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Eicosapentaenoic Acid (EPA) Production by *Mortierella alpina* ATCC 32222

PRATIMA BAJPAI,[1,2] PRAMOD K. BAJPAI,*[,1,2] AND OWEN P. WARD[1]

[1]Microbial Biotechnology Centre, University of Waterloo, Waterloo, Ontario, Canada N2L 3G1; and [2]Present address: Thapar Corporate Research & Development Centre, Patiala-147001, India

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ABSTRACT

Mortierella alpina ATCC 32222 grew well at 11°C, as well as at 25°C in a liquid medium containing glucose or linseed oil and yeast extract. High Eicosapentaenoic acid (EPA) yield was obtained at 11°C. *M. alpina* cells did not produce EPA at 25°C in the absence of linseed oil, whereas at 11°C, EPA accumulation was noted in the absence of linseed oil. When grown at 11°C for 10 d in a medium containing 2% linseed oil as carbon source, the mycelium yielded 435 mg/L EPA (20 mg EPA/g dry mycelia) with 5.1% in lipid fraction. By gradually increasing the concentration of linseed oil to 4%, yield of biomass and EPA were increased to 43 g/L and 596 mg/L, respectively.

Index Entries: Eicosapentaenoic acid; polyunsaturated fatty acid; *Mortierella alpina;* fungal lipid.

INTRODUCTION

EPA (5,8,11,14,17-*cis*-eicosapentaenoic acid) is a polysaturated fatty acid (PUFA) that is commonly found in marine animals and phytoplankton. It contains 20 carbon atoms with five double-bonds (20:5). The double-bonds are arranged with the last one located three carbon atoms from the end of the chain, i.e., methyl terminal, and, therefore, referred to as omega-3 or (n-3) fatty acid. The other members of omega-3 or (n-3)

*Author to whom all correspondence and reprint requests should be addressed.

family are docosahexaenoic acid (DHA) and α-linolenic acid. Although α-linolenic acid can be obtained from vegetable sources, e.g., linseed oil contains up to 58% α-linolenic acid, EPA and DHA are obtained from marine fish oils only.

The omega-3 fatty acids have been shown to be of major importance in the prevention and treatment of a range of human diseases or disorders, especially those related to the heart and circulatory system (*1–4*), the inflammatory area (*5–8*), and certain cancers (*9,10*). The content of omega-3 fatty acids in fish oils from each source varies depending on species of fish, seasons, and geographic location of catching sites, which in turn are determined by the availability and types of primary food chain, namely marine organisms (*11*). The supply of omega-3 fatty acids from fish is unlikely to meet future requirements and alternative sources are being sought (*11*). Several lower fungi of the class *Phycomycetes*, especially strains belonging to the genus *Mortierella*, produce substantial amounts of polyunsaturated fatty acids, eicosapentaenoic acid, arachidonic acid, and γ-linolenic acid, depending on species and cultural conditions (*12–15*). In our laboratory, preliminary screening of *Mortierella* strains for EPA production showed *M. alpina* ATCC 32222 to be an efficient producer of EPA. In this paper, we report the results of EPA production from this fungus.

MATERIALS AND METHODS

Chemicals

Standard fatty acids were purchased from Sigma Chemical Co., St. Louis, MO. Solvents and reagents were procured from Aldrich Chemical Co., Inc., Milwaukee, WI, and BDH Chemicals, Toronto, Ontario, Canada. Sugars and other media nutrients were obtained from Difco, Detroit, MI. Linseed oil supplied by Recochem Inc., Canada was procured locally. It contained (% w/w): palmitic acid, 4.9; stearic acid, 3.0; oleic acid, 19.5; linoleic acid, 14.5; α-linolenic acid, 53.5, and other acids, 3.7.

Microorganism and Culture Conditions

Mortierella alpina ATCC 32222 was maintained on 3% agar slants containing 20 g/L glucose and 10 g/L yeast extract and subcultured every 2 mo.

Mycelium, freshly grown on agar slants, was used to inoculate 250 mL Erlenmeyer flasks containing 50 mL of basal medium supplemented with 5 g/L yeast extract and 30 g/L linseed oil. 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 vol 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 $CuSO_4 \cdot 7H_2O$, 0.0005.

Analytical Procedures

Fungal biomass was recovered from liquid media by vacuum filtration or centrifugation at 3000*g*, followed by washing with acidified ether and distilled water and drying at 100°C for 12–16 h. The dried cells were weighed (20–40 mg) in teflon-lines screw-cap test tubes of 10 mL capacity, and the lipids were extracted according to the procedure of Bligh and Dyer (*16*). The extracted lipids were dried at 36°C under nitrogen atmosphere and then methylated using the method of Holub and Skeaff (*17*). Then, the fatty acid methyl esters were dissolved in 200 μL *n*-hexane and 1 μL sample was injected into a gas–liquid chromatograph (GLC) for analysis. The Shimadzu CR-601 GLC was connected with GC-14A data integrator. The GLC was fitted with megabore column DB-225 (chromatographic specialties, 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 Chemical Co. Pentadecanoic acid (C15:0) was used as internal standard.

RESULTS AND DISCUSSION

Production of biomass and EPA by *M. alpina* ATCC 32222 was first investigated at low temperature (11°C) as *Mortierella* fungi is reported to accumulate EPA at low-growth temperatures (*12*). Initial cultivation was carried out in HD medium of Hansen and Dostalek (*18*). It contained 30 g/L glucose and 5 g/L yeast extract in basal medium. The strain grew well in this medium and produced lipids that contained EPA. The EPA yield was 64 mg/L of culture broth and its content in biomass was 4.2 mg/g dry cells, which accounted for 1.9% of total lipids (Table 1). The fungus was also incubated in a medium (HD-G) that contained linseed oil at varied concentrations in place of glucose. Linseed oil (LO) contains α-linolenic acid as a major fatty acid (58%), which is a precursor for EPA synthesis, and facilitates EPA production. EPA content of biomass was found to change with LO concentration. Maximum EPA content in biomass, 20 mg/g dry cells, was observed in media containing 2% LO. The EPA content of lipids (5.2% w/w) was also highest in this medium. However, EPA yield per liter of culture, 596 mg/L, was highest when the medium contained 4% LO (initially, the medium contained 2% LO, after 48 h, 1% LO was added, and finally after 72 h, 1% more LO was added).

EPA production was also investigated at physiological growth temperature of 25°C. At this temperature in HD medium, the cells of *M. alpina* produced very high amount of arachidonic acid (ARA), but no EPA.

Table 1
Growth of *Mortierella alpina* ATCC 32222[a]

Parameter	HD	HD(-G) +2% LO	HD(-G) +3% LO	HD(-G) +(2+1+1)% LO*
Biomass (g/L)	15.2	21.7	30.3	43.2
Lipids in biomass (%w/w)	22.4	39.3	32.6	44.1
EPA: in biomass (mg/g)	4.2	20.1	14.3	13.8
in lipids (%w/w)	1.9	5.1	4.5	3.1
yield (mg/L)	64	435	446	596
Fatty acid spectra (%w/w)				
16:0	11.9	5.5	6.8	5.4
16:1	–	–	0.3	0.2
18:0	9.3	3.3	3.8	3.2
18:1	19.2	18.5	22.5	19.8
18:2	4.1	13.9	14.0	14.4
18.3	0.6	39.3	35.3	42.9
20:3	–	–	–	–
20:4	41.5	8.3	7.3	5.5
20:5	1.9	5.1	4.5	3.2
22:6	–	1.2	–	–
Others	11.5	4.9	5.5	5.4

[a]Its lipid and EPA yields and fatty acid spectra with and without linseed oil in HD medium at 11°C.

*Medium initially contained 2% LO; after 48 h, 1% LO was added and finally after 72 h, 1% more LO was added.

This is consistent with the findings of Shimizu et al. (*12*), who observed no EPA production at 20–28°C by strains of *M. alpina, M. hygrophila, M. elongata, M. exigua, M. parrispore.* When HD(-G) medium was supplemented with LO, EPA accumulation was noted at 25°C. However, the EPA content was lower than the values observed at 11°C. At 1% concentration of LO, the EPA content in biomass and in lipids was 10.2 mg/g dry cells and 2.45%, respectively, and yield of EPA was 187 mg/L (Table 2). Increase of LO concentration did not result in any increase in EPA content in biomass. However, the fraction of EPA in lipids was increased. At 3% concentration of LO, very high biomass production was obtained, which resulted in a high yield of EPA, 267 mg/L.

In the case of microscopic alga *Chlorella minutisima,* EPA production is reported to be stimulated at low-growth temperatures (*10*). Indeed, psychrophilic organisms with optimum growth temperatures below 20°C generally contain more highly unsaturated fatty acids. This may be as a result of the thermolabile nature of desaturases. Increased synthesis of unsaturated fatty acids at lower temperatures has been observed, not only in eukaryotic algae, but also in bacteria and blue green algae, yeasts, and

Table 2
Growth of *Mortierella alpina* ATCC 32222[a]

Parameter	HD	HD(-G) +1% LO	HD(-G) +2% LO	HD(-G) +3% LO
Biomass (g/L)	17.5	18.3	19.9	28.2
Lipids in biomass (%w/w)	17.8	41.7	26.3	30.9
EPA: in biomass (mg/g)	4.2	10.2	9.2	9.5
in lipids (%w/w)	nil	2.5	3.5	3.1
yield (mg/L)	nil	187	182	267
Fatty acid spectra (%w/w)				
16:0	9.0	7.8	7.7	7.1
16:1	-	0.3	0.3	0.3
18:0	6.8	4.6	4.3	4.0
18:1	8.3	26.2	25.2	23.4
18:2	7.3	12.9	13.1	13.8
18.3	-	30.0	31.6	34.6
20:3	2.5	0.4	0.5	0.5
20:4	54.1	5.8	5.9	5.2
20:5	0.0	5.1	4.5	3.1
Others	12.0	6.9	6.9	8.0

[a]Its lipid and EPA yields and fatty acid spectra with and without linseed oil in HD medium at 25°C.

bacteria (*18–23*). Increase in lipid unsaturation has been noted when some fungi are incubated at higher temperature (*24,25*), whereas in other cases, there appeared to be little relationship between culture temperature and fatty acid composition (*26*). In the case of *Mucor* and *Rhizopus* species, culture temperature reduction did not promote synthesis of more unsaturated fatty acids (*27*).

The data presented in this paper show that *M. alpina* ATCC 32222 is capable of accumulating high amount of EPA at low-growth temperature. The maximum yield, 435 mg/L, is comparable to EPA production by selected *Mortierella* strains obtained from IFO, Japan (*12*) or AKU, Japan (*13*), or NRRL (*28*).

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