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BIOTECHNOLOGICAL ASPECTS OF THE DEVELOPMENT OF A LIQUID FORMULATION OF MULTIFUNCTIONAL ENZYBIOTIC ANTISEPTIC

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Aim. Evaluationof biotechnological aspects of the production of a liquid formulation of the multifunctional antiseptic preparation of microbial origin, which can be typified as an enzybiotic; characterization of the enzymes pecificity of the studied formulation, stabilization methods, its ability to affect microbial biofilms.

Methods. Gel-filtration and electrophoresis were used to study the component composition and the specificity of the enzyme complex of the *Streptomyces albus* UN 44producer strain. Proteolytic and staphylolytic activities of individual fractions were determined. The *Pseudomonas aeruginosa* biofilm and its formation under the effect of various drug formulations werequantitatively evaluated by spectrophotometry.

Results: The stability of the liquid formulation of the enzybiotic biosynthesized by *S. albus* UN 44 was demonstrated. Activity of the formulation could be prolonged and additionally stabilizedby adding of 0.5% polyvinyl alcohol. Fractionation of the formulation enzyme complex usinggel-filtration revealed the presence of at least three proteinases of different molecular weights (80–100, 24–35 and 20 kDa) and lysoenzymes (18–22 kDa). The effectiveness of the developed liquid antibiotic formulation for the destruction and inhibition (8–10 folds) of the biofilm formation by clinical strains of *P. aeruginosa* was shown.

Conclusions: Thebroad spectrum, multidirectional mechanisms of antimicrobial and regenerative action of enzybiotic drug, and the possibility of its production directly from the biotechnological process determine the prospects of its manufacturing and use as a multifunctional surface antiseptic.

Key words: antibiotic, antimicrobial action, *Streptomyces albus*, liquid formulation, biofilms, pathogens.

Common features of the inflammatory processes (wounds, postoperative healing, burns) are the germination of microbial pathogens of various groups, formation of their biofilms and tissue necrotization [1–3]. All those complications, along with the problem of antibiotic resistance of pathogens, which has already became critical nowadays, complicate the treatment and determine the need for the development of multifunctional antiseptics. Therefore, modern compositions of antiseptic drugs most often combine antibiotics and enzymes or substances of chemical origin, whichensuresa wide spectrum of their antimicrobial action and high efficacy [4–6]. This approach was implemented, for example, in the commercially available drugs "Iruksol", "Iruksol-mono" (Germany), "Irucsan" (Ukraine), and the like.

However, the creation of such combined antiseptics involves the use of numerouspre-manufactured commercially available active ingredients, which requires long-timemanufacturing processand results in high costs of the drug production. A special feature of microbial biosynthesis is its capacity to simultaneously produce a complex of lysoenzymes and broad-spectrum antibiotics [7-9]. The advantages of biotechnological developments using microbial producers are the ability to obtain a ready-to-use for mulation of a multifunctional antiseptic preparation directly from the biotechnological production. Obviously, this approach is more cost-efficient, since it eliminates the need to use numerous pre-manufactured substances.

In addition to the strategy of treating multi-speciesand multi-resistant pathogens with combined antiseptics, drug developers and researchers have recently focused at a specific class of compounds called "enzybiotics" [10, 11]. This term is now applied to antimicrobial substances (bacteriocins, cathelicidins, bacteriophages, immunobiotics) with a specific mechanism of action. Many authors outlinethe advantages and broad prospects of such drugs, which can significantly increase the efficacy of antimicrobial effect, without causing the development of resistant pathogenforms. In fact, the largest group of enzybiotics is formed by enzymes that can disrupt specific bonds of the cell wall of microorganisms, such as muramidase, N-acetylglucosaminidase, amidase, peptidases, and some others. Known for quite a long time as bacteriolytic (lytic) enzymes, today they are also considered as objects for creating advanced antibiotics [12, 13].

Lytic enzymeshave long been found among the metabolites of the *Streptomyces albus* culture (originally *Streptomyces recifensis var. lyticus*), which stimulated long-term research and development of several antiseptic preparations for various purposes [14, 15]. Recently, however, a specific *S. albus* UN 44 strain (deposited as IMB Ac-5030) was established to have the capacity to synthesize a complex of antibiotics with high activity against *Candida albicans* [16].

Therefore, the aim of the presented work was to create a liquid formulation of the enzybiotic which could be synthesized by *S. albus* UN 44, and to evaluate its biopharmaceutical characteristics, and determine the prospects for using this drug as a multifunctional surface antiseptic for veterinary and medicine.

Materials and Methods

The producer strain of *Streptomyces albus* UN 44 from the museum collection of the Department of Industrial Biotechnology and Biopharmacy of Igor Sikorsky Kyiv Polytechnic Institute was used in the work. *S. albus* culture synthesizes a complex of biologically active substance sincluding glycosidases, lytic endopeptidases, muramidases, non-lytic proteinases, amylases, as well as antifungal and antibacterial drugs [14, 16].

Liquid formulation of the enzybioticdrug were produced according to the following scheme: cultivation of the producer was performed in 750 mL rolling flasks with 150 mL of nutrient medium based on glucose and soya flour, for 72 h at 28 ± 1 °C and stirring at 180 rpm [17]. After completion of the biosynthesis process, the biomass was separated by centrifugation, and the supernatant was sterilized, concentrated and purified from macromolecular compounds by microfiltration.

Fractionation of the formulation by gel-filtration was performed according to standard methods with the following process parameters: material — Sephadex (Superdex SF-75 (2 000–70 000 D), flow rate — 1 mL/min, pressure — 0.33 MPa, pH buffer 7.0 (0.1 M $(NH_4)_2HCO_3$), volume of the fractions — 5 mL. Adjacent fractions were combined according to protein concentration. *Electrophoretic analysis* of the drugproteins was performed by denaturing electrophoresis in 12.5% polyacrylamide gel at 10 mA and 100 V for 230 min using Techware PS 252-2 power supply (Sigma-Aldrich). The markerswere as follows(Mws in kDa): α-lactalbumin (Mw = 14.2), chicken egg albumin (Mw = 45.0), carbonic anhydrase (Mw = 29.0), bovine serum albumin (monomer Mw = 66.0 and dimer Mw = 132.0), urease (trimer Mw = 272.0 and hexamer Mw = 545.0)(Sigma MW-ND-500). At the end of the process, the developing gel plate was stained with amide black solution A-8181 (1% in 7%

acetic acid) (Sigma) and washed in 7% acetic acid.

As stabilizers of the experimental formulation, the methylcellulose, polyvinyl alcohol and polyacrylamidewere used, added at a concentration of 0.5%. The obtained samples of formulations were poured into glass vials and stored at 4–10 °C.

For the study of antimicrobial activity of the experimental formulation samples we used testcultures from the museum collection of the Laboratory of Medical Microbiology with the Museum of Pathogenic Human Microorganisms of SI "Institute of Epidemiology and Infectious Diseases. L. V. Gromashevsky National Academy of Medical Sciences of Ukraine": clinical strains of *Pseudomonas aeruginosa* (№ 233, № 430, № 452, № 183, № 278) and museum strain of *Staphylococcus aureus* ATCC 25923.

The lytic activity (LA) of the experimental formulation samples was determined by the turbidimetric method according the lysis ability of *S. aureus* suspension and was expressed in IU/mL. 1 IU unit of LA comprised the amount of enzyme that reduced the optical density of the testculture suspension by 0.001 per 1 min in 1 mL of the reaction mix [18, 19]. To 4 mL of the testculture suspension,0.2 mL of a sampleformulation was added and incubated for 15 min at 37 °C. As a control, 0.2 mL of distilled water was added to the test culture and incubated under the same conditions. The level of LA was determined by the difference in optical density of the suspension before and after incubation. The optical density was determined by a photocolorimeter KFK-3 at λ = 540 nm in a 0.5 cm cuvette againstdistilled wateras a background.

The proteolytic activity (PA) was determined using azocasein. The method included hydrolysis of the substrate by proteolytic enzymes, stopping the reaction by adding trichloroacetic acid, colorimetric determination of unprecipitated stained tyrosine-histidine-containing peptides [19]. The unit of activity was taken as the amount of enzyme that forms 1 μmol of free amino groups in 1 min.

The ability to destroy microbial biofilms of *P. aeruginosa* was determined according to the method of Romanova et al. [20]. The culture was grown in trypticase soy broth at 37 °C in flat-bottomed plates for enzyme-linked immunosorbent assay for 48 h to form a biofilm. After that test samples were added, and incubation was continued for 24 h. Then the contents of the wells were removed, the plates were washed with distilled water, filled with 1% alcohol solution of the dye violet crystal and kept for 45 min. The dye was removed, the wells were washed with distilled water, filled with 250 μl of ethyl alcohol and left for 45 min at room temperature. Stained ethylextracts were collected for spectrophotometric assay. The amount of formed biofilm was evaluated by the intensity of the ethylextractcolorationat 630 nm, and expressed in optical densityunits. To visually assess the biofilm formation, the cultures were incubated in a similar manner in plates on coverslips, which were then washed with distilled water and stained with gentian violet solution. The results were evaluated by light microscopy at 40-fold magnification.

Experimental data were statistically processed by conventional methods with the calculation of standard deviation, error of arithmetic mean;the differences between mean values were assesses using the "Biostat" softwareby the Student's *t*-test, taking into account the level of significance. Differences between mean values were

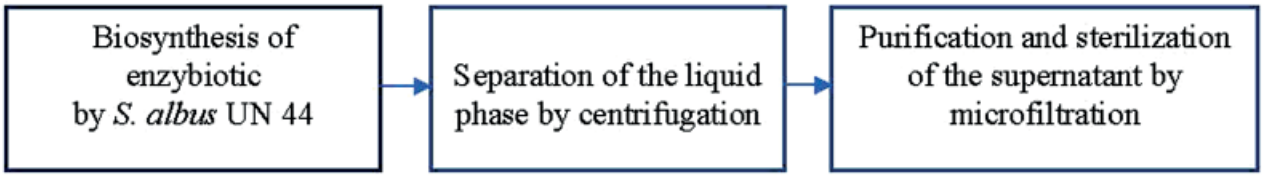

Fig. 1. **Stages of the producing the prototype liquid enzybiotic formulation from *S. albus* UN 44**

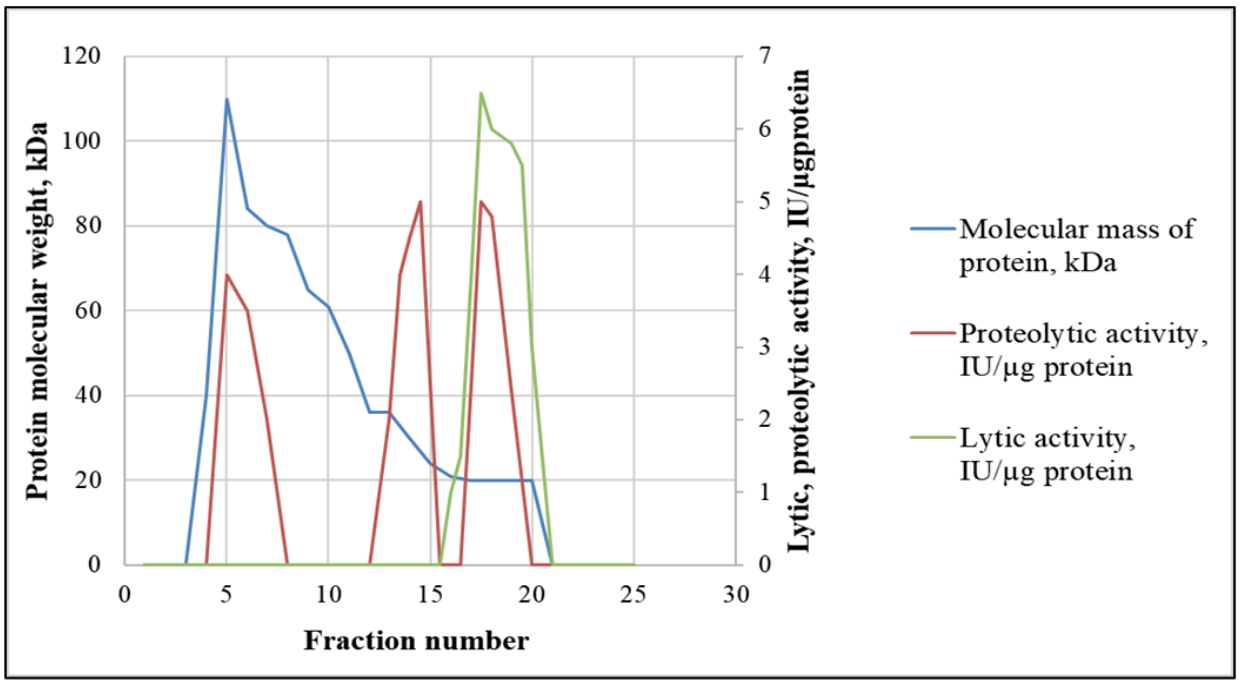

Fig. 2. **Profiles of *S. albus* UN 44 culture enzymes separated by gel-filtration and havingdifferent specificities**

considered significant at the significance level $P < 0.05$.

Results and Discussion

The method of lysoenzyme biosynthesis using the test *S. albus*culture, as well as the technology for producing a number of lyophilized (dry) formulations of enzybioticshad previously been developed by the authors using baromembrane method for product separation and purification [15, 17]. Baromembrane method is the efficient way to separate enzymes while minimizing the negative impact on the product structure and allowing simultaneous sterilizing of end product; the refore, this method was chosen to obtain a prototype of the liquid enzybiotic formulation (Fig. 1).

To determine the specificity of individual enzymes of *S. albus* enzybiotic complex, the sample was fractionated by gel-filtration using SUPERDEX SF-75, and the molecular weights of proteins in individual fractions were determined electrophoretically. The data shown on Fig. 2 indicate the presence of enzymes with molecular weights from 20 to 110 kDa in the enzyme complex of the producer strain.

Enzyme fractions with molecular weights of 80–100, 24–35 and 20 kDa were characterized by high proteolytic activity. The highest lytic activity of enzyme complex (toward *S. aureus*) was shown by a fraction of lysoenzymes with molecular weights of 18–22 kDa. Since the intended use of the developed formulation is an antiseptic drug, the specificity ofindividual enzymes of enzyme complex was not analyzed. It is obvious howeverthat lysoenzymes are not associated with the activity of proteinases or peptidases contained in the product fraction with molecular weights of 18–22 kDa. Their absence in other fractions of enzymes (80–100, 24–35 kDa) caused the absence of detectable lytic effect on cells of the testculture of *S. aureus*. The high proteolytic activity of the antiseptic formulation is important in the complex treatment of superficial wounds of various etiologies, as it causes the destruction of necrotic tissues, cleanses the wound and accelerates granulation and healing.

The advantages of liquid antiseptic formulations over dry dosage forms are their lower costs and the possibility to use without additional preparation of the solution; however, such formulation sinevitably have a shorter shelf life. Therefore, the stability of the experimental formulations during the storage, and the effectson the shelf-life duration of certain excipients, which wereadded in a concentration of 0.5%, were determined. The choice of this specific concentration and the substances themselves (polyacrylamide, methylcellulose, polyvinyl alcohol) was based on the analysis of similar developments, and their safety and potential ability to stabilize and prolong the action of biologically active substances.

The stabilities of the obtained native liquid formulation and formulations with added stabilizers were determined by the dynamics of lytic (staphylolytic) activity during the irstorage for 3 months at +4 °C (Fig. 3).

Some of the excipients led to the binding of the enzyme complex and subsequent decrease in its activity by 5 to 8% immediately after their addition (initial data are shown on the ordinate axis at “0” time point). Analysis of the staphylolytic activity of the formulations during theirstorage shows that the use of teste dexcipients had virtually no effect on lytic activity dynamics. In all variants, the decrease in the activity compared to the initial value in the same formulation was within the margin of error (5%), principally remaining at the same level.

It is obvious that for 3 months the lytic activity of the native liquid formulation remains stable, which makes it possible to predict an insignificant decrease in it during the year, and therefore the possibility of storing the liquid antiseptic drug for external use without the supplementation of stabilizers. However, polyvinyl alcohol, which had no negative effect on enzymatic activity, can be used as an auxiliary substance to increase the efficacy of the formulation. Thus, wecan, for example, anticipate the prolonged effect of the drug formulation

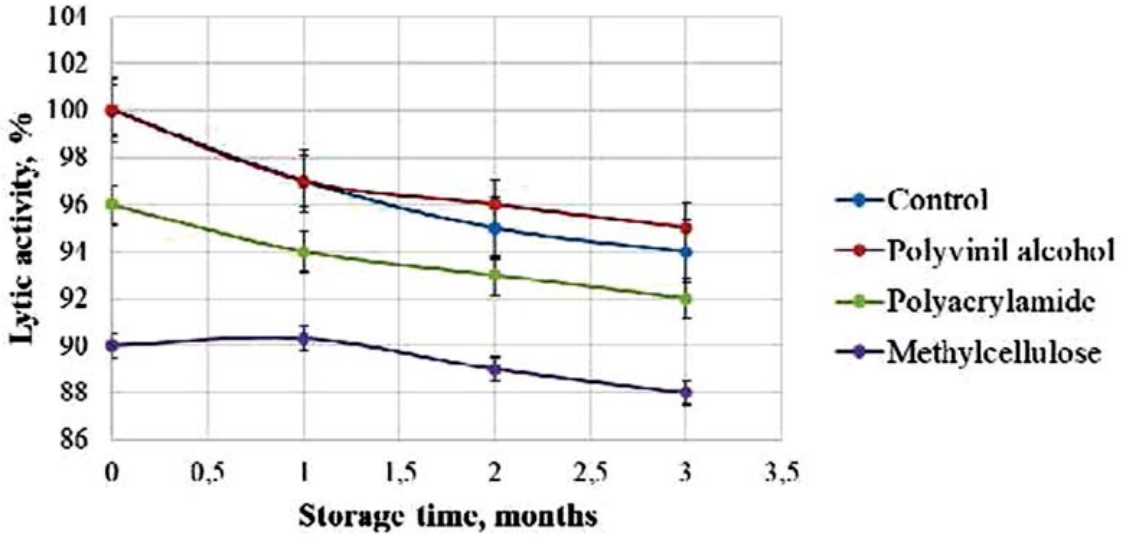

Fig. 3. **Dynamics of staphylolytic activity of liquid enzybioticformulations with various excipients**

containing those substances after application to the wound, since they will slow down the drying of the liquid drug formulation and facilitate its spread over skinsurface. The antimicrobial spectrum of this enzybiotic drug, shown in our previous studies, includes the main representatives of wound microflora, including *E. coli*, *S. aureus*, *P. aeruginosa*, *C. albicans* [15–17]. So, the liquid formulation of enzybioticsfrom*S. albus* UN44 can be considered as an efficient antiseptic drug in a formulation without a stabilizer, or as a prolongedliquid formulation with the supplementation of 0.5% of polyvinyl alcohol. Such formulations can be used to wash infected wounds or soak wipes rightbefore applying to the affected skinarea.

Since one of the most important problems in medical practice is the fight against infectious agents that form biofilms on medical instruments, after surgical interventions, etc., some of the developments are focused on the destruction of those structures. Thus, the combination of an antimicrobial enzyme and a fluoroquinolone antibiotic has been shown to cause a synergistic effect against *S. aureus*, which was based on the disruptionof the biofilm layer by theenzyme, and subsequent bactericidal effect of the antibiotic [7]. A similar mechanism was used in the development of a new drug Dispersin, which acts on biofilms by disruptingthe cementing substance of the biofilm matrix — poly-N-acetyl-glucosamine [21].

The ability to destroy *P. aeruginosa* biofilm and prevent its formation had previously been shown by us for other formulations of the enzybioticproduced by the studied culture (dry native Cytorecifen and immobilized Cytorecifen-M) [22]. This was the basis for determining such activity of the prototype enzybioticliquid formulation, which also contains a complex of antibiotics, and has primarilyantifungal effects (therefore referred to as

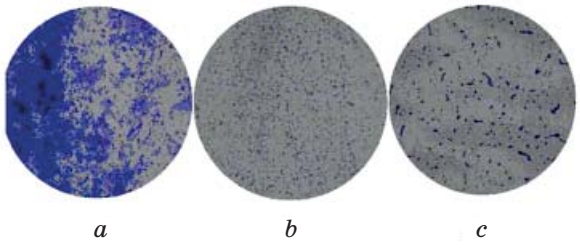

Fig. 4. **Effectof various enzybiotic formulations on the *P. aeruginosa* 278 biofilm formation:**
a — control; *b* — with Cytorecifen-M; *c* — with Streptofungin

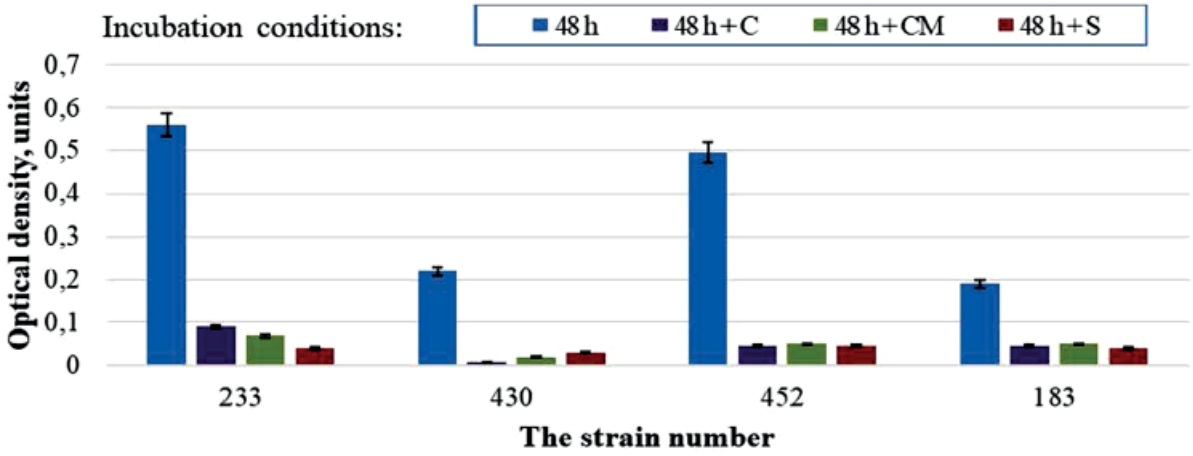

Fig. 5. **Quantitative assessment of the formation and destruction of biofilms formed by different *P. aeruginosa* strains:**
for 48 h incubation without test enzybiotic formulations (48 h), and with addition of Cytorecifen (C), Cytorecifen-M (CM), or Streptofungin (S) after 48 h and further incubation during 24 h

Streptofungin), and comparing its effectiveness with previously obtained enzybiotic formulations.

To that end, we used clinical strains of *P. aeruginosa* isolated from wounds of patients with infected surgeryarea. The effects of Cytorecifen, Cytorecifen-M and Streptofungin on the formation of *P. aeruginosa* biofilm were compared.

Analysis of antimicrobial action of various formulations of the *S. albus* enzybiotic, i.e. the suppression (Fig. 4) and destruction (Fig. 5) of the *P. aeruginosa* biofilm,showed their high efficiencies, which were expressed in the averagely 8–10–fold inhibition of biofilm formation, and the destruction of all studied clinical strains.

The use of tested enzybiotic formulations can be recommended in the treatment of infected wounds in veterinary and medicine (after appropriate safety studies), as well as for the treatment of reusable medical instruments and accessories, especially those that cannot be subjected to thermal sterilization. The latter is more related to the immobilized form of the antiseptic Cytorecifen-M, which has a prolonged effect and is more stable in use than native liquid formulations.

It was found that clinical strains of *P. aeruginosa* form a biofilm in amounts from 0.18 ± 0.015 units (strain № 183) to 0.56 ± 0.035 units (strain № 233) after 48 h of culture (Fig. 5).

Further incubation of teststrains together with various drug formulations resulted in significantly less biofilm formation. Thus, strain №430 cultivated for 24 hours in the presence of Cytorecifen formed a biofilm, which was smaller by 20.9 folds, № 452 — by 9.6 folds, № 233 — by 6.7 folds, and the strain № 183 — by 3.6 folds. Cytorecifen-M reduced biofilm formation by 9.8–3.6 folds, and Streptofungin — by 15.5–4.5 folds for different strains, as compared to their incubation without test formulations.

The data obtained indicate that all antiseptic formulations actively destroy and inhibit the formation of biofilms in tested clinical strains of *P. aeruginosa*, on average, 8–10 times. The shown strain-specificity of the tested enzybiotic formulations indicates the need for individual selection of drugs in the treatment of patients.

When analyzing the average amount of biofilm formed by *P. aeruginosa* strains during 24 h cultivation in the presence of the studied formulations, no significant difference in exposure was found. Therefore, we can state high efficacy of all studied formulations to prevent the formation of, and to destroy *P. aeruginosa* biofilms, including the proposed liquid formulation. Significant advantages of the liquid formulation of the enzybiotic, in addition to lower cost, prolonged action and ease of use, are its manufacturability. This drug formulation can be obtained directly from the biotechnological production, which will eliminate the stage of drying, and enables direct pouring of the formulation into vials of 100–200 mL.

Conclusions

Analyzing the previously shown antimicrobial profile of the *S. albus* UN44enzybiotic complex, it is obvious that the combined action of bacteriolytic enzymes and antifungal and antibacterial drugscan provide broad specificity of the antiseptic formulations based on it.The enzyme complex of the producer strain contains at least three proteinases of different molecular weights (80–100, 24–35 and 20 kDa) and lysoenzymes (18–22 kDa).

A liquid formulation of the multifunctionalenzybioticantiseptic Streptofungin based on lysoenzymes and antibiotics of *S. albus* UN44is proposed. The stability of the liquid enzybiotic formulation during the storage, and the possibility of shelf-life prolongation due to the supplementation of 0.5% polyvinyl alcohol were established. The ability of the enzybioticto inhibit 8–10 times the formation of, and to destroy *P. aeruginosa* biofilm was shown. Presence of proteinases in enzybiotic antiseptic determines its additional capacity to clean wounds from necrotic tissues, and to accelerate granulation and healing.

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БІОТЕХНОЛОГІЧНІ АСПЕКТИ РОЗРОБКИ РІДКОЇ ФОРМИ ПОЛІФУНКЦІОНАЛЬНОГО ПРЕПАРАТУ ЕНЗИБІОТИКА

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Мета. Вивчення біотехнологічних аспектів створення рідкої форми поліфункціональногоантисептику мікробного походження, що може бути віднесений до ензибіотиків: характеристика специфічності ензимів дослідного препарату, методів стабілізації, здатності впливати на мікробні біоплівки.

Методи. Для дослідження компонентного складу та специфічності ферментного комплексу продуценту *Streptomyces albus* UN 44 використовували гель-фільтрацію та електрофорез, визначали протеолітичну та стафілолітичну активність окремих фракцій. Кількісну оцінку біоплівки *Pseudomonas aeruginosa* та вплив на її формування дослідних зразків проводили за оптичною густиною спектрофотометрично.

Результати: Встановлена стабільність рідкої форми препарату, отриманого в результаті біосинтезу *S. albus* UN 44, а також можливість пролонгації дії та додаткової стабілізації за рахунок внесення 0,5% полівінілового спирту. Фракціонування ферментного комплексу продуценту гель-фільтрацією показало вміст щонайменше трьох протеїназ різної молекулярної маси (80–100, 24–35 та 20 kDa) та лізоензимів (18–22 kDa). Показана ефективність розробленого рідкого препарату ензибіотика щодо руйнування та пригнічення (у 8–10 разів) утворення біоплівок клінічних штамів *P. aeruginosa*.

Висновки: Широкий спектр, різноспрямовані механізми антимікробної та регенерувальної дії, можливість отримання препарату безпосередньо в ході біотехнологічного процесу обумовлюють перспективи його виробництва та застосування як поліфункціонального антисептика поверхневого призначення.

Ключові слова: ензибіотик; антимікробна дія; *Streptomyces albus*; рідка форма; біоплівки; патогени.