THE VIABILITY, POPULATION COMPOSITION, YIELD OF BIOMASS AND LIPIDS OF *Streptomyces canosus* CNMN-AC-02 STRAIN AFTER FREEZE-DRYING

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Abstract. The effect of freeze-drying temperature (-20°C, -50°C, -80°C) on viability, population composition, qualitative and quantitative composition of lipids in the biomass of the *Streptomyces canosus* CNMN-Ac-02 strain was studied. Storage of *Streptomyces canosus* CNMN-Ac-02 strain was performed on agar media Czapek with glucose and oatmeal agar. Freeze-drying was carried out using the equipment LabCONCO, protective media gelatin 2.5%+ sucrose 7.5%. The biomass was obtained before and after freeze-drying, after cultivation on liquid complex media M-I (basic source of carbon was corn flour) for 5 days at 27°C on agitator. The population composition, quantity of biomass and lipid content varies after freeze-drying. For long-term storage of this strain of *Streptomyces*, freeze-drying at range -20°C is recommended. This temperature range granted the best viability, productivity of biomass, lipid content and composition of the lipids fractions, similar before and after freeze-drying.

Keywords: Streptomycetes; freeze-drying; viability; biomass; lipids; lipids fractions.

INTRODUCTION

Analysis of the recent literature sources show that biotechnology industries need viable and stable cultures according to the biotechnological requirements. Therefore, the development of effective methods of preservation for long-term storage of microbial cultures – objects of biotechnology, represents practical and theoretical interest. Researchers are unanimous in the opinion that more effectively maintain of the viability and preservation of important physiological properties of strains for biotechnology relevant individual selection of optimal methods for long-term storage [2, 27, 28, 35].

The issue of safe preservation of microorganisms, preserving their viability and stability remains very current today, about the steady growth of the need to have strains of microorganisms which retains the original phenotypic characters important both for their identification and for use widely (obtaining certain metabolic products, regulation and combating pathogenic microorganisms to plants, animals and humans).

Besides increasing use of microorganisms, particularly the actinomycetes, characterized by a high degree of natural variability, require the development of effective conservation methods [14, 46, 48].

Actinomycetes are found everywhere in the air, freshwater lakes, seas, widespread in soil, which no doubt is a natural substrate for actinomycetes isolated in the greatest number and variety [11, 15, 26, 33].

Actinomycetes are bacteria, the "plane formed by the mycelia structure to more complex fungi eukaryotic feature, prokaryotic based on the characteristic chemical ultrastructure" [31].

There are numerous literary sources, which describe actinomycetes. Basic research of this group were made by Waksman (1959) [52]; Krasilnikov (1970) [33]; Otoguro (2009) [40]; Zhao (2009) [56]; Grishko (2010) [25]; Janso (2010) [30]; Gasanova (2013) [24] and others.

Wide use of actinomycetes as a source of bioactive substances requires a deep study of their biology. Preservation of viability and properties typical of actinomycetes is related to the difficulties caused by the high degree of variability of this group of microorganisms. Of great importance for the industry is to maintain biochemical potential of producing strain by applying the most effective ways to study its variability and finding the safest method of storage. To eliminate any factor of instability, particular attention is paid to the morphological appearance of strains and optimum conditions for cultivation and conservation [34, 43, 55].

Among actinomycetes, streptomycetes are the largest group, which in recent years are considered not only as a source of different chemical antibiotics but also other biologically active substances such as vitamins, enzymes, amino acids, plant hormones active substances that stimulate germination seeds, acts on growth processes, increase agricultural crop plants. That is why, in agricultural microbiology, directory called biotechnology of soil - which deals with the study and adjust the number of microorganisms in the soil and their metabolites work to optimize the grow productivity of agricultural plants, develop widely [50].

Biochemical studies have shown that the lipids are required components of the microbial cell. They form microbial cell energy reserves, structured cell membrane, which in turn determines the viability, resistance and adaptability of microorganism [42].

Streptomycetes have attracted attention as one of the reserves for preparing these substances because they are the producers of a wide range of physiologically active compounds: antibiotics, enzymes, hormones, vitamins, growth promoters, vaccines against infectious diseases of humans and animals, means of controlling insects and rodents - pest agriculture, etc. [3, 8, 21, 29, 41].

Studies by a number of authors show that among the substances that make up the cells of Streptomyces, a special role belongs lipids [1, 8, 38]. These compounds have various biological activities: antimicrobial (against some Gram-positive and Gram-negative bacteria, yeasts, and yeast-like fungi from the genus *Candida*), immunological, growth-stimulating,

etc. [8]. There are reports of anabolic and adjuvant properties of individual lipids [1]. In addition, the lipid fractions of actinomycetes possess antioxidant activity, and when administered intramuscularly increase the natural resistance and growth rate of piglets and reduce the cost of feed [8]. Particular attention attracted to researchers are phospholipids considered as dynamic components of biological membranes, supporting continuity and stability of membrane organization by delicately balanced decomposition reactions and resynthesize, as well as in terms of their use as bioantioxidants [8, 37].

The variability manifests itself in the nature of cultures growing colonies constituting the population. Colonies differ in shape, size, color, aerial and substrate mycelium, aerial mycelium of the degree and the ability to paint media. At the same culture can change the appearance of the colony: they can be smooth, bumpy, wrinkled. In some cases, colonies can be abundantly covered with aerial mycelium, in others it may be poorly developed or absent altogether. Pigmented culture may discolor [5, 10, 45].

One of the characteristic features of streptomycetes is great variability. Variability can be varied: individual, age, culture and species. Changes may be long and short-term nature with the formation of mutations and variations of long temporary deviations or modifications [10, 19, 33].

As is well known, the choice of storage of biological material is extremely important when working in microbiology and bio engineer, where microorganisms are used as models for studying [7, 20].

In this article are presents the results of studying the effect of freeze-drying at different temperatures on viability, population composition, yield of biomass and lipids of *Streptomyces canosus* CNMN-Ac-02 strain.

MATERIALS AND METHODS

The object of research is the actinomycetical strain *Streptomyces canosus*, identified by Krasilnikov N. A. as *S. canosus* 89 (1970). Strain obtained from the Collection of the Institute of Microbiology of the Academy of Sciences of Russia in 1992 and deposited in the National Collection of Non-pathogenic Microorganisms of Institute of Microbiology and Biotechnology of Academy of Sciences of the Republic of Moldova, as *Streptomyces canosus* CNMN-Ac-02. Strain is a producer of phytohormonal substances (auxins, gibberellins), essential and immunoactive amino acids, lipids which containing polyunsaturated fatty acids [8].

The strain was kept on agar media Czapek with glucose and oatmeal agar [6, 21].

Freeze-drying was carried out using the equipment LabCONCO in flacons, using protective media gelatin 2.5%+ sucrose 7.5%. Cultural properties (description of color, shape, size, profile) of colonies were studied by classical methods [4, 21].

Inoculum was cultivated on liquid mineral media Duloney, in Erlenmeyer flasks of 200 ml during 3 days at 27ºC on the agitator [49].

To obtain a biomass, inoculum in an amount of 8% was added to the flasks with liquid complex medium M-I (basic source of carbon was corn flour) of 200 ml for 5 days at 27°C on the agitator.

To determine the productivity, biomass has been separated from cultural liquid on a centrifuge (5000 r/min during 20 min). Quantity of absolutely dry biomass (ADB) was determined by a weight method [6].

The intracellular lipids were extracted from biomass by Folch method, modified in the laboratory [9].

Fractional composition of the lipids was determined by thin layer chromatography with „Sorbfil" plates (100x150 mm), in the solvent mixture hexane-diethyl ether-glacial acetic acid system (73:25:5), the quantity of each lipid fraction was determined using the method of densitometry [8, 9].

RESULTS

An important step in research is to verify the viability of the strain, because the dates obtained shown the efficiency of the method used and the consequences for the culture, for this to remain viable and continue to possess the qualities necessary for obtain biologically active substances.

To determine the viability rate before and after freeze-drying, at different temperature range, sowing *Streptomyces canosus* CNMN-Ac-02 strain on Petri dishes on agar media Czapek with glucose was made. Watching the growth of strain was made on 7-th, 10-th and 14-th day. Quantity and type of colonies was counted in 10^{-6} dilution by formula:

$$c = (\log UFC ml^{-1}_{fin} / \log UFC ml^{-1}_{in}) \times 100\%$$

where:

$\log UFC\ ml^{-1}_{in}$ - logarithm of the number of units forming colonies before freeze-drying;

$\log UFC\ ml^{-1}_{fin}$ - logarithm of the number of units forming colonies after freeze-drying or storage;

c – the viability of cultures (%) [39].

As could be seen in figure 1, was established that the viability of *S. canosus* CNMN-Ac-02 gradually decreases with the decrease of the thermal freeze-drying, this feature is characteristic of any microbial strain. The results of viability are: before freeze-drying - 100 % of colonies, after freeze-drying at range -20°C – 97.2 % of colonies, at -50°C – 96.6 % of colonies, and at -80°C – 92.9 % of colonies.

It was noted that after freeze-drying at range -20ºC, in population composition of *S. canosus* CNMN-Ac-02 strain, there is a third type of colonies, the color of the substrate mycelium is off-white and the color of the aerial mycelium is white. Dimensions of this type of colonies are between 1.0 and 2.0 mm.

Also was observed that the second type of colonies before freeze-drying with white aerial mycelium was transformed into white-gray color (Figure 2. **B**).

After freeze-drying at range -50ºC following observations were made: in the first type of colonies, aerial mycelium remained white, but the substrate mycelium was changed from white-gray color into off-white; in the second type of colonies, aerial mycelium turned from white to gray, but preserved color of substrate mycelium, which remained white-gray. Colony size remained practically at the same level, but the second type of colonies had increase from 1.0 mm - in 4.0-6.0 mm (Figure 2. **C**).

Comparing the composition of the population *S. canosus* CNMN-Ac-02 strain before and after freeze-drying at range -80ºC, the following observations were made: both types of colonies of aerial mycelium color remained the same (white), but the color of the substrate mycelium has undergone radical changes. In the first type of colonies, the substrate mycelium had white-gray color and after freeze-drying at range -80ºC – pale-yellowish; and at the second type of colonies the color transformed from white-gray into gray. It was also observed that the first type of colonies have increased in size from 4.0-7.0 mm - to 4.0-8.0 mm, but instead of the second type of colonies, were observed a new type of colonies with white aerial mycelium and that with the substrate mycelium - gray, with size 1.0-1.5 mm (Figure 2. **A**, **D**).

As could be seen in table 2, was determined that the amount of absolute dry biomass (ADB) produced by *S. canosus* CNMN-Ac-02 strain freeze-dried at range -80ºC, increased by 50% compared to the control, but after freeze-drying at range -20ºC and -50ºC it decreased by 25 and 35% respectively. The dates described strains before and after freeze-drying are shown in Table 1.

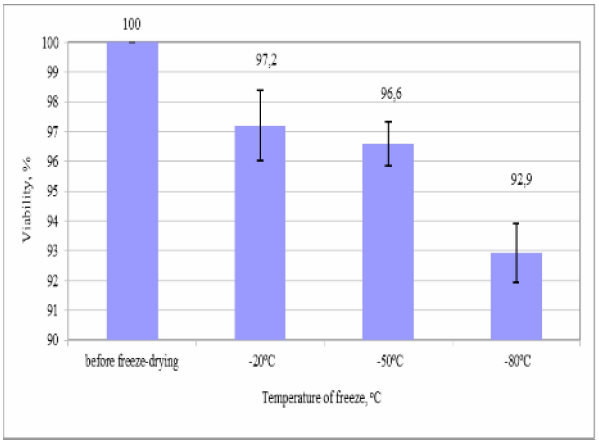

Figure 1. The Viability of *S. canosus* CNMN-Ac-02 strain before and after freeze-drying, %

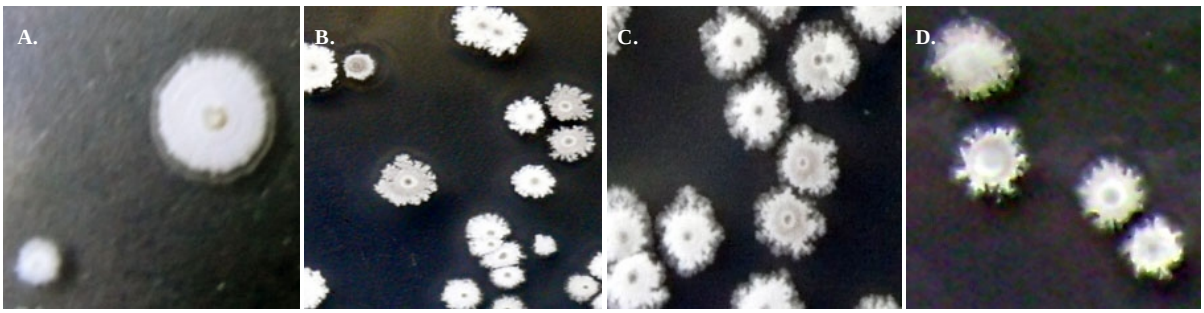

Figure 2. Colonies of *S. canosus* CNMN-Ac-02 strain before freeze-drying **A.** and after freeze-drying with temperatures of -20ºC **B.**, -50ºC **C.** and -80ºC **D.**

Table 1. Cultural characteristics of *S. canosus* CNMN-Ac-02 strain before and after freeze-drying

Temperature	Color of mycelium		Colony size, d (mm)	%
	Aerial	Substrate		
Before freeze-drying	White	White-gray	4.0-7.0	45.3
	White	White-gray	1.0	54.7
After freeze-drying at range -20°C	White	Off-white	4.0-6.0	79.3
	White-gray	White-gray	4.0-7.0	14.4
	White	Off-white	1.0-2.0	6.3
After freeze-drying at range -50°C	White	Off-white	4.0-7.0	89.5
	Gray	White-gray	4.0-6.0	10.5
After freeze-drying at range -80°C	White	Pale-yellowish	4.0-8.0	46.5
	White	Gray	1.0-1.5	53.5

Table 2. Quantity of biomass and lipids synthesized by *S. canosus* CNMN-Ac-02 strain

Temperature, °C	ADB, g/l	Lipids in ADB, g/l	Lipids, % in ADB
Before freeze-drying	2.197±0.06	0.136±0.01	6.19±0.09
After freeze-drying at range -20°C	1.536±0.05	0.0676±0.002	4.4±0.23
After freeze-drying at range -50°C	1.353±0.12	0.0525±0.002	3.88±0.15
After freeze-drying at range -80°C	3.16±0.08	0.1885±0.004	5.96±0.36

Was determined that the most big quantity of lipids was synthesized by strain before freeze-drying. The insignificant decrease in lipid quantity was observed after freeze-drying at range -80°C, but quantity of lipids decrease essential after freeze-drying at range -20°C and -50°C.

From the literature it is known that the population of microorganisms is not uniform, consists of several subpopulations, different by various physiological properties. Therefore, any factor or condition, controlled or uncontrolled, influencing the subpopulation that leads to increasing heterogeneity of culture, expressed in size, coloration, the ratio of colonies [36].

The dissociation of the population by morphological and physiological variants on different types, it is observed in most bacteria, actinomycetes of which are distinguished by a high degree of heterogeneity. It was established that the morphological polymorphism of *S. oligocarbohilus* sp. 5589 and features polymorphism correlated with antimicrobial activity and antibiotic resistance in the nature of dissociative and accompanied by specific genomic changes [16]. At the *S. violaceus* strain which is primary producer of lecithin also correlation was seen between the variability of some morphological features and properties of the antagonistic variants [32].

After cultivation of *S. canosus* CNMN-Ac-02 before and after freeze-drying at different temperatures, the population of the colonies as description submitted the following reports:

- before freeze-drying - two types of colonies, with white aerial mycelium color, different in size 4.0-7.0 mm and 1.0 mm, in the ratio of 45.3 and 54.7%;
- after freeze-drying at range -20ºC - three types of colonies, with white-gray and white aerial mycelium color, with size 4.0-6.0 mm, 4.0-7.0 mm and 1.0-2.0 mm, the ratio is 79.3%, 14.4% and 6.3%;
- after freeze-drying at range -50°C - two types of colonies with white and gray aerial mycelium color and size 4.0-7.0 mm and 4.0-6.0 mm, the ratio is 89.5 and 10.5%;
- after freeze-drying at range -80°C - two types of colonies, with white aerial mycelium color, size 4.0-8.0 mm and 1.0-1.5 mm, the ratio is 46.5 and 53.5%.

Determination of lipid content in the biomass before and after freeze-drying of this strain demonstrated that before and after freeze-drying at range -80°C, almost equal - 6.2 and 5.96%, which may be explained by population of identical content.

After freeze-drying of this strain at range -20ºC and at -50ºC, caused changes in the population, which is the main reason that influenced the reduce amount of lipids in biomass after its cultivation on complex liquid medium M-I.

So come in agreement with data from literary sources, it is noted that actinomycetes are distinguished by high population polymorphism. Causes and mechanisms influencing this phenomenon are currently under-researched and require additional research [22].

In figure 3 are presented the results of determining the amount of the major lipid fractions in lipid biomass of *S. canosus* CNMN-Ac-02 strain before and after freeze-drying at different temperatures.

It is evident that their number does not change after freeze-drying equally. For example, the phospholipids fraction after freeze-drying for all three modes decreased, but most of all - at -50°C (by 33%).

Number of sterols decreased slightly at -80°C (by 3%), and at -20°C and at -50°C noted their increase, especially after at -20°C (by 28%).

Triglycerides lipid fraction of biomass after freeze-drying at range -20°C decrease by 5%, while at -50°C and at -80°C noted its decrease (by 4% and 3%).

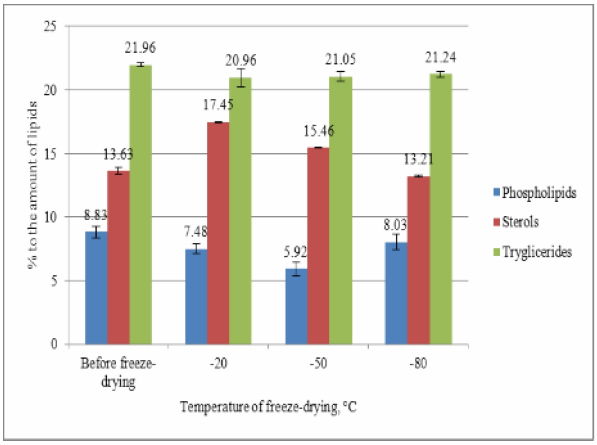

Figure 3. Quantity of the major lipid fractions in lipids of *S. canosus* CNMN-Ac-02 strain

Changes in the content of lipid fractions as mono and diglycerides in biomass of studied streptomycetes provided in figure 4.

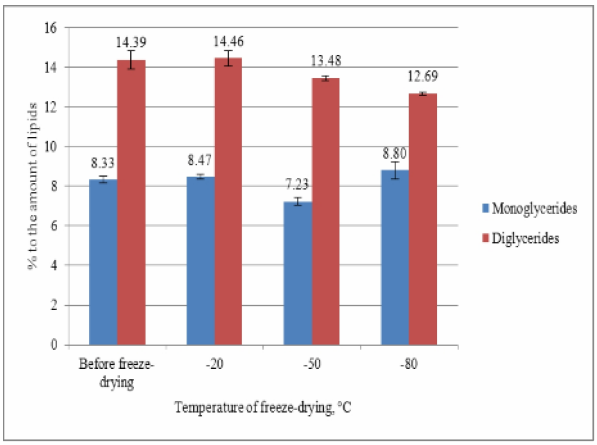

Figure 4. Quantity of mono- and diglycerides in lipids of *S. canosus* CNMN-Ac-02 strain

Could be seen a slight increase in fractions of monoglycerides after freeze-drying at range -20°C and -80°C (by 2% and 6%) and a decrease by 13% at -50°C.

Quantity of diglycerides in lipid biomass of *S. canosus* CNMN-Ac-02 strain after freeze-drying at range -20ºC and at -50ºC was almost identical to the control, was observed a significant decrease of this fraction after freeze-drying at range -80ºC (by 12%).

In the separation of lipid fractions by thin layer chromatography, was found a spot that corresponded to the total fraction of esters sterols and waxes. The smallest quantity was after freeze-drying at range -20ºC (26.68%), and most of all - after freeze-drying at range -50ºC (36.84%).

Typically, the separation of the lipid fraction of the gray group of streptomycetes was marked with presence of unidentified fraction, which is located between phospholipids and monoglycerides. After cultivation of *S. canosus* CNMN-Ac-02 strain on complex media M-I, it 5.8% to total lipid fraction. After freeze-drying at range -20ºC its share was less (by 23%), increased after freeze-drying at range -80ºC (by 16%) and almost absent after freeze-drying at range -50ºC.

DISCUSSIONS

From the literature it is known that long-term storage of microorganisms influence decrease in viability and change in composition of population. Thereupon, dominant phenotype may be substituted with other modified properties and productive activity [13].

Studies have shown that long-term storage of periodic transfer of *S. canosus* CNMN-Ac-02 strain resulted in a decrease in biosynthetic activity. Thus, it is noticeable that such a quantity of biomass after cultivation on complex media M-I declined from 4.37 g/l to 2,197 g/l (by 50%), according to Postolachi O. (2009). The lipid content in biomass remained almost at the same level - 6.22% and 6.19% [44].

However, were seen major changes in the amount of lipids fractions, thus, the number of phospholipids and sterols decreased by 25-40%, and triglycerides fractions has increased by 37%.

During the experiments it was observed that the method of storage of the studied strains influences not only the safety of biosynthetic activity, but most importantly on the composition of the population of a given microorganism.

Our dates are consistent with the results of studies on storage actinomycetes obtained by Russian and American scientists [23, 47] and show the need for further storage of the strain by another method - by freeze-drying, as recommended by scientists Egorov N. S., (2004) [21]; Detushev K. V., (2010) [17]; Vazhinskaya I. S., (2012) [51].

It is believed that the freeze-drying process often leads to the selection of the most resistant cells in the culture that may not possess the desired properties. Operating parameters of freeze-drying (freezing rate, drying temperature, drying rate, etc.), providing a significant impact on the viability of microorganisms [18].

Preservation of viability and properties typical of actinomycetes is about difficulties caused by the high degree of variability of this group of microorganisms [43].

Currently lipids are considered not only as a nutrient reserve, but also essential biological components of the cell. In connection with that phospholipids range from 40.0 up to 90.0% of total lipids, attention is increasingly being driven researchers study role in protecting cell membranes to various environmental actions. By changing the composition of membrane lipids can be reduced negative action of external factors [8, 53, 54].

There have been reports that the amount of lipids in microbial biomass correlates with the change of the morphological and physiological characteristics. The authors believe that the change in the hydrophobicity of the cell wall of microbial cell allows adapting more rapidly to the various environmental changes, including different types of organic substrate [12].

In conclusion, the results obtained can be used when choosing storage of actinomycetes strains for a long time in the collections, as established that periodic transfer actinomycetes significantly reduce the biosynthetic activity of the strains. Study of influence of freeze-drying on the viability, biomass productivity and lipid content and major lipid fractions can recommend for *Streptomyces canosus* CNMN-Ac-02 temperature range -20ºC, because when it awarded the best survival, productivity and similar lipid fraction composition as before freeze-drying.

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