Lipid and γ-Linolenic Acid Accumulation in Strains of Zygomycetes Growing on Glucose

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ABSTRACT: Strains of Zygomycetes, belonging to the genera *Zygorhynchus, Mortierella, Rhizopus, Mucor,* and *Cunninghamella,* when cultivated on glucose produced significant quantities of γ-linolenic acid (GLA). After exhaustion of the nitrogen source from the culture medium, all strains accumulated cellular lipids in concentrations ranging from 10 to 28% (oil/dry mycelium). However, in some strains after the depletion of the carbon source (glucose) from the culture medium, a reconsumption of the accumulated oil and synthesis of fat-free cell material was observed. Accumulation of large amounts of oil in the mycelium resulted in the production of oil with low GLA content. *Rhizopus stolonifer* strain LGAM (9)1, and *Cunninghamella* sp. strain LGAM (9)2 produced more than 30 mg GLA/g of dry cellular mass. *Cunninghamella* sp. accumulated 28.1% oil/dry cellular mass, which contained 11.9% GLA. The production of GLA was 260 mg/L of culture medium.

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KEY WORDS: γ-Linolenic acid, lipid accumulation-degradation, Zygomycetes.

The biochemistry of the lipid accumulation process has been extensively studied (1) in many yeasts and fungi. Numerous lipid-accumulating (oleaginous) microorganisms have been characterized for their ability to grow on different carbon sources, and to accumulate significant quantities of reserve lipid (2–5). However, the search for new oleaginous strains, mainly yeasts and fungi, is still of great scientific and industrial interest (6–8).

Oleaginous yeasts are used for production of common oil substitutes (4,9) and production of substitutes of exotic fats, such as cocoa-butter (10). The production of common oils from microorganisms is far away from large-scale application, because the cost of microbial oil production is presently very high and not competitive with agricultural production of common oils, for example, sunflower or soybean oil. The use of yeasts for the production of fats with special structure and composition, such as cocoa butter substitutes, may be a more realistic prospect. Production of oils containing polyunsaturated fatty acids, such as γ-linolenic acid (GLA) (C18:3n-6), from fungi (Zygomycetes) is the most obvious target. GLA is of great pharmaceutical interest as it has been used in the treatment of a number of diseases. Currently, evening primrose oil is the major commercial source of GLA, but its price is high. Zygomycetes, often cited in the literature for their competence in producing GLA, encompass the genera *Mucor*, *Mortierella*, *Rhizopus, Cunninghamella,* and *Zygorhynchus*. The substrates (carbon sources) used for this purpose have varied considerably. On a biochemical basis they can be divided into two general types: those metabolized as C6 compounds, such as glucose, other sugars and polysaccharides, and those metabolized as C2, such as acetic acid, ethanol, lipids and industrial derivatives of fats.

A common physiological property of the heterogeneous group of oleaginous microorganisms is the mechanism of lipid accumulation from glucose *via* the ATP:citrate lyase reaction. ATP:citrate lyase is an enzyme catalyzing the cleavage of citrate in the presence of CoA and ATP to acetyl-CoA and oxaloacetate (1). Furthermore, two physiological properties of theoretical and industrial interest have been observed in some Zygomycetes. The first is that degradation of the accumulated lipid is sometimes observed after the depletion of the carbon source in the growth medium (11), a property probably associated with the adaptation capability of Zygomycetes to survive in environments poor in carbon sources. The second is that the GLA concentration in the cellular fat decreased when the microorganism accumulated large quantities of lipid in the microbial mass (12).

In this work the growth and lipid accumulation of eight oleaginous strains of Zygomycetes were studied during growth on glucose in a nitrogen-limited medium. The fatty acid composition of the cellular lipid was determined, and the strains were compared for their growth yield and ability to produce GLA. Biochemical approaches to explain the behavior of the strains (lipid accumulation-degradation, lipid composition) were considered and discussed.

EXPERIMENTAL PROCEDURES

Microorganisms and culture conditions. Zygorhynchus moelleri BPIC 1703 and *R. stolonifer* BPIC 1676 were obtained from Benakion Phytopathological Institute of Kiphissia,

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Athens, Greece. *Zygorhynchus moelleri* MUCL 1430 was provided by the Mucotheque de l'Universite Catholique de Louvain, Belgium. The two fungi belonging to *Mortierella* species (*M. ramanniana* ATHUM 2922, and *M. isabellina* ATHUM 2935) were furnished by the Laboratory of Botany, Department of Biology, University of Athens. *Rhizopus stolonifer* LGAM (9)1 and *Cunninghamella* sp. LGAM (9)2 were isolated and identified in the Laboratory of General and Agricultural Microbiology, Department of Agricultural Biotechnology, Agricultural University of Athens. *Mucor rouxianus* CBS 120-08 was provided by Centraal Bureau voor Schimmelculture de Braan (Braan, The Netherlands). All strains were stored at 8°C on potato dextrose agar (Plasmatec Lab. Products Ltd., Dorset, England). Cultures were performed in 250-mL conical flasks containing 50 mL of a medium with the following composition (g/L): glucose (AnalaR®, BDH Lab. Supplies, Dorset, England), 30; $(NH_4)_2SO_4$ (AnalaR®), 1; KH_2PO_4 (AnalaR®), 7; Na_2HPO_4 (AnalaR®), 2; $MgSO_4 \cdot 7H_2O$ (Mallinckrodt®, Phillipsburg, NJ), 1.5; $CaCl_2 \cdot 2H_2O$ (AnalaR®), 0.1; $FeCl_3 \cdot 6H_2O$ (Merck, Darmstadt, Germany), 0.008; $ZnSO_4 \cdot 7H_2O$ (AnalaR®), 0.001; $CuSO_4 \cdot 5H_2O$ (AnalaR®), 0.0001; $Co(NO_3)_2 \cdot H_2O$ (May and Baker Ltd., Dagenham, England), 0.0001; $MnSO_4 \cdot 5H_2O$ (Mallinckrodt), 0.0001; yeast extract (Plasmatec Lab. Products), 0.5. pH after sterilization (121°C for 20 min) was 6 ± 0.1. Flasks were inoculated with 10 mL of a 2-d-old preculture on the above medium, containing 5 g/L glucose. All cultures were incubated in an orbital incubator (Gallenkamp, Leicestershire, England) at an agitation rate of 180 rpm and T = 28 ± 1°C).

Determination of microbial mass. Microbial mass was determined in its lyophilized form. Flasks were periodically removed from the shaker, and the mycelium was harvested by filtration, washed with deionized water, and frozen at –40°C before lyophilization in a Hetosicc, type OD 52 (Birkerod, Denmark) lyophilizer. Lyophilized samples contained less than 3% (w/w) water.

Analytical methods. Lyophilized samples were ground into a fine powder, and cellular lipids were extracted overnight in hexane (BDH Ltd., Poole, England), at room temperature. The process was repeated two more times. The extract containing the cellular lipids was dried over anhydrous $MgSO_4$ (Mallinckrodt®), and the solvent was removed by evaporation. Recovery of the cellular lipid was about 95% of the lipid extracted by the Folch method (13). However, hexane was chosen as solvent because its extract contained mainly neutral lipids (triglycerides).

Lipids were esterified according to the AFNOR method (14), and the resulting methyl esters were analyzed by gas–liquid chromatography (GLC), in a GLC Fisons 8000 (Milano, Italy), on a WCOT fused-silica, CP-Sil-88 column, 30 m, 0.32 mm i.d. The peaks were identified by comparison of the retention time of the unknown compounds with those of standard ones. Identification was verified by GLC–mass spectrometry (GC–MS) in a Fisons 8060, MD-800. For GC–MS analysis, a WCOT fused-silica, CP-Sil-88 column (30 m, 0.32 i.d.), was used. The mass spectrometer was operated in electron impact mode with the electron energy set at 70 eV.

Glucose concentration in the supernatant was determined by the Somogyi method (15). Ammonium sulfate concentration was evaluated with an ammonium-selective electrode (model SA720; Orion Inc., Boston, MA). Dissolved oxygen (DO) concentration was measured by a WTW microprocessor oximeter, Oxi 96 (Weilheim, Germany), in culture flasks; before sampling, the shaker was stopped and a WTW oxygen electrode EO 96 was placed into the flask. Then the shaker was switched on again and the measurement was taken after DO equilibration (usually in 5 min). In all cultures and harvest times DO was found to be ≥50% of the saturation value.

RESULTS AND DISCUSSION

Growth and lipid accumulation-degradation. All strains used in this study grew well on glucose in full aerobic conditions and accumulated significant quantities of oil. Oil was typically accumulated after depletion of the nitrogen source from the culture medium (data not shown). This property is commonly encountered in oleaginous yeasts and molds grown on carbohydrates, e.g., glucose. However, two different type of kinetics were observed. The first type was displayed by *Z. moelleri* 1703, *M. ramanniana,* and *M. isabellina*, and *R. stolonifer* (9)1 and 1676, in which the accumulated lipid was degraded after the exhaustion of the carbon source (glucose) from the medium (Fig. 1A). It appears that in these strains the

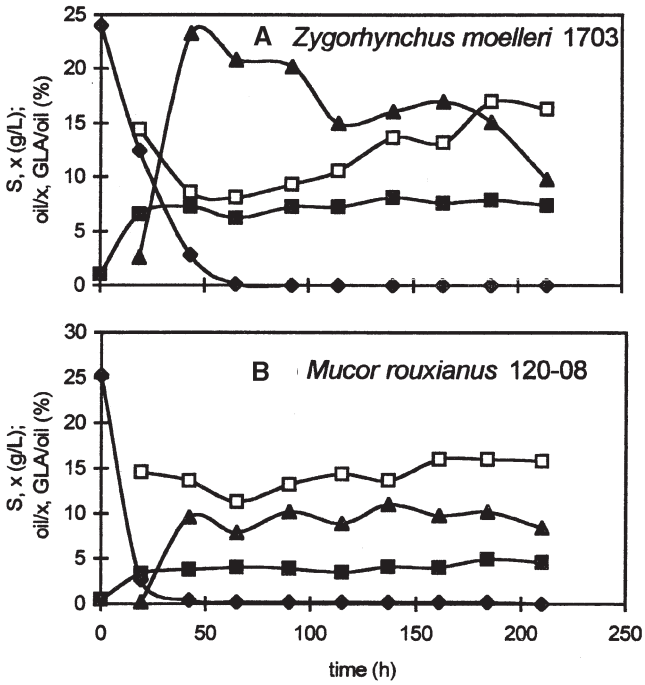

FIG. 1. Growth of total cellular mass (x, g/L, ■), glucose consumption (S, g/L, ◆), lipid accumulation (oil/x, %, ▲), and γ-linolenic acid concentration (GLA/oil, %, □) in (A) *Zygorhynchus moelleri* BPIC 1703 and (B) *Mucor rouxianus* CBS 120-08 (single determinations). Ammonium sulfate was exhausted 50 h after inoculation.

ammonium ions liberated from AMP, after the depletion of the nitrogen from the culture medium (1), allowed continued growth. Therefore, the accumulated lipid was used as carbon and energy source. Actually, fat-free material synthesis was clearly observed after glucose exhaustion from the medium (Table 1), whereas total biomass (e.g., fat-free material plus cellular oil), after 50 h of incubation, remained more or less constant (Fig. 1). The second type of kinetics was exhibited by *Z. moelleri* 1430, *Mucor rouxianus,* and *Cunninghamella* sp. In these cases no degradation of the storage lipid was observed (see, for example, Fig. 1B, Table 1). A possible explanation is that multiple limitations, besides nitrogen, may have occurred, as these strains seem to be incapable of assimilating stored lipid after glucose exhaustion from the medium.

Storage lipid degradation was also observed in oleaginous yeasts growing on glucose. Nevertheless, addition of an exogenous nitrogen source, and probably addition of other nutrients, was necessary to secure growth and, therefore, assimilation of storage lipid (16). Lipid degradation was also observed in the oleaginous fungus *Mucor circinelloides* growing on vegetable oil (11) and in the yeast *Yarrowia* (*Candida*) *lipolytica*, growing on animal fat (17). In all these cases, the onset of lipid turnover occurred rapidly after transition from nitrogen-limitation to carbon-starvation conditions. It was demonstrated that in oleaginous yeast this rapid turnover was mainly due to the control of ATP:citrate lyase, the key enzyme in the conversion of sugar to storage lipid, which supplies acetyl-CoA from citrate, and of isocitrate lyase, which catalyzes and regulates the simultaneous operation of the tricarboxylic acid cycle and the glyoxylate bypass (18). Isocitrate lyase is induced in microbial cells grown on C_2 compounds (such as ethanol or acetate), or on substrates that are degraded to C_2 compounds (such as lipids degraded *via* the β-oxidation pathway).

TABLE 1
Quantitative Data from the Cultures of Zygomycete Strains on Glucose[a]

Strain[b]	Fermentation time (h)	Glucose (g/L)	Fat-free cellular mass (g/L)	Cellular oil (g/L)
Z. moelleri	43	2.8	5.56	1.69
BPIC 1703	92	0.0	5.81	1.47
	214	0.0	6.66	0.72
Z. moelleri	29	14.4	3.86	0.23
MUCL 1430	50	9.8	3.22	0.68
	91	0.0	3.47	1.27
M. ramanniana	93	19.2	3.25	0.35
ATHUM 2922	166	6.2	4.91	1.19
	210	ND	5.11	0.69
M. isabellina	21	21	1.98	0.15
ATHUM 2935	92	0.0	5.32	3.34
	208	0.0	6.66	2.12
R. stolonifer	22	21.2	3.26	0.18
LGAM (9)1	47	0.0	3.60	1.37
	239	0.0	4.46	0.63
R. stolonifer	18	11.4	2.48	0.18
BPIC 1676	91	1.8	4.01	0.79
	191	0.2	4.19	0.61
Mucor rouxianus	19	2.7	3.33	0.01
CBS 120-08	90	0.2	3.48	0.40
	210	0.0	4.19	0.38
Cunninghamella	43	21.2	2.65	0.09
LGAM (9)2	140	10.4	4.57	0.83
	210	0.0	5.58	2.18

[a]In some strains cellular lipid degradation occurred after the exhaustion of glucose from the culture medium.
[b]*Z., Zygorhynchus*; *M., Mortierella*; *R., Rhizopus*; ND, not detected.

Since lipid degradation begins after depletion of the carbon source, this process should be independent of the nature of substrate (e.g., glucose or fats). However, storage lipid biosynthesis from glucose or other carbohydrates occurred after exhaustion of the nitrogen source. (Thus, lipid accumulation from glucose is considered as a secondary anabolic process.) Consequently, further microbial growth, and related cellular lipid degradation, depends on the rate of ammonium ion liberation from AMP as well as on the availability of other essential nutrients in the medium. Additionally, the rate of isocitrate lyase synthesis [enzyme strongly repressed from glucose (18)] could be the limiting step for lipid degradation. Therefore, storage lipid degradation should not be a common phenomenon in cultures growing on glucose. On the contrary, the accumulation of storage lipids from fats (used as carbon and energy sources) occurred in the first fermentation steps, regardless of nitrogen limitation (lipid accumulation from fats is considered as a primary anabolic process) (11,17). Accordingly, after the exhaustion of extracellular fat, the culture conditions (regarding mainly the nitrogen source availability) are still favorable for growth. Furthermore, isocitrate lyase levels are already high at this point, due to induction during growth on extracellular fat (18). For all these reasons, storage lipid degradation should be considered as a phenomenon regularly occurring in oleaginous microorganisms growing on fat substrates.

No matter what the carbon source used in the production of single-cell oil is, storage lipid degradation could be avoided by growing organisms on multiple nutrient-limited media (having at least two limited factors, e.g., nitrogen and magnesium). The industrial interest in this point is obvious mainly in batch or fed-batch cultures.

Composition of storage lipid during microbial growth. Oleic acid (C18:1) was the predominant fatty acid in all oils produced from the different strains and fermentation times. The concentration of this acid increased in the cellular lipid during growth and early stationary phase, but remained constant (usually 30–45%) in the cellular lipids (see, for example, Fig. 2). Palmitic acid (C16:0) was found in the cellular lipids of Zygomycetes in significant concentration (usually 20–25%). In some strains (*Z. moelleri* 1703, *M. ramanniana*, *M. isabellina*) the concentration of this acid showed a tendency to decrease during growth (data not shown).

GLA (C18:3n-6) was present in all oil produced, while α-linolenic acid was not. Indeed, in *Zygorhynchus* spp., *Rhizopus* spp., and *Cunninghamella* sp., GLA was found in large

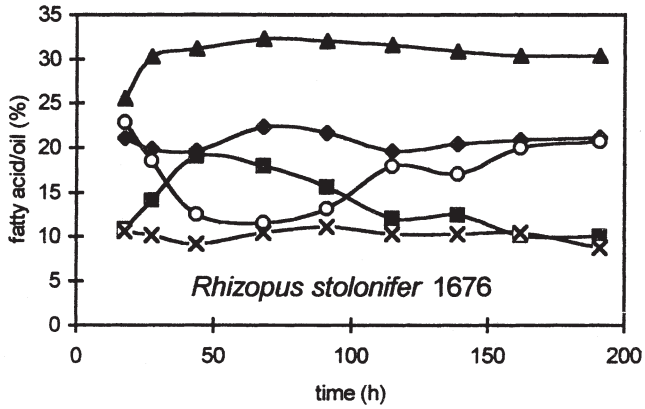

FIG. 2. Fatty acid composition of cellular lipids in *Rhizopus stolonifer* 1676 growing on glucose (determinations were carried out at least in duplicate). ◆, C16:0; ■, C18:0; ▲, C18:1; ×, C18:2; ○, GLA. For abbreviation see Figure 1.

concentrations (15–20%) in the produced oils. However, when large amounts of oil were accumulated in the mycelium, GLA was present in low concentrations (Fig. 1). Linoleic acid (C18:2) was found in lower concentrations than those of GLA and displayed only minor changes during microbial growth (Fig. 2). Finally, stearic acid (C18:0) was found in concentrations of 10–20%, and exhibited a tendency opposite that of GLA (the sum of C18:0 and GLA was about constant).

Physiological role of GLA in Zygomycetes. All Zygomycetes tested in this study had the tendency to produce oils with low GLA content when large amounts of oil were accumulated. However, during cellular oil degradation, the GLA concentration was considerably increased in the produced oil (Fig. 1). This indicates that conditions favoring GLA synthesis are not appropriate for lipid accumulation, and vice versa. Actually, it was found that GLA was produced during microbial growth, whereas oil was accumulated in the stationary growth phase. It is therefore probable that Zygomycetes synthesize limited quantities of GLA, needed for functioning cellular membranes. Furthermore, considering that the membrane surface is quantitatively related to the fat-free biomass, a correlation exists between the quantity of GLA synthesized by Zygomycetes and the quantity of fat-free material produced (Fig. 3).

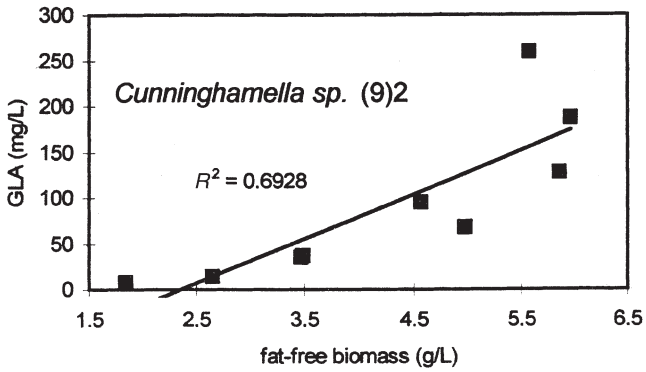

FIG. 3. Correlation of GLA (mg/L) vs. fat-free biomass (g/L) in *Cunninghamella* sp. LGAM (9)2 growing on glucose (R^2 = correlation coefficient). For abbreviaton see Figure 1.

The above finding and suggestions are in agreement with the results reported by Aggelis *et al.* (19), who demonstrated that polar (membrane) lipids from the mycelia of two *Mucor* spp. have compositions, degrees of unsaturation, and GLA contents similar to the storage mycelial lipid. It is therefore possible that storage lipid provides the growing mycelial membrane with the necessary amount of GLA. Analysis of sporangiospores showed that polar lipids were more saturated and contained low quantities of GLA compared to the reserve ones. Spores may need more saturated membranes, rigid to osmotic stress, whereas neutral lipids in spores should be more unsaturated in order to supply the new mycelial hyphae (produced from spore budding) with unsaturated fatty acids. In another work (20) it was demonstrated that polyunsaturated fatty acids are synthesized in the phospholipids of Zygomycetes and then slowly transferred into triacylglycerol pool.

Growth yield, cellular lipids, and GLA production. A comparison of the strains used in this experiment is given in Table 2. The strains *Z. moelleri* 1703, *Cunninghamella* sp., and *M. isabellina* grew well on glucose and gave satisfactory yields (more than 0.25 g of total biomass per g of glucose consumed). Especially, *M. isabellina* and *Cunninghamella* sp. accumulated significant quantities of oil (28.1% in dry cellular mass); *Z. moelleri* 1430 seemed to be able to accumulate large amounts of oil. However the gross production of oil (g/L of culture) was restricted due to the low biomass yield.

The concentration of GLA (GLA/oil) in the oils produced varied between 4.3 and 20%. In the cases where microorganisms showed good characteristics of growth (e.g., *Z. moelleri* 1703, *Cunninghamella* sp.), GLA was found in concentrations of 17 and 11.9%, respectively. This is encouraging as it is known that seeds of evening primrose plant (*Oenothera biennis* O.), presently used in the commercial production of GLA, contain 18–20% of oil, with a GLA concentration of 8–12% (GLA/oil) (8).

Yields of GLA expressed on the basis of glucose consumed (mg/g) were 8.5 and 8.4 for *R. stolonifer* 1676 and *Cunninghamella* sp., respectively, whereas the best yields of GLA on biomass (mg/g) were obtained from *R. stolonifer* (9)1 and *Cunninghamella* sp. (30.1 and 33.5, respectively). Finally, *Cunninghamella* sp. seemed to be a good producer of GLA, as 260 mg of GLA was produced per liter of the culture medium.

The yield obtained from *Cunninghamella* sp. is comparable to that given in the literature from cultures of Zygomycetes on glucose or other sugars. Strains of *Mortierella* spp. produced 15.7–38.9 mg GLA/g microbial mass (21). *Mucor rouxii* CBS 416.77, cultivated on cheap nitrogen and carbon sources, produced 17.8–31.5 mg GLA/g microbial mass (22). *Cunninghamella echinulata* CCRC 31840 cultivated on glucose produced 28 mg GLA/g dry cellular mass; under optimized culture conditions it produced 964 mg GLA/L of the culture medium (23).

The *Cunninghamella* sp. used in this study is therefore of special interest because it accumulates significant quantities

TABLE 2
Yields of Cellular Mass, Oil, and GLA from Zygomycetes Cultured on Glucose[a]

Strain	Growth yield, cellular oil, and GLA production							
	Fermentation time (h)	Total cellular mass (x, g/L)	Cellular oil (g/L)	GLA (mg/L)	Oil/x (%)	GLA/oil (%)	$Y_{GLA/Glc}$[b] (mg/g)	$Y_{GLA/x}$[c] (mg/g)
Z. moelleri BPIC 1703	187	7.9	1.19	203	15.1	17.0	8.5	25.7
Z. moelleri MUCL 1430	91	4.7	1.23	123	26.6	9.7	2.3	25.9
M. ramanniana ATHUM 2922	166	6.1	1.19	115	19.5	9.7	5.0	18.9
M. isabellina ATHUM 2935	140	10.5	2.94	127	28.1	4.3	5.8	12.1
R. stolonifer LGAM (9)1	120	5.1	1.00	153	19.8	15.2	4.2	30.1
R. stolonifer BPIC1676	162	5.4	0.80	159	14.7	20.0	8.5	29.4
M. rouxianus CBS 120-08	184	4.9	0.50	80	10.1	16.0	3.2	16.3
Cunninghamella LGAM (9)2	210	7.8	2.18	260	28.1	11.9	8.4	33.5

[a]Data obtained at the time when maximum GLA production (mg/L) was achieved.
[b]Yield of γ-linolenic acid (GLA) on glucose (Glc) consumed.
[c]Yield of GLA on total cellular mass (x) produced. For abbreviations see Table 1.

of lipid, and its GLA content is satisfactory. It also shows satisfactory growth and yields of cell material and cellular oil.

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