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EXPERIMENTAL ARTICLES

Dynamics of Spore Germination and Mycelial Growth of Streptomycetes under Low Humidity Conditions

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Abstract—At extremely low values of moisture pressure (–96.4 MPa; a_w 0.50), the spores of xerotolerant streptomycetes (*Streptomyces odorifer* and *S. rubiginosohelvolus*) germinated, their germ lengths increased, and lateral branching of the mycelium was observed after 5 days of incubation in a thin layer of agarized nutrient medium. At –22.6 MPa (a_w 0.86), the mycelium begins to branch after a 2-day incubation; over a 5-day incubation at –2.8 MPa (a_w 0.98), it goes through a reproduction cycle, which culminates in spore formation. The mathematical model approach enabled us to elucidate the behavioral patterns of *Streptomyces* spores in a thin layer of agarized nutrient medium at low humidity levels. The dynamics of spore germination is governed by the exponential law, which allows calculation of the average duration of the period a before spore germination, as well as the time needed for 50% of viable spores to germinate.

Key words: actinomycetes, *Streptomyces* spore germination, xerotolerance, moisture pressure, water activity

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The energy state of water is an important factor affecting the ability of microorganisms to grow [1]. This state may be expressed in terms of moisture pressure (*P*, megapascals, MPa) or water activity (a_w). Prokaryotes are much more demanding of moisture pressure than fungi, and most of them develop at *P* over –4 MPa (which corresponds to a_w 0.95) [1]. It is generally believed that –70 MPa (a_w 0.60) is the lowest *P* value, at which fungal growth occurs. The fungus *Xeromyces bisporus* is able to grow under these conditions [2–4]. However, the application of special techniques to study spore germination and mycelial growth of actinomycetes revealed that their spores are capable of germination (and their germ lengths increase) at lower *P* values (–96.4 MPa; a_w 0.50) [5–7]. In this case, the experimental conditions (spores on a glass surface) excluded nutrient inflow to the spores, so that the period of their development did not exceed 3 days.

The goal of this work was to study the patterns of spore germination and development in actinomycetes grown on a thin (0.6 mm) layer of agarized nutrient medium in equilibrium with the water vapor at a low moisture pressure.

MATERIALS AND METHODS

The subjects of this study were actinobacteria of the genus *Streptomyces, S. odorifer* strain 1 isolated from an enrichment culture of the cyanobacterium *Oscillatoria terebriformis* (Ag.) Elenk. Emend. and *S. rubiginosohelvolus* strain 1 isolated from apogeotropic roots of the relic cycad fern *Cycas micholitzii* (Tsytsyn Central Botanic Garden, Russian Academy of Sciences, Moscow) [5].

Various levels of water activity (a_w) were created in desiccators using saturated solutions of appropriate salts (Table 1): extremely low (–96.4 MPa; a_w 0.50); approximately comparable to the maximum adsorptive water capacity of soil (–22.6 MPa, a_w 0.86); and approximately comparable to the maximum hygroscopic moisture of soil (–2.8 MPa, a_w 0.98).

The specimen samples were obtained by application of one drop of the streptomycete monospore suspension (10^6 spores/ml) to a thin (0.6 mm) layer of 1.5% nutrient agar (Gauze 1 medium [8]) on slides kept in the desiccator in equilibrium with water vapor at the required saturation level. The drop was spread over an area of 1 cm^2. The specimen was dried to an air-dry state, and the average number of spores contained in

Table 1. Saturated salt solutions used for creating the moisture pressure in the desiccators

Salts	Moisture pressure, MPa	Relative air humidity, %	Water activity
$Ca(NO_3)_2$	–96.4	50	0.50
KCl	–22.6	86	0.86
K_2SO_4	–2.8	98	0.98

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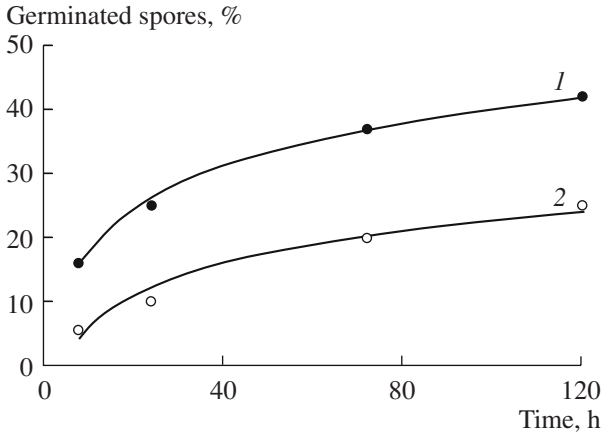

Fig. 1. Germination dynamics of streptomycete spores at a moisture pressure of –96.4 MPa (a_w 0.50): *Streptomyces odorifer* (*1*), *S. rubiginosohelvolus* (*2*).

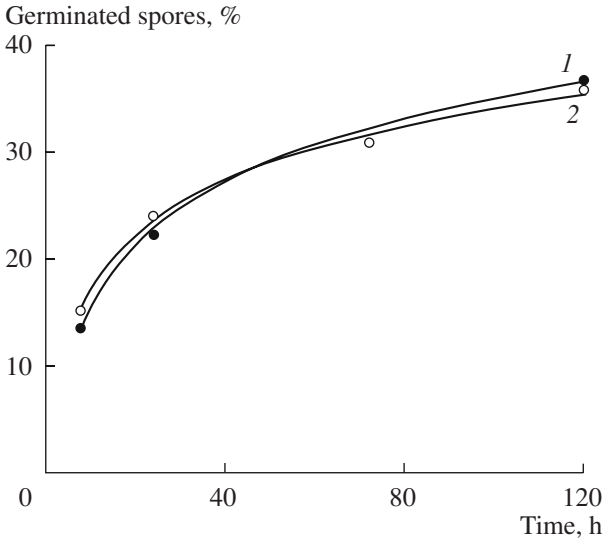

Fig. 2. Germination dynamics of streptomycete spores at a moisture pressure of –22.6 MPa (a_w 0.86). Designations: see Fig. 1.

30 microscope fields was then counted. The slides were then returned to the desiccators with the required level of air humidity and incubated in a thermostat at 28°C. The levels of air humidity in the desiccators were controlled with a Viking AB digital thermal moisture meter with an instrumental error of less than 1%. After 8-, 24-, 72-, and 120-h exposure, the slides were examined under a Zeiss Axiostar light microscope at ×1500 magnification. For each incubation period, germinated spores (with germ tubes) were enumerated, and the size of the germs was determined. For each subsequent exposure period, the measurements were carried out using new specimens from the desiccator.

RESULTS AND DISCUSSION

Even at an extremely low moisture pressure in the thin layer of the agarized medium (*P* –96.4 MPa; a_w 0.50) *S. odorifer* and *S. rubiginosohelvolus* spores germinated, which allowed us to term them xerotolerant microorganisms. The number of germinated spores was found to be highest in the case of *S. odorifer*: after 8-h incubation under these conditions, the amount of germinated spores reached 16% of the total number of spores (Fig. 1); after 120 h of incubation, it reached 42%. The rate of *S. rubiginosohelvolus* spore germination was lower at this level of moisture pressure. After 8-h incubation, the amount of germinated spores did not exceed 6%; by day 5 of incubation it was only 25% (Fig. 1).

At higher *P* (–22.6 MPa, a_w 0.86), the number of germinated spores of both strains increased and reached 36% of the total number of spores after 120-h incubation (Fig. 2).

At *P* –2.8 MPa (a_w 0.98), the rate of spore germination was the highest (especially in the case of *S. rubiginosohelvolus*): after 120-h incubation, 74% of the spores germinated (Fig. 3).

On the whole, the dynamics of spore germination of both *Streptomyces* strains at different humidity levels were similar: at the beginning of the experiment the germination rate was maximal and then gradually decreased; by the end of the experiment (after 120-h incubation), it asymptotically reached the lowest values close to zero.

These findings suggest that the dynamics of spore germination is governed by certain patterns. The works of Hattori [9] were devoted to the search for these patterns. He was the first to derive a theoretical equation (based on the Poisson distribution) describing the dynamics of colony formation on nutrient media.

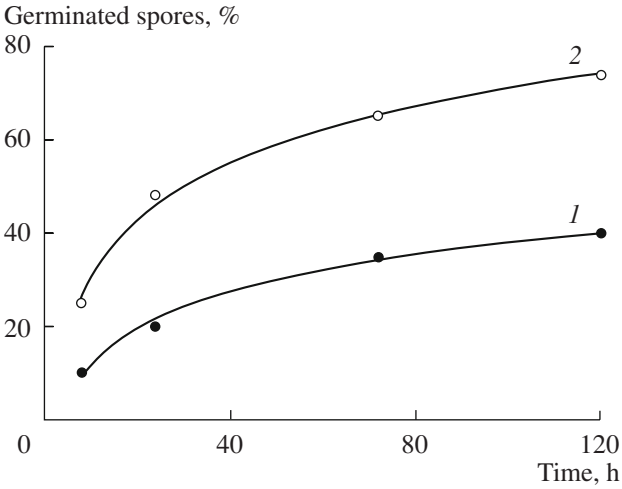

Fig. 3. Germination dynamics of streptomycete spores at a moisture pressure of –2.8 MPa (a_w 0.98). Designations: see Fig. 1.

Table 2. Dynamics of *Streptomyces* spore germination in the course of the experiment

Streptomycete	*P*, MPa	Time (h)			
		0	8	24	72
Streptomyces odorifer	–96.4	43/1.00	27/0.63	18/0.42	6/0.14
S. rudiginosohelvolus	–96.4	23/1.00	18/0.79	12/0.52	3/0.13
S. odorifer	–22.6	41/1.00	26/0.64	17/0.41	5/0.12
S. rudiginosohelvolus	–22.6	41/1.00	29/0.71	20/0.49	
S. odorifer	–2.8	73/1.00	48/0.66	25/0.34	9/0.12
S. rudiginosohelvolus	–2.8	40/1.00	30/0.75	20/0.50	5/0.12

Note: The numerator represents the average number of spores that remain ungerminated by the given time (N_t). $N_t = N_0 - N_a$, where N_a is the number of spores germinated by the given time, and N_0 is the number of spores at the beginning of the experiment (0 h). The denominator represents the N_t/N_0 values.

Table 3. Statistical analysis of the data on the germination dynamics of *Streptomyces* spores obtained during the experiment

Average N_t/N_0 value	1.00	0.70	0.45	0.12
Error of the average N_t/N_0 value (% of the average)	±0	±0.03 (±4%)	±0.03 (±7%)	±0.004 (±3%)
Logarithm of the average N_t/N_0 value, $\log(N_t/N_0)$	0	–0.15	–0.35	–0.90

Kozhevin [10] confirmed the high representativeness of this equation, which coincides completely with the equations describing the kinetics of the first order reactions, as well as the dynamics of radioactive decay [11, 12].

Judging from the results obtained by Hattori and Kozhevin [9, 10], these patterns are true for a number of microbiological objects. To understand whether they operate during the germination of actinomycete spores at low moisture pressure of the agarized medium, let us perform some necessary mathematical operations.

Let us assume that the number of spores (dN) germinating during a certain infinitesimal period of time (dt) increases with an increasing number (at the beginning of this time period) of viable ungerminated spores (N_t). This results in the following differential equation:

$$(dN/dt)_t = -N_t q, \quad (1)$$

where q is the constant rate representing the average probability of germination of each spore over any equivalent time period.

Upon integrating this equation and assuming that, at the beginning of the experiment ($t = 0$), the value N_0 for the number of viable spores (the spores that still remain ungerminated but will germinate by the end of the experiment), we get

$$N_t = N_0 e^{-qt}, \quad (2)$$

Taking the logarithm of the equation, we get:

$$\log(N_t/N_0) = -0.43qt. \quad (3)$$

To find out whether the dynamics of spore germination corresponds to this equation, a plot of the experimental data should be generated, with $\log(N_t/N_0)$, values on the abscissa and time on the ordinate. If the plot is in agreement with the equation, it gives a straight line with the tangent of the slope ratio to the time axis of $0.43q$.

To generate the plot, the N_t and N_0 values were to be determined. For this purpose, the numbers of spores $\{(N_a)_1, (N_a)_2, (N_a)_3$, and $(N_a)_4\}$ germinating over the successive time intervals (t_1, t_2, t_3, and t_4) were determined (Fig. 1–3). From the character of the dynamics, it is clear that almost all viable spores, which were spread on the medium surface at the beginning of the experiment, and which did not require any additional conditions for germination germinated under experimental conditions by the time t_4 (120 h); thus, $N_0 = (N_a)_4$. The number of spores N_t that remained ungerminated at the beginning of each time interval was then determined: $N_t = N_0 - (N_a)_t$ (Table 2). The obtained N_t values (N_1, N_2, etc.) were then divided by N_0. The N_t/N_0 ratios (N_1/N_0; N_2/N_0, etc.) obtained in all the experimental variants were relatively similar: the relative errors of the average values varied from 3 to 7% (Table 3). This fact suggests that different variants of the experiment are elements of the testing of a unified physical totality [12], and we may therefore operate with their average values for each stage of the experiment. The average N_t/N_0 values were found to be 0.7 for 8 h, 0.45 for 24 h, and 0.12 for 72 h (Table 3). The logarithms of each of these values were plotted along the $\log(N_t/N_0)_t$ axis against the corresponding values on the time axis (Fig. 4). The experimental points fell on a straight line.

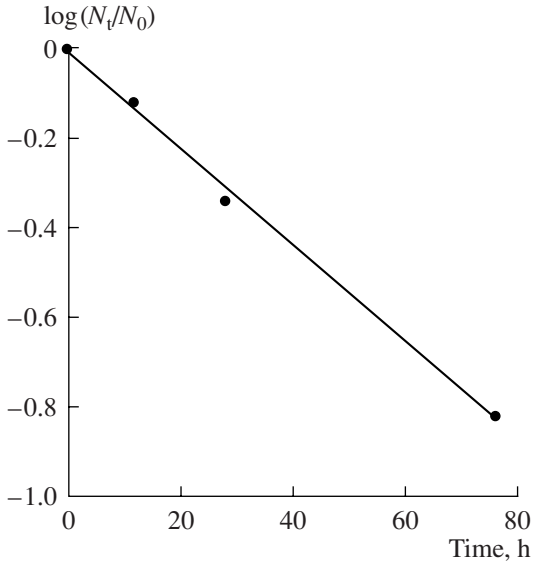

Fig. 4. Germination dynamics of streptomycete spores at different levels of moisture pressure (on average for the two *Streptomyces* cultures and three levels of moisture pressure). N_t, number of spores that remained ungerminated by the given time; N_0, number of viable spores at the beginning of the experiment (equals to the number of spores germinated after 120-h incubation).

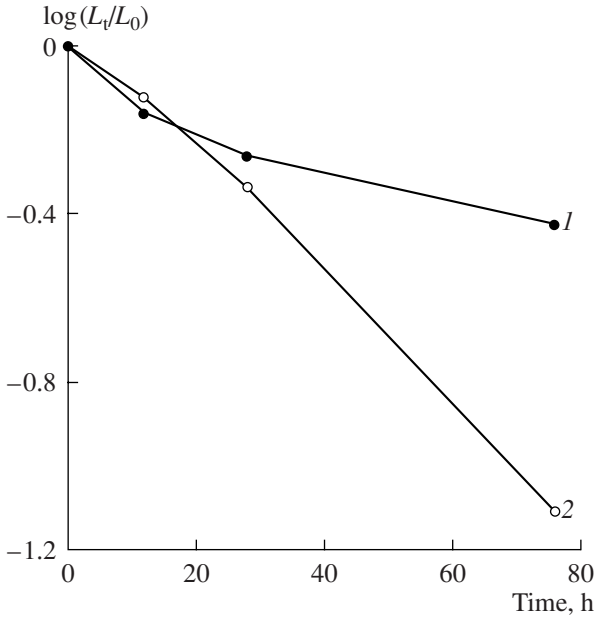

Fig. 5. Growth dynamics of streptomycetes mycelia at different levels of the medium humidity (on average for the two *Streptomyces* cultures at P –96.4 and –22.6 MPa). L_t, length of the mycelium that has yet to grow by the given time as compared to that after 120-h incubation; L_0, mycelium length after 120-h incubation. Designations: see Fig. 1.

This means that the obtained exponential equation adequately describes the process dynamics.

The tangent of the angle of the slope between the obtained straight line and the abscissa $[\log(N_t/N_0)_0 - \log(N_t/N_0)_t]/t$ is proportional to q. Substitution of the relevant data into the equation (3) gives:

$$q = -0.9/(-0.43 \times 72) = 0.029\ [1/\text{h}]. \quad (4)$$

Knowing the q value, we may calculate the average lifespan of the spores under experimental conditions before germination (S) [11, 12]:

$$S = 1/q = 34.5\ \text{h}. \quad (5)$$

The equation (2) is similar to the equation describing the dynamics of radioactive decay; therefore, the time it takes for 50% of viable spores to germinate ($t_{0.5}$) matched the half-life period of radioactive elements [11, 12]:

$$t_{0.5} = 0.7 \times 34.5 = 24.2\ \text{h}. \quad (6)$$

If the dynamics of mycelial growth is governed by the same law, it should be described by the following differential equation:

$$(dL_t/dt) = -L_t\, m, \quad (7)$$

where L_t is the length of the mycelium which "has yet to grow" by the given time and m is the constant rate (similar to q). Upon integrating we obtain:

$$L_t = L_0 e^{-mt}, \quad (8)$$

Taking the logarithm of the equation, we get:

$$\log(L_t/L_0) = -0.43mt. \quad (9)$$

It was demonstrated that this dependency was maintained during the whole experiment (120 h) with *S. rubiginosohelvolus* at P –96.4 and –22.6 MPa. The growth of *S. odorifer* hyphae followed this equation only during the first 8 hours, but then their growth rate decreased (Fig. 5). The different growth patterns of the studied cultures are probably associated with their genetic and physiological characteristics. For *S. rubiginosohelvolus* m = 0.028 [1/h], S = 36 h, and $t_{0.5}$ = 25 h.

As to the further development of streptomycetes, it is noteworthy that spores germinated on a thin agar layer at the lowest moisture pressure (P –96.4 MPa; a_w 0.50) and their germ lengths increased; in the case of *S. odorifer*, lateral branching of the mycelium was observed after 120-h incubation. At P –22.6 MPa (a_w 0.86), branching of the mycelia of both strains was observed after 72-h incubation. At P –2.8 MPa (a_w 0.98), not only branching was observed after 72 h, but also the formation of microcolonies by both strains. After 120-h incubation, *S. odorifer* produced macrocolonies with spores; that is, the strain went through the complete reproduction cycle from spore germination to production of new spores.

Hence, modification of the method based on the use of a nutrient substrate (a thin layer of 1.5% nutrient agar on slides), which is in equilibrium with the water vapor

in a desiccator, allowed us to conduct our experiment in a matter of 5 days. This enabled us to observe not only the spore germination and mycelium growth, but also lateral branching of the mycelia of both actinomycetes even at extremely low humidity (–96.4 MPa; a_w 0.5). The mathematical analysis of the available data on the dynamics of spore germination revealed that this dynamics is governed by the same laws as the first order reactions.

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