Production of the polyunsaturated fatty acids arachidonic acid and eicosapentaenoic acid by the fungus *Pythium ultimum*

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Several strains of species of the fungal genus *Pythium*, and of *Phytophthora cinnamomi*, were screened for content of the polyunsaturated fatty acids (PUFAs) arachidonic acid (AA) and eicosapentaenoic acid (EPA). The aim of the investigation was to establish alternative sources of these PUFAs, which are of importance in human nutrition. As a relatively prolific producer of EPA and AA, *P. ultimum* strain #144 was selected for a study of conditions that enhance their production over baseline levels that are present in the fungus when cultured for 6 d at 25 °C with rotary shaking (120 r.p.m.) in Vogel's medium containing sucrose as the carbon substrate. The levels of AA and EPA under these conditions were 133 ± 27 and 138 ± 25 mg l⁻¹ ($n = 5$), respectively. Maximal production of these fatty acids was accomplished by the following sequence of steps. (1) Incubate the cultures for 6 d after inoculation under the conditions described above. Then (2) add glucose to the cultures (2%, w/v, final concentration) and incubate for a further 6 d at 13 °C. Under these conditions, the AA content of the mycelium was 205% higher than baseline levels and the EPA content was 198% higher. (3) Allow the cultures to remain stationary for 10 d which increases the AA content to 253% above baseline levels and the EPA content by 236%. Using such a procedure, 322 mg AA l⁻¹ and 383 mg EPA l⁻¹ were produced.

Introduction

Beneficial health effects of consuming certain marine fish and fish oils are attributed to the presence of the ω -3 fatty acids 5,8,11,14,17-eicosapentaenoic acid (EPA) and 4,7,10,13,16,19-docosahexaenoic acid (DHA) (Sanders, 1988; Simopoulos, 1989). ω -3 fatty acids exhibit beneficial effects in coronary heart disease, hypertension, inflammation, arthritis, psoriasis, other autoimmune disorders and cancer (Simopoulos, 1989). At present, the oils of certain marine fish are the only commercial sources of EPA and DHA. This has prompted a search for other sources of such polyunsaturated fatty acids (PUFAs) (Yongmanitchai & Ward, 1989).

Possible alternate sources of the ω -3 fatty acids include certain algae (Erwin, 1973; Behrens *et al.*, 1989; Yongmanitchai & Ward, 1989), which are actually the dietary origin of such fatty acids in fish. Although restricted in occurrence, EPA appears to be more

widespread in nature than DHA. It has been reported in certain marine bacteria (Yazawa *et al.*, 1988) and some of the more primitive fungi such as the Oomycetes, including the pythiaceae species (*Pythium* and *Phytophthora*) (Shaw, 1966), and the Zygomycete genus *Mortierella* (Yamada *et al.*, 1987*a, b*).

Arachidonic acid (AA) is a biogenic precursor to a wide variety of biologically active eicosanoids such as prostaglandins and thromboxanes (Nelson *et al.*, 1982). This fatty acid is usually obtained from pig liver and adrenal glands (Ratledge, 1989). It appears to be more widely distributed than EPA and DHA, having been identified as a product of animals and numerous microorganisms such as certain protozoa, amoebae, algae and fungi. With few exceptions, AA distribution in fungi appears to be mainly in the more primitive species such as those belonging to the classes Hyphochytriomycetes, Chytridiomycetes and Oomycetes of the subdivision Mastigomycotina (Weete *et al.*, 1989), including *Pythium ultimum* (Kerwin & Duddles, 1989). It has also been found in *Mortierella* spp. (Yamada *et al.*, 1987*a, b*; Shinmen *et al.*, 1989). Low levels of AA have been reported (Radwan & Soliman, 1988) in species of some of the more advanced fungal genera, i.e. *Penicillium*,

Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FAME, fatty acid methyl ester; LO, linseed oil; PUFA, polyunsaturated fatty acid.

Aspergillus and *Fusarium*, when cultured on short-chain fatty acids.

Results of preliminary experiments in this laboratory indicated that certain *Pythium* species may have potential for the production of relatively high amounts of EPA and AA. The objective of the research reported here was to find a high EPA- and/or AA-producing strain(s) or species of *Pythium*, and to develop laboratory culture procedures for maximizing the production of these fatty acids.

Methods

Sources of fungi and culture conditions. *Pythium aphanidermatum* and *Pythium myriotylum* were obtained from M. C. Rush, Department of Plant Pathology and Crop Physiology, LSU, Baton Rouge, LA, USA; a complex of *Pythium irregulare* and *Pythium pareocandrum*, and *Pythium sylvaticum* were obtained from D. J. S. Barr (see Table 1). Otherwise, the sources of *Pythium ultimum* and *Phytophthora cinnamomi* are given in Table 1.

Mycelia of each strain were stored at -70°C in Vogel's medium containing 10% (v/v) glycerol. Unless noted otherwise, they were cultured for 6 d at 25°C in 250 ml Erlenmeyer flasks containing 100 ml Vogel's defined medium (Vogel, 1964) which has sucrose and ammonium nitrate as carbon and nitrogen sources, respectively. Cultures were agitated on a New Brunswick or Lab-line rotary incubator shaker at 120 r.p.m. Inoculations were made with 5 ml of blended mycelium from a 6-d-old culture grown as described above. Mycelia were harvested by suction filtration in a Buchner funnel, washed twice with 100 ml cold 0.1 M-potassium phosphate buffer (pH 7.0) each, and dried by lyophilization prior to extraction. In experiments where the fungus was grown at different temperatures beginning immediately after inoculation, the cultures used for inoculum were adapted to the requisite temperatures by two successive transfers and subsequent incubation for 6 d after each.

The effects of other nutrients on growth and lipid production was determined by growing the mycelia in Vogel's medium in which the sucrose was replaced by either 2% (w/v) glucose, 2% (w/v) maltose, or corn steep liquor (1% or 2%, v/v). The fungus was also cultured in Vogel's medium supplemented with 1% (v/v) bovine milk whey, 4% (v/v) V-8 (blended vegetables) juice or 1% (v/v) linseed oil. Also, potassium nitrate, urea or glycine were substituted for ammonium nitrate at equivalent nitrogen contents. In addition, mycelia were incubated in media with high carbon/nitrogen ratios or cultured in 2% (w/v) potato dextrose, 2% (w/v) malt dextrose and 1% yeast extract/2% dextrose media. With the exception of glucose in combination with low temperature (see below), none of these media components or treatments had an appreciable effect on total lipid content, or the AA and EPA content of the lipid, and therefore the data are not given.

To study the effects of glucose and temperature on EPA and AA production, mycelia were first grown in Vogel's medium for 6 d at 25°C , and then sterile glucose solution was added to the culture medium to a final concentration of 2%. The mycelia was then incubated for an additional 6 d at either 25°C or 13°C before harvesting. The controls received equal volumes of sterile distilled water in place of the glucose solution.

For the evaluation of data to determine conditions favouring AA and/or EPA production, values for growth, lipid and fatty acid contents were compared to 'baseline' data from fungi cultured in Vogel's medium under the conditions described above. Each experiment was done at least twice, and the data given in this paper are representative. The values given for each experiment are means of two to three

treatment replications. Overall baseline values for AA and EPA content of *P. ultimum* strain #144 are means of five separate experiments, and the standard deviations reflect variations among these experiments.

Extraction and analytical procedures. Lipid was extracted from mycelium (ca. 50 mg) using the procedure of Bligh & Dyer (1959). In the case of dry tissue, the mycelium was hydrated with 1.0 ml water prior to extraction. The amount of lipid extracted was measured gravimetrically.

A portion (ca. 5 mg) of the total lipid was methylated by heating the sample with 0.5 M-sodium methoxide in dry methanol for 20 min at 75°C according to the procedure provided by Applied Science Laboratories (Deerfield, IL, USA). The fatty acid methyl esters (FAMES) were analysed by gas-liquid chromatography as described previously (Weete *et al.*, 1989) except that the gas chromatograph was equipped with a 30 m $\times$ 0.25 mm fused silica capillary column (J. & W. Scientific) coated with DB-225 (50% cyanopropylmethyl, 50% methyl phenyl silicone). The injector and detector temperatures were 250°C , and the oven temperature was programmed from 180°C to 220°C at $1^{\circ}\text{C min}^{-1}$. FAMES were identified by comparison of their retention times relative to methyl heptadecanoate with those of authentic standards obtained from Applied Science Laboratories, and they were quantified by the internal standard method using the same standard. The identities of AA and EPA have been confirmed by mass spectrometry (data not shown).

Results and Discussion

Screening

Twenty-three strains of *Pythium ultimum*, seven strains of *Pythium aphanidermatum*, three other species of *Pythium* and *Phytophthora cinnamomi* were screened for growth, total lipid production, and their AA and EPA contents. Since values for the other species and strains (see Methods) were generally lower, only the results for *P. ultimum* are given. Total lipid production varied among strains from 0.75 g l^{-1} for strain #1786 to 2.01 g l^{-1} for #650 (Table 1). Several strains stood out from the others tested in terms of AA and EPA production, e.g. #128, #144, #418, #511, #583, #639, #640 and #650 at 110–230 mg AA l^{-1} and 150–220 mg EPA l^{-1} (Table 1). The AA/EPA ratios ranged from 0.44 to 3.00 (Table 1). The lipid from these strains contained more EPA than does Menhaden oil, e.g. lipid from strain #144 contained 70% more EPA than this fish oil. Although *Ph. cinnamomi* contained relatively high amounts of AA and EPA, the growth rate was slower than that of some of the most productive *Pythium* species, and therefore *Ph. cinnamomi* was not studied further.

Although Shaw (1966) suggested that the pythiaceae fungi are capable of producing the long-chain PUFAs AA and EPA, Bowman & Mumma (1967) were unable to detect these fatty acids in *P. ultimum*. They reported finding relatively high amounts of $\text{C}_{22:1}$ and $\text{C}_{22:2}$. More

Table 1. Total lipid, arachidonic acid (AA) and eicosapentaenoic acid (EPA) contents of *Pythium* species and strains, and of *Phytophthora cinnamomi*

All of the cultures were grown in Vogel's medium for 6 d at 25 °C with rotary shaking at 120 r.p.m., except for *Ph. cinnamomi* which was grown for 12 d under similar conditions. Values for *P. ultimum* strains # 128 to # 650 (denoted *a*) are means of two experiments and three replicates each. All the remaining values are means of three replicates of one experiment. The variation among replicates and experiment was less than 10%.

Species and strain #*	Total lipid (g l ⁻¹)	AA (mg l ⁻¹)	EPA (mg l ⁻¹)	AA/EPA ratio
<i>Pythium ultimum</i>				
128 ^a	1.46	140	150	0.93
144 ^a	1.94	230	160	1.44
319 ^a	1.49	70	100	0.70
406 ^a	1.68	120	90	1.33
418 ^a	1.78	110	210	0.52
425 ^a	1.45	80	130	0.62
433 ^a	1.90	140	150	0.93
447 ^a	1.27	100	150	0.67
471 ^a	1.25	90	80	1.13
511 ^a	1.90	190	210	0.91
583 ^a	1.33	140	140	1.00
600 ^a	1.13	40	40	1.00
612 ^a	1.44	100	100	1.00
634 ^a	1.60	90	100	0.90
638 ^a	1.68	100	90	1.11
639 ^a	1.55	150	190	0.79
640 ^a	1.83	170	220	0.77
1520 ^b	1.33	40	90	0.44
23-5 ^b	1.33	50	70	0.71
20-2 ^b	0.97	60	40	1.50
1417 ^b	0.81	50	40	1.25
1696 ^c	1.00	110	50	2.20
1786 ^c	0.75	90	30	3.00
650 ^a (var. <i>sporangiiferum</i>)	2.01	140	90	1.56
var. <i>ultimum</i> ^d	0.92	80	100	0.80
var. <i>sporogonium</i> ^d	0.52	20	20	1.00
<i>Phytophthora cinnamomi</i> ^e	1.12	110	140	0.79

* Sources of strains were as follows: *a*, D. J. S. Barr, Biosystematic Research Center, Central Experimental Farm, Ottawa, Canada; *b*, M. C. Rush, Dept. of Plant Pathology and Crop Physiology, Baton Rouge, LA, USA; *c*, E. Butler, Dept. Plant Pathology, University of California at Davis, CA, USA; *d*, A. J. Latham, Dept. of Plant Pathology, Auburn Univ., AL, USA; *e*, E. A. Curl, Dept. of Plant Pathology, Auburn Univ., AL, USA.

recently, however, Kerwin & Duddles (1989) reported both AA and EPA in this species.

Lipid and fatty acid contents as a function of culture age, temperature and shaker rate

Based on the results presented above, *P. ultimum* strain # 144 was selected for further study. When incubated at 13, 18 and 25 °C in Vogel's medium, the cultures reached

Table 2. Production of total lipid, arachidonic acid (AA) and eicosapentaenoic acid (EPA) by *P. ultimum* strain # 144 as a function of temperature

Cultures were incubated for 6 d at the respective temperatures. Values are means of two experiments with three replicates each. The variation among experiments and replicates was less than 8.0%.

Temperature (°C)	Biomass (g l ⁻¹)	Total lipid (g l ⁻¹)	AA (mg l ⁻¹)	EPA (mg l ⁻¹)
13	8.3	1.0	29	100
18	8.9	2.2	133	170
25	7.4	2.0	220	170

the stationary phase 4–6 d after inoculation. The inocula for these cultures had been pre-adapted at the respective temperatures. Although maximum biomass production at the different temperatures occurred in the order 18 °C > 13 °C ≥ 25 °C, the biomass per flask at 18 °C was only about 20% more than that at 25 °C (Table 2), and about 30% higher than that at 30 °C (data not shown). The lipid content increased rapidly during growth and did not change appreciably during the stationary phase, i.e. for a total of 10 d incubation. Cultivation at 18 °C or above favoured lipid accumulation compared to that at 13 °C, but the highest lipid content of 26% (on a dry weight basis) occurred after 6 d growth at 25 °C (Table 2).

The fatty acid composition changed with growth temperature (Table 3). The most notable differences were in the C_{18:1} content, which was 20% at 25 °C compared to 33% at 13 °C, and the AA content which decreased from 14.6% to 3.3% with the same temperature change (Table 3). The Δ mol⁻¹ value, an indication of the degree of unsaturation (see Table 3), of 1.6 was the same for total fatty acids from mycelium cultured at 13 °C and 18 °C, in spite of a higher C_{18:2} content and correspondingly less C_{18:1}, and a higher C_{18:3} and a lower C_{20:4} content at 18 °C compared to 13 °C (Table 3). The Δ mol⁻¹ value was 1.9 at 25 °C which was due mainly to the relatively high C_{20:4} content. Also, C_{20:1} was detected in cultures grown at the two higher temperatures, but not at 13 °C. Although the AA content increased progressively with increasing temperature, the relative proportion of EPA was similar at the three temperatures tested. However, although EPA production was essentially the same at about 170 mg l⁻¹ at 18 °C and 25 °C, it was 42% less at 13 °C (Table 2). AA production was progressively less by 87% from 25 °C to 13 °C. Because of the differential response of AA and EPA to temperature, the ratio of these two fatty acids in the oil may be regulated by temperature.

Table 3. Total fatty acid composition (weight%) of *Pythium ultimum* strain #144 as a function of cultivation temperature

Values are means of two experiments with three replicates each.
The variation among experiments and replicates was less than 5%.

Fatty acid	Temperature (°C)		
	13	18	25
C _{14:0}	9.3	9.1	7.0
C _{14:1} Δ ⁹	—	0.3	0.3
C _{16:0}	12.0	15.8	15.4
C _{16:1} Δ ⁹	6.4	6.3	4.8
C _{18:0}	2.0	1.4	2.0
C _{18:1} Δ ⁹	33.0	22.8	20.1
C _{18:2} Δ ^{9,12}	13.2	17.2	15.9
C _{18:3} Δ ^{6,9,12}	6.5	1.4	1.1
C _{18:3} Δ ^{9,12,15}	0.8	0.9	0.7
C _{20:0}	—	0.4	0.6
C _{20:1} Δ ¹¹	—	5.5	4.3
C _{20:2} Δ ^{11,14}	—	0.2	0.5
C _{20:3} Δ ^{8,11,14}	1.3	0.6	0.9
C _{20:4} Δ ^{5,8,11,14}	3.3	7.5	14.6
C _{20:5} Δ ^{5,8,11,14,17}	11.0	11.0	11.5
C _{22:1} Δ ¹³	0.0	0.1	0.1
Δ mol ⁻¹ *	1.6	1.6	1.9

* Calculated using the equation $\Delta \text{mol}^{-1} = 1(\text{monoenes}) + 2(\text{dienes}) + 3(\text{trienes}) + 4(\text{tetraenes}) + 5(\text{pentaenes})/100$.

Cultivation time (culture age) had little effect on the relative proportion of most fatty acids during 6 d growth at 25 °C. The noteworthy changes in fatty acid composition with time were a decrease in C_{18:1} and increases in AA and EPA after 2 d incubation, but these changes had little effect on the Δ mol⁻¹ value which was similar throughout the growth period at 1.7 to 1.8. Increasing the shaker rate from 120 to 300 r.p.m., at either 13 °C or 25 °C, beginning at inoculation, either had no effect or only a slight beneficial effect on biomass, total lipid, AA or EPA production (data not given).

When cultured under baseline (see Methods) conditions, *P. ultimum* strain #144 produced 133 ± 27 mg AA l⁻¹ and 138 ± 25 mg EPA l⁻¹ ($n = 5$). Both AA and EPA tended to be higher in the polar lipid fraction than in the neutral lipid fraction as a percentage of total fatty acids, but most of these PUFAs were found in the neutral lipid fraction because the neutral/polar lipid ratio was about 84:16 (data not shown).

Enhancement of AA and EPA production

With the exception of certain species and strains of the zygomycetous genus *Mortierella*, fungi have not been considered previously as potential sources of AA or EPA. Under conditions considered optimum for growth (20–28 °C), the *Mortierella* species studied produce no detectable EPA, but EPA production was induced by

cultivation at lower temperatures. Using a relatively high initial growth temperature for biomass production, and intermittent glucose feeding followed by a temperature shift to 12 °C, EPA production by strains of *M. alpina* was brought to 490 mg l⁻¹ (Yamada *et al.* 1987b; Shimizu *et al.*, 1988a). Certain species and strains of *Mortierella* are also capable of producing relatively large quantities of AA. For example, *M. elongata* 1S-4 cultured in 2% glucose/0.5% yeast extract medium produced 3.6 g AA l⁻¹ [80 mg (g⁻¹ dry wt)⁻¹] (Yamada *et al.*, 1987b), and strain 1S-5 cultured on 10% glucose/0.5% polypeptone/0.3% yeast extract medium produced 0.99 g AA l⁻¹ (Yamada *et al.*, 1987a). Unlike EPA production, AA production in *Mortierella* did not require a low temperature. The relative proportion of AA was increased from 31% of the total fatty acids to 70% by allowing the harvested mycelia to stand for 6 d (Shinmen *et al.*, 1989).

A series of preliminary experiments were done with the aim of enhancing AA and/or EPA production in *P. ultimum*. Some previous results indicated that although growth and total lipid were lower with glucose in place of sucrose as the carbon source, the relative proportion of EPA in the total fatty acid fraction was moderately increased. Also, although cultures grown at 13 °C beginning at inoculation contained less total lipid, AA and EPA were slightly enriched in the lipid at this temperature. Therefore, biomass was allowed to accumulate in Vogel's medium for 6 d at 25 °C, and then glucose at 2% final concentration was added to the culture medium. This was followed by an additional 6 d incubation that resulted in a 19% and 37% increase, on a mg l⁻¹ basis, in AA and EPA production, respectively, over that at 6 d with sucrose only. These values were increased to 99% and 129%, respectively, when glucose was added as before except that the cultures were incubated at 13 °C instead of 25 °C. The cultures had not been previously adapted to the lower temperature. Also, allowing the cultures (Vogel's medium, 25 °C) that had been incubated for 6 d with shaking to remain stationary for an additional 10 d increased AA and EPA production by 55% and 58%, respectively.

In the light of results from the separate experiments described above, experiments were done that incorporated the above conditions in an effort to maximize AA and EPA production. In a representative experiment, baseline values for AA and EPA production were 91 and 114 mg l⁻¹, respectively (Table 4). Extending the incubation time from 6 to 12 d at 25 °C had relatively little effect on lipid and AA or EPA production, i.e. a 32% decrease in AA and a slight increase in EPA. However, AA production and EPA production (mg l⁻¹) were increased by 14% and 68%, respectively, over the baseline values in cultures to which 2% glucose was

Table 4. Effect of temperature, glucose and agitation conditions on total lipid, arachidonic acid (AA) and eicosapentaenoic acid (EPA) contents of *Pythium ultimum*

All cultures were initially incubated in Vogel's medium at 25 °C under shake conditions. At 6 d after inoculation, some cultures received water and some glucose to a final concentration of 2%. These flasks were incubated for a further 6 d at either 25 °C or 13 °C. After a total of 12 d incubation, some cultures were harvested for analysis and others were incubated under stationary conditions for an additional 10 d prior to harvest. Baseline values were from mycelia cultured in Vogel's medium for 6 d at 25 °C, with rotary shaking at 120 r.p.m. Values are means of three replications of one representative experiment. The variation among replicates was less than 10%.

Temperature of second incubation (°C)	Incubation conditions	Dry wt (g l ⁻¹)	Total lipid (g l ⁻¹)	AA (mg l ⁻¹)	EPA (mg l ⁻¹)
25	Baseline values	7.57	1.1	91	114
	H ₂ O	7.14	1.2	62	126
	Glucose	9.12	1.6	104	191
	H ₂ O + 10 d	7.20	1.3	104	166
	Glucose + 10 d	9.13	1.8	214	292
13	H ₂ O	6.92	1.3	123	175
	Glucose	11.61	2.3	277	340
	H ₂ O + 10 d	7.09	1.3	136	165
	Glucose + 10 d	11.20	2.0	322	383

added and incubated for an additional 6 d at 25 °C (Table 4). The production of AA and EPA was increased by 35% and 54%, respectively, by transferring baseline cultures from 25 °C to 13 °C for the second 6 d incubation, and was increased by 204% and 198%, respectively, by incubating for the additional 6 d in the presence of glucose at 13 °C. Allowing the 12-d-old cultures without glucose, grown at either 25 °C or 13 °C, to remain stationary for a further 10 d did not result in as much AA or EPA as in the corresponding cultures not allowed the additional stationary incubation period, except in the case of the water controls at 13 °C where there was a slight increase in AA (Table 4). On the other hand, cultures with glucose added and allowed to stand as described above at 25 °C contained 135% and 156% more AA and EPA, respectively, than the baseline cultures. At 13 °C, glucose-supplemented cultures allowed to stand an additional 10 d produced 254% and 236% more AA and EPA, respectively, than the baseline cultures (Table 4).

With this strategy, we were able to stimulate EPA production by *P. ultimum* strain #144 from the baseline level of about 114 mg l⁻¹ (138 ± 25 mg l⁻¹, $n = 5$) to 340 mg l⁻¹. In order to reach this level of production, it was necessary to generate biomass using sucrose as the carbon source, and then stimulate additional EPA

production by adding glucose and incubating the cultures at a lower temperature. EPA production could be further enhanced to 383 mg l⁻¹ by allowing glucose-fed, low temperature-treated cultures to remain stationary for 10 d after the 12 d incubation under shake culture conditions. It has been found that glucose in a yeast and malt extract/peptone/salts medium gave the best mycelial and lipid yield in *Pythium irregulare*; the highest EPA yield in this species with cold temperature and glucose treatment was 112 mg l⁻¹ (E. E. Stinson, R. Vosacek and M. J. Durarity, personal communication). In this case, cultures were incubated at the low temperature beginning at inoculation and the cultures were not allowed to remain stationary prior to harvest. The apparent superiority of *Mortierella* spp. over *Pythium* spp. in EPA production appears to be due mainly to the higher growth capacity of the former rather than the amount of fatty acid produced per unit of biomass. For example, the enhanced level of EPA in *P. ultimum* strain #144 in this study was 22 mg (g dry wt)⁻¹ compared to 27–29 mg (g dry wt)⁻¹ for *M. alpina* (Shimizu *et al.*, 1988a, b).

It is well-known that poikilothermic micro-organisms adapt to lower temperature by increasing the degree of fatty acid unsaturation, presumably as a means of modulating membrane fluidity (Thompson, 1985). Therefore, it was unexpected that *P. ultimum* strain #144 did not respond accordingly during the most rapid growth phase when cultured at lower temperatures (<25 °C) beginning just after inoculation, i.e. relative EPA levels were unchanged and the AA content was the opposite of what might be expected at a lower temperature (Table 3). Considering the temperature effect alone, production of AA and EPA was enhanced only when the cultures were shifted to the lower temperature after growth had slowed substantially (stationary phase) which was unexpected in view of the results described above.

The higher AA and EPA contents resulting from this treatment were due mainly to an increase in total lipid. Shimizu *et al.* (1988b) attributed the stimulation of EPA production in *Mortierella* spp. to low temperature activation of enzyme(s) involved in EPA synthesis.

Shimizu *et al.* (1989) found that EPA production could be stimulated to 1.35 g l⁻¹ in *M. alpina* strain 20-17 by supplementing the culture medium with 1% linseed oil (LO). The rationale for this was that LO contains a relatively high amount of α -linolenic acid that would be expected to be converted to EPA. This was also tested with *P. ultimum* strain #144 but, although LO was a good cosubstrate for growth in that biomass production was greater than in Vogel's medium alone, the increase in EPA production was minimal and not proportional to the increase in biomass (data not shown). Since LO was

readily degraded and utilized for growth by *Pythium*, and α -linolenic acid was incorporated into phospholipids and therefore presumably available for elongation and desaturation (data not given), the reason for the inability of LO to promote EPA production in *P. ultimum* strain #144 is unclear.

AA production in *P. ultimum* strain #144 was also stimulated by the same conditions that promote EPA production in this fungus, with a maximum stimulation of 253% over baseline levels. Unlike EPA, which *Mortierella* spp. and *P. ultimum* strain #144 generally produced in similar amounts on a mycelial dry wt basis, AA production by *Pythium* [30 mg (g dry wt)⁻¹], at least under the conditions described here, was 62% less than that of *M. elongata* IS-4 (Yamada *et al.*, 1987b).

In conclusion, *P. ultimum* strain #144 and certain other strains produce relatively high levels of both AA and EPA under cultivation conditions optimum for growth. Production of these PUFAs can be enhanced substantially by altering the cultivation conditions as described herein. These results suggest that this fungal species (strains) may have potential for commercial development for the production of these fatty acids by fermentation techniques.

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