

INFLUENCE OF THE LONG-TERM PRESERVATION ON SOME BIOLOGICAL FEATURES OF THREE STREPTOMYCETES STRAINS, PRODUCERS OF ANTIBIOTIC SUBSTANCES

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Summary

The influence of long-term preservation in lyophilized state on the survival, the morphology and antibiotic activity of three streptomycetes strains Streptomyces galbus (F) subsp. achromogenes 695, Str. albogriseolus 444 and Streptomyces sp. T 741 was studied. It was established that viability of the strains depended not only on the conditions of lyophilization but also on the physiological state and strain features of the cultures. The spores of the strain 695, 444 and T- 741 remained viable 13, 21 and 8 years respectively. At the same time an increase of the polymorphism of the strains 695 and 444 and appearance of new morphological types was observed. The method of lyophilization was suitable also for long-term storage of antibiotic activity of the three strains. The activity was retained to 80-90% from initial one.

Introduction

The strains important for industry were usually obtained as a result of long-term selection. A reversion to the wild type accompanied with decreasing of biosynthetic activity had been very frequently observed. The storage of high productive variants at suitable conditions was one way to prevent the production of biological active substances

from sharp decreasing.

The aim of the present investigation was to study the influence of the duration and the maintenance conditions for preservation of the biological activity of three strains: *Streptomyces galbus* (F) subsp. *achromogenes* 695, *Str. albogriseolus* 444 and *Streptomyces sp.* T- 741, producers of antibiotic substances.

Materials and Methods

Microorganisms. The following strains were object of the investigation: *Streptomyces galbus* (F) subsp. *achromogenes* 695, a producer of antitumor antibiotic complex 695 [12] with high activity against Gram positive bacteria; *Str. albogriseolus* 444, a producer of antibiotic complex A-444 with antiviral, antibacterial and antifungal activity [11] and *Streptomyces sp.* T-741, a producer of bipharmycin, an antibiotic complex with antibacterial and antifungal activity [6]. The strains were placed at our disposal by the Department of Microbiology at the Sofia University "St. Kliment Ohridski". The initial cultures were maintained by periodical cultivation. These strains were also preserved by lyophilization and freezing in liquid nitrogen in the National Bank for Industrial Microorganisms and Cell Cultures (NBIMCC 885, 221 and 228).

The bacteria *Micrococcus luteus* NBIMCC 159 and *Bacillus subtilis* ATCC 6633 were used as test-microorganisms for determination of the antibiotic activity of the strains.

Nutritious media and conditions for cultivation. The streptomycetes strains were cultivated on solid mineral medium I of Gauze at al. [5], at temperature 28°C for 10-14 days. The strain 695 was grown in liquid

medium as described previously [14]. Test-microorganisms were cultivated on meat-peptone agar medium at temperature 37°C for 24-48 hours.

Lyophilization. The preservation medium was: sucrose (10 %), skimmed milk (5.5 %) and gelatin (1.5 %). The strains were freeze-dried according to the standard procedure used in NBIMCC. The lyophilized cultures were stored at temperature 4°C and were re-hydrated for 30 minutes at room temperature.

The viability and the population composition of the strains were determined by routine methods [4]. 60-70 single colonies were isolated after a natural spreading from every strain.

The antibiotic activity of 14-day cultures of the strains was determined according to the diffusive method on agar plate and the results were given in mm of diameter sterile zone [3]. *B. subtilis* ATCC 6633 was used as a test-microorganism for determination of antibiotic activity of the strains 444 and T-741 and *M. luteus* NBIMCC 159 – for the activity of the strain 695. Additionally, the antibiotic activity of the strain 695 was given in µg/ml according to Dmitrieva's table towards standard actinomycin D (1 µg/ml) [14].

Results and Discussion

Viability of the streptomycetes strains 695, 444 and T-741 after preservation in lyophilized state

The strain 695 was freeze-dried two times: in 1986 and in 1998 year. The survival of the spores right after the first lyophilization was 17.71 % (Table 1). After four and eight years of preservation in lyophilized state the number of viable spores retained in the same order (10^6) but after another five years (13-year storage) their quantity sharply decreased to 0.05% (Table 2). 86.73 % of the spores survived after

the second lyophilization. In fact, one year of storage did not result in changing of their viability (Table 1).

Only 0.42 % of the spores from the strain 444 survived after the freeze-drying (Table 1). All samples retained the same level of viability until 8-th year regardless of the investigated period. But the survival decreased sharply after 13-year preservation (Table 2).

Table 1. Influence of the lyophilization on the viability of the streptomycetes strains 695, 444 and T-741.

Strain	Year of conservation	Number of spores/ml		Survival (%)
		before	after	
695	1986	1.15×10^7	2.04×10^6	17.71
	1998	1.13×10^7	9.85×10^6	86.73
444	1986	2.91×10^7	1.21×10^5	0.42
741	1991	8.00×10^6	3.40×10^6	42.50

Table 2. Influence of the preservation duration on the viability of the freeze-dried streptomycetes strains 695, 444 and T-741.

Strain	Survival ^a (%)			
	Duration of the preservation (years)			
	4	5	8	13
695	98.03		97.05	0.05
444		80.32	78.69	12.29
741			96.47	

^a Survival according to data in Table 1, accepted as 100 %.

The lyophilization resulted in dying of 57.5 % of the spores of the strain *Streptomyces sp.* T-741 (Table 1). A large number of the survived spores remained viable also after 8-year preservation (Table 2).

The conditions of the freeze-drying, the preservation medium and the re-hydration of the strain 695 were the same at two lyophilization procedures (in 1986 and 1998) but there was a difference in the quantity of survived spores.

There are a lot of other factors that could influence the viability of the strain 695. One of them could be the physiological state of the culture (vegetative mycelium or spores) subjected to lyophilization. It has been shown that the streptomycetes spores are more resistant to temperature changes than vegetative mycelium [10]. It is necessary the culture to be mature and with good sporulation. The time for reaching that phase

varies for the different strains and it should be determined experimentally for every strain.

Another possible explanation for the differences in the viability of the strain 695 after two lyophilizations could be due to the clumping of spores and especially hyphal fragments of the streptomycetes when it was washed from agar surface. After lyophilizations, the clumps could be dispersed easily but unevenly during spreading on agar plates, so we get quite different viabilities. Furthermore, heterogeneous distribution of the hydrophobic spores and mycelial fragments as well as weak homogenization of the culture could give different numbers of colony-forming units per ampoule.

At the same time the spores that survived after freeze-drying retained their viability until the eighth year of the storage of all the three studied strains. Previously obtained data about the viability of

streptomycetes strains in lyophilized state by other authors were heterogeneous [8, 9, 15]. The viability had sharply decreased still after one-year preservation of the following strains: *Str. hygrosopicus*, *Str. coelicolor*, *Str. griseus* and *Str. lividans* 66 [15]. Other strains as *Str. spheroides* and *Str. fluorescens* [8] had possessed relatively high viability after

five-year storage whereas the strains as *Str. erythreus*, *Str. olivaceus*, *Str. venezuelae* [9] had survived and after twelve-year preservation.

Testing of other lyophilizates of the strains 444 and 741 proved presence of survived spores after 21 and 28 years respectively (data not shown).

Population composition of the strains 695 and 444 after preservation in lyophilized state

Our previous investigations showed that the population of the strain *Str. galbus* (F) subsp. *achromogenes* 695 characterized with high natural polymorphisms and consisted of four morphological types colonies varied mainly in the degree of a development of the aerial mycelium [13]. Those types of colonies were in ceaseless relationship and were able to change over from one type to another. The obtained results showed that the conditions of preservation did not influence considerably the population composition of the strain and the first morphological type remained basic for the population (Table 3). New morphological type of colonies with completely lack of aerial mycelium was established in the samples stored 13 years in lyophilized state. This fact could have a connection with the sharp decreasing of the viability of the spores established in these samples.

The strain *Str. albogriseolus* 444 characterized with polymorphisms as the most of streptomycetes. Four morphological types

were established in the population of the strain. The colonies of type **A** were big, protruding, white, with grained surface and crater in the centre; they went gray after 10 days of cultivation and it was difficult to separate them from agar medium. The colonies of type **B** varied in size; they were white, protruding with grained structure and went gray after 7 days of cultivation. The colonies of type **C** were small, flat with a peak in the center. The colonies of type **D** were flat gray with dark gray colored center, which could slightly protruding at some of the colonies (Fig. 1). The relative part of the separated types was: type **A** - 21.76 %, type **B** - 24.13 %, type **C** - 32.35 % and type **D** - 21.76 %. After spreading and plating, the types dissociated and changed over from one type to another but the correlation remained the same. Mainly the types **C** and **B** determined the characteristic features of the strain - color of the aerial mycelium and average antibiotic activity [1].

Table 3. Population composition of the strain 695 after preservation in lyophilized state.

Preservation (years)	Population composition (% from total quantity)				
	Morphological type				
	I	II	III	IV	New type
Initial culture	53.28	19.31	24.88	2.53	-
1	72.78	16.32	9.54	1.36	-
4	46.33	24.75	20.38	8.54	-
13	52.38	9.52	9.52	9.52	19.04



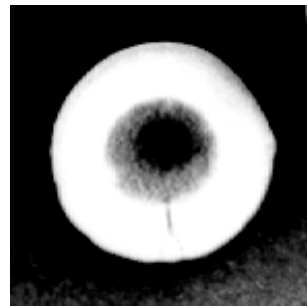
Type A



Type B



Type C

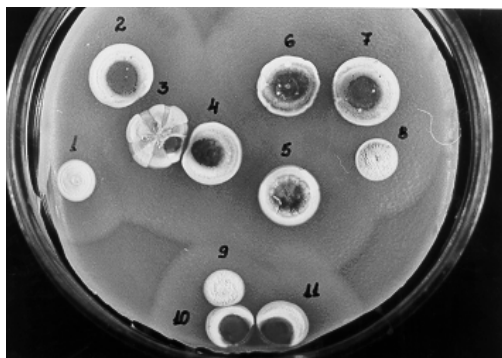


Type D

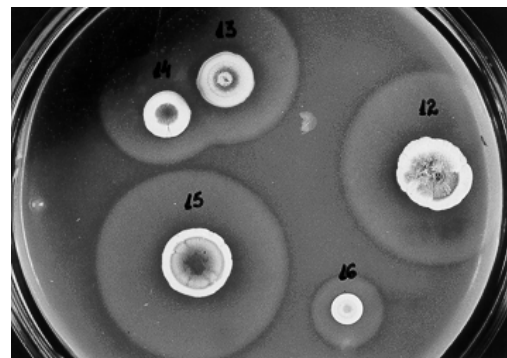
Fig. 1. Basic morphological types colonies of strain *Str. albogriseolus* 444.

After 13 years of preservation in lyophilized state it was not observed any significant change in the distribution of the morphological types in the population of the strain 444. The predominant part of the colonies belonged to the four morphological types typical for the strain (colonies 1, 2, 4, 7, 8, 9, 10, 11, 14 and 16) (Fig. 2). But single colonies, which did not belong to these basic types, came into view. The most frequently, they were big,

with slightly protruding cupola shaped center and several circles (type **E** - colony 6) (Fig.2 a) or with additional radial wrinkles (type **E₁** – colonies 5 and 15) (Fig. 2 a, b). Colonies with irregular shape and radial situated segments without aerial mycelium (type **F** – colonies 3 and 12) had been also observed (Fig. 2a and b). After plating these colonies dissociated into colonies of type **C**, **B** and single colonies from their initial morphological type.



a



b

Fig. 2. Morphological types colonies of strain *Str. albogriseolus* 444.

The advent of the new morphological types at the studied strains after lyophilization is not surprising considering the structural DNA changes which are typical for the streptomycetes and arise spontaneously or under the influence of different mutagenic factors, physiological conditions or as a

result of the method of cultivation and preservation [2]. The new arisen morphological variants are in relatively small quantity for both the strains. These variants dissociate into the basic morphological types after plating and do not cause a change in the correlation typical for the strains.

Antibiotic activity of the streptomycetes strains after preservation in lyophilized state

The separated morphological types of the strain 695 were characterized with definite antibiotic activity (Table 4). The representatives of the first morphological type had the highest antibiotic activity at the periodically cultivated culture as well as at lyophilized one. The results showed slightly decreasing of the antibiotic activity of the strain 695 after preservation in lyophilized state (Table 4 and 5). It could be avoided in practice if a selection of single colonies belonging to the first morphological type and retaining higher activity than the average for the strain was made.

The long-term preservation in lyophilized state did not influence significantly the average antibiotic activity of the populations of the strains 444 and T-741 (Table 6). The activity was 91.49 % and 88.55 % from the

initial one for the strain 444 correspondingly after 13 and 21-year storage. After plating of the lyophilized culture, single colonies with activity 100 % from initial one were isolated. They predominantly belonged to the morphological type **C** and **B**. The activity of the studied colonies from the types **E**, **E₁** and **F** was 4.72 % over the average activity of the population against *B. subtilis* ATCC 6633. After 8-year preservation, the lyophilized culture of the strain T-741 had almost completely retained biological activity – 98.48 % in comparison with the initial one.

Our results confirm the data that the lyophilization is a high effective method for preservation of the streptomycetes antibiotic activity, which had been described in several reports [7, 8, 15].

Table 4. Antibiotic activity of the morphological types colonies of the strain 695 after preservation in lyophilized state (test-microorganism *M. luteus* NBIMCC 159).

Preservation (years)	Average antibiotic activity (mm sterile zone)					
	Morphological type					Mixed culture
	I	II	III	IV	New type	
Initial culture	29	27	26	20	-	28
1	27	24	25	20	-	25
13	24	22.5	24	18.5	16	23

Table 5. Antibacterial activity of the strain 695 against *M. luteus* NBIMCC 159 after preservation in lyophilized state.

Preservation (years)	Antibacterial activity (µg/ml)	% compared to C
Initial culture (C)	140.0	100
1	134.4	96
4	142.3	101
13	120.0	86

Table 6. Antibacterial activity of the strains 444 and T-741 against *B. subtilis* ATCC 6633 after preservation in lyophilized state.

Strain	Preservation (years)	Average antibacterial activity	
		mm sterile zone	% compared to C
444	Initial culture (C)	61.3	100
	13	60	91.49
	21	67	88.55
741	Initial culture(C)	65	100
	8	60	98.48
	28	66	90.90

According to some authors, the variants without sporulation or proactinomycetes like colonies of the population are eliminated by lyophilization because of the less resistance of the vegetative mycelium to freezing and desiccation [10]. As a rule these variants have a low antibiotic activity. It is possible it becomes "improvement" of the qualitative composition of the population of the strains-producers at the expense of the dying of the

low active variants and increase of the relative content of the active colonies from the basic type. Moreover, it is established that the polymorphisms of the strains increases and new morphological types colonies with relatively high activity come into view. The combination of these factors could be a reason that the antibiotic activity of the populations retains almost the same as the initial level regardless of sharp decreasing of their viability.

Conclusions

The long-term preservation of the strains *Streptomyces galbus* (F) subsp. *achromogenes* 695 and *Streptomyces albogriseolus* 444 in lyophilized state resulted in increasing of their polymorphisms.

The method of lyophilization was suitable

for long-term storage of the strains *Streptomyces galbus* (F) subsp. *achromogenes* 695, *Streptomyces albogriseolus* 444 and *Streptomyces* sp. T-741. In lyophilized state they conserved their antibacterial activity over 80-90% from the initial one.

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ВЛИЯНИЕ НА ПРОДЪЛЖИТЕЛНОТО СЪХРАНЕНИЕ ВЪРХУ НЯКОИ БИОЛОГИЧНИ СВОЙСТВА НА ТРИ СТРЕПТОМИЦЕТНИ ЩАМА, ПРОДУЦЕНТИ НА АНТИБИОТИЧНИ ВЕЩЕСТВА

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Резюме

Проследено е влиянието на дългосрочното съхраняване в лиофилно състояние върху преживяемостта, морфологията и антибиотичната активност на три стрептомицетни щамове *Streptomyces galbus* (F) subsp. *achromogenes* 695, *Str. albogriseolus* 444 и *Streptomyces* sp. T 741. Установено е, че преживяемостта на щамовете зависи не само от условията на лиофилизация, но и от физиологичното състояние и щамовите особености на културата. Спорите на щамовете 695, 444 и 741 остават жизнеспособни съответно 13, 21 и 28 години. Едновременно с това се наблюдава повишаване на полиморфността на щамове 695 и 444 и появата на нови морфологични типове колонии. Методът на лиофилизация е подходящ и за дългосрочно съхраняване на антибиотичната активност на трите щамове, която се запазва на 80-90 % от изходната.