Lipids of *Haliphthoros philippinensis*: An Oomycetous Marine Microbe

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ABSTRACT: Lipids of the marine oomycetous microbe *Haliphthoros philippinensis* were characterized by chromatographic and spectroscopic techniques. Total lipid content of this organism was relatively low and not very responsive to manipulation of the culture conditions. Neutral lipid comprised 21% of the total lipid and the polar lipids were mainly phosphatidylcholine (44%), phosphatidylethanolamine (15%), and a ceramide-phosphorylethanolamine (19%). Palmitic (16:0) was the primary saturated fatty acid at 25% of the total fatty acids, and arachidonic acid (20:4n-6, ARA) and eicosapentaenoic acid (20:5n-3, EPA) were the major unsaturated fatty acids at 19 and 21%, respectively. Fucosterol was the principal sterol at 59% of the total sterols. The effects of several cultivation variables on growth and EPA production by this species were investigated. Among those tested, glucose and sodium glutamate were the most efficient carbon and nitrogen sources for growth, respectively. When the mycelium was cultivated for 6 d to produce biomass under optimal growth conditions, and then transferred to low temperature for an additional 13 d without glucose, the EPA content reached 31% of the total fatty acids and the yield was 203 mg/L. When the same experiment was performed with glucose supplementation during the low-temperature phase, EPA composed 27% of total fatty acids and yield reached 316 mg/L, or a 285% increase over that from mycelium cultured for 6 d at 24°C, and 56% over that cultured at 16°C for 13 d. ARA production did not respond accordingly.
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KEY WORDS: Arachidonic acid, eicosapentaenoic acid, fatty acids, fucosterol, *Haliphthoros philippinensis,* lipid, Oomycete, phospholipid, sphingolipid, sterols.

Health benefits derived from the dietary consumption of certain fish and oils therefrom are believed to be due to the presence of n-3 polyunsaturated fatty acids (PUFA) eicosapentaenoic acid (20:5 $\Delta^{5,8,11,14,17}$; EPA) and docosahexaenoic acid (22:6 $\Delta^{4,7,10,13,16,19}$; DHA) (1,2). Certain fish are the only commercial sources of EPA; but fish oil contains the mixture of n-3 fatty acids as well as the n-6 fatty acid arachidonic acid (20:4 $\Delta^{5,8,11,14}$; ARA), and it has a fishy odor. DHA from an alga is available commercially (Martek Biosciences, Inc., Columbia, MD).

It is well known that certain algae produce n-3 polyunsaturated fatty acids (3,4), and some of the relatively primitive fungi (Chytridiomycota and Mortierellaceae) are capable of producing ARA (5–7). Some species of *Mortierella* produce EPA, particularly at reduced cultivation temperatures (8). Other organisms no longer considered to be true fungi, e.g., Oomycetes, are capable of producing ARA and EPA (9–12). *Thraustochytrium* and *Schizochytrium* species produce DHA (13–17).

There is interest in finding alternate sources of EPA (18–20). Our efforts to find eukaryotic microbes that produce this nutritionally important PUFA resulted in the discovery of *Haliphthoros philippinensis* as an ARA- and EPA-producer. In this communication, we report the characterization of fatty acids, acyl lipids, and sterols from this organism, and conditions that favor PUFA production.

MATERIALS AND METHODS

Source of the fungus and culture conditions. Unless noted otherwise, *H. philippinensis* ATCC 58303 was cultivated at 24 or 28°C with rotary shaking (120 rpm) in 250-mL Erlenmeyer flasks containing 100 mL of semidefined medium (#790) as recommended by the American Type Culture Collection, referred to hereinafter as the base medium, that contained glucose (0.5%), yeast extract (0.1%) and peptone (0.1%) in synthetic seawater (SSW) (Sigma Chemical Co., St. Louis, MO). Inoculations were made with 5 mL blended mycelium from 6-d-old cultures. Mycelia were harvested by suction filtration in a Buchner funnel, washed with 100 mL SSW solution, dried by lyophilization, and stored at –20°C prior to analysis.

After determining the optimal culture conditions, including the source and concentrations of carbon, nitrogen, and other nutritional factors, the fungus was maintained in a SSW medium composed of 2% (wt/vol) glucose, 0.3% (wt/vol) yeast extract, and 1% (wt/vol) mineral solution (21), and transferred to fresh medium at 6-d intervals.

Lipid extraction and fatty acid analysis. Total lipid was extracted from dry cells (*ca*. 25 mg) using the procedure of Bligh and Dyer (22) as modified according to Kates (23), but

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with twice the solvent volume to cell dry weight ratio. All extraction and subsequent manipulations of the samples were conducted at low light intensity. Estimates of lipid content were calculated from gas chromatographic data when the methyl ester derivatives of fatty acids (FAME) were prepared directly from the cells after adding the fatty acid 23:0 as an internal standard (see below). These estimates were confirmed by gravimetric determinations from extractions of larger amounts of mycelium extracted as described above.

Thin-layer chromatography (TLC). Individual neutral lipids were separated from polar lipids by TLC on glass plates (20 cm × 20 cm) coated with a 250-μm layer of silica gel 60 (Whatman, Maidstone, NJ). The plates were developed twice in hexane/diethyl ether/acetic acid (79:20:4, vol/vol/vol) and, unless the lipids were to be recovered for further analysis, the individual lipid classes were visualized in iodine vapor. For the recovery of individual lipid classes, silica gel corresponding to the location of the lipids was removed from the plates and washed with chloroform/methanol (2:1, vol/vol). Polar and neutral lipid standards were obtained from Nu-Chek-Prep (Elysian, MN) and Avanti Polar Lipids (Alabaster, AL).

Sample derivatization and gas–liquid chromatography (GLC) analysis. For the preparation of methyl ester derivatives of the total fatty acids, a portion of total lipid (*ca.* 5 mg) was heated with 3.0 mL of methanolic-HCl at 80°C for 1 h, and FAME were then extracted with hexane (23). When the total fatty acids were derivatized directly without extraction of the lipid, 1.5 mL methanolic base (Supelco, Inc., Bellefonte, PA) was added to about 20 mg dry cells, the mixture was heated at 70°C for 15 min, and the FAME were then recovered as before (16). The FAME were analyzed by gas chromatography as described previously (5), except that the gas chromatograph (Varian 3300, Palo Alto, CA) was equipped with a 30 m × 0.25 mm fused-silica capillary column coated with DB-225 (50% cyanopropyl methyl, 50% phenyl silicone) (J&W Scientific, Folsom, CA). Injector and detector temperatures were 250°C, and the oven temperature was programmed from 140 to 160°C at 2°C/min, 160 to 180°C at 10°C/min, and then 180 to 220°C at 1°C/min. FAME were identified by comparison of their retention times relative to methyl tricosanoate (23:0) with those of authentic standards obtained from Nu-Chek-Prep, and they were quantified by the internal standard method using 23:0 as the standard.

To obtain the nonsaponifiable lipid, a portion of the total lipid (*ca.* 200 mg) was heated for 2.5 h in 8 mL of 95% ethanol containing 0.8 mL of 33% (wt/vol) KOH (23). The nonsaponifiable lipids were removed from the hydrolysate by washing it three times with 5 mL of hexane. The combined hexane washes were washed twice with 5 mL 3% KOH, twice with 5 mL H_2O, and then brought to dryness under a stream of nitrogen gas. The amount of nonsaponifiable lipid was determined gravimetrically.

Sterols in the nonsaponifiable fraction were analyzed by gas chromatography as their trimethylsilylether (TMS) derivatives prepared by heating a portion (*ca.* 20 mg) of nonsaponifiable lipid with 1 mL bis (trimethylsilyl) fluoroacetamide trimethylchlorosilane (Supelco Inc.) at 70°C for 25 min. The solvent was evaporated under nitrogen gas and the samples were diluted with hexane prior to analysis by GLC. The gas chromatograph (Varian 3400) was equipped with a 30-m fused-silica capillary SAC-5 [poly (5% diphenyl/ 95% dimethylsiloxane)] column (Supelco). Injector and detector temperatures were 300 and 310°C, respectively, and the oven temperature was 280°C isothermal. Sterols were quantitated using the TMS derivative of 5-dihydrocholesterol as the external standard.

GLC–mass spectrometry (MS). GLC-MS analysis of total sterols was performed using a VG 70E (Manchester, England) high-resolution mass spectrometer linked to a Varian 3700 gas chromatograph equipped with a 25 m × 0.25 mm DB-5 fused-silica capillary column as the sample inlet, and operated in the electron mode at 70 eV with the source temperature at 200°C.

Analysis of phospholipids. For ^{31}P nuclear magnetic resonance (NMR) analysis, a portion of total lipid (*ca.* 60 mg) was dissolved in 25 mL chloroform and washed with 25 mL Na^+/K-EDTA (0.2 M, pH 6.0) solution according to Seijo *et al.* (24). Emulsions formed during the washing process were disrupted by adding small amounts of KCl. The lower chloroform phase containing the lipid was collected and the solvent evaporated under nitrogen gas. The lipid obtained after washing was dissolved in 0.5 mL of chloroform (reagent grade) containing 5% benzene-d_6 and 3.1 mg of trimethylphosphate (TPP) as an internal reference, followed by the addition of 0.25 mL methanol (reagent grade) containing 0.2 M aqueous Cs-EDTA (pH 6.0) (25). The solution was mixed by vortexing, filtered through 0.2 μm Acrodisc nylon filters (Gellman Science, Ann Arbor, MI), and then transferred to a 5-mm NMR tube (Wilmad Glass Co., Buena, NJ). ^{31}P NMR analysis was conducted using a Bruker 250 AM system operated at 101.3 MHz.

The lipid classes were separated and quantified using an HPLC method recently used with other Oomycete species (26). The column was a LiChrosorb 5 Si 60 (3 × 100 mm) from Chrompack, Inc. (Raritan, NJ), with a solvent flow rate of 0.5 mL/min. The solvents were: A, hexane; B, isopropanol; and C, 0.04% triethylamine in water. The linear gradient timetable was: at 0 min, 100:0:0; at 5 min, 95:5:0; at 10 min, 85:15:0; at 15 min, 40:60:0; at 53 min, 40:51:9; at 68 min, 40:51:9; at 73 min, 40:60:0, at 78 min, 100:0:0; and at 100 min, 100:0:0 (%A:%B:%C, respectively). The HPLC system consisted of an ISCO (Lincoln, NE) Model 2350 Pump, an ISCO Model 2360 gradient programmer, and an Alltech-Varex (Deerfield, IL) Model Mark III Evaporative Light-Scattering detector, operated at a temperature of 40°C and a nitrogen flow rate of 1.7 L/min. The lipid extract was subjected to mild alkaline hydrolysis after removing the solvent by treating 5 mg of lipid with 1 mL 1.5 M methanolic KOH, 200 μL H_2O for 18 h, at 25°C. The reaction was then acidified to pH 2 with HCl, and the lipids were re-extracted with chloroform/methanol.

RESULTS

Growth and lipid composition. Haliphthoros philippinensis grew in liquid shake culture as white, thinly flocculant, irreg-

ular branching pseudofilaments containing numerous globular and crystal-like structures (Fig. 1A and B). Preliminary electron microscopic examination of the hyphae indicated that the globular structures were vacuoles (data not given).

When cultured in medium containing glucose (2%), sodium glutamate (0.338%), and mineral salts solution (1%) at 24°C, growth showed a typical pattern of biomass production 6 d after inoculation, with maximal biomass of 12.4 g/L (Fig. 2A). Total *O*-acyl lipid content decreased from 4.2% of the mycelial dry weight to 2.5% during rapid growth, but accumulated to 7.1% when biomass dry weight was decreasing (Fig. 2A). There was a reciprocal relationship between ARA and EPA during the first 3 d of incubation, after which time the relative proportions of each of these fatty acids was about 17% and remained relatively constant thereafter (Fig. 2B). Although the relative proportion decreased during the growth phase of culture development, EPA production increased by over 500% to 106 mg/L over the 12-d incubation (Fig. 2B).

The polar and nonpolar lipid contents of the total lipid from *H. philippinensis* were 79 and 21%, respectively. The results of ^{31}P NMR and HPLC analyses of the lipid were generally similar, with phosphatidylcholine (44% of the total lipid) and phosphatidylethanolamine (15%) as major components (Table 1). However, the ^{31}P NMR analysis indicated that phosphatidylglycerol (PG) was a major component; but the HPLC analysis suggested the presence of a sphingolipid which contains an unusual 19-carbon branched triunsaturated sphingoid (C19$\Delta^{4,8,10}$, 9-methyl long chain base) designated as cer-PE-2 (26). These lipids have similar HPLC retention times, so the sample was subjected to mild alkaline hydrolysis to remove the nonsphingolipid components. All of the individual polar lipids were removed from the sample except the peak corresponding to cer-PE-2, which represented 19% of the total lipid, indicating the presence of the sphingolipid rather than PG (Table 1). The remaining phospholipids included phosphatidylinositiol (1.9%) and phosphatidic acid (3.2%) (Table 1).

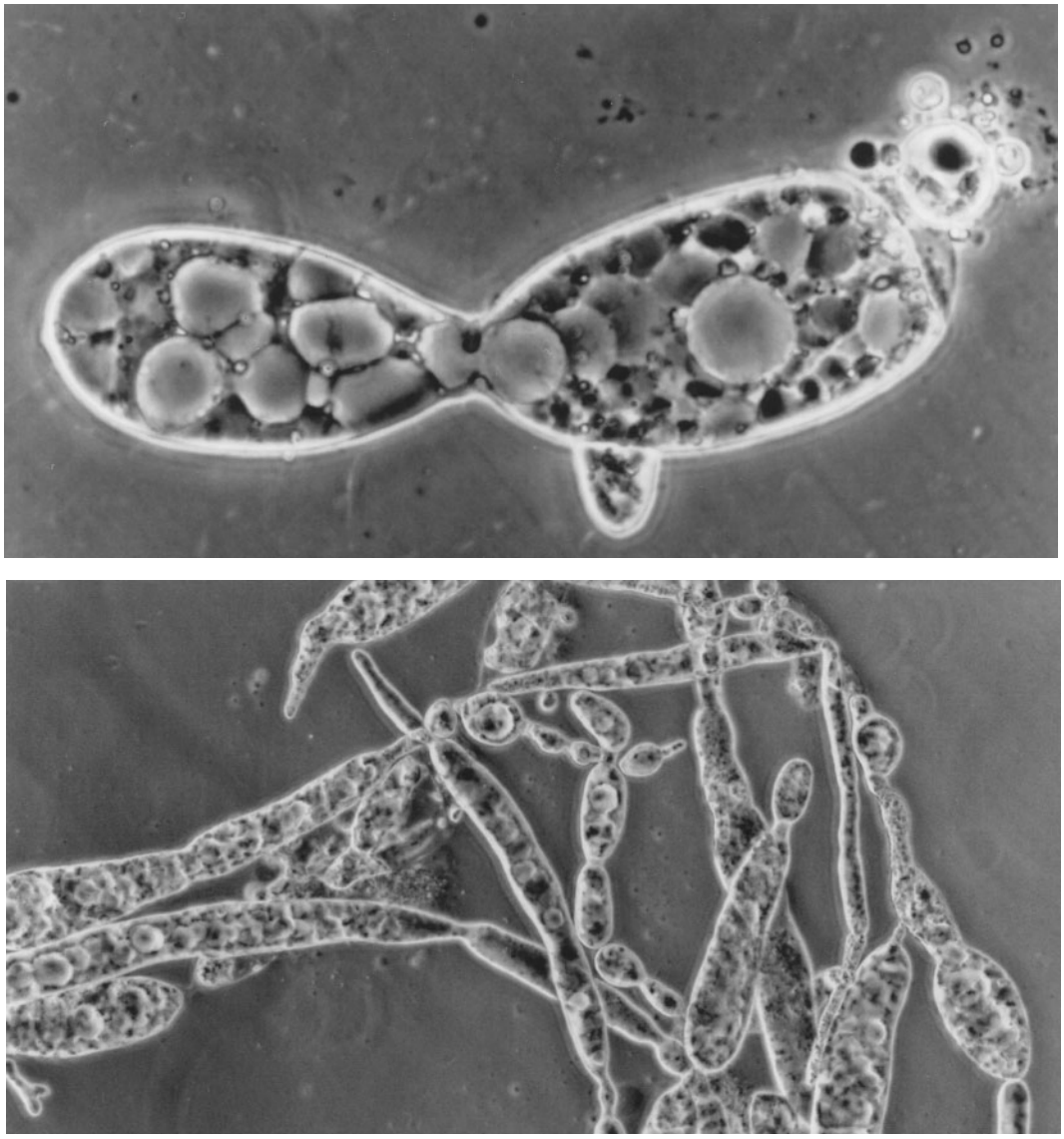

FIG. 1. Light (A = 40× and B = 10×) micrographs of *Haliphthoros philippinensis.*

TABLE 1
Total Neutral and Individual Lipid Composition of *Haliphthoros philippinensis*[a]

Lipid class	Mg lipid class/100 mg total lipid	
	Control	After alkaline hydrolysis
Nonpolar lipids	21.0	76.3
Phosphatidylethanolamine	15.0	0
Ceramide-phosphorylamine-2	19.7	14.1
Phosphatidylinositol	1.9	0
Phosphatidic acid	3.2	0
Phosphatidylcholine	44.3	0

[a]Data in this table were derived from the high-performance liquid chromatography analysis described in the Materials and Methods section.

The principal saturated fatty acid was palmitic acid (25% of the total fatty acids), and major unsaturated fatty acids were ARA (19%) and EPA (21%) (Table 2). Relative proportions of palmitic acid were higher in the sterol ester and triacylglycerol fractions, and ARA and EPA accumulated in the free fatty acid and polar lipid fractions (data not given).

The nonsaponifiable lipid represented 6.3% of the total lipid, and comprised mainly sterols. About 12 sterols were detected, three of which constituted 92% of the total sterols. The mass spectra of the two major sterols were very similar and resembled that of 24-ethyl-cholesta-5,24(28)-dienol with the molecular ion at *m/z* 484, base peak at *m/z* 386, and characteristic ion fragments at *m/z* 355 ($M^+ - 129$) indicating a double bond at C-5, 296 and 281 (27). The GLC retention time of the major sterol, constituting 59% of the total sterols, corresponded to that of an authentic standard of fucosterol {[24(28)*E*]-24-ethyl-cholesta-5, 24(28)-dienol]}. The other major sterol exhibiting a similar mass spectrum may be isofucosterol, which is the 24(28)*Z* isomer of fucosterol, and represented 28.5% of the total sterols. A third sterol at 4.5% of the total had a molecular ion at *m/z* 470, and major fragments at 386, 365, 341 ($M^+ - 129$), 296, and 129 (100%) indicating that it is 24-methylene cholesterol, a probable precursor to fucosterol.

TABLE 2
Fatty Acids of *Haliphthoros philippinensis*[a]

Fatty acid	Mol%
$C_{12:0}$	<0.1
$C_{14:0}$	2.2
$C_{14:1}$	<0.1
$C_{15:0}$	0.1
$C_{16:0}$	24.9
$C_{16:1}$	1.5
$C_{17:0}$	<0.1
$C_{18:0}$	2.5
$C_{18:1n-?}$	<0.1
$C_{18:1n-9}$	2.1
$C_{18:2}$	12.8
$C_{18:3n-6}$	0.6
$C_{18:3n-3}$	<0.1
$C_{20:0}$	1.8
$C_{20:1}$	0.3
$C_{20:2}$	5.5
$C_{20:3n-6}$	1.0
$C_{20:3n-3}$	0.3
$C_{20:4n-6}$ (ARA)	18.9
$C_{20:4n-3}$	—
$C_{20:5n-3}$ (EPA)	21.4
$C_{22:0}$	<0.1
$C_{22:4n-6}$	<0.1
$C_{22:5n-3}$	0.1
Others	3.7

[a]Grown at 24°C for 6 d in medium containing 2% glucose, 0.3% yeast extract, and 1% mineral salt solution. ARA, arachidonic acid; EPA, eicosapentaenoic acid.

Optimization and enhancement of biomass, total lipid, and EPA production. Biomass production and lipid content. Several sugars, complex carbohydrates and sugar alcohols were tested as carbon sources for biomass, lipid, and EPA production (Table 3). Among those sources tested, glucose, starch, and maltose were the most effective for biomass production, yielding 13.4, 12.6, and 12.2 g/L, respectively. Generally, mycelial lipid contents were similar for the various carbon sources ranging from 5.6 to 6.4%, except for sorbitol which was 3.7%.

Biomass production increased linearly with increasing glucose concentration up to 2%; lipid content was 7.5% of mycelial dry weight (Fig. 2A). Although biomass and EPA production were slightly higher at medium glucose contents (above 2%) (Fig. 3), total lipid content of the mycelium decreased progressively with increasing glucose concentration (data not given). The relative percentage of EPA dropped with increasing glucose concentration to 2%, and remained essentially constant at about 12% at higher glucose concentrations.

Various organic and inorganic nitrogen compounds were tested for their effects on growth and lipid production with glucose (2%) as the carbon source (Table 4). Generally, nutrients such as yeast extract, tryptone, and peptone gave good mycelial yields (13.4, 11.7, and 10.1 g/L, respectively); however, the total lipid content with these nutrients varied depending on the sources, ranging from 3.1 to 5.8% of the mycelial dry weight. Among the organic and inorganic nitrogen sources, growth and total lipid content (14.6 g/L and 6.1%, respectively) was greatest with sodium glutamate as the nitrogen source. From the above results, yeast extract and sodium glutamate were selected as the nitrogen sources used for culture maintenance and biomass production, respectively.

EPA production. As with biomass production, the highest yields of EPA were obtained with glucose, starch, and maltose at 102, 115, and 111 mg/L, respectively (Table 3). The highest relative percentage of EPA, 32%, was obtained with sorbitol as the carbon source, but biomass production was very low at 1.7 g/L. When determined as a function of glucose concentration in the medium, EPA yield was maximal (111 mg/L) at 2% glucose, and declined with increasing glucose concentration (Fig. 3).

EPA production varied considerably with the nitrogen source in the medium, from 35 mg/L with beef extract to 126 mg/L with sodium glutamate (Table 4). However, the highest relative percentage of EPA was obtained with NH_4NO_3 (32%) and NH_4Cl (30%). Adding sodium glutamate (1 to 20 mM) to medium containing NH_4NO_3 (0.2%) in an attempt to maximize biomass, total lipid, and relative percentage EPA resulted in reduced growth (data not shown). The relative pro-

TABLE 3
Effect of Carbon Sources on the Growth, Lipid, and PUFA Production by *Haliphthoros philippinensis*

Carbon source[a]	Biomass (g/L)[b]	Total lipid (%)[c]	Total lipid (g/L)	ARA (%)[d]	ARA (mg/L)[e]	EPA (%)[d]	EPA (mg/L)[e]
Glucose	13.4	5.8	0.78	14.1	109.9	13.1	101.9
Sucrose	2.2	5.6	0.13	18.7	23.4	29.6	37.0
Glycerol	8.4	6.4	0.54	13.6	73.0	18.3	98.0
Starch	12.6	5.8	0.73	16.1	118.2	15.6	114.6
Fructose	5.1	6.3	0.32	9.8	31.2	23.9	76.4
Maltose	12.2	5.9	0.72	15.2	109.6	15.4	111.0
Sorbitol	1.7	3.7	0.62	22.3	13.8	31.7	19.7
Xylose	N/G[f]						

[a]Yeast extract was used as a nitrogen source at 0.3% for each medium, each carbon source was used by 2% wt/vol, and the fungus was grown at 24°C for 6 d.
[b]Dry weight per liter of culture.
[c]Weight percentage.
[d]Molar percentage of total fatty acids.
[e]Amount of fatty acid produced per liter of culture medium.
[f]No growth was detected. PUFA, polyunsaturated fatty acids; for other abbreviations see Table 2.

portion of EPA was also relatively high with tryptone as the nitrogen source, but the lipid content was low, resulting in low EPA yield (74 mg/L) (Table 4).

ARA production. The relative percentage ARA and ARA production followed the same general trends as EPA with respect to carbon and nitrogen sources. High relative amounts of ARA in the lipid did not correlate with production. The highest amounts of ARA were 110, 118, and 110 mg/L with glucose, starch, and maltose as the carbon sources, respectively (Table 3). Similarly, the presence of yeast extract or sodium glutamate resulted in the highest ARA yields of 110 and 96 mg/L, respectively, compared to other nitrogen sources (Table 4).

Effects of temperature. To determine the effect of temperature on EPA production, mycelia were grown at 24°C for 6 d and the cultures were then transferred to either 13 or 16°C with and without glucose supplementation (2% w/w), and incubated for an additional 3 d. The shift to 16°C appeared to be more effective for increasing EPA production than 13°C (Table 5). At 16°C without glucose supplementation, EPA yield increased by 86% compared to the corresponding culture at 24°C, although biomass content of the culture was slightly less. With glucose supplementation, EPA yield increased by 129%. The shift to 13°C resulted in increased EPA

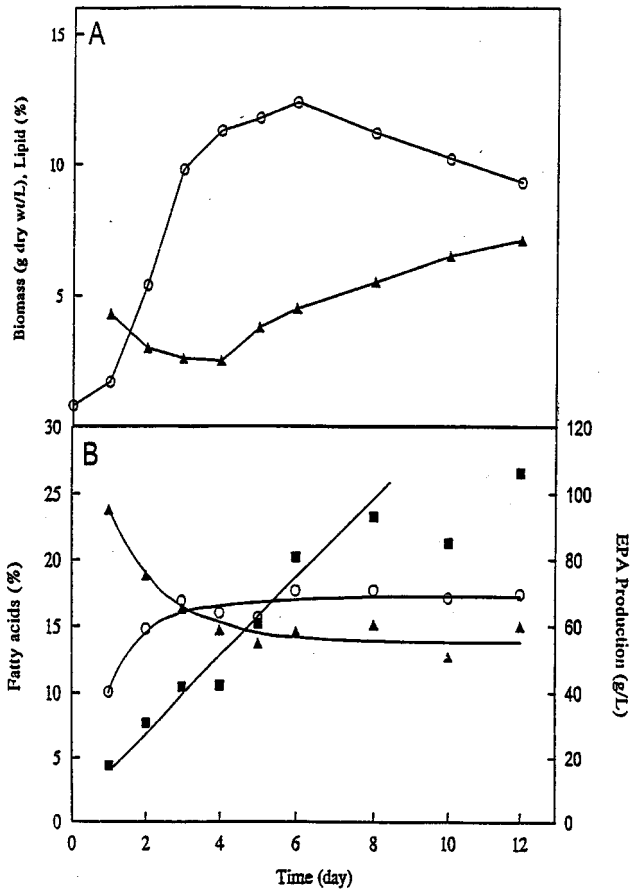

FIG. 2. (A) Changes in biomass production (-0-) and total lipid content (-▲-) of *Haliphthoros philippinensis* as a function of culture development. (B) Changes in the relative proportions of ARA (-▲-) and EPA (-0-), and EPA production (-■-) as a function of culture development. ARA, arachidonic acid; EPA, eicosapentaenoic acid.

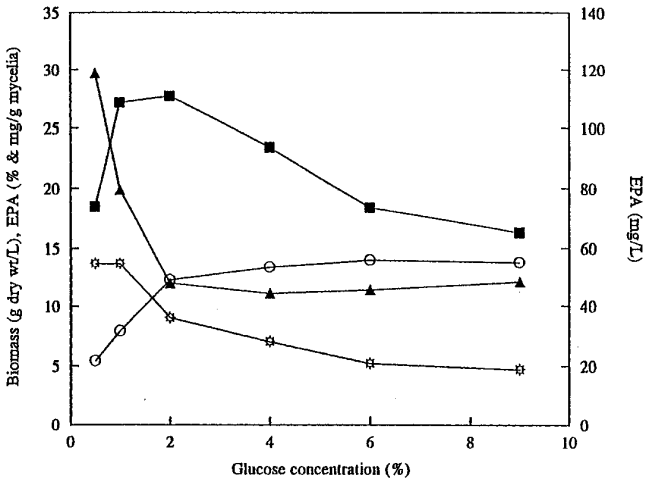

FIG. 3. Changes in biomass (-0-), relative percentage EPA (-▲-), EPA amount (-■-), and mycelial content of EPA (-✡-) as a function of glucose concentration in the culture medium. For abbreviations see Figure 2.

TABLE 4
Effect of Nitrogen Sources on the Growth, Lipid, and PUFA Production by *Haliphthoros philippinensis*[a]

Nitrogen source	Biomass (g/L)[b]	Total lipid (%)	Total lipid (g/L)	ARA (%)[d]	ARA (mg/L)[e]	EPA (%)[d]	EPA (mg/L)[e]
Sodium glutamate (50 mM)	14.6	6.1	0.89	10.8	96.2	14.1	125.7
Urea (25 mM)	3.7	5.7	0.21	17.4	36.1	17.5	36.4
NH_4NO_3 (25 mM)	5.5	5.1	0.28	17.2	48.2	31.9	92.1
NH_4Cl (50 mM)	2.4	5.4	0.13	17.2	21.8	29.8	38.0
Yeast extract (0.3%)	13.4	5.8	0.78	14.1	109.9	13.1	101.9
Tryptone (0.3%)	11.7	3.1	0.36	12.1	43.4	21.0	74.3
Peptone (0.3%)	10.1	4.2	0.43	14.8	62.8	14.9	63.2
Beef extract (0.3%)	5.8	2.5	0.14	11.4	16.1	25.4	35.8

[a]Growth conditions, abbreviations, and footnotes are the same as described in Table 2.

TABLE 5
Effect of Temperature on the Growth and Production of ARA and EPA by *Haliphthoros philippinensis*

Conditions	Temperature[b] (°C)	Biomass (g/L)	Total lipid (%)	Total lipid (g/L)	ARA (%)	ARA (mg/L)	EPA (%)	EPA (mg/L)
6-Day culture[a]		12.0	4.8	0.58	16.9	97.7	14.2	82.1
Without glucose	13	11.3	5.1	0.58	10.1	58.1	22.8	131.5
	16	10.6	5.2	0.55	11.3	62.2	26.2	144.8
	24	10.1	5.6	0.57	17.2	97.3	13.8	77.9
With glucose[c]	13	14.9	4.8	0.72	11.7	83.8	17.8	126.9
	16	15.2	5.7	0.87	12.8	110.9	20.9	181.4
	24	17.1	3.9	0.67	16.0	106.8	11.8	78.8

[a]Fungus was grown for 6 d at 24°C in medium composed of glucose (2%), sodium glutamate (20 mM), and Vogel's solution (1%).
[b]After culturing as described in footnote *a* for the 6-d culture, cultures were transferred to the respective temperatures and incubated for 3 d prior to harvesting the mycelium with or without glucose supplementation.
[c]Glucose was added to the culture to a final concentration of 2% after the initial 6-d incubation. For abbreviations see Table 2.

production by 61 and 69% with and without glucose supplementation, respectively. Cultivation at 13°C directly after inoculation rather than after a 6-d incubation period gave poor growth (1.6 g/L); however, the EPA content in the lipid was relatively high (30%).

The relative percentage of ARA was less at reduced temperatures than at the optimal temperature for growth with or without glucose supplementation, and ARA production was reduced 40 and 36% at 13 and 16°C without glucose supplementation, respectively, but was reduced only 14% with glucose at 13°C and was increased by 14% at 16°C (Table 5).

Extended incubation (mycelial aging). Based on the above results, 16°C was selected for determining the effect of extended incubation time at low temperature on EPA production with and without glucose supplementation. The culture was allowed to reach the stationary phase by growth in the control medium for 6 d at 24°C, and was then transferred to 16°C without glucose feeding and incubated for an additional 9 d. The relative percentage and yield of EPA increased progressively with time after transfer (Table 6) from 14 to 31%, and from 82 to 203 mg/L, i.e., increases of 116 and 147% over the control, respectively (Table 6). The ARA content of the lipid decreased by 33% during the first 3 d after transfer to 16°C, and remained relatively constant for the remainder of the incubation period (Table 6).

Similarly, when the same procedure as described above was followed, except with glucose supplementation after 6 d incubation, enhancement of EPA production was considerably greater than without glucose feeding. EPA yield increased with time from 181 to 316 mg/L, accounting for 284% increase over the control. Biomass production, total lipid content, and the relative amount of EPA content in the lipid also increased by 28, 65, and 88%, respectively, compared to the control. A time-dependent increase in ARA production at 16°C did not occur either with or without glucose supplementation, although ARA production was 42% higher with glucose than without after 9 d of incubation (Table 6). After 13 d of incubation, EPA production was 170% higher than ARA production with glucose (Table 6), and 89% higher without.

DISCUSSION

The lipid composition of *H. philippinensis* is consistent with what might be expected for members of the Oomycota. Moreau *et al.* (26) detected previously unreported ceramide-phosphorylethanolamines as major lipid components of *Pythium* and *Phytophthora* species, and we found that this lipid is also a major component of lipid from *H. philippinensis*. Since the occurrence of this sphingolipid was only very recently reported (26), it is not known whether it is unique to oomycetous organisms. However, it was not detected in *Thraustochytrium* sp., which is a member of the Labyrinthulomycota (unpublished data).

TABLE 6
Effect of Extended Culture Incubation at Low Temperature, and Glucose Supplementation on Growth, Total Lipid Content, and the Production of ARA and EPA by *Haliphthoros philippinensis*

Conditions	Time (d)[c] at 16°C	Biomass (g/L)	Total lipid (%)	Total lipid (g/L)	ARA (%)	ARA (mg/L)	EPA (%)	EPA (mg/L)
6-d Culture[a]		12.0	4.8	0.58	16.9	97.7	14.2	82.1
Without glucose	3	10.6	5.2	0.55	11.3	62.2	26.2	144.8
	6	11.1	6.0	0.67	10.2	68.5	28.9	193.2
	9	10.2	6.5	0.66	10.6	70.3	30.7	202.7
With glucose[b]	3	15.2	5.7	0.87	12.8	110.9	20.9	181.4
	6	15.3	5.7	0.88	11.9	104.1	26.6	233.3
	9	14.8	6.8	1.01	9.8	99.5	25.9	262.5
	13	15.0	7.9	1.18	9.9	117.4	26.7	315.6

[a]Fungus was grown for 6 d at 24°C in medium composed of glucose (2%), sodium glutamate (20 mM), and Vogel's solution (1%).
[b]After 6 d of incubation, glucose was added to the culture to a final concentration of 2%.
[c]After cultivation as described for the control (footnote *a*), cultures were transferred to 16°C and incubated for the periods indicated. For abbreviations see Table 2.

Like some other oomycetous organisms, such as *Pythium, Phytophthora* (10,11), and *Saprolegnia* (12), *H. philippinensis* produced both ARA and EPA. Also, the sterol composition of *H. philippinensis* is similar to that of sterol-producing oomycetous organisms in general, and members of the Saprolegniales in particular, with fucosterol as the principal sterol (28). To our knowledge, only *H. milfordensis* has been studied previously in this regard but, although fucosterol was detected as the major sterol of other oomycetous organisms in that study, fucosterol was not reported for this species (29). Instead, an unidentified C_{29} sterol along with stigmasta-5,22-dienol, ergost-5-enol, and 24-methylene cholesterol were reported for *H. milfordensis*.

Haliphthoris philippinensis does not meet most of the criteria for it to be a viable economic source of EPA. For example, although biomass production was marginal, the total lipid content was low (<8%) and, more importantly, the relative amount of triacylglycerols in the lipid was also relatively low (<15%) under optimal growth conditions for this organism. Furthermore, ARA was a substantial constituent of the lipid, and reached relative proportions almost equaling that of EPA under some growth conditions. *Haliphthoris philippinensis* is not an oleaginous organism in that it does not accumulate 20% or more lipid, at least under the conditions used in this study.

It is well known that some fungi and yeasts respond to certain culture manipulations whereby oil accumulation and PUFA enrichment in the oil are enhanced. Research in this regard with respect to EPA production has been conducted with relatively few primitive fungi. Although not common in zygomycetous fungi, EPA has been reported in *M. alpina* (30), *M. elongata* (6), and *Entomophthora obscura* (31). Where EPA production was either undetectable or relatively low at optimal growth temperatures, it was increased considerably by cultivation at less than optimal growth temperatures, e.g., *P. ultimum* (10), *P. irregulare* (11), and *Saprolegnia* sp. 28YTF-1 (12).

In this study, *H. philippinensis* was allowed to accumulate biomass to the stationary phase under optimal shake-culture growth conditions and was then subjected to reduced temperature, extended incubation, and glucose feeding as described previously by Gandhi and Weete (10) for *P. ultimum*. After shifting the mycelium from 24 to 16°C and incubating at the reduced temperature for 13 d without glucose feeding, EPA content of the lipid more than doubled to 31 mol%, which to our knowledge is higher than the relative amount of EPA previously reported for any fungus or stramenopile (Table 7). EPA content and yield with glucose feeding was 27 mol% of the total fatty acids and 316 mg/L, respectively, which is a 285% increase over that incubated for 6 d at 24°C, and 56% over that without glucose feeding. In spite of the substantial improvement in EPA production with reduced incubation temperature as described above, the relative amount of triacylglycerols in the total lipid remained relatively low at 21% (opposed to 13% at 24°C). Although several of the *Mortierella* strains studied in this regard are superior, EPA production by *H. philippinensis* is comparable to that by more closely related organisms (Table 7).

As noted above, a limitation of *H. philippinensis* as a potential commercial source of EPA is the presence of substantial relative amounts of ARA in the lipid. However, as we have reported with *P. ultimum* (10), there seems to be a reciprocal relationship between ARA and EPA content with respect to temperature, i.e., ARA formation is favored by optimal or relatively high cultivation temperatures and EPA production is favored by reduced temperatures, and this was the case for *H. philippinensis*. This relationship between ARA and EPA may offer opportunities to manipulate conditions to favor one over the other for desired PUFA composition.

ARA and EPA are formed *via* the n-6 and n-3 pathways with linoleic acid and α-linolenic acid, respectively, as initial precursors in the respective pathways. It is generally believed that crossover between these pathways does not occur. However, radiolabeled ARA was converted to EPA by *S. parasitica* (9) and *M. alpina* (31). Although indirect, the results of this study, such as the reciprocal relationship between ARA and EPA during the linear phase of growth and with reduced temperature, also suggest that the major pathway for EPA formation may be the Δ^{17} desaturation of ARA.

TABLE 7
EPA in Fungi, Stramenopiles, and Marine Bacteria

Species	EPA[a] (%)	EPA[a] (mg/L)	Reference number
Fungi			
Mortierella sp.	14	490	30
M. alpina			
IS-4	15	300	8
IS-4 12-Desaturase mutant	20	1000	38
20-17	5	1350	38
CBS 343.66	15	184	34
M. elongata NRRL 5513	15	610	6
Stramenopila			
Saprolegnia sp. 28YTF-1	—	176	12
Pythium ultimum #144	11	383	10
P. irregulare	25	(75)[b]	11
Haliphthoros philippinensis	27	316	This study
Bacteria			
SCRC-8132 (marine)	24	26	35
SCRC-2738 (from mackerel)	20	52	36

[a]Maximum values under enhancement conditions for EPA production.
[b]Calculated. For abbreviation see Table 2.

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