Journal of Industrial Microbiology, 13 (1994) 238–241

Published by The Macmillan Press Ltd

Production of docosahexaenoic acid by *Thraustochytrium roseum*

Zu yi Li and O.P. Ward

Microbial Biotechnology Laboratory, Department of Biology, University of Waterloo, Waterloo, Ontario, Canada N2L 3G1

(Received 20 January 1994; accepted 27 March 1994)

Key words: Docosahexaenoic acid; *Thraustochytrium*, Omega-3 fatty acids; Fermentation

SUMMARY

When three *Thraustochytrium* stains were cultivated in liquid media containing 2.5% starch and 0.2% yeast extract, initial pH 6.0, with shaking under fluorescent light for five days at 25 °C, similar biomass yields were observed (9.7–10.3 g L^{-1}). Contents of docosahexaenoic acid (DHA) in biomass varied: 0.15, 3.55 and 6.40% w/w for *T. striatum* ATCC 24473, *T. aureum* ATCC 34304 and *T. roseum* ATCC 28210, respectively. In further studies, *T. roseum* produced a maximum titer of 0.85 g of DHA per liter of culture broth. The DHA content of total lipids ranged from 46–49% w/w.

INTRODUCTION

The long-chain omega-3 polyunsaturated fatty acids (PUFAs), eicosapentaenoic acid (EPA, C-20:5) and docosahexaenoic acid (DHA, C-22:6) have been shown to have positive effects in the prevention or treatment of different cardiovascular disorders, such as arteriosclerosis and myocardiac infarct [10]. Recently, Akoh and Hearnsberger [1] demonstrated that diets high in *n*-3 PUFA, mainly EPA and DHA, have comparable effects in altering platelet lipid composition and blood-clotting factors, and may reduce the incidence of thrombosis. DHA is also essential for normal growth and functional development of the brain because it is a component of the membrane phospholipid [5]. DHA and EPA can be interconverted in vivo, that is, DHA can serve as a pool for EPA [9]. The current commercial source of DHA is fish and fish oils, which also contain EPA and other more saturated fatty acids. Because of the relatively low proportion of DHA in fish oil and the problems encountered in extraction and purification of omega-3 fatty acids, large-scale production of DHA is difficult. A survey of the literature [11] indicated that some microorganisms within the orders Saprolegniales and Entomorphorales, and especially strains of *Thraustochytrium*, produce significant quantities of DHA [2,6]. We have previously described conditions for production of DHA by *Thraustochytrium aureum* ATCC 34304 which yielded 0.511 g DHA per liter of culture broth [3]. Under these conditions, DHA content of total lipids was 51% w/w.

In the present study, we report levels of DHA produced by three species of *Thraustochytrium* and conditions for production of higher titers of DHA from *T. roseum* ATCC 28210.

Correspondence to: O.P. Ward, Microbial Biotechnology Laboratory, Department of Biology, University of Waterloo, Waterloo, Ontario, Canada N2L 3G1.

MATERIALS AND METHODS

Organisms

All *Thraustochytrium* sp. were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA).

Culture medium

The basal medium contained (g L^{-1}): NaCl, 25; $MgSO_4 \cdot 7H_2O$, 5; KCl, 1; KH_2PO_4, 0.1; $CaCO_3$, 0.2; $(NH_4)_2SO_4$, 0.2; $NaHCO_3$, 0.1; monosodium glutamate, 2.0.

Thraustochytrium species were maintained on 3% agar slants containing the basal medium supplemented with 2% w/v glucose and 0.2% w/v yeast extract. Cultures were subcultured every two months.

Culture conditions

Liquid cultures containing 50 ml of basal medium, supplemented with 2.5% w/v starch and 0.2% w/v yeast extract, in 250-ml Erlenmeyer flasks, were inoculated and incubated at 25 °C with orbital shaking at 250 r.p.m. for 2 days. A 5% w/v inoculum from these flasks was used to inoculate 250-ml Erlenmeyer flasks containing 50 ml of basal medium supplemented with carbon source and yeast extract as indicated in the Results section. Flasks were incubated for five days on an orbital shaker at 250 r.p.m. in light at 25 °C. The orbital shakers were Lab-line incubator shakers, model 3525 (Melrose Park, IL, USA), fitted with two 33-watt, 24-in fluorescent tubes in the lid at a distance of 14 in from the shaker platform.

Biomass determinations

Dry weight of biomass was determined by vacuum filtration or centrifugation of fungal cell suspension, washing

TABLE 1

Comparison of docosahexaenoic acid production by *Thraustochytrium* spp. and their cell fatty acid composition

Strain	Biomass ($g\ L^{-1}$)	Principal fatty acid composition (% w/w of total fatty acid)								DHA in biomass (% w/w)	Yield ($g\ L^{-1}$)
		16:0	18:0	18:1	18:2	18:3	20:4	20:5	22:6		
T. aureum ATCC 34304	9.7	33.84	12.05	23.59	3.09	1.28	0.64	1.40	32.85	3.55	0.34
T. roseum ATCC 28210	10.1	26.25	8.38	12.74	2.21	0.82	0.51	1.12	46.70	6.40	0.64
T. striatum ATCC 24473	10.3	33.92	8.21	48.21	3.32	0.24	0.35	0.25	0.75	0.15	0.014

The basal medium was supplemented with 2.5% starch.

it with 1% w/v NaCl and distilled water and drying it at 100 °C for 12–16 h.

Extraction and determination of lipids

Dried cells were weighed (20–40 mg) in 10-ml Teflon-lined screw cap test tubes of 10-ml capacity and the lipids were extracted according to the procedure of Bligh and Dyer [4]. The extracted lipids were dried at 36 °C under N_2, methylated using the method of Holub and Skeaff [7], the methyl esters being dissolved in 200 μl *n*-hexane and a 1-μl sample used for analysis. The Shimadzu (Kyoto, Japan) CR601 GLC was connected with GC-14A data integrator. The GLC was fitted with fused silica megabore column, 30 m in length and 0.52 mm internal diameter, coated with a 1-μm thickness of 25% cyanopropyl, 25% phenyl and 50% methylpolysiloxane (DB-225, Chromatographic Specialities, Brockville, Ontario, Canada) and a flame ionization detector. Helium was used as the carrier gas. The fatty acid ester peaks were identified and quantified using standard fatty acids supplied by Sigma Chemical Company (St Louis, MO, USA). Pentadecanoic acid (C15:0) was used as internal standard. Data are the averages of three determinations.

RESULTS AND DISCUSSION

Growth and fatty acid production by *T. roseum* ATCC 28210 was compared with the values obtained for two other strains, *T. aureum* ATCC 34304 and *T. striatum* ATCC 24473, cultured simultaneously for five days in the basal medium supplemented with 2.5% starch, as described in the Methods section. The results are presented in Table 1. Similar levels of biomass were produced by each of the cultures (9.7–10.3 $g\ L^{-1}$). *T. roseum* ATCC 28210 manifested a more dispersed morphology in culture than did the other strains and produced 0.64 $g\ L^{-1}$ of DHA. The DHA titer observed with *T. aureum* was 0.34 $g\ L^{-1}$ while *T. striatum* had a low DHA-producing ability. Neither *T. roseum* nor *T. aureum* produced significant amounts of other polyunsaturated fatty acids which has important implications for downstream processing.

The effect of carbon source, added to the basal medium, on biomass, lipid and DHA production by *T. roseum* ATCC 28210 is presented in Table 2. Canola oil and corn oil produced similar yields of biomass and lipid but the DHA content of the biomass was low. A negligible DHA titer was observed with sucrose as carbon source. DHA titers produced with glucose and starch as carbon source were 0.41 and 0.65 $g\ L^{-1}$, respectively. When the effect of starch concentration on growth and DHA production was investigated, biomass yield and DHA production increased with starch concentration up to a value of 2.5% starch (Table 3).

The effect of incubation temperature on biomass yield and production of DHA is illustrated in Fig. 1. The amount

TABLE 2

Effect of carbon source on DHA production by *T. roseum* ATCC 28210

Carbon source	Biomass ($g\ L^{-1}$)	Lipid in biomass (% w/w)	DHA		
			in biomass (% w/w)	in total fatty acid (% w/w)	yield ($g\ L^{-1}$)
Glucose	6.65	15.6	6.20	40.8	0.41
Sucrose	1.21	11.2	1.21	21.2	0.014
Starch	7.56	18.2	8.71	49.0	0.65
Corn oil	4.22	19.8	2.32	11.9	0.096
Canola oil	5.40	18.5	1.92	12.2	0.10

Carbon sources (2.5% w/w) were added to the basal medium.

TABLE 3

Effect of concentration of starch on the production of DHA by *T. roseum* ATCC 28210

Starch ($g\ L^{-1}$)	Biomass ($g\ L^{-1}$)	Lipid in biomass (% w/w)	DHA		
			in biomass (% w/w)	in total fatty acid (% w/w)	yield ($g\ L^{-1}$)
10.0	2.41	11.01	5.41	46.7	0.13
20.0	6.48	15.50	7.72	49.8	0.49
25.0	7.56	17.47	8.70	49.8	0.65
30.0	7.70	17.42	8.45	48.5	0.63

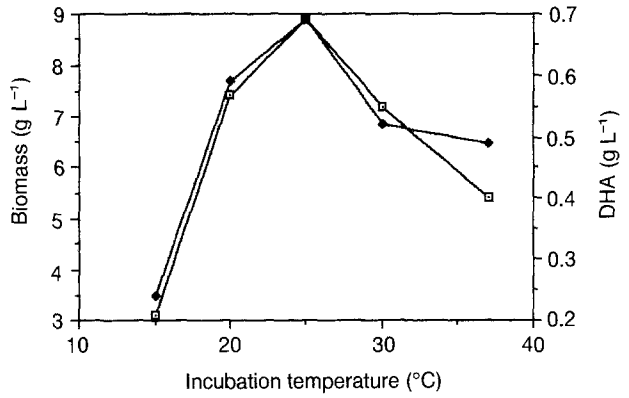

Fig. 1. Effect of the incubation temperature on the production of DHA by *T. roseum* ATCC 28210. The basal medium was supplemented with 2.5% starch; —⊡—, biomass ($g\ L^{-1}$); —♦—, DHA ($g\ L^{-1}$).

of DHA increased in parallel with cell growth and reached a maximum at 25 °C. Likewise, when the initial pH of the growth medium was varied in the range 4–9 (Fig. 2), maximum biomass and DHA titers were observed at an initial pH 6.0 with ratios of DHA to biomass remaining almost constant over that pH range.

Supplementation of the basal medium containing 2.5% starch, with 0.2% yeast extract, enhanced DHA production. The time course of cell growth and DHA production for *T. roseum* ATCC 28210 in this yeast extract-supplemented medium is presented in Fig. 3. Maximum biomass yield (9.8 $g\ L^{-1}$) was observed after five days cultivation. The amount of DHA increased in parallel with the cell biomass and reached the maximum on the fifth day (0.85 $g\ L^{-1}$, 85.9 $mg\ g^{-1}$ dry cells).

Kendrick and Ratledge [8] characterized the *n*-3 and *n*-6 polyunsaturated fatty acid components of molds, including *T. aureum* ATCC 34304 and *T. roseum* ATCC 28210, grown for 72 h in a minimal salts medium containing glucose and yeast extract in vortex-aerated 1-L bottles containing 800 ml medium. The biomass production and lipid content in biomass was much lower in *T. roseum* than in *T. aureum*. *T. roseum* biomass yield was 1.2 $g\ L^{-1}$ and the lipid content of the biomass was low (0.5% w/w). These contrasting results illustrate that differences in cultivation procedures profoundly affect growth and fatty acid production by these marine organisms.

To the best of our knowledge the DHA yields observed above for *T. roseum* are the highest titers reported for DHA production in microbial culture. Given the close correlation between biomass and DHA production observed in Fig. 3, there is potential to significantly enhance volumetric DHA

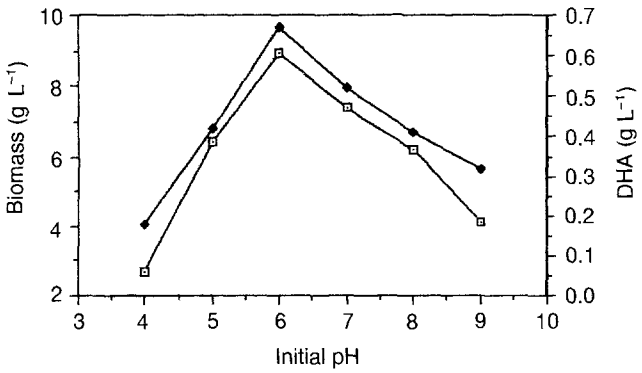

Fig. 2. Effect of the initial pH of the growth medium on the production of DHA by *T. roseum* ATCC 28210. The basal medium was supplemented with 2.5% starch; —⊡—, biomass ($g\ L^{-1}$); —♦—, DHA ($g\ L^{-1}$).

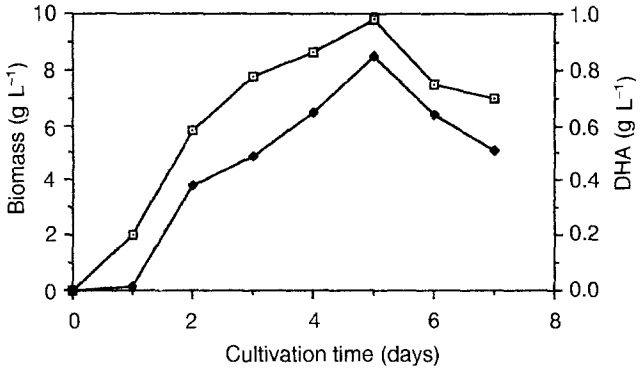

Fig. 3. Time course of growth and production of DHA by *T. roseum* ATCC 28210. The basal medium was supplemented with 2.5% starch + 0.2% yeast extract; —⊡—, biomass ($g\ L^{-1}$); —♦—, DHA ($g\ L^{-1}$).

titers in the culture by focusing on optimization of biomass production.

ACKNOWLEDGEMENTS

Support for this research by the Natural Sciences and Engineering Research Council of Canada is gratefully acknowledged.

REFERENCES

1 Akoh, C.C. and J.O. Hearnsberger. 1991. Effect of catfish and salmon diet on platelet phospholipid and blood clotting in healthy men. J. Nutr. Biochem. 2: 329.

2 Bajpai, P., P. Bajpai and O.P. Ward. 1991. Production of docosahexaenoic acid (DHA) by *Thraustochytrium aureum*. Appl. Microbiol. Biotechnol. 35: 706–710.

3 Bajpai, P., P. Bajpai and O.P. Ward. 1991. Optimization of production of docosahexaenoic acid (DHA) by *Thraustochytrium aureum* ATCC 34304. J. Amer. Oil Chem. Soc. 68: 509–514.

4 Bligh, E.G. and W.J. Dyer. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37: 911–917.

5 Connor W.E., N. Martha and D.S. Lin. 1990. Dietary effects on brain fatty acid composition, the reversibility of *n*-3 fatty acid deficiency and turnover of docosahexaenoic acid in brain, erythrocytes and plasma of rhesus monkeys. J. Lipid Res. 31: 237–247.

6 Haskins, R.H., A.P. Tulloch and R.G. Micetich. 1964. Steroids and the stimulation of sexual reproduction of a species of *Pythium*. Can. J. Microbiol. 10: 187–195.

7 Holub, B.J. and C.M. Skeaff. 1987. Nutritional regulation of cellular phosphatidylinositol. Meth. Enzymol. 141: 234–244.

8 Kendrick, A. and C. Ratledge. 1992. Lipids of selected molds grown for production of *n*-3 and *n*-6 polyunsaturated fatty acids. Lipids 27: 15–20.

9 Langholz, P., P. Anderson, T. Forskov and W. Schmidtsdorff. 1989. Application of a specificity of *Mucor miehei* lipase to concentrate docosahexaenoic acid (DHA). J. Am. Oil Chem. Soc. 66: 1120–1123.

10 Leaf, A. and P.C. Weber. 1988. Cardiovascular effects of *n*-3 fatty acids. N. Eng. J. Med. 318: 549–551.

11 Yongmanitchai, W. and O.P. Ward. 1989. Omega-3 fatty acids: alternative sources of production. Process Biochem. 24: 117–125.