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ScienceDirect
Enzyme and Microbial Technology 40 (2007) 1321–1327
ENZYME AND MICROBIAL TECHNOLOGY
www.elsevier.com/locate/emt

Compositional shifts in lipid fractions during lipid turnover in *Cunninghamella echinulata*

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Received 21 June 2006; received in revised form 26 September 2006; accepted 5 October 2006

Abstract

The oleaginous fungus *Cunninghamella echinulata* when cultivated on a tomato waste hydrolysate medium accumulated 7.8 g l^{-1} of reserve lipid, while, after the exhaustion of the carbon source in the growth environment, 44% of this lipid was consumed and 3.2 g l^{-1} of lipid-free biomass were synthesized. It was demonstrated that lipid fractions and individual lipid classes varied in amount, relative proportions and fatty acid profile during the turnover phase. Triacylglycerols (TAG) were preferentially consumed as their percentage proportion decreased from 26.6 to 6.9% (w/w) of lipid-free biomass, while TAG structures containing more unsaturated fatty acids were partially discriminated. Consequently, the relative proportion of γ-linolenic acid (GLA) increased in TAG from 9.2% (end of the lipogenic phase) to 15.3% (w/w), whereas C16:0 decreased from 22.7 to 15.6% (w/w). Concomitantly membrane polar lipid fractions were synthesized during lipid turnover. During the transition, glycolipids plus sphingolipids fraction was enriched in polyunsaturated fatty acids, especially in GLA, while phospholipids fraction was enriched in GLA but not in C18:2.

Keywords: Single cell oil; γ-Linolenic acid; *Cunninghamella echinulata*; Compositional shifts in lipid; Lipid turnover

1. Introduction

Oleaginous moulds are often considered for the production of single cell oil (SCO) rich in polyunsaturated fatty acids (PUFAs), either in submerged or in solid state fermentation systems [1–4]. The economics of these bioprocess become more favorable when zero or negative value waste substrates are utilized as carbon or nitrogen sources [4–7]. Among PUFAs γ-linolenic acid (GLA) is of particular interest owing to its selective anticancer properties [8,9].

Lipid turnover in oleaginous microorganisms typically commences when the cultivation medium is depleted of the carbon source [10–15]. Accordingly, the oleaginous mould *Cunninghamella echinulata* was reported to utilise its lipid reserves for the production of lipid-free biomass [16]. In *Saccharomyces cerevisiae*, a non-oleaginous yeast, it was shown that triacylglycerols (TAGs) are preferentially consumed during the turnover of reserve oil [17,18]. However, little is known about the composition of the reserve oil in oleaginous yeasts during the turnover and even less for oleaginous moulds. Thus, it was taken for granted that the same situation as in *S. cerevisiae* applies to oleaginous yeasts [10]. Furthermore, the changes in fatty acid composition of the main lipid fractions is of great interest, since it reflects the specificity of intracellular hydrolytic enzymes (i.e. lipases), which are responsible for lipid hydrolysis during lipid turnover [19].

Abbreviations: DAG, diacylglycerol; FA, fatty acid; FASF, fatty acid specific fluctuation (parameter used in the paper); FFAs, free fatty acids; G + S, glycolipids plus sphingolipids; GC, gas chromatography; GLA, γ-linolenic acid; MAG, monoacylglycerol; NL, neutral lipids; P, phospholipids; PUFAs, polyunsaturated fatty acids; RS, reducing sugars; SCO, single cell oil; TAG, triacylglycerol; TLC, thin layer chromatography; TWH, tomato wastes hydrolysate; UI, unsaturation index; x, total biomass; x_f, lipid-free biomass

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doi:10.1016/j.enzmictec.2006.10.005

In this paper, we have monitored the changes occurring in amounts of the various lipid fractions and individual lipid classes, as well as their fatty acid (FA) compositional shifts during lipid turnover in *C. echinulata*, hoping to get an insight of the process and to asses the physiological role of the GLA in the fungal growth.

2. Materials and methods

2.1. Microorganism and culture conditions

C. echinulata ATHUM 4411 was maintained on potato dextrose agar (PDA, Plasmatec, Dorset, UK) at 6 ± 1 °C. Growth medium contained tomato wastes hydrolysate (TWH) supplemented with (g l^{-1}): Glucose, 90; KH_2PO_4, 7; Na_2HPO_4, 2; $MgSO_4{\cdot}7H_2O$, 1.5; $CaCl_2{\cdot}2H_2O$, 0.1; $FeCl_3{\cdot}6H_2O$, 0.08; $ZnSO_4{\cdot}7H_2O$, 0.001; $CuSO_4{\cdot}5H_2O$, 0.0001; $Co(NO_3)_3{\cdot}H_2O$, 0.0001; and $MnSO_4{\cdot}5H_2O$, 0.0001. After sterilization (121 °C/20 min) flasks were inoculated with 1 ml of spore suspension containing 2×10^4 spores, which were produced by growing the strain on PDA for 8 days at 26 °C. All cultivation experiments were performed in 250 ml Erlenmeyer flasks, containing 50 ml of the above medium, incubated in a rotary shaker at 180 rpm and 28 °C.

TWH was obtained by mixing 1 kg of the solid tomato by-product with 3 l of diluted sulfuric acid [20]. The mixture was then autoclaved at 121 °C for 2 h. TWH was filtered through Whatman no. 1 paper and the pH of the filtrates was adjusted to 6 with a concentrated KOH solution.

2.2. Analytical methods

Total biomass (x) was harvested by filtration using a 0.09 mm stainless-steel sieve, washed with cold distilled water, frozen at −30 °C, freeze-dried for 24 h and then gravimetrically determined. Freeze-dried mycelia contained less than 3% water. Lipid-free biomass (x_f) was calculated after subtraction of cellular lipids from total biomass. Reducing sugars (RS) in the growth medium were measured by the dinitrosalicylic acid method [21] and expressed as glucose. Inorganic ammonium ion concentration (NH_4^+) was measured with a selective electrode (51927-00, Hach, Colorado, USA). Total nitrogen was determined by measuring the concentration of NH_4^+ with the selective electrode, after Kjeldalh digestion of the samples. A standard curve was plotted by measuring NH_4^+ concentration in bovine serum albumin (Sigma). Dissolved oxygen concentration and specific oxygen consumption rate were measured as described in [16], with a selective electrode (Oxi 200 Sensodirect, Lovibond, Dortmund, Germany). Dissolved oxygen was >70% (v/v) of the saturation value during all growth phases.

2.3. Lipid analysis of tomato waste hydrolysate

TWH was extracted first with hexane (3×) and then with diethylether (3×). The extracts were combined and the solvents were removed by evaporation. A portion of the extract was then transmethylated and analyzed by gas chromatography (GC), with margaric acid (C17:0) as an internal standard. The analysis showed that it contained no lipid.

2.4. Fungal lipid content determination

The freeze-dried mycelia were ground into a fine powder with an analytical mill and extracted three times with 100 ml of chloroform/methanol (2:1, v/v) for 48 h at room temperature (Folch et al. 1957) [22]. The lipid extract was washed with 0.88% (w/v) KCl (3× 20 ml) and dried over anhydrous Na_2SO_4; the solvent was removed by evaporation.

2.5. Fractionation of fungal lipids

A known weight of extracted lipid (approx. 100 mg) was dissolved in chloroform (1 ml) and fractionated by using a column (25 mm × 100 mm) of silicic acid (1 g), activated by heating overnight at 110 °C [23]. Successive applications of 1,1,1-trichloroethane (100 ml), acetone (100 ml), and methanol (50 ml) produced fractions containing neutral lipids (NL), glycolipids plus sphingolipids (G + S), and phospholipids (P), respectively. The weight of each fraction was determined after evaporation of the respective solvent. Lipid fractions were stored at −30 °C under a nitrogen atmosphere.

2.6. Analysis of fungal lipid fractions

All three-lipid fractions were analyzed by thin layer chromatography (TLC) using various solvent systems [24].

The separation of NL fraction (approx. 20 mg) was carried out using silica gel 60 plates 20 cm × 20 cm (Merck, Darmstadt, Germany), developed with petroleum ether (b.p. 60–80 °C)/diethyl ether/glacial acetic acid (70:30:3, v/v/v). After development, the plate was dried under vacuum and sprayed with 2′,7′-dichlorofluorescein. The bands corresponding to individual lipid classes, namely TAG, diacylglycerols (DAGs) and monoacylglycerols (MAGs), were scraped off and directly transmethylated for GC analysis, with margaric acid (C 17:0) added as an internal standard.

For G + S analysis TLC was developed with chloroform/methanol/ammonia (28%) (65:25:5, v/v/v). After development, the plate was dried and sprayed with α-napthol followed by sulfuric acid (95%) and heating at 120 °C [24]. P analysis was similarly carried out with chloroform/methanol/ammonia (28%) (65:25:5, v/v/v), the plate was dried and sprayed with phosphomolybdic acid followed by heating [24].

The identification of lipid classes was made by co-chromatography with authentic standards, while their quantification was made with margaric acid (C17:0) added as an internal standard before GC analysis. The unsaturation index was calculated as described by Certik et al [25].

2.7. Gas chromatography (GC) analysis

Trans-methylation of lipid extracts was performed according to the AFNOR method [26]. GC analysis was carried out on a Fisons 8060 device equipped with a CP-WAX 52 CB, Chrompack column (60 m × 0.32 mm) and a FID detector; helium was the carrier gas (2 ml/min). The analysis was run at 200 °C with the injector and detector at 250 °C. Fatty acid methyl esters were identified by reference to authentic standards.

3. Results and discussion

3.1. Lipid accumulation and turnover in C. echinulata

During growth and lipogenic phase the lipid content in *C. echinulata* grown on a medium comprised of TWH increases up to 25% of the x (Fig. 1). Although approx. 0.4 g l^{-1} of organic nitrogen (N) were measured in the growth environment, lipogenesis commenced, meaning that this N could not be assimilated. After lipogenesis induction, the carbon surplus was successfully transformed to lipid and 7.8 g l^{-1} of microbial oil were produced. Subsequent sugar exhaustion from the culture medium triggered lipid utilization (turnover phase) that commenced at 312 h when the lipid content in biomass started dwindling; at that time x_f was 24.8 g l^{-1}. After turnover completion at 380 h the lipid content decreased to 14% in x; no further growth or lipid utilization was observed after 380 h.

The utilization of the accumulated lipid was accompanied by new x_f production, which then reached the value of 28 g l^{-1}, while the amount of lipid was 4.4 g l^{-1}. Thus, 0.95 g of x_f was produced per g of lipid consumed. This value is lower than the theoretical yield of 1:1.7 (w/w) for the conversion of TAG to biomass [10] but is similar to the value reported for another oleaginous mould, namely *Mortierella isabellina* [16]. These

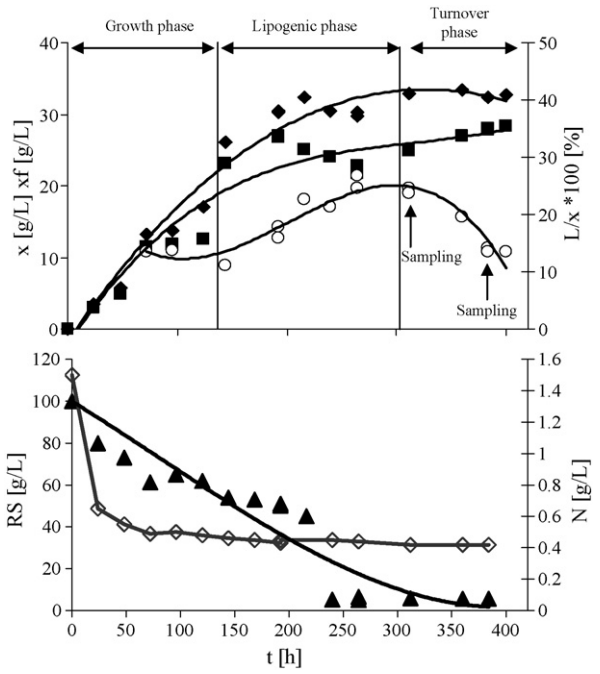


Fig. 1. Evolution of total biomass (x, g/l of cultivation medium—♦), lipid-free biomass (x_f, g/l of cultivation medium—■), lipid in total biomass [(L/x) × 100 (%)—◯), reducing sugars (RS, g/l of cultivation medium—▲) and total nitrogen (N, g/l of cultivation medium—◊) during growth of *Cunninghamella echinulata* on tomato waste hydrolysate. Two lots of independent cultures were conducted by using different inocula.

discrepancies may be attributed to the availability of intracellular nitrogen for the synthesis of new x_f. Utilization of storage lipid as carbon and energy source was also reported for a number of oleaginous Zygomycetes, when grown on synthetic media having glucose as carbon source [11].

3.2. Changes in the amounts of lipid fractions and classes during lipid turnover

Lipid analysis at the beginning of the turnover phase (indicated by an arrow in Fig. 1) showed that the percentage proportion NL/x_f was 29.6% (w/w) while the proportion of G + S/x_f fraction and P/x_f fraction were 1.1% and 0.5% (w/w), respectively (Table 1). The portion of NL fraction in total lipid was 92% (w/w) which indicated that most of the accumulated lipid proceeds as reserve lipid. Further analysis of NL fraction revealed that TAGs were the major constituents, accounting for 90% (w/w) of NL fraction, while DAGs and MAGs were minor components, at 6.4% and 0.6% (w/w) of NL fraction, respectively. Sterols and their esters were also present in very low quantities, while free fatty acids (FFAs) were found in traces. TAGs reside in lipid bodies along with other storage molecules, such as steryl esters [27,28]. G + S fraction comprised mainly of monogalactosylglycerol and digalactosylglycerol. Analysis of P fraction showed the presence of phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine. No further quantitative analysis were performed as these have been reported elsewhere [29].

Lipid analysis carried out after completion of lipid turnover (indicated by an arrow in Fig. 1) clearly demonstrated that NL were preferentially utilized against the other two fractions, since NL/x_f dropped from 29.6 to 12% (w/w) (Table 1), meaning that around 60% of NL were consumed. On the contrary, G + S/x_f and P/x_f proportions increased around two-fold, indicating that a portion of the reserve lipid was utilized for biosynthesis of polar lipids. Polar lipids are integral components of cell membranes, so their biosynthesis is interrelated with the production of new mycelia [30].

Among NL classes TAGs were preferentially utilized as their percentage proportion of the lipid-free biomass TAG/x_f, considerably decreased from 26.6 to 6.9% (w/w), corresponding to an important utilization of TAGs of 74%. Preferential TAG mobilization was also reported for oleaginous bacteria of the genus *Rhodococcus*, where TAGs were utilized more readily than stored polyhydroxyalkanoates [31]. The other two NL classes, DAG and MAG, increased their proportions to 4.4% and 0.72% (w/w), respectively.

Work with oleaginous microorganisms showed that under carbon limiting conditions the previously accumulated lipid was utilized for biosynthetic purposes [10,14–16]. This lipid was hypothesized to be TAG [10], though this was not checked by lipid analysis. Our data clearly demonstrate that stored TAGs were indeed catabolized, while FFAs produced from TAG hydrolysis, may be channeled to β-oxidation for energy provision. Alternatively, TAG hydrolysis products, namely DAG, MAG and FAs, may serve as precursors for polar lipid biosynthesis [17,18]. DAG and MAG are intermediates of TAG catabolism so the increase of their amount showed that their utilization rate was lower than their production rate. Likewise, the presence of FFAs in trace amounts after completion of lipid turnover indicated that FFAs were rapidly metabolized, after their release from TAG. Although both G + S and P fractions increased their

Table 1
Percentage proportions of lipid fractions and neutral lipid classes of the lipid-free biomass x_f after completion of lipid accumulation (312 h) and after completion of lipid turnover (384 h)

Physiological state	Percentage proportions of lipid fractions and classes to lipid-free biomass (%, w/w)					
	NL/x_f	TAG/x_f	DAG/x_f	MAG/x_f	G + S/x_f	P/x_f
Accumulation phase (312 h)	29.6	26.6	1.9	0.17	1.1	0.5
Turnover phase (384 h)	12.0	6.9	4.4	0.72	2.3	1.2

Two lots of independent cultures were conducted by using different inocula and samples were taken for analysis at time points indicated by arrows in Fig. 1. Data are presented as mean values from duplicate experiments. *Abbreviations*: NL, neutral lipids; TAG, triacylglycerol; DAG, diacylglycerol; MAG, monoacylglycerol; G + S, glycolipids + sphingolipids; P, phospholipids; FASF, fatty acid specific fluctuation; UI, unsaturation index.

amounts, we may not rule out the possibility that a portion of these fractions was degraded during lipid turnover. Likewise, production of new TAG from DAG and FFA or through acyl exchange between TAG and P should not be discarded [32]. Based upon these findings we tentatively put forward a schema (Scheme 1) depicting some of the possible pathways for lipid turnover.

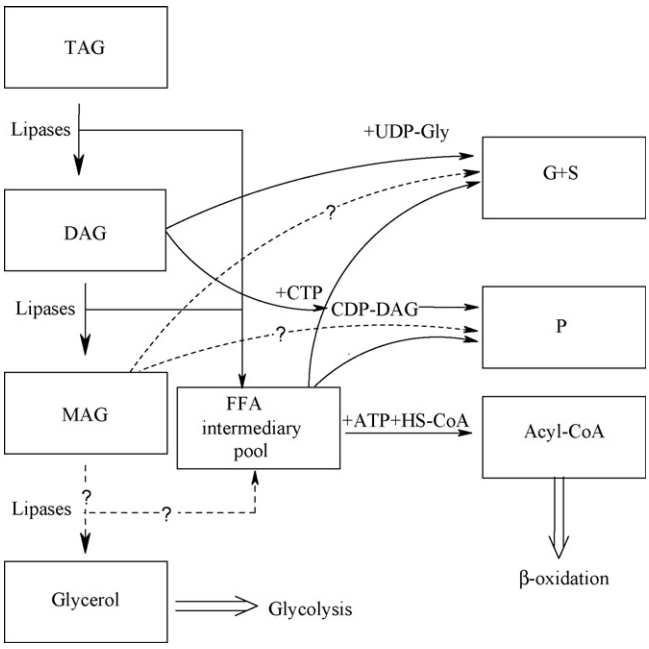
3.3. Changes in the fatty acid profile of lipid fractions and classes during lipid turnover

FA analysis was carried out after completion of the accumulation phase and after completion of lipid turnover (Table 2). In order to identify the specificity through individual FAs were elaborated in a given lipid fraction during the turnover phase, the parameter "*Fatty acid specific fluctuation*" (FASF) was used. FASF calculation was based on the following formula:

$$\text{FASF} = \frac{\text{FA}_\text{D}\% - \text{FA}_\text{A}\%}{\text{FA}_\text{A}\%}$$

where $\text{FA}_\text{D}\%$ was the percentage of an individual FA in a given lipid pool after completion of lipid turnover and $\text{FA}_\text{A}\%$ was its percentage in same pool after completion of lipid accumulation.

FASF describes the change in concentration of each FA in relation to the available substrate and provides a measure of the FA relative fluctuation to the lipid pool under consideration [33]. Negative values indicate that there was a relative FA decrease, while positive values that there was a relative FA increase in the lipid pool under consideration. Relative FA decrease should be attributed to one or more of the following: selective removal of the FA from the lipid pool, bioconversion of the FA in the



Scheme 1. Proposed pathways for lipid turnover in *Cunninghamella echinulata* (modified from [31]). Dashed lines: hypothetical movements of lipids among lipid pools. TAG: triacylglycerol; DAG: diacylglycerol; MAG: monoacylglycerol; G + S: glycolipids plus sphingolipids; P: phospholipids; FFA: free fatty acids. TAG was hydrolyzed to DAG, MAG and FFA. DAG may form P through cytidine diphosphate DAG (CDP-DAG), form G + S by reacting with uridine diphosphate glycoside (UDP-Gly), be hydrolyzed to MAG and FFA. MAG may form polar lipids after esterification with a FFA, producing DAG; newly formed DAG may then synthesize P or G + S. FFA may be channeled to β-oxidation after formation of acyl-CoA esters. Acyl-CoA esters may also exchange acyl residues with P.

Table 2
Fatty acid composition of *Cunninghamella echinulata* after completion of lipid accumulation and after completion of lipid turnover, and values of the parameter FASF

Physiological state	Lipid	Fatty acid concentration (%, w/w)					UI (Δ/mol)
		16:0	18:0	18:1	18:2	18:3	
Accumulation		21.7	6.9	48.5	13.4	9	1.02
Turnover	NL	15.6	5.3	43.6	17.8	17	1.30
FASF		−0.28	−0.23	−0.10	0.33	0.89	
Accumulation		22.7	6.5	48.2	13.3	9.2	1.02
Turnover	TAG	16.8	4.9	46.7	15	15.3	1.23
FASF		−0.26	−0.25	−0.03	0.13	0.66	
Accumulation		20.1	12.7	46.8	11.2	9.2	0.97
Turnover	DAG	12.9	3.5	39.7	23.1	20.7	1.48
FASF		−0.36	−0.72	−0.15	1.06	1.25	
Accumulation		21	5.2	42.1	15.8	15.7	1.21
Turnover	MAG	13.1	5.4	43.9	19.5	18.1	1.37
FASF		−0.38	0.04	0.04	0.23	0.15	
Accumulation		29.8	7.3	50.4	9.6	2.9	0.78
Turnover	G + S	17.8	4.4	41.9	18.9	15	1.25
FASF		−0.40	−0.40	−0.17	0.97	4.17	
Accumulation		20.5	2.5	41.8	24.7	10.2	1.22
Turnover	P	18.9	3.3	42.8	18.7	16.3	1.29
FASF		−0.08	0.32	0.02	−0.24	0.60	

Two lots of independent cultures were conducted by using different inocula and samples were taken for analysis at time points indicated by arrows in Fig. 1. Abbreviations as in Table 1. Data are presented as mean values from duplicate experiments.

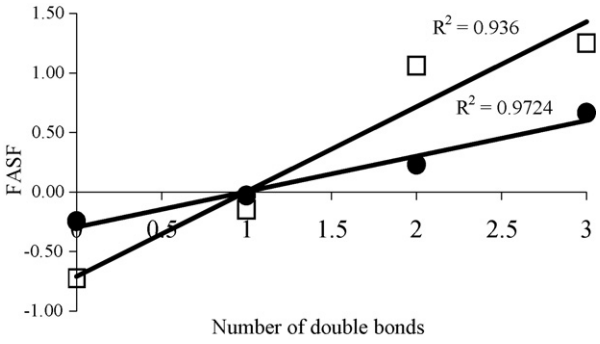


Fig. 2. Correlation between the number of double bonds of the C18 fatty acyl chain and fatty acid specific fluctuation (FASF) for triacylglycerol (●) and diacylglycerol (□).

pool, increase of the amounts of the other FAs residing in the specific pool. Likewise, relative FA increase should be attributed to one or more of the following: *de novo* biosynthesis of the FA in the pool, selective incorporation of the FA from other pools, removal of the other FAs residing in the specific pool.

In NL fraction the relative percentages of C16:0, C18:0 and C18:1 decreased while the percentages of PUFAs increased during the transition between the two phases. The same trend was observed among NL classes, except for MAG class, where the relative proportions of C18:0 and C18:1 remained constant during the transition. This resulted in an increase of the UI of NL fraction and their classes. However, the phenomenon was more intense in DAG class, where PUFAs almost doubled their percentages, causing an increase of the UI from 0.97 to 1.48.

FASF values for NL fraction indicate that the relative decrease of saturated FAs was greater than that of C18:1, while the relative increase of GLA was far greater than that of C18:2. In TAG class the same tendency was observed but the relative decrease of C18:1 and the relative increase of PUFAs' were reduced as compared to NL fraction. Since TAGs proved the major substrate of lipases, this would imply that the changes of their FA profile during the transition were mostly due to their selective hydrolysis. It should be considering that FASF reflects lipase specificity towards the various FAs located in TAG structures. A strong linear dependence of FASF on the number of double bonds of the C18 acyl chain was observed indicating that the order of TAG hydrolysis was conversely related to the unsaturation of acyl chains that participate in TAG structures (Fig. 2). This would then mean that GLA-containing TAGs were hydrolyzed slower than the other TAG molecular species. A similar discrimination was reported for some other microbial lipases, when added to a GLA-containing fungal oil [34]. Their selectivity was utilized for the enrichment of the oil in GLA, causing an increase of the oil's GLA content from 10 to 15% after the hydrolysis procedure.

Microbial TAG types are SUS, SUU and UUU (U = unsaturated FA, S = saturated FA), where C18:2 and GLA occupy the central position [35]. The enrichment of DAG in PUFAs (especially in GLA) indicated that the lipases of *C. echinulata* preferentially remove fatty acids esterified in 1,3 position (i.e. saturated and C18:1 FAs) from their substrate (TAG), as those from the Zygomycetes *Mucor javanicus* and *Rhizopus japonicus* [19]. Specific FA removal was also found for two *Mucor* strains when cultivated on mixtures of sugars and vegetable oils [36].

Taking into account the above suggested specificity of *C. echinulata* lipases, it would follow that 1,2 and 2,3 DAG produced during TAG hydrolysis should be more enriched in GLA, less in C18:2 and even less in C18:1. Thus, the incorporation of these newly formed DAG to the pre-existing DAG pool should result in a selective increase of FAs in DAG class. Indeed, these facts were depicted in the particularly high FASF values for PUFAs in DAG class and the very low FASF values for saturated and C18:1 FAs (Table 2). Accordingly, the linear correlation between FASF of the C18 acyl chain and the number of double bonds for DAG class further supported the suggested lipase specificity (Fig. 2).

In MAG, as opposed to the other two NL classes, there was a relative increase of C18:0 and C18:1, while the relative increase of GLA was the lowest among lipids. Newly formed MAG may originate from hydrolysis either of the preexisting DAG or of the DAG produced after TAG hydrolysis or both. This would then mean that the lipases' specificity towards DAG differs from their specificity towards TAG.

The above findings are in contrast with those reported for another member of Zygomycetes, *Mucor circinelloides*, when cultivated on C18:2-rich vegetable oil [14]. FASF calculation from the data reported in [14] showed that the order of FA preferential consumption was C18:2 > GLA > C18:1 > C16:0, while C18:0 accumulated in storage lipid. Furthermore, cultivation of the oleaginous yeast *Yarrowia lipolytica* on mixtures of stearin and hydrolyzed rapeseed oil showed that when storage oil was degraded, C18:1 (more readily) and C16:0 were preferentially consumed, while C18:0 once again accumulated in storage lipid [12,13]. Consequently, partially degraded storage oil in these two microorganisms was more saturated than the initial stored oil (i.e. UI of total lipids dropped from 1.4 to 0.96 in *M. circinelloides* [14]). On the contrary, partial degradation of the accumulated oil in *C. echinulata* resulted in an increase of the oil's unsaturation, as indicated by the increase of the UI of NL fraction from 1.03 to 1.3, with a higher GLA content. These observations may be indicative of the strain-dependent specificity of intracellular lipases and of the ability of these enzymes to modify the composition of the accumulated lipid.

FA profile of G + S fraction changed dramatically during the transition. The remarkably high percentages of C16:0 and C18:1 during accumulation decreased, along with C18:0, while C18:2 almost doubled its proportion; there was also a strikingly five-fold increase of the GLA percentage. These changes resulted in an increase of the UI, which was the lowest among lipids during accumulation, from 0.78 to 1.25. These very high percentages of C16:0 and C18:1 during the accumulation phase, which coincides with the stationary phase of growth, are in contrast with those previously reported when the same mould was cultivated on a carbon limited medium [29]. FASF values showed the relative decrease of saturated and C18:1 FAs, while there was a relative increase of C18:2 and GLA in G + S. The relative increase of GLA was the highest among lipids. Since there is no information as to G + S specific lipases for moulds, the changes in this fraction could be due to one or more of the following: *de*

novo biosynthesis of PUFAs, glycosylation of PUFA-rich DAG, incorporation of PUFAs from other pools (e.g. P fraction).

FA changes of P fraction were distinct from the other fractions: C16:0, C18:0 and C18:1 maintained a constant proportion, while the relative percentage of C18:2 decreased and that of GLA increased; a slight increase of the UI was also observed. FASF values for saturated FAs showed that there was a restricted relative decrease of C16:0, while the relative increase of C18:0 was the greatest among lipids. Turning to PUFAs, P was the only lipid that presented a relative decrease of C18:2, while there was a significant relative increase of GLA. P are the major site of PUFAs biosynthesis, from which PUFAs are transferred to the other lipid classes [32,37]. Hence, the relative decrease of C18:2 could be due to either its transport to other lipid classes or its biotransformation to GLA or both. On the other hand, the relative increase of GLA indicates that the rate of its biosynthesis (and possibly incorporation) was faster than the rate of its transport.

4. Conclusion

The data reported demonstrate that in *C. echinulata* triacylglycerols (TAGs) were preferentially degraded during storage oil turnover, concomitantly an increase of lipid-free biomass (x_f) was observed, suggesting that the TAG degradation resulted in the production of new x_f. Furthermore, TAGs containing γ-linolenic acid (GLA) were hydrolyzed with a slower rate than the other TAG molecular species. On the contrary, diacylglycerols (DAGs) and monoacylglycerols (MAGs) produced during TAG hydrolysis accumulated during the transition. Biosynthesis of new x_f was associated with the production of polar lipids, which most probably served as the integral parts of the newly formed mycelial membranes. The increase of GLA in glycolipid plus sphingolipid (G + S) fraction was the highest among lipids, indicating a possible role of G + S in GLA biosynthesis. Similarly, the enrichment of phospholipids (P) with GLA is in accord with the view that P are the major site of GLA biosynthesis.

Although degradation of lipid reserves is a well-established fact among oleaginous microorganisms the details of the process are yet to be elucidated. This would have then a dual interest: a better understanding of the physiology of oleaginous microorganisms and unambiguous implications in the production of single-cell oil. Unfortunately, while lipid accumulation is strongly related to the secondary metabolic growth, GLA biosynthesis seemed to be associated with the production of new biomass. Thus, culture conditions favoring lipid production do not favor GLA production [11].

Acknowledgements

Financial support was provided by the bilateral project between Greece and Slovakia: "Biotechnological valorization of agro-industrial materials by application of oleaginous microorganisms producing biologically active lipids". The Greek State Scholarship Foundation is acknowledged for the doctoral fellowship of Stylianos Fakas.

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