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NEW ANTIBIOTIC SUBSTANCES OF THE *STREPTOMYCES ALBUS* ENZYBIOTIC COMPLEX

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The aim. *Extraction and characterization of the metabolic products of antibiotic nature from bacteria Streptomyces albus, analysis of the antimicrobial complex from the perspective of an enzyme preparation development.* ***Methods.*** *Streptomyces albus UN 44, the producer of the bacteriolytic enzymes was used in the study. The antagonistic activity of the culture was determined by the streak plate method. The antibiotic activity was evaluated with the paper-disks method and with the bioautographic assay. Extracted antibiotic substances were analyzed with the thin-layer and liquid chromatography techniques coupled with mass spectrometry.* ***Results.*** *The compounds with antifungal activity were extracted from the S. albus UN 44. These compounds had a partition coefficient of 0.65 and an absorption maximum within 270 – 275 nm. The phenolic group was determined in their molecule too. The compounds were identified as bis(2-ethylhexyl)phthalate and 3-O-methylcyclopolic acid.* ***Conclusions.*** *For the first time there was detected the ability of the S. albus UN 44 to synthesize antibacterial and antifungal antibiotics that are related to phthalaldehyde derivatives.*

Keywords: Streptomyces albus, enzybiotics, antibiotics, bacteriolysins, antagonism, chromatography, antimicrobial spectrum.

Antimicrobial substances are used as a means of different purposes: medical and cosmetic, household and industrial disinfectants, preservatives for food products, etc. In this regard, the relevance of the search for new substances and the development of new antiseptics drugs is not reduced.

However, for a long time researchers have focused their attention on substances that exclude the development of the pathogen's resistance or are specific to pathogens resistant to most antibiotics. Another effective approach to the development of new antimicrobial agents is the combination of drugs with different mechanisms of action or specific to some of the features of the pathogen [1 – 3]. One of such features is an ability to create biofilms, which greatly increases the survival of cells under the influence of antiseptics [2]. Therefore, part of developments focuses precisely on the destruction of this structure. Shown that the combination of the antimicrobial enzyme and fluoroquinolone antibiotic resulted in a synergistic effect on *Staphylococcus aureus,* which is based on the destruction of the biofilm by the enzyme and the subsequent bactericidal action of the antibiotic [3]. The similar approach was used for the development of a new drug "Dispersin" that acts on the biofilms by destroying the cementing material of the biofilm matrix (poly-*N*-acetyl-glucosamine) [4].

In the publications mentioned above, as well as in many other studies and reviews of recent years, there was introduced a term "enzybiotic", which is being used now in relation to antimicrobials with a specific mechanism of action (bacteriocins, cathelicins, lysines, bacteriophages, immunobiotics) as well as to the preparations containing both enzyme and antibiotic [1, 5 – 7]. The authors determine the benefits and broad prospects of such drugs, which significantly improve the effectiveness of antimicrobials, without causing the emergence of resistant forms of pathogens.

In fact, the largest group of enzybiotics is formed by enzymes such as muramidase, N-acetylglucosaminidase, amidase, peptidases and some others which are able to destroy specific bonds of the cell wall of microorganisms. Bacteriolytic (lytic) enzymes, which have been known for a long time are now also mentioned as objects for the production of improved complex preparation [8]. Just these substances, the lytic enzymes, were found amongst the metabolites of *Streptomyces albus* (originally *S. recifensis var. lyticus*) a long time ago that became the basis for prolonged researches and for the development of a number of antiseptic drugs for various purposes [9, 10]. However, some other biological properties of this bacteria were discovered in recent years: stimulation of plant growth [11], antagonistic activity [10], a biofilm destruction activity against some microbial pathogens [12], and others like that. All this stimulated the authors of the current work to make an analysis of the metabolites of this bacteria by aiming at those ones that may have antibiotic activity. An additional, reason to expect the synthesis of such substances by this bacteria was its affiliation to streptomycetes, which are the widest industrial producers of antibiotics.

Therefore the aim of the current work was to extract and characterize those metabolites of *S. albus* that possess the antibiotic activity and analysis of the specificity of the antimicrobial complex as the basis for the development of the enzybiotic preparation.

Materials and methods. A producer of the complex of bacteriolytic enzymes *S. albus* UN 44 from the Museum of the Department of Industrial Biotechnology of Igor Sikorsky Kyiv Polytechnic Institute was used in the study. This bacterium is able to synthesize the enzyme complex, which includes glycosidases, lytic endopeptidases, muramidases, non-lytic proteinases, amylase [9, 10].

Antagonistic properties of this bacterium were studied against the reference strains from the Ukrainian collection of microorganisms at the Zabolotny Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine (Kyiv): *Bacillus subtilis* UCM B-901 (ATCC 6633), *B. subtilis* B-617, *B. cereus* B-908 (ATCC 11778), *Staphylococcus aureus* B-918 (ATCC 6538), *Kocuria* (previously *Micrococcus*) *varians* Ac-613, *Micrococcus flavus* Ac-634 (ATCC 9341); *Escherichia coli* B-906 (ATCC 25922), *Proteus vulgaris* B-905 (ATCC 6896), *Pseudomonas aeruginosa* B-900 (ATCC 9027), *Salmonella* Abony B-921 (NCTC 6017); *Candida albicans* Y-2681 (ATCC 10231), *C. cefyr* Y-899, *C. utilis* Y-984 (LIA-01).

S. albus UN 44 was grown on a liquid medium with glucose and soy flour in 750 ml Erlenmeyer flasks (250 ml of medium) at 28 °C for 96 hours [10].

The antagonistic properties of *S. albus* UN 44 were studied on a Gause's No.2 agar medium by the streak plate method. The bacterium was grown at 37° C for 24 h and, after that, the growth inhibition zones were determined [13]. Evaluation of antibiotic activity was done by the paper-disks methods and bioautographic assay. In the latter case, the thin-layer chromatography plate with a sample on it was used.

Extraction of antibiotic substances from the culture medium was carried out with chloroform in a ratio of 4:1, respectively. To determine the location of antibiotic of *S. albus* UN 44 in the biosynthesis process (namely, exogenously or endogenously) the culture fluid was separated by centrifugation and the antibiotics were extracted separately from the biomass of the cells and from the liquid phase (supernatant). Accordingly, were received an extract of biomass and supernatant, in which antagonistic activity was determined.

The Merk UV – 254 plates and chloroform:toluene:methanol at ratio 5:1:1 were used (a 10% acetic acid was used as a phase modifier) for the thin-layer chromatography. Plates were developed in iodine (I_2) vapors and the separation factor *Rf* was calculated as the ratio of the distance from the starting line to the center of the spot and the solvent, respectively [14]. The development of plates in 1% alcoholic $FeCl_3$ was used as a qualitative reaction to determine the presence of a phenol group in the structure.

The fractionation of the extracts was carried out with column chromatography. A glass column 320 × 26 mm was used. A silica gel with the Brockman activity equal to II and the particle size of 40/100 mkm was used as a sorbent. Solvents (n-hexane, chloroform, isopropanol, and ethanol) and their systems in order of increasing polarity were used to elute the active fractions from the column. The volume of the fractions for analysis was 5 ml. The absorption maxima of the extracts were determined compared to 96% ethyl alcohol with the spectrophotometer Specord (Germany) [15].

Chromatographic analysis of fermentation broth obtained after centrifugation was carried out by HPLC using liquid chromatograph Agilent 1200 with mass spectrometric detector Agilent G1956B. Chromatographic system was the following: column XDB-C18 (Zorbax 150 mm × 4.6 mm × × 5 mkm) with nonpolar octadecyl group (reversible phase), mobile phase – ACN:H_2O (55:45) and 0.5 M solution of ammonium acetate, temperature of the column was 30° C, flow rate 1 ml/min, isocratic regime, injection 5 µl.

The molecular mass of the substances was determined by the negative ionization mode using a mass spectrometric detector Agilent G1956B (USA), fixing the value of m/z [16]. Considering the conditions of ionization of compounds, the actual molecular masses of substances were reduced by 1 (which is the molecular weight of hydrogen). The structural formulas of substances, according to their molecular masses were determined by the database "Dictionary of natural products. ChemNetBase".

Statistical analysis of the results was performed with Microsoft Office Excel 2007 and Microsoft Office Excel 2017, BIOSTATE, as well as with other specific software that appropriate to the corresponding analysis described above. The tables show the average values. The differences between average values are significant at $p < 0{,}05$.

Results. The previously studied complex of antimicrobial metabolites from *S. albus* is represented by enzymes with staphylolytic activity [10 – 12]. The antagonistic activity of *S. albus* UN 44 was evaluated at the first stage of the study to determine the ability of the bacterium to the biosynthesis of antimicrobial metabolites of different nature, primarily antibiotics.

As a result, the antagonistic activity against two test-strains (*K. varians* and *C. albicans*) was revealed (Table 1).

Table 1

Antagonistic activity of *S. albus* UN 44

Test-strains	Growth inhibition zone, mm
Gram-positive bacteria	
Bacillus subtilis УКМ B-901	0
Bacillus cereus B-908	0
Staphylococcus aureus B-918	0
Kocuria varians Ac-613	9
Gram-negative bacteria	
Escherichia coli B-906	0
Proteus vulgaris B-905	0
Pseudomonas aeruginosa B-900	0
Salmonella enterica Abony B-921	0
Yeast	
Candida albicans Y-2681	16
Candida utilis Y-984	0

Higher activity against *C. albicans*, as well as the prevalence and complexity of candidiasis treatment gave reasons to choose this test organism for further researches.

It should be noted that the activity of extracts from the supernatant of *S. albus* UN 44 was almost twice higher the one from the biomass. The growth inhibition zones for *C. albicans* Y-2681 were 25 mm and 15 mm, respectively (Fig. 1).

This indicates the predominantly of synthesis of antibiotic substances by *S. albus* UN 44. Therefore, the supernatant extracts were used for further analyses with different chromatography approaches.

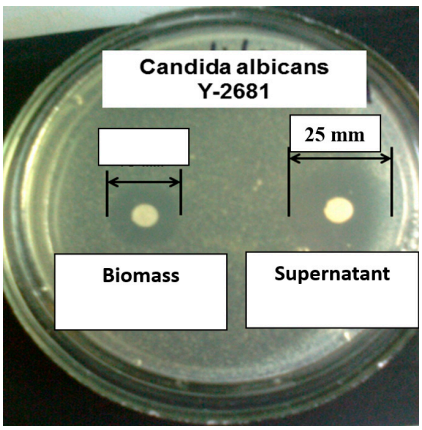

Fig. 1. Antagonistic activity of extracts from cells (biomass) and supernatant of *S. albus* UN 44

The main compound with a separation factor of 0.65 was revealed among the antibiotic substances by the thin-layer chromatography of the extracts (Fig. 2).

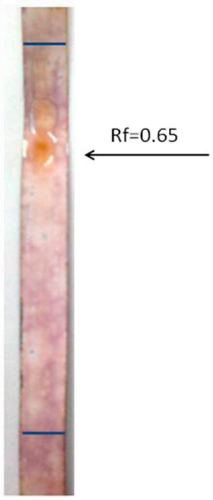

Fig. 2. Thin-layer chomatography of *S. albus* UN44 extracts

The brown-violet spots that developed in reaction with $FeCl_3$ indicated the presence of the phenolic group in the structure of a molecule of this substance. The bioautography was done to confirm the antibiotic activity of the compound. As a result, the growth inhibition zones of 22 – 23 mm for *C. albicans* Y-2681 were formed by the compound at the level of R_f = 0.65 that confirmed its antibiotic activity.

The absorption spectrum of the compound had a maximum at 270 – 275 nm and no absorption was marked in the visible region. Their fuzzy spectral characteristics, probably, were due to their low concentrations in the extracts. Subsequently, a step gradient column chromatography was used for isolation of antibiotic compounds.

For the next study of the antibiotic activity of the individual fractions of the extract, test-cultures that were representatives of the genera, which showed an antagonistic effect in the previous stage (*Kocuria* (*Micrococcus*) and *Candida*) were selected as well as a typical representative of the genus *Bacillus*, which, on the contrary, was not determined neither antagonism nor the action of antimicrobial enzymes of culture [9, 10]. In the latter case, the widespread ability of microbial producers to synthesize products of various nature with a different (complementary) antimicrobial spectrum of activity was tested. At the same time, the probability of the presence of other antibiotics (including antibacterial agents) that did not exhibit activity when cultivating the producer on the cups (due to low concentration) was taken into account, but was synthesized in deep conditions.

Fractions were active against *B. subtilis*, *C. cefyr* and *M. flavus* (Fig. 3): fractions No. 20 – 32 and No. 61 – 63 were active only against bacilli, and fractions No. 41 – 61 showed activity against bacilli, as well as against micrococci and yeast.

It is likely that there are at least two antibiotic compounds active against bacteria and fungi among the metabolites of *S. albus* UN 44 in the culture medium. This assumption is in agreement with the data on antagonistic properties of the bacterium (Table 1).

At the next stages we analyzed two main antibiotic compounds of different specificity. The active fractions isolated by column chromatography were used to determine the potential structure of antibiotics by LC/MS (Fig. 4, 5).

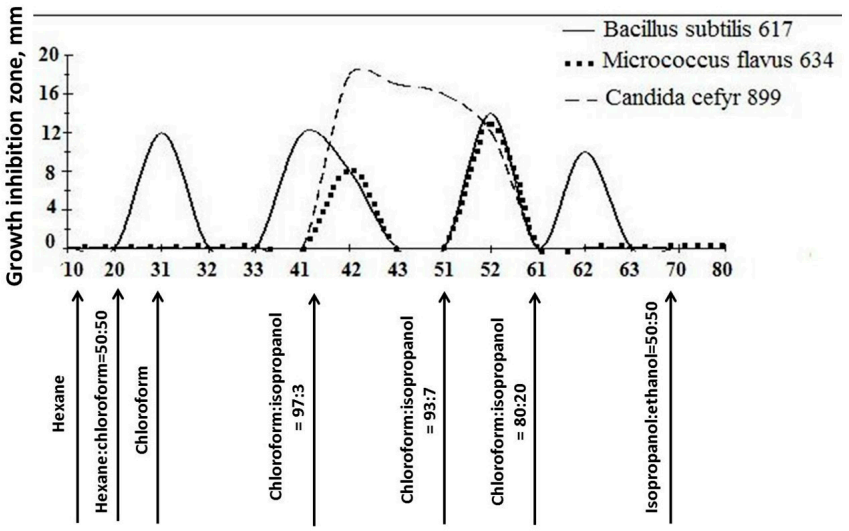

Fig. 3. Elution profiles of the extracts from the supernatant of *S. albus* UN 44 and the antibiotic activity of individual fractions

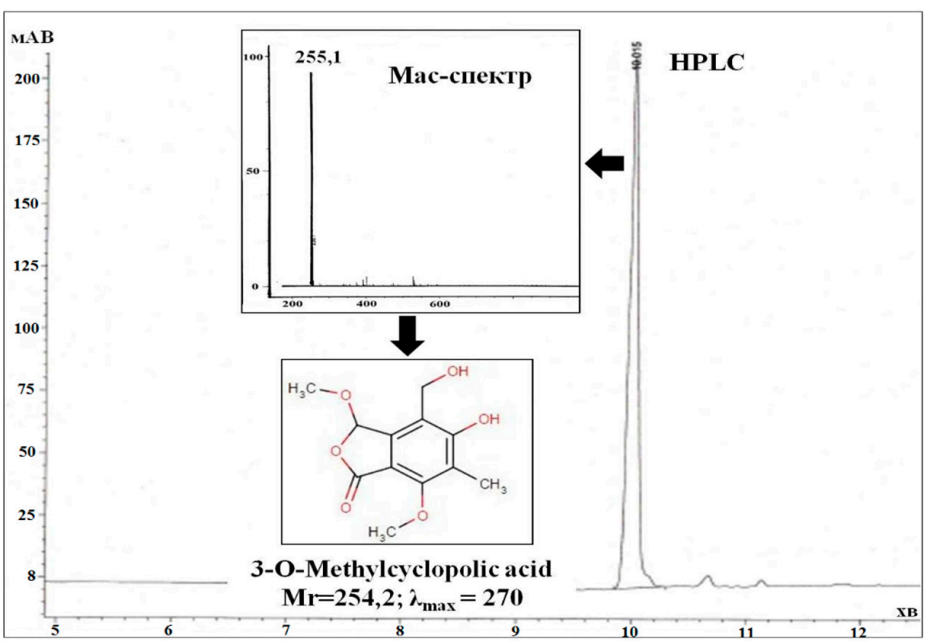

Fig. 4. Chromatography-mass spectrometric analysis of antibiotic compounds *S. albus* UN 44: 3-O-methylcyclopolic acid

Two compounds with molecular weights of 391,3 and 255,1 were identified. According to the ChemNetBase Mass Spectral Library, these compounds were classified as bis(2-ethylhexyl) phthalate and 3-O-methylcyclopolic acid, respectively.

Both antibiotics are derivatives of phthalic aldehyde, on the basis of which the antimicrobial preparation "Phthalazole", which is active against a number of opportunistic bacteria was created.

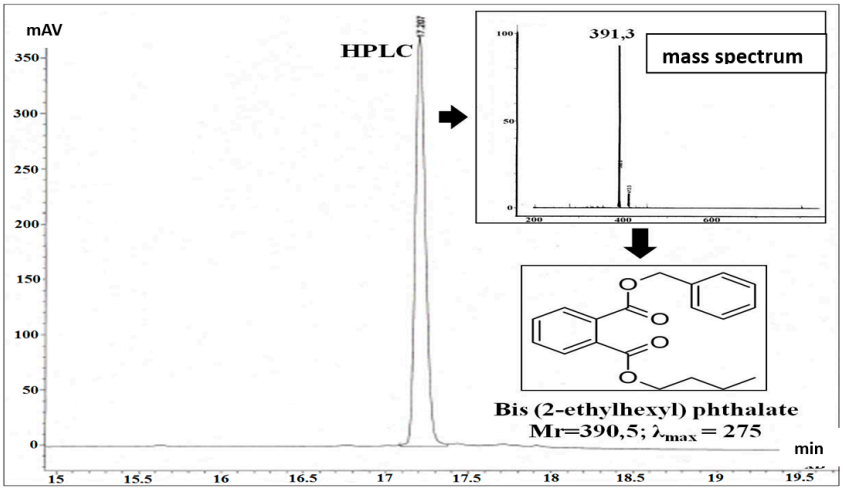

Fig. 5. Chromatography-mass spectrometric analysis of antibiotic compounds from *S. albus* UN 44: bis(2-ethylhexyl) phthalate

Discussion. *S. albus* is the object of researches in many studies since it produces a bacteriolytic enzyme complex, but its ability to synthesize metabolites with antibiotic activity and their identification were studied and done for the first time. It should be noted that the antibiotic spectrum of metabolites is fundamentally different from the spectrum of bacteriolysins and does not effective against most bacteria that are destroyed under the action of enzymes complex [9, 10]. In the previous work [10] we marked the suppression of the growth of test-strains under the action of crude enzyme preparation that contained all metabolites, including antifungal substances.

The main influence of isolated antibiotic compounds was against yeast, in particular, *C. albicans*. Such compounds were bis(2-ethylhexyl) phthalate and 3-O-methylcyclopolic acid. Cyclopolic acid is a dihydro-derivate of cyclopaldic acid that was isolated from *Penicillium ciclopium* [17, 18]. According to the literature, both compounds possess antifungal activity. Although the activity of cyclopolic acid is slightly lower, nevertheless it extends against the bacteria of the genus *Bacillus* as well. It was found that the antifungal properties of these compounds are related to the presence of an o-dialdehyde group in their structure, the reduction or oxidation of which with the conversion to phthalyl alcohol, formyl benzoic acid and dimethyl phthalate leads to complete loss of antibiotic activity.

Analysis of the antimicrobial profile of the enzybiotic complex from *S. albus* UN 44 showed that combined action of bacteriolytic enzymes along with antifungal and antibacterial properties can provide a broad specificity of drugs on their basis. Among the most resistant pathogens that can be destroyed by bacteriolysins (proteinases, muramidases, etc.) were *S. aureus*, *P. aeruginosa*, *P. rettgeri* [10], while antibiotics are able to inhibit the growth of *C. albicans* and species of the genus *Bacillus*. Obvious that from the technological point of view, it is possible to create preparations containing one or both antimicrobial metabolites (enzymes and antibiotics) in one production cycle because their different nature makes it possible to remove the protein components from the medium at first, and then extract the antibiotics.

Study of mutual influence of antibiotics and lytic enzymes showed the effectiveness of their combined use for the treatment of superficial wounds of different etiology and internal infections [19]. The special effect is due to the hydrolytic complexes containing enzymes of broad specificity: glucosaminidases and amidases possess high antibacterial activity, while proteinases purify wounds from necrotic tissue and promote surface granulation. Simultaneous action of antibiotics prevents further multiplication of individual cells that can survive and continue to be the source of the inflammatory process.

An important practical value for the development of an enzybiotic drug on the basis of antimicrobial metabolites from *S. albus* UN 44 rely on the ability of these enzymes to effectively destroy the biofilm of *P. aeruginosa* and prevents its formation [10]. Bacteria and fungi in biofilms may survive in the presence of antibiotics in quantities that are 500 – 1000 times exceeds their minimum inhibitory concentrations [20]. Therefore, the synergistic effect of enzymes and antibiotics may significantly decrease the effective dose of the latter, and as a consequence, lower the cost of the drug and prevent the development of the pathogen's resistance [21].

Thus, in the current study, for the first time, the ability of the *S. albus* UN 44 to the synthesis of the exogenous complex of antibacterial and antifungal antibiotics was revealed. The isolated antibiotic compounds were identified as bis(2-ethylhexyl) phthalate and 3-O-methylcyclopolic acid. The prospects for the development of a highly effective enzybiotic preparation based on enzymes and antibiotics of *S. albus* UN 44 were described.

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НОВІ АНТИБІОТИКИ ЕНЗИБІОТИЧНОГО КОМПЛЕКСУ *STREPTOMYCES ALBUS*

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Резюме

Мета роботи. Виділення та характеристика продуктів метаболізму антибіотичної природи *Streptomyces albus*, аналіз антимікробного комплексу продуцента в якості основи для розробки ензибіотичного препарату. **Методи.** В роботі використовували штам-продуцент бактеріолітичних ферментів *S. albus* UN 44. Антагоністичну активність культури визначали методом радіальних штрихів. Для оцінки антибіотичної активності використовували метод паперових дисків та біоавтографію. Виділені речовини антибіотичної природи аналізували методами тонкошарової та рідинної хроматографії і мас-спектрометрії. **Результати**. Зі штаму *S. albus* UN 44 виділена і охарактеризована сполука антифунгальної дії з фактором розділення 0,65, наявністю фенольної групи в молекулі та максимумом поглинання в області 270 – 275 нм. Виділені антибіотичні сполуки ідентифіковано як біс(2-етилгексил)фталат і 3-О-метилциклополова кислота. **Висновки**. Вперше встановлена здатність штаму *S. albus* UN 44 до синтезу антибактеріальних та антифунгальних антибіотиків, які відносяться до похідних фталевого альдегіду.

Ключові слова: Streptomyces albus, ензибіотики, антибіотики, бактеріолізини, антагонізм, хроматографія, антимікробний спектр.

НОВЫЕ АНТИБИОТИКИ ЭНЗИБИОТИЧЕСКОГО КОМПЛЕКСА *STREPTOMYCES ALBUS*

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Резюме

Цель работы. Выделение и характеристика продуктов метаболизма антибиотической природы *Streptomyces albus*, анализ антимикробного комплекса продуцента в качестве основы для разработки энзибиотического препарата. **Методы.** Работу проводили со штаммом-продуцентом бактериолитических ферментов *S. albus* UN 44. Антагонистическую активность определяли методом радиальных штрихов. Для оценки антибиотической активности использовали метод бумажных дисков и биоавтографию. Выделенные вещества антибиотической природы анализировали методами тонкослойной и жидкостной хроматографии, а также масс-спектрометрии. **Результаты.** Из штамма *S. albus* UN 44 выделены и охарактеризованы вещества,

имеющие фенольную группу в молекуле, фактор разделения 0,65, максимум поглощения 270 – 275 нм. Выделенные антибиотические вещества идентифицированы как бис(2-этилгексил)фталат и 3-О-метилциклополовая кислота. **Выводы.** Впервые установлена способность штамма *S. albus* UN 44 к синтезу антибактериальных и антифунгальных антибиотиков, которые относятся к производным фталевого альдегида.

Ключевые слова: Streptomyces albus, энзибиотики, антибиотики, бактериолизины, антагонизм, хроматография, антимикробный спектр.

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