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Effect of cultural conditions on production of eicosapentaenoic acid by *Pythium irregulare**

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SUMMARY

The effect of culture conditions upon lipid content and fatty acid composition of mycelia of *Pythium irregulare* was investigated with particular attention to increasing the yield of 5,8,11,14,17-eicosapentaenoic acid (20 : 5; ω-3) (EPA). All experiments were done by shake flask culture using a yeast extract + malt extract medium. The maximum growth rate was obtained at 25 °C, but maximum EPA production was obtained at 12 °C. The highest EPA production was 76.5 μg EPA/ml 13 days fermentation at 12 °C. Addition of glucose during fermentation increased the yield considerably. The highest yield was 112 μg/ml, obtained at 13 days fermentation with spiking on day 11. Fermentation time could be shortened by initial incubation at 25 °C for 2 days, followed by incubation at 12 °C for 6 days. The culture also produced arachidonic acid and other ω-6 polyunsaturated fatty acids. EPA production was also obtained with lactose or sweet whey permeate, a by-product of cheese manufacture that contains lactose as the main carbohydrate.

INTRODUCTION

Many reports have described beneficial health effects of dietary 5,8,11,14,17-eicosapentaenoic acid (20 : 5; ω-3) (EPA) in lowering the incidence of coronary heart disease and atherosclerosis [6,24]. Other studies have indicated beneficial effects of dietary EPA in other human diseases such as arthritis, renal disorders, psoriasis, non-insulin-dependent diabetes mellitus, and cancer [10,11,14,22,24]. If further medical research substantiates the value of dietary EPA to prevent any of the medical conditions that have been indicated, there would be a large potential market [23]. Pure EPA could be dispensed easily in pills as already done with fish oil capsules, or dispersed in food products such as mayonnaise with appropriate antioxidants [19].

Although the traditional source of EPA has been fish oil, processes involving the microalgae *Chlorella minutissima* for EPA production have been commercialized [16,17]. Production of EPA by *Mortierella* fungi has also been investigated [18]. EPA had been reported in many genera of the Oomycetes, the 'water molds' [1]: *Phytophthora infestans* [3,8]; *Pythium aphanidermatum* [13]; and *Saprolegnia parasitica* [5,12]. However, no information was available on overall EPA yields.

A preliminary investigation disclosed that one of these organisms, *Phytophthora infestans*, produces substantial quantities of EPA in undisturbed surface cultures [21]. Subsequent investigation has disclosed that several species of *Pythium* can be cultivated successfully in submerged culture and produce EPA [25]. This study was designed to investigate the effect of fermentation parameters on the EPA content of the mycelium of one of these species, *Pythium irregulare*, with the ultimate goal of assessing possible commercial production.

MATERIALS AND METHODS

Microorganism

The culture of *Pythium irregulare* ATCC 10951 was maintained on lima bean agar (LBA) and transferred every 3 weeks. To transfer the culture, a plug of LBA containing mycelium of the culture was placed on a Petri dish containing fresh LBA. The culture was grown at 25 °C for 2 days, and then stored at 15 °C for no longer than 3 weeks before transferring the culture again.

Media

Lima bean agar was prepared using a modification of the procedure of Bruck and Fry [2]. The ingredients (168 g Fordhook lima beans (frozen), 600 ml d H_2O, and 11.25 g

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agar) were homogenized at high speed for 1 min in a blendor, and then autoclaved. Large flasks were used to avoid problems with foams. The liquid medium used for experimental production of EPA was a modified 'YM' medium [26] consisting of yeast extract, 3.0 g/l; malt extract, 3 g/l; peptone, 5 g/l; glucose, 10 g/l; K_2HPO_4, 0.684 g/l, d H_2O to 1 l, pH to 6.0 (1 N HCl).

Fermentation

The inoculum was prepared by transferring a 1-cm^2 mycelial plug from a LBA culture to a 250-ml Erlenmeyer flask containing 100 ml of the sterile modified YM medium. The flask was incubated at 25 °C at 150 rpm for 2 days. The contents of the flask were transferred to a sterile 360-ml blender flask and homogenized 1 min at high speed. The experimental flasks were inoculated with 2-ml portions of the homogenate. The experimental flasks were 250-ml Erlenmeyer flasks containing 100 ml medium and were incubated on a rotary shaker at 150 rpm.

Experiments to establish optimal phosphate molarity and initial pH were performed at 25 °C for 5 days. Optimal phosphate molarity determination was performed at an initial pH 6.0. The molarity of phosphate was varied between 0 and 24 mM phosphate by the addition of K_2HPO_4, and the pH was adjusted to 6.0 by the addition of 1.0 N HCl. Determination of optimal initial pH was conducted with media that contained 3 mM K_2HPO_4. The pH was adjusted to values between 5.0 and 8.0 using either 1.0 N HCl or 1.0 N NaOH.

The effect of temperature on EPA production was studied at 12° and 25 °C. The 25 and 12 °C cultures were harvested and analysed daily over a period of 10 and 15 days, respectively.

The ability of the organism to utilize two carbohydrates was examined using media containing 1% glucose, 2% glucose, 1% lactose, 2% lactose, and 2.6% dehydrated sweet whey permeate. The latter contained 70% lactose (equivalent to 1.82% lactose in the media) plus additional minerals and growth factors from the whey.

The effect of supplying additional carbon substrate during fermentation was studied at 12 °C by adding 0.5 g glucose to 100-ml cultures at 2 days prior to harvest; another experiment involved adding two spikes to each sample, one at 2 days prior to harvest and the other at 4 days prior to harvest. This was done for samples harvested at 10, 13, and 15 days.

The effect of temperature shifts on EPA production was investigated by initially growing the mold in fermentation flasks at 25 °C for periods of 1, 2, and 3 days, after which the flasks were transferred to shaking platforms in 12 °C incubators and the fermentations were allowed to proceed. Samples were harvested from each after additional fermentation times of 6, 8, or 9 days at this lower temperature.

Analysis

The pH was measured at the end of the fermentation. The mycelia were harvested by filtering the suspension through a coarse-sintered glass funnel. The lipids were extracted by the procedure of Folch [4]. The semi-dry mycelia from the funnel were suspended in 50 ml of 2 : 1 $CHCl_3$: CH_3OH (v/v) and were homogenized for 1 min in a blender at high speed. The suspension was filtered through a coarse-sintered glass funnel. The volume of the extract was measured and readjusted to 50 ml, using the additional solvent that was needed as a rinse of the residue on the filter. The residue was dried in a desiccator, and the dry weight of this defatted residue recorded. The organic phase containing the lipids was washed with 10 ml of 0.37% KCl to remove water-soluble material, and the organic phase was evaporated to dryness and weighed. The dry lipid residue was redissolved in 2 : 1 $CHCl_3$: CH_3OH (v/v), and aliquots containing 20 mg of lipid residue were saponified with KOH/BF_3 and esterified with BF_3/MeOH. The fatty acid methyl esters (FAMES) were extracted with isooctane and analysed by capillary gas chromatography [20].

Gas chromatography

Gas chromatography was performed with a Hewlett Packard 5890 Gas Chromatograph equipped with a flame ionization detector and a split injector. The He flow rate was 0.92 ml/min. The fused silica capillary column was 30 m × 0.32 mm I.D., and was coated with 0.25 μm Supelcowax 10 (Supelco Inc; Bellefonte, PA) as the stationary liquid phase. The injector and detector temperatures were 220 °C. The FAMES were separated by the temperature programming sequence of isothermal operation at 180 °C for 16 min, then 3 °C/min to 240 °C, and isothermal operation at 240 °C for 10 min. Peak identities were determined by qualitative standards (Supelco) and confirmed by chemical ionization/mass spectrometry (Mass Spectroscopy Service Group, Center for Advanced Food Technology, Rutgers University, New Brunswick, NJ). Quantitation was by the method described by Slover [20]. Known amounts of heptadecanoic acid (17 : 0) to serve as internal standard were added to the aliquots of lipid extract prior to derivatization to FAMES.

RESULTS

Phosphate and pH

The results of the initial experiments to establish satisfactory phosphate concentration and initial pH are given

TABLE 1

Effect of phosphate molarity on EPA production at 25 °C in yeast extract/malt extract medium

mM K_2HPO_4 (pH = 6)	Final pH	Mycelia yield (mg[a]/ml[b])	Lipid yield (mg/ml)	EPA yield (μg/ml)	% EPA in lipid
0	5.16	2.2	0.47	31.6	6.69
3	5.09	2.2	0.52	30.5	5.90
6	5.15	1.6	0.40	24.4	6.08
12	5.35	1.0	0.29	14.8	5.14
24	5.59	1.2	0.40	18.0	4.50

[a] Dry weight of defatted residue.
[b] ml of culture medium.

in Tables 1 and 2. Optimum EPA production occurred with 0–3 mM added K_2HPO_4 with only minor differences in mycelia yield and EPA concentration (μg/ml) in this range of phosphate concentrations. Phosphate concentrations at levels 6 mM and above reduced EPA production (μg EPA/ml). Both yield of EPA/ml and percentage of EPA in lipid decreased at high phosphate concentrations (12–24 mM). In subsequent experiments, media were supplemented with 3 mM phosphate to ensure that phosphate would not be a limiting factor. There was no correlation between biomass or lipid yield and EPA production.

Table 2 shows the optimal initial pH for EPA production (μg EPA/ml) was between pH 6 and 7. EPA production in terms of μg EPA/ml was inhibited at initial pH = 8.0; the EPA content of the lipid extract also decreased. The remainder of the experiments described in this paper were done with media containing 3 mM K_2HPO_4, adjusted to an initial pH = 6.0.

Temperature and time

The effects of growth at 12 and 25 °C on mycelial mass and EPA production are depicted in Figs. 1 and 2, and Table 3. Maximum mycelial production was comparable at the two temperatures, but mycelial growth was more rapid at 25 °C (Fig. 1). In the 25 °C fermentation, the dry weight reached a maximum after 4 days, and declined slightly through the remainder of fermentation. Growth was slower during the 12 °C fermentation as the maximum mycelial dry weight was not reached until 7 days,

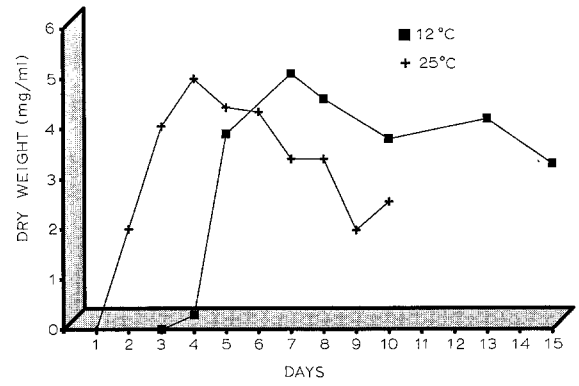

Fig. 1. Effect of temperature on growth of *P. irregulare* ATCC 10951 in submerged culture on a rotary shaker at 150 RPM using the modified yeast extract–malt extract media as described under MATERIALS AND METHODS. Growth was monitored by measuring the dry weight of the defatted residue.

TABLE 2

Effect of initial pH on EPA production at 25 °C in yeast extract/malt extract medium containing 3 mM K_2HPO_2

Initial pH	Final pH	Mycelia yield (mg[a]/ml[b])	Lipid yield (mg/ml)	EPA yield (μg/ml)	% EPA in lipid
5	5.14	1.6	0.38	23.1	6.08
6	5.09	2.0	0.52	30.4	5.85
7	5.44	2.9	0.59	35.1	5.95
8	5.42	2.1	0.47	12.6	2.68

[a] Dry weight of defatted residue.
[b] ml of culture medium.

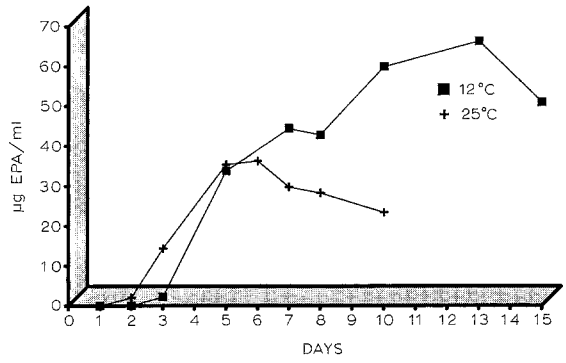

Fig. 2. Time course of EPA production by *P. irregulare* ATCC 10951 at 12 °C and 25 °C. The fermentations were conducted as described under MATERIALS AND METHODS. The results are the average of three replicates.

after which there was a gradual decrease in mycelial weight. Although both 12 and 25 °C ultimately gave comparable amounts of mycelia, higher EPA production was observed at 12 °C (Fig. 2). On a volume basis, maximum EPA yield at 25 °C was at 6 days; at 12 °C, maximum EPA yield was at 13 days.

An increase in pH to about 8.8 occurred at the end of the fermentation with both temperatures (Table 3). The EPA content of the mycelia proved to be variable, increasing in the 12 °C fermentation from 8.5 to 15.6 µg EPA/mg defatted residue by the end of the fermentation. The EPA content of the mycelium in the 25 °C fermentation increased from 3.97 µg EPA/mg defatted residue to 9 µg EPA/mg defatted residue at the end of the fermentation. The EPA content of the lipid fraction was higher at 12 °C (6–8%) than in the 25 °C fermentation, where the lipid extract contained 4–5% EPA.

Effect of carbohydrates

Different carbon sources were examined for their ability to promote growth and EPA production (Fig. 3 and Table 4). Glucose at 1% concentration was the most effective carbon source tested for promoting growth and EPA production. Increasing glucose concentration to 2% resulted in decreased growth and EPA production. Lactose and sweet whey permeate were also suitable for EPA production. Sweet whey permeate, which contains 70% lactose, is a by-product of the dairy industry, and would be an inexpensive alternative to purified lactose. The 2.6% sweet whey permeate media would be equivalent to 1.82% lactose. The sweet whey substrate was superior for EPA production than either lactose media. On a mycelial dry weight basis, the highest mycelial content of EPA was obtained with sweet whey permeate

TABLE 3

Time course of EPA production at 12 °C and 25 °C in yeast extract/malt extract medium containing 3 mM K_2HPO_4

Temperature	Days	Final pH	Lipid yield (mg[a]/ml[b])	% EPA in lipid
12 °C	3	6.12	0.22	0.98
	5	5.89	0.49	6.88
	7	6.73	0.53	8.23
	8	6.00	0.76	5.84
	10	7.81	0.99	6.05
	13	7.79	0.95	6.69
	15	8.83	0.62	8.83
25 °C	2	5.59	0.24	0.83
	3	6.67	0.42	3.40
	5	7.63	0.80	4.41
	6	7.83	0.69	5.25
	7	8.27	0.60	4.95
	8	8.54	0.56	5.04
	10	8.55	0.52	4.48

[a] Dry weight of defatted residue.
[b] ml of culture medium.

TABLE 4

Effect of carbon source on EPA production at 12 °C and 10 days

Carbon source	Final pH	Mycelia yield (mg[a]/ml[b])	Lipid yield (mg/ml)	% EPA in lipid
1% glucose	5.72	4.5	0.93	5.57
2% glucose	5.54	4.1	0.68	6.03
2.6% whey permeate	8.17	1.9	0.48	6.69
2% lactose	8.08	1.6	0.40	5.27
1% lactose	8.06	1.4	0.34	5.22

[a] Dry weight of defatted residue.
[b] ml of culture medium.

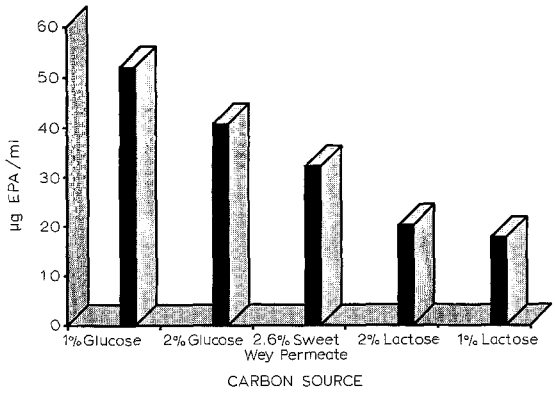

Fig. 3. Effect of carbon source on EPA production by *P. irregulare* ATCC 10951. The mold was grown at 12 °C under the conditions described under MATERIALS AND METHODS, except that the carbon source was varied as indicated.

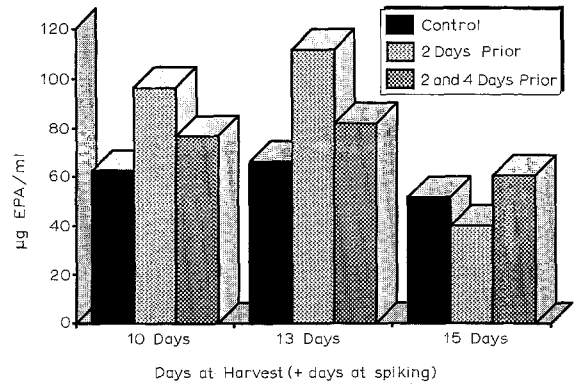

Fig. 4. Effect of glucose spiking of the growth medium on the EPA production by *P. irregulare* ATCC 10951. The organism was grown at 12 °C in submerged culture using a rotary shaker platform at 150 rpm. The control cultures were grown for 10, 13 and 15 days without spiking. The spiking was done at 4 days prior to harvest (i.e., on days 6, 9, and 11, respectively); and 2 days prior to harvest (i.e., on days 8, 11 and 13, respectively). The spikes were 0.5% glucose (0.5 g glucose/100 ml culture).

(16.9 µg EPA/mg defatted residue), and the lowest with 2% glucose (Table 4). The EPA content of the lipid showed little variation (5.2–6.7% EPA).

Effect of spiking

Glucose spiking gave large increases in EPA content in cultures harvested after 10 and 13 days when the cultures were spiked 2 days before harvest (Fig. 4 and Table 5). This procedure gave the highest EPA yield achieved to date (Fig. 4, 112 µg/ml; 26.67 µg/mg defatted residue).

Spiking the cultures with glucose on one occasion (2 days prior to harvest) proved to be superior to spiking the cultures twice (on 2 days and on 4 days prior to harvest). Spiked samples harvested at day 13 had higher EPA content than 10 days. The results shown in Fig. 4 are from spiking with 0.5% glucose (0.5 g glucose/100 ml medium). Spiking with larger amounts of glucose (1 g glucose/100 ml medium; not shown) resulted in decreased yield.

Effect of temperature shift

The results of shifting the temperature from 25° to 12 °C are shown in Fig. 5 and Table 5. Short periods of rapid growth at 25 °C followed by longer periods at 12 °C decreased fermentation time and still allowed high EPA production. The best combination was 2 days at 25 °C, followed by 6 days at 12 °C.

TABLE 5

Representative effects of fermentation parameters on EPA production in yeast extract/malt extract medium

	Final pH	Mycelia yield (mg[a]/ml[b])	Lipid yield (mg/ml)	% EPA in lipid
Effect of glucose spiking on cultures for 13 days at 12 °C				
No spiking	7.79	4.2	0.95	6.69
Spiked at day 11	7.40	4.2	2.01	5.57
Spiked at days 9 & 11	7.34	3.8	1.46	5.62
Effect of growing culture for 2 days at 25 °C, then shifting to 12 °C for 6 days				
	7.62	3.8	1.57	5.91

[a] Dry weight of defatted residue.

[b] ml of culture medium.

TABLE 6

Effects of fermentation parameters in fatty acid profiles

	Fatty acid profile of lipid extract, wt %															
	14:0	16:0	16:1	18:0	18:1.9	18:1.7	18:2.6	18:3.6	20:0	20:1.9	20:3.6	20:4.6 (ARA)	20:5.3 (EPA)	22:0	Unknown	100 × ARA/EPA
12 °C, isothermal																
5 days	9.39	18.59	5.90	2.75	13.37	1.84	15.27	1.78	1.18	2.97	2.34	4.06	15.69	1.25	3.53	25.88
7 days	9.90	19.57	11.62	2.17	20.26	3.14	11.42	0.90	0.65	3.17	1.10	3.34	8.44	0.62	3.48	39.57
8 days	10.25	19.19	10.57	2.25	17.86	2.86	12.44	1.04	0.68	2.9	1.3	4.08	10.1	0.66	3.41	40.40
10 days	9.23	16.56	5.74	1.13	15.78	3.33	13.97	0.91	0.35	3.11	1.31	7.12	12.29	0.47	8.10	57.93
13 days	10.07	15.47	5.56	1.54	11.40	2.61	16.21	1.14	0.67	3.56	1.70	7.41	15.20	1.01	5.56	48.75
15 days	10.51	13.87	0.20	0.85	8.20	2.09	15.90	1.20	0.46	4.00	1.78	10.55	17.27	0.61	11.80	61.09
25 °C, isothermal																
3 days	8.55	21.97	6.07	3.15	13.66	1.18	17.76	1.33	1.53	2.42	1.14	9.35	7.54	1.73	2.40	124.01
5 days	8.03	18.5	9.66	2.01	19.12	2.12	15.6	1.08	0.96	3.59	0.78	6.89	7.07	1.32	2.94	97.45
6 days	9.71	16.59	5.09	2.20	12.44	1.09	18.47	0.94	1.41	2.87	1.12	11.11	12.31	1.51	2.64	90.25
7 days	9.48	16.76	6.77	2.10	15.87	1.55	16.97	1.41	1.22	3.57	1.05	8.93	10.19	1.20	2.64	87.63
8 days	9.10	14.66	4.25	2.15	11.06	0.84	17.39	1.01	1.87	3.14	1.25	11.72	11.86	1.51	7.81	98.82
10 days	9.46	16.09	4.45	2.65	12.67	0.97	18.37	1.00	2.26	3.92	1.37	11.31	10.13	1.99	2.90	111.65
Effect of spiking on fatty acid composition (12 deg, 13 days, isothermal)																
spiked day 11	8.70	18.44	0.57	1.81	14.55	2.93	14.02	1.36	0.87	3.17	1.87	4.72	13.05	1.11	11.44	36.17
spiked, day 9 & 11	7.78	17.81	0.30	1.35	18.46	3.84	12.98	0.88	0.38	3.16	1.47	6.01	10.33	0.55	14.19	58.18
Effect of carbon source on fatty acid composition																
1% glucose (see above)																
2% glu	9.77	16.31	6.16	2.19	10.12	2.09	17.29	1.54	1.16	3.05	1.92	5.49	16.3	1.9	3.96	33.68
2.6 % whey permeate	6.76	16.65	9.48	0.72	9.59	3.2	14.97	1.08	0.34	3.51	1.85	8.5	19.12	0.46	3.69	44.46
2% lac	7.11	16.35	5.31	1.49	8.28	1.84	18.48	0.82	0.47	3.68	1.14	9.95	19.95	0.96	3.54	49.87
1% lac	7.16	17.00	5.14	1.69	8.01	1.81	18.36	0.86	0.62	3.76	1.38	9.18	19.93	1.17	3.22	46.06

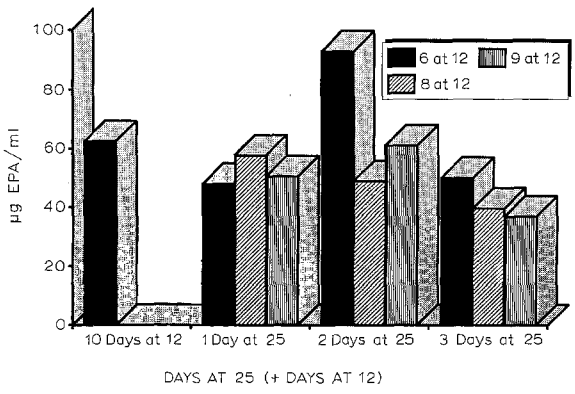

Fig. 5. Effect of EPA production of shifting the temperature during the fermentation from 25 °C to 12 °C. The cultures of *P. irregulare* ATCC 10951 were grown initially at 25 °C for periods of 1, 2 or 3 days. Following the initial growth at 25 °C, the flasks were transferred to a 12 °C incubator, and incubated for an additional time of 6, 8 or 9 days at this lower temperature.

Composition of lipid extract

Table 6 shows the contribution of individual fatty acids to the total fatty acid content of the lipid extract. EPA was generally the most abundant PUFA, comprising 7–19% of the total fatty acid content of the lipid extract. At 12 °C, the maximum EPA content, 17%, was reached at 15 days. At 25 °C, the highest EPA content attained, 12%, was reached at 8 days. Sweet whey permeate and the lactose media gave the highest EPA content of the lipid extracts, 19.92–19.95%. Higher concentrations of ARA, another PUFA having physiological activity, were obtained at 25 °C. Longer fermentation times also favored higher ARA concentration.

DISCUSSION

Submerged cultures of EPA-containing *P. irregulare* may be an attractive possibility as an alternative source of EPA when compared to fish oil or microalgae. The highest yield of EPA found in this study was 112 mg EPA/l. The yield of EPA reported by submerged culture of the microalgae *Chlorella minutissima* [15] was 8 mg/l. The yield of EPA by pond culture of the microalgae is unknown, but was reported to be low [17]. The EPA content of *P. irregulare* lipid compares favorably with that of fish oil (6.6% EPA for herring oil) [7].

As this study indicates, the EPA content of the mycelia is variable and is affected by the fermentation conditions. Conditions may be altered to increase both the total quantity of EPA as well as its concentration in the lipid extract. The optimum temperature found for EPA production by *P. irregulare* was 12 °C. Glucose was the most effective carbon source for high EPA content, followed by sweet whey permeate (containing 70% lactose). Spiking with glucose increased EPA content, and temperature shifts permitted shorter fermentation times.

Large amounts of ARA in the diet have been reported to have deleterious effects on health [9]. As the results of the analysis of the PUFAS shown in Table 5 indicate a significant amount of ARA relative to EPA, the ARA content should be carefully considered. The higher temperature (25 °C) resulted in formation of larger amounts of ARA relative to EPA. Formation of ARA increased at both 12 and 25 °C with increasing age of the culture. Purification of the product would probably be desirable to increase the EPA content and eliminate the ARA of the lipid fraction.

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