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The Effect of pH and Temperature on Arachidonic Acid Production by Glycerol-Grown *Mortierella alpina* NRRL-A-10995

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Abstract: Arachidonic acid (AA) has a wide range of applications in medicine, pharmacology, diet, infant nutrition, and agriculture, due to its unique biological properties. The microbiological processes involved in AA production usually require carbohydrate substrates. In this paper, we propose a method for AA production from glycerol, an inexpensive and renewable carbon substrate that is produced by the fungal strain, *Mortierella alpina* NRRL-A-10995. Our experimental results showed that the optimum pH values required for fungal growth and the production of lipids and AA were different and depended on the growth phase of the fungus. The AA production was shown to be extremely sensitive to acidic pH values and was completely inhibited at a pH of 3.0. The optimum temperature for AA production was 20–22 °C. Continuous cultivation of *M. alpina* occurred in a glycerol-containing medium, and growth limitations were implemented through the addition of nitrogen and the selection of optimal conditions (pH 6.0, 20 °C). This ensured that active AA production occurred (25.2% of lipids and 3.1% of biomass), with the product yield from the consumed glycerol being 1.6% by mass and 3.4% by energy.

Keywords: fungus *Mortierella alpina*; optimization; temperature; pH of medium; growth; arachidonic acid (AA)

1. Introduction

Arachidonic acid (20:4, 5,8,11,14-*cis*-eicosatetraenoic acid, AA) belongs to the ω-6 group of polyunsaturated fatty acids (PUFAs), and plays an important role in metabolic processes as a precursor to prostaglandins, leukotrienes, and a number of eicosanoids. AA is widely used as a pharmaceutical precursor and as a component of infant formula and dietary supplements due to its unique biological properties [1–3]. In recent years, the role of AA as an inductor (elicitor) of protective functions in plants has been studied in detail; the application of AA in very low concentrations has been shown to increase a plant's resistance to bacterial and fungal pathogens [4,5].

The main natural sources of AA are animal liver, adrenal glands, and egg yolk; however, the quantity of AA is so low that these sources cannot fulfill the growing requirements of this physiologically active acid. The limited supply of natural sources of AA necessitated the development of microbiological AA production with the use of highly effective strains–producers.

At present, microbial methods of AA production with the use of various strains of fungi belonging to the genus *Mortierella* have been developed in Europe, China, Japan, and the United States

(US) [3,6–13]. In these processes, carbohydrates are usually used as carbon substrates. However, the high production cost of the AA-containing microbial lipids led to an increased interest in the development of physiological methods which would optimize the fermentation processes through the use of highly effective strains–producers and inexpensive renewable carbon substrates.

In recent years, glycerol has been considered as a promising low-cost and renewable substrate for microbiological processes. Pure glycerol is produced by petrochemical synthesis, whereas raw glycerol is formed in large amounts as a by-product of biodiesel production through the transesterification of plant oils or animal fats with short-chain alcohols (principally methanol and, to a lesser extent, ethanol or butanol), in the presence of NaOH or KOH. As reported, the EU is the world's largest biodiesel producer, with a production capacity of 24.9 billion liters in 2016, which is expected to increase to 25.5 billion liters by 2017. Some of the major biodiesel-producing countries include the US, Brazil, Germany, France, Argentina, the Netherlands, and Indonesia [14]. Stoichiometrically, 10 wt % of glycerol is formed by this process. The current price of biodiesel-derived glycerol varied from US $0.06 to US $0.11 per pound [15]. On the basis of microorganisms grown on biodiesel-derived waste glycerol, researchers developed the method of production of 1,3-propanediol [16], biomass-enriched lipids [17–21], carbonic acids (in particular, pyruvic acid [22]), α-ketoglutaric acid [23,24], citric acid [16,25,26], and butanol [27]. However, few reports showed the satisfactory growth of fungi of the genera *Mucor*, *Cunninghamella*, *Thamnidium*, *Zygorhynchus*, and *Mortierella*, and similarly for lipid synthesis when cultivated on glycerol [15–19,21,28–33]. We have previously shown that both pure glycerol and glycerol-containing wastes of biodiesel production can be successfully used for AA synthesis by selected *M. alpina* strains [20,29]. It should be noted that the production of AA from glycerol—in contrast to that from glucose—is still limited by the lack of basic knowledge about the fermentation conditions that are essential for the overproduction of AA. Our previous study showed that the AA synthesis by the fungal strain, *M. alpina* LPM-301, was very sensitive to pH and was completely inhibited at a pH of 3.0 [33]. The question arose as to whether the extremely high sensitivity of the process of AA synthesis to acidic pH values is a specific feature of the individual fungal strain, or represents a common property of the AA producers.

The aim of this work was to study the effects of pH and temperature on the synthesis of lipids and AA by the fungal strain, *M. alpina* NRRL-A-10995, when it is grown on glycerol under nitrogen limitations.

2. Materials and Methods

2.1. Microorganisms and Chemicals

The study was carried out with the previously selected strain, *M. alpina* NRRL-A-10995, which was obtained from the ARS Culture Collection, United States [34,35].

All chemicals were of analytical grade (Mosreactiv, Moscow, Russia).

2.2. Media and Cultivation Conditions

The effect of pH on the growth of *M. alpina* NRRL-A-10995 and the synthesis of lipids and AA was studied during the batch cultivation of the producers in 750 mL flasks with 100 mL of medium containing 1.5 g L^{-1} of KNO_3, 2.0 g L^{-1} of KH_2PO_4, 0.15 g L^{-1} of $MgSO_4 \times 7H_2O$, 0.12 g L^{-1} of $CaCl_2 \times 6H_2O$, and 5.0 g L^{-1} of Difco yeast extract (BD Life Sciences, San Jose, CA, USA). The medium also contained trace elements, including 14.9 mg L^{-1} of $FeSO_4 \times 7H_2O$, 0.2 mg L^{-1} of $MnSO_4 \times 4H_2O$, 8.1 mg L^{-1} of $ZnSO_4 \times 7H_2O$, and 3.9 mg L^{-1} of $CuSO_4 \times 5H_2O$. The initial glycerol concentration was 30 g L^{-1}; subsequent addition of glycerol was performed as required. Cultivation was performed on a shaker (180–200 rpm) at 24 °C. The constant pH value was maintained through a daily addition of 5% H_2SO_4 or 5% NaOH. Fungi were grown at a pH of 6.0 for 4 days, then pH was adjusted to 3.0, 4.0, 5.0, 6.0, 7.0, or 8.0, and cultivation was continued for 7 and 14 days.

The effect of temperature on fungal growth, lipid synthesis, and AA production was studied during the continuous cultivation of the producer in a 2 L fermenter (KF-108 fermentation plant, Pushchino, Russia) with a working volume of 0.75 L. The medium contained 60 g L^{-1} of glycerol, 1.0 g L^{-1} of KNO_3, 2.0 g L^{-1} of KH_2PO_4, 0.15 g L^{-1} of $MgSO_4 \times 7H_2O$, 0.12 g L^{-1} of $CaCl_2 \times 6H_2O$, 5.0 g L^{-1} of Difco yeast extract, and trace elements, including 14.9 mg L^{-1} of $FeSO_4 \times 7H_2O$, 0.2 mg L^{-1} of $MnSO_4 \times 4H_2O$, 8.1 mg L^{-1} of $ZnSO_4 \times 7H_2O$, and 3.9 mg L^{-1} of $CuSO_4 \times 5H_2O$. The medium was added into a fermenter continuously at a rate of 5 mL h^{-1}; 120 mL of the culture broth was withdrawn when the medium volume in the fermenter reached 750 mL. The dilution rate and, correspondingly, the specific growth rate in the fermentation cycle between the samplings varied from 0.0079 to 0.0067 h^{-1}. At the beginning, the culture was grown through batch cultivation at 24 °C for 4 days, and then the medium input was switched on. The temperature (20, 22, 24, and 26, ± 0.1 °C) was maintained automatically. The values of pH (6.0 ± 0.1) and pO_2 (10–50% of saturation) were maintained automatically. The establishment of the steady-state condition was determined from the constant concentration of the residual glycerol. Experiments were repeated at the same dilution rates in a 5 L stirred-tank reactor (BIOSTAT B-PLUS, Sartorius, Göttingen, Germany) with a working volume of 2 L.

2.3. Assays

Biomass was determined gravimetrically; the culture broth was filtered through a paper filter; collected mycelium was washed with distilled water and dried at 105 °C to a constant weight. Glycerol was analyzed by gas-liquid chromatography (GLC) on a Chrom-5 chromatograph (Czech Republic) using a column (200 × 0.3 cm) packed with 15% Reoplex-400 on Chromaton N-AW (0.16–0.20 mm) under an isothermal regime (200 °C); argon was used as a carrier gas. The glycerol concentration was estimated by using a calibration curve.

To determine the fatty acid composition of lipids, mycelium was vacuum-dried at 70 °C to constant weight and subjected to direct transesterification at 80 °C for 3 h in a mixture of methanol:hydrochloric acid:chloroform (10:1:1 *v*/*v*), which was supplemented with heptadecanoic acid as an internal standard [36]. The resulting methyl esters of fatty acids (MEFAs) were thrice extracted from the reaction mixture with n-hexane, dried over sodium sulfate, evaporated on a vacuum evaporator, and analyzed by GLC. The identification of MEFAs was performed with the use of standard fatty acid mixtures (Serva, Heidelberg, Germany). The conditions of GLC were the same as those of glycerol analysis. The lipid content was calculated as the sum of fatty acids.

2.4. Calculations

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

$Y_{X/S}$ = X/S 100, where X is the biomass (g L^{-1}) and S is the glycerol consumed (g L^{-1}).

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

$Y_{L/S}$ = L/S 100, where L is the lipids (g L^{-1}) and S is the glycerol consumed (g L^{-1}).

The energy yield of the biomass from glycerol consumed (η_B, %) was calculated as follows:

$\eta_B = (Q_B/Q_S)\ Y_{X/S}$, where Q_B is the energy capacity of biomass (kJ g^{-1}), Q_S is the energy capacity of glycerol (kJ g^{-1}), and $Y_{X/S}$ is the mass yield of the biomass from the glycerol consumed (%). The energy capacity of glycerol (Q_S) was taken as 16.62 kJ g^{-1} [37].

The energy capacity of biomass (Q_B, kJ g^{-1}) was calculated as follows [37]:

Q_B = (0.3046 L) + 14.488, where L is the lipid content of the biomass (%).

The energy yield of lipids from the glycerol consumed ($\eta_{L/X}$, %) was calculated as follows:

$\eta_{L/X} = (Q_L/Q_S)\ Y_{L/S}$, where Q_L is the energy capacity of the lipids (kJ g^{-1}), Q_S is the energy capacity of glycerol (kJ g^{-1}), and $Y_{L/S}$ is the mass yield of the lipids from the glycerol consumed (%). The energy capacity of the lipids (Q_L) was taken as 38.42 kJ g^{-1} [37].

The yields of AA (both by mass and energy) were calculated in a similar way. The energy capacity of AA was taken as 38.78 kJ g^{-1} [37].

3. Results and Discussion

3.1. The Effect of pH

The effect of pH on the growth of *M. alpina* NRRL-A-10995 and the synthesis of lipids and AA was studied in the exponential and stationary phases. The strain, *M. alpina* NRRL-A-10995, was grown at pH 6.0 for 4 days; the pH was then adjusted to 3.0, 4.0, 5.0, 6.0, 7.0, or 8.0, and the cultivation was continued for 7 and 14 days (the exponential and the stationary phases, respectively). The required pH value was maintained by the daily addition of 5% H_2SO_4 or 5% NaOH into the culture broth.

The data on the effect of pH on the biomass accumulation, the lipids, and the production of AA, as well as the data on the yields of biomass, lipids, and AA from the glycerol consumed in the exponential phase of the growth of *M. alpina* NRRL-A-10995 are given in Table 1.

Table 1. The effect of pH on the yields of biomass, lipids, and arachidonic acid in *M. alpina* NRRL-A-10995 in the exponential growth phase.

Parameters	pH					
	3	**4**	**5**	**6**	**7**	**8**
Biomass (g L^{-1})	6.8	7.4	14.4	10.8	10.4	8.2
Lipids (% of biomass)	11.3	10.3	12.8	16.1	13.5	12.0
Lipids (g L^{-1})	0.77	0.76	1.76	1.74	1.40	0.98
Mycelium yield by mass ($Y_{X/S}$) (%)	33.3	33.0	37.8	38.8	37.9	30.3
Energy capacity of biomass (Q_B) (kJ g^{-1})	18.3	18.0	18.7	19.6	18.9	18.5
Mycelium yield by energy ($\eta_{X/S}$) (%)	33.9	33.0	39.4	42.3	39.8	31.2
Lipid yield by mass ($Y_{L/S}$) (%)	3.7	3.4	4.6	6.3	5.1	3.6
Lipid yield by energy ($\eta_{L/S}$) (%)	8.8	8.1	10.9	14.9	12.1	8.5
AA (% of lipids)	11.3	14.7	17.8	20.1	21.2	22.1
AA (g L^{-1})	1.27	1.52	2.27	3.24	2.87	2.64
AA yield by mass ($Y_{AA/S}$) (%)	0.086	0.112	0.320	0.230	0.290	0.217
AA yield by energy ($\eta_{AA/S}$) (%)	0.4	0.5	0.8	0.8	1.0	0.8

It was found that the optimal pH for *M. alpina* NRRL-A-10995 growth was 5.0 (biomass accumulation was 14.4 g L^{-1}). Over the pH range from 5.0–7.0, the biomass remained at a high level (10.4–10.8 g L^{-1}). When the pH increased to 8.0, the culture growth was inhibited and the biomass decreased to 8.2 g L^{-1}; when the pH dropped below 5.0, the growth was also strongly inhibited and at a pH of 3.0, the biomass accumulation was as low as 6.8 g L^{-1}.

The lipid content of biomass during the cultivation of *M. alpina* NRRL-A-10995 in glycerol-containing medium at different pH values varied between 10.3–16.1% and reached the maximum (16.1%) at pH 6.0. The cultivation at a pH of 7.0 and 8.0 resulted in a gradual decrease in the lipid amount, which comprised 13.5% and 12.0%, respectively. Acidic pH values (3.0–5.0) suppressed lipid synthesis; in this case, the lipid content of biomass did not exceed 12.8%. According to the data found in current literature, the lipid content of fungi grown on raw glycerol varied between 18.1–42.6% of biomass [16,31]; the maximum lipid amount (71.1%) occurred after *Thamnidium elegans* was cultivated on raw glycerol for 22 days [31]. It should be noted that these data were obtained for fungi, which were incapable of AA synthesis. There are scarce data on the lipid production by glycerol-grown AA producers. It was found that the lipid content of biomass in different *Mortierella* species grown on pure glycerol varied between 4.3–10.1% [38].

As seen from Table 1, the pH value showed no considerable effect on the mycelium yield by mass ($Y_{X/S}$) (30.3–38.8%). The energy capacity of biomass (Q_B) varied between 18.3–19.6 kJ g^{-1}; it was the highest at a pH of 6.0 and coincided with the maximum lipid content of biomass. The maximum mycelium yield by energy ($\eta_{X/S}$) (42.3%) was observed at a pH of 6.0. The lipid yield by mass ($Y_{L/S}$) reached the maximum (6.3%) at a pH of 6.0 and decreased under both acidic and alkaline conditions. Since the energy capacity of microbial lipids was 2.4-fold higher than that of glycerol, the maximum

lipid yield by energy ($\eta_{L/S}$), which characterizes the portion of energy passing from glycerol into lipids, was higher than the lipid yield by mass and reached the maximum (14.9%) at a pH of 6.0.

As seen from Table 1, the pH value showed considerable effect on the content of AA; it was the maximum (22.1%) at a pH of 8.0 and sharply dropped when the medium acidified. Over the pH range from 5.0–8.0, the AA accumulation remained at a high level (2.27–3.24% of biomass; 0.217–0.320 g L^{-1}). When the pH dropped below 5.0, the AA synthesis was strongly inhibited, and at a pH of 3.0, the AA accumulation was as low as 1.27% of biomass and 0.086 g L^{-1}. The AA yields by mass and energy were the highest (0.8–1.0 and 1.7–2.2%, respectively) at a pH range from 5.0–8.0 and decreased twofold at a pH of 3.0.

The data on the effect of pH on the accumulation of biomass, lipids, and AA, as well as the data on the yields of biomass, lipids, and AA from the glycerol consumed in the stationary phase of *M. alpina* NRRL-A-10995 are given in Table 2.

Table 2. The effect of pH on the yields of biomass, lipids, and arachidonic acid in *M. alpina* NRRL-A-10995 in the stationary phase.

Parameters	pH					
	3	**4**	**5**	**6**	**7**	**8**
Biomass (g L^{-1})	6.8	8.1	21.0	22.0	17.8	17.8
Lipids (% of biomass)	17.4	12.7	32.3	30.9	22.3	34.7
Lipids (g L^{-1})	18.1	18.0	18.3	19.0	19.4	18.7
Mycelium yield by mass ($Y_{X/S}$) (%)	17.5	12.7	32.9	32.6	24.6	36.1
Energy capacity of biomass (Q_B) (kJ g^{-1})	10.8	10.3	11.4	14.1	15.2	13.1
Mycelium yield by energy ($\eta_{X/S}$) (%)	0.73	0.83	2.39	3.10	2.70	2.33
Lipid yield by mass ($Y_{L/S}$) (%)	1.8	1.3	3.7	4.4	3.4	4.6
Lipid yield by energy ($\eta_{L/S}$) (%)	4.3	3.0	8.8	10.4	8.0	10.9
AA (% of lipids)	0	0.5	14.6	19.9	19.0	19.8
AA (g L^{-1})	0	0.13	1.72	2.83	2.67	2.65
AA yield by mass ($Y_{AA/S}$) (%)	0	0.008	0.367	0.622	0.516	0.463
AA yield by energy ($\eta_{AA/S}$) (%)	0	0.02	0.56	0.87	0.64	0.90

The most favorable pH for the mycelium growth was in a range of 5.0–6.0; biomass reached the maximum (21–22 g L^{-1}) on the 14th day of cultivation. Acidic pH values of 3.0 and 4.0 inhibited culture growth; on the 14th day, the biomass was 6.8 and 8.1 g L^{-1}, respectively, remaining almost at the same level as in the exponential phase (6.8 and 7.4 g L^{-1}, respectively) (Table 1). Considerable adaptation of the culture growth to an alkaline pH value (8.0) was observed; in the period from the exponential phase (7 days) to the stationary phase (14 days), biomass increased from 8.2 to 17.8 g L^{-1} (Tables 1 and 2).

The lipid content of biomass during the cultivation of *M. alpina* NRRL-A-10995 at different pH values varied in a range between 10.8–15.2% and reached the maximum at a pH of 7.0. Acidic pH values (3.0–5.0) somewhat suppressed lipid synthesis; the lipid content of biomass did not exceed 11.4% in the stationary phase (14 days). The mycelium yield by mass ($Y_{X/S}$) was the highest (30.9–32.3%) at a pH range of 5–6 and decreased under both acidic and alkaline conditions. The energy capacity of biomass varied from 18.1–19.4 kJ g^{-1} and correlated with the lipid content of biomass. The maximum mycelium yield by energy (32.6–32.9%) was observed at a pH range of 5.0–6.0. The lipid yield by mass ($Y_{L/S}$) reached the maximum (4.4%) at a pH of 6.0, slightly decreased at a pH of 5.0, 7.0, and 8.0 (3.7%, 3.4%, and 4.6%, respectively), and dropped to 1.8% and 1.3% at a pH of 3.0 and 4.0, respectively. The lipid yield by energy ($\eta_{L/S}$) reached the maximum (10.4%) at a pH of 6.0.

The AA synthesis was the maximum (19.0–19.9% of lipids; 2.65–2.83% of biomass; 0.463–0.622 g L^{-1}) at a pH range of 6.0–8.0, gradually decreased under acidic conditions, and was completely inhibited at a pH of 3.0. The AA yields by mass and energy were the highest (0.64–0.90% and 1.38–1.94%, respectively) at a pH range of 6.0–8.0 and decreased as the pH acidified.

As seen from Tables 3 and 4, the lipids of *M. alpina* NRRL-A-10995 contained saturated and unsaturated fatty acids, with the carbon chain length ranging from C_{14} to C_{20}.

Table 3. The effect of pH on the fatty acid composition (% of lipids) of *M. alpina* NRRL-A-10995 in the exponential phase.

Fatty Acids	pH					
	3	**4**	**5**	**6**	**7**	**8**
C_{14}	1.9	1.6	1.2	1.2	1.2	1.4
C_{15}	0.1	0.1	0	0.1	0.1	0.1
C_{16}	12.2	10.1	8.4	6.8	6.8	6.6
C_{18}	18.4	17.6	12.9	12.5	14.0	12.3
$C_{18:1}$	41.1	42.0	45.5	44.7	40.7	41.5
$C_{18:2}$	9.1	6.9	10.7	7.8	8.9	8.9
γ-$C_{18:3}$	4.9	5.6	0.3	5.6	5.4	6.1
C_{20}	0.4	0.4	3.1	0.3	0.2	0.3
$C_{20:1}$	0	0.3	0	0.4	0.3	0
$C_{20:2}$	0	0	0	0	0	0
$C_{20:3}$	0.6	0.7	0.0	0.6	1.2	0.7
$C_{20:4}$	11.3	14.7	17.8	20.1	21.2	22.1
$C_{20:4}/C_{18:2}$	1.24	2.13	1.66	2.57	2.38	2.48
$C_{18:2}/C_{18:1}$	0.22	0.16	0.23	0.17	0.21	0.21
$C_{18:1}/C_{18}$	2.23	2.38	3.52	3.57	2.90	3.37

Table 4. The effect of pH on the fatty acid composition (% of lipids) of *M. alpina* NRRL-A-10995 in the stationary phase.

Fatty Acids	pH					
	3	**4**	**5**	**6**	**7**	**8**
C_{14}	4.2	3.5	2.3	1.9	1.4	1.8
C_{15}	0.9	1.1	0.2	0.1	0.1	0.2
C_{16}	27.9	28.6	18.5	16.7	24.9	17.8
C_{18}	17.4	19.6	13.0	9.4	8.6	9.8
$C_{18:1}$	40.0	38.7	32.2	31.5	27.2	31.7
$C_{18:2}$	6.3	3.7	12.6	15.1	13.9	14.3
γ-$C_{18:3}$	1.6	trace	3.9	4.0	3.0	3.8
C_{20}	0.6	1.4	0.8	trace	trace	trace
$C_{20:1}$	trace	trace	trace	1.3	0.8	0.6
$C_{20:2}$	trace	trace	0.5	trace	trace	trace
$C_{20:3}$	1.03	2.4	1.3	0	1.2	0.3
$C_{20:4}$	0	0.5	14.6	19.9	19.0	19.8
$C_{20:4}/C_{18:2}$	0	0.14	1.16	1.32	1.37	1.38
$C_{18:2}/C_{18:1}$	0.16	0.09	0.39	0.48	0.51	0.45
$C_{18:1}/C_{18}$	2.30	1.97	2.48	3.35	3.16	3.23

The predominant fatty acids, besides AA, included oleic, palmitic, stearic, and linoleic acids, which varied in their quantities and their amount of AA synthesis, with their presumed quantities of AA varying in a range of 27.2–44.7%, 6.8–24.9%, 8.6–14.0%, and 7.8–15.1%, respectively. The AA content of lipids remained at a high level during cultivation at a pH of 6.0–8.0 in both the exponential and the stationary phases (20.1–22.1% and 19.0–19.9%, respectively). It should be noted that the amounts of direct AA precursors, gamma-linolenic (γ-$C_{18:3}$) and dihomo-gamma-linolenic ($C_{20:3}$) acids, remained low in all variants and did not exceed 2.4 and 6.1%, respectively. This can be explained by the rapid conversion of these acids into AA.

Literature data concerning the effect of the growth phase on the synthesis of polyunsaturated fatty acids (PUFAs) are contradictory. Taking into account the involvement of PUFAs in the function

of membranes, it was hypothesized that the biosynthesis of these acids should be associated with mycelial growth. However, this event was observed only in some Zygomycetes; e.g., *M. ramanniana*. On the contrary, the biosynthesis of PUFAs in *C. echinulata* continued after the cessation of growth, suggesting that the synthesis of PUFAs in this species is not a strictly growth-associated process [30]. We have previously found that the AA synthesis in the *M. alpina* strains, LPM-301 and NRRL-A-10995, depended considerably on the nature of growth-limiting components; AA synthesis reached a peak under growth limitations by glycerol and gradually decreased with the transition in culture to nitrogen limitations [20]. Therefore, it can be concluded that AA synthesis in these fungal strains is a growth-coupled process.

Information concerning the effect of pH on lipid production and PUFA synthesis in fungi is scarce; as a rule, the effect of pH had been tested only within a narrow range (from 5.5–7.0). It was shown that higher pH values increased the PUFA synthesis in *M. ramanniana var. angulispora* [39]. A two-stage pH control strategy was suggested to increase the AA production by the *M. alpina* mutant D20, in which the pH was maintained at 5.5 for the first 48 h and then shifted to 6.5 until the end of fermentation; using this strategy, the authors achieved the highest AA production from glucose (8.12 g L^{-1}), with a yield of 1.40 g $(L\ d)^{-1}$ [12].

The strong inhibition of AA synthesis in *M. alpina* NRRL-A-10995 under extremely acidic conditions (pH 3.0 and 4.0) is an observation in this study that is of great interest. Similar dependence has been previously demonstrated for another *M. alpina* strain (LPM 301); the AA synthesis in *M. alpina* LPM 301 was completely inhibited at a pH of 3.0 and was not restored after a subsequent pH adjustment to a pH of 6.0 [33]. Therefore, it can be suggested that this phenomenon is a common regularity associated with the inhibition of the activity (or synthesis) of enzymes involved in the AA synthesis under acidic conditions.

It is known that the 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 the substrate [32]. In particular, the ratio $C_{18:1}/C_{18}$ characterizes the activity of Δ-9-desaturase, which takes part in the conversion of stearic acid into oleic acid; 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; and the ratio $C_{18:2}/C_{20:4}$ characterizes the activity of a complex of enzymes responsible for the conversion of linoleic acid ($C_{18:2}$) into AA ($C_{20:4}$). As seen from Tables 3 and 4, the ratio $C_{18:1}/C_{18}$ correlates with a change in the AA content of lipids in the exponential and the stationary growth phase; under acidic conditions (pH 3.0–4.0), this ratio was decreased 1.4-fold, although it still remained at a rather high level; therefore, a change in the activity of Δ-9-desaturase could not be a reason for the inhibition of AA synthesis. At a pH of 3.0–4.0, the ratios $C_{18:2}/C_{18:1}$ and $C_{20:4}/C_{18:2}$ were decreased sharply in the stationary phase. It can be suggested that at low pH values, the activity (or synthesis) of Δ-12 desaturase and the complex of enzymes responsible for the conversion of linoleic acid ($C_{18:2}$) into AA ($C_{20:4}$) were inhibited, which resulted in the inhibition of AA synthesis under these conditions.

Thus, it was shown that the optimum pH values for the growth of *M. alpina* NRRL-A-10995, lipid accumulation, and AA synthesis were different and depended on the growth phase. In the course of a 7-day cultivation (exponential phase), the culture growth was the best at a pH of 5.0; lipid accumulation was the maximum at a pH of 6.0, whereas the AA content of lipids was the highest at a pH of 8.0 (Table 1). In the stationary phase (14 days), optimal pH values for the growth of *M. alpina* NRRL-A-10995, lipid accumulation, and AA synthesis were 5.0–6.0, 7.0, and 6.0–8.0, respectively (Table 2). Therefore, it can be recommended to maintain the pH between 5.0–6.0 in the exponential phase for optimal culture growth, and then to adjust pH to 8.0 in the stationary phase for optimal AA synthesis. The extremely high sensitivity of AA synthesis to acidic conditions is of practical interest since AA production is completely inhibited when the medium is acidified merely for a short period of time.

3.2. The Effect of Temperature

The effect of temperature on the growth of *M. alpina* NRRL-A-10995 and the synthesis of lipids and AA was studied during the continuous cultivation in a glycerol-containing medium at low specific growth rates (from 0.0067 to 0.0079 h^{-1}) and a pH of 6.0. The data on the effect of temperature on the production of biomass, lipids, and AA, as well as on their yields from the glycerol consumed are given in Table 5.

Table 5. The effect of pH on the yields of biomass, lipids, and arachidonic acid in *M. alpina* NRRL-A-10995 in the stationary phase.

Parameters	Temperature (°C)				
	20	**22**	**24**	**26**	**28**
Biomass (g L^{-1})	16.4	17.2	16.2	17.2	17.7
Consumed glycerol (g L^{-1})	30.0	31.2	32.5	33.2	34.0
Lipids (% of biomass)	12.2	11.5	16.3	19.3	22.2
Lipids (g L^{-1})	2.0	1.9	2.6	3.4	3.9
Mycelium yield by mass ($Y_{X/S}$) (%)	54.7	55.1	49.8	51.8	52.0
Energy capacity of biomass (Q_B) (kJ g^{-1})	18.5	18.3	19.6	20.5	21.3
Mycelium yield by energy ($\eta_{X/S}$) (%)	56.3	56.1	54.3	59.1	61.7
Lipid yield by mass ($Y_{L/S}$) (%)	6.6	6.1	8.0	9.9	11.4
Lipid yield by energy ($\eta_{L/S}$) (%)	15.6	14.5	19.0	23.4	27.0
AA (% of lipids)	25.2	20.8	19.6	18.9	18.2
AA (g L^{-1})	0.5	0.4	0.5	0.6	0.7
AA yield by mass ($Y_{AA/S}$) (%)	1.6	1.3	1.5	1.8	2.1
AA yield by energy ($\eta_{AA/S}$) (%)	3.4	2.8	3.2	3.8	4.5

It should be noted that temperatures ranging from 20–28 °C showed no marked effect on the biomass yield under the exponential growth of fungi. In all variants, the mass yield of mycelium from the glycerol consumed ($Y_{X/S}$) was in a range of 49.8–54.7%. Lipid accumulation was more sensitive to temperature than mycelium growth; it reached the maximum (22.2% of biomass) at 28 °C and gradually decreased to 11.5–12.2% as the temperature lowered to 20–22 °C. Our results obtained for the glycerol-grown continuous culture of *M. alpina* NRRL-A-10995 are in agreement with the data reported for the glucose-grown batch culture of *M. alpina*; the optimum temperature values for fungal growth and lipid synthesis were different and were 25 and 20 °C, respectively [40].

As seen from Table 5, the energy capacity of biomass (Q_B) correlated with the lipid content of biomass and increased from 18.5 to 21.3 kJ g^{-1} when the temperature increased from 20 to 28 °C. The energy yield of biomass ($\eta_{X/S}$) reached the maximum (59.1–61.7%) at 26–28 °C. The mass yield of lipids from glycerol consumed ($Y_{L/S}$) was maximal (11.4 %) at 28 °C and decreased to 6.1–6.6% at 20–22 °C. The values of the maximum mass yield of lipids from pure glycerol in *M. alpina* NRRL-A-10995 that was grown through continuous cultivation are comparable with those (6–15%) obtained for batch cultures of fungi *M. isabelina*, *Cunninghamela echinutalla*, and *Zygornunchus moelleri* grown on raw glycerol [16,29,30]. The maximum energy yield of lipids was higher than the mass yield of lipids and reached 27% in the continuous culture of *M. alpina* NRRL-A-10995 at 28 °C (Table 5).

As seen from Table 5, the AA content of lipids was the maximum (25.2%) at 20 °C and decreased to 18.2% at 28 °C. Since the lipid content of biomass reached the maximum at 28 °C, the AA production also reached the maximum (0.7 g L^{-1}) at 28 °C and slightly decreased with temperature alterations. The yield of AA from the glycerol consumed was at its highest at temperatures of 26–28 °C both by mass and by energy (1.8–2.1 and 3.8–4.5%, respectively). The study of the effect of temperature on lipid and PUFA synthesis by the oleaginous fungus *Entomophthora exitalis*, which was grown in a glucose-containing medium in a chemostat (nitrogen limitation, D 0.04 h^{-1}), showed that a decrease in temperature from 30 to 20 °C was accompanied by an increase in both the PUFA and the AA content

of total lipids (from 18 to 27% and from 8 to 19%, respectively); however, lipid accumulation remained constant at the temperature range between 26 and 30 °C [41].

It should be noted that in our experiments, a reverse correlation was observed between lipid and AA synthesis in *M. alpina* NRRL-A-10995 grown at different temperatures. Earlier, such a correlation had been revealed when the strain, *M. alpina* NRRL-A-10995, was grown in a batch culture at different glycerol concentrations [20]. This phenomenon can be due to a change in a ratio between functional lipids which are rich in AA and storage lipids. According to literature data, an inverse correlation between lipid accumulation and the amount of PUFA was revealed in fungi belonging to the genera *Cunninghamella* and *Mortierella* [3,31,32,42,43]. It has been suggested that fungi producing low amounts of PUFA have to accumulate a bulk of lipids to ensure adequate levels of PUFA, which is necessary for cell membrane function [31].

It should be noted that a stimulatory effect of lower temperatures on the synthesis of unsaturated fatty acids is a well-known phenomenon [3,41]. An increase in the unsaturation of lipids is considered to be an adaptive mechanism of microbial cells to maintain membrane fluidity at lower temperatures since a constant membrane fluidity is necessary for the functioning of membrane-bound enzymes, transport mechanisms, etc. [41]. It was found that the activities of delta-5 and delta-6 desaturases involved in PUFA synthesis were increased at lower temperatures [44]. The cultivation of the Mortierella fungi at low temperatures was widely applied for the optimization of AA production [6,45]. A temperature-shift strategy was developed to increase the AA production by glucose-grown *M. alpina*; a temperature of 25 °C was maintained for the first 108 h of cultivation and then it was switched to 20 °C. As a result, the lipid and AA production increased by 20% and 26%, respectively [40].

As seen from Table 6, the predominant fatty acids included AA (18.2–25.2%), palmitic acid (18.9–23.6%), stearic acid (8.8–15.5%), oleic acid (22.2–27.3%), and linoleic acid (8.8–16.7%). 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 their end products.

Table 6. The effect of temperature on the fatty acid composition (% of lipids) of *M. alpina* NRRL-A-10995 under continuous cultivation.

Fatty Acids	Temperature (°C)				
	20	**22**	**24**	**26**	**28**
C_{14}	1.7	1.8	1.8	1.7	1.4
C_{15}	0.2	0.2	0.3	0.2	0.5
C_{16}	18.9	21.3	22.6	21.5	23.5
C_{18}	11.0	12.9	12.4	15.5	8.8
$C_{18:1}$	23.9	22.1	27.3	23.9	29.9
$C_{18:2}$	15.0	16.7	12.6	12.9	8.8
γ-$C_{18:3}$	3.0	4.2	2.9	4.9	4.6
C_{20}	0.9	0	0.6	0.3	0.7
$C_{20:1}$	0	0	0	0.2	0.7
$C_{20:2}$	0.4	0	0	0	3.1
$C_{20:3}$	25.2	20.8	19.6	18.9	18.2
$C_{20:4}$	1.7	1.8	1.8	1.7	1.4

Thus, the continuous cultivation of *M. alpina* NRRL-A-10995 in a glycerol-containing medium under selected optimal conditions (growth limitation by nitrogen, pH 6.0, and 20 °C), ensured that active AA synthesis (25.2% of lipids and 3.1% of biomass) occurred, with the AA yield from glycerol consumed being1.6% by mass and 3.4% by energy.

It is well known that a great advantage of continuous cultivation compared to batch culture is that a specific growth rate and nutrient composition is maintained at a constant level during the steady-state period. Therefore, continuous cultivation is the most favorable method for investigating the effect of physical factors and the growth-limiting components on microbial metabolism. Since lipids are considered to be secondary metabolites, where active synthesis occurs after growth cessation, the

method of continuous cultivation of microorganisms is rarely used for lipid production. However, a number of studies were carried out with continuous cultures of oleaginous yeasts. In particular, a comparative investigation of lipid synthesis by the oleaginous yeast *Candida curvata*, which was grown in a xylose-containing medium either through continuous cultivation or in a batch culture, showed that lipid accumulation reached its maximum in batch culture (49% of biomass) and decreased to 37% in a chemostat under nitrogen limitation at the dilution rate of 0.05 h^{-1} [46]. The *Yarrowia lipolytica* yeasts that grew on industrial glycerol in a single-stage continuous culture were shown to produce high amounts of reserve lipids (up to 43% of biomass; 3.5 g L^{-1}), with a maximum volumetric productivity of 0.12 g lipid (L h)$^{-1}$ [19]. A comparison of the AA synthesis by *M. alpina* NRRL-A-10995 under continuous and batch cultivation in glycerol-containing media (Tables 1, 2 and 5) revealed that the maximum AA content of lipids under continuous cultivation (25.2%) was higher than that in batch culture (22.1% and 19.8% in the exponential and stationary phases, respectively), indicating that the AA synthesis in the fungal strain, *M. alpina* NRRL-A-10995, is a growth-associated process. The maximum value of mycelium yield from the glycerol consumed by mass (Ys) under continuous cultivation (55.1%) was also higher than that in batch culture (38.8 and 34.7% in the exponential and stationary phase, respectively). These results confirm that the continuous cultivation of fungi can be successfully applied for the AA production.

4. Conclusions

The obtained results indicate that glycerol, as an inexpensive and renewable substrate, can be successfully used as a carbon and energy source for the AA synthesis by *M. alpina* fungi under both batch and continuous cultivation. The optimum pH values for fungal growth and production of lipids and AA were shown to be different and depended on the growth phase. It was confirmed that the strict inhibition of AA synthesis by extremely acidic pH values (3.0 and 4.0) in *M. alpina* fungi is a general pattern and does not depend on the specific features of a fungal strain. This finding is of importance for industrial AA production, since even a short period of media acidification to a pH below 4.0 can completely inhibit AA synthesis. Continuous cultivation of *M. alpina* NRRL-A-10995 in a glycerol-containing medium under selected optimal conditions (growth limitation by nitrogen, a pH of 6.0, and 20 °C), ensured active AA synthesis and can be applied in AA production.

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