ISSN 0003-6838, Applied Biochemistry and Microbiology, 2015, Vol. 51, No. 2, pp. 242–248. © Pleiades Publishing, Inc., 2015.
Original Russian Text © E.G. Dedyukhina, T.I. Chistyakova, A.A. Mironov, S.V. Kamzolova, I.G. Minkevich, M.B. Vainshtein, 2015, published in Prikladnaya Biokhimiya i Mikrobiologiya, 2015, Vol. 51, No. 2, pp. 243–250.

The Effect of pH, Aeration, and Temperature on Arachidonic Acid Synthesis by *Mortierella alpina*

E. G. Dedyukhina[a], T. I. Chistyakova[a], A. A. Mironov[a, b], S. V. Kamzolova[a], I. G. Minkevich[a], and M. B. Vainshtein[a, b]

[a] *Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Moscow oblast, 142290 Russia*

[b] *Pushchino State Institute of Natural Sciences, Pushchino, Moscow oblast, 142290 Russia*

e-mail: dedeg@rambler.ru

Received May 22, 2014

Abstract—The effects of pH, aeration, and temperature on the growth of fungal strain *Mortierella alpina* LPM-301 and the synthesis of lipids and arachidonic acid in glycerol-containing medium were studied. Arachidonic acid production in the stationary growth phase was found to depend considerably on the pH value; it reached the optimum at pH 6.0 and was irreversibly inhibited at a pH of 3.0. The pO_2 values in a range from 10 to 50% showed no marked effect on mycelium growth or the synthesis of lipids and arachidonic acid. The temperature optimum for arachidonic acid production was 20–22°C. Under continuous cultivation, the amount of arachidonic acid reached 29.8% of lipids and 7.4% of biomass. The arachidonic acid yield from the glycerol consumed was 4.1% by mass and 8.8% by energy. It is suggested that arachidonic acid synthesis at an unfavorable pH and elevated temperatures was limited by the activity of Δ-12-desaturase and by the conversion of linoleic to arachidonic acid, respectively.

Keywords: arachidonic acid, biosynthesis, *Mortierella alpina*

DOI: 10.1134/S0003683815020040

Arachidonic acid (5,8,11,14-*cis*-eicosatetraenoic acid, **AA**) belongs to the ω-6 group of polyunsaturated fatty acids (**PUFA**). Because of its unique biological properties, it is widely used in agriculture as an elicitor of plant immunity to phytopathogens, in the food industry as an important component of dietary nutrition and infant formula, and in medicine [1–6]. The limited supply of natural AA sources (animal liver, adrenal glands, and egg yolk) dictated the necessity to develop microbiological AA production with the use of highly effective producer strains and low-cost renewable carbon substrates.

A microbiological method was previously developed for the selection of AA-producing strains of micromycetes. It is based on the application of acetylsalicylic acid as an inhibitor of AA metabolism [7]. Strains of *Mortierella alpina* were selected as active AA producers [8]; the AA production by these strains grown on glucose-containing medium under batch and continuous cultivation was investigated [9, 10]. It was shown that efficiency of microbiological processes using carbon substrates with different energy capacities can be evaluated on the basis of the material and energy balance of microbial growth [11]. A direct correlation between the lipid content, elemental composition, and energy capacity of the microbial biomass was established [12].

In recent years, glycerol has been applied as a promising low-cost and renewable substrate for microbiological processes [13–17]. Pure glycerol is produced by petrochemical synthesis, whereas raw glycerol is formed as a by-product of biodiesel production [18]. It was shown that micromycetes of the genus *Mortierella* were able to produce AA when grown on pure glycerol under submerged cultivation [19, 20] or in the course of hard-phase fermentation on oatmeal supplemented with 1–4% of glycerol [21, 22]. We have found that selected strains of *M. alpina* are able to use glycerol-containing by-products of the biodiesel industry as the sole source of carbon and energy for growth and AA synthesis [23].

According to the literature data, temperature is the factor that has the greatest influence on the synthesis of unsaturated fatty acids in microorganisms [3, 24–30]. It is considered that an increase in the unsaturation level of fatty acids in cytoplasmic membranes is an adaptive reaction of microorganisms to lowered environmental temperature [31]. Since fatty acid desaturation requires molecular oxygen, it can be assumed that the aeration level is essential for synthesis of unsaturated fatty acids in microorganisms [24, 32, 33]. It is believed that the pH usually showed no marked effect on lipid synthesis in eukaryotic microorganisms [3, 30]. There are few reports on an increase in PUFA synthesis in *M. raman-*

niana var *angulispora* at higher pH values [34]. No information is available on the effect of pH on AA synthesis in mycelial fungi.

The aim of this work was to study the effects of physical factors (pH, aeration, and temperature) on the growth of the previously selected, promising AA-producing strain *M. alpina* LPM-301, the lipid content, and AA synthesis under batch and continuous cultivation in medium containing pure glycerol as the carbon substrate.

MATERIALS AND METHODS

The study was carried out with strain *M. alpina* LPM-301, which was previously selected as an active AA producer [10].

The effect of pH on AA synthesis was studied under batch cultivation of producer in 750-mL flasks with medium (g/L): KNO_3, 1.5; K_2HPO_4, 1.5; KH_2PO_4, 0.7; $MgSO_4 \times 7H_2O$, 0.15; $CaCl_2 \times 6H_2O$, 0.12; yeast extract (Difco, United States), 5.0; trace elements (mg/L): $FeSO_4 \times 7H_2O$, 14.9; $MnSO_4 \times 4H_2O$, 0.2; $ZnSO_4 \times 7H_2O$, 8.1: $CuSO_4 \times 5H_2O$, 3.9. The initial glycerol concentration was 30 g/L; subsequent addition of glycerol was performed as required. Cultivation was performed on a shaker (180–200 rpm) at 28°C. A constant pH value was maintained by addition of 5% H_2SO_4. Fungi were grown at pH 6.0 for 7 days, then pH was adjusted to 3.0, 4.0, 5.0, 6.0, 7.0, or 8.0, and cultivation was continued for a further 7 days.

The effect of aeration on *M. alpina* LPM-301 growth, lipid synthesis, and AA synthesis was studied under batch cultivation in a 10-L ANKUM-2M fermenter (Russia) with a working volume of 7 L in medium (g/L): glycerol, 40; KNO_3, 1.5; KH_2PO_4, 2.0; $MgSO_4 \times 7H_2O$, 0.15; $CaCl_2 \times 6H_2O$, 0.12; yeast extract (Difco, United States), 5.0; trace elements (mg/L): $FeSO_4 \times 7H_2O$, 14.9; $MnSO_4 \times 4H_2O$, 0.2; $ZnSO_4 \times 7H_2O$, 8.1; $CuSO_4 \times 5H_2O$, 3.9. In the course of cultivation, the pH (6.0 ± 0.1) was maintained automatically by the addition of 5% H_2SO_4; the temperature was 28 ± 0.1°C. The concentration of dissolved oxygen (pO_2 of 5, 10, and 50% of saturation) was controlled by changing the air inflow rate. To avoid mycelium disruption, the agitation rate did not exceed 400 rpm.

The effect of temperature on fungal growth, lipid synthesis, and AA synthesis was studied under continuous cultivation of producer in the aforementioned conditions. The medium was added into a fermenter at a rate of 40 mL/h; the culture broth (1 L) was withdrawn when the medium volume in a fermenter reached 7 L. The dilution rate and correspondingly specific growth rate in the cycle between the samplings varied from 0.0067 to 0.0057 h^{-1}. The culture was grown under batch cultivation at 28°C for 3 days before the medium input was switched on. The establishment of steady-state conditions was determined from the constant concentration of residual glycerol. The values of pH (6.0 ± 0.1) and pO_2 (20–50% of saturation) were maintained automatically.

To determine biomass, the mycelium was separated from the culture broth by filtration through a paper filter and dried at 105°C to a constant weight. Glycerol was analyzed by gas-liquid chromatography (**GLC**) on a Chrom-5 chromatograph (Czech Republic) with a column (200 × 0.3 cm) packed with 15% Reoplex-400 on Chromaton N-AW (0.16–0.20 mm) under isothermal regime (200°C); argon was used as a carrier gas. Glycerol concentration was estimated by using a calibration curve.

To determine the fatty acid composition of lipids, the mycelium was vacuum-dried at 70°C to a constant weight and subjected to acid methanolysis [35]. The fatty acid methyl esters were analyzed by GLC and identified using the standard mixtures (Serva, Germany). The GLC conditions were the same as for glycerol analysis. The lipid content was calculated as the sum of fatty acids; heptadecanoic acid was used as an internal standard.

The mycelium yield by mass from the glycerol consumed ($Y_{X/S}$, %) was calculated as follows:

$$Y_{X/S} = (X/S) \times 100,$$

where X is biomass, g/L, and S is the glycerol consumed, g/L.

The lipid yield by mass from the glycerol consumed ($Y_{L/S}$, %) was calculated as follows:

$$Y_{L/S} = (L/S) \times 100,$$

where L is lipids, g/L, and S is glycerol consumed, g/L.

Biomass yield by energy from the glycerol consumed ($\eta_{X/S}$, %) was calculated as follows:

$$\eta_{X/S} = (Q_B/Q_S) Y_{X/S},$$

where Q_B is energy capacity of biomass, kJ/g, and Q_S is the energy capacity of glycerol, which is taken to be 17.96 kJ/g [11].

The biomass energy capacity (Q_B, kJ/g) was calculated as follows:

$$Q_B = 15.1 + 0.28 f_L,$$

where f_L is lipid content of biomass, %.

The lipid yield by energy from the glycerol consumed (η_L, %) was calculated by follows:

$$\eta_L = (Q_L/Q_S) Y_{L/X,}$$

where Q_L is energy capacity of lipids, which is taken to be 42.56 kJ/g [12].

Similarly, the AA yields by both mass and energy from glycerol consumed were calculated. The energy capacity of AA is taken to be 38.72 kJ/g [12].

RESULTS AND DISCUSSION

The effect of pH on AA synthesis was studied in the stationary phase of *M. alpina* LPM-301 growth. The strain was grown at a pH of 6.0 until the beginning of the stationary phase (7 days), and then the pH was main-

Table 1. Effect of pH in the stationary growth phase on biomass yield and the amounts of lipids and arachidonic acid in *M. alpina* LPM-301

Parameters	pH					
	3.0	4.0	5.0	6.0	7.0	8.0
Biomass, g/L	13.3	16.6	18.7	18.8	20.2	21.2
Mycelium yield by mass, % ($Y_{X/S}$)	28.7	25.5	25.5	25.9	24.3	26.2
Energy capacity of biomass (Q_B)	20.3	22.6	24.7	24.9	24.7	21.2
Biomass yield by energy, % ($\eta_{X/S}$)	32.5	32.1	35.2	35.9	33.4	31.0
Lipids, % of biomass	18.7	26.7	34.4	35.0	34.2	21.9
Lipids, g/L	2.5	4.4	6.4	6.6	6.9	4.6
Lipid yield by mass, % ($Y_{L/S}$)	5.4	6.8	8.8	9.1	8.3	5.7
Lipid yield by energy, % ($\eta_{L/S}$)	12.8	16.1	20.8	21.5	19.7	13.5
AA, g/L	0	0.5	1.4	1.8	1.7	1.1
AA, % of biomass	0	2.9	7.5	9.6	8.6	5.1
AA yield by mass, % ($Y_{AK/S}$)	0	0.7	1.9	2.5	2.1	1.3
AA yield by energy, % ($\eta_{AK/S}$)	0	1.6	4.1	5.3	4.5	2.9

tained at a level of 3.0, 4.0, 5.0, 6.0, 7.0, or 8.0 in the course of the stationary phase (from 7 to 14 days). The data on the effect of pH on the accumulation of biomass and lipids, as well as on yields of biomass, lipids, and AA from glycerol consumed in the stationary phase of *M. alpina* LPM-301 growth, are given in Table 1. Biomass increased with increasing pH value and reached 21.2 g/L at pH 8.0; the lipid biomass content reached the maximum (34.2–35.0%) at a pH of 5.0–7.0 and sharply decreased with pH changing to both acidic and alkaline values. The mycelium yield by mass ($Y_{X/S}$) was the highest (28.7%) at a pH of 3.0, which can be explained by negligible glycerol consumption in the stationary phase at an unfavorable pH value, although the biomass level did not decrease under these conditions. The energy capacity of biomass varied from 20.3 to 24.9 kJ/g and was the highest at a pH of 5.0–7.0, which coincided with the maximum biomass lipid content. The maximum mycelium yield by energy (35.2–35.9%) was observed at a pH of 5.0–6.0.

The lipid yield by mass ($Y_{L/S}$) reached the maximum (9.1%) at a pH of 6.0, slightly decreased at a pH of 5.0 and 7.0 (8.8 and 8.3%, respectively), and dropped to 5.4% at a pH of 3.0. It was previously found that the maximum lipid yield by mass for this strain grown on pure glycerol was 9.0% [20]. These values are comparable with those (6–15%) obtained for fungi *M. isabellina*, *Cunninghamella echinulata*, and *Zygorhynchus moelleri* grown in media with raw glycerol [15, 16, 36]. Theoretical calculations based on biochemical reactions of lipid synthesis revealed that the maximal possible lipid yield by mass from glycerol comprised 30% [16, 37, 38]. The observed low efficiency of glycerol conversion into lipids in *Zygomycetes* may be due to the imbalance in the regulation of enzymes involved in primary reactions of glycerol assimilation (glycerol kinase and 3-P-glycerol dehydrogenase) [36]. Since the energy capacity of microbial lipids was 2.4-fold higher than that of glycerol, the maximum lipid yield by energy, which characterizes the portion of energy passing from glycerol into lipids, was higher than the lipid yield by mass and reached 21.5% in *M. alpina* at a pH of 6.0 (Table 1).

As seen from Table 2, the lipids contained C_{14}–C_{22} acids with a prevalence of palmitic, stearic, oleic, linoleic acids, and AA. It should be noted that the amount of direct precursors of AA, γ-linolenic (γ-$C_{18:3}$ *n*-6) and dihomo-γ-linolenic ($C_{20:3}$ *n*-6) acids, remained low in all variants and did not exceed 2.2–3.1%, which can be explained by a rapid conversion of these acids into AA. The AA content of lipid was the maximum (27.3%) at a pH of 6.0, decreased to 23.3% at a pH of 8.0, and sharply dropped at medium acidifying. The AA synthesis was completely inhibited at a pH of 3.0 and was not restored after subsequent alkalization of medium to a pH of 6.0. It should be noted that the pH-dependent decrease in the AA level was accompanied by a decrease in the amount of linoleic acid and by an increase in the oleic acid content; therefore, it can be assumed that the conversion of oleic acid to linoleic acid was a limiting reaction in the AA synthesis. It is known that activities of desaturases involved in the synthesis of unsaturated fatty acids can be evaluated by measuring the ratio between fatty acids, which serve as the enzyme product and substrate [37]. In particular, the ratio $C_{18:2}/C_{18:1}$ characterizes the activity of Δ-12-desaturase, which is involved in the conversion of oleic into linoleic acid. In our experiments, the ratio $C_{18:2}/C_{18:1}$ completely correlated with a change in the AA content of lipids (Fig. 1). Thus, it can be suggested that the activity (or synthesis) of Δ-12-desaturase is inhibited under acidic

Table 2. Effect of pH in the stationary growth phase on the fatty acid composition (% of lipids) of *M. alpina* LPM-301

Fatty acids	pH					
	3.0	4.0	5.0	6.0	7.0	8.0
C_{14}	1.8	1.3	0.7	0.6	0.5	0.7
C_{15}	0.3	0.3	0.2	0.2	0.2	0.1
C_{16}	33.5	25.3	19.1	17.5	19.8	18.0
C_{18}	18.9	14.2	10.7	10.1	6.9	8.7
$C_{18:1}$	36.3	31.0	24.4	19.9	24.4	30.2
$C_{18:2}$	4.8	11.4	16.5	17.0	16.4	12.9
γ-$C_{18:3}$	0	1.8	2.6	3.1	2.7	2.4
C_{20}	1.4	0.8	0.5	0.5	0.4	0.4
$C_{20:1}$	1.9	1.2	0.7	0.5	0.5	0.7
$C_{20:2}$	0.1	0.4	0.6	0.7	0.6	0.4
$C_{20:3}$	0.1	0.8	1.8	2.2	1.9	1.6
$C_{20:4}$	0	10.9	21.7	27.3	25.3	23.3
C_{22}	0.8	0.6	0.5	0.4	0.4	0.5

conditions; this process is irreversible, since AA synthesis is not recovered after subsequent alkalization of the medium.

AA production reached the maximum (9.6% of biomass; 1.8 g/L) at a pH of 6.0 (Table 1). The AA yields by mass and energy were the highest (2.5 and 5.3%, respectively) at a pH of 6.0 and decreased with pH alteration; these changes were mostly pronounced under the medium acidification.

It is known that pH usually shows no considerable effect on fatty acid composition of lipids in eukaryotic microorganisms [3, 30]. However, there is information that the level of unsaturated fatty acids in lipids of *M. ramanniana* var. *angulispora* increased at higher pH values [34]. No data on the effect of pH on AA synthesis are available in literature. This study indicates that pH can be considered as a factor controlling the AA synthesis in mycelial fungi.

The effect of aeration (pO_2 of 5, 10, and 50% of saturation) on the growth of *M. alpina* LPM-301 and lipid and AA synthesis was studied under batch cultivation in a fermenter in glycerol-containing medium at 28°C with a pH of 6.0. It was found that pO_2 in a range from 10 to 50% of saturation had no considerable effect on biomass accumulation (8.7–9.8 g/L), biomass lipid content (23.8–23.9%), or the AA level of lipid (17.6–18.0 %). The growth limitation by oxygen supply was observed only at a pO_2 of 5%; under these conditions, biomass was decreased by 2.5 times.

The literature data on the effect of aeration on synthesis of unsaturated fatty acids in microorganisms are rather contradictory. It was revealed that the oxygen supply had no marked effect on synthesis of AA and other PUFA by fungi *Entomophthora exitalis* [24]. However, there is information that an increase in the oxygen concentration in the gas mixture up to 25% promoted a 60% increase in the AA amount per 1 L of medium in the case of *M. alpina* [32]. It should be noted that the application of this approach for industrial AA production needs economic estimation, since enrichment of the gas mixture with oxygen requires additional financial expenditures. The studies carried out with oleaginous yeast *Trichosporon pullulans* showed that the oxygen requirement of cells depended considerably on the concentration of iron ions in the medium and sharply increased under iron deficiency, possibly because of the impairment of cytochrome synthesis under these conditions [39]. The inconsistency of the literature data concerning the effect of

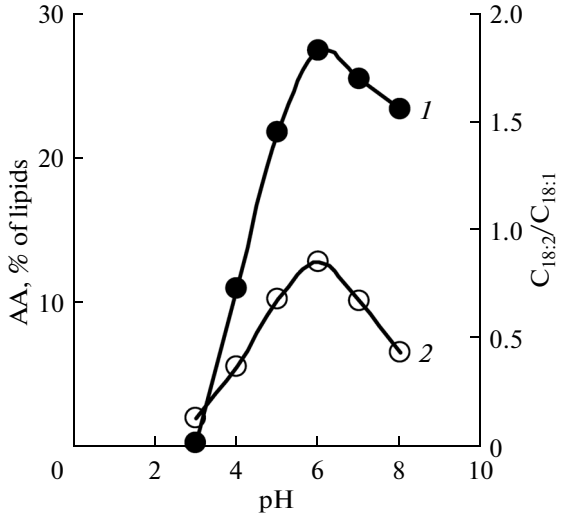

Fig. 1. Effect of pH on changes in (*1*) arachidonic acid level and (*2*) a ratio of $C_{18:2}/C_{18:1}$ acids in lipids of *M. alpina* LPM-301.

Table 3. Effect of temperature on the growth parameters and yields of lipids and arachidonic acid in *M. alpina* LPM-301 under continuous cultivation

Parameters	Temperature, °C				
	20	22	24	26	28
Biomass, g/L	11.1	13.2	14.1	17.4	8.4
Mycelium yield by mass, % ($Y_{X/S}$)	55.5	50.3	49.9	45.0	47.5
Energy capacity of biomass (Q_B)	22.2	21.9	22.5	23.1	20.9
Biomass yield by energy, % ($h_{X/S}$)	68.7	61.3	62.6	57.7	55.3
Lipids, % of biomass	25.4	24.3	26.5	28.4	20.8
Lipids, g/L	2.8	3.2	3.7	4.9	1.8
Lipid yield by mass,% ($Y_{L/S}$)	14.1	12.2	13.2	12.8	9.9
Lipid yield by energy, % ($\eta_{L/S}$)	33.4	28.9	31.3	30.3	23.4
AA, g/L	0.8	0.9	1.0	1.1	0.4
AA, % of biomass	7.4	7.2	6.8	6.6	4.3
AA yield by mass, % ($Y_{AK/S}$)	4.1	3.6	3.4	2.9	2.0
AA yield by energy, % ($\eta_{AK/S}$)	8.8	7.8	7.3	6.4	4.4

Table 4. Effect of temperature on the amounts of arachidonic acid and its precursors (% of lipids) in *M. alpina* LPM-301 under continuous cultivation

Acids	Temperature, °C				
	20	22	24	26	28
C_{18}	13.4	14.3	14.3	12.8	15.6
$C_{18:1}$	21.5	14.2	15.1	18.6	18.4
$C_{18:2}$	11.2	12.8	14.8	16.7	19.1
γ-$C_{18:3}$	3.6	3.9	3.3	2.6	4.8
$C_{20:3}$	1.9	4.8	4.3	2.6	2.2
$C_{20:4}$	29.3	29.8	25.8	23.1	20.6

aeration on the PUFA synthesis may be possibly due to the use of nonoptimal media and to the limited availability of iron ions.

The effect of temperature on the growth of *M. alpina* LPM-301 and the synthesis of lipids and AA was studied under continuous cultivation in glycerol-containing medium at low growth rates (0.006–0.007 h^{-1}), pH 6.0, and pO_2 of 20–50% of saturation. The optimum temperature for both mycelium growth and lipid synthesis was found to be 26°C (Table 3). Under these conditions, biomass accumulation was the highest (17.4 g/L) and decreased with a temperature shift to 20 and 28°C (11.1 and 8.4 g/L, respectively). The lipid content of biomass was the maximum (28.4%) at 26°C and decreased to 24.3–25.4% at a lower temperature (20–22°C) and to 20.8% at 28°C.

It is known that lowering of the cultivation temperature usually stimulated synthesis of unsaturated fatty acids in mycelial fungi [3, 24, 40, 41]. In our experiments, the AA content of lipids was the maximum (29.3–29.8%) at 20–22°C and decreased to 20.6% at temperature elevation to 28°C (Table 4).

In contrast to the effect of pH on fatty acid composition of lipids, a temperature change from the optimal level was accompanied by an inverse correlation between the amounts of linoleic acid and AA in fungal lipids; the level of linoleic acid gradually increased from 11.2 to 19.1% with temperature elevation from 20 to 28°C (Table 4). It can be assumed that AA synthesis under temperature elevation was limited by the conversion of linolieic acid into AA. As seen from Fig. 2, the ratio of acids AA/$C_{18:2}$ correlated with a change in the AA content of lipids. At the same time, the amounts of γ-$C_{18:3}$ and $C_{20:3}$ acids, direct precursors of AA, were low in all variants, which can be explained by their rapid conversion into end products. The data on the effect of temperature on growth of *M. alpina* LPM-301 and yields of lipids and AA are

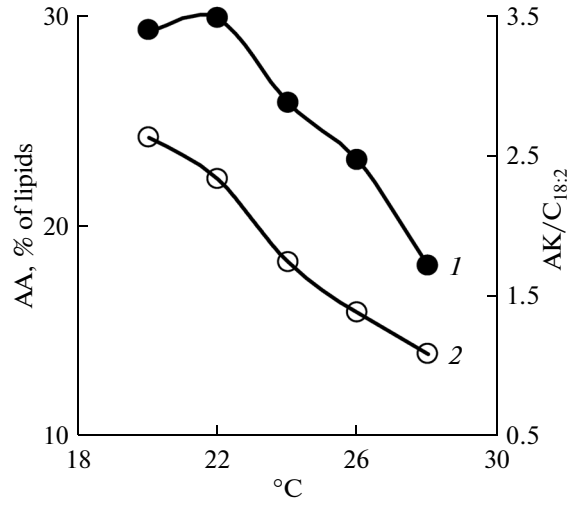

Fig. 2 Effect of cultivation temperature on (*1*) arachidonic acid content and (*2*) a ratio of AA/$C_{18:2}$ acids in lipids of *M. alpina* LPM-301.

given in Table 3. It should be noted that the biomass yields from glycerol consumed both by mass (45.0–55.0%) and by energy (55.3–68.7%) in all variants were almost twofold higher under the exponential growth of fungi than those under batch cultivation (Table 1). Under continuous cultivation, the highest lipid yields from glycerol consumed both by mass (14.1%) and by energy (33.4%) were observed at 20°C (Table 4), whereas the lipid yields by mass and energy (9.9 and 23.4%, respectively) at 28°C coincided with those revealed under batch cultivation (28°C, pH 6.0). The AA yields from glycerol consumed both by mass and energy reached the maximum (3.6–4.1 and 7.8–8.8%, respectively) at 20–22°C.

Thus, continuous cultivation of *M. alpina* LPM-301 in glycerol-containing medium under growth limitation by nitrogen at optimal values of pH (6.0) and temperature (20–22°C) ensured active AA synthesis (29.3–29.8% of lipid and 7.2–7.4% of biomass) with an AA yield from consumed glycerol of 3.6–4.1% by mass and 7.8–8.8% by energy. We have previously shown that the level of AA in lipids was increased to 43.5% with increasing glycerol concentration in the medium up to 80 g/L under batch cultivation of this strain [20]. This approach can be applied for continuous cultivation of a producer, although, in this case, a decrease in the AA yield from consumed glycerol is possible. It should be noted in this connection that a method for a 1.6- to 2-fold increase in the AA content of lipids (up to 57%) has been developed via the incubation of filtered *M. alpina* mycelium at room temperature for 7 days [23].

Thus, based on the results obtained, it can be concluded that pH and temperature are effective factors controlling the AA synthesis in mycelial fungi. Values of pO_2 in a range from 10 to 50% of saturation showed no marked effect on *M. alpina* growth and synthesis of lipids and AA. Continuous cultivation of a producer can be recommended for microbiological AA production.

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Translated by E.G. Dedyukhina