Microbiology, Vol. 73, No. 3, 2004, pp. 286–291. Translated from Mikrobiologiya, Vol. 73, No. 3, 2004, pp. 343–349.
Original Russian Text Copyright © 2004 by Semashko, Mikhailova, Lobanok.

EXPERIMENTAL ARTICLES

Growth Characteristics and Glucose Oxidase Production in Mutant *Penicillium funiculosum* Strains

T. V. Semashko, R. V. Mikhailova, and A. G. Lobanok

Institute of Microbiology, National Academy of Sciences of Belarus, ul. Kuprevicha 2, Minsk, 220141 Belarus

Received February 11, 2003; in final form, May 12, 2003

Abstract—The main parameters of growth and glucose oxidase production by the mutant *Penicillium funiculosum* strains BIM F-15.3, NMM95.132, and 46.1 were studied. The synthesis of extracellular glucose oxidase by these strains was constitutive and occurred following the phase of exponential growth. The mutant strains also synthesized extracellular invertase and cell-associated catalase and glucose oxidase. The syntheses of invertase, the cell-associated enzymes, and extracellular glucose oxidase were found to be maximum between 14 and 18 h, between 48 and 52 h, and by the 96th hour of cultivation, respectively. Among the mutants studied, *P. funiculosum* 46.1 showed the maximal rates of growth and glucose oxidase synthesis.

Key words: *Penicillium funiculosum*, mutant strains, enzyme biosynthesis, glucose oxidase.

Glucose oxidase (β-D-glucose:oxygen 1-oxidoreductase, EC 1.1.3.4) catalyzes the oxidation of β-D-glucose to β-D-glucono-δ-lactone and hydrogen peroxide. This enzyme is widely used in the food and chemical industries, medicine, and scientific research [1]. The main producers of glucose oxidase are mycelial fungi of the genera *Aspergillus* and *Penicillium*. Although the production and the properties of fungal glucose oxidases are well described [1], the regulation of glucose oxidase synthesis and its relationship to the growth of the producers are as yet poorly studied.

Previously, we studied the spontaneous and induced mutation of the glucose oxidase producer *Penicillium funiculosum* BIM F-15 and obtained three mutant strains (*P. funiculosum* BIM F-15.3, NMM95.132, and 46.1) [2–4]. The aim of this work was to study the main parameters of growth and glucose oxidase production by these mutant strains.

MATERIALS AND METHODS

Experiments were carried out with the mutant strains *Penicillium funiculosum* BIM F-15.3 [3], *P. funiculosum* NMM95.132 [2], and *P. funiculosum* 46.1 [4]. The mutants were maintained on Czapek agar.

The growth dynamics of fungal colonies was studied in the course of 7-day cultivation on Czapek agar. The radial growth rate of fungal colonies was calculated using the formula $K_r = \frac{dr}{dt}$, where dr is the radius of a colony and dt is time [5].

Submerged fungal cultures were grown for 96 hours at 24–28°C on a shaker (180–200 rpm) in 250-ml Erlenmeyer flasks with 50 ml of a medium containing (%) KNO_3, 0.8; KH_2PO_4, 0.1; $MgSO_4 \cdot 7H_2O$, 0.05; KCl, 0.05; $FeSO_4 \cdot 7H_2O$, 0.00005; $MnSO_4 \cdot 5H_2O$, 0.00026; sucrose, 6.0; and malt extract, 1.0. The initial pH of the medium was 5.0.

Aqueous suspensions containing (4–6) × 10^4 spores/ml of the mutant fungi cultivated at 24–26°C on Czapek agar for 7 days served as inocula. After cultivation, the mycelium was separated from the culture liquid by filtration, washed, dried at 105°C to a constant weight, and weighed.

Enzymatic activities were determined both in the culture filtrates and in cell homogenates. To obtain a cell homogenate, an aliquot (1 g wet weight) of the washed fungal mycelium was frozen in liquid nitrogen and disintegrated. The homogenate was diluted with 10 ml of water. The mixture was incubated on a shaker for 1.5 h and then centrifuged at 8000 *g* for 15 min. The supernatant was assayed for the activities of cell-associated glucose oxidase and catalase, which were expressed in U per milligram dry biomass.

The effect of carbon sources on the growth and glucose oxidase production by the *P. funiculosum* mutants was estimated using monosugars, disaccharides, and sugar alcohols.

Glucose oxidase was assayed by the spectrophotometric method based on the enzymatic conversion of benzoquinone to hydroquinone [6].

Catalase was assayed titrometrically [7].

The activity of invertase was determined in a mixture of 0.2 M sodium acetate buffer with pH 4.9 (2 ml), 0.5 M sucrose (1 ml), and an enzyme solution (0.8 ml). The blank solution contained 1 ml of distilled water

Table 1. The effect of various carbon sources on the synthesis of glucose oxidase by the *P. funiculosum* mutants

Carbon source, 4%	*P. funiculosum* BIM F-15.3				*P. funiculosum* NMM95.132				*P. funiculosum* 46.1			
	pH	biomass, mg/ml	glucose oxidase		pH	biomass, mg/ml	glucose oxidase		pH	biomass, mg/ml	glucose oxidase	
			U/ml	U/mg			U/ml	U/mg			U/ml	U/mg
Glucose	3.5	3.5	3.34	0.95	3.5	2.9	4.4	1.53	3.7	5.6	2.88	0.52
Xylose	4.8	2.3	1.53	0.66	5.5	4.7	0.80	0.17	5.6	4.4	0.62	0.14
Fructose	4.3	2.4	4.10	1.74	4.8	3.2	6.25	1.94	5.2	5.3	1.57	0.29
Lactose	5.2	1.3	traces	traces	6.4	1.9	traces	traces	6.9	1.5	traces	traces
Maltose	4.1	6.1	0.58	0.10	5.2	9.1	0.98	0.11	3.7	5.6	2.83	0.51
Sucrose	3.5	3.1	5.05	1.64	3.5	4.4	8.23	1.86	3.4	5.4	9.14	1.98
Glycerol	6.4	2.4	traces	traces	7.0	2.5	traces	traces	7.0	3.8	traces	traces
Mannitol	5.2	3.1	1.63	0.52	4.3	5.1	2.36	0.39	5.5	3.2	1.15	0.36

Table 2. The formation of hydrogen peroxide and the hydrolysis of sucrose by the *P. funiculosum* mutants

Cultivation time, h	*P. funiculosum* BIM F-15.3			*P. funiculosum* NMM95.132			*P. funiculosum* 46.1		
	reducing sugars, mg/ml	fructose, mg/ml	H_2O_2, μM	reducing sugars, mg/ml	fructose, mg/ml	H_2O_2, μM	reducing sugars, mg/ml	fructose, mg/ml	H_2O_2, μM
18	1.40	1.09	0	5.14	6.00	0	5.76	4.30	0
22	6.50	6.15	0.02	9.47	0.93	0.03	10.60	6.97	0.03
30	14.70	14.20	2.12	14.50	7.90	2.40	18.83	13.02	2.56
38	23.50	19.80	4.60	20.65	15.49	4.80	24.50	14.90	5.10
48	41.50	20.80	6.30	31.50	24.60	6.13	35.75	22.90	9.87
52	59.00	23.00	10.37	58.70	27.24	11.62	57.60	25.70	12.50
66	34.40	16.12	3.20	34.10	16.12	4.78	33.25	15.8	4.12
72	35.50	11.80	3.12	30.53	11.78	4.22	22.40	11.02	2.88
96	16.80	9.70	2.80	21.00	9.86	2.54	11.50	6.08	2.66

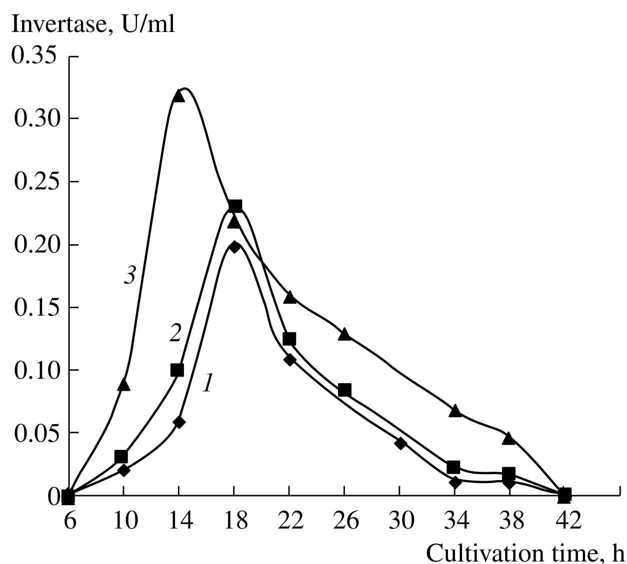

Fig. 1. The synthesis of invertase by the *P. funiculosum* mutants (*1*) BIM F-15.3, (*2*) NMM95.132, and (*3*) 46.1.

instead of the sucrose solution. The reaction mixture was incubated at 30°C for 10–20 min. One unit of invertase activity was defined as the amount of enzyme that converts sucrose with the formation of 1 μmol of reducing substances in 1 min at 30°C.

The activities of extracellular enzymes were calculated in units per milliliter of the culture liquid and, to characterize the mycelium productivity, per milligram dry weight of the mycelium.

Reducing substances were quantified with 3,5-dinitrosalicylic acid [8].

Fructose was assessed by the cysteine–carbazole method [9].

Hydrogen peroxide was determined in a mixture of 0.98 ml of 0.1 M citrate–phosphate buffer (pH 3.6), 0.04 ml of 0.1 M *o*-phenylenediamine, 0.175 ml of distilled water, 0.005 ml of 8.0×10^{-7} M peroxidase, and 0.8 ml of a culture liquid. The blank solution did not contain peroxidase. After incubation for 1 min, the optical density of the reaction mixture was measured at 455 nm with an SF-46 spectrophotometer. The concentration of hydrogen peroxide was determined by using a calibration curve.

The specific rates of growth (μ) and glucose oxidase synthesis (ε) were calculated by the formulas [10] $\mu = \frac{dx}{dtx}$, $\varepsilon = \frac{dE}{dtx}$, where x is the biomass and E is enzymatic activity.

The data presented here are the means of triplicate measurements performed at least in three independent experiments.

RESULTS AND DISCUSSION

It is known from the literature that the synthesis of glucose oxidase by mycelial fungi depends on the composition of the nutrient medium, especially, on the carbon source used for growth. The maximum production of glucose oxidase by the fungi *Alternaria alternata* [11], *Aspergillus niger* [12], *Penicillium variabile* [13], *P. notatum* [14], and *Talaromyces flavus* [15] was observed when they were grown on glucose, whereas some fungi actively synthesized glucose oxidase when grown on sucrose, molasses, or starch [16, 17].

As can be seen from data presented in Table 1, the three mutant *P. funiculosum* strains produced glucose oxidase when grown on all the carbon sources tested, which suggested a constitutive synthesis of this enzyme. The maximum production of glucose oxidase by all three mutants was observed when they were grown on glucose, sucrose, or fructose. In addition, *P. funiculosum* BIM F-15.3 actively produced glucose oxidase when grown on xylose or mannitol, and *P. funiculosum* 46.1 actively produced glucose oxidase when grown on maltose. The growth and enzyme production on lactose media were poor. The production of glucose oxidase by the mutant strains grown on glycerol was probably suppressed by a decrease in the pH of the medium during cultivation, which is in agreement with the data of Petruccioli and Federici [13] and Kusai *et al.* [16], who showed that the optimal pH for the synthesis of glucose oxidase by most fungi is 5.0–5.5.

Further studies into the production of glucose oxidase by the *P. funiculosum* mutants were carried out under the conditions of their submerged cultivation in the medium with sucrose as the sole carbon source. It is known that sucrose is metabolized by mycelial fungi with the aid of invertase, which hydrolyzes the disaccharide with the formation of glucose, fructose, and an amount of fructose-containing oligosaccharides [18]. The mutants *P. funiculosum* BIM F-15.3 and NMM95.132 actively synthesized invertase in the lag phase, beginning from the 6th hour of cultivation (Fig. 1). The proportion between the monosugars produced varied in the course of cultivation (Table 2). In the first hours of cultivation, the mutants actively consumed glucose. The maximum content of reducing sugars (glucose and fructose) was observed after 52 h of cultivation. By the end of the cultivation period (96 h), the culture liquid of *P. funiculosum* BIM F-15.3 and NMM95.132 contained, respectively, 16 and 21 mg reducing sugars per milliliter, including 9.7–9.86 mg/ml fructose.

The mutants *P. funiculosum* BIM F-15.3 and NMM95.132 actively grew beginning from the 18th hour of cultivation, whereas trace amounts of extracellular glucose oxidase were detected only after 30 h of growth. The maximum amounts of glucose oxidase in the culture liquids of BIM F-15.3 and NMM95.132

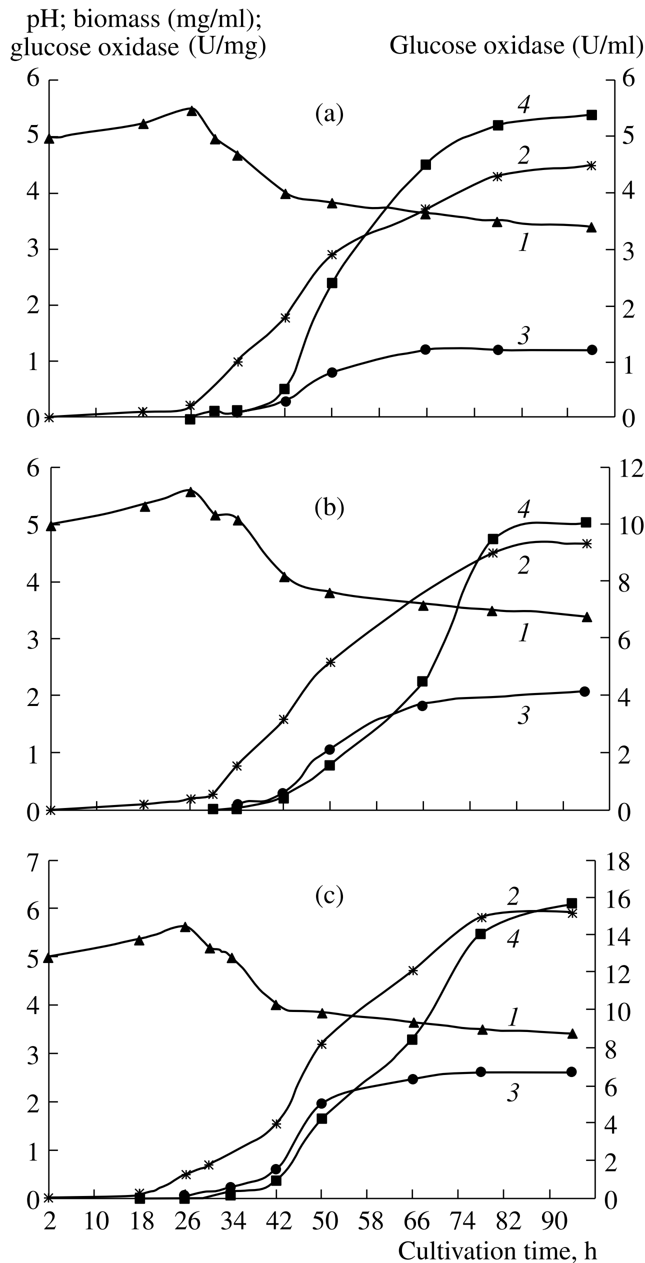

Fig. 2. The dynamics of growth and the synthesis of extracellular glucose oxidase by the *P. funiculosum* mutants (a) BIM F-15.3, (b) NMM95.132, and (c) 46.1: (*1*) pH; (*2*) biomass (mg/ml); (*3*) glucose oxidase, U/mg; (*4*) glucose oxidase, U/ml.

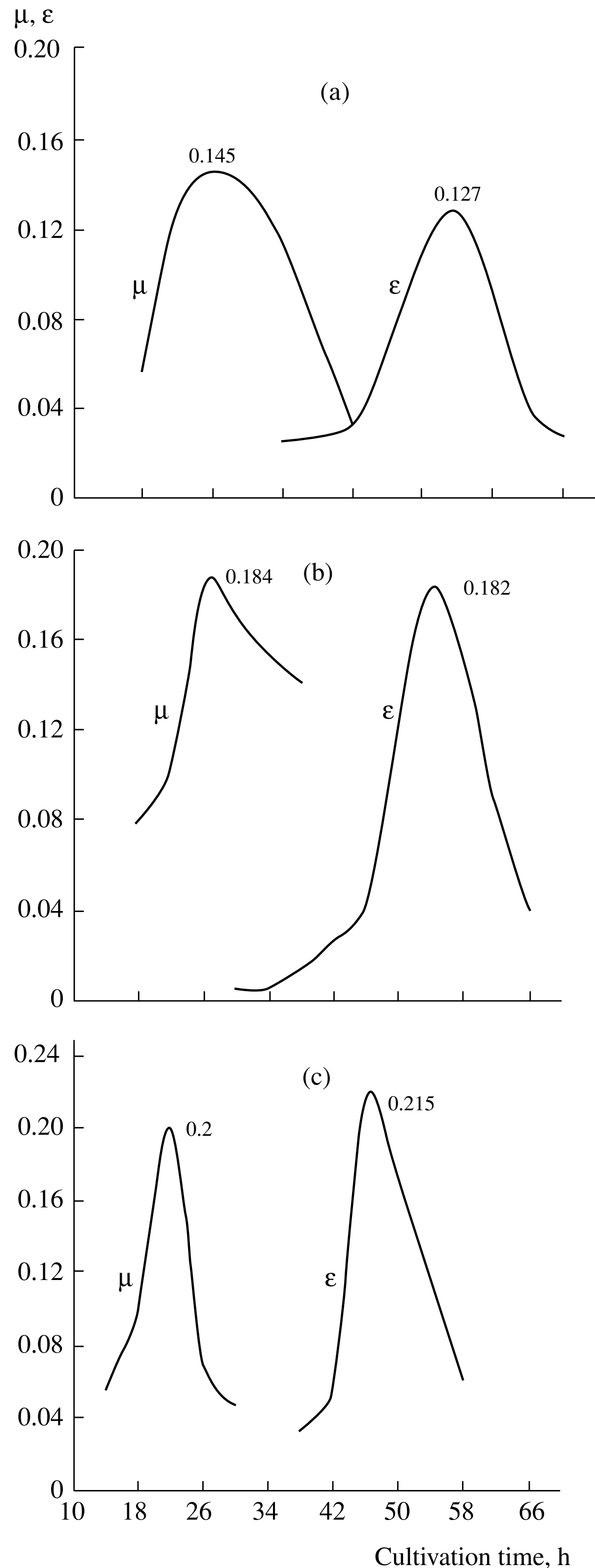

Fig. 3. The specific rates of growth (μ) and the synthesis of extracellular glucose oxidase (ε) by the *P. funiculosum* mutants (a) BIM F-15.3, (b) NMM95.132, and (c) 46.1. μ and ε are expressed in h^{-1} and U/(mg h), respectively.

(5.4 and 12.08 U/ml, respectively) were observed after 96 h of cultivation (Figs. 2a, 2b).

In the case of *P. funiculosum* BIM F-15.3, the specific rates of growth and glucose oxidase production reached maxima (μ_{max} = 0.145 h^{-1} and ε_{max} = 0.127 U/(mg h)) by the 26th and 52nd hour of cultivation, respectively (Fig. 3a). Consequently, glucose oxidase was synthesized by this mutant in the postexponential growth phase. Such a dynamics of synthesis

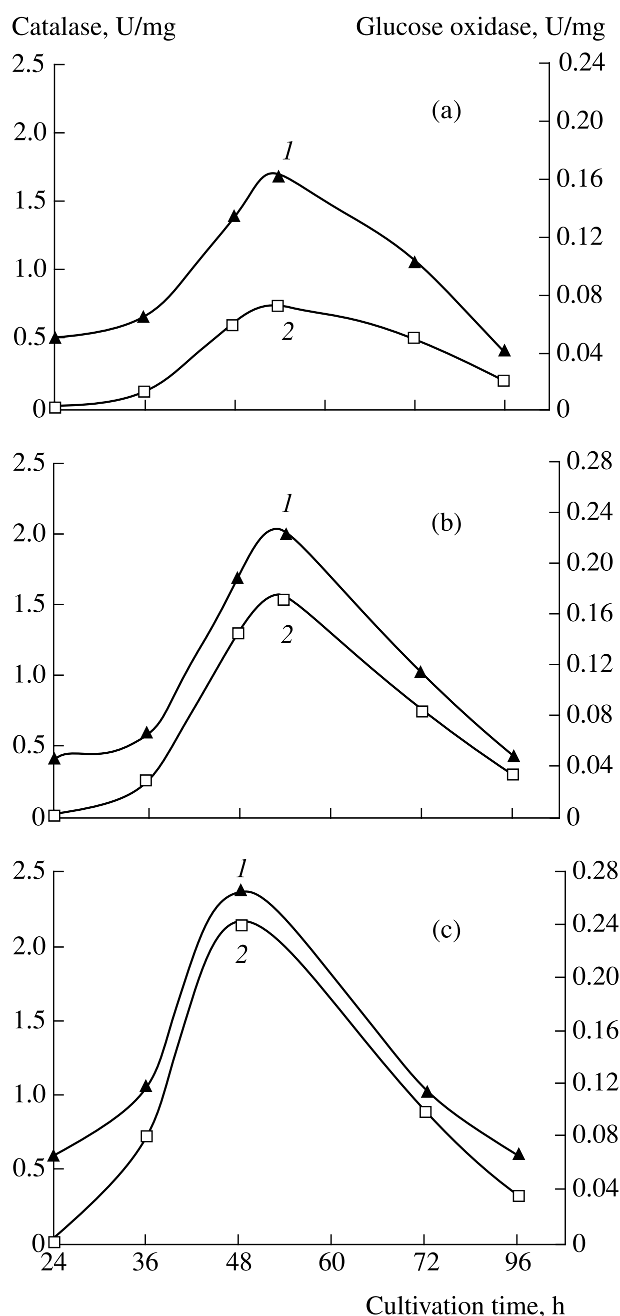

Fig. 4. The production of (*1*) cell-associated catalase and (*2*) cell-associated glucose oxidase by the *P. funiculosum* mutants (a) BIM F-15.3, (b) NMM95.132, and (c) 46.1.

is also typical of the hydrolytic enzymes of mycelial fungi [10, 19].

In the case of *P. funiculosum* NMM95.132, the specific rates of growth and glucose oxidase synthesis reached maximal values (μ_{max} = 0.184 h^{-1} and ε_{max} = 0.183 U/(mg hours)) by the 26th and 52nd hours of cultivation, respectively (Fig. 3b), being 26.9 and 44% greater than the corresponding parameters of *P. funiculosum* BIM F-15.3.

During the cultivation of the mutants, the initial decrease in the pH of the medium was followed by its increase. The increase in the pH coincided in time with the appearance of glucose oxidase in the medium (30 h of cultivation), which can be explained by the formation of gluconic acid, one of the end products of the reaction catalyzed by glucose oxidase. The concentration of the second product of the glucose oxidase reaction (hydrogen peroxide) in the culture liquids of *P. funiculosum* BIM F-15.3 and NMM95.132 initially increased but then (beginning from the 52nd hour of cultivation) rapidly decreased. According to the data available in the literature, many micromycetes synthesize extracellular catalase to decompose hydrogen peroxide [20]. Our studies showed that the *P. funiculosum* mutants did not produce extracellular catalase, but did produce cell-associated catalase and glucose oxidase. By the 52nd hour of cultivation, *P. funiculosum* BIM F-15.3 produced 1.34 U/mg cell-associated catalase and 0.07 U/mg cell-associated glucose oxidase and *P. funiculosum* NMM95.132 produced 2.04 U/mg cell-associated catalase and 0.17 U/mg cell-associated glucose oxidase (Fig. 4b).

The study of *P. funiculosum* 46.1 showed that this mutant began to grow after 14 h and to synthesize extracellular glucose oxidase after 26 h of cultivation (Figs. 1, 2c, 4c). The lag phase of this mutant lasted 22–26 h. The syntheses of invertase, extracellular glucose oxidase, cell-associated catalase, and cell-associated glucose oxidase by *P. funiculosum* 46.1 were 1.5, 1.7, 2.5, and 3 times more intense than those by *P. funiculosum* BIM F-15.3 (Figs. 1, 2c, 4c). The mutant 46.1 also consumed glucose and fructose more actively than did the mutants BIM F-15.3 and NMM95.132, as is evident from the low content of reducing sugars in the culture liquid of *P. funiculosum* 46.1 by the end of the cultivation period (Table 2).

The kinetic characteristics of growth and glucose oxidase production by *P. funiculosum* 46.1 (μ_{max} = 0.2 h^{-1} and ε_{max} = 0.21 U/(mg h)) were 35 and 58% higher than in the case of *P. funiculosum* BIM F-15.3 (Fig. 3c). The specific rates of growth and glucose oxidase production by *P. funiculosum* 46.1 reached maxima by the 22nd and 48th hours of cultivation, respectively.

It is known that the growth parameters of mycelial fungi in liquid and on solid media are linearly correlated [5]. The radial growth rate of fungal colonies is a function of the width of the peripheral growth zone on agar medium and the specific growth rate in liquid medium. Our experiments showed that the diameters of the 7-day-old colonies of *P. funiculosum* BIM F-15.3, NMM95.132, and 46.1 were 22–24, 26–28, and 29–32 mm, respectively (Fig. 5a). The mutants also differed in the radial growth rate of their colonies, which reached maxima by the fourth day of cultivation and then declined (Fig. 5b). The mutant *P. funiculosum* 46.1 exhibited the maximum radial growth rate of colonies.

Thus, the growth and the synthesis of glucose oxidase by *P. funiculosum* BIM F-15.3, NMM95.132, and

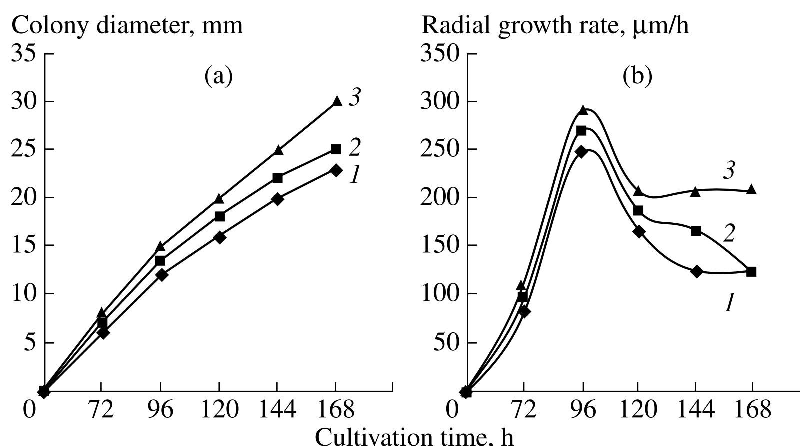

Fig. 5. (a) The diameter and (b) the radial growth rate of the colonies of (*1*) *P. funiculosum* BIM F-15.3, (*2*) NMM95.132, and (*3*) 46.1 grown on Czapek agar.

46.1 showed typical characteristics of the secondary metabolite production of microorganisms. Among the three mutants studied, *P. funiculosum* 46.1 had the highest rates of growth and glucose oxidase synthesis.

REFERENCES

1. Crueger, A. and Crueger, W., Glucose-Transforming Enzymes, *Microbial Enzymes and Biotechnology*, Fogarty, W.M. and Kelly, C.T., Eds., London: Elsevier Appl. Sci., 1990, pp. 177–226.
2. Semashko, T.V., Mikhailova, R.V., Lobanok, A.G., Moroz, I.V., and Osoka, O.M., Isolation and Characterization of *Penicillium funiculosum* Mutants with Enhanced Glucose Oxidase Production, *Mikrobiologiya*, 2000, vol. 69, no. 4, pp. 494–498.
3. Mikhailova, R.V., Semashko, T.V., Lobanok, A.G., and Osoka, O.M., Spontaneous Mutability of the Glucose Oxidase Producer *Penicillium funiculosum* BIM F-15, *Mikol. Fitopatol.*, 2001, vol. 35, no. 5, pp. 73–79.
4. Mikhailova, R.V., Semashko, T.V., and Lobanok, A.G., The Selection of *Penicillium funiculosum* Strains with Increased Glucose Oxidase Production, *Prikl. Biokhim. Mikrobiol.*, 2002, vol. 38, no. 3, pp. 273–277.
5. Pirt, S.J., *Principles of Microbe and Cell Cultivation*, Oxford: Blackwell, 1975. Translated under the title *Osnovy kul'tivirovaniya mikroorganizmov i kletok,* Moscow: Mir, 1978.
6. Markwell, J., Frakes, L.G., Brott, E.C., Osterman, J., and Wagner, F.W., *Aspergillus niger* Mutants with Increased Glucose Oxidase Production, *Appl. Microbiol. Biotechnol.*, 1989, vol. 30, no. 2, pp. 166–169.
7. Borisova, V.N., Oxygen-Activating Enzymes (Terminal Oxidases), *Metody eksperimental'noi mikologii* (Methods in Experimental Mycology), Bilai, V.I., Ed., Kiev: Nauk. Dumka, 1973, pp. 95–102.
8. Miller, G.L., Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar, *Anal. Chem.,* 1959, vol. 31, pp. 426–428.
9. Dische, Z. and Borenfreund, E., A New Spectrophotometric Method for the Detection and Determination of Keto Sugars and Trioses, *J. Biol. Chem.*, 1951, vol. 192, no. 2, pp. 583–587.
10. Terui, G., Kinetics of Hydrolase Production by Microorganisms, *Pure Appl. Chem.*, 1973, vol. 36, pp. 377–395.
11. Caridis, K.-A., Christakopoulos, P., and Macris, B.J., Simultaneous Production of Glucose Oxidase and Catalase by *Alternaria alternata, Appl. Microbiol. Biotechnol.*, 1991, vol. 34, no. 6, pp. 794–797.
12. Fiedurek, J., Glucose Oxidase Synthesis by *Aspergillus niger* GIV-10 on Starch, *Acta Microbiol. Pol.*, 1991, vol. 40, no. 3-4, pp. 197–203.
13. Petruccioli, M. and Federici, F., Glucose Oxidase Production by *Penicillium variabile* P16: Effect of Medium Composition, *J. Appl. Bacteriol*, 1993, vol. 75, pp. 369–372.
14. Roberts, E.S., Cain, C.K., Muir, R.D., Reithel, F.J., Gray, W.L., Van Bruggen, J.T., Homan, D.M., Kaltzman, P.A., Jones, L.R., and Doisy, E.A., Penicillin B, an Antibacterial Substance from *Penicillium notatum, J. Biol. Chem.*, 1943, vol. 147, pp. 47–58.
15. Kim, K.K., Fravel, R.D., and Papavizas, C.G., Production, Purification, and Properties of Glucose Oxidase from the Biocontrol Fungus *Talaromyces flavus, Can. J. Microbiol.*, 1989, vol. 36, pp. 199–205.
16. Kusai, K., Sekusu, I., Hagihara, B., Okuniki, K., Yamauchi, S., and Nakai, M., Crystallization of Glucose Oxidase from *Penicillium amagasakiense, Biochim. Biophys. Acta*, 1960, vol. 40, pp. 555–557.
17. Hatzinikolaou, D.C. and Macris, B.J., Factors Regulating Production of Glucose Oxidase by *Aspergillus niger, Enzyme Microb. Technol.*, 1995, vol. 17, no. 6, pp. 530–534.
18. Bilai, V.I., *Osnovy obshchei mikologiya* (Fundamentals of General Mycology), Kiev: Vishcha Shkola, 1974.
19. Lobanok, A.G., Mikhailova, R.V., and Sapunova, L.I., Kinetics of the Constitutive Synthesis of Polymethylgalacturonases by *Penicillium digitatum* as a Function of Carbon Source and pH, *Mikrobiologiya*, 1977, vol. 46, no. 5, pp. 920–925.
20. Kurakov, A.V., Kupletskaya, M.B., Skripnikova, E.V., and Somova, N.G., The Search for Micromycetes Producing Extracellular Catalase and the Study of Conditions Promoting Its Synthesis, *Prikl. Biokhim. Mikrobiol.*, 2001, vol. 37, no. 1, pp. 59–64.