γ-Linolenic Acid in Zygomycetous Fungi: *Syzygites megalocarpus*

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ABSTRACT: The fatty acids of over 150 species and isolates of zygomycetous fungi were analyzed, and it was found that γ-linolenic acid (GLA) composed 35 to 62% of the total fatty acids in several species, i.e., *Circenella simplex, Mucor indicus, Syzygites megalocarpus* (ATCC 18025), and *Zygorhynchus moellierie A* (UAMH 1556). Further study of *S. megalocarpus* showed that the total lipid content of the mycelium could be increased from 9.8% of the dry biomass to 20 to 25% when grown in a medium with a high carbon/nitrogen ratio. Under these conditions, the GLA content of the triacyglycerols increased during culture development, even during the stationary phase, but remained relatively constant in the phospholipid fraction. Nonsaponifiable lipid represented 4% of the total lipid, and the major sterol among 14 others detected was ergosterol at 52% of the total. Phospholipids composed 7% of the total lipid with phosphatidylethanol-amine and phosphatidylcholine representing 53 and 39% of the total, respectively.

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KEY WORDS: Ergosterol, fatty acids, γ-linolenic acid, phospholipids, sterols, *Syzygites megalocarpus,* zygomycota.

Recognition of the possible health benefits associated with consumption of the 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), and the n-6 arachidonic acid (20:4$\Delta^{5,8,11,14}$; ARA), has led to a large research effort to discover the physiological roles of these fatty acids and the dietary requirements for them (1). Certain fish oils are currently the main dietary sources of n-3 PUFA.

The n-3 and n-6 18 trienoic fatty acid isomers, α-linolenic (18:3$\Delta^{9,12,15}$; ALA) and γ-linolenic (18:3$\Delta^{6,9,12}$; GLA) are also nutritionally important, the former being an essential fatty acid found mainly in green plants that serves as a precursor to the n-3 20 and 22 PUFA (2). GLA is produced in mammalian systems where it is a precursor to ARA (2). Impairment of its formation has been associated with atopic eczema, diabetes, premenstrual syndrome, and other maladies (3). Supplementation of diets with this fatty acid has been suggested as a means of relieving symptoms of its deficiency (4). This fatty acid is available commercially in seed oils from black currant, evening primrose, and borage (5).

It has been recognized for many years that the distribution of 18 trienoic fatty acid isomers varies in fungi according to phylogenetic relationships, i.e., the more advanced taxa such as the Ascomycota and Basidiomycota produce the n-3 isomer, and the less advanced taxa (Zygomycota, Chytridiomycota) produce the n-6 isomer, and in some cases a mixture of the two isomers (6,7). Therefore, it might be expected that potential commercial sources of GLA may be found among the more primitive fungi. In this connection, we have examined the fatty acid composition of numerous species of zygomycetous fungi and are reporting the GLA content of 24 species and the lipid composition of the fungus *Syzygites megalocarpus,* which to our knowledge produces the highest proportion of GLA relative to other fatty acids than any other organism previously reported. *Syzygites megalocarpus*, previously known as *Sporodinia grandis*, is a mycoparasitic fungus found worldwide on decaying sporophores of various basidiomycetous fungi such as *Boletus, Lactorium,* and *Russula* (8), and is a single species genus belonging to the family Dicranophoraceae of the Mucorales (9).

MATERIALS AND METHODS

Source of the fungus and culture conditions. Syzygites megalocarpus strains 18025, 16776, 16777, 17778, and 11807 were obtained from the American Type Culture Collection (ATCC) (Bethesda, MD), strain 9909 was obtained from Institute for Fermentation (Osaka, Japan); and strain 372.39 was obtained from Central Bureau Voor Schimmel Cultures (CBS) (Baarn, The Netherlands). They were maintained on potato dextrose agar medium (Difco Laboratories, Detroit, MI) at 24°C and, unless noted otherwise, were cultured in 250 mL Erlenmeyer flasks containing 100 mL of 2% glucose and 1% yeast extract medium at 24°C with rotary shaking at 120 rpm. In some cases as noted, a mineral salts solution was added to the medium (10). Inoculations were made with 5 mL of blended mycelia from a 6-day-old culture grown as described previously. Mycelia were harvested by suction filtration in a Buchner funnel, washed twice with 100 mL of cold distilled water, dried by lyophilization, and stored at –20°C prior to extraction.

Lipid extraction and estimation of total acyl lipid. Total lipid was extracted from dry mycelia (*ca*. 25 mg) using the Bligh and Dyer procedure (11) that was modified according

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to Kates (12), but with twice the solvent volume to biomass dry weight ratio recommended by the latter [2 mL H_2O; 7.5 mL $CHCl_3$:MEOH (1:2, vol/vol); 9.5 mL $CHCl_3$: MEOH: H_2O (1:2:0.8, vol/vol/vol)]. All extraction and subsequent sample manipulations were conducted at low light intensity. The amount of extracted lipid was determined gravimetrically. Except as noted otherwise, all data on lipid content are based on extraction by the Bligh and Dyer procedure (11). In a separate experiment, two other solvent systems were tested for lipid extraction efficiency. For extraction procedure #1, mycelia were treated with 7 mL ethanol for 1 h and then 7 mL hexane for 1 h after removing the ethanol. The ethanol and hexane extracts were combined and the solvents evaporated under nitrogen gas. For extraction procedure #2, mycelia were treated with 7 mL chloroform/methanol (2:1, vol/vol) for 2 h and then again with the same solvent mixture for 10 min. The two extracts were combined and mixed after adding chloroform and water, and the chloroform layer containing the lipid was recovered. The chloroform was evaporated as before.

For monitoring changes over the course of culture development, the amount of acyl lipid was calculated from gas chromatographic data when the methyl ester derivatives of the fatty acids (FAME) were prepared directly from the mycelium after adding 23:0 as the internal standard (see following text).

Thin-layer chromatography (TLC). Individual neutral lipid classes 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, Clifton, 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 with iodine vapor. For the recovery of individual lipid classes, silica gel was removed from the plates and washed with chloroform/methanol (2:1, vol/vol). Individual phospholipid classes were separated by TLC as described above except that the plates were developed in chloroform/acetone/methanol/acetic acid/water (30:40:10:10:5, by vol). Lipid classes were identified by comparison of their R_f values with those of corresponding lipid standards (Nu- Chek-Prep, Elysian, MN).

Sample derivatization and gas–liquid chromatographic analysis (GLC). For the preparation of methyl ester derivatives of the total fatty acids, 1.5 mL methanolic base (Supelco, Inc., Bellefonte, PA) were added to 20 mg dry mycelia, the mixture was heated at 70°C for 15 min, and the FAME were then extracted with hexane. FAME were analyzed by gas chromatography as described previously (13), except that the gas chromatograph (Varian Model 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 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 (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 5 mL of 95% ethanol containing 0.5 mL of 33% (wt/vol) potassium hydroxide (KOH) (12). 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 and 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 were 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 (Hewlett-Packard model 5710) was equipped with a 25-m fused silica capillary DB-5 column (Applied Science Laboratories, State College, PA). Injector and detector temperatures were 300°C, and the oven temperature was 280°C isothermal. Sterols were quantified using the TMS derivative of 5-dihydrocholesterol as the external standard.

GLC–mass spectrometry. Gas chromatography–mass spectrometry (GC–MS) analysis of total sterols was performed using a VG 70E (Manchester, United Kingdom) 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.

^{31}P NMR analysis of phospholipids. A portion of total lipid (*ca.* 60 mg) was dissolved in 25 mL chloroform and washed with 25 mL Na^+/K-EDTA in a separatory funnel by shaking vigorously at 30-min intervals over a 4-h period. The Na^+/ K-EDTA (0.2 M, pH 6.0) solution was prepared according to Seijo *et al.* (14). Emulsions formed during the washing process were disrupted by the addition of KCl (*ca.* 2 g). 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). Cs-EDTA phospholipid NMR reagent was prepared as described by Meneses and Glonek (15). The solution was mixed by vortexing, then filtered through 0.2 μm Acrodisc nylon filters (Gellman Instruments Co., Ann Arbor, MI), and 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. Individual phospholipid classes were identified

by comparing chemical shift values of sample components, authentic phospholipid standards, and published values (16).

RESULTS AND DISCUSSION

GLA in zygomycetous fungi. Over 150 zygomycetous fungi representing 12 of the 16 families of the phylum Zygomycota were screened for the production of PUFA, and the fatty acid patterns in these fungi were typical of those expected for most fungi: for example 16:0 as the major saturated fatty acid and 18:1 or 18:2 being major unsaturated fatty acids (17). However, GLA represented 20% or more of the total fatty acids in 21 of the species tested (Table 1). GLA composed between 20 and 30% of the total fatty acids of most of these species, but *Circenella simplex, Mucor indicus, S. megalocarpus* (ATCC 18025), and *Zygorhynchus moellerie A* (UAMH 1556) contained 42, 35, 62, and 42%, respectively. These values are higher than those reported for fungi that have been evaluated previously as potential commercial sources of GLA [e.g., *Mortierella* species which contained up to only 25% GLA under the most favorable culture conditions (18)], and considerably higher than the current commercial sources of this fatty acid, e.g., seed oils of evening primrose, black currant, and borage, which contain 9, 16, and 24%, respectively (5). *S. megalocarpus* was selected for further study.

Total lipid and fatty acids of S. megalocarpus. Seven isolates of *S. megalocarpus* were screened for their ability to produce GLA when cultured for 6 d in yeast extract/dextrose (YD) (2% glucose, 1% yeast extract) medium at 24°C. Biomass production in these isolates ranged from 2.9 to 9.7 g/L and total lipid content ranged from 4.0 to 9.8% of the dry mycelium (Table 2). The fatty acid profiles for these isolates were relatively simple with 16:0 as the major saturated fatty acid and GLA ranging from 30 to 62% of the total fatty acids. *S. megalocarpus* ATCC 18025 contained 14:0 (1%), 16:0 (14%), 18:0 (1%), 18:1 (12%), 18:2 (10%), and n-6 18:3 (62%). To our knowledge, the latter value is the highest relative GLA content of any organism known.

After considerable testing of different carbon and nitrogen sources (data not given), we were able to increase the total lipid content of the ATCC 18025 isolate from about 10% to between 20 and 25% of the mycelial dry weight by culturing the fungus in medium with a relatively high carbon/nitrogen (C/N) ratio and an organic nitrogen source, i.e., 100 mL of 5% glucose, 0.15% sodium glutamate, and 1% stock mineral solution (10). Unless noted otherwise, further studies were conducted with mycelia cultured in this medium at 27 to 28°C. The lipid content of cells grown in the high C/N ratio medium was over twofold higher than when grown in the YD medium used in the initial screen, but the relative GLA content of the lipid was reduced to between 40 and 50% when grown in this medium.

The total lipid values reported here were obtained using the Bligh and Dyer (11) procedure for lipid extraction. Re-

TABLE 1
GLA Content of Selected Zygomycetous Fungi[a]

Fungi	I.D. number	Source	Biomass (g/L)	Total lipid (%)	GLA (%)
Absidia coerulea	1359B	CBS	1.4	8.0	26.3
*Actinomucor elegans**	100.13	ATCC	5.5	8.8	21.1
Cokeromyces recurvatus	13568	ATCC	5.6	10.8	20.4
Circinella simplex	142.35	CBS	4.7	6.0	42.0
Choanephora cucurbiratum		USDA-ARS**	5.1	10.0	27.6
Chaetostylum fresnii	11881	ATCC	9.1	2.0	30.1
Cunninghanella elegans	20230	ATCC	10.4	13.0	21.4
Dimargaris bacillispora	42702	ATCC	6.4	13.4	28.5
*Gongroella butleri**	1340	NRRL	2.1	7.4	26.7
Heliocostylum pulchrum	107.23	CBS	9.6	8.7	21.2
Mucor indicus	4855	ATCC	11.2	3.2	34.8
M. rouxii	24905	ATCC	8.3	11.6	25.0
*Parasitella parasiticus**	2501	NRRL	2.3	10.3	20.1
Phycomyces nitens	148.24	CBS	9.9	8.4	21.3
Radiomyces spectabilis	255.6	CBS	8.7	8.8	22.1
R. spectabilis	22871	ATCC	4.7	8.5	24.1
R. spectabilis	38964	ATCC	5.8	10.9	21.7
Syzygites megalocarpus	18025	ATCC	5.6	9.8	61.9
Thamnidium elegans	8997	ATCC	7.3	12.6	23.4
Utharomyces epallocaulus	329.73	CBS	1.7	10.6	30.9
*Zygorhynchus californiensis**	402.58	CBS	5.6	9.4	26.3
*Z. exponens**	404.58	CBS	5.9	10.4	31.0
*Z. moellerie**	296	UAMH	6.0	10.1	20.4
*Z. moellerie**	1556	UAMH	6.5	8.7	42.0

[a]*Cultures grown at 28°C; otherwise cultures were grown at 24°C as described in the Materials and Methods section.
**USDA-ARS at Peoria, IL. Abbreviation: GLA, γ-linolenic acid.

TABLE 2
Biomass Production, and Total Lipid and GLA Content of *Syzygites megalocarpus* strains[a]

Strain	Source	Biomass (g/L)	Total lipid (%)	GLA (%)
18025	ATCC	5.6	9.8	61.9
16776	ATCC	7.1	9.2	58.1
16777	ATCC	6.1	8.0	39.8
16778	ATCC	7.8	7.9	38.4
9099	IFO	2.9	7.9	40.4
372.39	CBS	9.7	4.0	58.8
11807	ATCC	5.8	7.4	29.8

[a]Culture conditions: 100 mL YD (2% glucose, 1% yeast extract) medium in 250 mL Erlenmeyer flasks with rotary shaking at 120 rpm at 24°C. See Table 1 for other abbreviation.

cently, Certik *et al.* (19) reported that other solvent systems give higher lipid extraction efficiencies from fungal mycelia containing GLA. Two additional procedures were used to extract the total lipid from *Syzygites* mycelium and it was found that the ATCC 18025 strain cultured in the high C/N medium contained 33 to 36% total lipid when extracted with these procedures compared to 22% using the Bligh and Dyer (11) procedure.

Biomass and total lipid content as a function of culture development. Biomass production peaked between 4 to 6 d after inoculation at about 3.6 g/L when incubated in the high C/N medium with 120 rpm rotary shaking. The total lipid content peaked between 7 and 8 d at 24% of the mycelial dry weight, and then remained essentially constant at that level through 14 d of incubation (Fig. 1). Beginning at 6 d after inoculation, the relative proportion of GLA in the lipid increased progressively from 38 to 50% of the total fatty acids through 14 d of incubation.

At 2 d after inoculation, the GLA content of the neutral (triacylglycerol) and polar lipid fractions was essentially the same at about 30% of the total fatty acids (Fig. 2). The GLA content increased from 28% in the triacylglycerols at 4 d after inoculation to 50% at 12 d after inoculation, and the GLA content of the polar lipid remained essentially constant. The timing of GLA increase in the triacylglycerols coincided with the increase in total lipid content with respect to culture development. Preliminary results suggested that cultivation at 20°C is near the optimum for the GLA content of the lipid, as opposed to 16 or 27°C. The relative percentage GLA was about 30% higher at this temperature compared to the others.

The substrate for desaturation of fatty acids beyond the C-9 position is believed to be the phospholipid derivative of the fatty acids (20). Therefore, the observed enrichment of GLA indicates that the Δ^6 and Δ^{12} desaturases, and an acyl transferase, remain active through the growth and stationary phases of culture development of this fungus.

Sterol composition. The nonsaponifiable fraction represented about 4% of the total lipid from *S. megalocarpus* ATCC 18025 when cultured for 6 d in the high C/N medium, and 12 to 14 sterols composed 85% of this lipid fraction with squalene representing 7.5%. Ergosterol [(22*E*, 24*R*) 24-methyl-cholesta-5,7,22-trienol] was the major sterol at 52% of

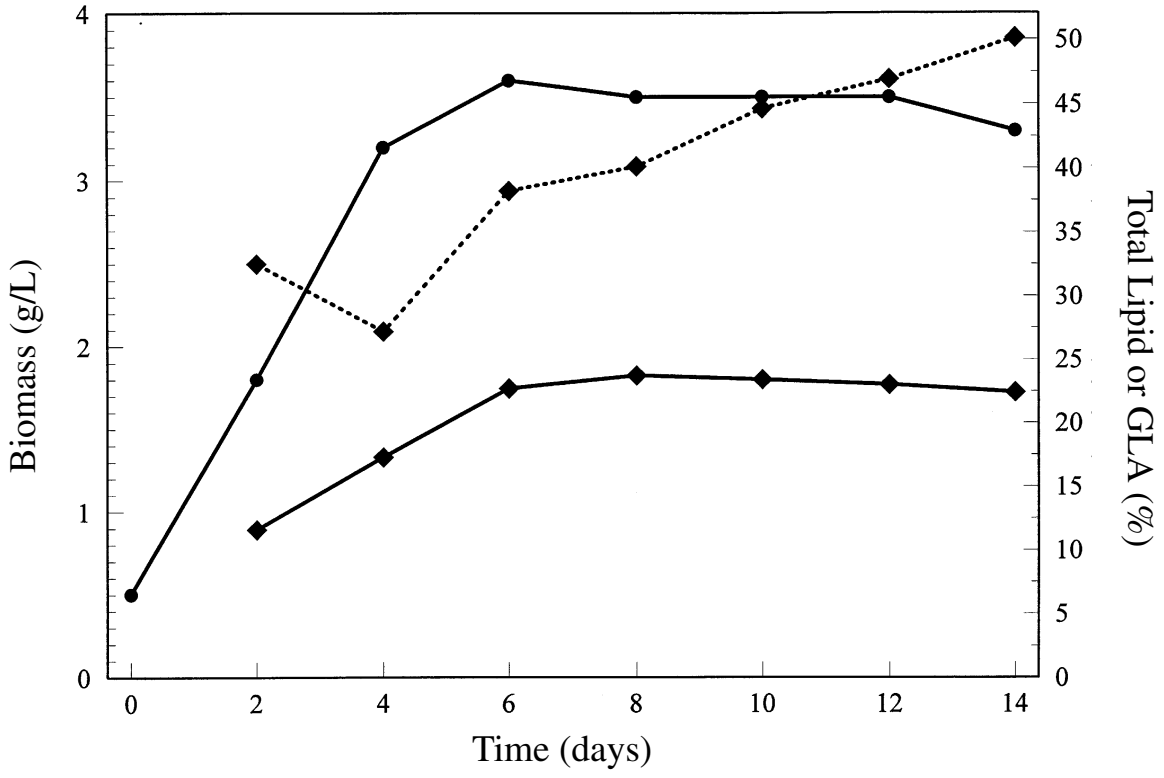

FIG. 1. Changes in biomass production, and total lipid and GLA content in *Syzygites megalocarpus* as a function of culture development. Biomass (●); total lipid (▲); GLA (--▲--) when grown in high carbon/nitrogen medium at 27°C (see Materials and Methods section). Abbreviation: GLA, γ-linolenic acid.

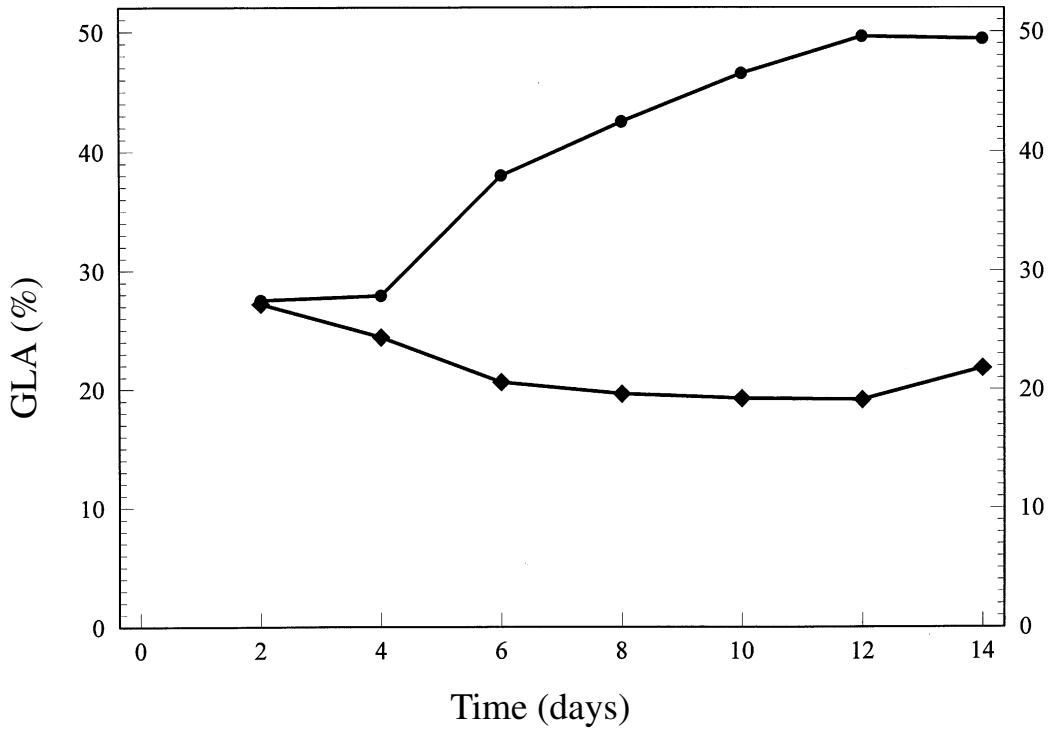

FIG. 2. Distribution of GLA between polar (▲) and triacylglycerols (●) in *Syzygites megalocarpus* as a function of culture development. See Figure 1 for abbreviation.

the total sterols. The GC retention time and mass spectrum of the TMS derivative of this sterol matched that of an authentic standard (Table 3). The second most abundant sterol at 18% was identified as ergosta-7,24(28)-dienol ($C_{28}\Delta^{7,24(28)}$) based on the molecular ion (M^+) at *m/z* 470, base peak at *m/z* 343, and strong fragment ion at *m/z* 386 (21) (Table 3). The spectra of this sterol matched that of an authentic standard. The third most abundant sterol at 5.3% was 22-dihydroergosterol ($C_{28}\Delta^{5,7}$) based on the M^+ at *m/z* 470 and strong *m/z* 337 ($M^+ - 131$), which is characteristic of the $\Delta^{5,7}$ double bond placement of TMS derivatives of 3β-hydroxy sterols (21). Several other 4- and 14-desmethyl sterols were detected as minor components along with several methyl sterols considered to be intermediates in ergosterol biosynthesis (22), such as lanosterol which composed 2.1% of the total sterols and 24-methylene dihydrolanosterol at 3.8% (Table 3). These sterols are typical of those found in most fungi of the order Mucorales (13,23).

Phospholipids. Phospholipid comprised 7.2% of the total lipid when the fungus was grown in high C/N medium. Although phosphatidylcholine is frequently the most abundant phospholipid in fungi (24), the phospholipid profile was similar to most fungi with phosphatidylethanolamine and phosphatidylcholine being the major components at 53 and 39% of the total, respectively (Table 4).

TABLE 3
Sterols of *Syzygites megalocarpus* ATCC 18025

MS scan #	Molecular ion (*m/z*)	Base peak (*m/z*)	Other major high mass fragments ions (*m/z*)	Identity	Relative percentage
885	468	363	378 ,337, 253	$C_{28}\Delta^{5,7,22}$ Ergosterol	52.1
911	466	466	378, 376, 361, 341, 337, 251	$C_{28}\Delta^{5,7,?,?,}$	1.6
927	470	470	455, 380, 365, 343, 337, 329, 255, 253, 227, 213	$C_{28}\Delta^{5,\text{ or }8,?}$	3.7
948	468	363	378, 337, 294, 253, 251	$C_{28}\Delta^{5,7,?}$	3.8
958	470	365	380, 339, 253	$C_{28}\Delta^{5,7}$ 22-Dihydroergosterol	5.3
974	470	343	455, 413, 386, 365, 303, 255	$C_{28}\Delta^{7,24(28)}$	18.4
1013	498	393	483, 341, 283, 255	$C_{30}\Delta^{8,24}$ Lanosterol	2.1
1031	484	484	469, 426, 411, 394, 379, 357, 295, 273, 271, 269, 257, 255, 241	Unknown	2.9
1087	512	407	497, 373, 309, 381, 255	$C_{31}\Delta^{8,24(28)}$ 24-Methylene-dihyrolanosterol	3.8
1107		425	440, 407, 341, 323, 273, 259	Unknown	1.6
Others					4.7

TABLE 4
Phospholipid Composition of Total Lipid from *Syzygites megalocarpus* ATCC 18025

Phospholipid	Chemical shift (ppm)	Composition (%)
Phosphatidylcholine	–0.84	39.1
Phosphatidylinositol + lysophosphatidylcholine	–0.32	5.9
Lysophosphatidylinositol	0.00	5.4
Phosphatidylethanolamine	0.08	52.6
Phosphatidic acid	0.36	2.6
Lysophosphatidylglycerol	1.08	0.7

In summary, the facts that *S. megalocarpus* accumulates triacylglycerol which becomes increasingly enriched with relatively high amounts of GLA with culture age, and that GLA is not accompanied by other polyunsaturated fatty acids such as ALA, ARA, EPA, or DHA, are favorable characteristics for this organism being a candidate as a commercial source of GLA. An estimate of GLA production under the conditions described is about 350 mg/L. However, further research will be required to overcome the apparent limitations to adequate biomass production and to increase oil accumulation without compromising GLA enrichment in the oil.

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