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EXPERIMENTAL ARTICLES

Isolation and Characterization of *Penicillium funiculosum* Mutants with Enhanced Glucose Oxidase Production

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Abstract—After the mutagenesis of *Penicillium funiculosum* with UV light and *N*-nitroso-*N*-methylurea, 83 of 2237 grown colonies were surrounded with increased zones of glucose oxidase diffusion. Analysis of the glucose oxidase activity of selected mutant strains grown in submerged cultures allowed 18 mutant strains to be obtained whose glucose oxidase activity was 5–153% higher (in a medium with glucose) and 4–83% higher (in a medium with sucrose) than that of the parent strain. Two of these mutant strains, UV6.31 and NMU95-132, possessed high glucose oxidase activity when grown in media with glucose or sucrose and produced large amounts of mycelia. The active and morphologically stable mutant *P. funiculosum* NMU95-132 was chosen for further selection work.

Key words: glucose oxidase, mutagenesis, *N*-nitroso-*N*-methylurea, UV light.

The practical application of enzymes is a constantly expanding area of biotechnology. In particular, glucose oxidase (β-D-glucose : oxygen-1 oxidoreductase, EC 1.1.3.4), which catalyzes the oxidation of β-D-glucose into β-D-glucono-δ-lactone, is widely used in the food and chemical industries, medicine, and scientific investigations [1–4]. Raising the biosynthetic activity of producers is an important stage of enzyme biotechnology. The employment of genetic engineering approaches made it possible to obtain highly active producers of glucose oxidase [5–7]. However, the traditional methods of induced mutagenesis employing various mutagens and their combinations are still successfully used for this purpose [8–11].

Earlier, we selected an active producer of glucose oxidase, *Penicillium funiculosum* G-15, and optimized its growth medium [12, 13]. The aim of the present work was to study the mutagenic effect of UV light and *N*-nitroso-*N*-methylurea (NMU) on *P. funiculosum* G-15 in order to obtain its mutant strains with enhanced glucose oxidase production.

MATERIALS AND METHODS

Penicillium funiculosum G-15 was obtained from the culture collection of the Institute of Microbiology, National Academy of Sciences of Belarus.

To prepare spores, the fungus was grown at 24–26°C on Czapek agar with 2% malt for 6 days. Three milliliters of spores suspended in water to a density of $5–6 \times 10^6$ spores/ml were placed in a petri dish ($d = 9$ cm) and irradiated with UV light from an OKN-IIM quartz lamp placed at a distance of 15 cm from the petri dish. The exposure time was 1, 2, 4, 6, 8, and 10 min.

P. funiculosum G-15 spores were treated with 0.05 and 0.1% NMU for 30–120 min under continuous stirring. After treatment, spores were collected by centrifugation at 10000 *g* for 15 min, washed several times with distilled water, and resuspended in 3 ml of distilled water.

The effect of mutagens was evaluated in terms of the survival rate of spores, the morphological and cultural properties of colonies, and the glucose oxidase activity of mutants.

The survival rate of fungal spores was determined by counting 3-day colonies grown on Czapek agar from single spores and expressed as percent of the control. To obtain colonies from single spores, the agar medium was inoculated with the serial dilutions of spore suspension.

To select a mutant with enhanced glucose oxidase production, mutagenized *P. funiculosum* G-15 spores were incubated for 48 h in liquid nutrient medium containing (%) KNO_3, 0.8, KH_2PO_4, 0.1; KCl, 0.05; $MgSO_4 \cdot 7H_2O$, 0.05, $FeSO_4$, 0.001; glucose, 1.0; and malt, 1.0 (pH 5.0). Outgrown spores were plated on a solid diagnostic medium, which allowed us to estimate the glucose oxidase activity by the diameter of the brown zones of *o*-dianisidine oxidation developed

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around the colonies [11]. Promising *P. funiculosum* mutants were grown in submerged mode in the liquid medium that was optimized for glucose oxidase production by the parent strain [13]. This medium was inoculated to a density of (4–6) × 10^4 spores/ml. The fungal mycelium grown for 96 h was collected by filtration, washed, dried at 105°C to a constant weight, and weighed. The culture liquid filtrate was used for the respective analyses.

Reducing sugars were determined with 3,5-dinitrosalicylic acid [14]; pH was measured potentiometrically.

The activity of glucose oxidase was measured by the spectrophotometric method [15] in a modification of Markwell *et al.* [16] and expressed either in U/ml culture liquid or in U/mg dry mycelium.

The morphological characteristics of mutant colonies were determined after their growth for six days on wort agar, Czapek agar, malt Czapek agar, Raistrik agar [17], and differential–diagnostic agar [11].

RESULTS AND DISCUSSION

The exposure of *P. funiculosum* G-15 spores to UV radiation for 1–10 min decreased their survival rate from 40.6 to 0.0027%. The maximum number of mutants with enhanced glucose oxidase synthesis was produced by UV irradiation corresponding to a 40.6% survival rate. According to data available in the literature, optimal survival rates for obtaining *Aspergillus niger* and *Penicillium variabile* mutants with enhanced glucose oxidase production are 33–78 and 52%, respectively.

The mutagenic effect of NMU depended on both its concentration and the time of treatment of the spore suspension; the latter factor primarily influenced the frequency rate of positive mutations. The maximum yield of mutants with improved glucose oxidase production (10.32 and 3.43%) was observed after 1.5-h treatment with 0.05 and 0.1% NMU, respectively.

It should be noted that the original population of *P. funiculosum* G-15 grown on the differential–diagnostic medium was uniform in the cultural and morphological properties of colonies: among the 200 colonies examined, only one colony differed from the others in size and color (i.e., the frequency rate of the appearance of morphologically altered colonies was 0.5%). Treatment with the mutagens gave rise to four colonial morphotypes. Morphotype I colonies were grayish green and flat. Colonies of morphotypes II and III were white and convex, either fluffy (morphotype II) or velvety (morphotype III) in consistence. Morphotype IV colonies were gray-brown, leathery, convex, and wrinkled.

As can be seen from Table 1, mutagenesis increased the number of morphologically altered colonies to 47.3–69.9%. The mutagenic efficiency of NMU was higher than that of UV light. The frequency rate of the appearance of morphotype II mutants under the action of 0.1% NMU was more than 60%. Morphotype III mutants appeared irrespective of the duration of NMU treatment. The maximum number of mutants of all morphotypes was observed after 60-min treatment with 0.1% NMU. The frequency rate of the appearance of the morphological mutants of *P. funiculosum* G-15 under the action of UV light was lower than under the action of NMU; the morphological diversity of mutants increased as the survival rate decreased from 1.65 to 0.021%.

Table 1. Effect of mutagens on the survival rate of *P. funiculosum* G-15 spores and the frequency rate of morphological mutants

Mutagen		Survival rate of spores, %	Frequency rate of morphological mutants, %			
			Morphotype I	Morphotype II	Morphotype III	Morphotype IV
UV:	1 min	40.62	52.7	47.3	0	0
	2 min	15.75	47.5	52.5	0	0
	4 min	1.65	46.3	50	3.7	0
	6 min	0.86	43.5	56.5	0	0
	8 min	0.24	47.4	48.4	4.2	0
	10 min	0.021	46.9	48.9	4.2	0
0.05% NMU:	60 min	22.81	46.8	50	0	0
	90 min	5.15	36.3	58.9	4.8	0
	120 min	0.14	31.8	62.8	5.4	0
0.1% NMU:	30 min	2.55	31.3	64.2	4.5	0
	60 min	1.34	32.9	61.9	5.2	0
	90 min	0.27	30.1	64.3	5.6	0.25
	120 min	0.027	31.5	63.7	4.8	0
Control		100	99.5	0.5	0	0

Based on the diameter of the glucose oxidase diffusion zone around colonies (a total of 2237 colonies were examined), we selected 37 UV-induced and 46 NMU-induced *P. funiculosum* mutants that had the maximum diameter of this zone. All the mutants selected turned out to belong to morphotypes I and II. Analysis of the glucose oxidase activity of these mutants grown in submerged mode led us to select 18 mutant strains whose glucose oxidase activity was 5–153% higher (in the medium with glucose) and 4–83% higher (in the medium with sucrose) than that of the parent strain. Among these mutants, only seven strains possessed glucose oxidase activity (calculated per 1 ml of culture liquid or per 1 mg of mycelium) that was 50% higher than that of the parent strain (these mutants are listed in Table 2).

Table 2. Production of glucose oxidase by *P. funiculosum* strains

Strain *P. funiculosum*	Carbon source*	pH	Biomass, mg/ml	Reducing sugars, mg/ml	Glucose oxidase activity			
					U/ml	% of the control	U/mg	% of the control
UV1.22	1	3.1	3.69	10.83	11.21	212	3.04	349
	2	3.1	5.92	21.80	6.90	111	1.17	101
UV1.25	1	3.1	3.61	11.08	9.49	180	2.63	302
	2	3.1	5.43	28.00	8.42	136	1.56	99
UV1.30	1	3.2	3.67	7.40	13.36	253	3.64	418
	2	3.2	5.35	28.70	6.61	107	1.24	105
UV6.31	1	3.3	3.41	7.20	8.23	156	2.42	278
	2	3.3	2.97	17.20	11.29	183	3.80	242
NMU95.132	1	3.2	3.21	10.08	6.38	121	1.99	228
	2	3.3	3.02	23.42	11.09	179	3.67	257
NMU95.173	1	3.2	3.08	10.83	6.01	114	1.95	224
	2	3.3	3.35	22.00	9.66	156	2.88	198
NMU95.243	1	3.3	3.98	14.40	5.87	111	1.47	170
	2	3.4	4.04	33.58	10.49	170	2.59	165
G-15 (control)	1	3.2	6.43	7.20	5.28	100	0.87	100
	2	3.2	3.94	32.67	6.18	100	1.57	100

* 1 indicates 4% glucose and 2 indicates 6% sucrose.

Table 3. Morphological characterization of mutant *P. funiculosum* strains

Diagnostic medium	*P. funiculosum* NMU95.132	*P. funiculosum* UV6.31
Raistrik agar	Colonies are yellow, fluffy, and convex, the reverse is orange, $d = 1.5$ cm	Colonies of morphotype I are yellow and flat, $d = 0.8$ cm; colonies of morphotype II are white, fluffy, and convex, $d = 1.2$ cm
Wort agar	Colonies are yellowish white, fluffy, and convex, $d = 1.2$ cm	Colonies of morphotype I are yellowish white, fluffy, and convex, $d = 1.2$ cm; colonies of morphotype II are white, fluffy, and convex, $d = 1.7$ cm; colonies of morphotype III are gray-green, fluffy, convex, and radially wrinkled, the edge is white, $d = 2.3$ cm
Czapek malt agar	Colonies are white, fluffy, and convex, the reverse is orange, $d = 2.0$ cm	Colonies of morphotype I are white, fluffy, convex, the reverse is yellow, $d = 2.0$ cm; colonies of morphotype II are gray-green, flat, the reverse is yellow, $d = 2.0$ cm
Czapek agar	Colonies are yellow with a white center, fluffy, convex, the reverse is yellow in the center and orange on the periphery, $d = 0.8$ cm	Colonies of morphotype I are yellow with a white center, fluffy, convex, the reverse is yellow in the center and orange on the periphery, $d = 0.8$ cm; colonies of morphotype II are gray-green, flat, the cone-shaped center is white, the reverse is yellow, $d = 1.0$ cm

In the course of submerged cultivation, the pH of the culture liquid of mutants decreased from 5.0 to 3.4–3.1, irrespective of the carbon source used. The accumulated biomass varied from 2.97 to 5.92 mg/ml and was maximum for mutant strains UV1.22, UV1.25, and UV1.30 grown on sucrose as the carbon source. All the mutants tested, except UV1.30 and UV6.31, consumed glucose more slowly than the parent strain. The glucose oxidase activity of these mutants was 11–153% higher (in the medium with glucose) and 7–83% higher (in the medium with sucrose) than that of the parent strain. If calculated per 1 mg of mycelium, the maximum glucose oxidase production was found in the mutants UV1.22 and UV1.30 grown on glucose (349 and 418%, respectively, as compared to the productivity of the parent strain). In the medium with sucrose, the most active producers of glucose oxidase were strains NMU95.132 and UV6.31. These strains were chosen for further

Table 4. Characterization of the morphological variants of *P. funiculosum* UV6.31

Morphological variant *P. funiculosum*	pH	Biomass, mg/ml	Glucose oxidase activity			
			U/ml	% of the control	U/mg	% of the control
UV6.31.1	3.2	3.55	9.65	211	2.73	343
UV6.31.2	3.1	4.22	1.64	36	0.37	47
UV6.31.3	3.4	5.04	2.46	54	0.46	57
G-15 (control)	3.1	5.61	4.58	100	0.79	100

selection, since they efficiently produced glucose oxidase on both glucose and sucrose (Table 2).

An important biotechnological characteristic of microbial producers is their genetic and phenotypic stability, which can be estimated by different methods: (1) analysis of the morphological stability of colonies grown on special agar media; (2) comparative analysis of cultures grown from the inocula taken from different parts of colonies (the method of DasGupta); and (3) analysis by the cytological (karyological) method [18].

Data on the population composition of mutants subcultured on four different media for 8 months are presented in Table 3. The population composition was studied by the first and third aforementioned methods, examining no less than 300 colonies in each mutant subculture (Table 3).

P. funiculosum NMU95.132 was found to be the most stable mutant strain. At the same time, the mutant strain *P. funiculosum* UV6.31 obtained by UV mutagenesis gave rise to three colonial morphotypes when grown on wort agar. Upon submerged cultivation, only one of the three UV6.31 morphotypes produced 111% more glucose oxidase than the parent strain (the glucose oxidase activity of this morphotype per 1 mg of mycelium was 243% higher than that of the parent strain) (Table 4).

Thus, UV light and NMU can be efficiently used for the mutagenesis of *P. funiculosum* to improve glucose oxidase production. These mutagens affect the morphological stability of *P. funiculosum*. The number of morphological mutants rises with the decreasing survival rate of spores. The maximum number of morphological mutants was observed after the 1.5-h treatment of fungal spores with 0.1% NMU. One-step induced mutagenesis allowed us to obtain 18 mutant strains of *P. funiculosum* with 1.5- to 2.5-fold improved glucose oxidase production. The morphologically stable mutant *P. funiculosum* NMU95.132 is chosen for further selection.

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