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EXPERIMENTAL ARTICLES

Activity of NAD-Dependent Isocitrate Dehydrogenase, Isocitrate Lyase, and Malate Dehydrogenase in *Mucor circinelloides* var. *lusitanicus* INMI under Different Modes of Nitrogen Supply

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Abstract—The growth and morphology as well as lipogenesis and activity of the enzymes of the tricarboxylic acid cycle and the glyoxylate cycle were studied in the fungus *Mucor circinelloides* var. *lusitanicus* INMI grown at various concentrations of urea (nitrogen source) added to the medium in different modes. It was shown that the maximum lipid content in the biomass was observed at a low (0.5 g/l) concentration of the nitrogen source, whereas the highest content of γ-linolenic acid in the lipids was detected at high (up to 4.0 g/l) concentrations of the nitrogen source. It was found that, when the feed-batch mode of nitrogen supply was used, the amount of γ-linolenic acid in total fatty acids was higher (up to 35%) than in the case of a single administration of the same amount of nitrogen source to the medium. The differences in the fatty acid composition and the unsaturation degree of the lipids from different subcellular fractions were demonstrated. The mycelium from the culture grown after a single administration of the nitrogen source was deformed to a great extent. The activities of the TCA cycle enzymes, NAD-dependent isocitrate dehydrogenase (IDH), and malate dehydrogenase (MDH) were lower than in the case of the feed-batch mode of urea addition, whereas the activity of isocitrate lyase (ICL), the key enzyme of the glyoxylate cycle, was higher. The coupling of the cell metabolism and the lipid composition of fungal cells and the process of cell differentiation in fungi depending on the conditions of nitrogen supply is discussed.

Key words: Mucor circinelloides, γ-linolenic acid, NAD-dependent isocitrate dehydrogenase, malate dehydrogenase, isocitrate lyase, morphogenesis.

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When the nitrogen source in the medium is depleted in a batch culture of oleaginous microorganisms, the biosynthesis of proteins and nucleic acids ceases, while the excessive carbon is metabolized with lipid accumulation. It is well known that urea as a source of nitrogen is an important regulatory factor involved in lipid biosynthesis. According to the results presented in the papers [1–3], it is rapidly consumed and catabolized to release NH_4^+. The increase in NH_4^+ content causes accumulation of mitochondrial citrate due to a decrease in the intracellular AMP concentration, which in turn leads to a decrease in the activity of AMP-dependent NAD^+ isocitrate dehydrogenase (IDH) in the mitochondria. Citrate is then transported across mitochondrial membranes in exchange for L-malate and then decomposed in the cell cytosol by ATP-citrate lyase with the resulting formation of acetyl-CoA, responsible for the synthesis of fatty acids, and oxaloacetate.

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It has been previously demonstrated that the regime of nitrogen supply to the fungus *Mucor circinelloides* var. *lusitanicus* INMI (i.e., a single administration of the nitrogen source to the nutrient medium at inoculation or multiple administration of urea in feed-batch mode) [4], as well as the C/N ratio in the medium [5], affect the synthesis of γ-linolenic acid. In the case of intermittent application of the nitrogen source (feed-batch mode), the content of γ-linolenic acid in lipids increases sharply; the lipid concentration in the biomass increases as well. An investigation of the activity of Δ6-desaturase, the enzyme responsible for the synthesis of γ-linolenic acid, revealed its increase in the mycelium of the mucor fungi grown in the feed-batch mode of urea addition [6].

In [7], it was demonstrated that the activity of Δ6-desaturase is associated with the ontogenetic stage of fungal growth. The maximum Δ6-desaturase gene expression in *Mucor rouxii* was detected during spore germination in the course of germ tube formation. The most intense synthesis of γ-linolenic acid in the fungus *M. circinelloides* var. *lusitanicus* INMI was observed at

the logarithmic stage of growth [8]. When, by the end of the exponential growth phase, the nitrogen source in the medium was depleted, the lipid content in the biomass increased, whereas the concentration of γ-linolenic acid decreased. In experiments with *M. rouxii*, it was demonstrated that the nitrogen source content in the medium affected the synthesis γ-linolenic acid; however, it did not affect the synthesis of oleic acid [9].

The synthesis of γ-linolenic acid is associated with the physiological state of the fungal mycelium and correlates with its respiratory activity and functioning of the enzymes of central metabolism (of the tricarboxylic acid (TCA) cycle), which are required to activate the desaturase system. The depletion of the nitrogen source leads to a decrease in the activity of the TCA cycle enzymes [10]. It is well known that the conditions favorable for the activation of the glyoxylate cycle enzymes (utilization of acetate or fatty acids as a carbon source, as well as cell differentiation) usually correlate with a low degree of lipid unsaturation and their content in basidiomycetes and aspergillum and mucor fungi [11–14]. It is therefore obvious that changes in the fatty acid composition of lipids of the fungal cells grown at different regimes of nitrogen supply may be due to the activity of certain enzymes. Along these lines, it was of particular interest to study the activity of the key enzymes of the TCA and glyoxylate cycles at various regimes of the nitrogen source administration during cultivation of the fungus *M. circinelloides* var. *lusitanicus* INMI, an active producer of γ-linolenic acid.

The goal of this work was to study the activities of the enzymes of the TCA and glyoxylate cycles (NAD-dependent IDH, MDH, and ICL), as well as the content of γ-linolenic acid, during growth of the fungus *M. circinelloides* var. *lusitanicus* INMI at various concentrations of the nitrogen source administered using different modes of application.

MATERIALS AND METHODS

Microorganism. The fungal strain *Mucor circinelloides* var. *lusitanicus* INMI (=VKM F-306 D) was obtained by selection at the Winogradsky Institute of Microbiology, Russian Academy of Sciences.

Cultivation conditions. The culture was grown at 27°C for 72–96 h on a shaker (130 rpm) in two-liter flasks with 500 ml of the medium (pH 7.0) containing the following (g/l): glucose, 60.0; urea, 2.0; NaCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; K_2HPO_4, 1.0; $ZnSO_4 \cdot 7H_2O$, 0.05; $FeSO_4 \cdot 7H_2O$, 0.01; and yeast extract, 0.5. In some experiments, the nitrogen source (urea) was added once at inoculation; in other experiments, 0.5-g portions of urea were added per 500 ml of the medium at inoculation with subsequent addition of 0.25 g/500 ml in the following two days (feed-batch mode). During the experiments aimed at determining the effect that various concentrations of the nitrogen source have on fungal growth and lipid production by fungal cells, urea was added to concentrations of 0.5, 2.0, and 4.0 g/l.

The nitrogen content in the culture liquid was determined by the Kjeldahl method; the glucose concentration was determined using Fehling's solution.

Microscopy. The morphology of the fungus was examined under an Axio Imager.D1 light microscope (Carl Zeiss, Germany) at ×630 magnification.

Obtaining of cell-free extracts. The mycelium was frozen in liquid nitrogen, disrupted by extrusion in a high-pressure homogenizer analogous to a French press desintegrator, and resuspended in K-Na phosphate buffer (0.05 M, pH 7.2). Cell wall fragments were removed from the cell-free extract by centrifugation; the resultant lipid film was removed from the surface; and the supernatant was used for determination of enzymatic activity.

The enzymatic activities of isocitrate lyase (ICL, EC 4.1.3.1), NAD-dependent isocitrate dehydrogenase (IDH, EC 1.1.1.41), and malate dehydrogenase (MDH, EC 1.1.1.37) in the cell-free extracts were determined according to the techniques described in [15–18] on a Specord UV-VIS spectrophotometer (Jena, Germany) at 324 nm (for ICL) and 340 (for IDH and MDH). The enzymatic activity was expressed in μmol of the reaction product (NADH) or its derivative, glyoxylate phenylhydrazone, formed min^{-1} (mg protein)$^{-1}$. The protein content was determined by the Lowry method. The presented results are an average of at least three independent measurements.

Isolation of cell fractions. The mixed membrane/mitochondrial and microsome fractions were obtained by differential centrifugation of the cell-free extract. To precipitate the mixed membrane/mitochondrial fraction, the extract free of lipid granules was centrifuged at 25000 *g* for 1 h. Trace quantities of lipid granules were removed from the supernatant surface; to precipitate the microsome fraction, the supernatant was then centrifuged at 100000 *g* for 1 h.

Lipids were extracted using the Folch method [19]; their amounts were determined gravimetrically.

Gas-liquid chromatography. To obtain methyl esters of fatty acids, lipids were subjected to acid methanolysis at 80°C for 1.5 h in a methanol : acetyl chloride mixture. Methyl esters of fatty acids (MEFA) were extracted with hexane and analyzed by GLC in the isothermal mode at 170°C in a Model 3700 chromatograph (Russia), equipped with a column with 17% diethylene glycol succinate on Chromosorb W, at a carrier gas (helium) flow of 40 ml/min. The quantity of γ-linolenic acid was expressed as a percentage of total fatty acids. The unsaturation degree of the lipids was expressed as the number of double bonds per 100 molecules of fatty acids.

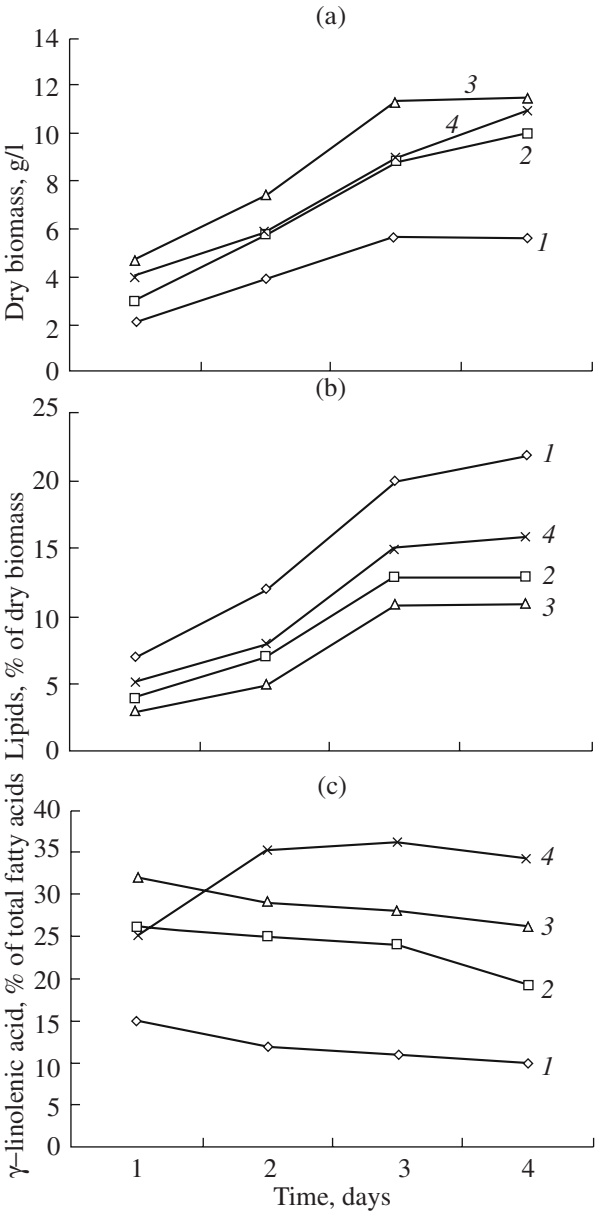

Fig. 1. Accumulation of biomass (a), lipids (b), and γ-linolenic acid (c) in the fungus *M. circinelloides* var. *lusitanicus* INMI after the single addition (*1–3*) and feed-batch application (*4*) of urea to the medium. Total content of the nitrogen source: (*1*) 0.5 g/l; (*2* and *4*) 2.0 g/l; and (*3*) 4.0 g/l.

RESULTS AND DISCUSSION

Figure 1 shows the data on the growth and lipogenesis of the fungus *M. circinelloides* var. *lusitanicus* INMI at various concentrations of the nitrogen source administered using different modes of application.

The amount of biomass obtained on the medium containing various concentrations of urea added once at inoculation, as well as the content of γ-linolenic acid in the lipids (Fig. 1a, *1–3*, and Fig. 1c, *1–3*), were in direct relationship to the urea content. After 48-h incubation, the difference in biomass yield between experimental

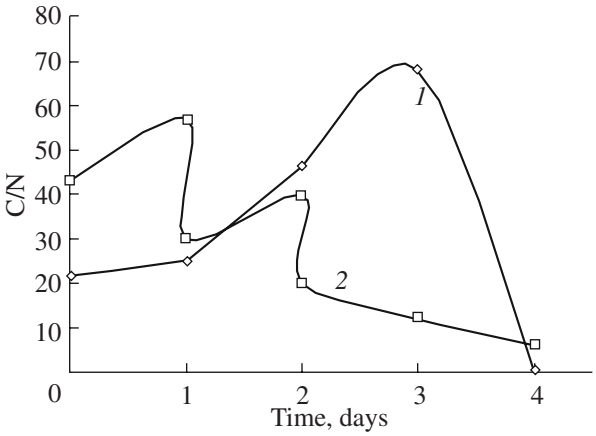

Fig. 2. The C/N ratio in the nutrient medium under different conditions of nitrogen supply: (*1*) single administration and (*2*) feed-batch mode.

variants was 40%; the difference in the content of γ-linolenic acid in total fatty acids was 50%. The maximum amount of lipids present in the biomass (Fig. 1b; *1*) was accumulated at a low concentration of the nitrogen source, while the maximum content of γ-linolenic acid in the lipids was observed in the biomass grown at high urea concentrations (Fig. 1c, *3*).

In the case of intermittent application of the nitrogen source (2 g/l), the amount of biomass and its lipid content were slightly higher than in the case of a single administration of the same dose of urea to the medium (Fig. 1a and 1b; *2* and *4*). The content of γ-linolenic acid in the lipids increased sharply (Fig. 1c; *2* and *4*). Three-step addition of the nitrogen source resulted in a rise in the proportion of γ-linolenic acid to more than 35%.

Figure 2 shows the C/N ratio in the cultivation media (the nitrogen source concentration was 2 g/l). Intermittent application of the nitrogen source (Fig. 2; *2*) altered the regime of its consumption, as compared to the single addition of urea to the medium (Fig. 2; *1*). This prevented the depletion of nitrogen and changed the C/N ratio dynamics during the cultivation. In one day of cultivation, the C/N ratio and, apparently, the content of acetyl-CoA were high enough to promote lipid accumulation in the mycelium. After the subsequent addition of nitrogen (at the end of days one and two of cultivation), the C/N ratio decreased to a level that promoted the production of γ-linolenic acid against the background of the constant consumption of glucose.

The observation of the morphological changes in the fungal cells grown at different modes of nitrogen supply (the nitrogen source concentration was 2 g/l) did not show any significant changes in the mycelium by the end of day one (Fig. 3a and 3b). The hyphae were long, even, nonseptate, and undistorted. When the feed-batch mode of urea addition was applied, the mycelium contained a large amount of lipid granules (Fig. 3b), prob-

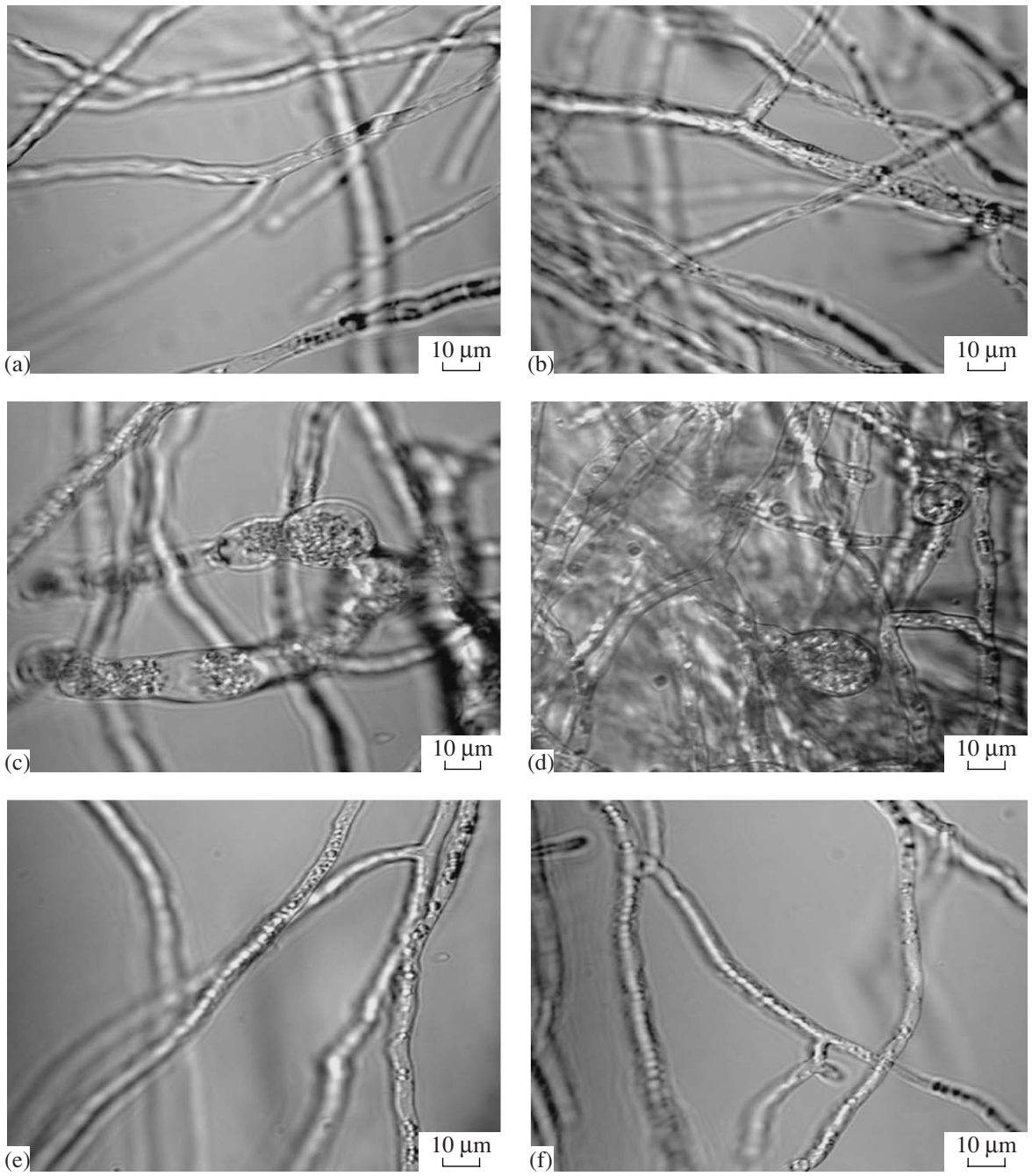

Fig. 3. Changes in the morphology of the cultures of *M. circinelloides* var. *lusitanicus* INMI grown under different conditions of nitrogen supply: (a) 1 day, single addition of urea; (b) 1 day, feed-batch mode; (c, d) 3 days, single addition of urea; (e, f) 3 days, feed-batch mode.

ably resulting from the higher initial C/N level in the medium. By the end of day three of cultivation, the mycelium grown after a single administration of the nitrogen source was distorted; the apexes of its hyphae were swollen; arthrospores, septate fragments of the mycelium, as well as regions containing degraded cytoplasm, were observed (Fig. 3c and 3d). When the feed-batch mode of the addition was applied, the mycelium was not deformed (Figs. 3e and 3f) and resembled the mycelium of a young culture (Fig. 3b).

The morphological changes that depend on the cultivation mode have been also detected in other fungi. In the case of *Aspergillus niger*, a citric acid producer, the deformation of hyphae was observed at the end of ordinary batch cultivation, whereas no morphological changes or spore formation were detected at the feed-

Table 1. Enzymatic activities of NAD-dependent isocitrate dehydrogenase, malate dehydrogenase, and isocitrate lyase under different conditions of nitrogen supply in the culture of the fungus *M. circinelloides* var. *lusitanicus* INMI (3 days)

Modes of nitrogen source application	Enzymatic activity, μmol/(min × mg protein)		
	NAD-dependent IDH	MDH	ICL
Single application	1.62	2.02	0.08
Feed-batch mode	2.35	4.00	0.04

batch mode of ammonium source addition [20]. In [21], the effect of cultivation conditions (batch or chemostat) on the *Kluyveromyces marxianus* morphology was demonstrated. In a chemostat, the culture grew in the form of pseudohyphae, whereas it grew as yeastlike cells in ordinary batch culture. According to the published data [22, 23], nitrogen and carbon starvation resulted in a decrease in such key morphological indices as the length and thickness of hyphae and the hyphal growth unit of *Penicillium chrysogenum*, which points to hyphae fragmentation. Moreover, in the culture grown under nitrogen starvation conditions, an increase in the amount of empty sections in the hyphae was observed.

Hence, our microscopic examinations and investigations into the fatty acid composition demonstrated that the three-day culture of *M. circinelloides* var. *lusitanicus* INMI grown at the feed-batch mode of nitrogen supply was morphologically younger than the culture of the same age but grown after a single administration of the nitrogen source. The observed pronounced morphological changes were accompanied with changes in the content of γ-linolenic acid.

The data on the enzymatic activities of NAD-dependent IDH, MDH, and ICL associated with the TCA and glyoxylate cycles are shown in Table 1. In this phase of development of the culture (three days) grown after a single administration of the nitrogen source, the activity of IDH and MDH was lower than in the case of the feed-batch mode, whereas the activity of ICL was higher. It is known that glucose causes catabolite inactivation of ICL [24, 25]. This is a possible reason why the ICL activity was low in both experimental variants. In the paper [10], dealing with the oleaginous fungi *Mucor circinelloides* and *Mortierella alpina* grown under nitrogen limitation, it was demonstrated that the activity of NAD-dependent IDH was regulated by AMP concentrations, which decreased as a result of the increase in the AMP deaminase activity. As a consequence, the IDH activity decreased as well, causing a series of biochemical reactions resulting in lipid accumulation.

Hence, under nitrogen starvation conditions, the flow of carbon through the glyoxylate cycle increased, whereas the carbon flow through the TCA cycle decreased. Glucose induced repression of NAD-dependent enzymes, including NAD-dependent IDH [26]. When the nitrogen source was applied in a feed-batch mode, the functioning of the TCA cycle was more active, as demonstrated by the more intense IDH activity.

Figure 2 shows that, in the case of the intermittent application of the nitrogen source on the third day of incubation of *M. circinelloides* var. *lusitanicus*, depletion of nitrogen was not detected and, therefore, the activity of the TCA cycle enzymes, including MDH, remained unchanged. It is known from the literature [14] that the MDH activity affects the duration of the lipid synthesis in the course of fungal development. It seems likely that this enzyme is particularly important for the production of NADH in the cytoplasm and mitochondria. It is possible that the high lipid content determined in the biomass was due to this phenomenon.

Both NAD-dependent IDH and MDH are enzymes associated with the mitochondrial membranes. They are essential for cell respiration [27] and, therefore, for fatty acid desaturation. ICL is associated mainly with microsomes. The fatty acid composition of the cell fractions (microsomes and the mixed membrane/mitochondrial fraction) isolated from the mycelium grown at different modes of nitrogen supply is shown in Table 2. When the feed-batch mode was applied, the proportion of γ-linolenic acid was higher in both subcellular fractions then in the case of a single administration of the nitrogen source to the medium. In the microsome fraction, the unsaturation degree of the lipids was virtually the same in both variants due to the higher level of saturated fatty acids. In the mixed membrane/mitochondrial fraction of the mycelium grown in the feed-batch mode, both a high level of γ-linolenic acid and higher unsaturation of the lipids were detected. Formation of fatty acids, especially of γ-linolenic acid, is probably intensified under conditions favorable for the functioning of the enzymes of the TCA cycle, which provides the building blocks, coenzymes, and energy for biochemical synthesis.

Thus, the physiological state of the mycelium associated with the functioning of the main metabolic cycles (TCA and glyoxylate) has a significant effect on the synthesis of γ-linolenic acid. In the case of intermittent application of the nitrogen source, the culture remains physiologically young as compared to the culture grown after a single administration of the nitrogen source. After 72-h incubation, the glyoxylate cycle activity was higher in the case of single administration of the nitrogen source than in the case of feed-batch mode. In fungal cells, this phenomenon may be related to the processes of cell differentiation and conidia formation [7, 25, 28]. *M. circinelloides* var. *lusitanicus* does not form spores in liquid media; however, it produces arthrospores, and the mycelium undergoes pronounced morphological changes. It is probable that an increase in the activity of the glyoxylate cycle enzymes

Table 2. Fatty acid composition of total lipids of subcellular fractions of the mycelium of *M. circinelloides* var. *lusitanicus* INMI grown under various conditions of nitrogen supply (% of total fatty acids, 3 days)

FA	Membranes and mitochondria		Microsomes	
	Single addition of the nitrogen source, 2.0 g/l	Feed-batch mode of nitrogen addition, 2.0 g/l	Single addition of the nitrogen source, 2.0 g/l	Feed-batch mode of nitrogen addition, 2.0 g/l
$C_{14:0}$	3.33	2.15	3.10	3.03
$C_{15:0}$	0.56	0.68	1.90	Traces
$C_{16:0}$	23.79	23.55	28.52	36.51
$C_{16:1}$	9.94	1.57	9.47	Traces
$C_{18:0}$	0.85	0.34	0.56	Traces
$C_{18:1}$	24.07	27.65	23.06	20.71
$C_{18:2}$	15.17	11.43	9.88	8.09
γ-$C_{18:3}$	22.12	32.94	24.26	31.35
Unsaturation degree, Δ/100 molecules	130.71	150.90	128.37	130.94

may be considered an index of the degree of cell differentiation in fungi.

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