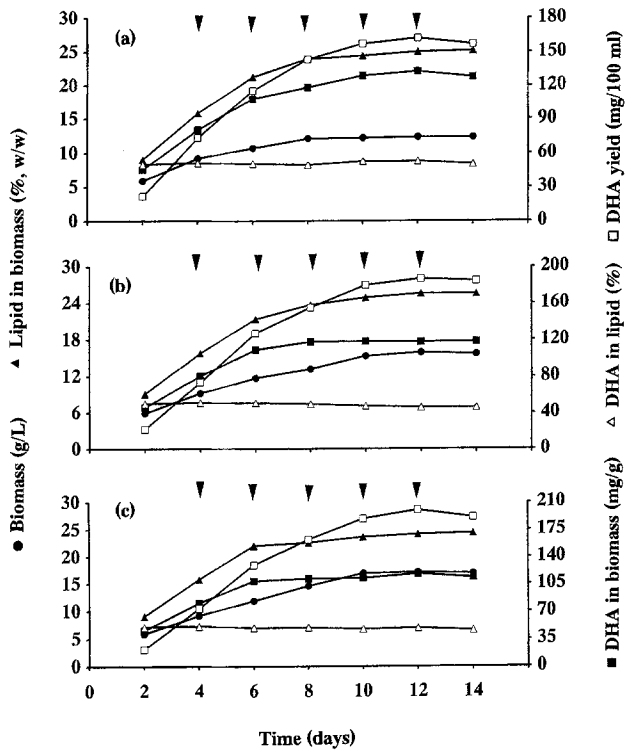

Figure 3 Effect of starch and nutrient supply on DHA production by *Thraustochytrium roseum* ATCC 28210. The culture was incubated in Medium No. 6 containing 25 g L^{-1} starch at 25°C on a rotary shaker (200 rpm). Additional starch or nutrients (at 40% level of the initial) were supplied after 4 days at 2-day intervals as indicated by arrowheads: (a) addition of starch (10 g L^{-1}); (b) addition of starch (10 g L^{-1}) and yeast extract (0.8 g L^{-1}); and (c) addition of starch and nutrients. In the case of (c), supplemented medium contained starch, 10 g L^{-1}; sodium glutamate, 0.8 g L^{-1}; yeast extract, 0.8 g L^{-1}; $(NH_4)_2SO_4$, 0.08 g L^{-1}; KH_2PO_4, 0.08 g L^{-1}; and $MgSO_4$, 2 g L^{-1}. ●, Biomass (g L^{-1}); ▲, Lipid in biomass (%, w/w); ■, DHA in biomass (mg g^{-1}); △, DHA in lipid (%); □, DHA yield (mg per 100 ml).

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