

Production of eicosapentaenoic acid by the filamentous fungus *Pythium irregulare*

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Abstract. Because of the diversity of their lipids and fatty acid biosynthetic pathways, lower fungi may find utilization as sources of omega-3 or other polyunsaturated fatty acids (PUFA). Production of eicosapentaenoic acid (EPA) by the filamentous fungus, *Pythium irregulare*, has been demonstrated in 14-l fermentors. Sweet whey permeate (lactose) was preferred over glucose as a substrate for production of a high-EPA-content lipid. Characterization of the lipid indicated that approximately 90% of the EPA was contained in the neutral lipid fraction. A specific productivity of 24.9 mg EPA/g dry biomass was achieved at 14°C, at which temperature the lipid contained 25.2% EPA and 54.2% PUFA. This is the highest mycelial EPA content for a fungal lipid that has been reported in the literature.

Introduction

The role of 5,8,11,14,17-*cis*-eicosapentaenoic acid (EPA) in lowering blood triglyceride levels, the potential for blood clot formation, and thus, purportedly, the risk of coronary heart disease, as well as its involvement in inflammation responses (Simopoulos 1989), has led to research to identify potential sources of this omega-3 fatty acid. Putative uses of EPA-containing fats or oils would be as a food additive or feedstock for further purification and concentration for pharmaceutical applications. Fish oils, the current commercial supply of EPA, are relatively low in EPA content, possess objectionable tastes and odors, and require processing to remove cholesterol and small amounts of potentially toxic impurities.

As alternative sources, most attention has been focused on marine microalgae, for example, *Chlorella minutissima* (Seto et al. 1984) but EPA production in fungi of the genera *Mortierella* (Shimizu et al. 1988)

and *Pythium* (Gandhi and Weete 1991) has also been reported. Microbial sources of EPA and other polyunsaturated fatty acids (PUFA) have been recently reviewed (Radwan 1991). *P. irregulare* is a filamentous fungus of the Oomycetes group of the lower Phycomycetes family of fungi. It was identified as a potential organism for EPA production from a specific screening of selected genera of fungi (Wessinger et al. 1990).

Industrial use of fungi for EPA production would require design of a process that would include fermentation, mycelium separation from the fermentation broth, and lipid extraction from the fungal mycelium. Few published studies have examined EPA production by filamentous fungi in fermentors; Kendrick and Rattledge (1992) recently investigated polyunsaturated fatty acid (PUFA) production in *Entomophthora exitalis*. The purpose of this study was to determine the effect of fermentation variables on growth rate, specific EPA productivity, and lipid production in fermentors operated in an airlift mode. An additional objective was to evaluate a lactose-based medium for EPA production by *P. irregulare*. Sweet whey permeate, a major dairy industry by-product, is a plentiful, inexpensive source of lactose. Its increased utilization in new products or processes could ameliorate a significant disposal problem of the dairy industry.

Materials and methods

Organism/media. Cultures of *P. irregulare*, ATCC 10951, were maintained on corn meal agar slants. The media employed in fermentation experiments were as follows. (1) Sweet whey permeate (SWP) medium: spray dried sweet whey permeate powder (Clofine Dairy Products, Linwood, N.J., USA), 13 g/l; glucose, 3 g/l; yeast extract, 3 g/l. (2) Corn steepwater (CSW) medium: spray-dried sweet whey permeate powder, 13 g/l; glucose, 3 g/l; corn steepwater (50% solids, Corn Products, Englewood Cliffs, N.J., USA), 1.64 g/l. (3) Glucose (GLU) medium: glucose, 10 g/l; yeast extract, 3 g/l. The pH of all media was adjusted to 6.5 prior to autoclaving. The lactose content of SWP and CSW media was 10 g/l. In media containing sweet whey permeate, a concentrated solution was autoclaved separately and added to the remaining ingredients.

Fermentations. Fermentations were carried out in a 14-l New Brunswick Scientific MagnaFerm bench-top fermentor from which the interior baffles and agitation devices had been removed to allow operation in an airlift-type mode. Foaming was controlled either mechanically or by automatic addition of a commercial antifoam. Temperature was controlled by circulation of water from a constant temperature bath through the fermentor cooling coil. The aeration rate was 8–10 standard l/min for a fermentor working volume of 10 l. Fermentations were conducted as fed-batch by periodic addition of a concentrated sweet whey permeate solution. The inoculum consisted of 500 ml of a macerated 3-day-old culture grown at 24°C in the fermentation medium. Maceration was carried out in a Waring-type blender for 30 s at low speed. Lactose and glucose consumption rates were determined by a linear fit of the data during the growth phase of the fermentation.

Analytical. Lactose and glucose analyses were performed as described previously (Wessinger et al. 1990).

The fatty acid composition of mycelial lipids was determined on a lyophilized sample of mycelia extracted according to the procedure of Folch et al. (1957). The crude lipid extract was dissolved in CHCl_3 and methylated by the procedure of Slover and Lanza (1979). Quantification as the weight % of fatty acid methyl esters (FAME) was conducted on a Hewlett Packard 5890 gas chromatography equipped with a 30 m Supelcowax 10 M capillary column using temperature programming between 180–240°C (180°C for 16 min, 3°C/min to 240°C, 2 min at 240°C). Standard FAME mixtures and an internal standard, heptadecanoic acid, provided the basis for quantification.

For lipid fractionation, a crude mycelial extract was obtained after co-current milling and solvent extraction (with 3/2 (v/v) hexane-isopropanol) of the wet, filtered mycelia grown on SWP medium. The crude mycelial extract was the material that remained after evaporation of the extraction solvent. This extract was further extracted with 2/1 (v/v) chloroform-methanol and dried under N_2 to obtain the mycelial lipids. A weighed sample of lipid was dissolved in CHCl_3 and applied to the bottom of a silica gel TLC plate along with known standards at the edges of the plates. After separation with the chromatography solvent, hexane/diethyl ether/formic acid (70/30/1), the standards were sprayed with phosphomolybdic acid (5% in ethanol), dried, and heated for visualization. Individual lipid fractions were scraped from the plates, eluted with 1/1 chloroform-methanol (methanol for polar lipids), dried, and weighed.

The lipid fractions were derivitized by dissolution in CHCl_3 , saponification with KOH/BF_3 , and esterification with BF_3 /methanol. The FAMES were extracted with isooctane and quantified by gas chromatography as above. In this procedure, a known amount of the internal standard, heptadecanoic acid, was added to each fraction before derivatization.

Results

The results of preliminary experiments demonstrated that the airlift mode of operation in comparison to the standard stirred tank configuration produced a relatively dispersed mycelial morphology and minimized growth on submerged surfaces. Growth occurred as small flocs, not pellets. However, some attachment did occur on interior surfaces and above the liquid level. The attached growth caused fouling of dissolved O_2 (DO) electrodes and precluded accurate, on-line measurement of DO and biomass concentrations. The lactose consumption rate was used to examine the effects of fermentation variables on the growth rate of *P. irregularis* in these experiments. The course of a typical

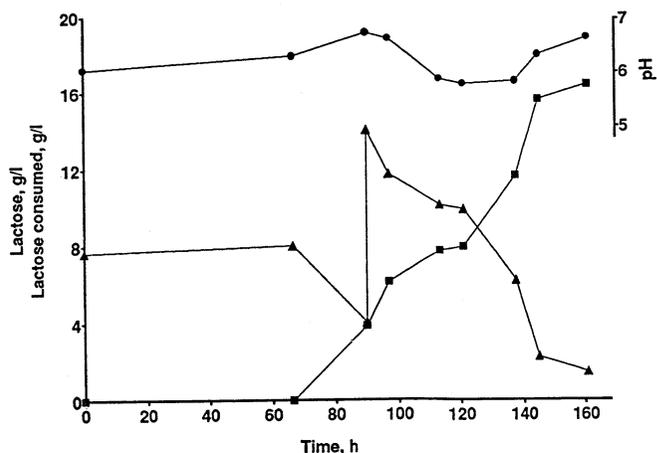


Fig. 1. Fermentation of sweet whey permeate medium by *Pythium irregularis* at $T=22^\circ\text{C}$. Lactose (as sweet whey permeate) was added at 90 h: \blacktriangle , lactose; \blacksquare , lactose consumed; \bullet , pH

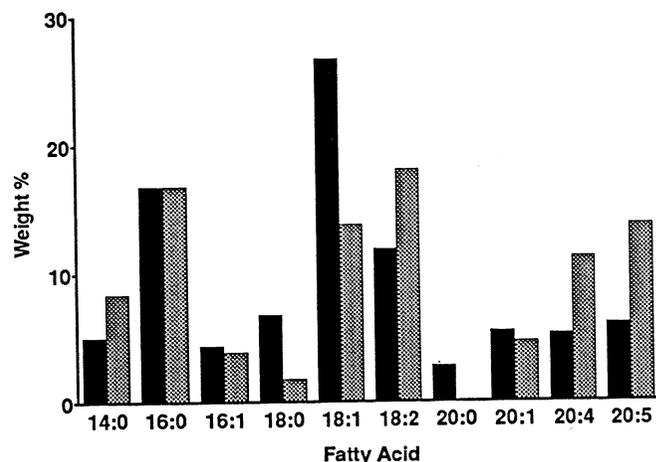


Fig. 2. Effect of fermentation medium on lipid fatty acid distribution: \blacksquare , GLU medium; \square , SWP medium. See text for media

fermentation is depicted in Fig. 1. Growth on lactose began after the glucose originally present in the medium, added to minimize the length of the lag phase, was exhausted.

The results of fermentation experiments are presented in Table 1. Shake-flask experiments (data not shown) had determined that on a N equivalent basis CSW and SWP media could support equal biomass yields. However, results of fermentor studies demonstrated the statistically significant ($P=0.05$) inferiority of CSW medium with regard to lactose consumption rate and EPA specific productivity, defined as yield of EPA per unit of dry biomass (w/w), in lactose-based media. While the disaccharide lactose is generally considered to be a poor substrate for most fungi, comparison of SWP and the glucose-based GLU media on a glucose-equivalent basis (galactose is not metabolized by *P. irregularis*) revealed that the lactose consumption rate was approximately 70% of the glucose consumption rate.

Table 1. Eicosapentaenoic acid (EPA) production by *Pythium irregulare* in 10-l fermentations

Culture ^a medium	T (°C)	Lactose consumption rate (g/l/h)	Lipid content (g/100 g dry wt)	Lipid fatty acid distribution (wt %)		EPA specific productivity (mg EPA/g dry wt)
				EPA	PUFA	
SWP	22	0.18	9.8	16.5	47.0	16.2
		0.23	14.4	14.0	43.6	20.2
		0.20	7.5	21.3	55.8	16.0
		0.18	12.7	13.8	45.4	17.5
CSW	22	0.16	8.8	11.6	35.5	10.2
		0.11	6.3	10.4	34.2	6.6
		0.17	11.7	9.6	34.1	11.2
SWP	14	0.12	8.5	24.1	54.0	20.5
		0.09	9.9	25.2	54.2	24.9
GLU	22	0.14 ^b	17.9	6.2	25.7	11.1
		0.13 ^b	34.1	6.1	24.8	20.8

PUFA, polyunsaturated fatty acids

^a Media compositions defined in text^b Glucose consumption rate as g glucose/l per hour**Table 2.** Effect of fermentation temperature on fatty acid distribution of *P. irregulare* lipids^a

Major fatty acids	Fermentation temperature (°C)	
	22	14
14:0	8.4	6.8
16:0	16.8	13.4
16:1	3.9	2.7
18:0	1.8	1.8
18:1	13.8	9.3
18:2	18.1	15.6
20:1	4.7	5.2
20:4	11.3	10.4
20:5	13.8	25.2

^a Fermentation conditions described in text**Table 3.** Composition of *P. irregulare* lipids^a

Lipid class	wt. %	FA distribution (% of total)	
		EPA	PUFA
Polar	10	4	4
Monoglycerides	10	1	1
Diglycerides	10	8	8
Triglycerides	53	63	65
Free fatty acids	15	22	19
Sterols	3	1	2

FA, fatty acids

^a Extracted from mycelia grown on SWP medium at 20°C under the conditions described in text

Additionally, lipid production and the lipid fatty acid distribution were substantially different in GLU medium. While average lipid production increased by over 100%, the EPA and PUFA contents decreased dramatically and EPA productivity remained generally unchanged. The complete fatty acid profiles of lipid

produced by growth on SWP and GLU media are presented in Fig. 2.

It is well known that cultivation of many fungi at reduced temperatures increases lipid unsaturation (Niedleman 1987). In *P. irregulare* fermentations carried out at 14°C in SWP medium, the lactose consumption rate decreased by approximately 50% but the maximum lipid EPA and PUFA contents were obtained, 25% and 54%, respectively. Fatty acid distributions of the lipid produced at 22 and 14°C are compared in Table 2.

Further characterization of *P. irregulare* mycelial lipids consisted of fractionation into standard classes and determination of fatty acid profiles for each class. Results of a typical fractionation of lipid from mycelia grown on SWP medium are given in Table 3. Neutral lipids, mainly triglycerides, predominated, comprising 88% of the lipid weight, which accounted for 94% of the EPA.

Discussion

The strategy for medium development for *P. irregulare* fermentations consisted of utilizing a complex nitrogen source, which would also provide all essential micronutrients together with sweet whey permeate providing lactose as the primary carbon substrate. Yeast extract was utilized as the nitrogen source, since average EPA specific productivity in the CSW medium decreased 44% from that in the SWP medium. Yeast extract was found to be the preferred nitrogen source for EPA production by a *Mortierella* species (Bajpai et al. 1992).

There were obvious differences in the fermentations conducted in GLU and SWP media. Lactate and acetate were products of the acidogenic GLU fermentation (data not shown) but were not produced in SWP fermentations, which required no pH control. Whereas, on a biomass basis, EPA production in the two me-

dia was not significantly different, the EPA content of the lipid grown on SWP medium was substantially higher (14–21% vs 6%), which has an important bearing on potential uses of the lipid, as discussed later. Gandhi and Weete (1991) reported no differences in lipid content or EPA production when *P. ultimum* was grown on sucrose, maltose, or glucose. Lactose was not investigated. Besides its beneficial impact on *P. irregulare* lipid fatty acid profiles, SWP medium provided a good growth medium as the relative growth rate (as represented by the sugar consumption rate) in SWP medium was approximately 70% of that on GLU medium.

The major effect of temperature (14°C vs 22°C) on EPA production by *P. irregulare* in the experiments reported here was an increase in the EPA content of the mycelial lipids, approx. 25% vs 14–21% under similar conditions. In shake-flask studies with other fungi, Shimizu et al. (1988) reported that EPA production only occurred in *Mortierella alpina* below 28°C in this high-arachidonic acid (20:4) producer and lipid content was constant. Reduced temperature (13°C) induced changes in the fatty acid composition of *P. ultimum* lipids (Gandhi and Weete 1991) but the EPA content was unaffected and both lipid content and EPA yield dropped substantially as the incubation temperature was decreased from 25 to 13°C. The differences in methods of cultivation (shake flask vs fermentor), growth medium, and other cultivation conditions make comparison of these results among organisms difficult. Kendrick and Ratledge (1992) recently demonstrated that temperature rather than accompanying changes in growth rate or DO levels was the predominant factor in the degree of lipid unsaturation in the fungus *Entomophthora exitalis*.

For industrial use of microbial-derived sources of EPA, properties such as the type of lipid and the distribution of EPA among the lipid classes become important in the extraction of lipid from microbial biomass and its subsequent processing. The high percentage of neutral lipids in *P. irregulare* mycelial lipids, typically 80–85%, is advantageous in solvent extraction operations for recovery of lipid from fungal mycelia. A high neutral lipid content was also reported in a different strain of *P. irregulare* (Bhatia et al. 1972). Compared to other fungi being studied for EPA production, mycelial lipid from *P. irregulare* as reported in the present study had the highest EPA fatty acid content. Maximum values reported were 25.2% (this study), 13.5% for *M. alpina* (Shimizu et al. 1988), 15.1% for *M. elongata* (Bajpai et al. 1992), and 11.5% for *P. ultimum* (Gandhi and Weete 1991). A high EPA content would be an important parameter in purification of a microbial oil to yield a product with an enhanced EPA and/or PUFA content.

The maximum biomass-specific productivities of the fungi cited above are approximately equivalent for *P. irregulare*, *M. alpina*, and *P. ultimum*, at 24.9, 29, and 22 mg EPA/g dry weight, respectively. The productivity of *P. irregulare* was measured in fermentor experiments, not shake-flask studies as were those for *M. al-*

pina and *P. ultimum*, and thus may not be directly comparable due to differences in growth morphology that could affect lipid production and composition. Many studies have been conducted on improvement of lipid formation or degree of lipid (poly)unsaturation in microorganisms (e.g. Neidleman 1987). Interestingly, for *P. irregulare*, *M. alpina*, and *P. ultimum* the effects of cultivation variables were quite different. In SWP medium, EPA productivity in *P. irregulare* was relatively unaffected by cultural conditions whereas increased EPA productivity in *P. ultimum* and *M. alpina* was attributed to increased lipid production and activation of desaturase enzymes at low temperature, respectively. Improvements in fermentor volumetric EPA productivity by *P. irregulare* could best be obtained by increased biomass yields, which for fermentations on SWP medium were in the range 3–4 g dry wt/l.

In summary, the effects of major fermentation variables on EPA production by the filamentous fungus, *P. irregulare*, have been investigated in 14-l fermentors. Whereas lactose is not often considered as a substrate for fungal growth, fermentation in a medium based on sweet whey permeate significantly increased the EPA content of the mycelial lipids. Results have demonstrated that this organism has the capability of producing a lipid with the highest EPA content (25%) of any fungal mycelial lipid reported in the literature.

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