

# Recovery of Eicosapentaenoic Acid from Fungal Mycelia by Solvent Extraction

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Utilization of lipids containing eicosapentaenoic acid (EPA) produced by microorganisms requires processes for their efficient recovery from microbial cells. Recovery of EPA from mycelia of the fungus *Pythium irregulare* by solvent extraction with hexane-isopropanol (HIP) in a pilot-plant colloid mill was investigated. Extraction efficiencies of 96% for lipid and EPA were achieved with a 3:2 (vol/vol) HIP mixture by milling wet, filtered mycelia for 5 min at a solvent/dry solids ratio of 100 L/kg. The process yielded a crude extract that contained up to 96% lipid and an EPA content as high as 24% (with no selectivity for EPA).

**KEY WORDS:** Colloid mill, fatty acid, hexane, isopropanol, lipid, polyunsaturated fatty acid, *Pythium irregulare*.

5,8,11,14,17-*cis*-Eicosapentaenoic acid (EPA) is an omega-3 polyunsaturated fatty acid, 20:5, which has beneficial effects on blood lipid profiles and the body's responses to inflammation. As summarized by Simopoulos (1), the consensus of nutritional and medical studies indicates that EPA lowers blood serum triglyceride levels, reduces the degree of platelet aggregation (involved in clot formation) and is anti-inflammatory. Findings such as these suggest potential applications for EPA in the food and pharmaceutical industries.

The current commercial source of EPA is fish oil, but its relatively low EPA content and objectionable taste and odor have driven research into new sources. EPA has been identified in the lipids of microalgae (2-4) and filamentous fungi (5-7). Production of a high-EPA lipid (ca. 25% EPA) by the filamentous fungus *Pythium irregulare* has been reported (7). Microbial production of "specialty" lipids or unusual fatty acids with potential commercial applications has mostly been focused on organism selection and optimization of cultural conditions. Research on extraction systems amenable to industrial recovery of lipids from yeast cells or fungal mycelia has been concentrated in two areas—cell disruption, such as by bead mills (8,9), or liquid shear devices, such as homogenizers (10). Oil production by yeast has been reviewed by Davies (11). Recently, supercritical fluid extraction has been investigated for fungal oil extraction (12,13) and for EPA recovery from fungal mycelia (14).

A process for the recovery of EPA from filamentous fungal mycelia should have the following attributes: efficient lipid extraction at mycelial lipid contents of 10-15%, common in nonoleaginous fungi; use of nontoxic solvents or those already in industrial use; and suitability of conventional cell disruption equipment. The objectives of this study were to develop a solvent system for efficient lipid extraction from mycelia of the EPA-producing filamentous fungus *P. irregulare* and to determine the effect of operating conditions for solvent extraction on lipid and EPA recovery in a pilot-plant colloid mill.

## EXPERIMENTAL PROCEDURES

**Fermentations.** *Pythium irregulare*, ATCC 10951, was maintained on corn meal agar slants. The growth medium consisted of 13 g/L spray-dried sweet whey powder (Clofine Dairy Products, Linwood, NJ), 3 g/L yeast extract (Difco, Detroit, MI) and 3 g/L glucose, pH 6.5 (adjusted prior to autoclaving). Inocula for fermentations were 5% (vol/vol) cultures of a 3-day culture grown at 24°C and macerated in a stainless-steel Waring blender for 30 s at low speed. Fermentations were conducted in either a 14-L New Brunswick Scientific MagnaFerm fermentor (Edison, NJ) or a 70-L pilot plant fermentor (Abec, Bethlehem, PA) under conditions described previously (7). Harvesting of the mycelia was accomplished by filtration through Whatman #1 filters (Maidstone, England) and washing the mycelial cake with deionized water. The wet mycelia were packaged under vacuum in polyethylene bags and stored frozen.

Due to the large number of experiments required, mycelia from several different fermentation runs were utilized throughout this study. All experiments relative to the investigation of a particular variable were conducted with mycelia from the same fermentation. The ranges of the important mycelial variables were: lipid content (dry basis), 7-14%; specific EPA content, 8-23 mg EPA/g dry wt.

**Solvent extraction.** Experiments to evaluate candidate solvents and solvent mixtures were conducted in a bench-top Waring blender. The solvent systems tested (mixture compositions are reported as volume ratios) are listed in Figure 1. Individual solvents were evaluated alone and in combination with water at two levels, 5 and 50%. Lyophilized mycelium (300-500 mg) was successively extracted with two 75-mL volumes of solvent for one minute each, the mixture was filtered through a sintered-glass filter to remove the mycelium, and the filtrate was centrifuged to effect phase separation (if required). The organic phase was dried under a flow of nitrogen, and the extracted matter was collected with isoctane. The total extracted matter was determined after evaporation of the isoctane under N<sub>2</sub>. The results were normalized to the lipid and EPA contents, determined on a mycelial sample that was extracted by the procedure of Folch *et al.* (15).

Batch pilot-plant solvent extractions were performed in an Eppenbach colloid mill (Gifford-Wood Co., Hudson, NY), the feed hopper and milling sections of which are schematically depicted in Figure 2. The hopper and recirculation pipe were stainless-steel, and the hopper was covered with a removable rubber cover to minimize solvent evaporation. Wet, filtered mycelium and the solvent mixture were initially mixed in a large beaker before being introduced into the feed hopper. The temperature range during extraction experiments was 22-30°C. The initial dry weight of mycelium for an experiment was calculated from a dry-weight determination on a sample of the filtered, wet mycelium, or, in some experiments, by direct measurement of the dried mycelium and extracted matter at the conclusion of the experiment. Amounts of

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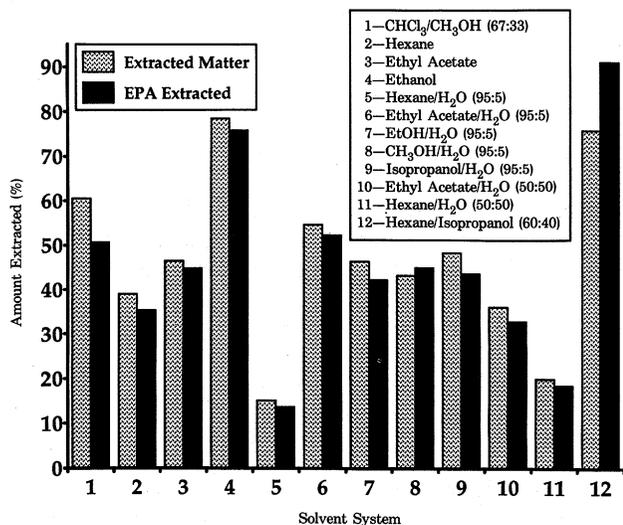


FIG. 1. Solvent extraction of *Pythium irregulare* mycelia. EPA, eicosapentaenoic acid.

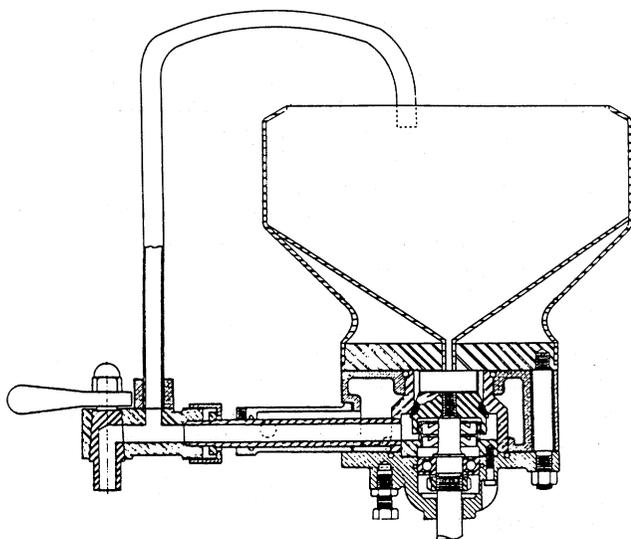


FIG. 2. Eppenbach colloid mill (Gifford-Wood Co., Hudson, NY). Feed hopper, milling section and recirculation pipe are shown.

fungal mycelium and solvent used in colloid mill experiments were: mycelium, 6–20 g dry wt (moisture content of mycelium was approximately 85%); solvent, 1.2–2.0 L. Samples were withdrawn through the sampling valve on the recirculation line. At the conclusion of the run, the solvent was separated from the mycelium by filtration through a sintered-glass filter and evaporated under a flow of N<sub>2</sub>. The crude extract was collected with isooctane and weighed after evaporation to dryness under N<sub>2</sub>.

**Analytical.** Lipid contents of mycelia and crude extract samples were determined by the procedure of Folch *et al.* (15). The extracted lipid was dissolved in CHCl<sub>3</sub> and methylated according to Slover and Lanza (16). Lipid fatty

acid distributions were determined by capillary gas chromatography as described in O'Brien *et al.* (7).

## RESULTS

The screening results of twelve solvent systems for EPA recovery from *P. irregulare* mycelia are presented in Figure 1. Eleven candidate systems were compared to solvent system #1, the solvent system utilized in the method of Folch *et al.* (15), which is a widely accepted standard for lipid extraction from biological tissue. Based on the criterion of amount of EPA extracted, 3:2 (vol/vol) hexane-isopropanol (HIP) was deemed the best solvent system of those studied. While comparable in total matter extracted, ethanol was rejected because of an unacceptably large amount of nonlipid matter extracted (11.3 vs. 21.9% of the total extracted matter for HIP and ethanol, respectively). All further studies were conducted with the HIP solvent system.

Pilot-plant experiments with the colloid mill were conducted to determine the effects of processing variables on extraction of lipid and EPA. Extraction is essentially independent of the milling time in the range of 0.5–10 min (Fig. 3). The points represent data from two separate experiments. Efficiencies of extraction of EPA and lipid (based on the starting mycelia) averaged 76.9% (range 60.9–85.9) and 72.1% (range 55.2–81.8), respectively. The lipid extraction efficiency computation is based upon the "Folch" extraction methodology (the solvent system as well as the extraction equipment used), and efficiencies greater than 100% are possible if the HIP solvent system and the colloid mill are a more effective lipid extraction system.

The solvent/solids ratio (L solvent/kg dry mycelium) is the major operational parameter affecting lipid extraction in the colloid mill. Results of pilot-plant experiments are presented in Table 1. The maximum amount of EPA and lipid is extracted at a solvent/solids ratio in the range 80–130 L/kg dry mycelium. In this range, the extraction efficiencies for EPA and lipid were 63.5–97.2 and 58.2–95.3%, respectively.

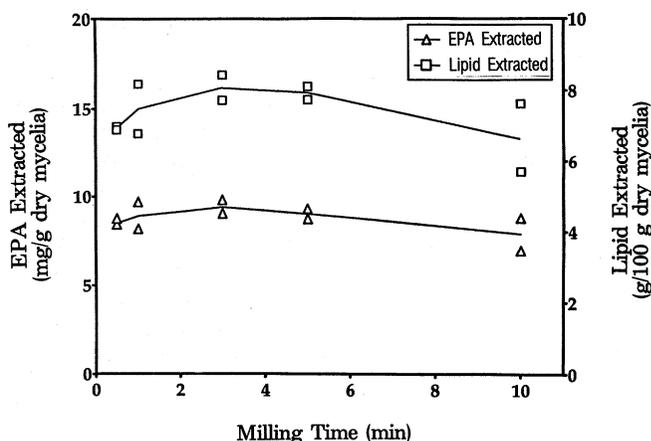


FIG. 3. Effect of milling time on extraction of lipid and eicosapentaenoic acid (EPA) from fungal mycelia by hexane-isopropanol. Extraction was conducted in colloid mill with 3:2 (vol/vol) hexane-isopropanol.

## RECOVERY OF EICOSAPENTAENOIC ACID

TABLE 1

Effect of Solvent/Dry Solids Ratio on Lipid Extraction from Fungal Mycelia<sup>a</sup>

Solvent ratio L/kg dry mycelia	Mycelia before extraction		Crude extract		Extraction Efficiency (%)	
	EPA (mg/gdw)	Lipid (g/100 gdw)	EPA (mg/gdw)	Lipid (g/100 gdw)	EPA	Lipid
35.9	9.60	10.22	4.98	5.01	51.9	49.0
42.5	9.13	11.04	4.76	5.31	52.1	48.1
79.8	9.35	10.03	7.74	8.37	82.8	83.4
85.6	9.13	11.04	5.82	6.43	63.8	58.2
124.8	9.35	10.03	7.61	8.61	81.4	85.8
133.7	9.35	10.03	9.07	9.56	97.0	95.3
197.4	9.60	10.22	5.59	5.72	58.2	56.0
198.3	9.60	10.22	5.96	5.95	62.1	58.2

<sup>a</sup>Extractions were performed in a colloid mill with 3:2 (vol/vol) hexane-isopropanol. EPA, eicosapentaenoic acid.

TABLE 2

Pilot-Plant Extraction Experiments<sup>a</sup>

Run number	Mycelia before extraction			Crude extract			Extraction efficiency (%)		
	Lipid (g/gdw)	EPA (mg/gdw) (%FA <sup>b</sup> )		Lipid (%)	Lipid <sup>c</sup> (g/gdw)	EPA <sup>c</sup> (mg/gdw)	%FA	Lipid	EPA
1	.0670	8.01	12.1	65.0	.0687	7.54	11.0	103	94
2	.0794	14.2	17.8	96.0	.0631	12.9	20.5	80	91
3	.088	15.8	18.1	94.3	.102	19.86	19.6	116	126
4	.088	15.8	18.1	96.3	.093	19.04	20.6	106	121
5	.0923	22.80	24.7	63.9	.0458	10.80	23.5	50	47
6	.095	17.6	18.5	92.3	.099	17.60	17.8	104	100
7	.095	17.6	18.5	96.0	.112	21.48	19.1	118	122
8	.1017	18.05	17.8	92.3	.0838	15.9	18.9	82	88
9	.1118	9.22	8.2	88.7	.1228	9.33	7.6	110	101
10	.1118	9.22	8.2	92.2	.0802	5.93	7.4	72	64
11	.1118	9.22	8.2	91.7	.1215	9.11	7.5	109	99
12	.1305	11.07	8.5	93.9	.1168	9.92	8.5	90	90

<sup>a</sup>Eppenbach Colloid Mill (Gifford-Wood Co., Hudson, NY), 22–30°C, 3:2 (vol/vol) hexane-isopropanol, 5 min milling time, solvent/solids ratio = 100 L/kg dry mycelium.

<sup>b</sup>Weight % EPA in fatty acids (FA). See Table 1 for other abbreviation.

<sup>c</sup>Amount extracted per g dry mycelia.

A series of pilot-plant runs was conducted to examine the characteristics of the extracted lipid and to determine extraction efficiencies of EPA and lipid under optimal conditions. The results are summarized in Table 2. Mycelia with lipid contents in the range 6.7–13.05%, which lipid contained 8.2–24.7% EPA, served as the starting materials for the experiments. Neutral lipids, predominantly triglycerides, comprise 88% of the mycelial lipid of this organism (7). Extraction efficiencies for lipid and EPA averaged 95%.

## DISCUSSION

The HIP solvent mixture has been shown earlier (17) to be an effective system for lipid extraction from biological tissues. When used as an extraction solvent for wet mycelia, as in this study, the addition of water to the system and its effects on processing variables must be considered. The presence of water has been considered essential for extraction of phospholipids from fungi (18) that are present, even though at a low level, in the lipids of *P. irregularis* (7). For mycelia at 15% solids and a solvent/solids ratio of 100 L/kg dry solids, the water content of the solvent after extraction is approximately 5%

(volume basis), at which composition only one phase exists, rendering a decanting step unnecessary. Finally, both hexane and isopropanol have long histories of safe use in the food industry.

The high degree of mechanical shear, provided by the colloid mill, yielded a fine slurry, which was easily separated by vacuum filtration into a solvent phase and mycelial cake. No emulsion was observed in the solvent phase in any experiment. As demonstrated in Figure 3, lipid extraction was rapid, on the order of one minute. The efficiency of EPA extraction was reasonably uniform in the range of 80–140 L solvent/kg dry solids (Table 1). The reasons for the decrease in extraction efficiency at a ratio of 200 are unclear but may be due to poorer mechanical shearing action at these more dilute conditions. In addition, the presence of variable amounts of water during these experiments was not studied. While the solvent/solids ratio at maximum EPA extraction efficiency, 134 L/kg dry solids, appears high, especially in comparison to a similar process reported for oil extraction from an oleaginous yeast by hexane-ethanol in which the ratio was 6 L/kg (11), the problems associated with emulsion formation and the need for multiple phase separation are avoided. In addition, it is difficult to compare

extraction conditions for oleaginous organisms to those for fungi, such as *P. irregulare*, with lipid contents of approximately 10%.

Other methods that have been proposed for extraction of microbial lipids involve either supercritical fluids or, as part of a process for the production of a yeast oil (11), a similar solvent (hexane), with extraction also occurring in a milling operation. From a processing point of view, supercritical fluid extraction can be characterized by high equipment costs and (usually) the need for expensive drying of the biomass. Advantages are the ease of solvent-lipid separation and the minimal waste streams generated. In the process described herein, conventional equipment is used and no drying of mycelium is required. However, solvent recovery *via* distillation is complicated by the formation of an isopropanol-water azeotrope. In the yeast oil process, drying of the biomass was required to eliminate the difficulties associated with emulsion formation. But extraction with hexane alone leads to a simpler and less costly solvent recovery operation.

Pilot-scale extraction of *P. irregulare* wet mycelia, produced under a variety of experimental conditions in 14 and 70 L fermentors (Table 2), demonstrated, with one exception (run #5) in which an unusually low percentage of lipid was extracted, that solvent extraction with 3:2 HIP in a colloid mill is an effective method for EPA recovery. The average extraction efficiencies for lipid and EPA, 95%, are similar to yields that have been reported for extraction of fungal mycelia by supercritical solvents, i.e., 85–90% for supercritical CO<sub>2</sub> (plus hexane or ethanol) extraction of *Mortierella* species (13) and 89% with supercritical CO<sub>2</sub> and ethanol for extraction of *Saprolegnia parasitica* (14).

The quality of the crude extract resulting from the solvent extraction operation is an important consideration in the downstream processing of the extract to a final product. Lipid contents of the extracts produced in the pilot-plant runs (Table 2) ranged from 64–96%, indicating that a reasonably pure lipid product can be obtained by this process. Because the extraction efficiencies for lipid and EPA are similar in each experiment, the extraction opera-

tion is neutral in regard to selectivity for EPA. Thus, even for mycelial lipids with as much as 25% EPA as has been reported for *P. irregulare* (7), EPA recovery by extraction with HIP in an apparatus such as a colloid mill could be accomplished with no diminution of EPA content, thereby yielding a high-EPA lipid product for potential industrial applications.

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