ANTIMICROBIAL ACTIVITY OF *Streptomyces levoris* CNMN-Ac-01 AFTER LONG-TERM STORAGE BY SUBCULTURING ON DIFFERENT COMPOSITION MEDIA

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Abstract. The paper deals with the results of a research of changes in antimicrobial activity of strain *Streptomyces levoris* CNMN-Ac-01 on synthetic and complex media after long-term storage by subculturing (10 years). Antimicrobial activity was determined by the disk diffusion method, as test cultures were chosen opportunistic pathogenic bacteria and fungi spread in the Republic of Moldova. It was experimentally proved that the strain lost antimicrobial activity to a greater extent in relation to opportunistic pathogenic fungi, especially after cultivation on a complex medium (by 20.0-23.5%), while on a synthetic medium Czapek it is less (8.37-22.2%). It was established that the strain isolated from the soil of the Republic of Moldova differs from other strains of the same species in its ability to inhibit the growth of such fungi as *Aspergillus flavus*, *Penicillium expansum* and strains of the genus *Fusarium*. Studied strain has the ability to synthesize not only antibiotics levorin and levoristatin, but also probably, and other substances with antifungal activity.

Keywords: *Streptomyces levoris*; antimicrobial activity; subculturing; long-term storage; synthetic media; complex media.

INTRODUCTION

After the discovery in 1940 of valuable medicinal drug penicillin, the concept of "antibiotic" or "antibiotic substance" became firmly established in science and in daily life of people. For a relatively small history of the existence of the concept of "antibiotic", it was interpreted by researchers in different ways. The correct, most likely, definition was given by Egorov N.S.: "Antibiotics are specific products of vital activity or their modification, possessing high physiological activity in relation to certain groups of microorganisms (bacteria, fungi, algae, protozoa) or against malignant tumors that selectively retard their growth or completely inhibit the development" [14]. The authors note, that antibiotics have a high biological activity against susceptible microorganisms, for example, they even in very low concentrations show a high physiological effect. They possess the selectivity of action, and a number of antibiotics along with antibacterial properties may exhibit an immunomodulatory effect or act as inhibitors of enzymes that inactivate practically significant antibiotic substances [4, 5, 17, 34, 45, 55, 65]. There is an opinion, that antibiotics are not intermediate metabolic products of the organism (metabolites), but final products of metabolism which accumulated inside the cell and released into the environment [14].

One of the most important problems of modern medicine is the search for new antibiotics, in connection with the manifestation of resistance by pathogens [1, 41, 48]. Synthesis of antibiotic substances by microorganisms is only one of the forms of microbial antagonism. The biosynthesis of antibiotic substances is a specific feature of a species or even a strain of microorganisms, resulting from their evolutionary development as one of the adaptive features [57].

It is known that actinobacteria often simultaneously synthesize several polyene antibiotics that are similar in their physicochemical properties. Were obtained mutants differed by ratio in the culture supernatant of levorin and levoristatin. Of particular interest are antibiotics with different composition, which affects the chemotherapeutic activity, toxicity and stability of antibiotics, that is, improves the quality of the drug [11, 33, 43, 64].

According to many researchers, the synthesis of antibiotics by microorganisms after laboratory cultivation conditions does not manifest itself in all organisms: only 40-70 % of streptomycete strains have antibiotic activity, and the rest are inactive. However, under appropriate cultivation conditions, the so-called inactive strains of streptomycetes are able to produce antibiotic substances in varying degrees in laboratory conditions [14, 27, 36, 61].

The most significant factors affecting the manifestation of the antibiotic properties of microorganisms include: the composition of the medium, its active acidity, cultivation temperature, methods of joint cultivation of two or more types of microorganisms, etc. By the nature of the composition, all culture media can be divided into 2 main groups: natural media of indefinite composition and synthetic media. The advantage of natural media of indefinite composition is that many representatives of different types of microorganisms are growth well on them and accumulate biomass, since they contain all the components necessary for the growth and development of the microbial cell. However, it should be borne in mind that some strains of streptomycetes growth well on different by composition natural media, accumulating abundant biomass, but did not produce antibiotic substances or in small quantities under these conditions. In addition, the composition of natural media is not constant because of the not standardized composition of the vegetable or animal ingredients.

Therefore, to obtain comparable results and especially to study the physiological and biochemical characteristics of the microorganism, synthetic media are used, contained in their composition certain chemically pure ingredients taken at precisely specified concentrations [21, 37, 46, 49, 54].

There are a lot of scientific sources about the effect of nutrient media on growth, biomass accumulation, synthesis of antibiotics and other biologically active substances of the strain *S. levoris*. Kuznetsov V.D. et al. considered the most favorable medium for obtaining a complex of lytic enzymes is medium with maize content [30]. To obtain highly active producers of levorin and amphotericin, Shabas et al., chose the Czapek medium with starch and with various additives. In order to obtain lipoteichoic acid from this strain, the authors cultivated it on a medium with peptone and glucose [44, 52]. Other authors emphasized the importance of microelements in the development of microorganisms which are producers of antibiotics, in particular, the cultivation of *S. levoris* with took into account the microelement composition of the medium (Fe, Cu and As) and analyzed its relationship with the main parameters of antibiotic production [38, 53].

Microorganisms, and especially actinobacteria, are very variable during conventional storage methods [20]. Often a loss of activity is observed during the cultivation of microorganisms on rich media and with frequent subculturing. By changing the composition of the medium, it is possible to direct the biosynthetic activity of microorganisms for obtain previously known biologically active substances, change their proportion and their activity [14]. For the directed synthesize of an antibiotic, various methods of intervention in the metabolism of microorganisms are used:

1 – change in the composition of the cultivation medium;

2 – specific inhibitors are introduced into the culture medium;

3 – the nature of the metabolism of the microorganism associated with the modification of the structure of the synthesize of the antibiotic can be changed as a result of obtaining the certain mutants;

4 – properties of antibiotic substances can be changed as a result of exposure to these antibiotics of one of the microorganisms or enzymes produced by them;

5 – possibility to change the nature of the metabolism by applying a combination of the factors listed above, as example, the use of appropriate mutants with the simultaneous introduction of specific precursors or inhibitors into the medium for their development [14, 51].

Thus, the purpose of the paper was determination the safety of biosynthetical activity (antimicrobial substances synthesis) of the studied strain of *Streptomyces levoris* CNMN-Ac-01 on different nutrient media, after subculturing on synthetic medium Czapek with glucose for 10 years.

MATERIALS AND METHODS

As object of the research served strain *Streptomyces levoris* CNMN-Ac-01 isolated from the samples of soil of central part of Republic of Moldova. The strain was isolated by classic method (Koch), on starch ammonia agar medium (SAA) [39].

Strain was stored by subculturing, using agar medium Czapek with glucose. After 10 years of storage, antimicrobial activity was determined.

For carried researches were used agar media Czapek ($NaNO_3$, K_2HPO_4, $MgSO_4*7H_2O$, KCl, $FeSO_4$, agar, source of carbon – glucose, pH – 7.0-7.3); SAA (K_2HPO_4, $MgSO_4$, NaCl, $(NH_4)_2SO_4$, $CaCO_3$, agar, source of carbon – soluble starch, pH – 7.0-7.4); and complex media M-I ($CaCO_3$, baker's yeast, source of carbon – corn flour, pH – 7.0); SP-I (NaCl, $CaCO_3$, source of carbon – glucose, soybean flour, maize flour, pH – 7.2-7.4).

The strain was cultivated for 2 weeks on mentioned media in thermostat at temperature of 28ºC in Petri dishes for obtaining continuous lawn. During growth strain synthesized substances with antimicrobial properties diffused in agar medium. Antimicrobial activity was determined by disk diffusion method [15].

The following test cultures were used: opportunistic pathogenic bacteria – *Paenibacillus alvei*, *Bacillus larvae*, *Bacillus subtilis*, *Staphylococcus aureus, Clavibacter michiganensis* 13^a, *Xanthomonas campestris* 8003, *Erwinia carotovora* 8982; and opportunistic pathogenic fungi – *Ascosphaera apis, Aspergillus flavus, Aspergillus niger, Fusarium solani, Fusarium oxysporum, Fusarium graminearum*, *Penicillium expansum*, *Candida albicans* [3, 10, 15, 16, 22, 56, 63].

Test cultures of fungi grew up on a wort agar of 5.0ºBlg (pH – 5.8-6.0), and test cultures of bacteria – on a potato agar (pH – 7.0-7.5) [15].

Mentioned test cultures cause various diseases of crops and animals being widely spread in Republic of Moldova.

RESULTS

By this way, were obtained next results: as could be seen in table 1 and 2, at the beginning of the experiment, the strain grown on the agar medium Czapek with glucose, more actively suppressed the growth of some test cultures of bacteria than of fungi. The growth inhibition zones of opportunistic pathogenic bacteria varied between 13.0-20.3 mm and for filamentous fungi 12.0-19.5 mm (except for *A. flavus* with growth inhibition zones up to 24.0 mm). Subculturing to a lesser extent reduced antibacterial activity of strain against opportunistic pathogenic bacteria (by 3.95-5.56 %), while the ability to inhibit the growth of opportunistic pathogenic fungi such as *A. flavus, A. niger*, *F. solani*, *F. graminearum* decreased by 8.37-22.2 %.

After the growth of the strain on the medium SAA antibacterial activity was very small (10.0-11.0 mm – growth inhibition zones of test bacteria) and practically remained at the same level, whereas antifungal activity lack at all in relation to the test cultures of the used fungi in the experiments.

As a result of growth of the studied strain on agar complex medium M-I, antibacterial activity was not the same and decreased by 6.67-11.2 % in relation with test cultures of opportunistic pathogenic bacteria. In these experiments antifungal activity in relation with test cultures of opportunistic pathogenic fungi, decreased in activity to a greater extent up to 20.0 %.

After growing on another medium of complex composition – SP-I, it was noticed that the metabolites of the studied strain after subculturing for a long time caused growth retardation of opportunistic pathogenic bacteria with less activity than at the beginning of the experiment (by 6.51-14.3 %), while antifungal activity of this strain appeared only in relation to 3 out of 7 filamentous fungi selected as test cultures, and the decrease in activity was more significant - up to 23.5 % in case of *P. expansum* (Table 2). Obtained results in our experiments are consistent with the literature data: on the synthetic medium, the spectrum of the test cultures is greater relative to which the studied strain exhibits antimicrobial activity (antibacterial and antifungal activity) than cultivated on media of complex composition (M-I and SP-I).

It was also experimentally established that storing of *S. levoris* CNMN-Ac-01 for a long time (10 years) by subculturing causes a decrease in antibiotic activity both against to test bacteria and filamentous fungi, and the antifungal activity decreases to a greater extent. In addition, it was found that the strain *S. levoris* CNMN-Ac-01 isolated from the soil of R. of Moldova has the ability to retard the growth of some filamentous fungi, but not as actively as the strain of same species from the Collection of Vinogradski S.N. Institute of Microbiology. There are also differences in the description of the antibacterial activity: according to Krasilnikov N.A., strain *S. levoris* does not act on bacteria or suppresses 1-2 types of Gram-positive bacteria (*Bacillus idosus*, *Mycobacterium luteum*) [27]. Ukrainian scientists noted that strain *S. levoris* stored in their collection is an antagonist of Gram-positive bacteria and yeasts, without mention of fungi [60]. According to Egorov N.S., *S. levoris* synthesize a complex of antibiotics. In the process of selection of the producer of levorin, a strain with increased antibacterial activity was obtained, in the mycelium of which there is a second non-polyene antibiotic,

Table 1. Antibacterial activity of *S. levoris* CNMN-Ac-01 after 10 years storage by subculturing

Test culture	Year of experiment	Diameter of growth inhibition zones of test cultures, mm			
		Czapek	SAA	M-I	SP-I
P. alvei	2008	17.0 ± 1.1	12.0 ± 0.7	19.0 ± 0	20.3 ± 0.7
	2018	–	–	–	–
B. larvae	2008	13.0 ± 1.1	0	0	10.0 ± 0
	2018	–	–	–	–
B. subtilis	2008	14.3 ± 0.7	0	0	0
	2018	–	0	0	0
S. aureus	2008	0	0	9.0 ± 0	0
	2018	0	–	0	0
C. michiganensis 13[a]	2008	20.3 ± 0.7	0	22.5 ± 1.1	24.5 ± 1.5
	2018	19.5 ± 1.5	0	20.0 ± 1.1	21.0 ± 0
X. campestris 8003	2008	18.5 ± 0	10.0 ± 0	15.5 ± 0	16.3 ± 0.7
	2018	17.5 ± 1.1	10.0 ± 0	14.0 ± 0	14.5 ± 1.1
E. carotovora 8982	2008	18.0 ± 0	11.0 ± 0	11.5 ± 1.1	12.3 ± 1.1
	2018	17.0 ± 0	10.5 ± 1.1	10.5 ± 0	11.5 ± 1.1

– the experiment has not been done

Table 2. Antifungal activity of *S. levoris* CNMN-Ac-01 after 10 years storage by subculturing

Test culture	Year of experiment	Diameter of growth inhibition zones of test cultures, mm			
		Czapek	SAA	M-I	SP-I
A. apis	2008	12.0 ± 0	0	18.0 ± 1.1	14.5 ± 1.1
	2018	–	0	–	–
A. flavus	2008	24.0 ± 1.1	0	0	0
	2018	21.0 ± 0	0	–	–
A. niger	2008	14.0 ± 0	0	17.5 ± 1.1	18.0 ± 0.7
	2018	12.0 ± 1.1	0	14.0 ± 0	14.0 ± 1.1
F. solani	2008	12.0 ± 0	0	0	0
	2018	11.0 ± 0	0	0	0
F. oxysporum	2008	12.0 ± 1.1	0	0	0
	2018	11.0 ± 0	0	0	0
F. graminearum	2008	18.0 ± 0	0	0	0
	2018	14.0 ± 1.1	0	0	0
P. expansum	2008	19.5 ± 1.1	9.8 ± 1.2	17.5 ± 1.1	18.3 ± 1.7
	2018	14.0 ± 1.1	0	14.0 ± 0	14.0 ± 1.1
C. albicans	2008	14.7 ± 0.7	0	13.0 ± 1.1	15.0 ± 0
	2018	11.3 ± 1.7	0	11.0 ± 0	13.0 ± 1.1

– - the experiment has not been done

levoristatin. The author emphasizes that the levorin synthesized by their strain is less active in relation to filamentous fungi than against yeast-like fungi (genus *Candida* and others) [14].

During studies by Georgian scientists of biological properties of actinobacteria – producers of antibiotics, a strain was found close to the species described by Krasilnikov N.A., which has antagonistic properties against phytopathogenic fungi – *Rhizoctonia* sp., *F. solani* and Gram-positive bacteria (*Staphylococcus aureus*), but weakly acted against yeast and not acted against Gram-negative bacteria, rhizobia, actinobacteria and mycobacteria. It was found that antibiotic compounds produced by this strain are high-molecular substances of protein nature, thermostable, hydrophilic, and resistant to O_2 and a wide pH range [42].

Studied the antagonistic activity of some actinobacteria isolated from the Kazbegi region (Khevi) against phytopathogenic bacteria, the authors also emphasized the significant role in the formation of substances with antimicrobial activity related to the composition of the nutrient medium [35].

Thus, our data are consistent with the literature: long-term storage on the agar medium Czapek allowed to keep the studied strain active, and the decrease in antibacterial activity after 10 years of storage was small – 3.95-5.56 % (with growth on the Czapek medium) and 6.51-14.3 % (with growth on the complex media M-I and SP-I). The decrease in the antifungal activity of the studied strain manifested itself to a greater extent when the strain was cultivated on complex media (by 20.0-23.5 %) and slightly less when cultivated on Czapek medium. Analysis of the change in the antimicrobial activity of the strain *S. levoris* CNMN-Ac-01 isolated from soil of R. of Moldova during long-term storage and subculturing confirmed the assumption we had previously said, that one of the distinguishing features of the strain is the ability to synthesize not only levorin, but also and levoristatin or any other substance with antifungal activity. After growth on the synthetic medium of Czapek with glucose, the strain had the ability to inhibit the growth of not only Gram-positive and Gram-negative bacteria, but also representatives of the genus *Penicillium*, *Aspergillus* and *Fusarium*. According to Egorov N.S., the strain of *S. levoris*, which synthesize levorin, is less active against filamentous fungi [14]. *S. levoris* CNMN-Ac-01, isolated from the soil of the central part of Republic of Moldova, rather actively delayed the growth of *A. flavus* (growth inhibition zones up to 21.0 mm). There was less activity against to representatives of the genus *Fusarium* (growth inhibition zones between 11.0-14.0 mm).

DISCUSSION

Since the time of the research of Koch R. (1843-1910) and up to present days, microbiology is based on one of the basic principles – working with pure cultures of microorganisms [32]. At the same time, modern microbiology, especially industrial microbiology, has accumulated many examples showing that the process of obtaining a particular product of vital activity is more active in mixed cultures, that is, with the joint development of several (most often two) types of microorganisms [37]. Thus, an increase in the biosynthesis of *S. levoris* – levorin is observed with the joint cultivation of streptomycete with the yeast-like fungus *Candida tropicalis* [31, 47].

For our research aimed at enhancing the biosynthetic activity of the strain *S. levoris* CNMN-Ac-01 isolated from the soil of R. of Moldova, which is considered not as an active producer of a specific antibiotic levorin, but as a strain that synthesize complex substances such as amino acids, lipids, substances with antimicrobial properties and compounds that have a stimulating effect on the seeds of a number of agricultural plants are more interesting to scientists about the stimulating effect growth and antibiotic biosynthesis of organic acids, primarily – succinic acid [2, 14, 26, 23, 29].

Such facts from the literature emphasize the possibility of using the method of co-cultivation of 2 mutant strains of *S. nursei* which lost their ability to nystatin biosynthesis: their co-cultivation provided the formation of nystatin in the same amount as during the development of the original active strain [14, 59].

Widely known in the literature is such a phenomenon as a significant decrease in antibiotic activity or its complete loss in streptomycetes isolated from natural substrates during long-term storage in laboratory conditions. Co-cultivation of strains of streptomycetes with some fungi of the genus *Penicillium* or with soil bacteria restores the ability to produce antibiotics or stimulates its accumulation by those strains that did not synthesize them. The study of the causes of the stimulation of the antibiotics production by the strain *S. coelicolor* under the influence of the vital activity of the bacterium *B. rusticus* and fractions of the culture supernatant showed that the stimulation is associated with the fraction of volatile acids. Egorov N.S. also notes that in a mixed culture, not only the selection of strains is essential, but also their quantitative ratio in the medium [14]. An increase in the production of levorin by about 40-50 % is observed when 1-4 % of yeast-like organisms of the genus *Candida* are pre-grown for 48 hours added to the *S. levoris* producer strain [31, 62].

In a series of publications about the biphasic cultivation of *S. levoris*, the effect of the organic phase, the accumulation of biomass and proteolytic enzymes, depending on the composition of the medium and especially the presence of paraffins, Dymshitz et al. described the cultivation of the producer as carried out on a complete fermentative medium containing maize flour, soy flour, hydrol, NaCl, $CaCO_3$, as well as on synthetic medium containing starch and salts - $(NH_4)_2SO_4$, $MgSO_4$, $CaCO_3$, KCl and KH_2PO_4. The

experiments showed that the total amount of biomass in the experimental and control samples was the same, but in the experiment the conditional transition of culture to idiophase was carried out by 1 day earlier, which is a favorable point, in particular, helps to reduce the stationary phase, when the synthesis of the antibiotic levorin occurs [7-9].

A huge amount of new data about the world of microorganisms are scattered at present in various laboratories of the earth. Many well-known collections deal with isolates, and in the context of preserving microbial diversity, an important problem is mainly the variability of isolated and maintained cultures, which confirms the need to strengthen the research of microorganisms *in situ* [6, 12, 25].

It is known that microorganisms of various systematic groups can differ significantly in their sensitivity to the same conditions and storage time. None of the many methods of storing cultures known to date can be regarded as universal. The choice of method of storing biological material is extremely important when carried out any work in the field of microbiology, molecular biology and bioengineering, where microorganisms are used as models for research. Each strain requires an individual approach regarding media, cultivation conditions and especially storage [13, 14, 18].

The experience of long-term storage of industrial strains shows that, during long-term storage, in addition to the loss of cell viability, a process of population variability is observed, when the dominant phenotype is replaced by another with altered initial properties and productive activity [28, 58]. In some phytopathogenic micromycetes, a loss of pathogenicity and ability to produce secondary metabolites as a result of long-term storage by subculturing was observed [24]. To reduce these undesirable effects, was proposed to apply for prolonged storage and subculturing of actinobacteria on the most suitable for this media – SP-I (soy medium), on which spontaneous variability reduced till 2-3 types, when on Gause or Waksman media can be up to 6-7 types [50]. To maintain actinobacteria *Streptomyces* sp. 1618, the authors recommended using the medium based on oatmeal broth by subculturing 1.5 years with a reapplication after 5 years [19]. For long-term storage of the producer of antibiotic litmofungin, was used Czapek medium with glycerin [40].

In conclusion, in order to maximize the preservation of antimicrobial activity, the studied strain of *S. levoris* CNMN-Ac-01 is preferably stored and cultivated on a synthetic medium, as well as to identify ways to increase it by the example of the above recommendations.

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Received: 11 February 2020
Accepted: 24 March 2020
Published Online: 26 March 2020
Analele Universităţii din Oradea, Fascicula Biologie
http://www.bioresearch.ro/revistaen.html
Print-ISSN: 1224-5119
e-ISSN: 1844-7589
CD-ISSN: 1842-6433
University of Oradea Publishing House