ISSN 0026-2617, Microbiology, 2015, Vol. 84, No. 3, pp. 365–369. © Pleiades Publishing, Ltd., 2015.

EXPERIMENTAL ARTICLES

Effect of Cultivation Conditions on Production of Secondary Metabolites by *Penicillium citrinum*

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Received November 13, 2014

Abstract—Medium composition, cultivation temperature, and pO_2 had a regulatory effect on production of ergot alkaloids (EA) and quinocitrinines (QC) by the fungal strain *Penicillium citrinum* VKM F-4043 D. Maximal accumulation of EA and QC occurred at 24 and 28°C, respectively. Maximal EA synthesis occurred at 40% pO_2, while at 10–20% the conditions were optimal for QC synthesis. Production of these metabolites depended directly on the concentration of the carbon source in the medium. Addition of 2.5% NaCl did not inhibit EA and QC accumulation.

Keywords: fungi, *Penicillium citrinum*, ergot alkaloids, quinocitrinines, formation, secondary metabolites

DOI: 10.1134/S0026261715030017

Filamentous fungi, as well as other microorganisms, synthesize structurally diverse, biologically active compounds. Formation of secondary metabolites by producers is controlled by the regulatory mechanisms affected by changing environmental conditions. Since the conditions favorable for the maximum rate of a producer do not necessarily favor the synthesis of secondary metabolites, a balance between the growth rates and the maximum product yield is required [1, 2]. In the production of antibiotics, this problem is solved by using two-stage processes. The conditions for rapid vegetative growth and for efficient product formation are created at the first and second stage of cultivation, respectively (the tropho- and idiophasic kinetics). Growth of the overwhelming majority of fungal producer strains of the genus *Penicillium* in a synthetic medium with mannitol, succinic acid, mineral salts, and ammonium nitrogen (optimal for secondary metabolite synthesis) was accompanied by the synthesis of nitrogen-containing compounds [3]. It is known that the genes involved in the synthesis of secondary metabolites are clustered, and these clusters are controlled by transcription regulators (DNA-bound proteins) having either specific effects on the genes within the cluster or on the genes of the common metabolic pathways [4]. Secondary metabolite biosynthesis involves multifunctional enzyme complexes: a polyketide synthase, a nonribosomal peptide synthetase, and their hybrid [5]. Expression of the genes of secondary metabolism depends on the medium composition, cultivation conditions, metabolic products, and trace elements. During the cultivation of producers, production of the bioactive compounds is sometimes substantially enhanced by optimizing the physical (temperature, pH, aeration) and chemical factors (medium components, precursors, inducers, etc.) [6].

Penicillium citrinum VKM F-4043 D isolated from ancient permafrost deposits forms two classes of biologically active compounds: clavine ergot alkaloids (EA) (epoxyagroclavine-I and agroclavine-I) and quinoline alkaloids, quinocitrinines (QC) [7, 8].

The goal of this work was to study the effects of cultivation temperature, pO_2, and the concentrations of NaCl and the carbon substrate (mannitol) on the formation of secondary metabolites by *P. citrinum* VKM F-4043 D.

MATERIALS AND METHODS

The fungus *P. citrinum* VKM F-4043 D used in the work was provided by the All-Russian Collection of Microorganisms, the Institute of Biochemistry and Physiology of Microorganisms of the Russian Academy of Sciences (VKM). The effects of growth temperature (20, 24, and 28°C) and dissolved oxygen concentration (pO_2 10, 20, 40, and 80% of air saturation at 24°C) were studied during the cultivation of the fungus in a 10-L ANKUM-2M fermenter (Biopribor, Russia) with a 6-L working volume. Constant temperature and the pH changes were recorded automatically. The pO_2 values were regulated by rotational velocity of the stirrer (300–400 rpm) and by air supply varying from 0.01 to 0.5 L/L min according to the pO_2-meter reading. The fungus was cultivated in a mineral medium containing the following (g/L of dis-

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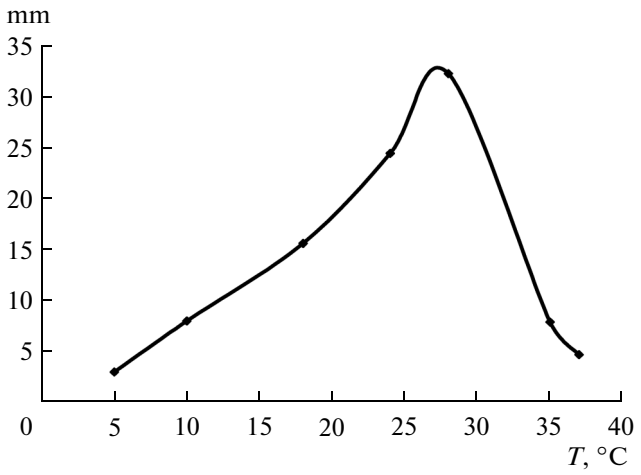

Fig. 1. Effect of temperature on the diameter (mm) of *P. citrinum* colcolonies (day 7).

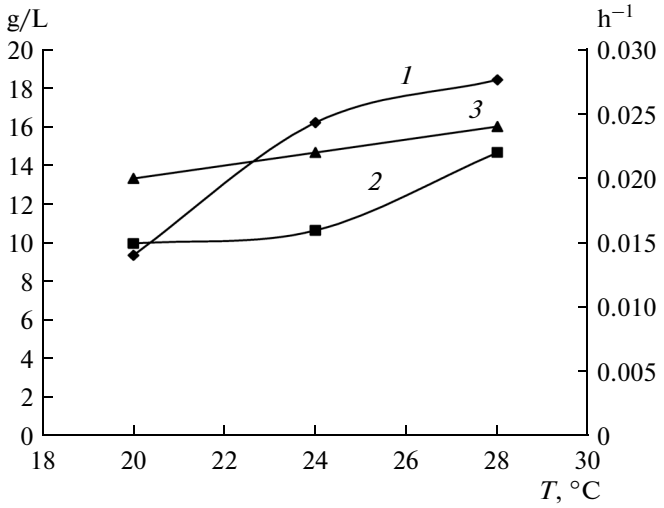

Fig. 2. Effect of cultivation temperature on the maximum growth parameters of *P. citrinum*: biomass, g/L (*1*); μ_1, h^{-1} (*2*); and μ_2, h^{-1} (*3*).

tilled water): mannitol, 50.0; succinic acid, 5.4; KH_2PO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.3; $ZnSO_4 \cdot 7H_2O$, 0.005; pH was adjusted to 5.4 with a concentrated ammonia solution.

The effect of the substrate concentration on secondary metabolite production was investigated at 20 or 50 g/L of mannitol. The effect of 2.5% NaCl was studied at a mannitol concentration of 20 g/L with or without the addition of $ZnSO_4 \cdot 7H_2O$ (5 mg/L). The fungus was grown in a submerged culture in flasks (750 mL) with 150 mL of the medium at 24 ± 1°C on a shaker (220 rpm) for 13 days. The media were inoculated with conidia ($1–2 \times 10^7$ cells/mL) washed of a 14-day culture grown on wort agar slants with 0.05% Tween-80 solution. The fermenter was inoculated with a 6-day mycelium (10% of the medium volume) grown as a submerged culture in flasks with the medium used for cultivating the producer in a fermenter. *P. citrinum* was grown for 7 days on the Czapek yeast extract agar (CYA) at 5, 9, 18, 25, 28, and 37°C to investigate the influence of cultivation temperature on the linear growth rate. The content of fungal biomass in submerged culture was determined daily by the biomass dry weight. The culture was examined under a light microscope (Jena, Germany). Secondary metabolites were analyzed daily in the culture liquid filtrate.

The metabolites were isolated from the culture liquid filtrate by threefold chloroform extraction as described [7] and from mycelium by extraction with chloroform–methanol (1 : 1). The extract from the biomass was diluted with distilled H_2O and extracted thrice with chloroform at acidic and alkaline pH values. Chloroform extracts were dried over anhydrous Na_2SO_4 and evaporated under vacuum. The extracts were analyzed by thin-layer chromatography on Silica gel (Silica gel 60 F_{254}, Merck, Germany) in the chloroform/methanol/25% NH_4OH (conc.) (90 : 10 : 0.1) and (80 : 20 : 0.2) systems. The substances were detected by UV absorption and after spraying the plates with the Ehrlich reagent for EA detection and with the Dragendorff reagent for QC identification. The amounts of EA and QC were determined by spectrophotometry [7].

RESULTS AND DISCUSSION

Investigation of the effect of temperature on the linear growth rate of *P. citrinum* on solid medium was carried out within the range from 5 to 37°C with an optimum of 28°C (Fig. 1). Cultivation of the fungus on the mineral medium in the fermenter at 28, 24, or 20°C confirmed that the optimal growth temperature was 28°C, when the highest values of the growth parameters (biomass yield (x), the maximum specific growth rates on succinate (μ_1) and mannitol (μ_2)) were observed (Fig. 2). When the fungus was cultivated at 20 and 24°C, these values were considerably lower than at 28°C. Within the studied temperature range, μ_1 and μ_2 decreased in proportion to the decrease in the cultivation temperature. Thus, the *P. citrinum* strain isolated from a permafrost soil sample 1.8–3 million years old [9] may be classified as a mesophile.

The effect of cultivation temperature on secondary metabolite production in *P. citrinum* was assessed by determining the maximum concentration of metabolites in the culture liquid, the maximum specific rate of their biosynthesis and productivity, and the yield of metabolites per unit of biomass. The *P. citrinum* cultivation temperature was shown to have different effects on EA and QC biosynthesis. The maximum values of EA biosynthesis were observed during the cultivation of *P. citrinum* at 24°C (Fig. 3). It decreased at both higher and lower values of the cultivation temperature. On the contrary, the maximum values of QC biosyn-

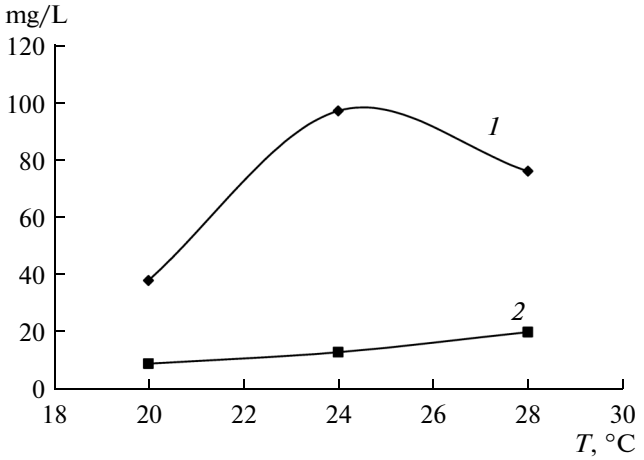

Fig. 3. Effect of cultivation temperature on the maximum accumulation of EA (*1*) and QC (*2*) in the medium (mg/L).

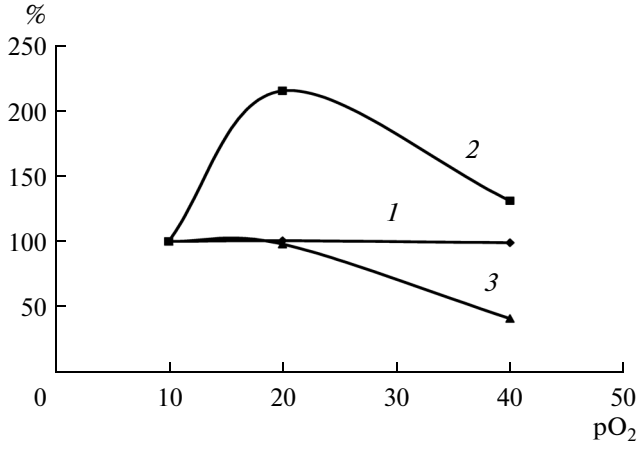

Fig. 4. Effect of pO_2 on the maximum growth parameters of *P. citrinum* (percentage of the respective values at pO_2 10%): biomass (*1*); μ_1 (*2*); and μ_2 (*3*).

thesis increased with the cultivation temperature and reached maximum values at 28°C. Thus, QC production was shown to be more intensive at the optimal growth temperature (28°C), while the optimal temperature for QA production was 24°C.

The effects of different pO_2 values (10, 20, 40, and 80% of air saturation at 24°C) were studied during cultivation of the fungus in the fermenter. With high initial aeration of the medium (air feeding, 0.50 L/L min; shaker, 400 rpm), pO_2 values did not go down below 80% during 72 h of observation. No biomass growth occurred under these conditions; the differentiation of hyphae and the formation of penicilli and conidia were observed in 24 h after the beginning of fermentation. At lower initial aeration of the medium (0.01, 0.08, and 0.15 L/L min), the pO_2 values decreased to 10, 20, and 40%, respectively, in 24 h and were maintained at these levels during further cultivation. Under these conditions, no differentiation of the mycelium was observed throughout the experiment. The biomass yield was the same (17.7 g/L). It was shown that the maximum specific growth rates on succinate (μ_1) and mannitol (μ_2) were more than twofold higher at pO_2 20% compared to other tested pO_2 values (Fig. 4). Thus, these data show that the optimal pO_2 value for the culture growth is 20% of aeration. The lack of oxygen at pO_2 10% limits the culture growth on succinate, while the excess of oxygen at pO_2 40% inhibits fungal growth on both substrates. The inhibition of fungal growth by oxygen substantially increases at pO_2 80%.

The pO_2 values maintained throughout the cultivation process influenced the biosynthesis of EA and QC by the fungus *P. citrinum* (Fig. 5). EA concentration was 36% higher at pO_2 40% compared to pO_2 10%. The effect of pO_2 on QC production was the opposite: QC accumulation was 35% lower at pO_2 40% compared to pO_2 10 and 20%. Thus, pO_2 values can be used for regulating the composition of the synthesized secondary metabolites: EA and QC production conditions are optimal at pO_2 40% and pO_2 10–20%, respectively.

When the fungus was cultivated in the medium with 50 g/L mannitol, its growth was diauxic with the second lag phase on day 6–7. Conidia formation and microcyclic conidiogenesis observed in the medium with 20 g/L mannitol were determined by carbon and energy deficiency in the medium. Mannitol concentration influenced the yield of biomass. When the fungus was grown in the medium with 20 g/L mannitol, the biomass yield was 2.4 times lower compared to 50 g/L of the substrate (see table). Thus, in the presence of two carbon sources in the medium, the optimal concentration of the second carbon substrate (mannitol) was shown to be 50 g/L.

The dependence between mannitol concentration and the values of EA and QC production was revealed

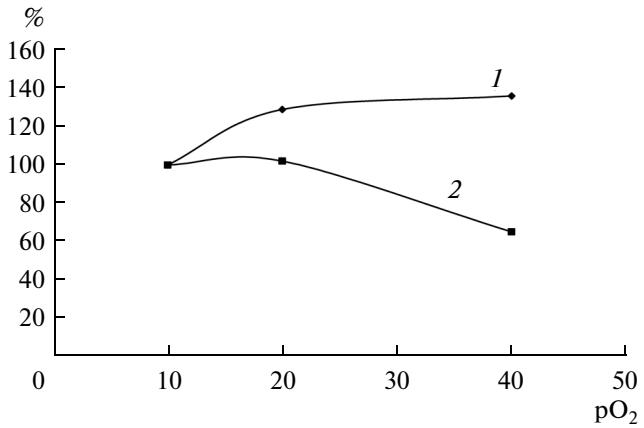

Fig. 5. Effect of pO_2 on the maximum accumulation in the medium of EA (*1*) and QC (*2*) (percentage of the respective values at pO_2 10%).

Table. Effect of mannitol and NaCl concentrations on the maximum parameters of growth and secondary metabolite biosynthesis in the fungus *P. citrinum*

Medium components, g/L			Index									
Mannitol	NaCl	Zn^{2+}	pH	Biomass, g/L	μ_1, h^{-1}	μ_2, h^{-1}	EA, mg/L	q_{EA} mg/g day	$Y_{EA/x}$, mg/g	QC, mg/L	q_{QC} mg/g day	$Y_{QC/x}$, mg/g
50	0	0	4.4	12.4	0.022	0.012	37	1.9	3.0	14	1.1	1.1
20	0	0	6.8	5.2	0.025	0.015	9	0.7	1.7	9	0.4	1.7
	25	0	4.6	3.8	0.025	0.014	2.4	0.7	0.7	6	0.3	2.1
	25	0.001	4.0	7.7	0.046	0.019	38	1.6	5.2	7	0.2	1.2

(table). In the presence of 20 g/L mannitol in the medium, accumulation of EA and QC in the culture liquid was 4.1 and 1.6 times less and the maximum specific rates of EA (q_{EA}) and QC (q_{QC}) biosynthesis were 2.7 times lower compared to the medium with 50 g/L mannitol. With 20 g/L mannitol, $Y_{EA/x}$ decreased 1.8 times and $Y_{QC/x}$ increased by 50% compared to the medium containing 50 g/L mannitol.

Investigation of the effect of 2.5% NaCl on the growth of *P. citrinum* and production of alkaloids by the fungus showed that addition of this salt to the medium resulted in a 1.4-fold decrease in biomass yield compared to the control, without any significant effects on the maximum specific growth rates on succinate (μ_1) and mannitol (μ_2) (table). The decrease in biomass yield was undoubtedly associated with the increase in the amount of energy the culture spends on the maintenance of the necessary intracellular osmotic pressure [10]. Under these conditions, the maximum accumulation of EA and QC in the culture liquid was 3.8- and 1.3-fold lower than in the control, respectively, but the maximum specific rates of EA and QC biosynthesis remained unchanged (table), indicating the absence of inhibition of the enzymes of EA and QC biosynthesis by 2.5% NaCl.

Previously, it has been shown that zinc ions stimulate the EA and QC production in *P. citrinum* [7]. The values of the growth parameters of the fungus increased upon addition of zinc ions to the medium with NaCl. The biomass yield doubled and the maximum specific growth rates on succinate (μ_1) and mannitol (μ_2) increased 1.8- and 1.4-fold, respectively, compared to the medium without zinc (table). In this medium, the maximum accumulation of EA and QC in the culture liquid was 15.8- and 1.2-fold higher, respectively, than in the medium without zinc. In the presence of NaCl, zinc ions stimulated only EA production but had a negative effect on QC production. The q_{EA} and $Y_{EA/x}$ values increased 2.3- and 7.4-fold, respectively, while q_{QC} and $Y_{QC/x}$ decreased by about 40% in the presence of both salt and the trace element, compared to the medium containing NaCl alone (table). Since the energy spent on maintenance of the concentration gradients between the mycelium and the medium was the same in the zinc-containing medium, it may be supposed that zinc ions activated oxidative metabolism. Zinc ions are known to activate one of the glycolytic enzymes: glucose-1,6-diphosphate aldolase. It should be noted that EA concentration in the medium containing 20 g/L mannitol, 2.5% NaCl, and zinc ions reached the level observed in the medium with 50 g/L mannitol (38 mg/L), while the levels of QC were lower by 47 and 10%, respectively.

Cultivation temperature is one of the essential factors influencing the intensity and direction of secondary metabolite biosynthesis. A decrease in the cultivation temperature of *Aspergillus parasiticus* resulted in the shift of the biosynthesis of aflatoxin B_1 towards aflatoxin G_1 [11]. In *P. roqueforti*, roquefortine was not formed at the temperature below 12°C [12].

Our research showed the cultivation temperature to have a regulatory effect on the synthetic activity of *P. citrinum*. The production of QC was more intensive when the fungus was grown at the optimal growth temperature (28°C), while EA production was more intense at the temperature below the optimum (24°C). These differences may be due to different functions of the metabolites in the producing organism. QC can be considered as antibacterial protection of the culture, which increases in the case of favorable growth conditions. QCs were shown to be efficient against gram-positive and gram-negative bacteria, yeasts, and fungi, as well as cytotoxic for tumor cells [8]. It is supposed that the production of ergot alkaloids is associated with reserving the primary metabolites that can be utilized by the culture for cell growth processes in case of deficiency, e.g., of tryptophan [3].

Low solubility of oxygen in the nutrient media, which depends on temperature, concentration of nutrients, etc., often limits the growth and biosynthetic processes. The necessary condition of any microbiological process is determination of the aeration mode for the cultivation of the producer, since different producers of biologically active substances have peculiar requirements for oxygen concentration [13]. Investigation of the effect of different pO_2 values on EA and QC production by *P. citrinum* revealed the optimal conditions for EA and QC accumulation to

develop at pO_2 values of 40 and 10–20%, respectively. Various oxygenases requiring molecular oxygen for their activity are known to be involved in ergot alkaloid synthesis [1]. This fact probably accounts for the higher pO_2 values needed for EA production.

Many fungi of the genus *Penicillium*, including the species *P. citrinum*, are tolerant to different NaCl concentrations [14]. It gives them an ecological advantage of competitiveness when they colonize some habitats, e.g., foodstuffs with the high concentrations of osmolytes. Production of the secondary metabolites is considered to be associated with the adaptation of producers to the environment. This suggestion was confirmed by the data on changes in the profiles of secondary metabolites in response to variation of the medium composition. NaCl added to the cultivation medium of *P. thymicola* at a concentration above 2.5% inhibited the synthesis of yellow pigments and stimulated the formation of fumiquinazolines [15]. In *P. verrucosum*, NaCl affected the regulation of citrinin and ochratoxin accumulation [16]. Predominant formation of ochratoxin A containing a chlorine atom in its molecule was observed at high salt concentrations, suggesting partial involvement of this metabolite in chlorine ion homeostasis in a fungal cell. Addition of NaCl to the medium for *P. citrinum* resulted in lower accumulation of biomass, EA, and QC in the culture liquid. At the same time, the maximum specific rates of EA and QC production did not change, demonstrating the absence of inhibition of the enzymes of EA and QC synthesis by NaCl. Production of nitrogen-containing compounds is probably not associated with the regulatory mechanism ensuring fungal growth at a high osmolarity of the medium.

Thus, the medium composition, cultivation temperature, and pO_2 were shown to have regulatory effects on the production of ergot alkaloids (EA) and quinocitrinines (QC) by the fungus *P. citrinum* VKM F-4043 D. The temperature of 24°C was optimal for EA biosynthesis, while the maximum accumulation of QC took place at 28°C. The maximal biosynthesis of EA was observed at pO_2 40%, while the conditions for QC production were optimal at pO_2 10–20%. The metabolite production directly depended on the carbon substrate concentration in the medium. Addition of 2.5% NaCl did not inhibit the accumulation of EA and QC.

REFERENCES

1. Rehachek, Z. and Sajdl, P., *Ergot Alkaloids: Chemistry, Biological Effect, Biotechnology*, Praha: Academia, 1990.
2. Cairns-Fuller, V., D. Aldred, D., and Magan, N., Water, temperature and gas composition interactions affect growth and ochratoxin A production by isolates of *Penicillium verrucosum* on wheat grain, *J. Appl. Microbiol.*, 2005, vol. 99, pp. 1215–1221.
3. Kozlovsky, A.G., Zhelifonova, V.P., and Antipova, T.V., Fungi of the genus *Penicilium* as producers of physiologically active compounds, *Appl. Biochem. Microbiol.*, 2013, vol. 49, no. 1, pp. 1–10.
4. Fox, E.M. and Howlett, B.J., Secondary metabolism: regulation and role in fungal biology, *Curr. Opin. Microbiol.*, 2008, vol. 11, pp. 481–487.
5. Keller, N.P., Turner, G., and Bennett, J.W., Fungal secondary metabolism—from biochemistry to genomics, *Nature Rev. Microbiol.*, 2005, vol. 3, pp. 937– 947.
6. Calvo, A.M., Wilson, R.A., Bok, J.W., and Keller, N.P., Relationship between secondary metabolism and fungal development, *Microbiol. Mol. Biol. Rev.*, 2002, vol. 66, no. 3, pp. 447–459.
7. Kozlovsky, A.G., Zhelifonova, V.P., and Antipova, T.V., The fungus *Penicillium citrinum*, isolated from permafrost sediments, as a producer of ergot alkaloids and new quinoline alkaloids quinocitrinines, *Appl. Biochem. Microbiol.*, 2005, vol. 41, no. 5, pp. 499–502.
8. RF Patent no. 2386692, 2008.
9. Ozerskaya, S., Kochkina, G., Ivanushkina, N., and Gilichinsky, D., Fungi in permafrost, in *Permafrost Soils, Soil Biology*, Margesin, R., Ed., Berlin: Springer, 2009, vol. 16, pp. 85–95.
10. Pirt, S.J., *Principles of Microbe and Cell Cultivation*, Oxford: Blackwell, 1975.
11. Schmidt-Heydt, M., Rüfer, C.E., Abdel-Hadi, A., Magan, N., and Geisen, R., The production of aflatoxin B1 or G1 by *A. parasiticus* at various combinations of temperature and water activity is related to the ratio of *aflS* to *aflR* expression, *Mycotoxin Res.*, 2010, vol. 26, no. 4, pp. 241–246.
12. Scott, P.M., Roquefortine, in *Mycotoxins—Production, Isolation, Separation and Purification*, Betina, A.B., Ed., Amsterdam: Elsevier, 1984, pp. 463–468.
13. Barberel, S.I. and Walker, J.R., The effect of aeration upon the secondary metabolism of microorganisms, *Biotechnol. Genet. Eng. Rev.*, 2000, vol. 17, pp. 281–323.
14. Frisvad, J.C., Halotolerant and halophilic fungi and their extrolite production, in *Adaptation of Life at High Salt Concentrations in Archaea, Bacteria and Eukarya, Cellular Origin, Life in Extreme Habitats and Astrobiology*, 2005, Rensing, pp. 425–439.
15. Zhelifonova, V.P., Antipova, T.V., and Kozlovskii, A.G., Biosynthesis of fumiquinazolines by the fungus *Penicillium thymicola, Appl. Biochem. Microbiol.*, 2012, vol. 48, no. 3, pp. 302–306.
16. Schmidt-Heydt, M., Graf, E., Stoll, D., and Geisen, R., The biosynthesis of ochratoxin A by *Penicillium* as one mechanism for adaptation to NaCl rich foods, *Food Microbiol.*, 2012, vol. 29, pp. 233–241.

Translated by E. Makeeva