Journal of Industrial Microbiology, 9 (1992) 11–18
Elsevier

SIM 00380

Optimisation of culture conditions for production of eicosapentaenoic acid by *Mortierella elongata* NRRL 5513

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(Received 25 February 1991; accepted 23 September 1991)

Key words: Eicosapentaenoic acid; *Mortierella elongata*; Omega-3 fatty acids

SUMMARY

When *Mortierella elongata* NRRL 5513 was cultured in shake flasks at 25 °C, mycelial growth reached a stationary phase at 48 h but maximum eicosapentaenoic acid (EPA) production was observed at 6 days. When incubated at 11 °C, EPA production also continued to rise during the stationary phase of growth, reaching a maximum after 10 days. An initial culture pH of 6.1 was found to be optimum for EPA production. The effect of temperature on EPA production was dependent on medium constituents. In glucose and linseed oil supplemented media, optimum temperature for EPA production was 11 and 15 °C respectively. A maximum EPA yield of 0.61 g/l was obtained in linseed oil (2%), yeast extract (0.5%) supplemented basal medium. Maximum EPA content as a percentage of lipids (15.12%) was observed when the latter medium was supplemented with 0.25% urea.

INTRODUCTION

Eicosapentaenoic acid (EPA) has been shown to be of major importance in the prevention and treatment of a range of human diseases and disorders. EPA produces beneficial nutritional or pharmacological effects, especially with respect to three areas: the heart and circulatory system [6, 14, 23], the inflammatory area [12, 16, 29] and cancers of mammary and colon tissue [4, 15]. The predicted market for omega-3 fatty acids is $790 million [24].

The major commercial source of EPA is from fish and fish oil [27]. Fish oil concentrates are considered unattractive because they contain substantial amounts of undesirable fatty acids and cholesterol [19, 26]. Consequently, alternative sources of EPA are being sought, especially from algae [17, 27, 28] and from fungi of the order Mucorales [9, 19, 22]. When strains of *Mortierella alpina* and *Mortierella elongata* were screened for EPA production, *M. elongata* NRRL 5513 was found to produce the highest EPA yield when incubated at 11 °C for 10 days in the presence of 2% linseed oil [1]. In the present report, studies have been carried out on optimisation of culture conditions with respect to EPA production by *M. elongata* NRRL 5513.

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MATERIALS AND METHODS

Stock cultures

Mortierella elongata NRRL 5513 was maintained on 3% agar slants containing 20 g/l glucose and 10 g/l yeast extract and subcultured every two months.

Culture conditions

Mycelium, freshly grown on agar slants, was used to inoculate 250-ml Erlenmeyer flasks containing 50 ml of basal medium supplemented with 30 g/l linseed oil and 0.5 g/l yeast extract. Flasks were incubated at 25 °C on an orbital shaker, set at 300 rpm, to produce an inoculum for EPA production cultures. Liquid EPA production cultures also contained the basal medium with supplements and was dispensed in 50-ml volumes in 250-ml Erlenmeyer flasks, inoculated with 5% inoculum and incubated on an orbital shaker at 300 rpm. The basal medium consisted of (g/l): KH_2PO_4, 2.4; KNO_3, 1; $CaCl_2 \cdot 2H_2O$, 0.1; $MgSO_4 \cdot 7H_2O$, 0.5; $FeCl_3 \cdot 6H_2O$, 0.015; $ZnSO_4 \cdot 7H_2O$, 0.0075 and $CuSO_4 \cdot 5H_2O$, 0.0005 [9]. Medium used for fungal surface growth and EPA production contained 3% agar, basal medium and linseed oil supplement. This medium was inoculated with a loopful of mycelium from the inoculum cultures grown in flasks.

Each data point in shake flask experiments reflects the average of a minimum of four determinations, based on the analysis of replicate samples from two separate flasks. Variations in values observed between flask pairs was less than 7%. In the case of agar plates, larger variations were

observed. Therefore, triplicate plates were inoculated simultaneously, and at least two plates were utilised for analysis. The third plate was only used if the analytical results of the first two plates were more than 10% apart.

Analytical procedures

Fungal biomass was recovered from liquid media by vacuum filtration (using Whatman filter paper, grade GF/F having 0.7-mM size pores) or centrifugation at 3000 × *g*, followed by washing with 50 ml ether acidified with 0.5 ml of 2NHCl and then with 50 ml distilled water [20] and drying at 100 °C for 12–16 h. This washing protocol was developed by Shimizu et al. [20] for washing *M. alpina* mycelium recovered from linseed oil containing cultures prior to recovery of EPA from the mycelium. Fungal biomass was recovered from agar cultures by scraping with spatula.

Extraction and determination of lipids

The dried cells were weighed (20–40 mg) in teflon-lined screw cap test tubes of 10 ml capacity in duplicate and the lipids were extracted according to the procedure of Bligh and Dyer [3]. The extracted lipids were dried at 36 °C under nitrogen atmosphere and then methylated using the method of Holub and Skeaff [10]. Then the fatty acid methyl esters were dissolved in 200 µl *n*-hexane and an 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, St. Louis, MO. Pentadecaenoic acid (C15 : 0) was used as internal standard.

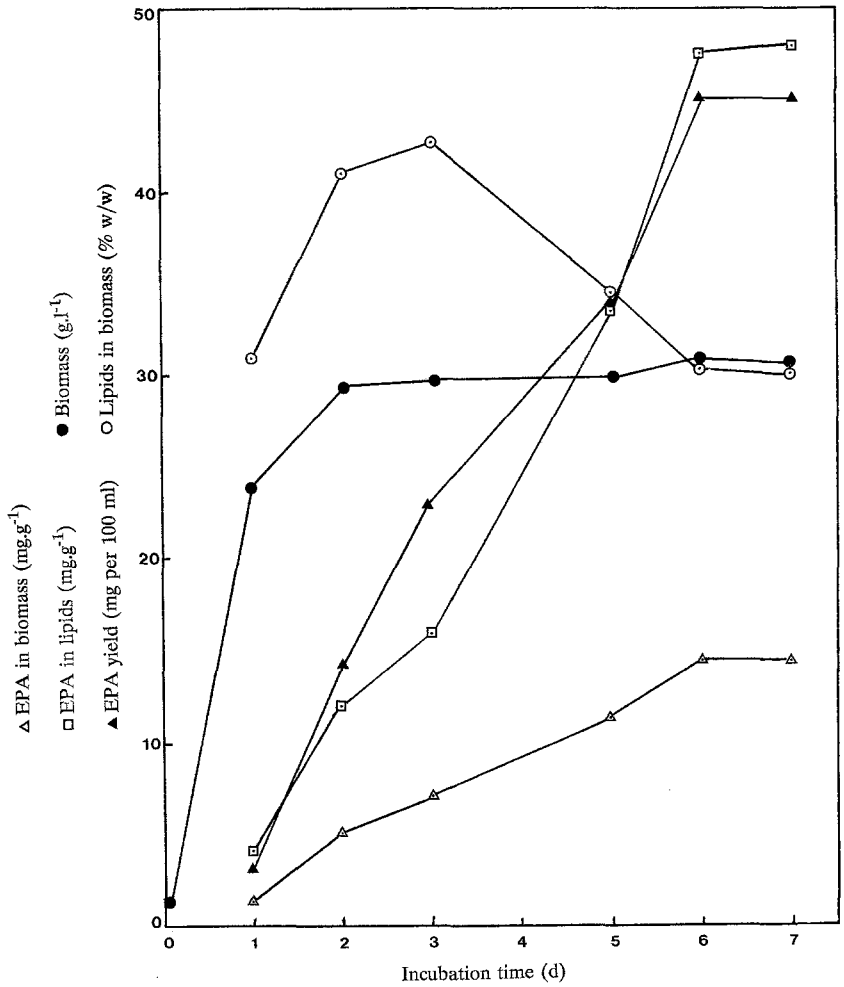

Fig. 1. Pattern of production of EPA, biomass and lipid by *Mortierella elongata* NRRL 5513 at 25 °C in shake flask culture. Medium: basal medium + 3% linseed oil.

Chemicals

Standard fatty acids were obtained from Sigma, St. Louis, MO. Solvents and reagents were obtained from Aldrich, Milwaukee, WI and British Drug House, Toronto, Ontario. General medium constituents were obtained from Difco, Detroit, MI, and linseed oil was supplied by Recochem Inc., Canada. This linseed oil contained (%, w/w): palmitic acid, 4.9; stearic acid, 3.0; oleic acid, 19.5; linoleic acid, 14.5; α-linolenic acid, 53.3; and other acids 3.7.

RESULTS

In preliminary studies on this organism, cultivated at 11 and 25 °C, maximum EPA was produced in the basal medium containing 2.5 and 3% linseed oil respectively. The patterns of biomass development, and production of lipids and EPA, under these conditions in submerged culture were monitored and the results are presented in Fig. 1 and 2. At 25 °C, fungal growth and lipid production in biomass was essentially complete after 48 h and, although biomass remained relatively constant up to 8 days a decline in lipid is observed after 3 days. EPA content of biomass increased during the stationary phase of growth from 4.89 mg/g after 48 h to a plateau of 14.5 mg/g after 6 days. EPA content of lipid and overall EPA yields reached maxima of 4.76% w/w and 0.45 g/l respectively after 6 days (Fig. 1).

When incubated at 11 °C, biomass and lipid content in biomass was maximum after 8 days. However, EPA content of biomass and overall EPA yield increased to 22.12 mg/g and 0.55 g/l after 10 days (Fig. 2). These levels were about 51 and 22% higher, respectively, than the corresponding EPA levels observed at the higher temperature.

The time course of biomass growth and EPA production was also monitored in agar surface cultures. At 25 °C, maximum biomass and lipid content of biomass was observed after 15 days after which biomass and lipid content declined (Fig. 3A). EPA content of biomass reached a maximum after 27 days while EPA content in lipids continued to increase to completion of the experiment at 36 days. When agar cultures were first incubated at 25 °C for 24 h and then incubated at 4 °C (Fig. 3B) a similar pattern was observed. Biomass and lipid content of biomass was maximum after a 20-day incubation whereas a higher EPA content was observed in biomass and lipids after a more prolonged incubation (36 days). The maximum EPA

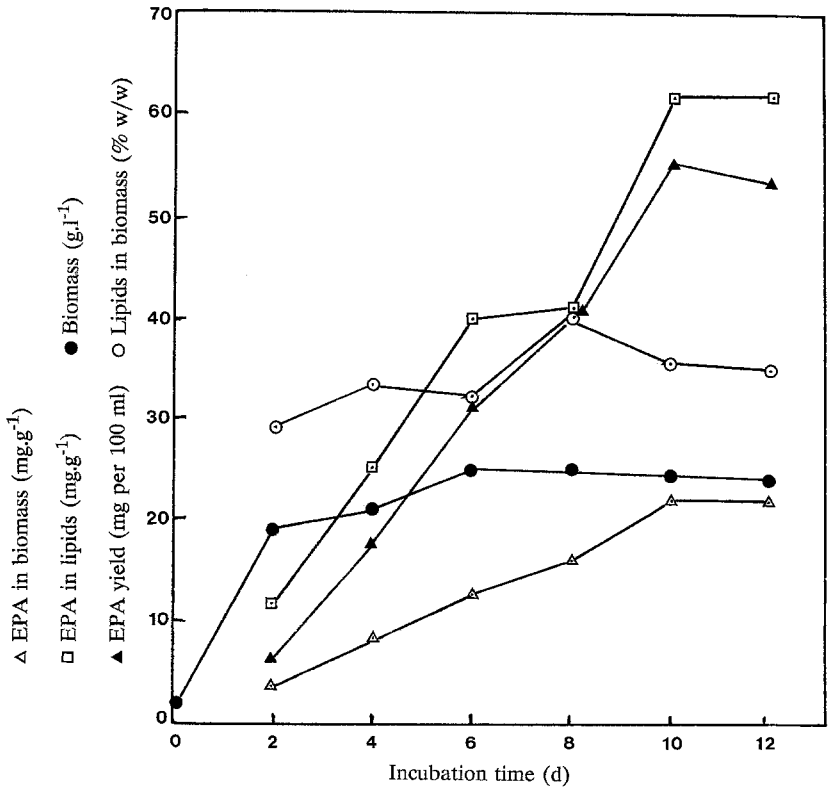

Fig. 2. Pattern of production of EPA, biomass and lipid by *M. elongata* NRRL 5513 at 11 °C in shake flask culture. Medium: basal medium + 2.5% linseed oil.

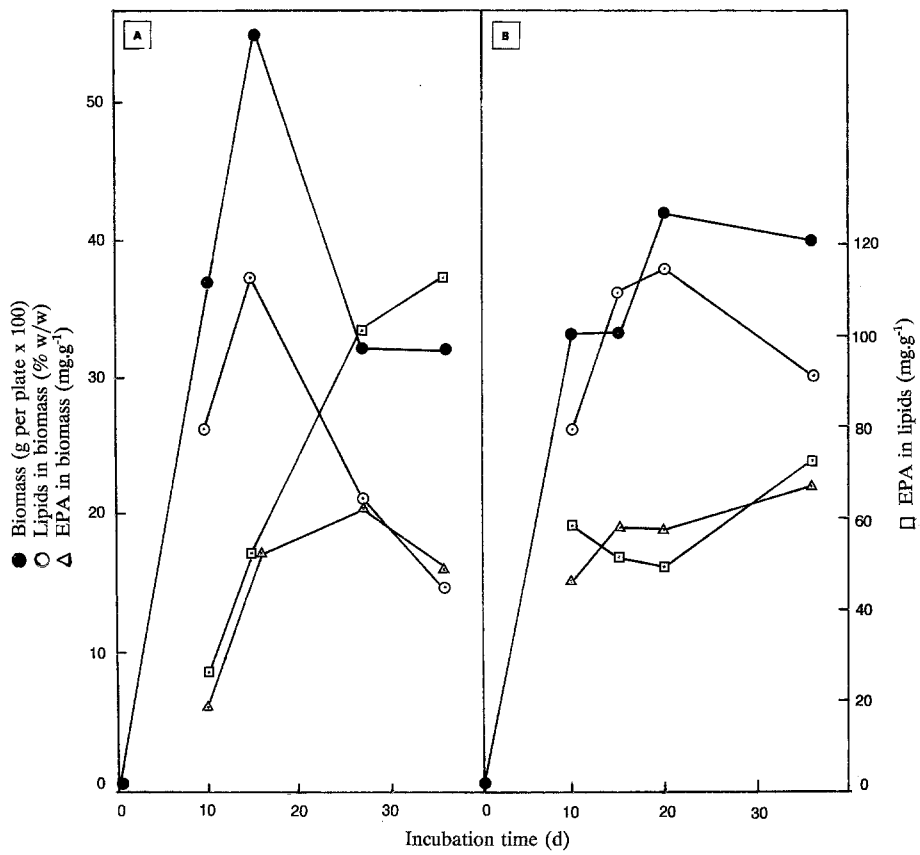

Fig. 3. Pattern of production of EPA, biomass and lipid by *M. elongata* NRRL 5513 in surface agar cultures at (A) 25 ° and (B) 11 °C. Medium: 2% agar + basal medium; linseed oil, 3% at 25 °C and 2.5% at 11 °C.

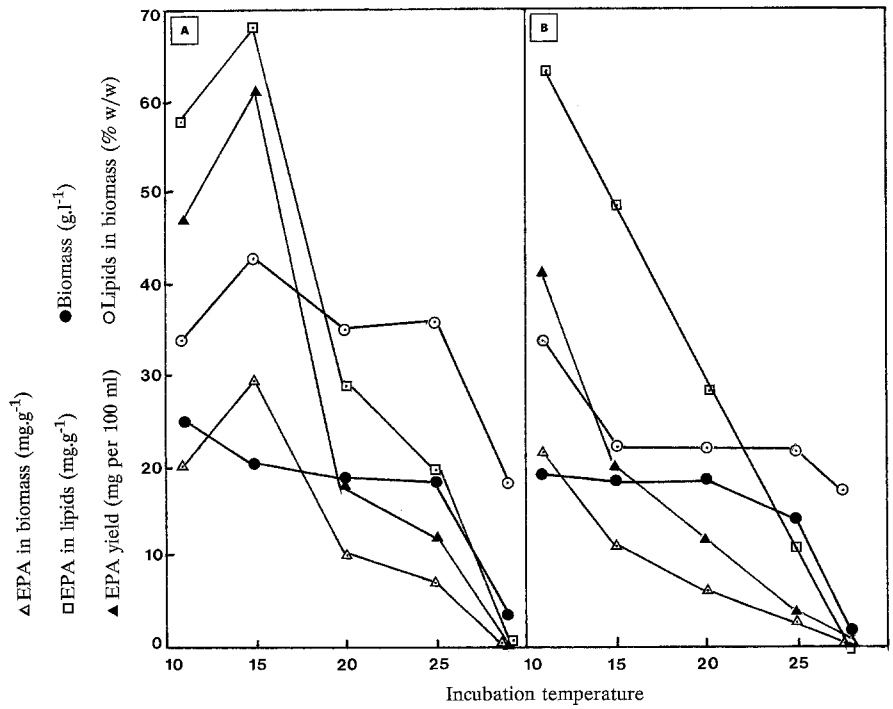

Fig. 4. Effect of temperature on EPA and lipid production by *M. elongata* NRRL 5513 with (A) linseed oil and (B) glucose as carbon sources, in shake-flask cultures. Medium: basal medium + carbon source.

TABLE 1

Effect of nitrogen source on EPA, lipid and biomass production by *M. elongata* NRRL 5513. Medium: Basal medium + 3% linseed oil + 0.5% nitrogen source. Incubation temperature: 25 °C. Incubation time: 6 days.

Nitrogen Source (5 g/l)	Biomass (g/l)	Lipids in biomass (% w/w)	EPA		
			in biomass (mg/g)	in lipids (% w/w)	yield (g/l)
Peptone	24.70	33.89	8.59	2.54	0.212
Tryptone	23.77	30.27	12.57	4.15	0.299
Malt extract	19.60	27.30	2.53	0.93	0.050
Yeast extract	24.01	30.50	14.51	4.76	0.450

TABLE 2

Effect of initial culture pH on EPA, lipid and biomass production by *M. elongata* NRRL 5513 in shake flask culture. Medium: Basal medium + 3% linseed oil + 0.5% yeast extract. Incubation temperature: 25 °C. Incubation time: 6 days.

pH	Biomass (g/l)	Lipids in biomass (% (w/v)	EPA		
			in biomass (mg/g)	in lipids (% w/w)	yield (g/l)
4.8	27.36	31.68	11.07	3.50	0.303
5.6	22.10	31.53	12.57	3.99	0.278
6.1	24.01	39.57	13.80	3.85	0.333
6.8	25.22	37.04	9.30	2.51	0.235
7.8	22.70	37.86	10.30	2.72	0.234

content of biomass and lipids observed at 25 °C was considerably higher in agar surface cultures than in liquid flask cultures whereas the maximum EPA values observed at 4 °C in surface culture were similar to the lower temperature submerged culture values.

The effect of nitrogen source on growth and EPA production was investigated by incorporating 0.5% of each nitrogen source into the basal medium supplemented with 3% linseed oil. Maximum EPA was produced in the medium containing yeast extract (Table 1). The effect of initial culture pH on EPA production was tested in the pH range 4.8–7.8. An initial pH of 6.1 was optimum for EPA production (Table 2).

In preliminary studies, *M. elongata* was found to produce EPA in the basal medium supplemented with linseed oil at both 11 and 25 °C but only produced EPA at the lower temperature in glucose supplemented media. The effects of temperature on production of EPA in linseed oil and glucose supplemented media were therefore investigated (Fig. 4). In linseed oil supplemented medium, opti-

TABLE 3

Effects of urea and linseed oil concentrations on EPA, lipid and biomass production by *M. elongata* NRRL 5513 in shake flask culture. Medium: Basal medium + 0.5% yeast extract supplemented with urea and linseed oil. Incubation temperature: 15 °C. Incubation time: 10 days.

Linseed oil conc. (% v/v)	Urea conc. (% w/v)	Biomass (g/l)	Lipids in biomass (% w/v)	EPA		
				in biomass (mg/g)	in lipids (% w/w)	yield (g/l)
2.0	0.00	20.55	43.20	29.51	6.83	0.606
2.0	0.05	19.97	24.98	23.02	9.22	0.460
2.0	0.15	19.55	11.98	16.23	13.55	0.317
2.0	0.25	19.20	7.01	10.61	15.12	0.204
2.5	0.25	24.67	13.99	12.84	9.17	0.317
3.0	0.25	29.66	22.06	14.58	6.61	0.432
3.5	0.25	29.60	21.12	16.96	8.03	0.502

TABLE 4

Fatty acid profiles of *M. elongata* mycelia from shake flask cultures producing (a) maximum EPA yields and (b) maximum EPA content in lipids. Incubation temperature: 15 °C. Incubation time: 10 days.

	(a)*	(b)**
16:0	6.00	7.40
16:1	traces	traces
18:0	2.64	1.67
18:1	12.34	17.71
18:2	13.77	12.29
18:3	37.22	24.60
20:4	5.20	16.37
20:5	6.83	15.12
Others	10.00	4.84

* Basal medium + 2% linseed oil + 0.5% yeast extract.
** Basal medium + 2% linseed oil + 0.5% yeast extract + 0.25% urea.

mum temperature for EPA production was 15 °C whereas the maximum EPA in glucose supplemented medium was produced at 11 °C. EPA produced in linseed oil supplemented medium at 15 °C amounted to 29.5 mg/g biomass, 6.8% w/w of lipid and 0.61 g/l of culture broth. The effect of linseed oil concentration and supplementation of the medium with urea is illustrated in Table 3. Maximum yield of EPA was obtained at a linseed oil supplement of 2% while increasing urea concentration significantly reduced lipid content in biomass and overall has a negative effect on EPA yields. However, a dramatic increase in EPA content of lipids was observed by supplementation of the medium with 0.25% urea. EPA accounted for 15.12% of total lipids. Fatty acid profiles in mycelia of cultures producing maximum EPA yields and in cultures producing highest EPA content in lipids are compared in Table 4.

DISCUSSION

In studying factors affecting EPA production, two parameters are important in pursuing an optimisation strategy. Maximizing EPA yield per litre has an impact on the reduction of the fermentation cost while optimizing EPA percentage in lipids has an important bearing on EPA recovery costs. The priority parameter for ultimate process optimisation requires a detailed economic process analysis after a downstream processing protocol has been developed.

In general, in these growth studies, it was observed that the percentage of EPA in lipids continued to rise after net lipid synthesis has ceased. Wassef [26] noted that the amount of lipid produced by a given species depends on the developmental stage of growth of that species. In the case of *Aspergillus nidulans*, fat formation in surface cultures was also found to accelerate at the later stages of growth, after which fat content decreased [21]. This later decrease in fat content has also been observed in our studies with *M. elongata*. Extending the fermentation time to achieve a decrease in lipid content and an increase in the percentage of EPA in lipids, while increasing fermentation costs, would reduce the complexity and cost of EPA recovery.

The higher EPA content of biomass and lipids observed at 25 °C in agar surface cultures compared to submerged cultures may be due to increased oxygen tension in surface as compared to submerged cultures. Increased oxygen tension elevated the unsaturated fatty acid content of fungi of the order Mucorales [22] and the desaturase enzymes required for production of the unsaturated fatty acids require molecular oxygen as cofactor [25]. However, the prolonged incubation times required to achieve higher yields of EPA in surface cultures makes this cultivation method much less practical than submerged culture from an economic standpoint.

An initial culture pH of 6.1 was found to be optimal for EPA production. Optimum initial culture pH for arachidonic acid production by *M. alpina* was in the range 6.0–6.7 [2]. It was noted that, as incubation temperature increased, EPA content in biomass and in lipids dramatically declined. However, this also resulted in an extension of the fermentation time from 6 to 10 days so that overall EPA productivity was higher at the higher temperature. Generally, lower temperatures have been found to result in an increase in lipid unsaturation [8, 11, 26]. These relationships between the level of lipid unsaturation and cultivation temperature may be due to the thermolabile nature of desaturases, which are reported not to operate at higher temperatures [5].

Increasing urea content in the medium resulted in a dramatic decrease in lipid and EPA accumulation. This may be explained by the general observation that lipid accumulation is favoured by an increasing C : N ratio and is indeed often triggered on depletion of nitrogen. Nitrogen content of the medium has been reported also to affect the proportion of saturated to unsaturated fatty acids in a variety of microorganisms, including fungi [7, 13, 18].

Culture conditions were established in which EPA content of lipids amounted to 15.12%. The percentage of EPA in lipids reported in cultures of different strains of *M. alpina* ranged from 3.2–5.4% [19]. However, *M. alpina* produced a maximum yield of 0.87 g EPA per litre of culture compared to a maximum yield of 0.61 g/l observed with *M. elongata* NRRL 5513.

ACKNOWLEDGEMENTS

Support for this research by the Natural sciences and Engineering Research Council of Canada is gratefully acknowledged. O.P. Ward is holder of an NSERC Industrial Research Chair, co-sponsored by Allelix Biopharmaceuticals Inc., Canada.

REFERENCES

1 Bajpai, P., P.K. Bajpai and O.P. Ward. 1990. Eicosapentaenoic acid (EPA) production by fungi. Abstr. Soc. Indust. Microbiol. Conf. on Novel Microbial Metabolites, Sarasota, October 1990, p. 27.

2 Bajpai, P.K., P. Bajpai and O.P. Ward. 1991. Arachidonic acid production by fungi. Appl. Environ. Microbiol. 57: 1255–1258.

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

4 Braden, L.M. and K.K. Caroll. 1986. Dietary polyunsaturated fat in relation to mammary carcinogenesis in rats. Lipids 21: 285–288.

5 Brock, T.D. 1967. Life at high temperatures. Science 158: 1012–1019.

6 Dyerberg, J. 1968. Linoleate-derived polyunsaturated fatty acids and prevention of atherosclerosis. Nutr. Rev. 44: 125–134.

7 Erwin, J. 1973. Comparative biochemistry of fatty acids in eucaryotic microorganisms. In: Lipids and Biomembranes of Eucaryotic Microorganisms (Erwin, J.A., ed.), pp. 41–143. Academic Press, New York.

8 Gregory, M. and M. Woodbine. 1953. Microbiological synthesis of fat. J. Exp. Bot. 4: 314–318.

9 Hansson, L. and M. Dostalek. 1988. Effect of culture conditions on mycelial growth and production of γ-linolenic acid by the fungus *Mortierella ramanniana*. Appl. Microbiol. Biotech. 28: 240–246.

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

11 Kates, M. and P.-O. Hagen. 1964. Influence of temperature on fatty acid composition of psychrophilic and mesophilic *Serratia* species. Can. J. Biochem. 42: 481–488.

12 Lands, W.E.M. 1986. The fate of polyunsaturated fatty acids. In: Health Effects of Polyunsaturated Fatty acid in Seafoods (Simopoulos, A.P., R.R. Kifer and R.E. Martin, eds.), pp. 319–351, Academic Press Inc., Orlando.

13 Marr, A.G. and J.L. Ingraham. 1962. Effect of temperature on the composition of fatty acids in *Escherichia coli*. J. Bacteriol. 84: 1260–1267.

14 Mortensen, J.Z., E.B. Schmidt, A.H. Nielson and J. Dyerberg. 1983. The effect of n-6 and n-3 polyunsaturated fatty acids in homostasis, blood lipid and blood pressure. Thromb. Haemostas. (Stuggart). 50: 543–546.

15 Reddy, B.S. and H. Maruyama. 1986. Effect of dietary fish oil on aroxymethane induces colon carcinogenesis in male F344 rats. Can. Res. 46: 3367–3370.

16 Robinson, D.R., J.D. Prickett, G.T. Makoul, A.D. Steinberg and R.B. Colvin. (1986). Dietary fish oil reduces progression of established renal disease in (NZBX NZW)F1 mice and delay renal disease in BXSB and MRL/1 strains. Arth. Rheum. 29: 539–546.

17 Seto, A., H.L. Wang and C.W. Hesseltine. 1984. Culture conditions affect on eicosapentaenoic acid content of *Chlorella minutissima*. J. Am. Oil Chem. Soc. 61: 892–894.

18 Shaw, R. 1966. The polyunsaturated fatty acids of microorganisms. Adv. Lipid Res. 4: 7–174.

19 Shimizu, S., Y. Shinmen, H. Kawashima, K. Akimoto and H. Yamada. 1988. Fungal mycelia as a novel source of eicosapentaenoic acid. Biochim. Biophys. Acta 150: 335–341.

20 Shimizu, S., H. Kawashima, K. Akimoto, Y. Shinmen and H. Yamada. 1989. Microbial conversion of oil containing α-linolenic acid to an oil containing eicosapentaenoic acid. J. Am. Oil Chem. Soc. 66: 342–347.

21 Singh, J. and T.K. Walker. 1956. Changes in the composition of the fat of *Aspergillus nidulans* with age of culture. Biochem. J. 62: 286–289.

22 Sumner, J.L., E.D. Morgan and H.C. Evans. 1969. The effect of growth temperature on the fatty acid composition of fungi in the order Mucorales. Can J. Microbiol. 15: 515–520.

23 Urakaze, M., T. Hamazaki, Y. Soda, M. Miyamoto, F. Ibuki, S. Yano and A. Kumagai, 1986. Infusion of emulsified trieicosapentaenoyl-glycerol into rabbits. The effects on platelet aggregation, polymorphonuclear, leukocyte adhesion and fatty acid composition in plasma and platelet phospholipids. Thromb. Res. 44: 673–682.

24 Van der Wende, L.A. 1988. Omega-3 fatty acids: algal and fish fatty acids could net $790 million, but markets need backing of medical industry. In: Biomarkets: 33 Market Forecasts for Key Product Areas, pp. 199–203, Technical Insight Inc., New Jersey.

25 Walker, P. and M. Woodbine. 1975. The biosynthesis of fatty acids. In: The Filamentous Fungi, Vol. 2: Biosynthesis and Metabolism. (Smith, J.E. and D.R. Berry, eds.), pp. 136–158. Edward Arnold. London.

26 Wassef, M.K. 1977. Fungal lipids. In: Advances in Lipid Research (Paoletti, A. and D. Kritchevsky, eds.), 15, 159–232. Academic Press, New York.

27 Yongmanitchai, W. and O.P. Ward. 1989. Omega-3 fatty acids: Alternative sources of production. Proc Biochem. 24: 117–125.

28 Yongmanitchai, W. and O.P. Ward. 1991. Growth of and Omega-3 fatty acid production by *Phaeodactylum tricornutum* under different culture conditions. Appl. Environ. Microbiol. 57: 419–425.

29 Ziboh, V.A., K.A. Cohen, C.N. Ellis, C. Miller, T.A. Hamilton, K. Kragballe, C.R. Hydrick and V.J. Voorhees. 1986. Effect of dietary supplementation of fish oil on neutrophil and epidermal fatty acids: Modulation of clinical course of psoriatic subjects. Arch Dermatol 122: 1277–1282.