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ELECTRONIC INFORMATION SYSTEMS IN BIOTECHNOLOGY

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The aim of the work was to generalize and analyze the use of electronic information systems in biotechnology in order to create new versions of these systems. The publications concerning the systems of different types for solving the problems in biotechnology were studied. Similar systems which traditionally belong to biology and medicine were classified. The prospects of their application for development of more advanced electronic systems were considered.

Key words: bioinformatics, electronic information systems, databases.

The progress of contemporary biotechnology-based production in the world is unprecedented. This happened due to the achievements in areas of molecular studies and modeling, discoveries in the field of pharmacology, studies of disease characteristics, achievements in the clinical aspect of health care and, on other hand, due to the intensive development of information technologies (IT) and computer technologies (CT), mainly on the basis of the databases (DB), which gave the opportunity to work with great volumes of obtained digitized results. The notion "information system" (IS) means any system, that is capable to receive, to process, to memorize, and to transmit the information according to classic definition [1, 2]. In our research, like other authors, we distinguished two types of information systems [1, 2]. So, "information system" may mean: 1 — living systems in nature, we suggest to call them "nIS" (natural information systems), and 2 — technical information systems, respectively "tIS" (technical information systems), namely this publication is dedicated to the observation of the latter ones. During the last decade the "hybrid" ISs have been developed also; they unite characteristics of nIS and tIS [1]. Present review about the elaboration of modern electronic information systems (ISs) on the basis of databases (DBs) for biotechnology (and biology in general), we have done after the analysis of more than 370 modern sources (since 2000 year), as well as number of earlier works; in general, relevant publications have been reviewed over the past 35–30 years. Below in this article the results were published of such author's original works and analysis of IS use in biotechnology and linked biological and medical disciplines [1–37]. Some other authors' works with the description of tISs for these spheres are in [38–68].

Below the results of our first classification of modern ISs for the facilitation of future ISs' versions construction were suggested; and these ISs can be used in biotechnology. It should be noted that scientific and technical publications about novel tISs (technical information systems) often contain a number of deficiencies. Most of them announced only the development of any tIS and give it superficial characteristic which should announce a novelty. But they omitted numerous technical characteristics of the systems, the principles of their functioning, and etc.; all these complicate the analytical research. Despite of this, we have analyzed the experience of modern ISs constructing, which can be applied for successful works in biotechnology. Today all such tISs are network-based [1, 10, 11, 22, 25, 41, 42, 48, 54, 60–62, 67, 69–94]

and linked with DBs [95–127]. Content for these databases was obtained usually from the results of biological and medical observations and experiments [4, 5, 10, 12–14, 17, 22–44, 47–49, 61, 68, 71, 74, 78–81, 85, 86–88, 92, 95–97, 106, 112, 117, 119–151].

Further each of the mentioned tISs we have characterized briefly.

The first works on the development of tIS with databases for biotechnology, other biological and medical sciences. The importance of IT developments for all biological branches and, first of all, for biotechnology, has often been highlighted during contemporary international forums with the participation of biologists, managers and IT professionals. One of the first important forums — VLDB 2000 Conference — had happened on June, 2000. VLDB 2000 was dedicated to the prediction of the ways of IT development for the biology and medicine; for the control of situation with biodiversity and ecosystems [1, 10]. It was noted that the study of biodiversity and ecosystems refers to those sciences that can (and must be) fully formalized and informative, despite the fact that the objects of their study are extremely complex. The new branch "Informatics in Biodiversity and Ecosystems" (BDEI — BREI) was officially started at this meeting. It was emphasized that the diversity of living organisms in nature is an impressive feature of our reality. As a result of biodiversity existence, the people of the Earth were provided with clean air and water, food, homes, medicine, and etc. Finally, the biodiversity based on the existence of ecosystems gives billions dollars to national economies of different countries, either directly, through the agriculture, forestry, fishing, or as a result of protecting crops, pest control practices, soil restoration, carbon dioxide removal, nitrogen fixation, environment perfection. Obviously, this was one of the most important areas of vital importance; and it is important in terms of science, education, economics and government control. Therefore, the development of electronic ISs, which would enable to realize the scientific study of the nature at the modern level, educational work and rational economic development of nature with the support of governments, was one of the most important tasks of that time. It was emphasized that for 2000-th year humanity has not had yet sufficiently good IT/CT tools for the solution of such problems. Therefore, it was necessary to invent a new generation of such means, including means of satellite environmental control and computer processing of obtained biological information. At the same time it was necessary to solve the problem of matching and ordering into the linked DBs the entire volume of biological data of various natures, both obtained by digital and classical means during the centuries. This task was extremely complicated, since in electronic databases in ISs should be ordered the latest digitized data at the level of molecular biology, genetics, biochemistry with its processes and reactions, as well as the data from field observations of species, observations of changes in the environment, data of classical taxonomy (and they should correlate with each other!) At the same time, these data should be in such form, that they can be processed, analyzed and compared jointly. One such IT/CT tool for this period was called the "Super Blue Gene", it was the supercomputer which IBM started to develop on 2000 for the analysis of thousands of protein molecules composition of various species living on the Earth.

Classification of information systems with databases for biology and medicine. Primary there were no specific information systems developed specifically for biotechnology — it was economically unjustified. The first developed tISs were accepted from both spheres, either medicine or biology, for all the works in biotechnology. Later some of such tISs were modernized and adapted for biotechnological tasks. Therefore, below we offer the classification of tISs developed for medicine and biology that became the resource of the data and procedures for biotechnological works. Let's observe the examples of various modern ISs with DBs in biology and medicine, information about which was published since 2000. Despite the diversity of such systems (and, accordingly, despite the diversity of publications), it was possible to distinguish certain well-defined types of ISs among them. It should be noted that such classification in the finished form in the scientific and technical literature until 2008 did not exist; it is original and the author made it on the basis of materials of about 166 publications.

Presence of large variety of tISs' versions in modern medicine was due to a good funding of the works related to medicine and health protection in the world. Thus, among the ISs with DBs for medicine one can distinguish (Fig. 1): medical ISs of general purposes, expert systems, electronic systems for working with images, electronic systems for working with medical documents, systems for scientific purposes, library medical systems, electronic

educational systems in medicine, electronic medical databases.

To the first positions in this list we placed the systems, information about which was published in the most numerical contemporary scientific and technical sources. For example, the group "Medical ISs of general purposes" included large and complex medical ISs developed for hospitals, medical research centers, etc. They united large numbers of sectors, DBs, electronic libraries and other services, sometimes complex networks with information defense and etc. The developers payed great attention to them, respectively, because of health care and health protection purposes. Such ISs were of the greatest demand of users, often embodied in practice in modern hospitals, laboratories and etc. So, these ISs felt into our section "Medical ISs of general purpose". This can be explained by the practical needs of clinical medicine, which requires the most of such versions.

When viewed the list of ISs classification from the top to the end, the number of publications corresponding to one specified type of the system decreases, and in the section "Electronic medical databases" felt to the lowest number of publications. This does not mean that electronic medical DBs were not important in medical practice. Such pattern could also be explained by the fact that the procedure for creating of such databases is standard, described in university textbooks, and perhaps the developers of such DBs did not see them as novelty, required for scientific publications.

Describing the classification of electronic ISs in biological sciences, it was necessary to tell that such ISs types can also be successfully applied in biotechnology. Placing these ISs types in hierarchy (Fig. 2), we followed the same principle as above: the more publications contain modern scientific and technical sources about this type of systems; respectively, the higher its name was in our list. Similar types of systems in biology and medicine we marked with the same hatch (for example, "Electronic systems for working with images" are marked by a square hatch for all branches; Fig. 1, 2). Regarding to the classification of ISs in biology, most of them were scientific ISs with DBs according to their purposes and the developers paid the main attention to them. Such situation looked like similar to "Medical ISs of general purpose" from Fig. 1. At the same time, such scientific systems in biology also performed educational functions, so, they are (and may be called) "Educational ISs". Further, according to the attention of developers, and consequently, according to the number of developed systems were electronic libraries in biology and biological databases. There were relatively not numerical publications about the systems designed for the work with images. Abovementioned could not be applied to systems for the work with images in anatomy, cytology — such systems were numerous, quite thoroughly developed, because they support some medical operations (for example, surgeons) and computer diagnostics are based on them also. But in our classification, they were at the third position from the top in the

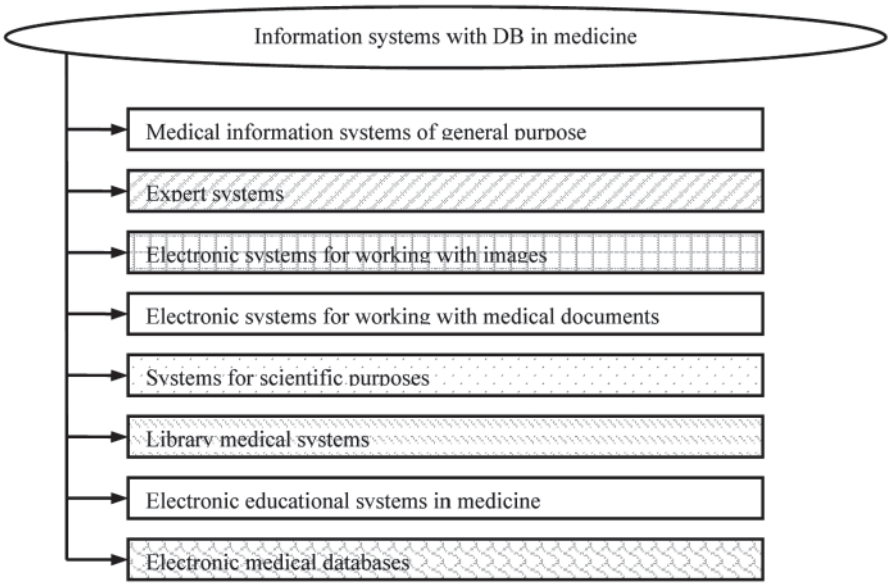

Fig. 1. **Information systems with databases for medicine that could be applied in biotechnology [1]**

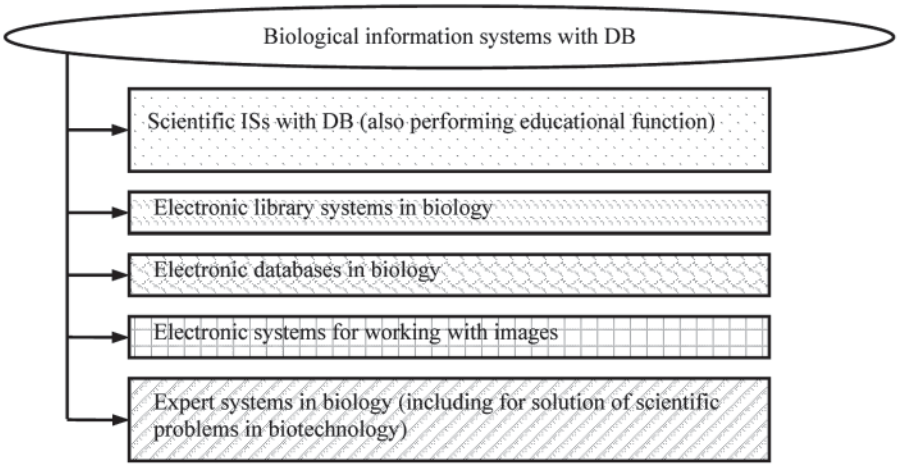

Fig. 2. **Biological information systems with databases that could be applied in biotechnology [1]**

classification of ISs for medicine, because they are necessary for the needs of this sphere; so, they were first developed and applied with equal intensity in medicine and in biology. Computer expert biological systems were the most close to the biotechnological practice, for example, for the estimation of pests' presence on fields, for bee diseases identification, and etc. Thus, the classification of ISs for biology could be presented as: scientific ISs with DBs (also performing educational function), electronic library systems in biology, electronic databases in biology, electronic systems for the work with images, expert systems in biology (including ones for solution of scientific problems in biotechnology) (Fig. 2).

Information systems with databases in biotechnology and some related biological sciences. Let's observe the examples of ISs with databases in the fields of biology, such as: 1 — biotechnology, and namely, those areas where data were used to study the genome, molecular biology and pathophysiology, 2 — related branches of biology, in which biotechnological works are also represented: biodiversity, ecology, adaptive biology. Our attention to the designed electronic ISs in these spheres was due to the fact that the author has her own researches in them during long years.

Technical information systems (tISs) in biotechnology, molecular biology, pathophysiology, genome studies. Some time ago, the achievements in these areas of biology were limited by enough low level of individual genes and biological molecules studies, but accelerated development of computer and ISs for biotechnology made it possible to solve such important tasks as, for example, computer scans of thousands samples per day, high-resolution detection systems (thousands of data points, for example) and the creation of appropriate electronic ISs. In the United States, the National Center for Biotechnology Information (NCBI) has a collection of databases with genetic sequencing data, decoded protein structures, and other biologic information that are updated constantly, and expanding exponentially.

NCBI published the 146th issue of GenBank, this is a publication on the gene sequences database, which contains an information on 42, 734, 478 gene sequences (February 15, 2005). Before there the results of the full decoding of human genome were published (February, 2001). These events had demonstrated two the most prominent achievements in these areas over the previous 10 years. An outstanding feature was that a large set of biological data has been constantly and completely digitized and recorded into the memory of computer domains of electronic ISs for the first time in the world scientific practice, which became an influential factor of the progress in this field. Today, in addition to human genome, there was continuous work on decoding the genomes of many other living organisms, such as mammals, agricultural, viral and bacterial organisms, which gives humanity fundamental knowledge about the information basis of living systems. Another fundamental discovery of modern biotechnology had become the "high-capacity" scan of genome, in which the integrativity (polymorphism of a separate nucleotide) and the activity (gene expression profiling) for each gene in the same genome can be recorded from the same sample.

This was done by using a technology of DNA milieu platform that can detect tens of thousands of genes, using a small functional system like a post stamp, with data immediately

recorded into computer's memory. Information coming from the laboratories working with DNA milieu platforms contained thousands of gene-specific measurements per day. So, the general result of the work with the use of such revolutionary technologies also depended on the level of used integrated ISs for the analysis and recording of the data, as well as from the computer system for this gene-specific detection (hybridization).

In the United States, the Food and Drug Administration (FDA) has published guidelines for the development of biotechnological methods, including such as DNA milieu technology platforms, for their use in genetic prognostication and diagnosis of humans; there were indicated that such methods actually initiated a revolution in medicine, which became "more personalized" with their use.

Progress in biochemistry and genome studies faced new challenges, stimulated the development of other branches of science, interdisciplinary research. For example, in [1, 100] it was demonstrated that as a result of the necessity of processing of a huge amounts of experimental data obtained using modern automated experiments in biochemistry, molecular biology, there was a need to create a new set of computer methods and optimization techniques. The peculiarity of such situation was that many of the important problems arising from the researches in computer biochemistry and gene analysis could be formulated in terms of certain combinatorial optimization problems in specially constructed graphs. The authors of the article developed such approaches and offered them to solve new biological problems. The methods used in bioinformatics have been called "the use of mathematical, statistical and computer tools for analyzing of biological data". These were sorting of sequences, phylogenetic trees, predictions of structures, and various modeling methods. Later the recognition was added to this set. Speaking about the main fields of application of computer methods in modern gene and molecular biology, ones told about the study of DNA chains sequences formation, collection of these chains, mapping of genomes, comparison of sequences and analysis of phylogenetic relationships.

The natural extension of IT/CT application in the study of genome and molecular biology phenomena became the field of study and correction of metabolic disorders with IT use [1, 101, 102, 107]. The study of metabolic disorders was focused on the research of metabolic mechanisms, the search for effective treatment methods, and improvement of clinical diagnosis during the XX century. The successes that have been achieved in deciphering of gene sequences and associated metabolic data, the success in the development of ISs based on the databases of genome and molecular biology research results, elaboration of database on protein and other biomolecules important in terms of metabolism, allowed ones to transfer some of the developed computer methods to the field of study of metabolism and its disorders. In this article the analytical strategies for bioinformatics were used to process current data both at genome level and at level of metabolism, and then the results were combined to explain certain metabolic disorders. For the analysis of biomedical data in disorders the PathAligner Internet Information System was used, and the results were shown on example of urea cycle disorders.

The "Petri net" model was designed to assess the regulation both at the gene level and at the metabolic level. To explain the regulation of the urea cycle, the transcription factors and signaling pathways were also analyzed. It is known that gene/metabolic defects often cause metabolic blockade and lead to the metabolic disorders. In accordance with the current state of development of methods and concepts of bioinformatics for the analysis of metabolic disorders, it was necessary to understand first the ways of reactions that are effected by encoded gene information (directly or indirectly) and to find out how the modification of the reaction phases and the depletion of metabolites reserves affect the overall response of the system of reactions. For the solution of these problems the methods of biomedical information search and system modeling were used. The PathAligner Web-based system was used to allow users to navigate easily in genetic and metabolic related information. The "Petri net" methodology was used to create a model of biomedical system. For example, in the system of urea cycle modeling, a huge volumes of the data from different databases were used; as a result of which the authors described the metabolic paths and mechanisms, regulatory schemes, and etc. Subsequently, the authors wanted to extend these methods to the study of rheumatoid arthritis and other diseases. The design and analysis of signal network allowed the authors to verify the integrity of the data set. The "base network" constructed by the authors was useful for the next simulation, forecasting and comparison of various cellular systems. The obtained

graphical results and the results of dynamic modeling made it possible to understand intuitively the logic of cellular metabolism. Thus, the applied methods of bioinformatics could be considered as non-invasive methods for studying of the functions of genetic and metabolic systems.

The authors of [102] believed that on the basis of their developed methods of bioinformatics, the method for integrating of the data and procedures for the modeling of "Petri net" will be following: 1 — to integrate biological and biomedical data; 2 — to create functional structure models; 3 — to predict genetic predisposition to pathologies, disease detection, improved diagnosis, development of new drugs, toxicology; 4 — to use alternative, compensatory metabolic paths instead of damaged, and etc. As a result, the scientists would be able to use such system analysis, for example, to prevent metabolic disorders and comprehensive testing of drugs before their traditional laboratory testing. The authors considered that their main achievement is to integrate various levels of metabolic data using the existing methods of bioinformatics and computer-based methods on the basis of the databases. They believed that the use of IT/CT can simulate a general scenario of how biomedical systems work, which clinical manifestations can have changes in genes and, consequently, the false changes in the paths of chemical reactions (which follow gene changes); so, what measures are necessary for the prevention of various human pathologies, including oncopathology [110, 111]. Examples of such works using the newly created tISs could be illustrated by Figures 3, 4. Thus, in [110] (Fig. 3), as a result of processing of experimental data from elaborated databases in the corresponding tIS, the authors had found "clusters enriched for genes involved in both cell cycle regulation and cell division, which is biologically reasonable in a cancer orientated dataset". Than they have evaluated their method on both synthetic and gene expression analysis problems.

In other publication [111] (Fig. 4) using the tIS with linked databases with the obtained experimental data, and as a result of automatic processing of these data, the authors wrote that their results provide a novel molecular stratification of the breast cancer population, derived from the impact of somatic copy number aberrations on the transcriptome.

Information systems developed in other spheres of biology, data from which were used in biotechnology: biodiversity, ecology, zoology

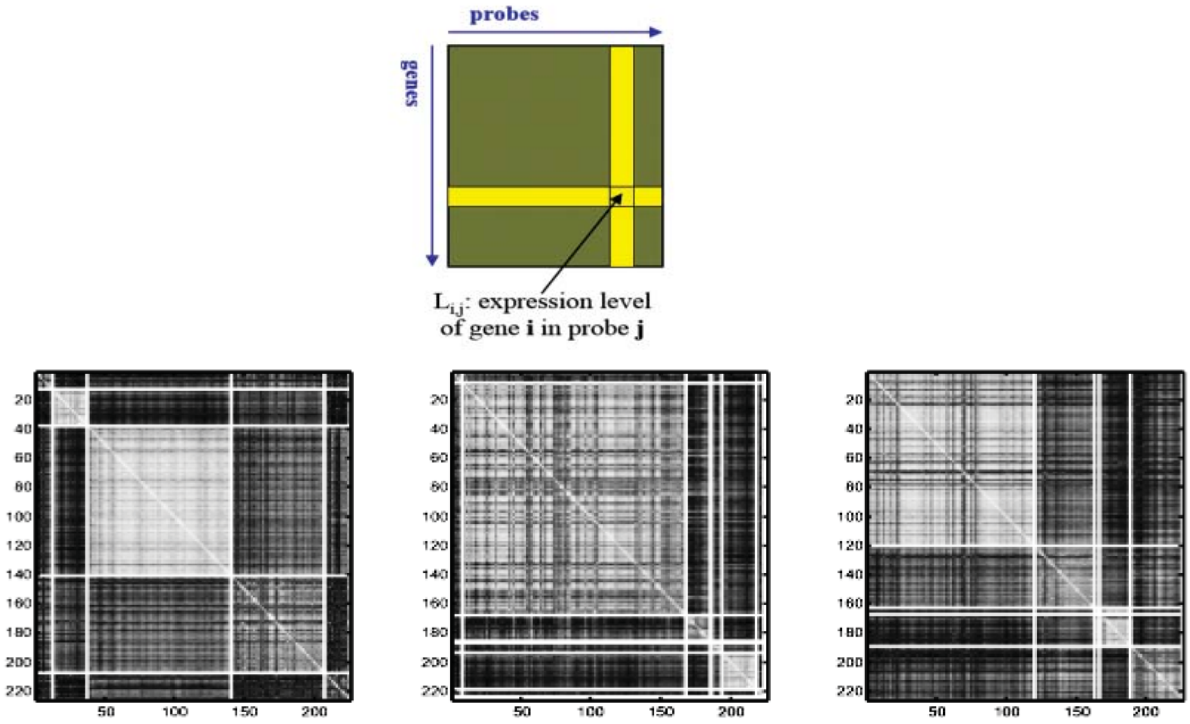

Fig. 3. **Clustering of covariance structure (explanations see in text) [110]**
Above: The gene expression matrix. $L_{I,j}$: expression level of gene I in probe j.
Below. Left: k-means using correlation distance. *Middle*: agglomerating of hierarchical clustering using average linkage and correlation distance. *Right*: DPVC MCMC

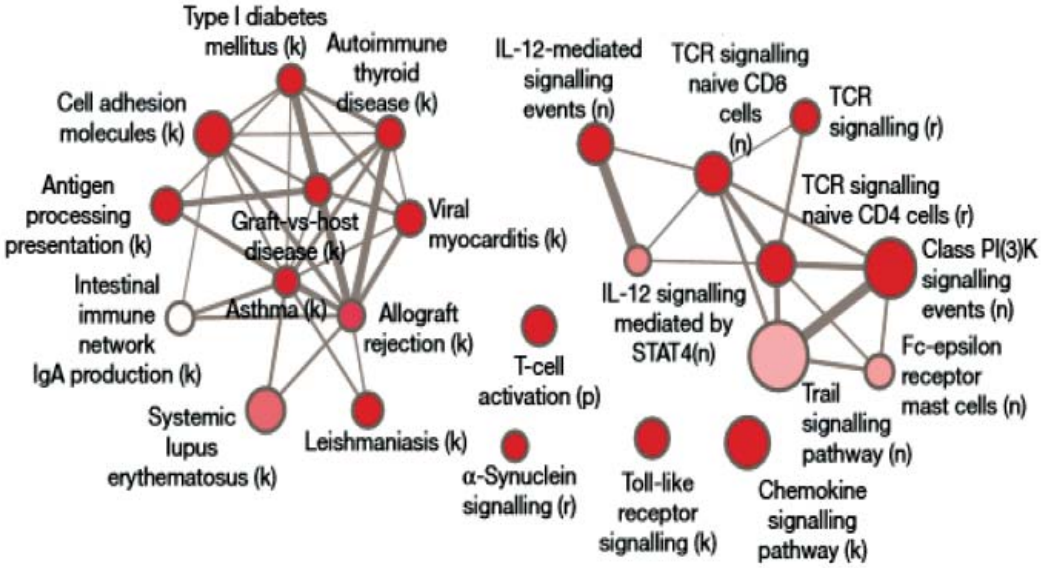

Fig. 4. **Trans-acting aberration hotspots modulate concerted molecular pathways (explanations see in text) [111]**

[113, 114, 117, 120–122, 134–136, 139–141, 146, 147]. In this chapter let's review the experience of elaboration of ISs based on the databases about insects and some other living organisms. The development of such tISs started in the late 1990-th [112]. These systems were named EuroLOOP and BioNET [112]. EuroLOOP was suggested as database about the insects and some other living organisms in Europe. BioNET was developed as similar database system but of a larger scale which, in addition to the databases about European organisms, included collections and data analysis from organisms in some African countries. Later BioNET became a well-known global system. In these two systems developers have acquired the first experience in construction of informational biological systems, taking into account the specifics of biological objects and data. This experience has been used further by ISs' developers in other countries.

Later and more perfect examples of development and use of electronic databases in Internet with a purely academic purpose were following two projects [113, 114]. The "Tree of Life" Web project [114] was a good electronic resource for finding of information, relevant links and hyperlinks on animal and plant diversity (http://tolweb.org). This project satisfied the needs of one who interested in information about the certain groups of organisms. The project was started in early 1980-th by David Maddison and later it was supported by scholars and amateurs throughout the world. The site was structured in the form of a tree with roots, branches and leaves. So-called page-branches were branched off, reflecting the fact that some groups of organisms can be further subdivided into subgroups due to the certain differences in their genotypes. Unlike such groups, phylogenetic groups that formed leaves (and, respectively, in ISs, leaves-pages) corresponding to the terminal groups that are not subdivided into subgroups in nature [114].

If one could go to any page ("branch" or "leaf"), one can find a brief overview of the most important characteristics of a certain group of organisms. In addition, one can access a collection of relevant scientific articles, other accessible Web-resources, notes and comments with additional information and so-called "Treehouses" [114], which were designed for the children as a teaching Web-resource. For the most pages the pictures of described organisms — colored illustrations there were presented also.

If the previous databases about the insects were first developed for the Euro-African region, the following two electronic DBs about insect pathogens were visualized in Internet on 1996 [87]. They were developed in the United States and Japan, and they are updated by the material mainly from these regions. For example, in the United States there were already many databases about the different groups of living organisms of this country, such as ANIMAL INFO — Animal Bytes; this was a site for various organisms of animal world, from worms to mammals.

Later the development of IS with living organisms databases started in India, where a technically more complex BODHI system [117] with biodiversity data was elaborated (Fig. 5). The works were conducted under the supervision of Biotechnology Department of the Ministry of Science and Technology with the support of India Government [117]. In electronic databases of this system there should be accumulated the plant world information. The BODHI authors considered as it unique characteristic the possibility to store different types of the data at different levels: from molecular level to organism level. These data also included taxonomic characteristics, geographic distribution, genetic sequences, and others. For the work with these data some specific indexing strategies were developed and used to enable these data access. A special processor was designed to optimize the execution of queries. The BODHI system was an integrative database system, it was an object-oriented system in which complex objects form a subordinate hierarchy and sequences that are similar with biodiversity domain in nature.

BODHI was considered to be the first system of nature in which such integrative approach and data aggregation are implemented at different levels of hierarchy systems [117]. The system was based on licensed and accessible software and it is provided free of charge. It was implemented on the basis of PC Pentium-III with the operating system Linux. Access to the data in database was carried out on the basis of the structures described in technical literature. For example, the Path-Dictionary and Multi-key Type can accelerate the access in inheritance and aggregation hierarchies, while R* -tree and Hilbert R-tree were used to control spatial queries. BODHI server was compliant with ODMG, it supports OQL/ODL queries, and data modeling interface. In order to permit biologists intuitively easier to work with this system, BODHI also supported access through a Web client-server model, where the client generated its requests through the HTTP protocol and CGI-bin scripts, and results are displayed using the browser interface. And finally, the server was "XML-friendly" , the object-results are displayed in XML format, which allows their visualization (Fig. 5) [117].

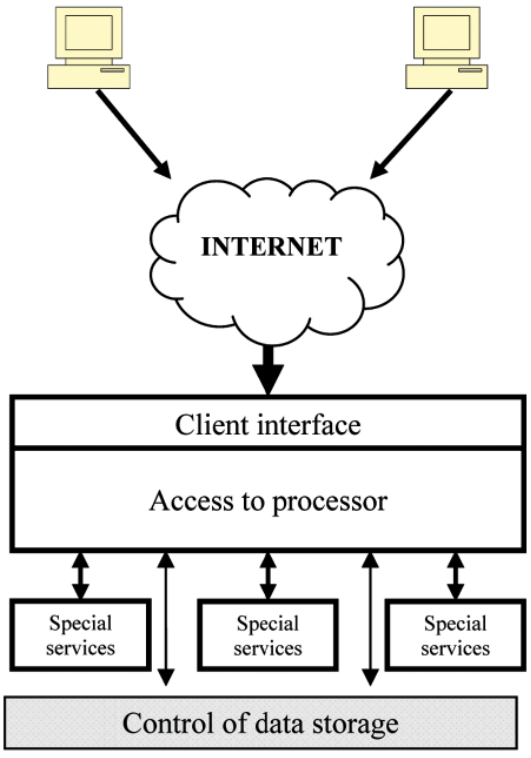

Fig. 5. **Scheme of BODHI system architecture (Bedathur) [117]**

Developers created BODHI not only as database system by itselves, it plays a more important role. It is also a central repository that provides users with a common platform for information exchange, decision making, packet visualization, and etc. The role of BODHI developers is similar to the role played by the Management Information Base (MIB) system in the management of networks in telecommunications.

Information systems with databases about biological objects developed for the global networks. Contemporary electronic databases with access to the Internet with information about living organisms were the tISs, designed during the last 25 years. They were designed either for academic purposes — to maximize the accumulation of information on groups of living organisms, or for the needs of economy, in particular, for conducting of ecological monitoring, including monitoring of polluted environments. Today all such tISs are network-based and linked with databases [1, 10, 11, 25, 41, 42, 60–62, 67, 69–71, 90–94, 96–113, 116, 119–126, 133–140, 143–146].

Let's observe several tISs, in which the numerous electronic collections (they are ordered into the databases about the biological organisms) were connected into the one network, uniting segments in different world regions. A number of databases united into such a system should contain information on the types of bioorganisms, their variability, biodiversity, which during monitoring are able to reflect the effect of environmentally harmful influences on these bioorganisms [1, 12, 17, 22, 25, 29, 98–100, 108, 117]. Data from already

developed networks with electronic databases demonstrate the high variability of taxonomic groups on a global scale (for example, information from the Global Biodiversity Information Facility), the variability of taxonomic groups in different regions of the Earth (for example, the European Natural History Specimen Information Network) and the variability of areas of the specific taxonomic groups distribution on a planet scale (for example, the Mammal Networked Information System — MANIS) [113]. In frameworks of these projects, the data ordered in disparate electronic databases developed by implementing institutions were linked over the Internet using modern network technologies. In such a way the data of separate and previously disparate electronic collections were actually merged into a single, large-scale centralized collection. So, the user could, in response to one request to receive an information simultaneously from all collections of institutions participated in these projects. These networks contained information about 20–40% of species (approximately 60 million electronic records) up to 2008 [113]. Consequently, since the basic information about numerous species had already been ordered in the electronic database, it was necessary to do the further steps — the addition to biological data the relevant geographic information, standardization, detection of errors and the completion of already developed databases with information about the new species from databases of other networks. The authors considered that fulfillment of these tasks in the context of already developed IS would greatly improve the quality of all ISs in general. Thus, during the creation of the MANIS database, the electronic records from geographic databases were recorded into it, for example, with the information about latitude and longitude, and in process of geographic location determining an error was estimated [113].

Standardization of taxonomic information — the presentation of generally accepted non-conflicting names of species — is another difficulty, if to take into account that information about these species is distributed (and comes to the user) from/to geographically remote compartments of the global network, and such information may differ in databases created in different regions of the world. The work on the collecting and recording of taxonomically correct information about different species is of priority task, since the practical value of the whole project depends on the validity of such information. Consequently, a lot of efforts would be necessary for taxonomic electronic information correction on a global scale, taking into account also the fact that taxonomy studies are constantly ongoing worldwide, and therefore, such information in electronic databases should be constantly updated and standardized [1, 113].

The similarity of collection contents and observational data in such databases, including Darwin Core (http://speciesanalyst.net/docs/dwc/) and the group that solve the task of access to biological data collections (Task Group on Access to Biological Collection Data-ABCD; http://www.bgbm.org/TDWG/ CODATA/ Schema/default.htm) [1, 113] allowed ones to search for ordered information and to get it from different data sets. In the framework of some projects, standardization of taxonomic information, which has been distributed in networks, has already been carried out. Such projects included the Integrated Taxonomic Information System (http://www.itis.usda.gov/), Species2000 (http://www.sp2000.org/) and the Electronic Directory of Names of Famous Organisms (Electronic Catalog of Names of Known Organisms — ECAT; http://www.gbif.org/prog/ecat/prog). The databases for these projects were developed in close cooperation between taxonomists and researchers in other fields that provided their data. For data transmission through the Internet within these projects, freely available software was developed by Distributed Generic Information Retrieval (DiGIR; http://digir.sourceforge.net/) for the Darwin Core project and BioCASE Data Transfer Protocol (http: // www.biocase.org/) for the ABCD project. These client-server protocols provided a single source of access to the data received from the few data sources [1].

Information systems with databases, which combine characteristics of both medical and biological ones. In many recent publications it has been demonstrated that development of ISs with databases in medicine and biology is gradually reducing the distance between biological sciences and medicine [1, 120, 132]. This happened due to number of reasons, but mainly because of the fact that many stored data can be used both in clinical practice and in biological research [1]. Besides of this, such branch as biotechnology occupied intermediately positions between these sciences by themselves. Thus, the library system BioMedNet, which contains abstracts of well-known biological and medical journals, is used by biotechnologists, physicians and biologists as well. The same one can say about the library system Academic Press and

Springer, which contain a large number of full-text journals, necessary for biologists and doctors both. To the database with information about proteins and decoded gene sequences apply many professionals — biotechnologists, doctors, specialists from many other biological specialties. In article [120] it was noted that this situation led to the introduction in the United States, primarily at Columbia University, of new courses in the educational process, which combine biological and medical informatics. After the analysis of the state of the problem, the course "Theory and Methods of Biomedical Informatics" was developed there and approved for studying. A similar course was also studied in the Stanford University. The article [132] outlined the latest for these years' methodologies for the development of ISs with databases. These methodologies were based on ontological principles, the developments of ISs with "mixed" information were used widely; and they combined both the databases with gene information and clinical databases. New ontological methods for development of such ISs were needed to facilitate the information search, access to it, and obtain it from the remote Internet resources. One of the areas of application of new ISs developments are biotechnology and biomedicine. These spheres, on expert opinions, would lead soon to new scientific achievements and would accelerate the works on the program "Human Genome Project". The development of such newest ISs in biotechnology and biomedicine were being conducted by European Commission programs, for example, the INFOGENMED project [1].

Types of information systems that occupied intermediately positions between biology and medicine. As an example of such systems we can observe a neurophysiology; moreover, many professionals in biotechnology are interested in the data obtained in this sphere. Neurophysiology as a branch is at the "intersection" between medical and biological sciences. So, some peculiarities of ISs' classification there were similar to both biology and medicine (let's compare figures 1, 2, 6) [1]. Sure, it did not have "Medical ISs of general purpose" group because there is not need in their development — such tasks are important for medical practice only. However, the neurophysiology deals with the solution of purely scientific problems, so developed electronic systems here have to facilitate their solution. There are also the need to develop own large databases and electronic library systems to store numerous experimental neurophysiological results and data from literary sources. So, in the classification list for neurophysiology, some items would coincide with the list for medical ISs, and some — with biological ISs. As a result, the general classification for the field of neurophysiology acquired the following form (Fig. 6): expert systems in neurophysiology (including ones for the solution of scientific problems), electronic systems for the work with images, electronic library systems in neurophysiology, and electronic databases in neurophysiology [1].

We could forecast that classification list of ISs types for biotechnology will demonstrate similar regularities as ones for neurophysiology, because this sphere is also at "intersection" between biology and medicine; but final correct conclusions we will be able to make after the upcoming additional studies.

Thus, at the current level of development of computer technologies in the medical and biological practice both older ISs according to time of their development, as well as more recent ideas, methods, technologies and developments coexist successfully [1]. Their role and the attention given to their application are determined by practical activity. The reasons of the fast development of ISs with databases in these spheres are the burst-like

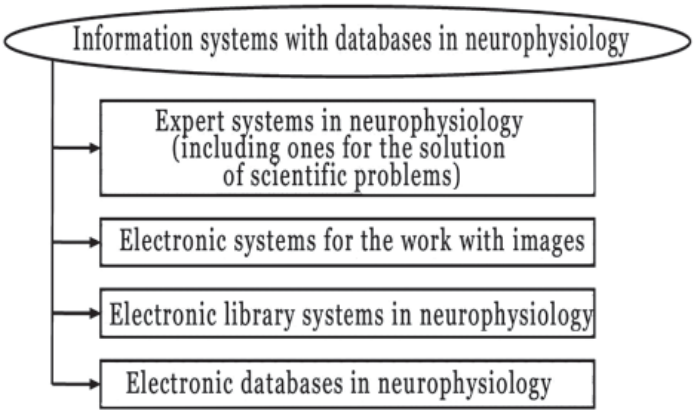

Fig. 6. **Information systems with databases in neurophysiology [1]**

increase in the amount of input data, which requires the appropriate development of database management systems and ISs.

Medical ISs obtain better funding then biological ones usually, the period of their intensive development was during the last 22–25 years. Consequently, medical ISs demonstrate the greater number of varieties in comparison with biological ones [1]. The first biological ISs were developed on 1980–1990-th, but the period of their intensive development was the last 15–16 years. Among the biological ISs, the first ones are those related to industry (biotechnology), medicine (for physiology, brain biology, etc.) and business (database with information on biological medicinal products, sales sites, and etc.) With regard to such biological ISs, which contain purely academic information, the classification of living organisms, taxonomy data and etc., their examples are not numerical today in comparison with medical ones [1], and their constructing is waiting for their developers still.

Comparing two classifications of modern electronic information systems — in medicine and biology, we can make some conclusions (Fig. 1, 2), basing on publications [1–132].

1. The medical ISs were characterized by the greatest variety and necessity for the practice.

2. Electronic ISs in biology were characterized by the greater proximity to scientific research.

3. Examining the numerous works on the development of various ISs in medicine and biology, we saw that developers are focused on the development of: 1) medical ISs of general purpose; 2) electronic library systems; 3) electronic systems for working with documents; 4) expert systems.

4. In parallel with these types of systems, the following types of ISs were presented in the publications of world scientific and technical literature: 1) electronic systems for working with images; 2) systems for scientific purposes; 3) electronic teaching systems, and etc.

The latter conclusion reflects the fact that the procedure of electronic teaching systems constructing is well developed and does not require special publications, then the elaboration of electronic systems for the work with images and systems for scientific purposes solves often the unique priority tasks, which also results less number of publications with the description of such systems. As example of biological ISs the BODHI system we observed more attentively. It was an integrative database system, an object-oriented system in which complex objects form a subordinate hierarchy and sequences that are similar with biodiversity domains in nature [117].

So, in present publication we generalized and analyzed the experience of electronic information systems with databases use; and such systems may be used for tasks solution in biotechnology. We examined ISs from numerical scientific and technical publications, suitable for the solutions of different tasks in biotechnology. Then we classified such systems, which traditionally refer to both biological and medical sciences. Further we observed different examples of such information systems, as well as systems that have characteristics both medical and biological in order to facilitate the invention of future more advanced electronic information systems for biotechnological purposes.

REFERENCES

1. *Klyuchko O. M.* Information and computer technologies in biology and medicine. *Kyiv: NAU-druk*. 2008, 252 p. (In Ukrainian).
2. *Piatigorsky B.Ya., Zaitman G.A., Cherkasky V. L., Chinarov V.A.* Automatic electrophysiological experiment. *Kyiv: Nauk. dumka*. 1985. 216 p. (In Russian).
3. *Klyuchko O. M.* On the mathematical methods in biology and medicine. *Biotechnol. acta.* 2017, 10(3), 31–40. https://doi.org/10.15407/biotech10.03.031
4. *Klyuchko O. M.* Application of artificial neural networks method in biotechnology. *Biotechnol. acta*. 2017, 10(4), 5–13. https://doi. org/10.15407/biotech10.04.005
5. *Klyuchko O. M.* Cluster analysis in biotechnology. *Biotechnol. acta.* 2017, 10(5), 5–18. https://doi. org/10.15407/biotech10.05.005
6. *Klyuchko O. M.* Technologies of brain images processing. *Biotechnol. acta.* 2017, 10 (6), 5–17. https://doi.org/10.15407/biotech10.05.005
7. *Tasseff R. A.* Mathematical Models in Biotechnology. *PLoS One,* 5:e8864, 2010.
8. *Mata-Alvarez J.* Mathematical Modeling in Biotechnology. *Biotechnology*. V. II. 2004. https://www.eolss.net/sample-chapters/C17/ E6-58-02-04.pdf
9. *Gordeev L. S.* Mathematical Modeling in Chemical Engineering and Biotechnology. *Theoretical Foundations of Chemical Engineering.* 2014, 48 (3), 225–229. https://link.springer. com/article/10.1134/S0040579514030099
10. *Schnase J.L., Cushing J., Frame M.* Information technology challenges of biodiversity and ecosystems informatics. *Information systems*. 2003, 28 (4), 339–345.

11. *Rana B. K., Insel P. A.* G-protein-coupled receptor websites. *Trend. Pharmacol. Sci.* 2002, 23 (11),535–536. doi: http://dx.doi.org/10.1016/ S0165-6147(02)02113-2
12. *Patent* 1370136 USSR, МКИ C12N 5/00. The method for dissociation of hippocampal cells. Klyuchko E. M., Tzyndrenko A. Ya. Priority: 31.01.1986; Issued: 30.01.1988, Bull. No 4, 3 p.
13. *Sigworth F.* Single channel registration. *Moskva: Mir*, 1987. 448 p. (In Russian).
14. *Kostyuk P. G.* Mechanisms of electrical excitability of nerve cells. *Moskva: Nauka,* 1981, 208 p. (In Russian).
15. *Klyuchko O. M., Paskivsky A. O., Sheremet D. Y.* Computer modeling of some nanoelements for radio and television systems. *Electr. Contr. Syst.* 2012, 3 (33), 102–107. (In Ukrainian).
16. *Klyuchko O. M., Hayrutdinov R. R.* Modeling of electrical signals propagation in neurons and its nanostructures. *Electr. Contr. Syst.* 2011, 2 (28), 120–124. (In Ukrainian).
17. *Trinus K. F., Klyuchko E. M.* Mediators influence on motoneurons retrogradly marked by primulin. *Physiol. J.* 1984. 30 (6), 730–733 (In Russian).
18. *Aralova N. I., Klyuchko O. M., Mashkin V. I., Mashkina I. V.* Algorithmic and program support for optimization of interval hypoxic training modes selection of pilots. *Electr. Contr. Syst.* 2017, 2 (52), 85–93.
19. *Aralova N. I., Klyuchko O. M., Mashkin V. I., Mashkina I. V.* Mathematic and program models for investigation of reliability of operator professional activity in "Human-Machine" systems. *Electr. Contr. Syst.* 2017, 1 (51), 105–113.
20. *Aralova N. I., Klyuchko O. M., Mashkin V. I., Mashkina I. V.* Mathematical model for research of organism restoring for operators of continuously interacted systems. *Electr. Contr. Syst.* 2016, 3 (49), 100–105.
21. *Aralova N. I., Klyuchko O. M., Mashkin V. I., Mashkina I. V.* Investigation of reliability of operators work at fluctuating temperature conditions. *Electr. Contr. Syst.* 2016, 2 (48), 132–139.
22. *Plakhotnij S. A., Klyuchko O. M., Krotinova M. V.* Information support for automatic industrial environment monitoring systems. *Electr. Contr. Syst.* 2016, 1 (47), 19–34.
23. *Onopchuk Yu. M., Aralova N. I., Klyuchko O. M., Beloshitsky P. V.* Mathematic models and integral estimation of organism systems reliability in extreme conditions. *Electronics and control systems*. 2015, 4 (46), 109–115.
24. *Onopchuk Yu. M., Aralova N. I., Klyuchko O. M., Beloshitsky P. V.* Integral estimations of human reliability and working capacity in sports wrestling. *Journal of the Engineering Academy*. 2015, V. 3, P. 145–148. (In Russian).
25. *Klyuchko O. M., Shutko V. N., Navrotskyi D. O., Mikolushko A. M.* The set of program models for ecological monitoring technical system based on principles of biophysics. *Electr. Contr. Syst.* 2014, 4 (42), 135–142.
26. *Klyuchko O. M., Sheremet D. Yu.* Computer simulation of biological nanogenerator functions. *Electr. Contr. Syst.* 2014, 2 (40), 103–111.
27. *Klyuchko O. M., Shutko V. N.* Computer modeling of auto-oscillating phenomena in neuron complexes. *Electr. Contr. Syst.* 2014, 1 (39), 127–132.
28. *Klyuchko O. M., Sheremet D. Yu.* Computer modeling of biologic voltage-activated nanostructures. *Electr. Contr. Syst.* 2014, 1 (39), 133–139.
29. *Beloshitsky P. V., Klyuchko O. M., Onopchuk Yu. M.* Radiation damage of organism and its correction in conditions of adaptation to highmountain meteorological factors. *Bulletin of NAU*. 2010, V.1, P. 224–231. (In Ukrainian).
30. *Beloshitsky P. V., Klyuchko O. M., Onopchuk Yu., Makarenko M. V.* Estimation of psycho-physiological functions of a person and operator work in extreme conditions. *Bulletin of NAU*. 2009, No 3, P. 96–104. (In Ukrainian).
31. *Beloshitsky P. V., Klyuchko O. M., Onopchuk Yu., Kolchinska A. Z.* Results of research of higher nervous activity problems by Ukrainian scientists in Prielbrussie. *Bulletin of NAU*. 2009, No 2, P. 105–112. (In Ukrainian).
32. *Beloshitsky P. V., Klyuchko O. M., Onopchuk Yu.* Results of research of structural and functional interdependencies by Ukrainian scientists in Prielbrussie. *Bulletin of NAU*. 2009, No 1, P. 61–67. (In Ukrainian).
33. *Beloshitsky P. V., Klyuchko O. M., Onopchuk Yu.* Results of research of highlands factors influence on health and longevity by Ukrainian scientists in Prielbrussie. *Bulletin of NAU*. 2008, No 4, P. 108–117. (In Ukrainian).
34. *Onopchuk Yu. M., Klyuchko O. M., Beloshitsky P. V.* Development of mathematical models basing on researches of Ukrainian scientists at Elbrus. *Bulletin of NAU*. 2008, No 3, P.146–155. (In Ukrainian).
35. *Beloshitsky P. V., Klyuchko O. M., Onopchuk Yu.* Results of research of adaptation problems by Ukrainian scientists in Prielbrussie. *Bulletin of NAU*. 2008, No 1, P. 102–108. (In Ukrainian).
36. *Beloshitsky P. V., Klyuchko O. M., Onopchuk Yu.* Results of research of hypoxia problems by Ukrainian scientists in Elbrus region. *Bulletin of NAU*. 2007, No 3–4, P. 44–50. (In Ukrainian).

37. *Beloshitsky P. V., Klyuchko O. M., Onopchuk Yu*. Results of medical and biological research of Ukrainian scientists at Elbrus. *Bulletin of NAU*. 2007, No 2, P. 10–16. (In Ukrainian).
38. *Belan P. V, Gerasimenko O. V., Tepikin A. V., Petersen O. H*. Localization of Ca++ extrusion sites in pancreatic acinar cells. *J. Biol. Chem.* 1996, V. 271, P. 7615–7619.
39. *Belan P., Gardner J., Gerasimenko O*. Extracellular Ca++ spikes due to secretory events in salivary gland cells. *J. Biol. Chem.* 1998, V. 273, P. 4106–4111.
40. *Jabs R., Pivneva T., Huttmann K*. Synaptic transmission onto hyppocampal glial cells with hGFAP promoter activity. *J. Biol. Chem.* 2005, V. 118, P. 3791–3803.
41. *Gavrilovich M*. Spectra image processing and application in biotechnology and pathology. Dissertation for Ph.D. *Acta Universitatis Upsaliensis. Upsala.* 2011, 63 p.
42. *Perner P., Salvetti O*. Advances in Mass Data Analysis of Images and Signals in Medicine, Biotechnology, Chemistry and Food Industry. *Third International Conference, Leipzig, (Germany): Springer, 2008, Proceedings.* 2008, 173 p.
43. *Baert P., Meesen G., De Schynkel S., Poffijn A., Oostveldt P. V*. Simultaneous in situ profiling of DNA lesion endpoints based on image cytometry and a single cell database approach. *Micron.* 2005, 36 (4), 321330. https://doi.org/ 10.1016/j.micron.2005.01.005.
44. *Berks G., Ghassemi A., von Keyserlingk D. G.* Spatial registration of digital brain atlases based on fuzzy set theory. *Comp. Med. Imag. Graph*. 2001, 25 (1), 1–10. https://doi.org/10.1016/S0895-6111(00)00038-0.
45. *Nowinski W. L., Belov D.* The Cerefy Neuroradiology Atlas: a Talairach–Tournoux atlas-based tool for analysis of neuroimages available over the internet. *NeuroImage.* 2003, 20(1), 50–57. https:// doi.org/10.1016/S1053-8119(03)00252-0.
46. *Chaplot S., Patnaik L. M., Jagannathan N. R.* Classification of magnetic resonance brain images using wavelets as input to support vector machine and neural network. *Biomed. Signal Process. Control*. 2006, 1 (1), 86–92. https:// doi.org/10.1016/j.bspc.2006.05.002.
47. *Kovalev V. A., Petrou M., Suckling J*. Detection of structural differences between the brains of schizophrenic patients and controls. *Psychiatry Research: Neuroimaging*. 2003, 124 (3), 177–189. https://doi.org/10.1016/S0925-4927(03) 00070-2.
48. *Araújo T*. Classification of breast cancer histology images using Convolutional Neural Networks. *PloS One*. 2017, 12 (6), e0177544.
49. *Vecht-Lifshitz S. E., Ison A. P*. Biotechnological applications of image analysis: present and future prospects. *J. Biotechnol*. 1992, 23 (1), 1–18.
50. *Toga A. W., Thompson P. M*. The role of image registration in brain mapping. *Image Vis. Comput*. 2001, 19 (1-2), 3–24.
51. *Carro S. A., Scharcanski J*. A framework for medical visual information exchange on the WEB. *Comput. Biol. Med.* 2006, V. 4, P. 327–338.
52. *Chakravarty M. M., Bertrand G., Hodge C. P., Sadikot A. F., Collins D. L*. The creation of a brain atlas for image guided neurosurgery using serial histological data. *NeuroImage.* 2006, 30(2), 359–376. doi: 10.1016/ j.neuroimage.2005.09.041.
53. *Dikshit A., Wu D., Wu C., Zhao W*. An online interactive simulation system for medical imaging education. *Comp. Med. Imag. Graph.* 2005, 29 (6), 395404. https://doi.org/10.1016/j.compmedimag.2005.02.001.
54. *Singh R., Schwarz N., Taesombut N., Lee D., Jeong B., Renambot L., Lin A. W., West R., Otsuka H., Naito S., Peltier S. T., Martone M. E., Nozaki K., Leigh J., Ellisman M. H*. Real-time multi-scale brain data acquisition, assembly, and analysis using an end-to-end. *OptIPuter Fut. Gener. Comp. Syst.* 22 (2006), 1032–1039.
55. *Stefanescu R., Pennec X., Ayache N*. Grid powered nonlinear image registration with locally adaptive regularization. *Med. Imag. Anal.* 2004. 8 (3), 325–342.
56. *Ma Y., Hof P. R., Grant S. C., Blackband S. J., Bennett R., Slatest L., McGuigan M. D., Benveniste H*. A three-dimensional digital atlas database of the adult C57BL/6J mouse brain by magnetic resonance microscopy. *Neuroscience*. 2005, 135 (4), 12031215. doi: 10.1016/j.neuroscience.2005.07.014.
57. *Yu-Len Huang*. Computer-aided Diagnosis Using Neural Networks and Support Vector Machines for Breast Ultrasonography. *J. Med. Ultrasound*. 2009,17 (1), 17–24.
58. *Prachi Damodhar Shahare, Ram Nivas Giri*. Comparative Analysis of Artificial Neural Network and Support Vector Machine Classification for Breast Cancer Detection. *Int. Res. J. Engineer. Technol. (IRJET)*. 2015, 2 (9).
59. *Natrajan R., Sailem H., Mardakheh F. K., Garcia M. F., Tape C. G., Dowsett M., Bakal C., Yuan Y*. Microenvironmental heterogeneity parallels breast cancer progression: a histology–genomic integration analysis. *PLoS medicine*. 2016, 13(2), e1001961. https://doi.org/10.1371/journal.pmed.1001961.
60. *Klyuchko O. M*. Brain images in information systems for neurosurgery and neurophysiology. *Electr. Contr. Syst.* 2009, 3(21), 152–156. (In Ukrainian).

61. *Klyuchko O. M.* Using of images' databases for diagnostics of pathological changes in organism tissues. *Electronics and control systems.* 2009, 2 (20), 62–68. (In Ukrainian).
62. *Klyuchko O. M.* Elements of different level organization of the brain as material for electronic databases with images. *Electronics and control systems.* 2009, 1 (19), 69–75. (In Ukrainian).
63. *Steimann F.* On the representation of roles in object-oriented and conceptual modelling. Data& Knowedge Engineering. 2000, 35 (1), 83–106.
64. *Klyuchko O. M., Managadze Yu. L., Pashkivsky A. O.* Program models of 2D neuronal matrix for ecological monitoring and images' coding. *Bulletin of the Engineering Academy*. 2013, No 3–4, P. 77–82. (In Ukrainian).
65. *Klyuchko O.M., Piatchanina T.V., Mazur M.G.* Combined use of relation databases of images for diagnostics, therapy and prognosis of oncology diseases. "*Integrated robototechnic complexes*". *X IIRTC-2017 Conference Proceedings.* P. 275–276. (In Ukrainian).
66. *Shutko V. M, Shutko O. M., Kolganova O. O.* Methods and means of compression of information. *Kyiv: Nauk. dumka.* 2012. 168 p. (In Ukrainian).
67. *Jecheva V., Nikolova E.* Some clusteringbased methodology applications to anomaly intrusion detection systems. *Int. J. Secur. Appl*. 2016, 10 (1), 215–228. http://dx.doi.org/10.14257/ijsia.2016.10.1.20.
68. *Iakovidis D. K., Maroulis D. E., Karkanis S. A.* Texture multichannel measurements for cancer precursors' identification using support vector machines. *Measurement*. 2004, V. 36, P. 297–313. 2004.09.010 https://doi. org/10.1016/j.measurement.
69. *Nguyen H.Q., Carrieri-Kohlman V., Rankin S.H., Slaughter R, Stulbarg M. S.* Internet-based patient education and support interventions: a review of evaluation studies and directions for future research. *Comp. Biol. Med.* 2004, 34 (2), 95–112. doi: 10.1016/S0010- 4825(03)00046-5.
70. *Jézéquel P., Loussouarn L., Guérin-Charbonnel C., Campion L., Vanier A., Gouraud W., Lasla H., Guette C., Valo I., Verrièle V. Campone M.* Gene-expression molecular subtyping of triple-negative breast cancer tumours: importance of immune response. *Breast Cancer Res*. 2015, 17 (1), 43. https:// doi. org/10.1186/s13058-015-0550-y.
71. *Bozhenko V. K.* Multivariable analysis of laboratory blood parameters for obtaining diagnostic information in experimental and clinical oncology. The dissertation author's abstract on scientific degree editions. *Dc. Med. Study. Moscow*. 2004. (In Russian).
72. *Ko J. H., Ko E. A., Gu W., Lim I., Bang H., Zhou T.* Expression profiling of ion channel genes predicts clinical outcome in breast cancer. *Mol. Cancer*. 2013, 12 (1), 106. doi: 10.1186/1476-4598-12-106.
73. *Kawai M., Nakashima A., Kamada S., Kikkawa U.* Midostaurin preferentially attenuates proliferation of triple-negative breast cancer cell lines through inhibition of Aurora kinase family. *J. Biomed. Sci*. 2015, 22 (1), 48. doi: 10.1186/s12929-015-0150-2.
74. *Uhr K., Wendy J. C., Prager-van der Smissen, Anouk A. J. Heine, Bahar Ozturk, Marcel Smid, Hinrich W. H. G hlmann, Agnes Jager, John A. Foekens, John W. M. Martens.* Understanding drugs in breast cancer through drug sensitivity screening. *SpringerPlus*. 2015, 4(1), 611. doi: 10.1186/ s40064-015-1406-8.
75. *Onopchuk Yu. M., Biloshitsky P. V., Klyuchko O. M.* Development of mathematical models based on the results of researches of Ukrainian scientists at Elbrus. *Bulletin of NAU*. 2008, No 3, P. 146–155. (In Ukrainian).
76. *Ankur Poudel, Dhruba Bahadur Thapa, Manoj Sapkota*. Cluster Analysis of Wheat (Triticum aestivum L.) Genotypes Based Upon Response to Terminal Heat Stress. *Int. J. Appl. Sci. Biotechnol*. 2017, 5 (2), 188–193. doi: http://dx.doi.org/10.3126/ijasbt. v5i2.17614.
77. *Zaslavsky L., Ciufo S., Fedorov B., Tatusova T.* Clustering analysis of proteins from microbial genomes at multiple levels of resolution. *BMC Bioinform*. 2016, 17 (8), 276. Published online 2016 Aug 31. doi: 10.1186/s12859-016-1112-8.
78. *Zhou J., Richardson A. J., Rudd K. E.* EcoGene-RefSeq: EcoGene tools applied to the RefSeq prokaryotic genomes. *Bioinformatics*. 2013, 29 (15), 1917–1918. Published: 04 June 2013. doi: 10.1093/bioinformatics/btt302.
79. *Zhang J., Wu G., Hu X., Li S., Hao S.* A Parallel Clustering Algorithm with MPI — MKmeans. *J. Comput*. 2013, 8 (1), 1017. doi: 10.1109/ PAAP. 2011.17.
80. *Tatusova T., Zaslavsky L., Fedorov B., Haddad D., Vatsan A., Ako-adjei D., Blinkova O., Ghazal H*. Protein Clusters. *The NCBI Handbook [Internet]. 2nd edition*. Available at https:// www.ncbi.nlm.nih.gov/books/ NBK242632.
81. *Anderson J.G.* Evaluation in health informatics: computer simulation. *Comp. Biol. Med.* 2002, 32 (3), 151–164. https:// doi.org/10.1016/ S0010-4825(02)00012-4.
82. *Aruna P., Puviarasan N., Palaniappan B.* An investigation of neuro-fuzzy systems in psychosomatic disorders. *Exp. Syst. Appl*. 2005, 28 (4), 673–679. https://doi. org/10.1016/j. eswa.2004.12.024.
83. *Bange M. P., Deutscher S. A., Larsen D., Linsley D., Whiteside S.* A handheld decision

support system to facilitate improved insect pest management in Australian cotton systems. *Comp. Electron. Agricult*. 2004, 43 (2), 131–147. https:// doi.org/10.1016/j.compag.2003.12.003.

84. *Beaulieu A.* From brainbank to database: the informational turn in the study of the brain. *Stud. Hist. Phil. Biol. Biomed. Sci.* 2004, V. 35, P. 367–390. https://doi.org/10.1016/j.shpsc.2004.03.011.
85. *Horn W.* AI in medicine on its way from knowledge-intensive to data-intensive systems. *Artif. Intell Med. Elsevier.* 2001, 23 (1), 512. https://doi.org/10.1016/S0933-3657(01)00072-0.
86. *Brake I.* Unifying revisionary taxonomy: insect exemplar groups. *Abstr. XV SEL Congr. Berlin (Germany)*. 2007.
87. *Braxton S. M., Onstad D. W., Dockter D. E., Giordano R., Larsson R., Humber R. A.* Description and analysis of two internet-based databases of insect pathogens: EDWIP and VIDIL. *J. Invertebr. Pathol*. 2003, 83 (3), 185–195. doi: 10.1016/S0022-2011(03)00089-2.
88. *BreauxA.,CochraneS.,EvensJ.,MartindaledM., Pavlike B., Suera L., Benner D.* Wetland ecological and compliance assessments in the San Francisco Bay Region, California, USA. *J. Environm. Manag*. 2005, 74 (3), 217–237.
89. *Budura A., Cudré-Mauroux P., Aberer K.* From bioinformatic web portals to semantically integrated Data Grid networks. *Fut. Gener. Comp. Syst.* 2007, 23 (3), 281–522. doi: 10.1016/j.jenvman.2004.08.017.
90. *Burns G., Stephan K. E., Ludäscher B., Gupta A., Kötter R.* Towards a federated neuroscientific knowledge management system using brain atlases. *Neurocomputing*. 2001, V. 3840, P. 1633–1641. https://doi.org/10.1016/S0925-2312(01)00520-3.
91. *Butenko S., Wilhelm W. E.* Clique-detection models in computational biochemistry and genomics. Eur. J. Oper. Res. 2006, 173 (1), 117. https://doi.org/ 10.1016/j.ejor.2005.05.026.
92. *Carro S. A., Scharcanski J.* Framework for medical visual information exchange on the WEB. Comp. Biol. Med. 2006, 36 (4), 327–338. doi: 10.1016/ j.compbiomed.2004.10.004.
93. *Chau M., Huang Z., Qin J., Zhou Y., Chen H.* Building a scientific knowledge web portal: The NanoPort experience. *Decis. Support Syst.* 2006. https://doi.org/10.1016/j.dss.2006.01.004.
94. *Chen M., Hofestädt R.* A medical bioinformatics approach for metabolic disorders: Biomedical data prediction, modeling, and systematic analysis. *J. Biomed. Inform*. 2006, 39 (2), 147–159. https://doi. org/10.1016/j.jbi.2005.05.005.
95. *Chli M., De Wilde P.* Internet search: Subdivision-based interactive query expansion and the soft semantic web. *Appl. Soft Comp.* 2006. https://doi.org/10.1016/j.asoc.2005.11.003.
96. *Despont-Gros C., Mueller H., Lovis C.* Evaluating user interactions with clinical information systems: A model based on human– computer interaction models. *J. Biomed. Inform*. 2005, 38 (3), 244–255. https://doi. org/10.1016/ j.jbi.2004.12.004.
97. *Marios D., Dikaiakos M. D.* Intermediary infrastructures for the World Wide Web. *Comp. Networks*. 2004, V. 45, P. 421–447. https:// doi.org/ 10.1016/j.comnet.2004.02.008.
98. *Dimitrov S. D., Mekenyan O. G, Sinks G. D., Schultz T. W.* Global modeling of narcotic chemicals: ciliate and fish toxicity. *J. Mol. Struc.: Theochem*. 2003, 622 (12), 63–70. https://doi.org/10.1016/S0166-1280(02)00618-8.
99. *Dong Y., Zhuang Y., Chen K., Tai X.* A hierarchical clustering algorithm based on fuzzy graph connectedness. Fuzzy Sets. Syst. 2006, V. 157, P. 1760–1774. https:// doi.org/10.1016/j.fss.2006.01.001.
100. *Duan Y., Edwards J. S., Xu M. X.* Web-based expert systems: benefits and challenges. Inf. *Manag*. 2005, 42 (6), 799811. https://doi.org/10.1016/j.im.2004. 08.005.
101. *Essen van D. C.* Windows on the brain: the emerging role of atlases and databases in neuroscience. *Curr. Opin. Neurobiol.* 2002, 12 (5), 574–579. https://doi.org/10.1016/S0959-4388(02)00361-6.
102. *Fellbaum C., Hahn U., Smith B.* Towards new information resources for public health From Word Net to Medical Word Net. *J. Biomed. Inform*. 2006, 39 (3), 321–332. doi: 10.1016/j.jbi.2005.09.004.
103. *Ferraris M., Frixione P., Squarcia S.* Network oriented radiological and medical archive. *Comp. Physics Commun.* 2001, V. 140, P. 226–232. https://doi.org/10.1016/S0010-4655(01)00273-9.
104. *Flower D. R., Attwood T. K.* Integrative bioinformatics for functional genome annotation: trawling for G protein-coupled receptors. Reviews 55 Semin. *Cell. Dev. Biol*. 2004, 15 (6), 693–701. doi: 10.1016/j.semcdb.2004.09.008.
105. *Fink E., Kokku P. K., Nikiforou S., Hall L. O., Goldgof D. B., Krischer J. P.* Selection of patients for clinical trials: an interactive webbased system. *Art. Intell. Med*. 2004, 31 (3), 241–254. doi: 10.1016/j.artmed.2004.01.017.
106. *Fitzpatrick M. J., Ben-Shahar Y., Smid H. M., Vet L. E., Robinson G. E., Sokolowski M. B.* Candidate genes for behavioural ecology. *Trend Ecol. Evol*. 2005, 20 (2), 96–104. doi: 10.1016/j.tree.2004.11.017.

107. *Fox J., Alabassi A., Patkar V., Rose T., Black E.* An ontological approach to modelling tasks and goals. *Comp. Biol. Med.* 2006, V. 36, P. 837–856. https://doi.org/10.1016/j.compbiomed.2005.04.011.
108. *Fu Zetian, Xu Feng, Zhou Yun, Shuan X. Z.* Pig-vet: a web-based expert system for pig disease diagnosis. *Fuzzy Sets. Syst.* 2006. https://doi.org/10.1016/j.eswa.2005.01.011.
109. *Gaulton A., Attwood T. K.* Bioinformatics approaches for the classification of G-protein-coupled receptors. *Curr. Opin. Pharmacol.* 2003, 3 (2), 114–120. doi: 10.1016/S1471-4892(03)00005-5.
110. *Palla K., Ghahramani Z., Knowles D. A.* A nonparametric variable clustering model. *Adv. Neural. Inform. Proc. Syst.* 2012, 2987–2995.
111. *Curtis C. L., Shah S. P., Chin S. F., Turashvili G., Rueda O. M., Dunning M. J., Speed D., Lynch A. G., Samarajiwa S., Yuan Y., Gräf S., Ha G., Haffari G., Bashashati A., Russell R., McKinney S.; METABRIC Group, Langerød A., Green A., Provenzano E., Wishart G., Pinder S., Watson P., Markowetz F., Murphy L., Ellis I., Purushotham A., Børresen-Dale A. L., Brenton J. D., Tavaré S., Caldas C., Aparicio S.* The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature*. 2012, 486 (7403), 346–352.
112. *Proceedings* of the second EuroLOOP Workshop, Amsterdam, 27–30 March 1995.
113. *Graham C. H., Ferrier S., Huettman F., Moritz C., Peterson A. T.* New developments in museum-based informatics and applications in biodiversity analysis. *Trend. Ecol. Evol.* 2004, 19 (9), 497–503. https://doi. org/10.1016/ j.tree.2004.07.006.
114. *Anokhin B., Hemmrich G.* Zoology www guide. *Zoology*. V. 108, Is. 4, 2005, P. 673–679.
115. *Hirano S., Sun X., Tsumoto S.* Comparison of clustering methods for clinical databases. *Inform. Sci*. 2004, 159 (34), 155–165. https:// doi.org/ 10.1016/j.ins.2003.03.011.
116. *Hong Yu., Hatzivassiloglou V., Rzhetsky A., Wilbur W. J.* Automatically identifying gene/ protein terms in MEDLINE abstracts. *J. Biomed. Inform*. 2002, 35 (56), 322–330. https://doi. org/10.1016/S1532-0464(03)00032-7.
117. *Bedathur S. J., Haritsa J. R., Sen U. S.* The building of BODHI, a bio-diversity database system. *Inform. Syst.* 2003, 28 (4), 347367. https://doi.org/ 10.1016/S0306-4379(02)00073-X.
118. *Hsi-Chieh Lee, Szu-Wei Huang, Li E. Y.* Mining protein–protein interaction information on the internet. *Exp. Syst. Appl. Elsevier*. 2006, 30 (1), 142–148. https://doi. org/10.1016/j.eswa.2005.09.083.
119. *Young R.* Genetic toxicology: web–resources. *Toxicology*. 2002, 173 (1–2), P. 103–121.
120. *Johnson S. B., Friedman R.* Bridging the gap between biological and clinical informatics in a graduate training program. *J. Biomed. Inform*. 2007, 40 (1), 59–66. Epub. 2006 Mar 15. doi: 10.1016/j.jbi.2006.02.011.
121. *Kaiser M., Hilgetag C. C.* Modelling the development of cortical systems networks. *Neurocomputing*. 2004, V. 5860, P. 297–302. https://doi.org/ 10.1016/j.neucom.2004.01.059.
122. *Kane M. D., Brewer J. L.* An information technology emphasis in biomedical informatics education. *J. Biomed. Inform*. 2007, 40 (1), 67–72. https://doi.org/10.1016/j. jbi.2006.02.006.
123. *Kannathal N., Acharya U. R., Lim C. M., Sadasivan P. K.* Characterization of EEG. A comparative study. *Comp. Meth. Progr. Biomed*. 2005, 80 (1), 17–23. https://doi.org/10.1016/j.cmpb.2005.06.005.
124. *Koh W., McCormick B. H.* Brain microstructure database system: an exoskeleton to 3D reconstruction and modelling. *Neurocomputing*. 2002, V. 4446, P. 1099–1105. https:// doi.org/10.1016/S0925-2312(02)00426-5.
125. *Koh W., McCormick B. H.* Registration of a 3D mouse brain atlas with brain microstructure data. *Neurocomputing*. 2003, V. 5254, P. 307–312. https://doi.org/10.1016/ S0925-2312(02)00793-2.
126. *Kulish V., Sourin A., Sourina O.* Human electro encephalograms seen as fractal time series: Mathematical analysis and visualization. *Comp. Biol. Med*. 2006, 36 (3), 291–302. doi: 10.1016/j.compbiomed.2004.12.003.
127. *Lubitz von D., Wickramasinghe N.* Network-centric healthcare and bioinformatics: Unified operations within three domains of knowledge. *Exp. Syst. Appl*. 2006, 30 (1), 11–23. https://doi. org/10.1016/j.eswa.2005.09.069.
128. *Martin-Sanchez F., Iakovidis I., Norager S., Maojo V., de Groen P., Van der Lei J., Jones T., Abraham-Fuchs K., Apweiler R., Babic A., Baud R., Breton V., Cinquin P., Doupi P., Dugas M., Eils R., Engelbrecht R., Ghazal P., Jehenson P., Kulikowski C., Lampe K., De Moor G., Orphanoudakis S., Rossing N., Sarachan B., Sousa A., Spekowius G., Thireos G., Zahlmann G., Zvárová J., Hermosilla I., Vicente F. J.* Synergy between medical informatics and bioinformatics: facilitating genomic medicine for future health care. *J. Biomed. Inform*. 2004, 37 (1), 30–42. doi:10.1016/j.jbi.2003.09.003.
129. *Masseroli M., Visconti A., Bano S. G. Pinciroli F.* He@lthCo-op: a web-based

system to support distributed healthcare co-operative work. *Comp. Biol. Med.* 2006, 36 (2), 109–127. doi:10.1016/j.compbiomed.2004.09.005.

130. *Moon S., Byun Y., Han K.* FSDB: A frameshift signal database. *Comp. Biol. Chem.* 2007, 31 (4), 298–302. doi: 10.1016/j.compbiolchem.2007.05.004.
131. *Orgun B., Vu J.* HL7 ontology and mobile agents for interoperability in heterogeneous medical information systems. *Comp. Biol. Med.* 2006, 36 (78), 817–836. https://doi.org/10.1016/j.compbiomed.2005.04.010.
132. *Pérez-Rey D., Maojo V., García-Remesal M., Alonso-Calvo R., Billhardt H., Martin-Sánchez F., Sousa A.* Ontofusion: Ontology-based integration of genomic and clinical databases. *Comp. Biol. Med.* 2006, 36 (78), 712–730. doi: 10.1016/j.compbiomed.2005.02.004.
133. *Krishtal O.A., Kiskin N.I., Tsyndrenko A.Ya., Klyuchko E.M.* Pharmacological properties of amino acid receptors in isolated hippocampal neurons. *In: Receptors and ion channels. Ed. By Ovchinnikov Y.A., Hucho F. Berlin-New York: Walter de Gruyter,* 1987, P. 127–137.
134. *Klyuchko E. M., Klyuchko Z. F., Beloshitsky P. V.* Some adaptation characteristics of insects in mountains of Prielbrussie. *Nalchik (Russia), "Hypoxia: automatic analysis of hypoxic states of healthy people and sick ones".* 2005, V. 1, P. 137–140. (In Russian).
135. *Klyuchko Z. F., Klyuchko E. M.* Analysis of taxonomic structure of moth fauna (Lepidoptera: Noctuidae s.l.) of Ukraine according to monitoring data. *Eversmannia.* 2012, 3 (33), 41–45. (In Russian).
136. *Klyuchko Z. F., Klyuchko E. M.* Moth (Lepidoptera: Noctuidae s. l.) of Chercasska region of Ukraine according to results of many-year monitoring. *Eversmannia.* 2014, No 37, 32–49. (In Russian).
137. *Gonchar O., Klyuchko O., Seredenko M., Oliynyk B.* Correction of metabolic disorders at hypoxia by new pharmacological preparations. *Mater. 3 FEPS Congress. Nice (France),* 2003, P. 228.
138. *Seredenko M., Gonchar O., Klyuchko O., Oliynyk S.* Peculiarities of prooxidant — antioxidant balance of organism under hypoxia of different genesis and its corrections by new pharmacological preparations. *Acta Physiologica Hungarica. Budapest (Hungary).* 2002, 89 (1–3), 292.
139. *Klyuchko O., Klyuchko Z., Lizunova A.* Electronic Noctuidae database: some problems and solutions. *Proceed. 16th European Congress of Lepidopterology. Cluj (Romania),* 2009, P. 31–32.
140. *Klyuchko O., Klyuchko Z., Lizunova A.* Noctuidae fauna of Ukrainian Karpathy: results of monitoring (1956–2008). *Proceed. 16th European Congress of Lepidopterology. Cluj (Romania),* 2009. P. 31.
141. *Klyuchko O. M., Beloshitsky P. V.* Investigation of insect adaptation characteristics in Prielbrussie in 2004–2005. *Mater. VIII World Congress of International Society for Adaptive Medicine (ISAM). Moskva (Russia).* 2006, P. 165–166.
142. *Beloshitsky P. V., Klyuchko O. M.* Contribution of Sirotinin's school into adaptation medicine. *Mater. VIII World Congress of International Society for Adaptive Medicine (ISAM). Moskva (Russia),* 2006. P. 158.
143. *Klyuchko O. M., Klyuchko Z. F.* Ukrainian Noctuidae Database. *Mater. XIV SEL Congress. Roma (Italy),* 2005, P. 49.
144. *Klyuchko Z. F., Klyuchko O. M.* Noctuidae (Lepidoptera) of Donbass, Ukraine. *Mater. XIV SEL Congress. Roma (Italy),* 2005, P. 41–42.
145. *Beloshitsky P., Klyuchko O., Onopchuck Yu., Onopchuck G.* Mathematic model for hypoxic states development for healthy people and ones with ischemic heart disease. *High altitude medicine and biology: Mater. ISMM Congress. Beijing (China),* 2004, V. 5, P. 251.
146. *Beloshitsky P., Klyuchko O., Kostyuk O., Beloshitsky S.* Peculiarities of high mountain factors influence on organism. *High altitude medicine and biology: Mater. ISMM Congress. Beijing (China),* 2004, V. 5, P. 250.
147. *Klyuchko O. M., Kiskin N. I., Krishtal O. A., Tsyndrenko A. Ya.* Araneidae toxins as antagonists of excitatory amino acid responses in isolated hippocampal neurons. *X School on biophysics of membrane transport. Szczyrk (Poland),* 1990, V. 2, P. 271.
148. *Akaike N., Kawai N., Kiskin N. I., Krishtal O. A., Tsyndrenko A. Ya., Klyuchko O. M.* Spider toxin blocks excitatory amino acid responses in isolated hippocampal pyramidal neurons. *Neurosci. Lett.* 1987, V. 79, P. 326–330.
149. *Gonchar O., Klyuchko O., Beloshitsky P.* Ways of myocardial metabolic disorders correction at hypoxia by new pharmacological preparations. *High altitude medicine and biology: Mater. ISMM Congress. Beijing (China).* 2004, V. 5, P. 249.
150. *Gonchar O., Klyuchko E., Mankovskaya I.* Role of complex nucleosides in the reversal of oxidative stress and metabolic disorders induced by acute nitrite poisoning. *Ind. J. Pharmacol.* 2006, 38 (6), 414–418.

151. *Gonchar O., Klyuchko E., Seredenko M., Oliynik S.* Corrections of prooxidant — antioxidant homeostasis of organism under hypoxia of different genesis by yackton, new pharmacological preparation. *Acta Physiol. Pharmacol. Bulg.* 2003, V. 27, P. 53–58.

ЕЛЕКТРОННІ ІНФОРМАЦІЙНІ СИСТЕМИ В БІОТЕХНОЛОГІЇ

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Метою роботи було узагальнити та проаналізувати використання електронних інформаційних систем у біотехнології для створення нових версій цих систем. Опрацьовано публікації стосовно систем різних типів, придатних для вирішення проблем у біотехнології. Подібні системи, які традиційно належать до біології і медицини, було класифіковано. Розглянуто перспективи їх застосування для розроблення в подальшому більш досконалих електронних систем.

Ключові слова: біоінформатика, електронні інформаційні системи, бази даних.

ЭЛЕКТРОННЫЕ ИНФОРМАЦИОННЫЕ СИСТЕМЫ В БИОТЕХНОЛОГИИ

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Целью работы было обобщить и проанализировать использование электронных информационных систем в биотехнологии для создания новых версий этих систем. Изучены публикации относительно систем разных типов для решения проблем в биотехнологии. Подобные системы, которые традиционно относятся к биологии и медицине, были классифицированы. Рассмотрены перспективы их применения для разработки в дальнейшем более совершенных электронных спстем.

Ключевые слова: биоинформатика, электронные информационные системы, базы данных.

EXPERIMENTAL ARTICLES

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SYNTHESIS AND CHARACTERIZATION OF POLY(D,L-LACTIC-CO-GLYCOLIC)ACID MICROPARTICLES LOADED BY DIPHTHERIA TOXOID

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The aim of the study was to synthesize poly(D,L-lactide-co-glycolide) particles — PLGA and obtain their complexes with a non-toxic diphtheria toxin recombinant fragment subunit B fused with enhanced green fluorescent protein EGFP-SubB; to characterize the main physical and chemical properties of the resulting complexes.

Two types of the diptheria toxoid loaded PLGA microparticles were obtained: particles with immobilized diphtheria toxoid (PLGA 1) and particles with encapsulated diphtheria toxoid (PLGA 2). Micropartcles obtaines were characterized for antigen loading, particle size, polydispersity index and in vitro antigen release.

Key words: diphtheria toxoid, poly (lactic-co-glycolic-acid) microparticles.

Diphtheria toxin (DT) is one of the most important pathogenic factors of diphtheria bacilli *Corynebacterium diphtheriae* [1–4]. It consists of single polypeptide chain, which can be broken into two subunits A and B. Subunit A possess catalytic activity, and subunits B is responsible for binding to diphtheria toxin receptor HB-EGF (heparin binding EGF-like grows factor) on cell surface [5].

Diphtheria is an infectious disease that affects only humans. Highly infectious disease can be transmitted from human to human airborne. This disease was very dangerous and killed amount of humans until vaccination era. Vaccination by diphtheria toxoid is one of the precautions that can be taken against this disease [6].

Vaccination with the diphtheria toxoid was discovered by Ramon Gaston during the 1920s has been used in medical practice. Currently, vaccination against this disease depends on cultivation of *C. diphtheriae* bacilli in bio-secure conditions and inactivation of toxin by chemical methods. New generation more effective vaccines need to be developed. In the last few decades the studies to develop a recombinant vaccine against diphtheria focused on obtaining inactive mutants of DT-toxoids. For this reason, using adjuvants in order to obtain a more robust immune response is aimed.

Particle carriers are effective delivery systems for antigens and increase the antigen uptake on cellular level. They are effective on controlled antigen release and can protect the antigen integrity from degradation [7].

Fortunately, modern nanotechnology provides new scientific means for mucosal immune response regulation. Biodegradable and biocompatible polymers, like poly(D,L lactide-co-glycolide) (PLGA), are widely used for the design of mucosal immunizing tools. PLGA, which one of the most widely used co-polymer, is a biopolymer approved by FDA and has several advantages such has inert properties in physical environments, degradability in biological environments, biocompatible properties and being able

to be degraded into non-toxic products [8]. Importantly, that the way of particle preparation plays an important role in PLGA biodegradation and antigen release.

It is believed, that strong induction of antitoxic immunity in mucosal tissues can protect the body against the infection. All current diphtheria vaccines have parenteral route of administration. Such vaccines results in formation of high serum antitoxin levels in the blood, predominantly IgG class. However, most important role in the mucosal surfaces protection is playing secretory IgA antibodies [7]. Undoubtedly, oral administration of antigens would be the most patient-friendly way of immunization. However, the efficacy of free antigens oral administration is limited by their degradation in the gastrointestinal tract and poor absorption by M-cells (microfold cells) in the follicle-associated epithelium of the Peyer's patches, which play the major role in stimulating mucosal immunity [8].

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In this study, diphtheria toxin subunit B, genetically fused to EGFP, encapsulated PLGA micro-particles were synthesized for using vaccine models. Synthesized microparticles were characterized by scanning electron microscopy (SEM), loading yields and antigen release under acidic pH were measured.

Materials and Methods

Materials

In the work there were used: acrylamide, N,N′-methylenebisacrylamide from AppliChem GmbH (Germany); 2-mercaptoethanol from Helicon (Russia); plastic Petri dishes from Greiner BioOne (Austria); kanamycin, glucose from "Kyivmedpreparat", Arterium Co. (Ukraine); poly(lactic-co-glycolic acid) — PLGA, polyvinyl alcohol (PVA), dichloromethane (DCM), fetal bovine serum (FBS), LB medium, NaN_3, RPMI-1640 medium with L-glutamine, sodium dodecyl sulphate (SDS), stock solution of amphotericin B, penicillin G and streptomycin for cell culture, Trisma base, Triton X-100, urea from Sigma (USA); imidazole from Shanghai Synnad (China); KCl, NaCl, Na_2HPO_4, NaOH, KH_2PO_4 from Miranda-C (Ukraine); bovine serum albumin (BSA), isopropyl β-D-1-thiogalactopyranoside (IPTG), nickel-nitrilotriacetic acid-agarose (Ni-NTA agarose), prestained protein markers for gel electrophoresis from Thermo Fisher Scientific (USA).

All chemical reagents were of analytical grade. Ultra-pure water was acquired from Adrona Water Purification system.

Antigen preparation

The recombinant proteins used in this study: EGFP, EGFP-SubB, were obtained as previously reported [11]. Briefly, bacterial culture of Escherichia coli BL 21 (DE3) Rosetta (Novagen, Reno, NV USA) transformed by corresponding genetic constructions was grown at 37 °C under intensive stirring (250 rpm) up to extinction A600 — 0.5–0.7. Expression of the proteins was triggered via incubation with 1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) up to 3 hours at 30 °C under intensive stirring (250 rpm). Recombinant protein was purified by the Ni^{2+}-NTA-agarose column chromatography with imidazole elution gradient and stored in PBS, pH = 7.2.

Electrophoretic separation of proteins

Electrophoresis in polyacrylamide gel with sodium dodecyl sulfate was performed following the modified method by H. Schagger [12].

Preparation of Microparticles

Two types of PLGA particles were prepared and characterized: particles with immobilized diphtheria toxoid (PLGA 1) and particles with encapsulated diphtheria toxoid (PLGA 2).

(1) The PLGA particles of the first type (PLGA 1) were prepared by solvent displacement method [13]. Briefly, 3 ml of 0.5% w/v PLGA (lactide:glycolide — 65:35) (Sigma-Aldrich Co., St. Louis, USA) in acetone were added drop-wise to 30 ml of pure distilled water under magnetic stirring. Acetone was evaporated overnight at room temperature.

Afterwards particles suspension was filtered through 10 μm filter. PLGA 1 particles were collected by centrifugation at 12,000 g for 3 min and resuspended in PBS contained protein (1% w/w PLGA). The emulsion was stirred overnight on magnetic stirrer at 4 °C. Afterwards, the particles were collected by centrifugation for 20 min at 12 000 rpm the pellet was resuspended in 1 ml of PBS and stored at 4 °C.

(2) The PLGA particles of the second type (PLGA 2) were prepared by previously reported method [14] with some modifications (double emulsification using solvent evaporation method). Briefly, 2 ml of 0.5% w/v PLGA (lactide:glycolide — 50:50) in methylene chloride were sonicated with 2 ml of protein solution (1 mg/ml) in 0.5% PVA by ultrasound homogenizer LabsonicM (Sartorius, Germany) for 2 min at an amplitude of 90%, and a duty cycle of 0.9 s. Afterward, the water-in-oil emulsion was added to 8 ml of 3% PVA solution and sonicated in ice bath for 10 min. Obtained emulsion was stirred overnight at room temperature on a magnetic stirrer for evaporation of the organic solvents. Afterwards, the particles were collected by centrifugation for 20 min at 12 000 rpm the pellet was resuspended in 1 ml of PBS and stored at 4 °C.

Characterization of Microparticles

In this study, obtained microparticles were detailed analyzed by following parameters: encapsulation efficiency (EE), drug loading (DL), particle size (Z-ave) and polydispersity index (PDI).

Antigen immobilization measurement

The electrophoretic separation of the obtained particles was carried out in a polyacrylamide gel with sodium dodecyl sulfate using the modified Schagger H. technique [12]. The effectiveness of antigen immobilization on the particles was determined by electrophoregram with densitometric method using TotalLab TL120 software. The effectiveness of antigen immobilization on the particles was determined by electrophoregram by densitometric method using Origin 8.0 (OriginLab Corporation, USA) and Fiji (Open Source software project) [15]. The effectiveness of immobilization was calculated based on the ratio of protein concentrations in the solution before and after conjugation with the particles.

EE was determined by using of the supernatants obtained after centrifugation for each of microparticles and determined using the formula given below:

$$EE\ (\%) = \frac{AA - AN}{AA} \times 100,$$

where AA — the amount of polypeptide initially added (mg);
AN — the amount of non-encapsulated polypeptides (mg).

The DL of polypeptide was calculated using the formula given below:

$$DL\ (\%) = \frac{AA - AN}{AM} \times 100,$$

where AA — the amount of polypeptide initially added (mg);
AN — wherehe amount of non-encapsulated polypeptides (mg);
AM — Amount of prodused particles.

Particle Size and Polydispersity Index of Microparticles

Determination of particle size was carried out using two different methods: Nanoparticle Tracking Analysis (NTA) using NanoSight NS300 (Malvern Instruments, UK) and dynamic light scattering (DLS) using Zetasizer Nano ZS Analyzers (Malvern Instruments, UK).

SEM Measurements of Microparticles

Particles size and morphology were studied using a scanning electron microscopy method (SEM) performed on a Mira 3 Tescan microscope, with an accelerating voltage of 10 kV, other parameter is shown on the pictures. To avoid sample charging during SEM investigation, samples was previously coated by thin conductive layer using Gatan Pecs, in this work we use 20 nm of Au/Pd.

In vitro antigen release studies

To determine the effect of the medium pH on the antigen release rate, the PLGA particles loaded with the recombinant antigen EGFP-SubB were incubated at pH 1.2 in an environment that simulates the acidity of the gastric juice (solution of hydrochloric acid at pH 1.2) and as a control at pH 7.4 in phosphate buffer solution (PBS). The microparticles suspension was incubated in a shaking incubator (100 rpm) at a stable temperature, close to the temperature of the human body — 37 °C. Samples were removed from solutions at various time intervals: after 15 min, 30 min, 60 min, 120 min and 240 min. Since the particles were loaded with fluorescent

recombinant antigen, the determination of their degree of degradation was carried out using Flow cytometry.

Results and Discussion

Encapsulation Efficiency and Drug Loading of Microparticles

A prerequisite for successful immunization and the development of an effective immune response is the sufficient amount of introduced antigen. In view of this, an important indicator is the effectiveness of the antigen sorption of particles.

We synthesized PLGA-based microparticles and conjugated them with the protein in two different techniques, which allowed obtaining two varieties of PLGA particles: particles with immobilized diphtheria toxoid (PLGA 1) and particles with encapsulated diphtheria toxoid (PLGA 2).

The effectiveness of antigen immobilization on the particles was determined by electrophoregram by comparing the amount of proteins initially added and in the samples containing the non-encapsulated proteins (supernatant after centrifugation). Densitometric analysis of electrophoresis results was performed using Fiji and OriginLab software. The effectiveness of immobilization was calculated based on the ratio of protein concentrations in the solution before and after conjugation with the particles. Obtained results are given in Table.

It can be seen that the EI was higher for particles synthesized by the second method (PLGA 1 — 72%, PLGA 2 — 90%). At the same time, by the flow cytometry it was demonstrated that 99% of the PLGA 1 particles are loaded with EGFP-SbB, while only 92% of PLGA 2 particles are bound to the protein. According to the obtained results, it can be said that the loading is more effective in encapsulating antigens within the particle, but the amount of particles containing the protein in its composition is slightly less in such synthesis technique.

Particle size Analysis of Microparticles

Also, produced microparticles were analyzed for particle size and polydispersity index (PDI) The particle sizes were slightly different according two distinct techniques (NTA — number based, the software tracks individual particles; DLS — scattering intensity weighted), however demonstrate similar patterns. The size distributions of the produced microparticles and PDI values were summarized in Fig. 1 and in Table.

NTA data showed that the particle sizes of PLGA 1 ranges from 50 nm to 510 nm, most of the particles had a size of 81.4 nm. The particle sizes of PLGA 2 ranges from 70 nm to 400 nm, most of the particles had a size of 155.9 nm. The mean particle sizes were 192.8 nm and 183.8 nm for PLGA 1 and PLGA 2, respectively (Fig. 2).

DLS data also showed that the mean PLGA 1 particles size was 203.3 nm and the

Microparticles properties including EE, DL, particle size and PDI

Type of particles	EE (%)	DL (%)	Mean Particle Size NTA (nm)	Mean Particle Size DLS (nm)	PDI
PLGA 1	725	7	183.8	203.3	0.143
PLGA 2	90	8	192.8	211.6	0.115

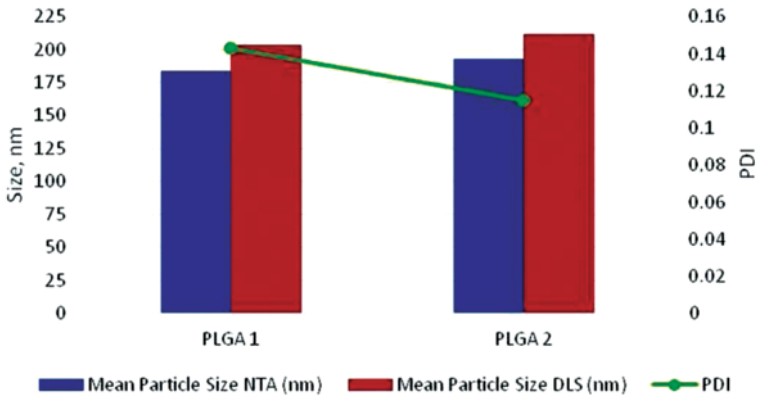


Fig. 1. **Z-ave of PLGA 1 and PLGA 2 particle sizes according two distinct techniques:** NTA and DLS (bars); and PDI (line)

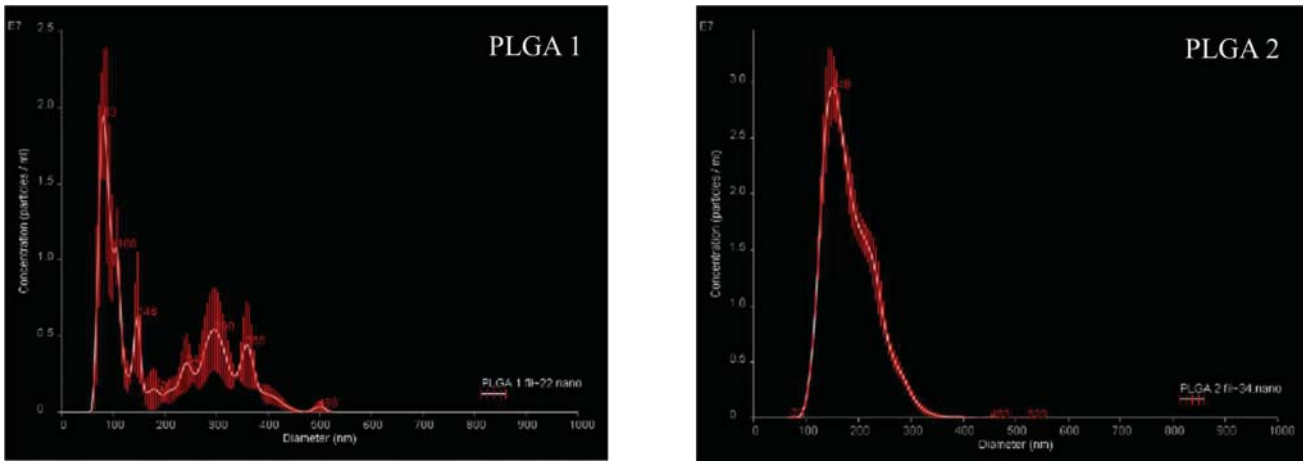


Fig. 2. **Particle size distribution of PLGA 1 and PLGA 2 respectively according NTA technique**

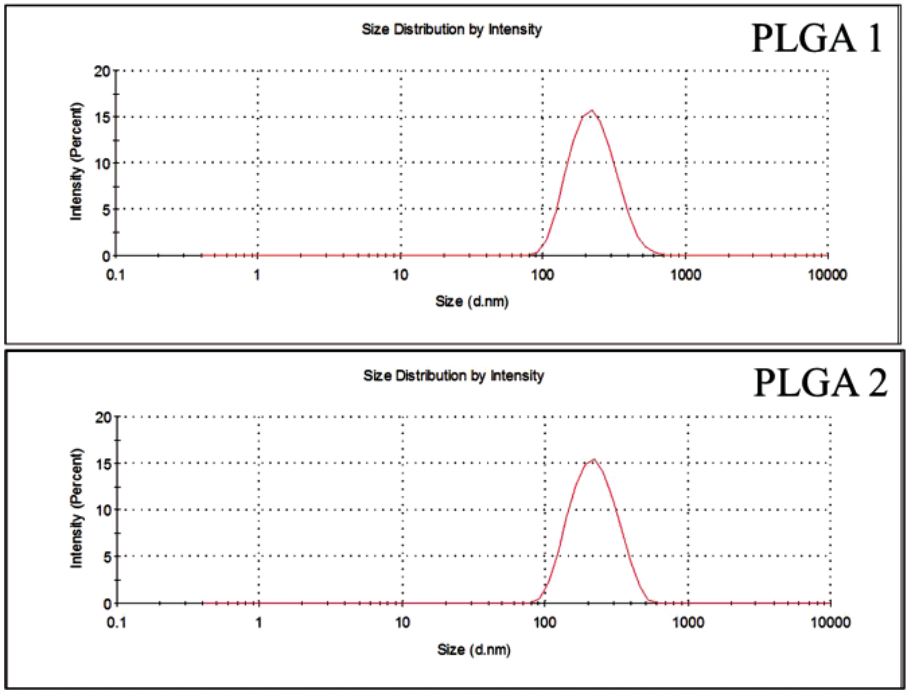


Fig. 3. **Particle size distribution of PLGA 1 and PLGA 2 respectively according DLS technique**

mean PLGA 2 particles size was 211.6 nm (Fig. 3). Moreover, the obtained particles demonstrated similar oval to round shape. The slight difference in particle size estimation is not unexpected as they are two different techniques.

The size distributions are similar for both particles and there is no agglomeration of the particles.

SEM-Analysis of Microparticles

Microparticles synthesized using different methods were analyzed morphologically by scanning electron microscopy (SEM). Obtained SEM images were shown in Fig. 4, *A*, *B*.

The results of electron microscopy of PLGA particles confirmed the results presented above. As can be seen from the pictures (Fig. 4), single particles have similar round correct shape, and their sizes are within the size determined by the data of NTA and DLS.

In vitro Release of Microparticles

To determine the effect of the medium pH on the antigen release rate, the PLGA particles loaded with the recombinant antigen EGFP-SbB were incubated in an environment that simulates the acidity of the gastric juice (solution of hydrochloric acid at pH 1.2) and as a control in a phosphate buffer solution (pH 7.8). Fig. 5 illustrates the dependence of the particle degradation process on incubation time in different environments.

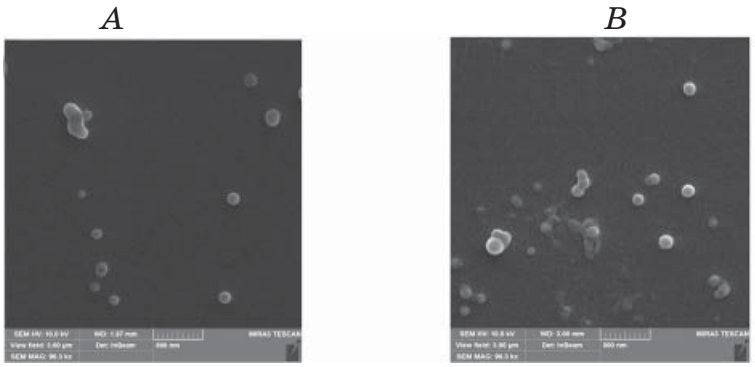
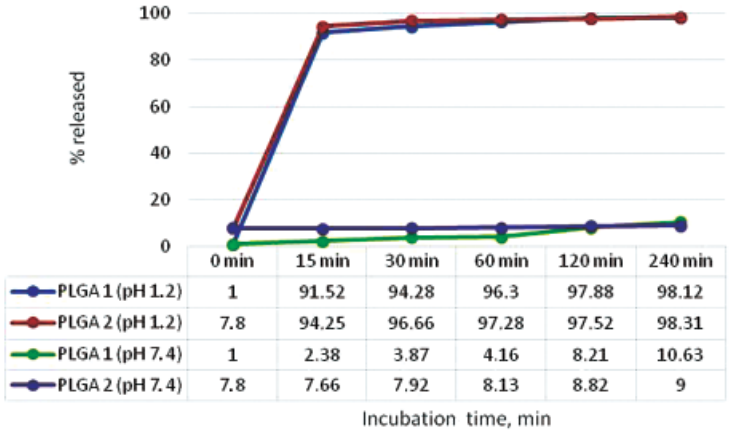


Fig. 4. ***A*** **— SEM image of PLGA 1 microparticles;** ***B*** **— SEM image of PLGA 2 microparticles**



	0 min	15 min	30 min	60 min	120 min	240 min
PLGA 1 (pH 1.2)	1	91.52	94.28	96.3	97.88	98.12
PLGA 2 (pH 1.2)	7.8	94.25	96.66	97.28	97.52	98.31
PLGA 1 (pH 7.4)	1	2.38	3.87	4.16	8.21	10.63
PLGA 2 (pH 7.4)	7.8	7.66	7.92	8.13	8.82	9

Fig. 5. ***In vitro*** **release of EGFP-SubB antigen from PLGA particles in solution of hydrochloric acid (pH 1.2) and phosphate buffer (pH 7.4).** Significant compared to control results pH 7.4 ($P \leq 0.05$)

It has been demonstrated that at the beginning of the experiment, 99% of the PLGA 2 particles were loaded with EGFP-SbB protein. There were not observed significant changes in the groups where the incubation was carried out in a buffer environment at pH 7.8. Striking effects were observed after the incubation of PLGA 2 particles in a medium with high acidity. Similar, but even more critical situation was with PLGA 1 particles.

Demonstrated differences in the properties of synthesized particles may have an influence on the immunogenicity of the antigen in their use for oral immunization. The sizes of both particles resemble bacteria size that could increase probability of particles interaction with M-cells. Obtained results can be used for developing a new tool for per os immunization against diphtheria. It can be also expected that the results of our study may be useful for the development of new delivery systems for other means, such as nucleic acids, drugs, vitamins, antigens etc. to the entire body.

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REFERENCES

1. *Labyntsev A. J., Korotkevych N. V., Manoilov K. J., Kaberniuk A. A., Kolybo D. V., Komisarenko S. V.* Recombinant Fluorescent Models for Studying the Diphtheria Toxin. *J. Bioorg. Chem.,* 2014, 40(4), 433–442, doi: 10.7868/S0132342314040083
2. *Freeman V. J. Morse I. U.* Further observations on the change to virulence of bacteriophage-infected a virulent strains of Corynebacterium diphtheria, *J. Bacteriol.*, 1952, 63(3), 407–414.
3. *McEvoy P., Polotsky Y., Tzinserling V. A., Yakovlev A. A.* The pathology of diphtheria. *J. Infect. Dis.* 2000,181(1), 116–20.
4. *Collier R. J.* Understanding the mode of action of diphtheria toxin: a perspective on progress during the 20th century. *Toxicon.* 2001, 39, 1793–803.
5. *Chudina T. O., Labintsev A. J., Romaniuk S. I., Kolybo D. V., Komisarenko S. V.* Changes in proHB-EG F expression after functional activation of the immune system cells, *Ukr. Biochem. J.* 2017, 89 (6), 33–40. doi: 10.15407/ubj89.06.031
6. *Adler N. R., Mahony A., Friedman N. D.* Diphtheria: forgotten, but not gone. *Inter.n Med. J.* 2013, 43(2), 206-210. doi: 10.1111/imj.12049.
7. *Couvreur P.* Nanoparticles in drug delivery: past, present and future. *Adv. Drug. Deliv. Rev.* 2013, 65(1), 21–23. doi: 10.1016/j.addr.2012.04.010.
8. *Hirenkumar K. Makadia, Steven J. Siegel.* Poly Lactic-co-Glycolic Acid (PLGA) as Biodegradable Controlled Drug Delivery Carrier. *Polymers (Basel).* 2011, 3(3), 1377–1397. doi:10.3390/polym3031377
9. *Kaberniuk A. A., Labyntsev A. I., Kolybo D. V., Oliynyk O. S., Redchuk T. A., Korotkevych N. V.,*

Horchev V. F., Karakhim S. O., Komisarenko S. V. Fluorescent derivatives of diphtheria toxin subunit B and their interaction with Vero cells. *Ukr. Biokhim. Zh.* 2009, 81(1): 67–77. (In Ukrainian).
10. *Schägger H., von Jagow G.* Tricinesodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* 1987, 166(2), 368–379.
11. *Davies G, ed.* Vaccine adjuvants: methods and protocols. *N.Y.: Humana Press*, 2010.
12. *Mohammad Tariq, Md. Aftab Alam, Anu T. Singh, Zeenat Iqbal, Amulya K. Panda, Sushama Talegaonkar.* Biodegradable polymeric nanoparticles for oral delivery of epirubicin: *In vitro, ex vivo*, and *in vivo* investigations. *Colloids and Surfaces B: Biointerfaces,* 2015, 128, 448–456. doi: 10.1016/j.colsurfb.2015.02.043.
13. *Tariq M., Alam M. A., Singh A. T., Iqbal Z., Panda A. K.* Talegaonkar S. Biodegradable polymeric nanoparticles for oral delivery of epirubicin: In vitro, ex vivo, and in vivo investigations. *Colloids Surf. B Biointerfaces.* 2015, 128, 448–456. doi: 10.1016/j.colsurfb.2015.02.043.
14. *Schägger H., von Jagow G.* Tricinesodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* 1987, 166 (2), 368–379.
15. *Schindelin J., Arganda-Carreras I., Frise E., Kaynig V., Longair M., Pietzsch T., Preibisch S., Rueden C., Saalfeld S., Schmid B., Tinevez J. Y., White D. J., Hartenstein V., Eliceiri K., Tomancak P., Cardona A.* Fiji — an Open Source platform for biological image analysis. *Nat. Meth.* 2012, 9 (7), 676–682. doi: 10.1038/nmeth.2019.

СИНТЕЗ І ХАРАКТЕРИСТИКА МІКРОЧАСТИНОК НА ОСНОВІ ПОЛІ(ЛАКТИД-КО-ГЛІКОЛІДУ), НАВАНТАЖЕНИХ ДИФТЕРІЙНИМ ТОКСОЇДОМ

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Мета роботи — синтезувати частинки на основі полі(лактид-ко-гліколіду) — PLGA й одержати їх комплекси з нетоксичним фрагментом дифтерійного токсину, міченим зеленим флуоресцентним протеїном (EGFP-SubB); охарактеризувати певні фізико-хімічні властивості отриманих кон'югатів і дослідити вплив модифікації поверхні.

Було синтезовано два типи частинок PLGA: з іммобілізованим антигеном на поверхні частинок — PLGA 1 та з інкапсульованим всередині частинок — PLGA 2. Отримані мікрочастинки характеризували за навантаженням антигену, розміром частинок, індексом полідисперсності та вивільненням антигену *in vitro*.

Ключові слова: дифтерійний токсоїд, мікрочастинки полі(лактид-ко-гліколевої) кислоти.

СИНТЕЗ И ХАРАКТЕРИСТИКА МИКРОЧАСТИЦ НА ОСНОВЕ ПОЛИ(ЛАКТИД-КО-ГЛИКОЛИДА), НАГРУЖЕННЫХ ДИФТЕРИЙНЫМ ТОКСОИДОМ

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Цель работы — синтезировать частицы на основе поли(лактид-ко-гликолида) — PLGA и получить их комплексы с нетоксичным фрагментом дифтерийного токсина, меченным зеленым флуоресцентным протеином (EGFP-SubB), охарактеризовать определенные физико-химические свойства полученных конъюгатов и исследовать влияние модификации поверхности.

Были синтезированы два типа PLGA частиц: с иммобилизованным антигеном на поверхности частиц — PLGA 1 и с инкапсулированным внутри частиц — PLGA 2. Полученные микрочастицы характеризовали по нагрузке антигена, размеру частиц, полидисперсному индексу и высвобождению антигена *in vitro*.

Ключевые слова: дифтерийный токсоид, микрочастицы поли(лактид-ко-гликолевой) кислоты.

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RECOGNITION OF *Mycobacterium tuberculosis* ANTIGENS MPT63 AND MPT83 WITH MURINE POLYCLONAL AND scFv ANTIBODIES

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The goal of this study was to characterize serum immunoglobulin G (IgG) antibody responses during experimental immunization of laboratory mice by purified recombinant proteins MPT63, MPT83 of *Mycobacterium tuberculosis* and artificial fusion protein MPT83-MPT63 and obtain the recombinant single chain variable fragments of antibodies (scFv) against these antigens.

This study demonstrates that the humoral immune response to MPT63, MPT83, MPT83-MPT63 fusion protein and equimolar set of MPT63 and MPT83 was highly different. For each antigen, serum antibody levels were evaluated by a cutoff value based on optical density index. A crucial role of MPT83 for immunogenicity of chimeric protein and/or cocktail of individual antigens under conditions of immunization of laboratory animals.

We obtained also specific scFv antibodies against MPT63 and MPT83. These antibodies can be used for the development of the system for quantitative determination of antigens as well as for their biological properties investigation.

Thereby, based on the results of the immune response and mycobacterial proteins antigenicity we showed highly immunogenicity properties of N-terminal part of MPT83 antigen for enhencement of ELISA sensitivity. We suggest MPT83-MPT63 fusion protein as a potential candidate on the role of antigenic substance for the serological diagnosis of tuberculosis.

Key words: tMPT63, MPT83, antigens, polyclonal antibodies, scFv, diagnostic.

Tuberculosis (TB) is the widespread infectious disease of human being and animals. In 2015, there were an estimated 10.4 million new (incident) TB cases worldwide and an approximately 1.6 million death [1]. Early diagnosis of TB is crucial to prevent the spread of the disease in the community. Immunological methods measure specific cellular or humoral responses of the host to detect presence of infection or disease. They do not require a specimen from the site of infection unlike reference standard in TB diagnosis such as bacteria culture. Moreover, it can take up to 6–8 weeks to isolate *Mycobacterium tuberculosis* [2], while serological tests take a few hours.

An important step for constructing of new diagnostic kits based on ELISA technique is the selection of the optimal high immunogenic substances for specific, accurate and sensitive determination of antibodies to the pathogen.

Difficulties in tuberculin skin test administration and interpretation often lead to false results [3]. An accurate serological test could provide rapid diagnosis of TB and in a suitable format would be particularly useful both as a replacement for laboratory based tests and for extending TB diagnosis to lower levels of health services, especially those without on-site laboratories [4]. Serological tests that use various *M. tuberculosis* antigens such as secretory proteins, heat shock proteins, lipopolysaccharide and peptides have been developed [5]. But despite numerous studies of the genome and proteomics of *Mycobacteria* only few ELISA kits are available for wide clinical practice use. This is most often due to negligent attitude to the choice of an antigenic substance or even with the use of total lysates of *M. tuberculosis* or protein purified derivate (PPD). As a consequence, significant variation of

sensitivity and specificity indicators, which will not allow the developed product to be used in practice or/and false positive results will be observed in antibody-based diagnostic tests due to the exposure to environmental non-tuberculous *Mycobacteria* (NTM) or prior Calmette-Gu rin bacillus (BCG) vaccination [6].

Recombinant antigens of *M. tuberculosis* and *M. bovis* strains MPT63 and MPT83 obtained from *E. coli* expression system do not differ from serologic characteristics from such own proteins of the causative agents[7]. In addition, these antigens were found only in the representatives of *M. tuberculosis complex*, and not found or expressed in of atypical mycobacteria strains [7]. This is important to overcome the false positive results caused by NTM.

MPT63, a major secreted 16 kDa protein from *M. tuberculosis*, has been shown to have immunogenic properties and has been implicated in virulence. According to literature data MPT63 cause inflammatory processes due to degranulation of mast cell with following release histamine and hexaminidase [8].

MPT83 is a lipoprotein which undergoes acylation and glycosylation and associated with bacilli cell wall by myristyl tail [9]. MPT83 is one of the ligands of toll like receptor 2 (TLR-2) [10]; also, it was described as an adhesion factor [11]; as inducer of apoptosis of infected macrophages by activating the TLR2/p38/COX-2 signaling pathway [12].

Classic BCG vaccine has been used worldwide to prevent TB disease in infants and children, but it has demonstrated limited and variable effectiveness in preventing pulmonary TB in adolescents and adults. Immunisation studies in mice indicated that MPT83-MPT83 are highly immunogenic with adjuvant. The use of alternative antigens and additional approaches for vaccine development urgently needed to protect against TB. Nevertheless, these results establish a novel platform for development of antigenic substances with inherent immunogenic characteristics that are desirable in vaccines.

It follows that the antigens chosen by the researchers are serologically valuable for use as an antigenic substance to create new diagnostic ELISA kit. In the present study, we have evaluated humoral responses in mice, TB suitable animals, to immunodominant antigens of *M. tuberculosis* MPT63 and MPT83, their cocktails and chimeric protein MPT83-MPT63 obtained as a result of the fusion of genetic sequences of individual antigens in one open reading frame. Furthermore, in this study we obtained the recombinant murine scFv to MPT63 and MPT83. Antibodies are an important tool both for studying of biological activity of mycobacterial antigens and developing of test-systems for quantitative measurement of proteins in fluids as biomarkers of TB pathogenesis. Also the recombinant antibodies may be used for the affine purification of these antigens. Thus, it has been received polyclonal and scFv antibodies, which resemble the characteristics of monoclonal antibodies and may be used for further improve existing TB diagnostics.

Statistical data analysis. The data were statistically treated using standard MO Excel and Origin 8.0 software. To compare the data in two groups we used Student's *t*-criterion test. The difference was considered statistically significant for $P < 0.05$.

Matherials and Methods

Matherials and reagents: bovine serum albumin (BSA), Complete and Incomplete Freund's Adjuvants (Sigma, USA), Ni-NTA agarose (Qiagen, Germany), imidazole, isopropyl-β-D-1-thiogalactopyranoside (IPTG) (Thermo Scientific, Lithuania), bacterial culture medium LB (Sigma, USA), skim milk powder (Fluka, Switzeland), coomassie G250, ammonium persulfate (APS), urea, acrylamide, N,N′-Metilenbisakrilamid, 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma, USA), conjugate of IgG horseradish peroxidase (HRP) (Thermo Scientific, Lithuania), tris (hydroxymethyl) aminomethane (Sigma, USA), tricine, molecular weight markers for protein gel electrophoresis (Thermo Scientific, Lithuania), β-mercaptoethanol, Tween-20 (Helicon, Russia), kanamycin, chloramphenicol, H_2O_2 ("Kyivmedpreparat", Ukraine), glycerol, KCl, NaCl, Na_2HPO_4, NaOH, KH_2PO_4, ("Miranda-C", Ukraine), sodium dodecyl sulfate (SDS) (Sigma, USA). A 96-well microtiter plates for enzyme-linked immunosorbent assay (Greiner Bio One, Great Britain) were used.

Recombinant proteins expression

Bacterial cultures with recombinant proteins expressed *E.coli* cells were grown at 37 °C under aeration conditions (250 rpm) up to A_{600} — 0.3–0.5 in the LB medium with 50 mg/l of kanamycin, 170 mg/l of chloramphenicol and 1% glucose. After this, cells were precipitated and resuspended in fresh LB medium with kanamycin (50 μg/ml)

and an inducer of expression of IPTG in a concentration of 1 mM. Target proteins expression has been performed during 3–4 hours at 30 °C under strong aeration conditions (250 rpm), after what cells have been precipitated by centrifugation at 3300 g during 15 min.

Immobilized-metal affinity on-column chromatography of polyHis-tag proteins

Column containing affine sorbent has been equilibrated with wash buffer (50 mM Na_2HPO_4, (pH 8.0), 0.5 M NaCl) with 6 or 8 M urea. Cell precipitates were resuspended in a same buffer (1 ml buffer solution per precipitate from 50 ml of bacterial culture). Samples have been sonicated by ultrasonic homogenizer LabsonicM (Sartorius, Germany). Cell's wall residues have been precipitated by centrifugation under 10 000 *g* during 20 min, and a preequilibrated Ni^{2+}-NTA agarose column has been filled by supernatant.

Renaturation by washing the column with step by step decrease of urea concentration (8 M → 6M → 4 M → 2 M → 0 M) in wash buffer (50 mM Na_2HPO_4, pH 8.0; 0.5 M NaCl) has been performed for obtaining soluble recombinant proteins. Proteins were eluted by wash buffer containing 250 mM imidazole without urea. Protein for further procedures was dialyzed against PBS (0.14 M NaCl, 0.03 M KCl, 0.011M Na_2HPO_4, 0.002 M KH_2PO_4, pH 7.2).

MPT83 expressed *E. coli* cells were resuspended in PBS or urea free wash buffer and were treated and centrifuged as described above. MPT83 protein extraction was carried out in nondenaturating conditions. For higher protein yield, the technique has been modified through use DNAse (10 U/ml), 1% Triton X-100 and lysozyme for better *E. coli* cell's walls and nucleoids dissociation as performed according to [13].

Electrophoretic separation of proteins

Determination of protein concentration by tricine SDS-PAGE analysis with TotalLab TL120 software were performed according to [14, 15].

Immunization of experimental animals

BALB/c 4-month female white mice (30–35 g of body mass) were used in the experiments. All animals had unlimited access to animal chow and tap water throughout the study.

The experiments are consistent with the requirements of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986) and with ethical norms as laid down in the laws of Ukraine.

Primary and booster injections of antigens were intraperitoneal as emulsions in CFA (1^{st}) or IFA (2^{nd}, 3^{rd}) administered in dose 1,52 nmol per animal (n = 8–10 for each group) at intervals of 2 weeks. The level of specific antibodies to mycobacterial antigens in the sera of immunized mice was determined by ELISA after 7^{th} day since last immunization.

Enzyme-linked immunosorbent assay (ELISA)

As antigens were used appropriate recombinant proteins of *M.tuberculosis* MPT63, MPT83, artificial fusion protein and mix set of separate component and BSA (negative control). Monoclonal antibodies against marker sequence fused with scFv (E-tag) (Amersham Bioscience, USA) were used to determine scFv antibodies; and anti-mouse IgG HRP were used as the secondary antibodies. TMB was used as chromogenic substrate. The color reaction was quantified by measuring the absorbency at 490 nm.

Construction of immune phage library

Total RNA, which was used as a matrix in cDNA synthesis, was isolated from the spleen tissue of immunized with MPT63 or MPT83 mice by TRI Reagent (Sigma, USA). The sequences encoding the variable domains of the light and heavy immunoglobulin chains (V_H andV_L) were amplified with the set of specific primers designed according to [16]. The high-fidelity polymerase Accu*Taq* LA DNA Polymerase (Sigma, USA) was used for amplification. Nucleotide sequences V_H and V_L were assembled by SOE-PCR (splicing by overlap extension PCR). DNA sequences of scFv were inserted into the phagemide vector p*CANTAB*-5E by *Sfi*I and *Not*I restriction sites. The ligase mixture was used for transformation of *E. coli* XL1-blue by the electroporation.

Isolation of phage particles

The cells transformed with scFv phagemids were infected with phage-helper M13K07 and incubated overnight. The phage cells were precipitated with a solution of PEG/NaCl (20% PEG-6000, 2.5 M NaCl) as described previously [16].

Selection of scFv antibodies

The positive clones against target antigens MPT63 and MPT83 were selected by the phage display method as described before [17].

Colonies lift assay

For pre-selection of positive clones, the colonies were transplanted into Petri dishes with a 2YT agar medium containing 2% glucose and ampicillin to a final concentration of 100 μg/ml. The prints of the colonies were

carried over on a nitrocellulose membrane (Amersham Bioscience, USA), which was previously incubated in a 5% non fat milk in PBS 60 min at 37 °C.

At the same time on the membranes of the identical size were immobilized with mycobacterial proteins MPT63 and MPT83 (incubation overnight in 10 ml of a solution in PBS, an antigen concentration of 10 µg/ml, with next blocking in a 5% solution of skim milk during 60 min). The immobilized by antigens nitrocellulose membranes were transferred to 2YT agar medium containing 100 µg/ml ampicillin and 1 mM IPTG and incubated overnight after 30 °C. After that, the membranes were treated by classic Western blot analysis using anti-E-tag and anti-mouse IgG-HRP conjugates. Inprints have been detected by 3,3′-Diaminobenzidine tetrahydrochloride (DAB) chromogenic substrate.

Results and Discussion

One of the most important characteristic of different type antibodies is their specificity, i. e., the capacity to bind with target proteins avoiding cross-reaction with other antigens. Moreover, for ELISA test diagnostics is ultimate need the use of antigens which have a number of serologically important epitopes that would provide interaction with antibodies and increase the sensitivity of the method.

Obtaining of recombinant proteins MPT63, MPT83 and MPT83-MPT63 fusion

Proteins expression was performed in the culture *E. coli* BL21 (DE3) Rosetta (Novagen, USA), transformed by pET24a- or pET28a-based (Novagen, USA) genetic constructs. Purification of recombinant proteins MPT63, MPT83 and MPT83-MPT63 fusion performed with metal affinity chromatography on Ni^{2+}-NTA column. Taking into the account that MPT63 and MPT83-MPT63 fusion were insoluble, procedure of refolding was performed. Analysis of protein fractions after the refolding was performed on 10% SDS-PAGE (Fig. 1).

Characterization of antigenicity

This study demonstrates that the humoral immune response to MPT63, MPT83, MPT83-MPT63 fusion protein and equimolar set of MPT63 and MPT83 is highly distinguished. ELISA measurements of mice serum polyclonal antibodies against differently administrated antigens are shown in Fig. 2. It was shown absence of specific antibodies titers to

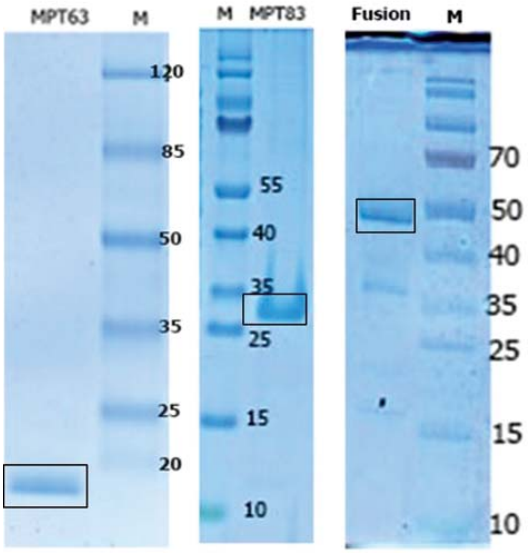

Fig. 1. **Electrophoregram of used proteins:** MPT63, MPT83 and fusion (MPT83-MPT63); M — molecular mass markers, kDa. Bands corresponding to the target protein are indicated with a rectangular frame

recombinant antigens of *M.tuberculosis* in mice before immunization (Fig. 2).

For each antigen, serum antibody levels were evaluated by using a cutoff value based on optical density index (ODI), a ratio between OD obtained for a test serum samples collected after immunization and OD obtained for a serum samples collected from the same animal at an initial, preimmunization time point [18]. Optimal serum dilution was chosen 1:16 000. Lower antibody titers was found against MPT63 (ODI ≥ 15,6), higher — against MPT83-MPT63 fusion (ODI ≥ 66,1). Anti-MPT83 and anti-(MPT63+MPT83 mixture) sera demonstrate practically the same (ODI ≥ 55,4) (Fig. 3).

Crossreactivity of polyclonal IgG amoung M. tuberculosis antigens

Mycobacterial antigens MPT63 is secretory protein and MPT83 is myristylited and in bacilli associate with cell wall. Obtained by us recombinant analogues of these antigens potentially could show cross-reactivity due to same *E.coli* expression system and encoding polyHis tag in genetic constructs for affinity isolation and purification of antigens. It was considered that as a result of protein eluates contamination with *Escherichia* components, recombinant antigens are able show cross-reactivity to different types of antisera. MPT83 and MPT63 antigens not recognized with anti-MPT63 (OD 0,056±0,02) and anti-MPT83 (OD 0,027±0,014) sera respectively (Fig. 4).

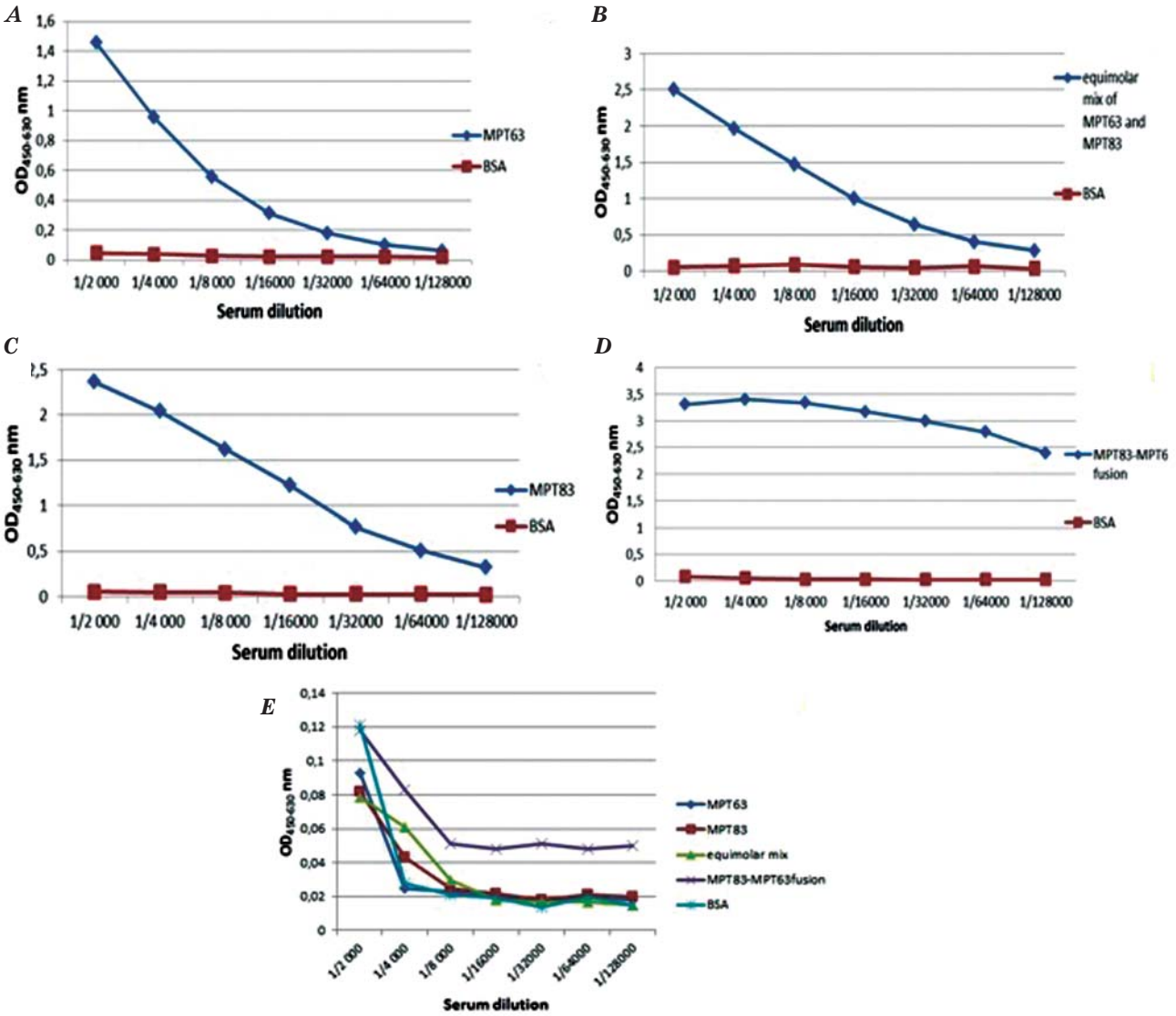

Fig. 2. **IgG level to the target recombinant proteins MPT63 (*A*), cocktail of MPT63+MPT83 (*B*), MPT83 (*C*), MPT83-MPT63 fusion (*D*) in the sera of immunized mice and pre-immunized sera (*E*)**

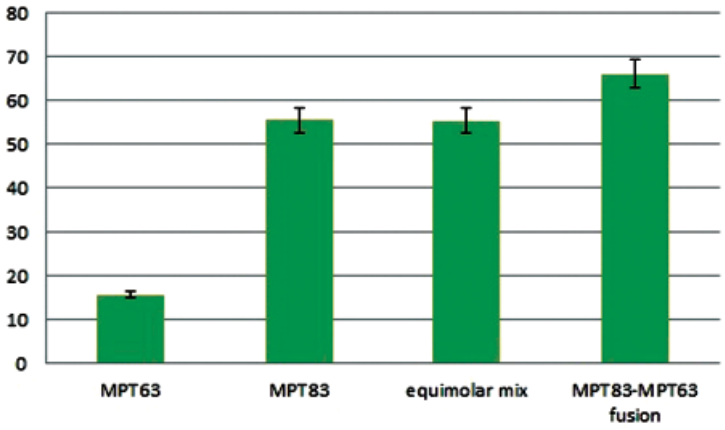

Fig. 3. **Optical density index for each antigen substance**
ODI = $OD_{(postimmunization)}/OD_{(preimmunization)}$

Moreover, we showed a crucial role of MPT83 for the chimeric protein MPT83-MPT63 and heterogeneous set of proteins in immune response (Fig. 4): anti-MPT83-MPT63 fusion (OD 3,96±0,037) and anti-(MPT+63MPT83) (OD 0,049±3,123) sera characterizes by a high level of IgG against MPT83 antigen than to MPT63 (OD 0,05±2,536 and 0,011±0,487 for anti-MPT-83 MPT63 fusion and anti-(MPT+63MPT83) sera respectively). Similar experiments were conducted to recognize all types of antigens and their combinations with different sera (Fig. 4).

Chimeric protein based on Fascycline-like domain of MPT83 (MPT83 $FLD_{115-220aa}$) and MPT63 was obtained previously [19] and was used for development of efficient TB diagnostic for cattle [20]. Unlike MPT83(full)-MPT63 fusion which was used for this experiments it incomplete analogue did not retain the primary protein structure and as a result restricted sterically range of serologically important determinants of both antigens. As a result, Fascycline-like domain of MPT83 fused to MPT63 did not contain all serologically important determinants of new synthetized MPT83(full)-MPT63 protein. As we can see (Fig. 5, *A*) this antigen, as in the case with full-size MPT63-MPT83 (Fig. 5, *B*), was better recognized by anti-MPT83-MPT63 serum, however anti-MPT63 antiserum was better recognized short MPT83–MPT63, while antiserum anti-MPT83 was better recognized the full-size MPT63-MPT83. Thus, it has been proven a special role of MPT83 for immunogenicity of fusion antigens and significance of spatial organization new antigenic composition (MPT83-MPT63) and it plasticity of serologically important epitopes exposure.

Immune library of murine recombinant antibodies

Total RNA (2–5 μg/μl, $A_{260}/A_{280} \geq 1,6$) from splenocytes of immunized mice which were characterized by highest immune

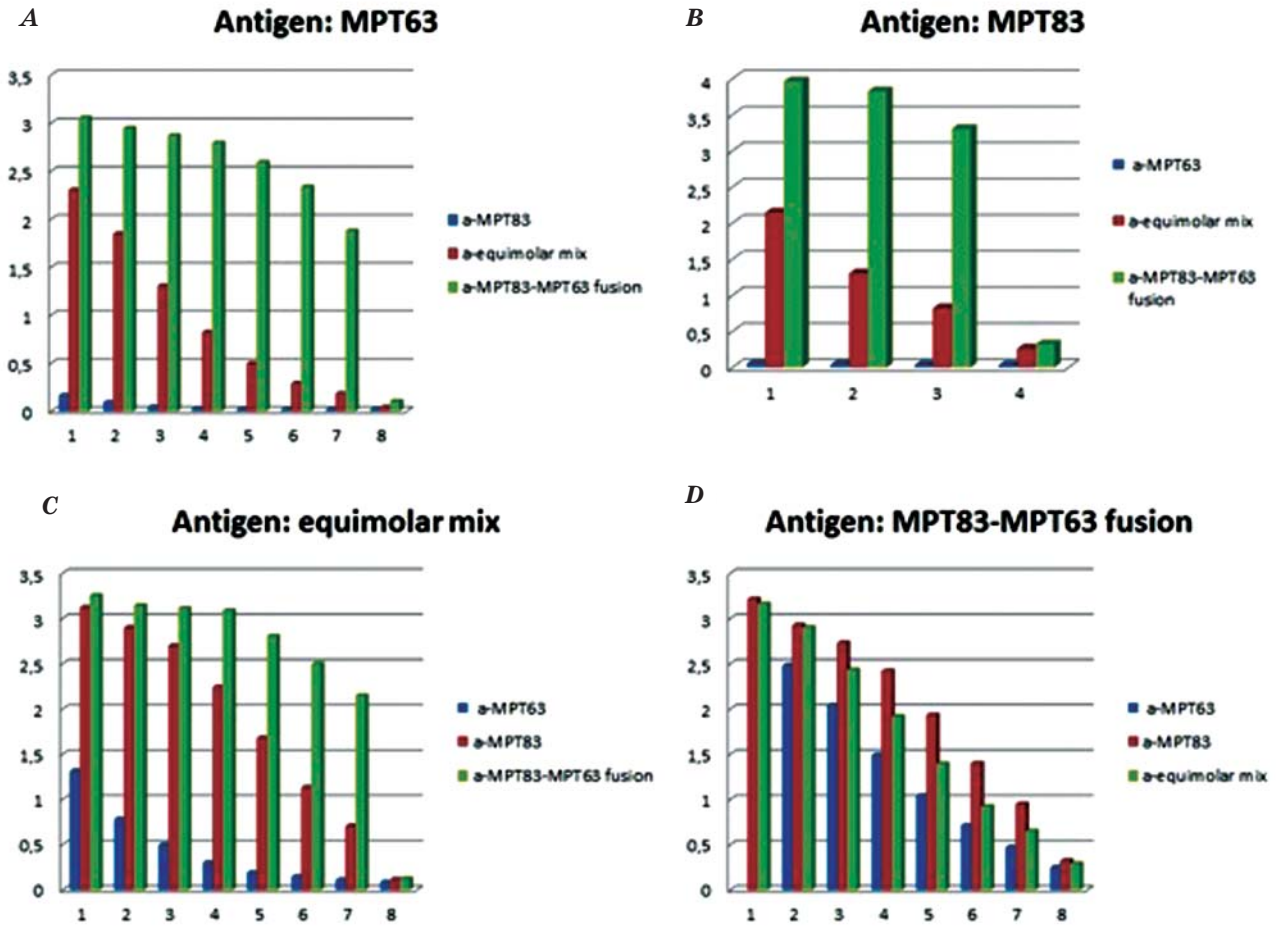

Fig. 4. **Results of cross reactivity among four types of antigen substances:**
A — MPT63; *B* — MPT83; *C* — (MPT63+MPT83 mix); *D* — MPT83-MPT63 fusion with all types of antisera without of own

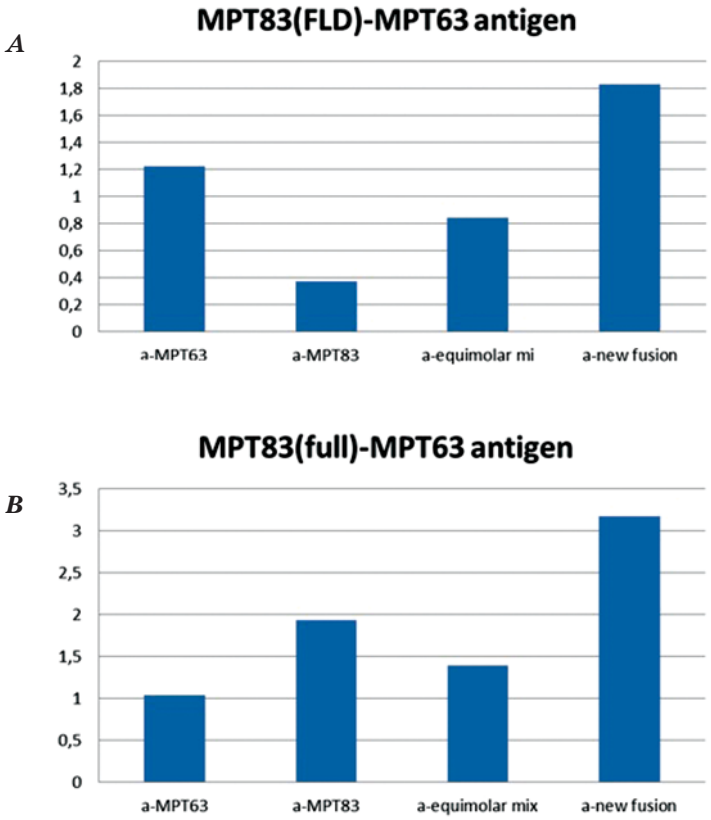

Fig. 5. **IgG recognition results of MPT83(FLD)-MPT63 fusion (*A*) or MPT83(full)-MPT63 fusion (*B*) antigens with 4 types of antisera**

response to MPT63 and MPT83 were used as the matrix for reverse transcriptase reaction for cDNA obtaining with next amplification of sequences encoding the variable domains of the heavy and light immunoglobulin chains. Electrophoresis on agarose gel showed 380 and 400 b.p. PCR products corresponding in size to V_L and V_H respectively (Fig. 6, *A*). The purified V_H and V_L were assembled in a single step of assembly PCR as described in [16]. Genes encoding scFv and phagmide p*CANTAB*-5E vector were treated by restriction endonucleases *Sfi*I and *Not*I with next fusion by T4 DNA lygase. *E. coli* XL1-blue was chosen as a host cells.

Selection and characteristic of MPT63 and MPT83 specific scFvs

For isolation of high specific scFv against mycobacterial antigens we have developed a modified selection scheme described in[17] (we specifically do not focused at the routine antibody selection scheme that has been repeatedly and in detail described previously). After selection of library against MPT63 and MPT83 few obtained colonies studied by the method of Lift Assay. At the same time, most of the colonies were negative (Fig. 6, *B*). Also, several of the positive clones were tested by immonoenzyme assay. In order to confirm that the isolated clones were highly specific to MPT63 or MPT83 we analyzed their interaction with a number of control antigens. It has been confirmed that appropriate scFv antibodies recognize MPT63 or MPT83 and do not cross-react among themselves and did not react with BSA or milk casein (Fig. 6, *C*).

In spite of the fact that the obtaining antibodies were not characterized by a high affinity constant unlike polyclonal antibodies obtained due mice immunization, they are more specific and reminiscent monoclonal antibodies. Our next step will be a more detailed selection of antibodies that can be used as research tools for mycobacterial antigens or even as components of test systems as positive controls.

Improved tools for TB detecting are urgently needed. In this prospective study, efforts were made to evaluate the immunodiagnostic potential of the secretory protein MPT63 and MPT83 lipoprotein of *M. tuberculosis* for developing a novel ELISA-based serodiagnostic test employing an individual, fusion or cocktail of two (16 and 22.6 kDa) recombinant proteins to enhance the sensitivity of the immunoassay, and attempts were also made to check the specificity of the all variants of antigens substances.

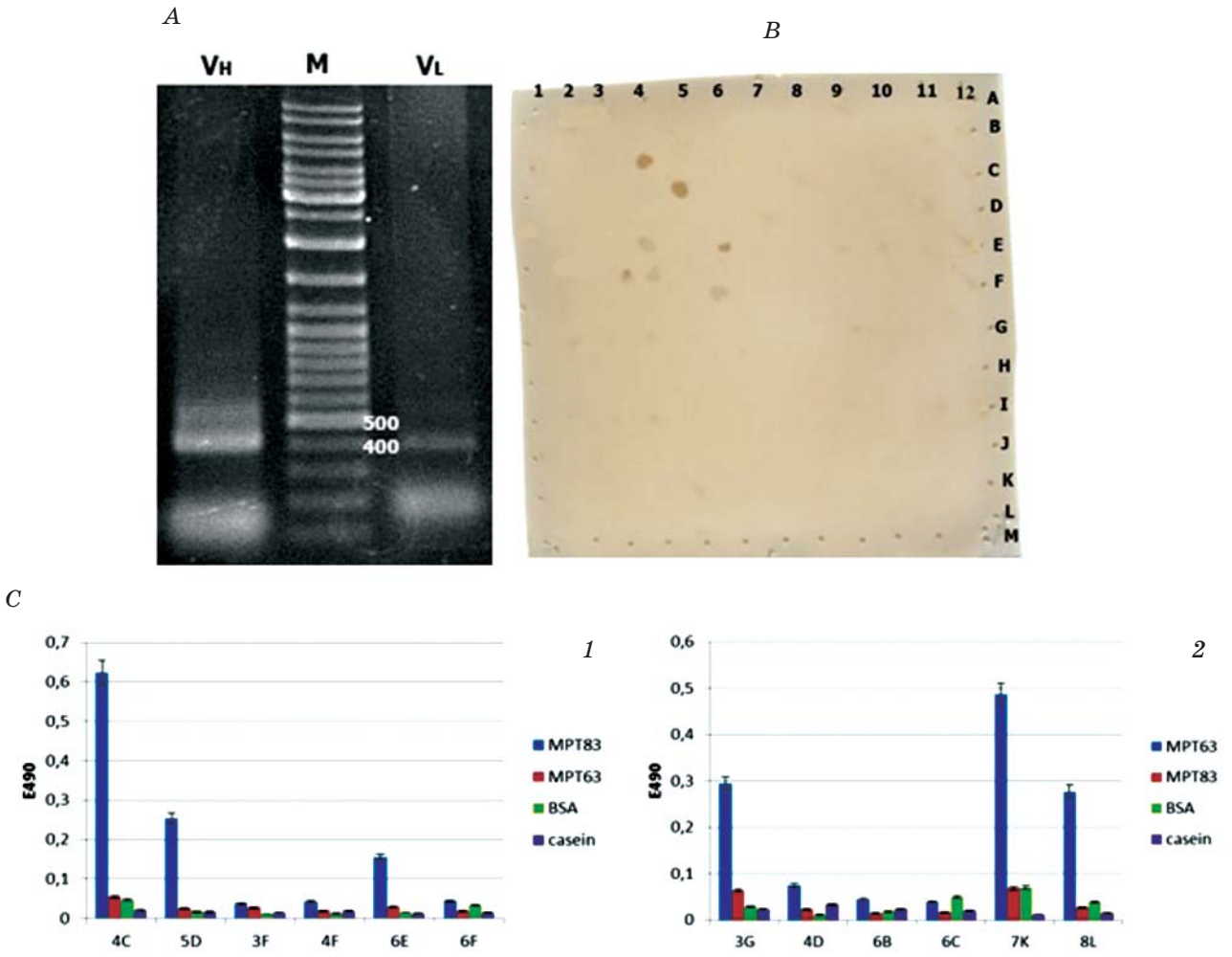

Fig. 6. ***A*** **— PCR-amplification of VH- and VL-domains;** ***B*** **— Analysis of** ***E. coli*** **expressed scFv against MPT83 colonies with Lift Assay method;** ***C*** **— ELISA results of periplasmic extracts containing scFv antibodies against MPT83 (*1*) and MPT63 (*2*) from positive (Lift Assay)** ***E. coli*** **clones**

Our findings clearly demonstrate that for new TB diagnosis ELISA test MPT83-MPT63 fusion protein as plate coating antigen was the best candidate among tested antigens. This chimeric antigen as a cocktail of individual MPT63 and MPT83 characterized by higher immunogenic and antigenic properties than MPT63 or MPT83 individual immune response. But unlike serological diagnostics based on a mixture of antigens or mycobacterial lysate, our proposed MPT83-MPT63 fusion antigen guarantees the reproducibility of the results, since it uses as a homogeneous antigenic substance on a solid-state carrier. Moreover, for antigens mixture, each antigen must be tested separately, which leads to an increase in the cost of the analysis, the timing of its implementation and it's not very adequate evaluation of the results.

It should be noted that due to high level of antigenicity and immunogenicity of fusion protein, it can detect trace amounts of antibodies to the pathogen. Also, the use of two completely non-homologous proteins MPT63 and MPT83 with different effects and hitherto known functions increases its value for the identification of biomarkers of TB infection. The absence of *mpt63* and *mpt83* genes in NTM strains allows the use of this antigen for the diagnosis both *M. tuberculosis* and *M. bovis* (strains that cause pulmonary TB) infected patients.

Thus, based on the results of anti-serum recognition of various proteins and their cocktails, we have chosen a fusion protein MPT83-MPT63 for TB and health patients screening by ELISA for further offer of new diagnostic kit.

Conflicts of interest

No potential conflict of interest relevant to this article was reported.

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REFERENCES

1. World Health Organization. Global tuberculosis report 2017. Available at: http://www.who.int/tb/publications/global_report/en/. (acsessed, February, 2018).
2. *Nema V.* Tuberculosis diagnostics: challenges and opportunities. *Lung India*. 2012, 29(30), 259–266. doi: 10.4103/0970-2113.99112.
3. *McNerney R., Cunningham J., Hepple P., Zumla A.* New tuberculosis diagnostics and rollout. *International Journal of Infectious Diseases*. 2015, 32, 81–86. doi: 10.1016/j.ijid.2015.01.012.
4. World Health Organization. Commercial serodiagnostic tests for diagnosis of tuberculosis: policy statement. 2011, WHO Library Cataloguing-in-Publication Data. Available at http://apps.who.int/iris/bitstream/10665/44652/1/9789241502054_eng.pdf.
5. *Banerjee S., Gupta S., Shende N., Kumar S., Harinath B.* Serodiagnosis of tuberculosis using two ELISA systems. *Ind. J. Clin. Biochem.* 2003, 18(2), 48–53.
6. *Tiwari D., Tiwari R. P., Chandra R., Bisen P. S., Haque S.* Efficient ELISA for diagnosis of active tuberculosis employing a cocktail of secretory proteins of *Mycobacterium tuberculosis*. *Folia Biologica*. 2014, 60, 10–20.
7. *Redchuk T. A., Korotkevich N. V., Kaberniuk A. A., Oliinyk O. S., Labyntsev A. Iu., Romaniuk S. I., Kolibo D. V., Busol V. A., Komisarenko S. V.* Statistical analysis of the distribution of the antibody levels to *Mycobacterium bovis* antigenes for bovine tuberculosis diagnostics. *Cytol Genet*. 2010, 44(5), 280–285.
8. *Muñoz S., Hernández-Pando R., Abraham S. N., Enciso J. A.* Mast cell activation by *Mycobacterium tuberculosis*: mediator release and role of CD48. *J. Immunol*. 2003, 170(11), 5590–5596.
9. *Wiker H. G.* MPB70 and MPB83 — major antigens of *Mycobacterium bovis*. *Scand. J. Immunol*. 2009, 69(6), 492–499.
10. *Chambers M. A., Whelan A. O., Spallek R., Singh M., Coddeville B., Guerardel Y., Elass E.* Non-acylated *Mycobacterium bovis* glycoprotein MPB83 binds to TLR1/2 and stimulates production of matrix metalloproteinase 9. *Biochem. Biophys. Res. Commun*. 2010, 400(3), 403–408.
11. *Becker K., Haldimann K., Selchow P., Reinau L., Molin M., Sander P.* Lipoprotein glycosylation by protein-O-mannosyltransferase (MAB_1122c) contributes to low cell envelope permeability and antibiotic resistance of *Mycobacterium abscessus*. *Front Microbiol*. 2017, 8, 1–12. doi: 10.3389/fmicb.2017.02123.
12. *Wang L., Zuo M., Chen H., Liu S., Wu X., Cui Z., Yang H., Liu H., Ge B. Mycobacterium tuberculosis* lipoprotein MPT83 induces apoptosis of infected macrophages by activating the TLR2/p38/COX-2 signaling pathway. *J. Immunol*. 2017, 198(12), 4772–4780. doi: 10.4049/jimmunol.1700030.
13. *Manoilov K. Yu., Labyntsev A. Yu., Korotkevych N. V., Kolibo D. V., Komisarenko S. V.* Interaction of recombinant diphtheria toxoids with cellular receptors *in vitro*. *Biotechnol. acta*. 2016, 9(3), 44–51. doi: 0.15407/biotech9.03.044.
14. *Siromolot A. A., Oliinyk O. S., Kolibo D. V. Komisarenko S. V. Mycobacterium tuberculosis* antigens MPT63 and MPT83 increase phagocytic activity of murine peritoneal macrophages. T*he Ukr. Biochem. J*. 2016, 88(5), 62–70. doi: 10.15407/ubj88.05.062.
15. *Schägger H., Jagow G.* Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem*. 1987, 166(2), 368–379.
16. *Oliinyk O. S., Palyvoda K. O., Lugovskaya N. E., Kolibo D. V., Lugovskoy E. V., Komisarenko S. V.* Recombinant single chain variable fragments antibodies (scFv) against Pro^{144}-Leu^{155} fragment of human protein C. *The Ukr. Biotech. J*. 2015, 87(2), 88–94.
17. *Oliinyk O. S., Kaberniuk A. A., Kolibo D. V., Komisarenko S. V.* Isolation and characterization of recombinant single chain variable fragment antibodies (scFv) against human heparin-binding EGF-like growth factor. *Biotekhnolohiia*. 2012, 5(6), 47–56. (In Ukrainian).
18. *Lyashchenko K. P., Pollock J. M., Colangeli R., Gennaro M. L.* Diversity of antigen recognition by serum antibodies in experimental bovine tuberculosis. *Infection and Immunity*. 1998, 66(11), 5344–5349.
19. *Redchuk T. A., Korotkevich N. V., Kaberniuk A. A., Oliinyk O. S., Labyntsev A. Yu., Romaniuk S. I., Kolibo D. V., Komisarenko S. V.* Recombinant chimera protein MPB63-MPB83 as perspective antigen for diagnostic of tuberculosis. *Biotekhnolohiia*. 2010, 3(5), 50–56. (In Ukrainian).
20. *Siromolot A. A., Redchuk T. A., Solodiankin O. S., Kolibo D. V., Gerilovich A. P.,*

Komisarenko S. V. The trial of experimental test system for the specific diagnostics of cattle tuberculosis. *Biotechnol. acta.* 2016, 9(4), 14–18. https://doi.org/10.15407/biotech9.04.014

РОЗПІЗНАВАННЯ АНТИГЕНІВ *Mycobacterium tuberculosis* MPT63 ТА MPT83 ПОЛІКЛОНАЛЬНИМИ І scFv АНТИТІЛАМИ МИШІ

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Метою дослідження було охарактеризувати імуноглобулін G (IgG) опосередковану відповідь за умов експериментальної імунізації лабораторних мишей очищеними рекомбінантними протеїновими препаратами *Mycobacterium tuberculosis* MPT63, MPT83, еквімолярним коктейлем протеїнів та штучним злитим протеїном MPT83-MPT63, а також отримати рекомбінантні одноланцюгові варіабельні фрагменти антитіл scFv до MPT63 та MPT83 проти цих антигенів.

Дослідження показало, що гуморальна імунна відповідь до MPT63, MPT83, химерного протеїну MPT83-MPT63 та еквімолярної суміші MPT63 і MPT83 істотно відрізняється. Для кожного антигену рівні сироваткових антитіл оцінювали, використовуючи значення обрізання на основі індексу оптичної щільності. Доведено вирішальну роль MPT83 для імуногенності химерного протеїну та/або коктейлю окремих антигенів за умов імунізації лабораторних тварин.

Отримано специфічні scFv антитіла проти MPT63 та MPT83, які можуть бути використані для розроблення системи для кількісного визначення антигенів, а також вивчення їхніх біологічних властивостей.

Було показано високоімуногенні властивості N-кінцевої ділянки MPT83, що підвищує чутливість ELISA, й запропоновано використовувати химерний протеїн MPT83-MPT63 як перспективний кандидат на роль антигенної субстанції для серологічної діагностики туберкульозу.

Ключові слова: антигени MPT63, MPT83, поліклональні антитіла, scFv, діагностика.

РАСПОЗНАВАНИЕ АНТИГЕНОВ *Mycobacterium tuberculosis* MPT63 И MPT83 ПОЛИКЛОНАЛЬНЫМИ И scFv-АНТИТЕЛАМИ МЫШИ

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Целью исследования было охарактеризовать иммуноглобулин G (IgG) опосредованный ответ в условиях экспериментальной иммунизации лабораторных мышей очищенными рекомбинантными протеиновыми препаратами *Mycobacterium tuberculosis* MPT63, MPT83, эквимолярным коктейлем протеинов и искусственным слитым протеином MPT83-MPT63, а также получить рекомбинантные одноцепочечные вариабельные фрагменты антител scFv к MPT63 и MPT83 против этих антигенов.

Исследование показало, что гуморальный иммунный ответ к MPT63, MPT83,слитому протеину MPT83-MPT63 и эквимолярной смеси MPT63 и MPT83 существенно отличается. Для каждого антигена уровни сывороточных антител оценивали, используя значения обрезания на основе индекса оптической плотности. Доказана решающая роль MPT83 для иммуногенности химерного протеина и/или коктейля отдельных антигенов при иммунизации лабораторных животных.

Получены специфические scFv антитела против MPT63 и MPT83, которые могут быть использованы для разработки системы для количественного определения антигенов, а также для изучения их биологических свойств.

Показаны высокоиммуногенные свойства N-концевого участка MPT83, что повышает чувствительность ELISA, и было предложено использовать химерный антиген MPT83-MPT63 в качестве перспективного кандидата на роль антигенной субстанции для серологической диагностики туберкулеза.

Ключевые слова: антигены MPT63, MPT83, поликлональные антитела, scFv, диагностика.

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MATHEMATICAL MODELLING OF OPTIMIZATION NUTRIENT MEDIUM COMPOSITION FOR ENTOMOPATHOGENIC BACTERIA STRAIN *Bacillus thuringiensis* 87/3 CULTIVATION

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The aim of the research was to develop an optimal liquid medium composition for bacteria strain *B. thuringiensis* 87/3 which is the most favorable for the identification the potential production of biologically active components. In this work we have used new entomopathogenic bacteria strain *B. thuringiensis var. thuringiensis (Bt H1)* № 87/3 selected *in vitro*, isolated from the larvae of leaf-eating insects natural populations of *Leptinotarsa decemlineata* Say (L_4). Nutrient medium for cultivation of *Bt* 87/3 was optimized on base a full-fledged experiment, according to the Box-Behnken (3^3) plan. Optimumal concentrations were investigated in the medium of carbon source (15 g/l of glucose), nitrogen (10 g/l of corn extract) and phosphorus-containing inorganic salts (1,5 g/l of diammonium phosphate). When this strain grown in periodic conditions at 30 °C, the number of viable cells reached $4{,}4 \cdot 10^9$ cells/ml. Nutrient medium that was optimized can be recommended for cultivation of *Bacillus thuringiensis* 87/3 in production conditions.

Key words: optimization, cultivation, *B. thuringiensis var. thuringiensis*, nutrient medium.

Biological pesticides is one of the most promising alternatives over conventional chemical pesticides, which offers less or no harm to the environments and biota. A large range of microorganisms such as bacteria, viruses, fungi, and protozoans have since been identified as potential candidates for use in biocontrol strategies against insect pests. The dominant position among the complex of entomopathogenic microorganisms known in the world and used in the protection of plants occupy entomopathogens belonging to the species *Bacillus thuringiensis (Bt)*[1,2]. There are more than 80 serovariants of *Bt*, selectively specific to the definite groups of host insects belonging to the order *Lepidoptera*, *Diptera, Coleoptera, Hymenoptera, Orthoptera, Hemiptera, Isoptera, Mallophaga, Thisanoptera, Nematoda, Acari* [3].

Serological *Bt* variants produce different entomotoxins, their synthesis in many respects depends on the conditions of cultivation. Toxigenicity of microorganisms can be changed by biotechnological procedures (changing the conditions of cultivation) and thus affect metabolism in general [4]. The most important stage in the production of bacterial preparations is obtaining the maximum production of delta endotoxin in a minimum time of cultivation with a maximum economic effect.

The aim of the research was to develop an optimal liquid medium composition for bacteria strain *B.thuringiensis 87/3*, which are the most favorable for the identifiying the potential production of biologically active components.

Materials and Methods

Research was conducted on the base of the National University of Life and Environmental Sciences of Ukraine, department of biotechnology and biodiversity; Institute of Food Biotechnology and Genomics of the National Academy of Sciences of Ukraine, department of industrial and food biotechnology. The subject of researches

was entomopathogenic bacteria strain *B. thuringiensis var. thuringiensis (Bt H1) №87/3* selected in vitro, isolated from the larvae of leaf-eating insects natural populations of *Leptinotarsa decemlineata* Say (L_4). The qualitative and quantitative composition of medium components was determined on the basis of a priori information analysis.

The task of full-fledged experiment, according to the Box-Behnken (3^3) plan is based on obtaining a mathematical model of the *Bacillus thuringiensis 87/3* development process and its subsequent use in nutrient medium optimizing.

The planning of an experiment allows not only to vary all the operating factors during the process, but also to obtain a quantitive assessment of the main factors and the effects of interaction between them [5]. Optimization is possible using methods of steep climbing, as well as research of the desirability function of the resulting factor. The influence of different medium composition on the growth of microorganisms was investigated during active experiment process. The average level for *Bacillus thuringiensis 87/3* cultivating was corn extract 10 g/l, diammonium phosphate 1.5 g /l, glucose 15 g/l (Table 1).

Cultivation was carried out in Erlenmeyer's flasks on biotechnology shaker with termoplatform (220 rev./min, the temperature 30 degrees °C during 72 hours). Medium, of volume 50 ml, the amount of inoculum — at least 4.0% by volume of the medium (the titer of colony forming units, CFU, 4.2–4.4 billion/ml of the culture liquid, which was determined by inoculation on agar and counting in Goryaev chamber).

For the experiment conduction it was decided to investigate three factors and their three levels. In this case, the number of experiments that must be carried out can be calculated by the formula (1):

$$N_e = (N_\Phi)^p, \qquad (1)$$

where N_e — the number of experiments, p — the number of levels factors.

Since formula (1) shows that 27 experiments are needed to solve optimization problems. The plan for a full-fledged experiment with the specified levels and factors, as well as the function of the experiment response, has been formed. In order to reduce the impact on the results of the response, the experiments were carried out in random sequence. The processing of the experiment results began with regression analysis: the model was built and unknown coefficients were determined:

$$Y = (b_0 + b_1 \cdot X1 + b_2 \cdot (X1)^2 + b_3 \cdot X2 + \\ + b_4 \cdot (X2)^2 + b_5 \cdot X3 + b_6 \cdot (X3)^2) \cdot 10^9 \qquad (2)$$

It was determined that the effects of the interaction factors are practically absent, and therefore they were not included in the general view of the model (2). The determination of unknown constant coefficients was performed using the least squares method. The obtained model coefficients were calculated using the following formulas:

$$b_0 = \sum_{i=1}^{N} Y_i / N; \quad b_j = \sum_{U=1}^{N} Y_i X_{ji} / N;$$

$$b_{j^2} = \sum_{i=1}^{N} Y_i (X_{ji})^2 / N.$$

Description of factors and response using a mathematical model (2) is characterized by a determination coefficient, which must be not less than 0.95, for qualitative description of the research object, this coefficient is calculated by the formula,

$$R^2 = 1 - \frac{\sigma^2_{з.р.}}{\sigma^2_Y} = 1 - \left(\sum_{i=1}^{N} (Y_i - \hat{Y}_i)^2 / \sum_{i=1}^{N} (Y_i - \bar{Y})^2\right), \qquad (3)$$

where $\sigma_{з.р.}{}^2$, $\sigma_Y{}^2$ — dispersion of residues regression, response; Y_i, $\bar{Y}$, $\hat{Y}_i$ — actual, average, estimated value of response.

Table 1. **Formation of factors and their levels for the experiment**

Factors	Levels			
	Lower	Main	Upper	Variation Step
X1 — corn extract, g/l	5	10	15	5
X2 — diammonium phosphate, g/l	0,5	1,5	2,5	1
X3 — glucose, g/l	5	15	25	10

The standard error which characterizing the standard deviation of the studied regression coefficients from the mean value is calculated by the formula:

$$S_{b_{j^r}} = \sqrt{\frac{\sum_{i=1}^{N}(Y_i - \hat{Y}_i)^2}{\sum_{i=1}^{N}(X_j - \bar{X}_j)^2} \cdot \frac{1}{n-2}}, \quad (4)$$

where n — sample size.

The statistical significance of the regression coefficient is estimated according to Student's criterion. In this case, compare the calculated with the table value for a given confidence level significance of 0.05 and freedom degrees calculated:

$$\left|t_{\alpha,f}\right| = \left|\frac{b_{j^r}}{S_{b_{j^r}}}\right| \geq t_{\alpha/2,f.table,} \quad (5)$$

where b_{jr} — estimated regression coefficients, α — confidence probability 0.95, f — freedom degree. With a significant regression coefficient, the student's calculated criterion is over than tabular.

The calculation of the marginal deviation error was established from the following calculations:

$$\Delta_{jr} = t_{\alpha,f.table} \cdot S_{b_{jr}} \quad (6)$$

Determination of the confidence interval for each regression coefficient was conducted according to the inequality:

$$b_{jr} - \Delta_{jr} \leq b_{jr} \leq b_{jr} + \Delta_{jr} \quad (7)$$

The correspondence of the mathematical model to the experimental data was determined by Fisher's criterion (F). In this case, the calculation criterion should be over than the tabular one:

$$F = \frac{\sigma_X^2}{\sigma_Y^2} = \frac{R^2}{1-R^2} \cdot \frac{f_2}{f_1} \geq F_{\alpha,f.table,}$$

where $\sigma_X^2 = \left(\sum_{i=1}^{N}(X_i - \bar{X})^2\right)/f_1$ — variance factor; $f_1 = k_b$ — freedom degree; k_b — the coefficients number of the regression model; $\sigma_Y^2 = \left(\sum_{i=1}^{N}(Y_i - \bar{Y})^2\right)/N - f_1 - 1$ — response variance, N — number of experiments, R^1 — determination factor.

To solve the optimization problem, the method of analyzing the desirability function of Harrington was used [6].

The analysis of this experiment plan was carried out using portable software (Statistica 10.0.1011.0, CD-key 42347678921334567692).

Results and Discussion

Bacillus thuringiensis grows slowly and spores weakly on media with a known composition containing glucose and salts. Growth can be enhanced by the addition of amino acids or casein hydrolyzate, but if not balanced the nitrogen medium content with an appropriate source of carbon and energy, such as glucose, sporulation will remain low.

Optimization of cultivation conditions can be based on a combination of experimental and mathematical modeling with a computational experiment, which contains an important stage — the definition of a mathematical model that characterizes the connection of the optimization parameter with the main factors. Using such a simplified model allows conclusions accelerating about the significance of various medium components, its qualitative and quantitative composition [7]. The mathematical method of experiment planning allows us to reasonably approach the nutrient medium constructing, making its more economical and technological.

The nutrient medium for cultivation was optimized by the composition of sources of carbon and nitrogen feed, as well as on the content of micro elements. We used the cabbage broth as the basis for preparing the nutrient medium. Cabbage is a vegetable raw material with a unique composition: sugar, pectin, starch, fiber, proteins, pantothenic acid, tartronic acid, carotene, vitamins (C, P, B, PP, K, D and U), micro- and macroelements (K, Na, Ca, Mg, Fe, P, S, Cl, also Co, F,I, Mo, Cu, Zn, Si). To increase the yield of heat-resistant spores and the amount of endotoxin, the nutrient medium was enriched with corn extract, amino acids and mineral salts (Mg, Mn, diammonium phosphate).

Using mathematical method of experiment planning, the nutrient medium was optimized for the cultivation of a new technological strain *B. thuringiensis 87/3* in the conditions of biolabs low tonnage production. The nutrient medium optimization was carried out in the alternation of the maximum and minimum values contents of the media components [8–10]. Regression analysis of experimental results is reflected in Table 2.

Table 2. **Regression analysis of experimental results**

$R^2 = 0.98081$ — regression model determination coefficient of the experimental data						
Coefficients of regression (factors)	**Regression coefficients**	**Standard error**	**Student's coefficient**	**Level of significance p.**	**Trust interval — 95%**	**Trust interval — 95%**
b0	1.006898	0.096511	10.4330	0.000015	0.805579	1.208217
X1	0.092667	0.017812	5.2025	0.000043	0.055511	0.129822
$(X1)^2$	–0.002444	0.000881	–2.7731	0.011733	–0.004283	–0.000606
X2	1.667778	0.067323	24.7727	0.000028	1.527344	1.808212
$(X2)^2$	–0.494444	0.022037	–22.4373	0.000011	–0.540412	–0.448477
X3	0.161000	0.006732	23.9145	0.000034	0.146957	0.175043
$(X3)^2$	–0.005494	0.000220	–24.9331	0.000016	–0.005954	–0.005035

Analyzing the table 2 data it is possible to conclude that all the included factors are statistically significant, as evidenced by their level of significance, a model describing the development of bacteria *Bacillus thuringiensis 87/3* using the studied components. Substitute the table data in the general form of the regression equation:

To assess the adequacy of this model, a dispersion analysis of experimental data and a Fisher's criterion evaluation were performed. The dispersion analysis implementation is reflected in Table 3.

Table 3 shows that the mathematical model included factors (1) adequately describe the investigated process of optimizing the composition of nutrient medium, since the significance level p for each factor is below the permissible level.

The response surfaces to the bacterial colonies development by titration of the investigated process with the reflection of the values factors and the scale of desirability are presented in Fig. 1. Levels of function response to the desirability scale are shown in Fig. 2. Visually analyzing the graphics data, it is possible to clearly state that the optimal composition of the nutrient medium is present in the studied ranges of values factors X1, X2, X3. Implementation of the nutrient medium optimization, according to the developed model, is possible with the help of the desirability function [8]. To determine the nutrient medium optimal composition maximum interval of the investigated response is formed. This takes into account the response surface (Fig. 1) and its maximum levels, set the zero level to $4.11 \cdot 10^9$ cells/ml, and a maximum of $4.41 \cdot 10^9$ cells/ml. Under these conditions it is possible to find the required maximal response values to the desirability function. Implementation of the determination procedure optimization is presented in Fig. 3.

Fig. 3 shows that the optimal components variant is at the intersection of the maximum value of the desirability function in the specified interval of each factor. In this case, rational limits and corresponding optimal values factors that make up the *B. thuringiensis* nutrient medium are determined. For corn extract, this is the interval [8.75 ... 11.25 g/l], and the optimum is 10 g/l; for diamonium phosphate is the interval [1.4 ... 2.0 g/l], and an optimum is 1.5 g/l; for glucose it is the interval [12.5 ... 17.5], and the optimum — 15 g /l.

Table 3. **Dispersion analysis of experimental results**

Factors	**Median deviation**	**Degree of freedom**	**Dispersion**	**Fisher criterion**	**Trust interval p**
X1	0.884830	2	0.442415	218.0776	0.000010
X2	2.079207	2	1.039604	512.4473	0.000035
X3	1.837785	2	0.918893	452.9457	0.000019

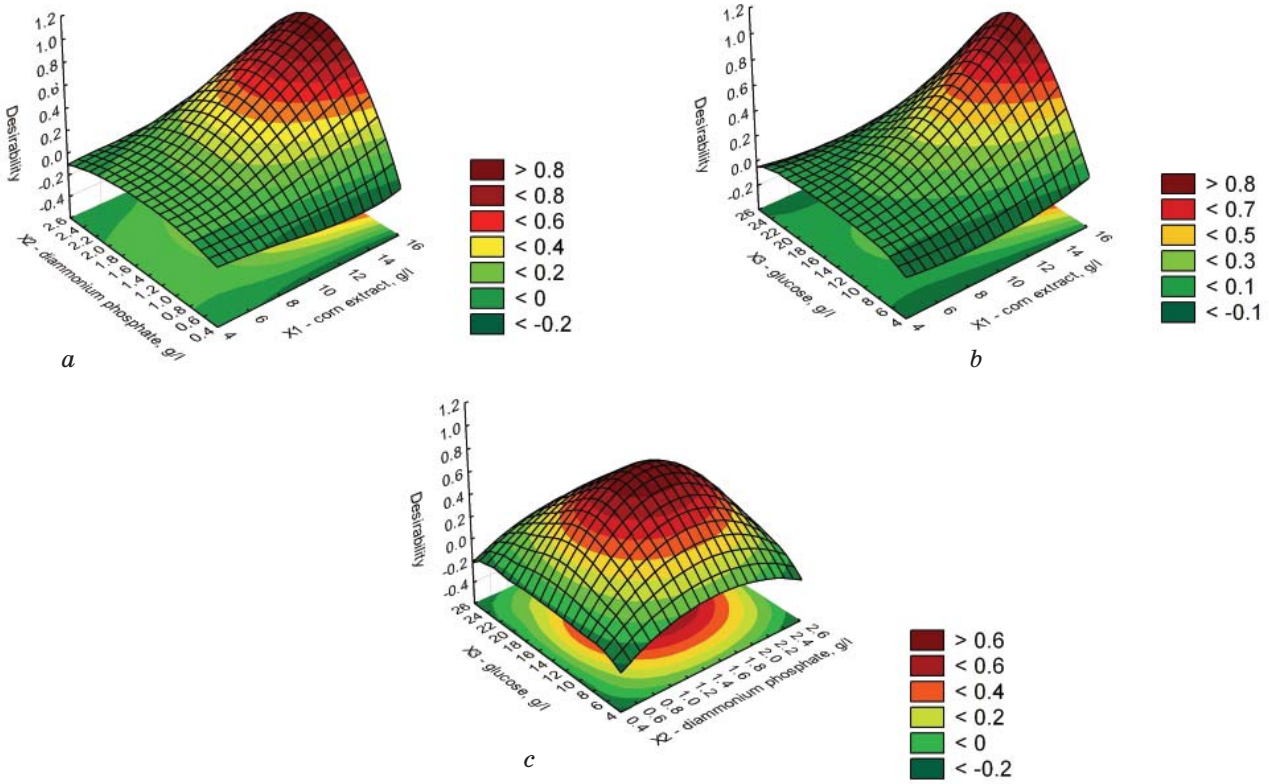

Fig. 1. **Schedule of surfaces response on the desirability scale of the investigated process:** *a* — Y_d(X1, X2); *b* — Y_d(X1, X3); *c* — Y_d(X2, X3)

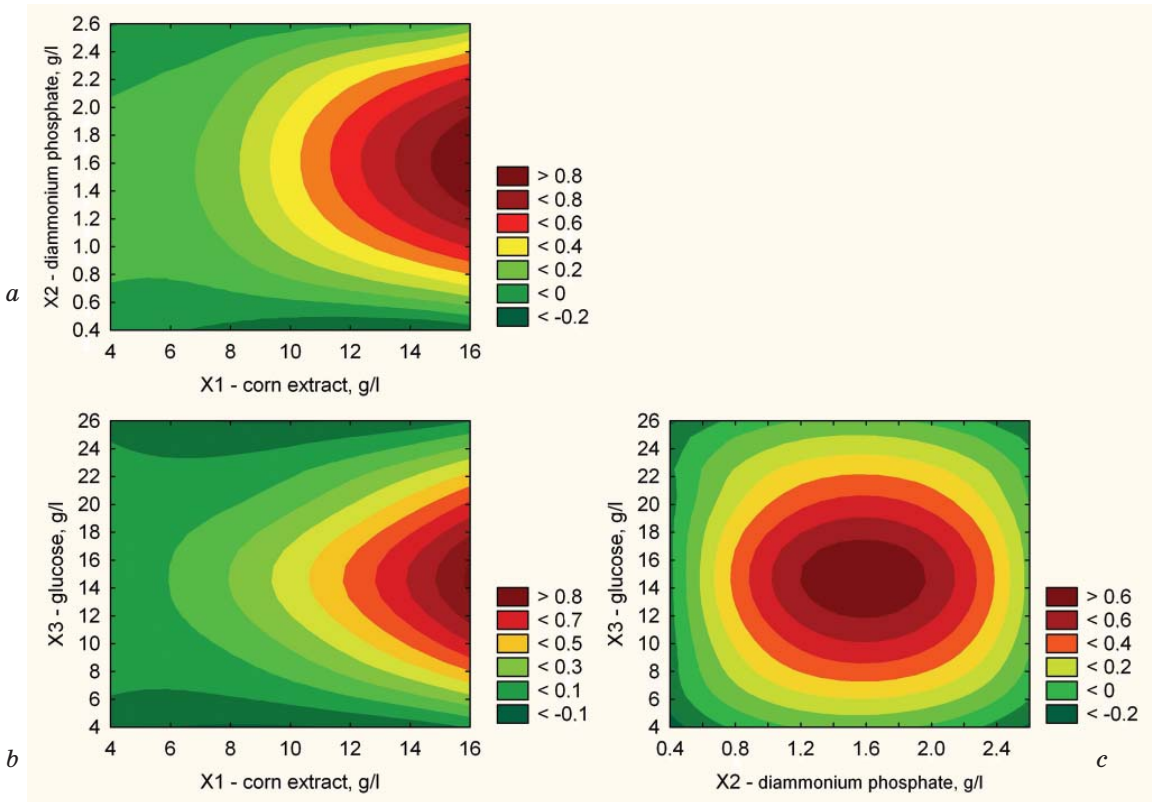

Fig. 2. **Graph of the investigated process response level:** *a* — Y_d(X1, X2) = const; *b* — Y_d(X1, X3) = const; *c* — Y_d(X2, X3) = const

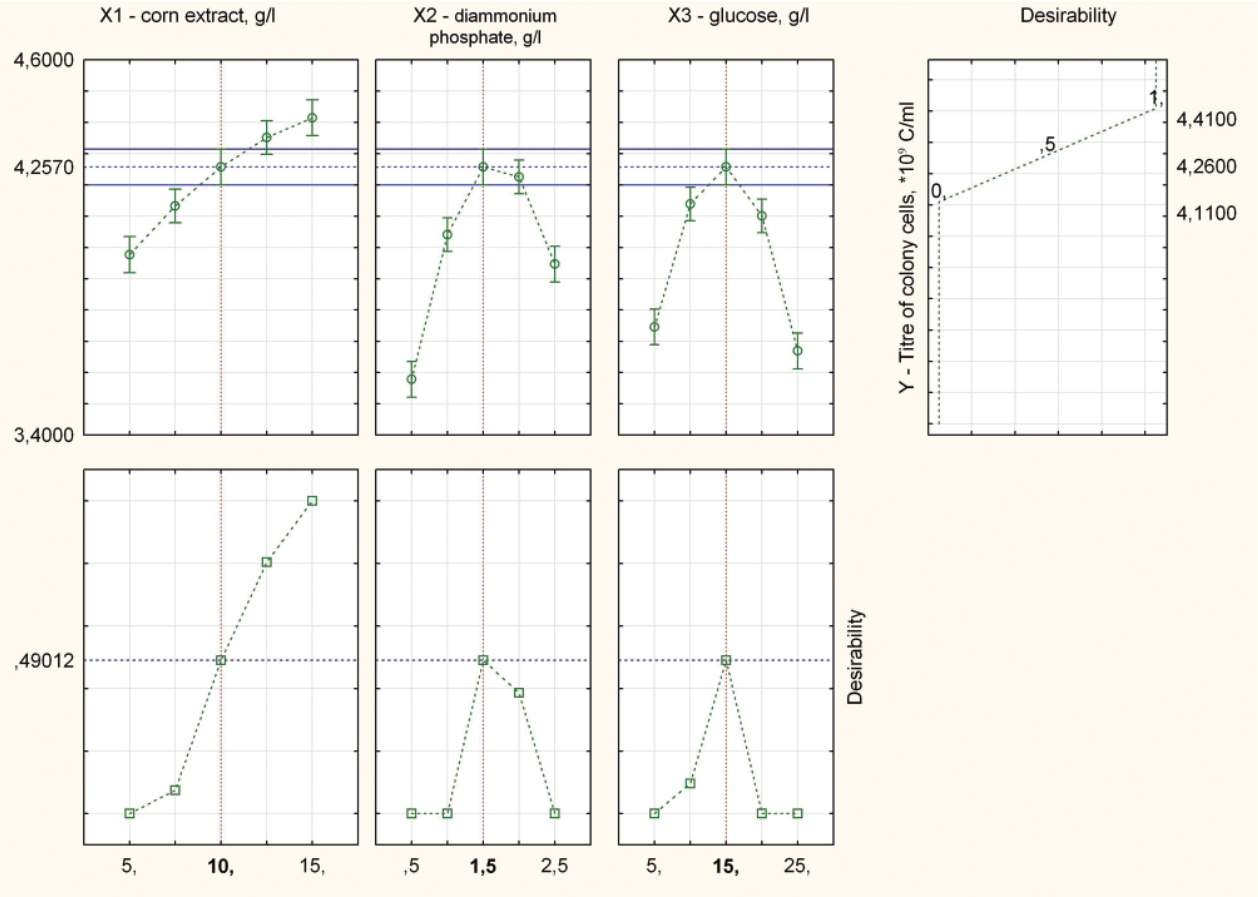

Fig. 3. **Graphic representation of the nutrient medium optimal composition finding procedure**

Using the method of mathematical experiment planning the nutrient medium was optimized for the cultivation of a new technological strain *B. thuringiensis* 87/3. The results of the studies indicate that optimized medium is intended in laboratory conditions and provides the ability to obtain a high yield of viable cells in 24 hours of cultivation (the titre of the metabolic spore-crystalline complex is up to 4.4 billion/ml of culture liquids). The nutrient medium proposed composition is much cheaper than laboratory medium, which are widely used for cultivation microorganisms of this species and can be recommended for using in laboratory and production conditions.

REFERENCES

1. *Kandyibin N. V.* Mikrobiokontrol chislennosti nasekomyih i ego dominanta *Bacillus thuringiensis. Moskva, Sankt-Peterburg, Pushkin: Nauchnoe izdanie* "*Innovatsionnyiy tsentr zashchity rasteniy*" . 2009, 252 p.
2. *Lamenha C. Bacillus sphaericus* and *Bacillus thuringiensis* to Insect Control. Process Development of Small Scale Production to Pilot-Plant-Fermenters. *Federal University of Pernambuco, Brasil.* 2012, 613–627.
3. *Roh J. Y., Choi J. Y., Li M. S., Jin B. R., Je Y. H. Bacillus thuringiensis* as a specific, safe and effective tool for insect pest control. *J. Mol. Biol.* 2007, 17, 547–559.
4. *Biryukov V. V., Kantere V. M.* Optimizatsiya periodicheskih protsessov mikrobiologicheskogo sinteza. *Moskva: Nauka*. 1985, 296 p.
5. *Avignone-Rossa C., Arcas J., Mignone C. Bacillus thuringiensis* growth, sporulation and δ-endotoxin production in oxygen limited and non-limited cultures. *World J. Biotechnol.* 1992, 8, 301–304.
6. *Harrington E. C.* The Desirability Function. Industrial Quality Control. 1965, 494–498.
7. *Mason R., Gunst R., Hess J.* Statistical Design and Analysis of Experiments With Applications to Engineering and Science. *John Wiley &Sons, Inc.* 2003, 585–586.
8. *Anderson R. K. I., Jayaraman K.* Impact of balanced substrate flux on the metabolic process employing fuzzy logic during the cultivation of *Bacillus thuringiensis* var. Galleriae. *World J. Microbiol. Biotechnol.* 2005, 21, 127–133. doi: 10.1007/s11274-004-3043-1.

9. *Kraemer-Schafhalter A., Moser A.* Kinetic study of *Bacillus thuringiensis var. israelensis* in lab-scale process. *Bioproc Eng*. 1996, 14, 139–144.

10. *Popovic M., Liu W., Iannotti E. L., Bajpai R. K.* A mathematical model for vegetative growth of *Bacillus thuriniensis*. *Eng. Life Sci.* 2001, 2, 85–90.

МАТЕМАТИЧНЕ МОДЕЛЮВАННЯ ОПТИМІЗАЦІЇ СКЛАДУ ЖИВИЛЬНОГО СЕРЕДОВИЩА ДЛЯ КУЛЬТИВУВАННЯ ШТАМУ ЕНТОМОПАТОГЕННИХ БАКТЕРІЙ *Bacillus thuringiensis* 87/3

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Метою дослідження було розробити математичне моделювання оптимального складу рідкого живильного середовища для бактеріального штаму *B. thuringiensis* 87/3, що є оптимальним для отримання біологічно активних компонентів. У роботі використовували нові ентомопатогенні бактерії штаму *B. thuringiensis var. thuringiensis (Bt H1)* № 87/3, селекціоновані *in vitro*, що їх виділено з личинок природних популяцій листогризних комах *Leptinotarsa decemlineata* Say (L_4). За допомогою повнофакторного експерименту за планом Бокса-Бенкіна (3^3) оптимізовано живильне середовище для культивування штаму *Bacillus thuringiensis* 87/3. Визначено оптимальні концентрації в середовищі джерел вуглецю (15 г/л глюкози), азоту (10 г/л кукурудзяного екстракту) та фосфоровмісних неорганічних солей (1,5 г/л діамонію фосфату). Під час культивування цього штаму в періодичних умовах за 30 °С кількість життєздатних клітин досягала $4{,}4 \cdot 10^9$ КУО/мл. Оптимізоване середовище можна рекомендувати для культивування штаму *Bacillus thuringiensis* 87/3 у промислових умовах.

Ключові слова: оптимізація, культивування, *B. thuringiensis* var. *thuringiensis*, живильне середовище.

МАТЕМАТИЧЕСКОЕ МОДЕЛИРОВАНИЕ ОПТИМИЗАЦИИ СОСТАВА ПИТАТЕЛЬНОЙ СРЕДЫ ДЛЯ КУЛЬТИВИРОВАНИЯ ШТАММА ЭНТОМОПАТОГЕННЫХ БАКТЕРИЙ *Bacillus thuringiensis* 87/3

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Целью исследования было разработать математическое моделирование оптимального состава жидкой питательной среды для бактериального штамма *B. thuringiensis* 87/3, оптимальный для получения биологически активных компонентов. В работе использовали новые энтомопатогенные бактерии штамма *B. thuringiensis var. thuringiensis (Bt H1)* № 87/3, селекционированные *in vitro*, выделенные из личинок природных популяций листогрызущих насекомых *Leptinotarsa decemlineata* Say (L_4). С помощью полнофакторного эксперимента по плану Бокса-Бенкина (3^3) оптимизирована питательная среда для культивирования штамма *Bacillus thuringiensis* 87/3. Определены оптимальные концентрации в среде источников углерода (15г/л глюкозы), азота (10 г/л кукурузного экстракта) и фосфорсодержащих неорганических солей (1,5 г/л диаммония фосфата). При культивировании данного штамма в периодических условиях при 30 °С количество жизнеспособных клеток достигала $4{,}4 \cdot 10^9$ КОЕ/мл. Оптимизированную среду можно рекомендовать для культивирования штамма *Bacillus thuringiensis* 87/3 в промышленных условиях.

Ключевые слова: оптимизация, культивирование, *B. thuringiensis* var. *thuringiensis*, питательная среда.

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POLYMORPHISM OF SOME TRANSCRIPTION FACTOR GENES RELATED TO DROUGHT TOLERANCE IN WHEAT

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The aim of the research was to study polymorphism of preselected gene loci of three transcription factors (*TaNAC2a*, *TaWRKY2*, and *TaWRKY19*) and the Late Embryogenesis Abundant *(LEA)* proteins dehydrin (*Td29b*) related to wheat drought tolerance. The genes structure and chromosome location were established via bioinformatics tools. It is stated that *TaWRKY2* and *TaWRKY19* genes were comprised of 4 exons and 3 introns located on 2BS and 1DS chromosome arms, respectively; *TaNAC2a* — 2 exons and 1 intron 7AS; *Td29b* — single exon gene 3AS. Using polymerase chain reaction, no polymorphism was observed. Polymorphic bands were detected for *TaWRKY2* locus. The screening of the distribution of the revealed polymorphic loci was carried out for a set of wheat and rye varieties, old landraces and interspecific hybrids. The polymorphism of *TaWRKY2* locus indicated the presence of some other possible alleles of the gene. The obtained data are importantfor further investigations of wheat drought tolerance.

Key words: *Triticum* spp., polymerase chain reaction, transcription factors, *TaNAC2a*, *TaWRKY2*, *TaWRKY19*, *LEA*, *Td29b*, drought tolerance.

Common wheat (*Triticum aestivum* L.) is very important widely grown crop used for bread baking, food and animal feed. Wheat yield is the third largest cereal production in the world, after maize and rice [1]. In consequence of methods of modern plant breeding, numerous varieties with increased productivity were obtained. However, due to the recent undesired climate changes and global warming, the selection of drought-tolerant germplasm donors must be constantly monitored to include them in contemporary breeding programs [2]. Marker-assisted selection (MAS) based on DNA markers can be effectively applied in the process of such selection [3–6]. While different types of DNA sequences can be employed for this purpose.

First DNA marker systems to study drought tolerance in plants were based on non-coding DNA sequences — RAPD (Random Amplified Polymorphic DNA) [7, 8], SSR (Simple Sequence Repeats) [9, 10] and ISSR (Inter Simple Sequence Repeats) [8] etc. Presently, attention mostly attracted to target encoding gene sequences, which play a great role in plant response to stress factors. These genes predominantly represented with transcriptional factors (TFs) and dehydrin genes [11].

In present work, our aim was to study DNA polymorphism of preselected gene loci of three transcription factors (*TaNAC2a*, *TaWRKY2*, *TaWRKY19*) and the LEA dehydrin (*Td29b*) related in their expression response to wheat drought tolerance.

WRKY transcription factors represent family of proteins that have *WRKY* domain (approximately 60 amino acids), involving the conserved *WRKYGQK* domain and a zinc-finger-like motif [12, 13]. These proteins are of great importance for biotic and abiotic stress responses [14, 15]. Overexpression of *TaWRKY2* as well as *TaWRKY19* increased dehydration stress tolerance in transgenic

Arabidopsis plants [12]. It was also found out that *TaWRKY2* overexpressing plants had enhanced *STZ* and *RD29B* gene expressions due to temperate binding to the loci from *RD29B STZ-1* and *STZ-2* locus of *Arabidopsis*. As to *TaWRKY19* transgenic plants, they had higher expression levels of *DREB2A*, *RD29B*, *Cor6.6* and *RD29A* genes [12].

Another *TF* family that highly introduced in common wheat is represented with proteins containing a highly conserved *NAC* domain at the N-terminus and a variable transcriptional regulation domain at the C-terminus [16, 17]. Overexpression of different *TaNAC* responded to enhanced biotic and abiotic tolerance [18, 19]. It was postulated in the [16], that *TaNAC2a* transgenic plants of tobacco had extremely increased drought tolerance.

Special role in response to dehydration stress relates to dehydrin proteins, which help plant cell cope with osmotic changes. The number of dehydrins were described in wheat [20, 21]. Late Embryogenesis Abundant (*LEA*) proteins belong to above mentioned group of proteins and can be candidate for wheat improvement [22]. It was reported [23] that *LEA* proteins accumulation enhanced stress tolerance protecting plant cells against dehydration. It was also described the importance of *Td29b* dehydrin in common wheat, which synthesis was highly induced by dehydration.

Materials and Methods

The subject of the study was a set of wheat cultivars of Ukrainian and foreign origin (25 and 36, consequently), a set of 52 old wheat species, distant and interspecific hybrids, 4varieties of rye.

BLAST searches and sequence analyses were implemented by BLASTn on the *Triticum aestivum* genome (https://blast.ncbi.nlm.nih.gov/ and https://wheat-urgi.versailles.inra.fr). The schemes of exon–intron structures were obtained by employing the online Gene Structure Display Server bioinformatic tools (http://gsds.cbi.pku.edu.cn/) from both coding sequence (CDS) and genomic sequences [24].

Total DNA was isolated from one kernel with the modified CTAB method [25]. Polymerase chain reaction (PCR) of 20 µl included 0.5 µM of forward and reverse primers each (Metabion, Germany), 1× Reaction Buffer B (Solis BioDyne, Estonia), 2 mM $MgCl_2$, 0.2 µM of each deoxyribonucleoside triphosphate (Thermo Fisher Scientific, USA), 1 unit of FIREPol® DNA Polymerase (Solis BioDyne, Estonia), 30 ng of total plant DNA. Primer sequences for loci *TaNAC2a*, *TaWRKY2*, *TaWRKY19* and *Td29b* used in the study and PCR conditions are indicated in the Table 1. The CDS accessions in the GenBank are HM027575.2 (*TaNAC2a*), EU665425.1 (*TaWRKY2*), EU665430.1 (*TaWRKY19*) and AJ890139.1 (*Td29b*).

The PCR products were separated by means of electrophoresis in 2% agarose gels in lithium borate buffer, 0.1 µg/ml ethidium bromide [26]. Gels were visualized in UV-light with a photosystem Canon EOS 600D. GelAnalyzer 2010 software was applied to identify the size of amplified fragments (http://www.gelanalyzer.com). Frequencies for each combination of amplified fragments were calculated according to [27].

Results and Discussion

As it was denoted above, data on CDS only are available for those three studied transcriptional factors (*TaNAC2a, TaWRKY2,* and *TaWRKY19*) and the dehydrin (*Td29b*). Thus, we managed to predict the exon-intron

Table 1. **Primer sequences and PCR conditions**

TF gene	Primer sequences 5′–>3′	PCR conditions
TaNAC2a	F: GGTAGTGCGGTGCTTCCAAT R: TGAATGTTGTTGCTCGTCCC [16]	94 °C — 30 s; 58 °C — 30 s 72 °C — 30 s; 35 cycles
TaWRKY2	F: GGCGCTGCCGACGTCATCTT R: AGCAGAGGAGCGACTCGACGA [12]	94 °C — 30 s; 58 °C — 30 s 72 °C — 30 s; 35 cycles
TaWRKY19	F: AGGGAAGCATACGCATGACGTGC R: GGCGAGATCGTTCAGAATGGCTGT [12]	94 °C — 30 s; 60 °C — 30 s 72 °C — 30 s; 35 cycles
Td29b	F: CGCACCCAGCTAGTAAGTTCG R: CCCAGCCCAGTAATAACCCAT [23]	94 °C — 30 s; 53 °C — 30 s 72 °C — 30 s; 35 cycles

structure and location of their genes by means of alignment via BLAST tools.

Having carried out every CDS alignments in the database of wheat whole genome shotgun contigs, the gene structures and chromosomal location were defined for three studied transcription factors (*TaNAC2a, TaWRKY2, TaWRKY19*) and the dehydrin (*Td29b*) (Fig. 1) in accordance with [28]. Hence, *TaWRKY2* and *TaWRKY19* have similar structure of 4 exons and 3 introns (Fig. 1, *A, B*), though, they are situated in different chromosomes (*TaWRKY2* — short arm of 1D chromosome; *TaWRKY19* — short arm of 1B). Both primer pairs applied in the following DNA polymorphism study hybridized at the end of the fourth exon. The gene of TF TaNAC2a comprises of 2 exons and 1 intron (Fig. 1, *C*) and allocates at the short arm of 7A chromosome. The primer pair for this gene locus annealed at the central part of exon 2. It was established, that Td29b gene might have referred to single exon gene (Fig. 1, *D*). Its location is the short arm of 3A chromosome.

Molecular genetic study

To study DNA polymorphism of the selected loci of 4 genes (three TF — *TaNAC2a*, *TaWRKY2*, *TaWRKY19*; and the dehydrin gene *Td29b*) a set of 25 Ukrainian and 37 international wheat accessions from Global Wheat Program of the International Maize and Wheat Improvement Center (CIMMYT) and the Wheat Germplasm Bank was collected. By means of applying primer pairs and PCR conditions indicated in the Table 1, we observed no polymorphism for gene loci *TaNAC2a*, *TaWRKY19* and *Td29b*. There was one fragment amplified only for each sample — fragment of approximately 227 base pairs (bp) for *TaNAC2a* gene locus, 160 bp for *TaWRKY19*, 86 bp for *Td29b* (Fig. 2).

Following the amplification of total genomic DNA of all common wheat varieties of Ukrainian and foreign origin, there were two fragments detected for each sample. We observed three different genotypes in the studied *TaWRKY2* locus. The first one

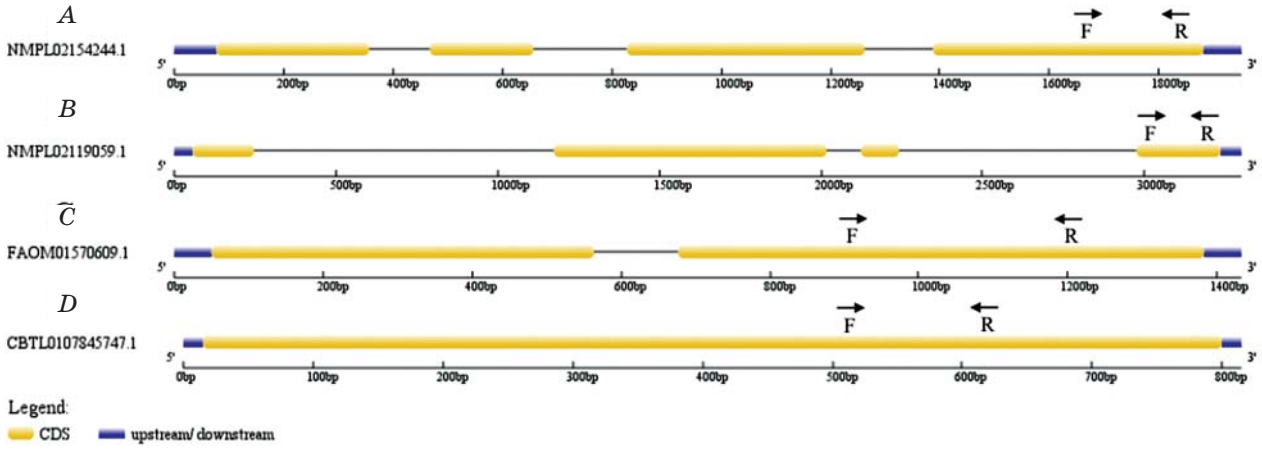

Fig. 1. **Prediction of gene exon-intron structure:**
A — TaWRKY2; *B* — TaWRKY19; *C* — TaNAC2a; *D* — Td29b

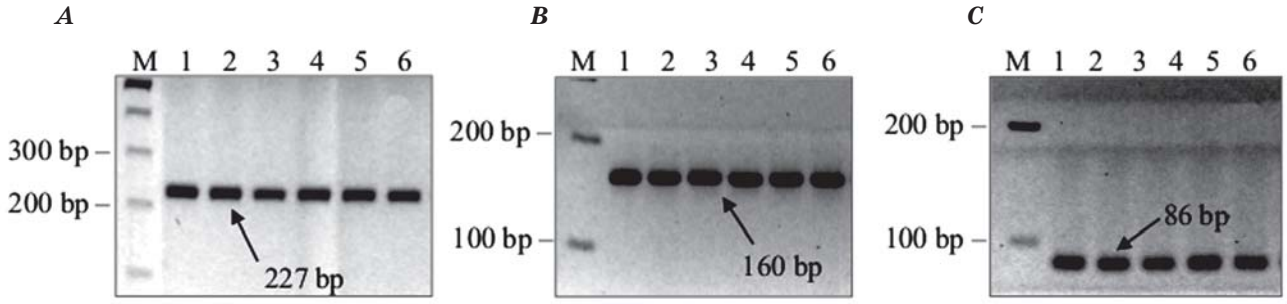

Fig. 2. ***The electrophoregrams denoting PCR-products segregation of DNA marker systems***:
A — TaNAC2a; *B* — TaWRKY19; *C* — Td29b
Lanes 1–6 — common wheat varieties (Glenlea, Comanche, Wilbur, Granero Inta, Tobarito M 97, V-17);
M — marker of molecular weight GeneRuller™ DNA Ladder Mix

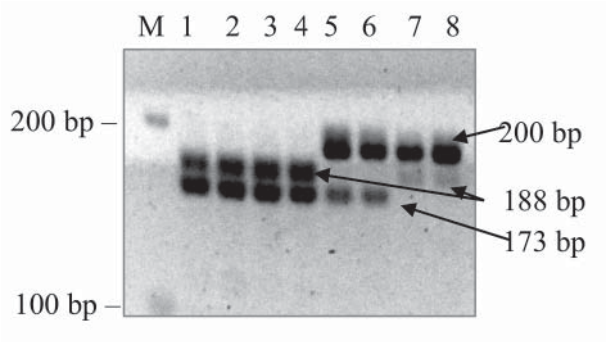

Fig. 3. **The electrophoregram depicting DNA polymorphism of *TaWRKY2* locus:** Lanes 1 — Odeska 267; 2 – Poliska 90; 3 — Darunok Podillia; 4 — Podolianka; 5 – Astarta; 6 — Kryzhynka; 7 — Sotnytsia; 8 – Zolotokolosa; M — molecular weight marker GeneRuller™ DNA Ladder Mix

represented amplified fragments of 173 and 188 bp, second — 188 and 200 bp, the third — of 173 and 200 bp (Fig. 3). Moreover, Ukrainian varieties showed greater diversity, than those foreign ones. The frequencies for each allele of amplified fragments among Ukrainian varieties are 0.52 (173+188 bp), 0.28 (173+188 bp) and 0.2 (173 + 200 bp). On the contrast, a set of amplified fragments 188+ +200 bp was not observed among 36 foreign varieties obtained from the CIMMYT. In addition, only two samples (Millaleau Inia and Tobarito M 97) possessed 173+200 bp pattern. Thereafter, frequencies for these two allele of amplified fragments among the CIMMYT varieties were 0.944 (173+188 bp) and 0.056 (173+200 bp). The results on the DNA polymorphism study of *TaWRKY2* locus are indicated in the Tables 2 and 3.

Table 2. **Detected DNA polymorphism of *TaWRKY2* locus in Ukrainian varieties**

Variety	Originator	Amplified fragment, bp	Variety	Originator	Amplified fragment, bp
Astarta	IPPG NASU	173, 200	Poliska 90	NSC "IA NAAS"	173, 188
Bohdana	IPPG NASU	173, 188	Shchedrivka Kyivska	IPPG NASU NSC "IA NAAS"	173, 188
Bunchak	PBGI NCSCI NAASU	173, 188	Slavna	IPPG NASU	188, 200
Darunok Podillia	IPPG NASU	173, 188	Smuhlianka	IPPG NASU	188, 200
Drevlianka	n.a.	173, 188	Solomiia	IPPG NASU	173, 188
Favorytka	IPPG NASU	173, 200	Sonata	Institute of Field and Vegetable Crops, Novi Sad, Serbia	173, 200
Hileia	IPPG NASU	188, 200	Sotnytsia	IPPG NASU	188, 200
Kryzhynka	RMIW NAASU IPPG NASU	173, 200	Spasivka	IPPG NASU	188, 200
Natalka	IPPG NASU	173, 188	Vesnianka	IPPG NASU	188, 200
Novokyivska	IPPG NASU	173, 200	Yatran 60	IPPG NASU	173, 188
Odeska 267	PBGI NCSCI NAASU	173, 188	Yednist	PBGI NCSCI NAASU	173, 188
Pereiaslavka	IPPG NASU	173, 188	Zolotokolosa	IPPG NASU	188, 200
Podolianka	IPPG NASU	173, 188			

Note: IPPG NASU — Institute of Plant Physiology and Genetics, National Academy of Sciences of Ukraine; PBGI NCSCI NAASU — Plant Breeding and Genetics Institute — National Center of Seed and Cultivar Investigation, the National Academy of Agrarian Sciences of Ukraine; RMIW NAASU — The V.M. Remeslo Myronivka Institute of Wheat, the National Academy of Agrarian Sciences of Ukraine; NSC "IA NAAS" — National Scientific Centre "Institute of Agriculture of the National Academy of Agrarian Sciences of Ukraine"; n.a. — not available. http://www.wheatpedigree.net, State register of plant varieties suitable for distribution to Ukraine of the Ministry of Agrarian Policy and Food of Ukraine http://www.sops.gov.ua/reestr-sortiv-roslin.

Table 3. **Detected DNA polymorphism of TaWRKY2 locus in varieties from germplasm collections of the CIMMYT**

Variety	Locality	Originator*	Year of registration	Amplified fragment, bp
1	2	3	4	5
AC Vista	Canada (Saskatchewan)	Agriculture and Agri-Food Canada Semi-arid Prairie Agricultural Research Centre, Swift Current	1996	173, 188
Albis	Switzerland (Zurich)	Federal Research Station for Agronomy	1983	173, 188
Anza	Mexico, USA (California)	California Agricultural Experiment Station	1971	173, 188
Batavia	Australia (Queensland)	Queensland Wheat Research Institute	1991	173, 188
Caribo	Germany	Heidenreih, Bad-Schwartau	1968	173, 188
Cenad-512	Romania	n.a.	1958	173, 188
Comanche	USA (Kanzas)	Kansas Agricultural Experiment Station	1942	173, 188
D-12	Peru	n.a.	1972	173, 188
Excalibur	Australia (South-Australia)	RAGT	1990	173, 188
Gabo	Australia (New-South-Wales)	University of Sydney Plant Breeding Institute, Cobbitty	1942	173, 188
Glenlea	Canada (Manitoba)	University of Manitoba	1972	173, 188
Grande-Del-Monte	Venezuela	n.a.	n.a.	173, 188
Granero Inta	Argentina	Inta	1987	173, 188
Inia-F-66	Mexico	INIA, CIMMYT	1966	173, 188
Iskamish-K-2-Light	Afghanistan	n.a.	1975	173, 188
Janz	Australia (Queensland)	Queensland Wheat Research Institute	1989	173, 188
Katunga	Australia (Victoria)	n.a.	1992	173, 188
Ke Feng 2	China (Heilongjiang)	Keshan WRI	1979	173, 188
Kimmo	Finland	n.a.	1941	173, 188
Klein Favorito	Argentina	E. Klein	1920	173, 188
Kulin	Australia (Western-Australia)	Department of Agriculture, W.A.	1986	173, 188
Manital	Italy	Samoggia Luigi, Bologna	1981	173, 188
Millaleau Inia	Chile	INIA, CIMMYT	1982	173, 200
Recital	France	Benoist	1986	173, 188
Rokycanska sametka	Czechoslovakia	n.a.	1899	173, 188
Safed Lerma	India	Indian Agricultural Research Institute	1967	173, 188
Sakha 69	Egypt	Agricultural Research Center, Giza	1980	173, 188
Svenno	Sweden	W. Weibull	1953	173, 188
Talimka	Kyrgyzstan	Kirgizskaya GSS	1940	173, 188
Tobarito M 97	Mexico	CIMMYT	1997	173, 200

Table 3. End

1	2	3	4	5
Tobarito M 97	Mexico	CIMMYT	1997	173, 200
Tselinnaya-Yubileinaya	Kazakhstan	Kazakhskiy NII zernovogo khozyaystva	1988	173, 188
V-17	Mexico	CIMMYT	1968	173, 188
Wilbur	USA (Oregon)	W.J. Mariner	1897	173, 188
Zambesi	Zimbabwe	Salisbury AES	1963	173, 188
Zerdakia	Iraq	n.a.	n.a.	173, 188
Zirka	Ukraine	Plant Breeding and Genetics Institute, National Academy of Agrarian Sciences of Ukraine	1984	173, 188

Note: n.a. — not available; * — from the Genetic Resources Information System for Wheat and Triticale (http://www.wheatpedigree.net/) provided by Vavilov Research Institute of Plant Industry (VIR) and International Maize and Wheat Improvement Center (CIMMYT).

According to CDS sequence of *TaWRKY2* (GenBank ID EU665425.1), the primer pair for this TF locus is likely to amplify the fragment of 188 bp long. Such a fragment was observed through the study; however, not all the wheat samples possessed it. Consequently, there must be an indel mutation, which is likely to form another allele.

The old wheat landraces is the source of potential genes of interest which can be of great value for common wheat improvement in modern breeding programs. Thus, the following screening of a number of wheat landraces and interspecific hybrids was carried out. The data were indicated in the Table 4. As it can be seen from the table, most of them carried fragments of 173+188 bp (46 among 52 samples, frequency — 0.88). On the other hand, all the 3 fragments (173, 188 and 200 bp) were amplified from 2 wheat accessions (*T. spelta* var. *duhamelianu* Baulaender and

Table 4. **Detected DNA polymorphism of *TaWRKY2* locus in old wheat species, distant and interspecific hybrids**

Species/Hybrid/Cross	Subspecies	Country of originator	Amplified fragment, bp
1	2	3	4
AD	*T. persicum*/*Ae. tauschii*	Japan	173, 188
AD	*T. dicoccum*/*Ae. speltoides*	Azerbaijan	173, 188
AD	*Ae. ventricosa*/*T. dicoccum*	Russia	173, 188
AD	*T. aestivum*/*Ae. comosa*	Russia	173, 188
AD 217	*T. timopheevii*/*Ae. umbellulata*	Japan	173, 188
AD 7	*T. ispahanicum*/*Ae. cylindrical*	Azerbaijan	173
AD 8	*T. dicoccum*/*Ae. triuncialis*	Azerbaijan	173, 188
Aegilotricum cylindroaestivum	*Aegilops cylindrica*/*T. aestivum*	Armenia	173, 188
Haynatricum	*T. dicoccum*/*Dasypyrum villosum*	Russia	173, 188
PAH-31	*T. dicoccum*/*T. monococcum*	Russia	173, 188
PEAH	*T. dicoccum*/*Ae. tauschii*	Russia	173, 188
T. dicoccum Schuebl.	var. *rufum*	Sweden	173, 188

Table 4. Продовження

1	2	3	4
T. dicoccum Schuebl.	var. *aeruginosum*	Azerbaijan	173, 188
T. dicoccum	var. *aeruginosum* Runo	Russia	173, 188
T. dicoccum	var. *serbicum* Polba 3	Russia, Udmurtia	173, 188
T. dicoccum	var. *dicoccum*	Ukraine	173, 188
T. dicoccum	var. *nigroajar*	Ethiopia	173, 188
T. dicoccum	var. *rufum*	Ukraine	173, 188
T. dicoccum	var. *aeruginosum*	Russia, Dagestan	173, 188
T. dicoccum	var. *semicanum*	Germany	173, 188
T. dicoccum	Polba Kokchetavska	Kazakhstan	173, 188
T. dicoccum	var.*vasconicum* Crjunella	Spain	173, 188
T. dicoccum	var. *rufum*	Spain	173, 188
T. dicoccum	var. *atratum*	Poland	173, 188
T. dicoccum	n.a.	Kazakhstan	173, 188
T. dicoccum Schuebl.	var. *serbicum*	Belarus	173, 188
T. dicoccum Schuebl.	var. *dicoccum*	n.a.	173, 188
T. dicoccum Schuebl.	var. *serbicum* Chervona krasa	Belarus	173, 188
T. dicoccum Schuebl.	var. *haussknachtianum* Bolshaia holova	India	173, 188
T. dicoccum Schuebl.	var. *loganse* Polba Kokchetavska	n.a.	173, 188
T. dicoccum Schuebl.	var. *volgense* Vernal	USA	173, 188
T. dicoccum Schuebl.	var. *aeruginosum*	Armenia	173, 188
T. dicoccum Schuebl.	var. *serbicum*	Russia	173, 188
T. dicoccum Schuebl.	var. *volgense*	Russia	173, 188
T. dicoccum Schuebl.	var. *haussknachtianum*	Kazakhstan	173, 188
T. dicoccum Schuebl.	var. *haussknachtianum*	Azerbaijan	173, 188
T. dicoccum Schuebl.	var. *aeruginosum* Runo	Russia	173, 188
T. ispahanicum	var. *ispahanicum*	Iran	173, 188
T. kiharae	*T. timopheevii* × *Ae.tauschii*	Japan	173, 188
T. macha	var. *palaeoimereticum*	Georgia	173, 188
T. sinkajae	var. *sinskajae*	Russia	173
T. spelta	var. *album*	Canada	173, 188
T. spelta	var. *caeruleum* CDC Zobra	Canada	173, 188
T. spelta	var. *griseoturanorecens*	Tajikistan	173, 188
T. spelta	var. *duhamelianum*	Poland	173, 188
T. spelta	var. *duhamelianum* Baulaender	Germany	173, 188, 200
T. spelta	var. *duhamelianum* Frankenkorn	Germany	173, 188

Table 4. End

1	2	3	4
T. spelta	var. *caeruleum*	Azerbaijan	173, 188
T. spelta	var. *album*	Canada	173, 188
T. vavilovii	var. *vavilovii*	Armenia	173, 188, 200
T. hexapolonicum	n.a.	Armenia	173, 200
Tritordeum 1199/09	*T. durum*/*Hordeum chilense*	Spain	188

T. vavilovii var. *vavilovii*) representing frequency 0.04 only. Three wheats (frequency — 0.06) had only one type of fragment (173 or 188 bp). The only sample (frequency — 0.02) (*T. hexapolonicum*) had fragments of 173+200 bp.

Additionally, four rye verities (Avgust, Khmarka, Remington and Stoir) were tested for polymorphism in *TaWRKY2* locus. As a result, the only fragment of 200 bp was detected in each sample. This fact shows that other cereal crops might have the *TaWRKY2* gene too.

The study of the genes, that impact greatly on drought response, is of great value for wheat improvement in present-day plant breeding programs. The current research reveals knowledge on DNA polymorphism of three transcriptional factors (*TaNAC2a*, *TaWRKY2*, *TaWRKY19*) and the dehydrin (*Td29b*) genes which can be applied for MAS. During the analysis the gene structure and chromosomal location were established. Thus, *TaWRKY2* and *TaWRKY19* genes comprised of 4 exons and 3 introns (2BS and 1DS, respectively); *TaNAC2a* — 2 exons and 1 intron (7AS); *Td29b* — single exon gene (3AS).

In the result of this study, no polymorphism was observed for gene loci *TaNAC2a*, *TaWRKY19* and *Td29b* by means of preselected primer pairs. In contrast, polymorphic bands were detected for *TaWRKY2* locus that did not correspond to CDS from GenBank. This fact indicated the presence of some other possible alleles of the gene.

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REFERENCES

1. *Food Outlook*: Biannual report on global food markets. November 2017. Available at: http://www.fao.org/3/a-I8080e.pdf.
2. *Kaur V., Singh S., Behl R. K.* Heat and drought tolerance in wheat: Integration of physiological and genetic platforms for better performance under stress. *Ekin J.* 2016, 2 (1), 1–14.
3. *Pokhylko S. Yu., Troyanovska A. V., Stepanenko A. I., Urbanovich O. Yu., Dugan O. M., Rybalka O. I., Morhun B. V.* Studies of bread wheat genotypes with transferred gene GPC-B1 of Triticum turgidum ssp. dicoccoides. *Fact. Exp. Evol. Org.* 2016, 18, 132–136. (In Ukrainian).
4. *Stepanenko O. V., Stepanenko A. I., Kuzminskiy Ye. V., Morgun B. V.* Identification of Psy1 gene alleles responsible for carotenoid accumulation in wheat grains. *Biotechnol. acta*. 2017, 10 (2), 57–66. https://doi.org/10.15407/biotech 10.02.057.
5. *Lakhneko O. R., Stepanenko A. I., Morgun B. V.* Genotyping *Triticum aestivum* L. cultivars of Ukraine with microsatellite markers. *Fact. Exp. Evol. Org*. 2016, 19, 61–63.
6. *Kosová K., Vítámvás P., Urban M. O., Kholová J., Prášil I. T.* Breeding for enhanced drought resistance in barley and wheat — drought-associated traits, genetic resources and their potential utilization in breeding programmes. *Czech J. Genet. Plant Breed.* 2014, 50 (4), 247–261.
7. *ElSayed A. I., Rafudeen M. S.* Molecular marker assisted for recognition drought tolerant in some of bread wheat genotypes.

J. Crop Sci. Biotech. 2012, 15 (1), 17–23. doi: 10.1007/s12892-011-0051-1.
8. *El-Sayed O. E., Ibrahim H. F.* RAPD and ISSR markers related to drought tolerance of regenerated plants in wheat double haploids and varieties. *Middle East. Russian J. Plant Sci. Biotechnol*. 2008, 2 (2), 44–51.
9. *Kim S. H., Kim D. Y., Yacoubi I., Seo Y. W.* Phenotypic and genotypic analyses of drought tolerance in Korean and Tunisian wheat cultivars. *Plant Breed. Biotech*. 2014, 2 (2), 139–150. doi: 10.9787/PBB.2014.2.2.139.
10. *Sadat S., Saeid K. A., Bihamta M. R., Torabi S., Salekdeh S. G. H., Ayeneh G. A. L.* Marker assisted selection for heat tolerance in bread wheat. *World Appl. Sci. J*. 2013, 21 (8), 1181–1189. doi: 10.5829/idosi.wasj.2013.21.8.2866.
11. *Rana R. M., Rehman S. U., Ahmed J., Bilal M.* A comprehensive overview of recent advances in drought stress tolerance research in wheat (*Triticum aestivum* L.). *Asian J. Agr. Biol*. 2013, 1 (1), 29–37.
12. *Niu C.-F., Wei W., Zhou Q.-Y., Tian A.-G., Hao Y.-J., Zhang W.-K., Ma B., Lin Q., Zhang Z.-B., Zhang J.-S., Chen S.-Y.* Wheat WRKY genes TaWRKY2 and TaWRKY19 regulate abiotic stress tolerance in transgenic Arabidopsis plants. *Plant Cell Environ*. 2012, 35, 1156–1170. doi: 10.1111/j.1365-3040.2012.02480.x.
13. *Okay S., Derelli E., Unver T.* Transcriptome-wide identification of bread wheat WRKY transcription factors in response to drought stress. *Mol. Genet. Genomics*. 2014, 289 (5), 765–781. doi: 10.1007/s00438-014-0849-x.
14. *Zhu X., Liu S., Meng C., Qin L., Kong L., Xia G.* WRKY transcription factors in wheat and their induction by biotic and abiotic stress. *Plant Mol. Biol. Rep*. 2013, 31 (5), 1053–1067. doi:10.1007/s11105-013-0565-4.
15. *Pandey S. P., Somssich I. E.* The role of WRKY transcription factors in plant immunity. *Plant Physiol*. 2009, 150 (4), 1648–1655. doi: 10.1104/pp.109.138990.
16. *Tang Y. M., Liu M. Y., Gao S. Q., Zhang Z., Zhao X., Zhao C. P., Zhang F. T., Chen X. P.* Molecular characterization of novel TaNAC genes in wheat and overexpression of TaNAC2a confers drought tolerance in tobacco. *Physiol. Plant*. 2012, 144, 210–224. doi:10.1111/j.1399-3054.2011.01539.x.
17. *Nakashima K., Yamaguchi-Shinozaki K., Shinozaki K.* The transcriptional regulatory network in the drought response and its crosstalk in abiotic stress responses including drought, cold, and heat. *Front. Plant Sci.* 2014, 5, 1–7. doi: 10.3389/fpls.2014.00170.
18. *Xue G. P., Way H. M., Richardson T., Drenth J., Joyce P. A., McIntyre C. L.* Overexpression of TaNAC69 leads to enhanced transcript levels of stress up-regulated genes and dehydration tolerance in bread wheat. *Mol. Plant*. 2011, 4 (4), 697–712. doi: 10.1093/mp/ssr013.
19. *Xia N., Zhang G., Liu X. Y., Cai G. L., Zhang Y., Wang X. J., Zhao J., Huang L. L., Kang Z. S.* Characterization of a novel wheat NAC transcription factor gene involved in defense response against stripe rust pathogen infection and abiotic stresses. *Mol. Biol. Rep*. 2010, 37 (8), 3703–3712. doi: 10.1007/s11033-010-0023-4.
20. *Hassan N. M., El-Bastawisy Z. M., El-Sayed A. K., Ebeed H. T., Alla M. M. N.* Roles of dehydrin genes in wheat tolerance to drought stress. *J. Advanced Res*. 2015, 6, 179–188. doi: 10.1016/j.jare.2013.11.004.
21. *Min D.-H., Zhang X.-H., Xu Z.-S.; Zhao Y., Chen Y., Li L.-C., Chen M., Ma Y.-Z.* Induction kinetics of a novel stress-related LEA gene in wheat. *Plant Mol. Biol. Rep*.2012, 30 (6), 1313–1321. doi: 10.1007/s11105-012-0446-2.
22. *Amara I., Zaidi I., Masmoudi K., Ludevid M., Pagès M., Goday A., Brini F.* Insights into late embryogenesis abundant (LEA) proteins in plants: from structure to the functions. *Amer. J. Plant Sci*. 2014, 5 (22), 3440–3455. doi: 10.4236/ajps.2014.522360.
23. *Ali-Benali M. A., Alary R., Joudrier P., Gautier M.-F.* Comparative expression of five Lea Genes during wheat seed development and in response to abiotic stresses by real-time quantitative RT-PCR. *Biochim. Biophys. Acta*. 2005, 1730, 56–65. doi: 10.1016/j.bbaexp.2005.05.011.
24. *Hu B., Jin J., Guo A.-Y., Zhang H., Luo J., Gao G.* GSDS 2.0: an upgraded gene feature visualization server. *Bioinformatics*. 2015, 31 (8), 1296–1297. doi: 10.1093/bioinformatics/btu817.
25. *Murray M., Thompson W.* Rapid isolation of high molecular weight plant DNA. *Nucl. Acids Res*. 1980, 8 (19), 4321–4325.
26. *Singhal H., Ren Y. R., Kern S. E.* Improved DNA electrophoresis in conditions favoring polyborates and Lewis acid complexation. *PLoS One*. 2010, 5 (6), 1–6. doi: 10.1371/journal.pone.0011318.
27. *Akinina G. E., Dugar V. N. Popov V. N.* Statistical analysis of genetic data using software ARLEQUIN, PHILYP, CLANN, STRUCTURE. *Kharkiv: The Plant Production Institute named by V. Ya. Yuryev, NAAS*. 2014, 100 p. (In Russian).
28. *International Wheat Genome Sequencing Consortium (IWGSC)*. A chromosome-based draft sequence of the hexaploid bread wheat (*Triticum aestivum*) genome. *Science*. 2014, 345 (6194), 1251788. doi: 10.1126/science.1251788.

ПОЛІМОРФІЗМ ГЕНІВ ДЕЯКИХ ТРАНСКРИПЦІЙНИХ ФАКТОРІВ, ЩО ПОВ'ЯЗАНІ З ПОСУХОСТІЙКІСТЮ ПШЕНИЦІ

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Метою дослідження було вивчити поліморфізм попередньо відібраних локусів генів трьох транскрипційних факторів (*TaNAC2a*, *TaWRKY2*, *TaWRKY19*) та протеїн пізнього ембріогенеза *(LEA)* дегидрину (*Td29b*), пов'язаних зі стійкістю пшениці до посухи. Структуру генів та хромосомну локалізацію було встановлено за допомогою біоінформаційних підходів. З'ясовано, що гени *TaWRKY2* та *TaWRKY19* складаються з 4 екзонів і 3 інтронів, локалізованих на плечах 2BS та 1DS хромосоми, відповідно; *TaNAC2a* містить 2 екзони та 1 інтрон 7AS. Ген *Td29b* містить один екзон 3AS. У результаті використання полімеразної ланцюгової реакції не було виявлено поліморфізму для локусів генів *TaNAC2a*, *TaWRKY19* та *Td29b* за допомогою попередньо відібраних пар праймерів. Проте для локусу *TaWRKY2* виявлено поліморфні фрагменти. Скринінг поширення поліморфних локусів проводили для набору сортів пшениці та жита, давніх пшениць та міжвидових гібридів. Поліморфізм локусу *TaWRKY2* свідчить про наявність деяких інших алелів цього гена. Ці дані є важливими для подальших досліджень посухостійкості пшениці.

Ключові слова: *Triticum* spp., полімеразна ланцюгова реакція, фактори транскрипції, *TaNAC2a*, *TaWRKY2*, *TaWRKY19*, *LEA*, Td29b, посухостійкість.

ПОЛИМОРФИЗМ ГЕНОВ НЕКОТОРЫХ ТРАНСКРИПЦИОННЫХ ФАКТОРОВ, СВЯЗАННЫХ С ЗАСУХОУСТОЙЧИВОСТЬЮ ПШЕНИЦЫ

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Целью исследования было изучение полиморфизма предварительно отобранных локусов генов трех транскрипционных факторов (*TaNAC2a*, *TaWRKY2*, *TaWRKY19*) и протеин позднего эмбриогенеза (*LEA*) дегидрина (*Td29b*), связанных с устойчивостью пшеницы к засухе. Структура генов и хромосомная локализация определены с помощью биоинформационных подходов. Установлено, что гены *TaWRKY2* и *TaWRKY19* состоят из 4 экзонов и 3 интронов, 2BS и 1DS хромосомы, соответственно; *TaNAC2a* содержит 2 экзона и 1 интрон 7AS. Ген Td29b состоит из одного экзона 3AS. В результате использования полимеразной цепной реакции не было выявлено полиморфизма для локусов генов *TaNAC2a*, *TaWRKY19* и *Td29b* с помощью предварительно отобранных пар праймеров. Однако для локуса *TaWRKY2* обнаружены полиморфные фрагменты. Скрининг распространения полиморфных локусов проводили для набора сортов пшеницы и ржи, древних пшениц и межвидовых гибридов. Полиморфизм локуса *TaWRKY2* свидетельствует о наличии других аллелей этого гена. Эти данные важны для дальнейших исследований устойчивости пшеницы к засухе.

Ключевые слова: *Triticum* spp., полимеразная цепная реакция, факторы транскрипции, *TaNAC2a*, *TaWRKY2*, *TaWRKY19*, LEA, *Td29b*, засухоустойчивость.

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APPLICATION OF AERATION-OXIDATIVE JET-LOOPED SETUP FOR BIOLOGICAL WASTEWATER TREATMENT

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The purpose of the study was the design of an aerator-oxidizer and optimal parameters of its work to ensure the conditions of mixing and dissolution of oxygen, in which there is no disturbance of the state of activated sludge. The testing of the work of the aeration-oxidation setup of rotor type with the use of a sludge mixture with different design of aerator-oxidizers and in different operating modes was performed. The assessment of the effect of processing on the state of the activated sludge was carried out according to standard parameters: sludge index, chemical oxygen demand. In addition, the number and state of activated sludge organisms were evaluated. The results of qualitative and quantitative analysis of activated sludge before and after processing in the setup are presented. The parameters in which the activated sludge functions in satisfactory mode are revealed.

Key words: activated sludge, wastewater, aerator-oxidizer, sludge index.

The effectiveness of biological wastewater treatment depends on many factors: the dose of activated sludge, temperature, pH, daily wastewater consumption, etc. However, the most important and most energy-consuming is the degree of sewage saturation with oxygen. In the process of sewage aeration, pneumatic systems are widely used, which provide sufficient concentration of dissolved oxygen in the sludge mixture and its mixing. The search for ways to saturate the sludge mixture with air oxygen with reduced energy consumption remains an urgent problem [1, 2]. One of these methods is the use of hydromechanical systems of aeration. It is known that such aeration systems have lower specific energy expenditure compared with more commonly used pneumatic systems [3, 4]. Loop reactors with jet mixing are not widely used in biological treatment of sewage. However, it is known that such reactors create favorable conditions for the effective dissolution of oxygen in water. The induced air in the conditions of intense mixing and turbulent flows is dispersed in the form of microbubbles, greatly increasing the phase separation surface, which facilitates its dissolution [5]. However, the passage of sludge mixture through a centrifugal pump and high velocities of passage through pipelines can cause mechanical damage to microorganisms of activated sludge [6, 7]. In addition, the sedimentation properties of activated sludge deteriorate as a result of mechanical damage to the activated sludge, which complicates its further separation [8].

The purpose of the study is to search for the design of an aerator-oxidizer and rational parameters of its work to ensure the mild conditions of oxygen mixing and dissolution, in which there is no violation of the state of activated sludge.

The objectives of the study are:

– estimation of the stability of sludge mixture parameters at different setup modes and aerator-oxidizer design;

– determination of the efficiency of contaminants removing by the criteria of chemical oxygen demand (COD).

Materials and Methods

The research was carried out on the basis of the experimental aeration-oxidative jet-looped setup at the Institute of Engineering

Thermophysics of the National academy of sciences of Ukraine (Fig. 1). Oxygen saturation of the treated medium and its mixing takes place in the aerator-oxidizer, which is rotary-pulsating apparatus (RPA). The research was carried out using two aerator-oxidizers of different design.

The working volume of the first aerator-oxidizer is 0.0014 m^3. The main element of this device is the rotary-pulsating knot (RPK_1), which consists of two rotors, connected in a single rotor knot (RK), and stator. The rotors have the following design parameters: internal radius of a small rotor R_{sr} = 56 mm, of a large rotor R_{lr} = 65 mm; the dimensions of the slits a = 3 mm; height h_{sl} = 5 mm; angle between them is 6°; number m = 60. The gap between the rotor and the stator in the RPK is δ = 0.15 mm. Structural parameters of the stator are as follows: internal radius R_{st} = 61 mm; the dimensions of the slits a = 3 mm; height h_{sl} = 5 mm; angle between them is 6°; number m = 60.

The second type of aerator-oxidizer, with a volume of 0.0014 m^3, contains RPK_2 consisting of one rotor and a stator. The structural parameters of the rotor are: internal radius R_{int} = 40 mm; external radius R_{ext} = 70 mm. The rotor contains 12 round holes with a diameter d_h = 12 mm. Structural parameters of the stator: internal radius $R_{st.int}$ = 70 mm; external radius $R_{st.ext}$ = 75 mm. The number of holes is 20. The gap between the rotor and the stator is δ = 0.3 mm.

As a parameter characterizing the conditions of medium processing in the RPA and can serve for different structures of the RPA comparison, the rate of the flow shift was chosen, which is determined by the formula:

$$\nu = \frac{\omega \cdot R}{\mu}, s^{-1}, \quad (1)$$

where ω is the angular velocity of the rotor, s^{-1}; R is the radius of rotor, m; μ is the gap thickness between the cylinders, m.

In the work, the determination of the doses of activated sludge by dry substance a and volume V, and sludge index I were performed.

The dose of activated sludge was calculated by the formula:

$$a = \frac{(m_2 - m_1)}{v} \cdot 1000, \text{ g} / \text{dm}^3. \quad (2)$$

Determination of the dose of activated sludge by volume lie in gravity sedimentation of the sludge mixture (V_s = 200 cm^3) for 30 min with subsequent determination of the volume V_{sl}, which takes the sludge after sedimentation, and recalculated to 1 dm^3:

$$V = \frac{v_{sl}}{v_s} \cdot 1000, \text{ cm}^3 / \text{dm}^3 \quad (3)$$

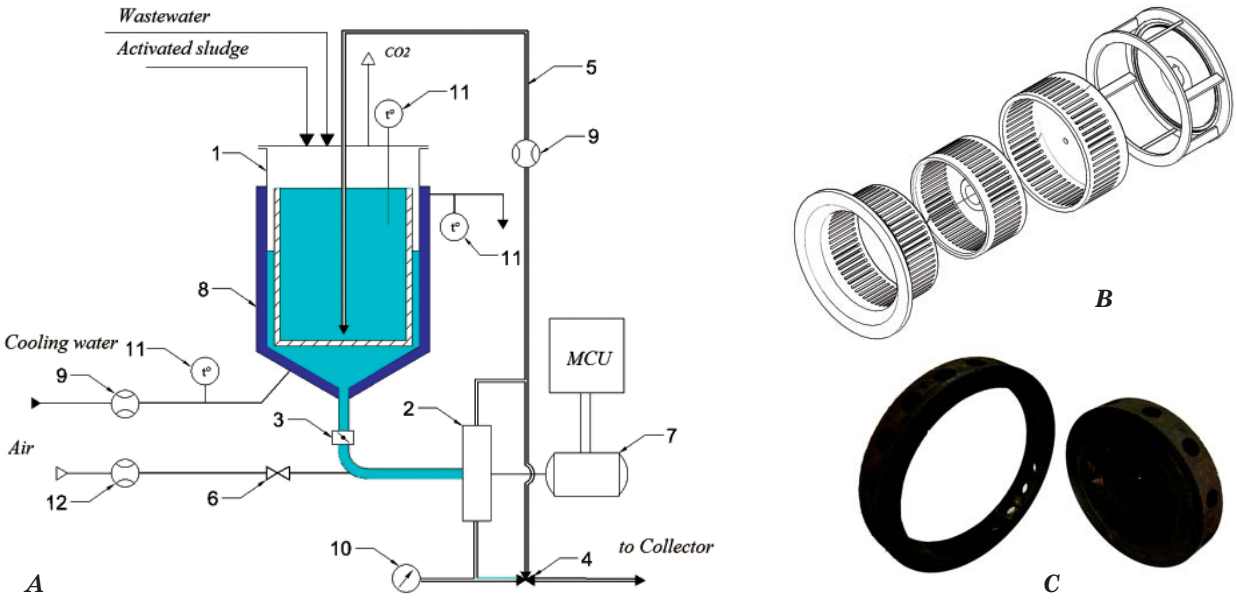


Fig. 1. **Aeration-oxidative jet-looped setup:**
A — the chart; *B* — RPK 1; *C* — RPK 2.
1 — storage capacity with internal cylinder; *2* — aerator-oxidizer; *3* — damper; *4* — three-way valve; *5* — recirculation pipeline; *6* — two-way valve; *7* — engine; *8* — cooling shirt; *9* — flow meter; *10* — manometer; *11* — thermometer; *12* — air flow meter; MCU is a management and control unit

In order to determine the dose of activated sludge a, the sludge mixture of volume V = 50 cm^3 was collected, which was filtered on a pre-dried and weighed paper filter "white tape" of mass m_1 and dried to constant weight m_2 at 105 °C for six hours.

The sludge index is defined as the ratio of activated sludge dose of volume V to its dose a of dry matter:

$$I = \frac{V}{a}, \text{ cm}^3 / \text{g} \qquad (4)$$

Investigation of activated sludge was carried out using a trinocular microscope of XSP-139TP Ulab model, equipped with an eyepiece with an increase of ×10 and lenses with magnifications of ×10, ×20 and ×40. In addition, the Carl Zeiss Axio Imager microscope was used.

To calculate the number of groups of activated sludge organisms, the method of "Calibrated drop" [9] was used. A sample of 150 cm^3 of sludge mixture was collected, 0.1 cm^3 of fluid was collected from it after preliminary mixing with a micropipette, placed on object glass and covered with 18×18 mm cover glass. Three such specimens were made. In each specimen, the number of organisms was counted in 10 fields of vision under a microscope with an increase of ×100. The number of organisms D in 1 cm^3 of sludge mixture was determined by the formula:

$$D = \frac{S \cdot d}{\pi \cdot r^2 \cdot \rho}, \text{ cell} / \text{cm}^3, \qquad (5)$$

where d is the number of organisms in one field of view (the arithmetic mean of the examined fields of view); r is radius of field of view in mm; S is the area of cover glass in mm^2; ρ is the volume of used liquid, cm^3.

Active sludge for research was taken from the sludge chamber after the secondary settling stations of Bortnitskaya aeration station in Kyiv. It represents an association of microorganisms such as bacteria, mushrooms, actinomycetes, diatoms, green microalgae, as well as protozoa and some multicellular animals (flagellates, sarcodes, infusoria, worms, rotifers, etc.). Microphotographs of activated sludge are shown in Fig. 2.

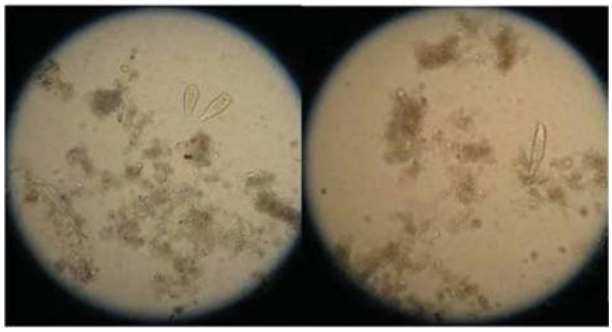


Fig. 2. **Microphotography of activated sludge with magnification of ×200**

The studies were conducted in two replicates. In both samples, the sludge is moderately-laden, no filamentous bacteria, but infusoria of the genera: *Paramecium, Vorticella, Epistylis, Euplotes;* rotifers of the genera*: Habrotrocha, Epipheres, Rotaria, Pleurotrocha* are present. In the second sample, the estimated number of organisms was determined, where the number of infusoria was 3 335 individuals per cm^3, and the number of rotifers — 205 individuals per cm^3. The both samples of activated sludge parameters are presented in Table 1. For further investigation of the purification effect by the COD parameter, a new sample of activated sludge was selected: the dose of sludge in volume is 880 cm^3/dm^3. Sludge index is 110 cm^3/g.

For a qualitative assessment of sewage pollution, a standard method for chemical oxygen demand determining for 2 hours was used — COD.

The volume of sludge mixture was 30 dm^3 with a sludge concentration of 2.5 g/dm^3, which was obtained by diluting the activated sludge with settled tap water (when evaluating the parameters of activated sludge in the first stage of the research) and with sewage (in determining the degree of sewage treatment) in the apparatus before the start of experiments.

Results and Discussion

Table 1 shows the initial parameters of activated sludge, which was selected for research conducting at Bortnitskaya aeration station.

The first 3 experiments were aimed at rational parameters of setup work and its design finding. The following two studies were conducted to assess the depth of sewage treatment by COD index.

The first study of the parameters of activated sludge in the aeration-oxidative jet-looped setup was carried out at a rate of flow shift of $112 \cdot 10^3$ s^{-1}, which is minimal for this apparatus. The temperature of the sludge mixture was 21.7 °C. The treatment time was 40 minutes. Sampling was performed every 10 minutes. An aerator-oxidizer with RPK_1 was used. The results are presented in Table 2 and in Fig. 3.

Table 1. **Parameters of activated sludge selected at Bortnitskaya aeration station**

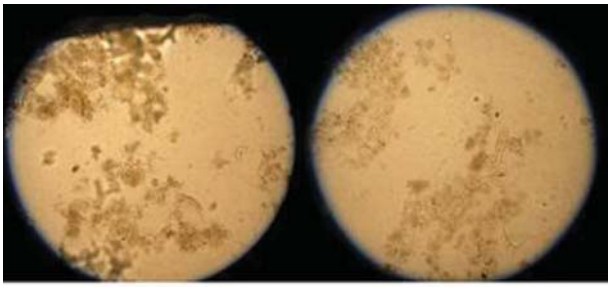
Samples №№	Dose of activated sludge, a, g/dm^3		Average dose of activated sludge, a_{av}, g/dm^3	Dose of activated sludge V, cm^3/dm^3		Average dose of activated sludge V_{av}, cm^3/dm^3	Sludge index, I, dm^3/g
	I	II		I	II		
1	8.28	8.46	8.37	925	935	930	111.11
2	7.3	8.54	7.92	950	960	955	120.58

Note: 1 and 2 are successive samples; I and II are parallel samples.

Table 2. **Parameters of activated sludge after processing with a flow shift rate of 112·10^3 s^{-1}**

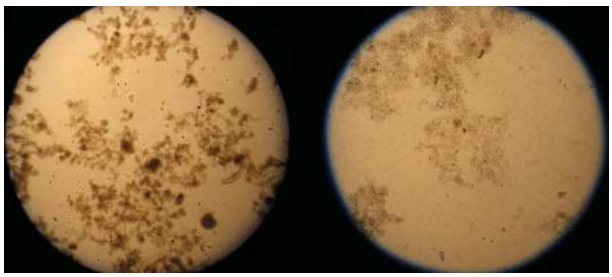
Samples №№	Dose of activated sludge, a, g/dm^3		Average dose of activated sludge, a_{av}, g/dm$_3$	Dose of activated sludge V, cm^3/dm^3		Average dose of activated sludge V_{av}, cm^3/dm^3	Sludge index, I, dm^3/g
	I	II		I	II		
C	0.8	0.74	0.77	60	65	62.5	81.17
4	0.68	0.74	0.71	60	65	62.5	88.02

Note: C — control, 4 — sample taken after 40 min of setup working; I and II are repeated experiments.



A *B*

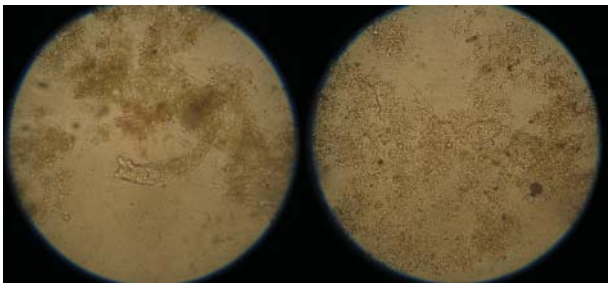
Fig. 3. **Microphotography of activated sludge with magnification of ×200:**
A — control; *B* — the 4th sample



A *B*

Fig. 4. **Microphotography of activated sludge with magnification of ×100:**
A — control; *B* — the 4th sample

The second study of activated sludge in setup was carried out at a rate of flow shift of 140·10^3 s^{-1}. The temperature of the sludge mixture was 21.7 °C. An aerator-oxidizer with RPK$_1$ was used. Processing time was 40 minutes. Sampling was performed every 10 minutes. The results are presented in Table 3, in Fig. 4 and Fig. 5.



A *B*

Fig. 5. **Microphotography of activated sludge with magnification of ×400:**
A — control; *B* — the 4th sample

The third study of activated sludge in the setup was carried out at a rate of flow shift of 70·10^3 s^{-1}. The temperature of sludge mixture was 21.7 °C. An aerator-oxidizer with RPK$_2$ was used. Processing time was 28 minutes. The results are presented in Fig. 6 and Fig. 7.

The following studies were conducted using an aerator-oxidizer with RPK$_2$.

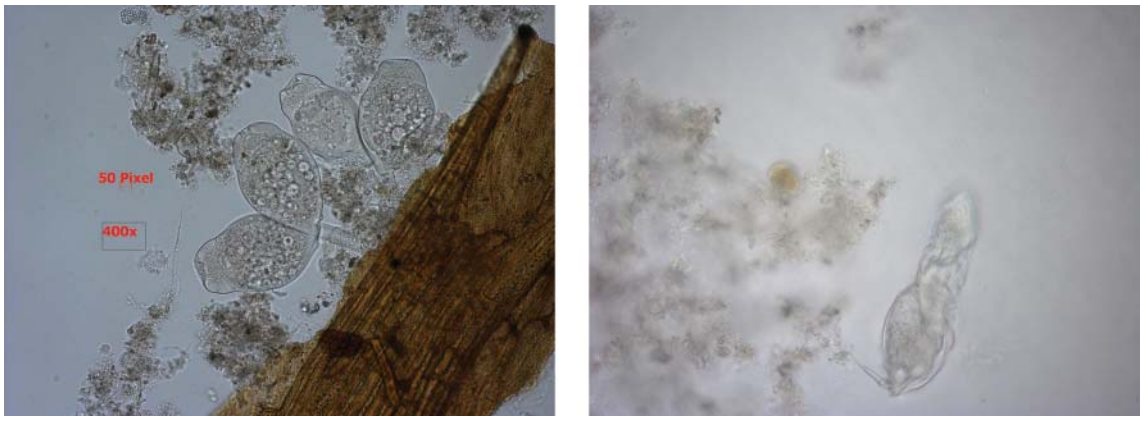
The fourth biological wastewater treatment experiment was conducted at a rate of flow shift of 70·10^3 s^{-1}. The temperature of the sludge mixture was 24 °C. Processing time was 4 hours. Sampling was carried out before the study, at the 2nd hour, at the 3rd hour and at its completion. The results are presented in Table 4.

The fifth experiment on biological wastewater treatment was carried out with a reduced rate of flow shift — 56·10^3 s^{-1}. The temperature of the sludge mixture was 24 °C. Processing time was 4 hours. Sampling was carried out before the study, at the 2nd hour, at the 3rd hour and at its completion. The results are presented in Table 4.

Table 3. **Parameters of activated sludge after processing with the rate of flow shift of $70 \cdot 10^3$ s^{-1}**

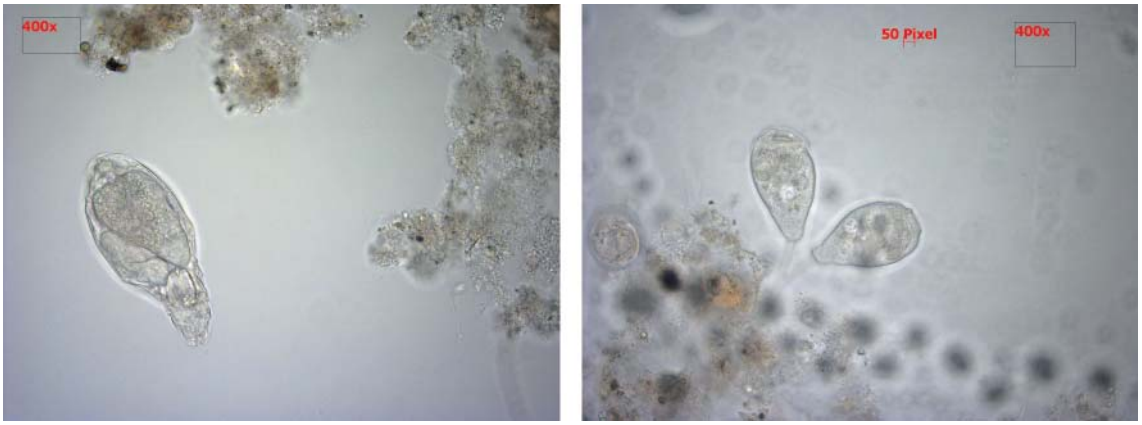
Samples №№	Average dose of activated sludge V_{av}, cm^3/dm^3	Average dose of activated sludge, a_{av}, g/dm^3	Sludge index, I, dm^3/g	Time from the start of the setup working t, min
C	82.5	0.64	128.91	0
1	90	0.61	147.54	10
2	90	0.56	160.71	20
3	90	0.52	173.08	30
4	77.5	0.47	164.89	40

Note: see note to the Table 2.



A *B*

Fig. 6. **Microphotography of activated sludge with magnification of ×400:**
A — control; *B* is a sample



A *B*

Fig. 7. **Microphotography of activated sludge with increase of ×400:**
A — control; *B* is a sample

Studies performed using an aerator-oxidizer with RPK_1, demonstrate the constancy of activated sludge parameters at a minimum angular rate of setup.

During the study of activated sludge at maximal angular rate, the destruction of activated sludge flakes and the death of eukaryotes were detected according to the results of microscopy (Fig. 5, *B* and Fig. 6, *B*). Significant growth of sludge index was noticed (Table 3).

Thus, in the third study with the usage of aerator- oxidizer with RPK_2, it was established that the parameters of activated sludge at maximum angular rate of the setup were unchanged.

Table 4. **Effect of sewage treatment in the setup**

Experiment	Sample №№	Processing duration, *t*, h	COD, mg O_2/dm^3
I	control	0	200
	2	1	–
	3	2	160
	4	3	220
	5	4	240
II	control	0	113
	2	1	180
	3	2	167
	4	3	167
	5	4	–

During the fourth study, the crushing of activated sludge flakes starting from the first hour of the experiment was observed. At the 3rd hour of the experiment, there was a complete destruction of activated sludge flakes and the death of infusoria and rotifers — the remnants of the outer shells of these organisms are present. Further destruction of particles in the sludge mixture has led to an increase of pollution by the index of COD.

During the fifth study, the sludge retained viability throughout the experiment, but still there was a crushing of activated sludge from the first hour of treatment, which also led to the re-contamination of sewage.

It should be noted that secondary contamination of sewage apparently was the result of a fairly harsh conditions of the experiment. Thus, when the rate of flow shift was $70 \cdot 10^3$ s^{-1} and the volume of sludge mixture was 30 dm^3, the frequency of its passing through the slotted holes of RPK aerator-oxidizer was 240 times per hour, or four times per minute, that does not meet the actual processing on treatment facilities.

It is found that using the second option of aerator-oxidizer provides soft mixing and aeration mode of the mixture that does not lead to the destruction of sludge and significant changes in its parameters (sludge index, sludge flakes integrity, protozoa and multicellular animals by samples microscopy). Further research will be directed to study the dynamics of wastewater biological treatment, removal efficiency of organic pollution and rational parameters of the purification process finding.

REFERENCES

1. *Sablii L. A., Bunchak O. M., Zhukova V. S., Rossinskyi V. M.* Equipment and engineering in bioenergy and water treatment and safety management. *Rivne. National University of Water and Environmental Engineering.* 2016, 356 p. (In Ukrainian).
2. *Sablii L. A.* Physical, chemical and biological treatment of highly concentrated waste water. *Rivne: National University of Water and Environmental Engineering.* 2013, 291 p. (In Ukrainian).
3. *Bloor J. C., Anderson G. K., Willey A. R.* High rate aerobic treatment of brewery wastewater using the jet loop reactor. *Wat. Res.* 1995, 29 (5), 1217–1223.
4. *Abdel-Aziz M. H., Amin N. K., El-Ashtoukhy E. S. Z.* Removal of heavy metals from aqueous solutions by liquid cation exchanger in a jet loop contactor. *Hydrometallurgy*. 2013, 137, 126–132.
5. *Petruccioli M., Cardoso Duarte J., Eusebio A., Federici F.* Aerobic treatment of winery wastewater using a jet-loop activated sludge reactor. *Proc. Biochem.* 2002, 37 (8), 821–829.
6. *Park B., Hwang G., Haam S., Lee Ch., Ahn Ik-Sung, Lee K.* Absorption of a volatile organic compound by a jet loop reactor with circulation of a surfactant solution: performance evaluation. *J. Hazard. Mater.,* 2008, 153 (1–2), 735–741.
7. *Eusébio A., Mateus M., Baeta-Hall L., Saagua M. C., Tenreiro N., Almeida-Vara E., Duarte J. C.* Characterization of the microbial communities in jet-loop (jacto) reactors during aerobic olive oil wastewater treatment. *Intern. Biodeterior. Biodegrad.* 2007, 59 (3), 226–233.
8. *Patil M. S., Usmani G. A.* Laboratory scale study of activated sludge process in jet loop reactor for waste watertreatment. *J. Engineer. Res. Applicat.* 2014, 4 (5), 68–74.
9. *Kutikova L. A.* Fauna of aerotanks. *Sankt-Peterburg: Nauka.* 1984, 264 p. (In Russian).

ВИКОРИСТАННЯ АЕРАЦІЙНО-ОКИСНЮВАЛЬНОЇ УСТАНОВКИ РОТОРНОГО ТИПУ ДЛЯ БІОЛОГІЧНОГО ОЧИЩЕННЯ СТІЧНИХ ВОД

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Метою дослідження був пошук конструкції аератора-окиснювача та оптимальних параметрів його роботи для забезпечення умов перемішування та розчинення кисню, за яких не відбуватиметься порушення стану активного мулу.

Проведено випробування роботи аераційно-окиснювальної установки роторного типу з використанням мулової суміші з різними конструкціями аераторів-окиснювачів і за різними режимами роботи. Оцінювання впливу обробки на стан активного мулу здійснено за стандартними показниками: муловий індекс, хімічне споживання кисню. Також оцінено кількість і стан організмів активного мулу. Наведено результати якісного й кількісного аналізів активного мулу до та після обробки в установці. Виявлено параметри, за яких активний мул функціонує в задовільному режимі.

Ключові слова: активний мул, стічні води, аератор-окиснювач, муловий індекс.

ИСПОЛЬЗОВАНИЕ АЭРАЦИОННО-ОКИСЛИТЕЛЬНОЙ УСТАНОВКИ РОТОРНОГО ТИПА ДЛЯ БИОЛОГИЧЕСКОЙ ОЧИСТКИ СТОЧНЫХ ВОД

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Целью исследования был поиск конструкции аэратора-окислителя и оптимальных параметров его работы для обеспечения условий перемешивания и растворения кислорода, при которых не будет происходить нарушение состояния активного ила.

Проведены испытания работы аэрационно-окислительной установки роторного типа с использованием иловой смеси с различными конструкциями аэраторов-окислителей и в разных режимах работы. Оценку влияния обработки на состояние активного ила осуществляли по стандартным показателям: иловый индекс, химическое потребление кислорода. Также оценивали количество и состояние организмов активного ила. Приведены результаты качественного и количественного анализов активного ила до и после обработки в установке. Выявлены параметры, при которых активный ил функционирует в удовлетворительном режиме.

Ключевые слова: активный ил, сточные воды, аэратор-окислитель, иловый индекс.

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NECESSITY OF TRANSLOCATION DOMAIN FOR REALISATION OF CYTOSTATIC EFFECT OF NON-TOXIC DERIVATIVES OF DIPHTHERIA TOXIN

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The aim of the work was to evaluate *in vitro* the cytostatic effect of recombinant fragments of the non-toxic point mutant of diphtheria toxin — CRM197, which was suggested as a potent medication for treatment of triple negative breast cancer. For this purpose, non-toxic recombinant derivatives of diphtheria toxin — CRM197, subunit B SbB and receptor domain Rd had been isolated by Ni-NTA agarose affinity chromatography and their effect on the growth of individual colonies of triple negative breast cancer MDA–MB–231 cells were characterized by such parameters as average colony area, perimeter and circularity index. According to the obtained results, CRM197 and SbB, whose molecules contain the translocation domain Td, exhibited the same cytostatic effect against MDA–MB–231 cells, reducing the area and perimeter of individual colonies. Rd protein did not affect the last two parameters that characterize the size of the colonies, but changed the form of the margin of colonies, as evidenced by an increase in the circularity index.

It is supposed that Td may be involved in the implementation of cytostatic action due to its inherent pore-forming activity in relation to lipid membranes. It is concluded that Rd and Td, unlike the catalytic domain of diphtheria toxin, play important roles in the implementation of the cytotoxic properties of CRM197, while SbB consisting of Rd and Td is the structural DT fragment of smallest molecular weight that can be used as the analog of CRM197.

Key words: CRM197, diphtheria toxin, HB-EGF, toxoid, triple negative breast cancer.

CRM197 protein (cross-reacting material 197) is a non-toxic point mutant of diphtheria toxin (DT) produced by *C7 Corynebacterium diphtheriae* strain which was lysogenically transformed by a mutated corynephage β, containing the altered tox^+ gene. Mutated phage named $\beta 197^{tox-}$ was obtained by nitrosoguanidine treatment of toxigenic C7(β) *C. diphtheriae*, containing prophage β, during the lytic phase induced by UV-light exposure. Then *C7(–) C. diphtheriae* cells were transformed by the resulted phage mutants and obtained lysogenic clones were tested for production of non-toxigenic material that was cross-reacting with anti-DT polyclonal antibodies [1]. Clone 197 which produced crossreacting with DT protein, was found to synthesize a DT polypeptide chain with a single Gly52Glu mutation [2] which leads to an almost complete loss of the catalytic activity of A-subunit of DT. Apart from this single mutation, protein CRM197 structure is absolutely the same as that of the native toxin: its molecule contains subunit A with a non-active catalytic or C-domain and subunit B which comprises functional and entire receptor or R-domain (Rd) and translocation or T-domain (Td).

It should be noted that the ability of native DT to inhibit the growth of malignant cells in resistant to toxin mice has been already known for a relatively long time [3]. The non-toxic to DT-sensitive species, CRM197 turned out to be a promising agent in applying to humans. It has been demonstrated that this toxoid effectively inhibits the growth of human malignant cells *in vivo* in nude mice model [4–6] and increases survival of patients with progressive cancer [7–9]. There are a lot of evidence that CRM197 is effective in suppressing the cancer of breast [6, 10, 11], oral cavity [12], stomach [13], immune cells [14] and ovaries [4, 5].

Nowadays, CRM197 is already undergoing clinical trials for introduction in the therapeutic practice of human cancer treatment [8]. Production of CRM197 in *Escherichia coli* greatly facilitated the large-scale manufacturing of this protein [15–17].

However, as a medicine for intraperitoneal administration, CRM197 possess essential disadvantages, as it preserves almost the full immunogenicity of the native DT. This means, that after the first few administrations, like native DT, toxoid CRM197 will provoke a strong immune response and its molecules will be eliminated fast from the bloodstream. Besides, some parts of the CRM197 molecule may be functionally superfluous, as they do not participate in the implementation of its cytostatic effect and may have potential side effects. We suppose that subunit A of CRM197 may be of a low necessity in the realization of its anticancer properties.

Recombinant fragments of CRM197 with smaller molecular weight can be less immunogenic than full molecule. Besides, these fragments could be constructed to contain only functionally beneficial structural parts. The anticancer potentials of different structural parts of the CRM197 molecule have not yet been studied. The aim of the present work was to evaluate the cytostatic effect of recombinant non-toxic fragments of DT molecule for finding a compound of a smaller molecular size, which preserve the most of the anticancer properties of entire CRM197.

The effect of CRM197 on tumors is implemented by the interaction of this protein with growth factor HB-EGF. It was demonstrated that HB-EGF is often overexpressed in the transformed cells and that soluble form of HB-EGF (sHB-EGF) promotes the development of a malignant phenotype. Today, the gene of HB-EGF is considered to be strongly responsible for chemotherapy resistance [18]. Production of HB-EGF promotes cell surveillance and development of signs of oncogenic transformation. It is generally accepted that treatment with CRM197 leads to decrease in cell proliferation, because when sHB-EGF is bound to CRM197, it is unable to interact with its natural cell receptor EGFR [13,18,19].

There are some findings that sHB-EGF and its transmembrane precursor can be especially important targets for CRM197 in breast cancer therapy [10]. In the case of triple-negative breast cancer, there is no increase in expression of estrogen, progesterone and HER2 receptors in cells, which are targeted by conventional hormonal therapy (such as tamoxifen or aromatase inhibitors) or therapies that target HER2 receptors (Herceptin). However, there is an evidence that triple-negative breast cancer can be effectively suppressed by CRM197 [6, 11, 19]. HB-EGF-targeted therapy for triple negative breast cancer is of a high relevance, as it can sufficiently increase surveillance in patients with this type of oncology. Thus, in the present study, it was decided to analyze the effects of DT toxoids in relation to triple negative MDA-MB-231 cell line, derived from the human organism.

Previously in the department of molecular immunology at Palladin Institute of Biochemistry of the NAS of Ukraine was created a set of *pET24(a)*+-based plasmid genetic constructions for production of different non-toxic structural DT derivatives in *BL21 Rosetta* (*DE3*) *E. coli*: CRM197 – the product of site-specific mutagenesis of native tox^+ gene from PW8 *C. diphtheriae* strain [20]; SbB – subunit B of DT which contain the entire Td and Rd of DT [21]; Rd – entire receptor domain of toxin possessing no other structural parts. All of the above listed recombinant DT toxoids retain the ability to bind proHB-EGF on the surface of mammalian cells [22–25] and have been used in present work to evaluate the cytostatic effect of toxin recombinant derivatives produced and purified from *E. coli* on malignant cells.

Materials and Methods

Materials and reagents. In present work there were used: acrylamide, ammonium persulfate, N,N,N′,N′-tetramethylethylenediamine, N,N′-methylenebisacrylamide, phenylmethylsulfonyl fluoride (AppliChem GmbH, Germany); chloramphenicol, kanamycin (Arterium Co., Ukraine); eukaryotic cell culture Petri dishes (Greiner Bio One, Great Britain); centrifugal filters with 10 kDa nominal molecular weight limit (Merk, Germany); KCl, KH_2PO_4, NaCl, Na_2HPO_4, NaH_2PO_4, NaOH, β–mercaptoethanole (β–ME) ("Miranda-C", Ukraine); crystal violet, imidazol, sodium deoxycholate (Shanghai Synnad, China); amphotericin B, foetal bovine serum (FBS), lyzozyme from chicken egg, paraformaldehyde, penicillin G, RPMI-1640 media, streptomycin, LB media, tricine, tris-hydroxymethyl aminomethane, Triton X-100, urea (Sigma Aldrich, USA); human recombinant DNAse I, isopropyl β-D-1-thiogalactopyranoside, molecular weight markers for protein gel electrophoresis nickel-

nitrilotriacetic acid agarose (Ni-NTA) (Thermo Fisher Scientific, USA).

Production of CRM197, SbB and Td in E. coli cells. Creation of the *pET24a(+)*-based genetic constructs for expression of CRM197 and SbB was described in [20,21]. Creation of *pET24a(+)-Rd* construct for expression of Rd was carried out as described in [20]. *E. coli BL21 Rosetta (DE3)* cells containing the appropriate expression vectors were cultivated on LB media with kanamycin and chloramphenicol at 37 °C under rotation (250 rpm). Expression was induced by isopropyl β-D-1-thiogalactopyranoside and conducted at 30°C under rotation (250 rpm).

Bacterial peptidoglycan cell walls were digested by addition of 10 mg/ml lysozyme to bacteria suspension in 50 mM TrisHCl, 1 mM EDTA, 100 mM NaCl, 0.01 M phenylmethylsulfonyl fluoride, pH = 8.0 at 4 °C. Then concentrations of $MgCl_2$ and $CaCl_2$ in resulted suspensions were brought to 25 mM and 55 mM respectively, and protoplast membranes were destroyed by addition of 40 mg/ml of sodium deoxycholate. Bacterial DNA was digested by DNAse I at room temperature until suspension become non-viscous. Then the insoluble fraction of cell lysate containing inclusion bodies was collected by centrifugation and washed by resuspension in the initial cell solubilizing buffer solution containing 0,5% Triton X-100.

Proteins CRM197, SbB and Rd were extracted from the water-insoluble fraction of bacterial cell lysate by 8 M urea in 250 mM NaH_2PO_4, 2.5 M NaCl, 10 mM imidazole and 10 mM β-ME, pH = 8.0. Purification by imidazole elution gradient and refolding by decreasing urea and β-ME concentration were carried out on Ni-NTA agarose. Eluted samples were dialyzed against phosphate buffered saline (PBS), 0.01 M Na_2HPO_4, 0.002 M KH_2PO_4, 0.137 M NaCl, 0.003 M KCl, pH 7.4, and concentrated. The purity and concentration of the target proteins in resulted samples were estimated on tricine SDS-PAGE [26] electrophoregrams by gel densitometry using Fiji software.

Cultivation of cancer cells. MDA-MB-231 cells were obtained from the Bank of cell lines from human and animal tissues of Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of the National Academy of Sciences of Ukraine. Cells were maintained at 37 °C and under 5% CO_2 on RPMI-1640 media with 10% FBS, 0.3 g/l Lglutamine, and antibiotics: 100 mg/l streptomycin 10 000 U/l penicillin G and 250 mg/l amphotericin B.

Colony assay. To obtain individual colonies, 5.5×10^3 of MDA-MB-231 cells were seeded on plastic 60 mm Petri dishes on RPMI-1640 with 10% FBS. Next day, when individual cells were already attached to the plastic surface, the medium was changed to RPMI-1640 with 5% FBS and 0.254×10^{-6} M of a recombinant DT derivative of interest. Equal volumes of filtrates from the same protein samples, obtained by gravity concentration on the semi-permeable membrane, were used as controls. The absence of protein in the obtained controls was also demonstrated by SDS-PAGE. Cultivation in presence of DT recombinant derivatives was carried out during 7 days until colonies visible to the naked eye were formed. The medium was changed to fresh every 2–3 days. After 7 days, cells were fixed by 5% of paraformaldehyde in PBS for 40 min at room temperature and stained with 0.2% crystal violet solution for 3 h at 37 °C under rotation (60 rpm). Excess of crystal violet dye was removed by washing 3 times in deionized H_2O under 37 °C and rotation (60 rpm). Petri dishes were dried and scanned at 720 dpi, 48-bit color (Fig. 2). Size, perimeter and circularity of colonies on resulted pictures were analyzed by Fiji software.

Area of colonies was calculated in pixels, perimeter — in arbitrary units. Shape descriptor of Fiji, that calculates object circularity, uses the formula:

$$Circularity = 4*pi*(Area/Perimeter\hat{}2),$$

that allows differentiating objects with same values of area and perimeter, but with different shapes. A circularity value of 1.0 indicates a perfect circle. A circularity value of 1.0 indicates a perfect circle. As the value approaches 0.0, it indicates an increasingly elongated polygon.

Statistical data analysis. The mean values were calculated on the basis of 3 independent experiments in each case. Error bars represent the standard deviations or respective mean values. The *t*-test for experimental and control groups with a significance level of 0.05 was carried by Origin9 software.

Results and Discussion

Characterisation of resulted protein samples by gel electrophoresis. After the lysis of *E. coli* cells, proteins CRM197, SbB and Rd were found in the insoluble fraction of the lysate, which suggest the accumulation of studied proteins in inclusion bodies. The effectiveness of *in vitro* renaturation (refolding, [22–28]) of fluorescently labeled diphtheria toxoids after urea extraction from the inactive water-

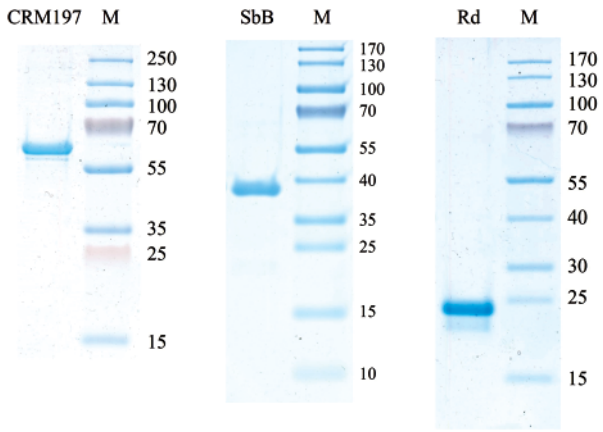

Fig. 1. **SDSPAGE electrophoregram of CRM197, SbB and Rd samples:**
M — molecular weight markers

insoluble state was demonstrated previously in [22–25]. SDS-PAGE showed the high purity and concentration of targeted proteins in resulted samples (Fig. 1).

The growth of single MDA-MB-231 colonies in presence of DT recombinant derivatives. Previously, it has been shown that recombinant SbB at a concentration of 1.28×10^{-6} M exhibits a sufficient cytotoxic effect on human histiocytic lymphoma cell line U937, which expresses a large amount of sHB-EGF [26–29]. In the present study, we decided to compare cytotoxicity of different DT derivatives which retain the receptor-binding ability in relation to HB-EGF. It was shown that presence of 0.254×10^{-6} M of recombinant CRM197, SbB and Rd in the culture media allow the single MDA-MB-231 cells to grow into colonies. Studied DT derivatives affected size and shape of MDA-MB-231 colonies arising from the single cells on the flat surface.

It was found that toxin derivatives which contain Td (CRM197 and SbB) are more effective in inhibiting the growth of MDAMB231 colonies than toxin derivatives which do not contain Td (Rd). Parameter of colony area which characterize the growth of cancer cells, reduced significantly compared to controls in the presence of proteins CRM197 and SbB (Fig. 3). The perimeter changes were generally in accordance with the area of the colonies. Compared to each other, CRM197 and SbB have approximately the same inhibitory effect on colony area and perimeter. In relation to controls, Rd did not affect the size and perimeter of the resulted MDA-MB-231 colonies. Nevertheless, Rd has still exerted a significant influence on cells which resulted in the change of the colony shape.

To characterize the shape changes under the influence of recombinant DT derivatives, we use the parameter of circularity. Circularity — is a value that shows how the shape of a particular object approaches the shape of an ideal circle. In the case of colonies of eukaryotic cells, circularity characterizes the asymmetry and boarder of colonies which possibly may reflect the intercellular organization and interactions on the surface in 2D. Recombinant Rd significantly increased the circularity of growing MDA-MB-231 colonies.

Decrease in circularity of colonies can be a sign of a malignant phenotype since more disorganized cells on the surface should also have the increased ability to migrate and reduced capacity to form and maintain the intercellular contacts. It is naturally to assume that cells, forming colonies with a smaller circularity, can exhibit an increased ability of metastic invasion *in vivo*. Thus, it was decided that the increase in the circularity of MDA-MB-231 colonies in 2D culture in presence of Rd may be an indicator of reduced expression of malignancy in Rd-treated cells.

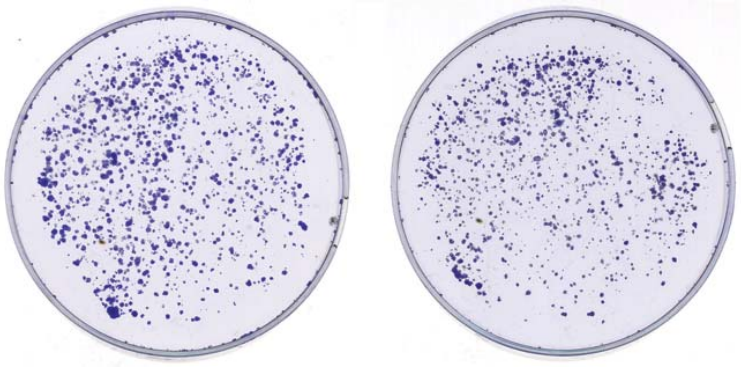

Fig. 2. **MDAMB231 colonies, obtained from the single cells attached to the plastic surface and formed under:**
the presence of 10 mkg/ml (0.254 mkM) of SbB (right) and the equal volume of PBS (left) after 7 days of culturing. Fixation with paraformaldehyde and crystal violet staining

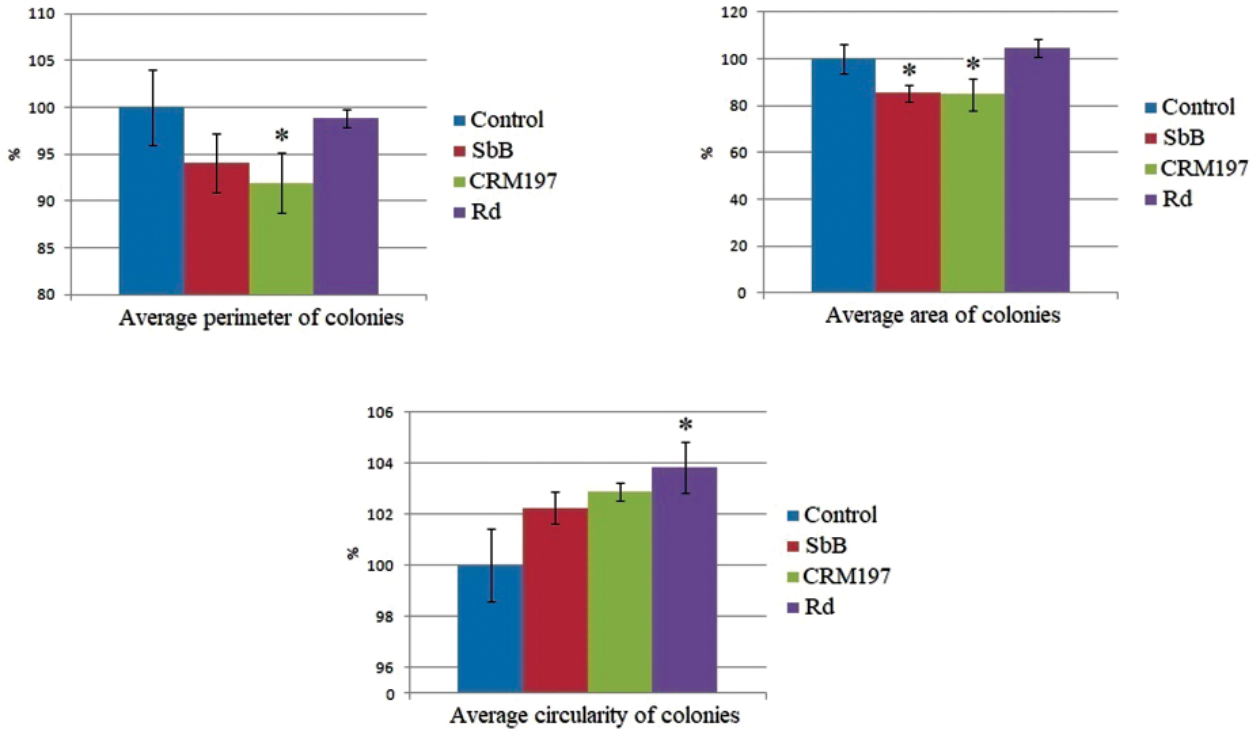

Fig. 3. **The average size, perimeter and circularity of MDA-MB-231 colonies formed under:** the influence of equimolar concentrations of CRM197, SbB and Rd. Asterisks indicate when the *p*-values of *t*-test for control and experimental groups are less than 0.05

The most common hypothesis about the CRM197 antitumor effect is that this toxoid blocks the ability of sHB-EGF to bind its natural receptor EGFR. Besides, the affinity of the CRM197–HB-EGF interaction is of a relatively high value, which makes the mentioned protein complex stable from the reverse dissociation [10,13]. Inactivated by a toxoid, sHB-EGF is removed from the intercellular space, which leads to a weakening of the sHB-EGF-dependent paracrine and autocrine stimulation of cell proliferation. The role of the CRM197 interaction with the transmembrane proHB-EGF in the realization of its cytostatic effect has not been sufficiently investigated.

According to our results, it is suggested that Td is sufficiently involved in the realization of the total cytostatic effect of CRM197. The influence of Td on cell proliferation may suggest that recombinant DT derivatives can affect cells by their Td due to the inherent ion-conductive and pore-forming properties of this domain regarding lipid membranes [30, 31]. The data on Td-influence on cell growth which was obtained in present work is in a good agreement with the previous our data on the characterization of ion-conductive properties of CRM197 and SbB [32, 33], which strongly suggest that function of Td in *E. coli*-derived and *in vitro* renaturated recombinant products is well reproduced.

Td pores are permeable to K^+, Na^+ and H^+ [34]. The ionic conductivity caused by Td may interfere with the balance of ions and protons between cytoplasm and endosome lumen [24, 25]. As a result, enzymes that require highly acidic environment are prevented from the activation, endosome maturation slows down [25] and degradative endocytic pathway in cells becomes disrupted. The greater amount of proHB-EGF receptor is located on the surface of cancer cells, the more endosome pathway of cells is affected. Disorder in general physiological cellular condition due to the influence of Td may ultimately result in proliferation slowdown.

Thus, our results support the idea that CRM197 and other DT toxoids can realize their tumor suppressive effects by two interdependent mechanisms: 1. sHB-EGF-dependent, when cells affected indirectly by depletion of proliferative soluble signal in intercellular space; and 2. proHB-EGF-dependent, when cells affected directly by functional pore-forming Td of DT.

Obtained results suggest that non-active subunit A of CRM197 is not required for

cytostatic action of this toxoid and Rd alone is not so effective in suppression of cancer cell proliferation as DT fragments that contain the Td. Thus, we recommend the use of SbB produced by *E. coli* for cancer therapy, as the closest functional analog of CRM197 that contains no functionally superfluous structural parts with potential side effects.

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REFERENCES

1. *Uchida T., Pappenheimer A. M., Greany R.* Diphtheria toxin and related proteins. I. Isolation and properties of mutant proteins serologically related to diphtheria toxin. *J. Biol. Chem.* 1973, 248 (11), 3838–3844.
2. *Giannini G., Rappuoli R., Ratti G.* The amino-acid sequence of two non-toxic mutants of diphtheria toxin: CRM45 and CRM197. *Nucleic Acids Res.* 1984, 12 (10), 4063–4069.
3. *Buzzi S., Maistrello I.* Inhibition of growth of Erlich tumors in Swiss mice by diphtheria toxin. *Cancer Res.* 1973, 33 (10), 2349–2353.
4. *Tang X.-H., Deng S., Li M., Lu M.-S.* Cross-reacting material 197 reverses the resistance to paclitaxel in paclitaxel-resistant human ovarian cancer. *Tumour Biol.* 2016, 37 (4), 5521–5528. doi: 10.1007/s13277-015-4412-0.
5. *Yagi H., Yotsumoto F., Sonoda K., Kuroki M., Mekada E., Miyamoto S.* Synergistic anti-tumor effect of paclitaxel with CRM197, an inhibitor of HB-EGF, in ovarian cancer. *Int. J. Cancer.* 2009, 124 (6), 1429–1439. doi: 10.1002/ijc.24031.
6. *Nam S.O., Yotsumoto F., Miyata K., Fukagawa S., Odawara T., Manabe S., Ishikawa T., Kuroki M., Yasunaga S., Miyamoto S.* Anti-tumor Effect of Intravenous Administration of CRM197 for Triple-negative Breast Cancer Therapy. *Anticancer Res.* 2016, 36 (7), 3651–3657.
7. *Buzzi S., Rubboli D., Buzzi G., Buzzi A. M., Morisi C., Pironi F.* CRM197 (nontoxic diphtheria toxin): effects on advanced cancer patients. *Cancer Immunol. Immunother. CII.* 2004, 53 (11), 1041–1048. doi: 10.1007/s00262-004-0546-4.
8. *Nam S. O., Yotsumoto F., Miyata K., Suzaki Y., Yagi H., Odawara T., Manabe S., Ishikawa T., Kuroki M., Mekada E., Miyamoto S.* Pre-clinical Study of BK-UM, a Novel Inhibitor of HB-EGF, for Ovarian Cancer Therapy. *Anticancer Res.* 2014, 34 (8), 4615–4620.
9. *Tsujioka H., Fukami T., Yotsumoto F., Ueda T., Hikita S., Takahashi Y., Kondo H., Kuroki M., Miyamoto S.* A possible clinical adaptation of CRM197 in combination with conventional chemotherapeutic agents for ovarian cancer. *Anticancer Res.* 2011, 31 (7), 2461–2465.
10. *Lian C., Ruan L., Shang D., Wu Y., Lu Y., Lü P., Yang Y., Wei Y., Dong X., Ren D., Chen K., Liu H., Tu Z.* Heparin-Binding Epidermal Growth Factor-Like Growth Factor as a Potent Target for Breast Cancer Therapy. *Cancer Biother. Radiopharm.* 2016, 31 (3), 85–90. doi: 10.1089/cbr.2015.1956.
11. *Yotsumoto F., Oki E., Tokunaga E., Maehara Y., Kuroki M., Miyamoto S.* HB-EGF orchestrates the complex signals involved in triple-negative and trastuzumab-resistant breast cancer. *Int. J. Cancer.* 2010, 127 (11), 2707–2717. doi: 10.1002/ijc.25472.
12. *Dateoka S., Ohnishi Y., Kakudo K.* Effects of CRM197, a specific inhibitor of HB-EGF, in oral cancer. *Med. Mol. Morphol.* 2012, 45 (2), 91–97. doi: 10.1007/s00795-011-0543-6.
13. *Sanui A., Yotsumoto F., Tsujioka H., Fukami T., Horiuchi S., Shirota K., Yoshizato T., Kawarabayashi T., Kuroki M., Miyamoto S.* HB-EGF inhibition in combination with various anticancer agents enhances its antitumor effects in gastric cancer. *Anticancer Res.* 2010, 30 (8), 3143–3149.
14. *Kunami N., Yotsumoto F., Ishitsuka K., Fukami T., Odawara T., Manabe S., Ishikawa T., Tamura K., Kuroki M., Miyamoto S.* Antitumor effects of CRM197, a specific inhibitor of HB-EGF, in T-cell acute lymphoblastic leukemia. *Anticancer Res.* 2011, 31 (7), 2483–2488.
15. *Mahamad P., Boonchird C., Panbangred W.* High level accumulation of soluble diphtheria toxin mutant (CRM197) with co-expression of chaperones in recombinant Escherichia coli. *Appl. Microbiol. Biotechnol.* 2016, 100 (14), 6319–6330. doi: 10.1007/s00253-016-7453-4.

16. *Dukhovlinov I. V., Fedorova E. A., Bogomolova E. G., Dobrovolskaya O. A., Chernyaeva E. N., Al-Shekhadat R. I., Simbirtsev A. S.* Production of recombinant protein CRM197 in Escherichia coli. *Russ. J. Infect. Immun.* 2015, 5 (1), 37. doi: 10.15789/2220-7619-2015-1-37-44.
17. *Stefan A., Conti M., Rubboli D., Ravagli L., Presta E., Hochkoeppler A.* Overexpression and purification of the recombinant diphtheria toxin variant CRM197 in Escherichia coli. *J. Biotechnol.* 2011, 156 (4), 245–252. doi: 10.1016/j.jbiotec.2011.08.024.
18. *Wang F., Liu R., Lee S. W., Sloss C. M., Couget J., Cusack J. C.* Heparin-binding EGF-like growth factor is an early response gene to chemotherapy and contributes to chemotherapy resistance. *Oncogene*. 2007, 26 (14), 2006–2016. doi: 10.1038/sj.onc.1209999.
19. *Zhou Z. N., Sharma V. P., Beaty B. T., Roh-Johnson M., Peterson E. A., Van Rooijen N., Kenny P. A., Wiley H. S., Condeelis J. S., Segall J. E.* Autocrine HBEGF expression promotes breast cancer intravasation, metastasis and macrophage-independent invasion *in vivo*. *Oncogene*. 2014, 33 (29), 3784–3793. doi: 10.1038/onc.2013.363.
20. *Labyntsev A.J., Korotkevych N.V., Manoilov K.J., Kaberniuk A.A., Kolybo D.V., Komisarenko S.V.* Recombinant fluorescent models for studying the diphtheria toxin. *Russ. J. Bioorg. Chem.* 2014, 40 (4), 401–409. doi: 10.1134/S1068162014040086.
21. *Kaberniuk A. A., Oliinyk O. S., Redchuk T. A., Romaniuk S. I., Kolybo D. V., Komisarenko S. V.* Cloning of recombinant subunits of *Corynebacterium diphtheriae* diphtheria toxin and their expression in *Escherichia coli*. *Dopov. Nats. akad. nauk Ukr.* 2008, 3, 160–166. (In Ukrainian).
22. *Kaberniuk A. A., Labyntsev A. I., Kolybo D. V., Oliĭnyk O. S., Redchuk T. A., Korotkevych N. V., Horchev V. F., Karakhim S. O., Komisarenko S. V.* Fluorescent derivatives of diphtheria toxin subunit B and their interaction with Vero cells. *Ukr. Biokhim. Zh.* 2009, 81 (1), 67–77. (In Ukrainian).
23. *Labyntsev A. I., Korotkevich N. V., Kaberniuk A. A., Romaniuk S. I., Kolybo D. V., Komisarenko S. V.* Interaction of diphtheria toxin B subunit with sensitive and insensitive mammalian cells. *Ukr. Biokhim. Zh.* 2010, 82 (6), 65–75. (In Ukrainian).
24. *Labyntsev A. J., Kolybo D. V., Yurchenko E. S., Kaberniuk A. A., Korotkevych N. V., Komisarenko S. V.* Effect of the T-domain on intracellular transport of diphtheria toxin. *Ukr. Biochem. J.* 2014, 86 (3), 77–87.
25. *Labyntsev A. J., Korotkevych N. V., Kolybo D. V., Komisarenko S. V.* Effect of diphtheria toxin T-domain on endosomal pH. *Ukr. Biochem. J.* 2015, 87 (4), 13–23.
26. *Schägger H., von Jagow G.* Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* 1987, 166 (2), 368–379.
27. *Schindelin J., Arganda-Carreras I., Frise E., Kaynig V., Longair M., Pietzsch T., Preibisch S., Rueden C., Saalfeld S., Schmid B., Tinevez J. Y., White D. J., Hartenstein V., Eliceiri K., Tomancak P., Cardona A.* Fiji: an open-source platform for biological-image analysis. *Nat. Methods*. 2012, 9 (7), 676–682. doi: 10.1038/nmeth.2019.
28. *Rudolph R., Lilie H.* In vitro folding of inclusion body proteins. *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* 1996, 10 (1), 49–56.
29. *Korotkevych N. V., Labyntsev A. I., Kaberniuk A. A., Oliĭnyk O. S., Romaniuk S. I., Kolybo D. V., Komisarenko S. V.* Cytotoxicity of the B subunit of diphtheria toxin to human histocytic lymphoma U937. *Ukr. Biokhim. Zh.* 2009, 81 (4), 69–80. (In Ukrainian).
30. *Donovan J. J., Simon M. I., Draper R. K., Montal M.* Diphtheria toxin forms transmembrane channels in planar lipid bilayers. *Proc. Natl. Acad. Sci. U. S. A.* 1981, 78 (1), 172–176.
31. *Kagan B. L., Finkelstein A., Colombini M.* Diphtheria toxin fragment forms large pores in phospholipid bilayer membranes. *Proc. Natl. Acad. Sci. U. S. A.* 1981, 78 (8), 4950–4954.
32. *Manoilov K. Y., Gorbatiuk O. B., Usenko M. O., Shatursky O. Y., Borisova T. O., Kolybo D. V.* The characterization of purifed recombinant protein CRM197 as a tool to study diphtheria toxin. *Dopov. Nats. acad. nauk Ukr.* 2016, 9, 124–133. doi: 10.15407/dopovidi2017.02.088. (In Ukrainian).
33. *Manoilov K. Y., Gorbatiuk O. B., Usenko M. O., Shatursky O. Y., Borisova T. O., Kolybo D. V., Komisarenko S. V.* The characterization of purified recombinant fragment B as a tool to study diphtheria toxin. *Dopov. Nats. acad. nauk Ukr.* 2017, 2, 88–99. doi: 10.15407/dopovidi2016.09.124. (In Ukrainian).
34. *Papini E., Sandoná D., Rappuoli R., Montecucco C.* On the membrane translocation of diphtheria toxin: at low pH the toxin induces ion channels on cells. *EMBO J.* 1988, 7 (11), 3353–3359.

НЕОБХІДНІСТЬ ТРАНСЛОКАЦІЙНОГО ДОМЕНУ ДЛЯ РЕАЛІЗАЦІЇ ЦИТОСТАТИЧНОГО ЕФЕКТУ НЕТОКСИЧНИХ ПОХІДНИХ ДИФТЕРІЙНОГО ТОКСИНУ

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Метою роботи було оцінити *in vitro* цитостатичну дію рекомбінантних фрагментів нетоксичного точкового мутанту дифтерійного токсину — CRM197, який було запропоновано як потенційний препарат для лікування трійчасто-негативного раку грудної залози. Із цією метою з використанням методу металоафінної хроматографії на Ni-NTA агарозі виділили рекомбінантні похідні дифтерійного токсину — CRM197, SbB (субодиницю B) і рецепторний домен Rd та дослідили їх вплив на ріст поодиноких колоній клітин трійчасто-негативного раку грудної залози людини MDA-MB-231 за такими показниками, як площа, периметр та індекс циркулярності. Одержані результати показали, що CRM197 і SbB, які містили у складі молекули транслокаційний домен Td, однаковою мірою виявляли цитостатичний ефект стосовно клітин MDA-MB-231, зменшуючи площу та периметр поодиноких колоній. Протеїн Rd не впливав на останні два параметри, які характеризують розмір колоній, однак змінював форму їхнього краю, про що свідчить підвищення індексу циркулярності. Ймовірно, що Td може брати участь у реалізації цитостатичної дії через притаманну йому пороутворювальну активність щодо ліпідних мембран. Зроблено висновок, що Rd і Td, на відміну від каталітичного домену дифтерійного токсину, відіграють важливу роль у реалізації цитотоксичних властивостей CRM197, а SbB, яка складається з Rd і Td, є структурним фрагментом дифтерійного токсину з найменшою молекулярною масою, і її можна використовувати як аналог CRM197.

Ключові слова: CRM197, дифтерійний токсин, HB-EGF, токсоїд, трійчасто-негативний рак грудної залози.

НЕОБХОДИМОСТЬ ТРАНСЛОКАЦИОННОГО ДОМЕНА ДЛЯ РЕАЛИЗАЦИИ ЦИТОСТАТИЧЕСКОГО ЭФФЕКТА НЕТОКСИЧЕСКИХ ПРОИЗВОДНЫХ ДИФТЕРИЙНОГО ТОКСИНА

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Целью работы была оценка *in vitro* цитостатического действия рекомбинантных фрагментов нетоксичного точечного мутанта дифтерийного токсина — CRM197, который был предложен как потенциальный препарат для лечения тройчато-негативного рака грудной железы. С этой целью с использованием метода металлоафинной хроматографии на Ni-NTA агарозе выделены рекомбинантные производные дифтерийного токсина — CRM197, субъединица B SbB, рецепторный домен Rd и исследовано их влияние на рост одиночных колоний клеток тройчато-негативного рака молочной железы человека MDA-MB-231 по таким показателям, как площадь, периметр и индекс циркулярности. Полученные результаты показали, что CRM197 и SbB, которые содержали в составе молекулы транслокационный домен Td, в равной степени проявляли цитостатический эффект по отношению к клеткам MDA-MB-231, уменьшая площадь и периметр отдельных колоний. Протеин Rd не влиял на два последних параметра, которые характеризуют размер колоний, однако изменял форму их края, о чем свидетельствует повышение индекса циркулярности. Вероятно, Td может принимать участие в реализации цитостатического действия за счет присущей ему порообразующей активности по отношению к липидным мембранам. Сделан вывод, что Rd и Td, в отличие от каталитического домена дифтерийного токсина, играют важную роль в реализации цитотоксических свойств CRM197, а SbB, состоящая из Rd и Td, является структурным фрагментом дифтерийного токсина с наименьшей молекулярной массой, и ее можно использовать в качестве аналога CRM197.

Ключевые слова: CRM197, дифтерийный токсин, HB-EGF, токсоид, тройчато-негативный рак грудной железы.

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PECULIARITIES OF ANTIBIOTIC BATUMIN ACTION ON BIOFILM FORMATION BY *Staphylococcus aureus* AND *Pseudomonas batumici*

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The aim of the work was the research of antibiotic batumin action on the biofilm formed by staphylococci and on the process of the biofilm formation by strain-producer *Pseudomonas batumici*. The minimal inhibiting concentration (MIC) of batumin was studied according to The Clinical and Laboratory Standards Institute standards — CLSI. Biofilm formation was studied using photometric O'Toole method with flatbed photometer at wavelength 540 nm. The batumin penetrated into biofilms of staphylococci and reduced the biomass of the formed biofilm of *Staphylococcus aureus* and *S. epidermidis* at the antibiotic concentration of 0.5 μg/ml, which was only twice higher than MIC of batumin for planktonic cells. The degree of biofilm changes in the presence of batumin differed for different staphylococcus strains. However, these differences did not depend on strains sensitivity or their resistance to the antibiotic.

The ability of batumin strain-producer to form the biofilm and the ability of antibiotic to stimulate this process in concentrations of 1 and 10 μg/ml was established for the first time. In addition, it was found that in the *Pseudomonas batumici* B-321 planktonic culture in the presence of 10 μg/ml of batumin, the increase in length of producer cells on average by 15% was noted.

The obtained data shed the light on batumin antimicrobic action on the staphylococci biofilm. It can be argued that is a perspective for treatment of staphylococcal infections. It enabled to consider batumin as a promising drug for the staphylococcal infections treatment.

Key words: batumin, *staphylococci*, biofilm.

The polyketide antibiotic batumin synthesized by the producer strain *Pseudomonas batumici* has a high selective activity against staphylococci [1,2], which determines the prospects for its medical application in the treatment of staphylococcal infections and the control of the nasal carriage of this pathogen [3]. Data obtained in recent years indicate that 95 to 99% of microorganisms in natural habitats exist in the form of biofilms. It is known that bacterial biofilms are involved in the pathogenesis of chronic infections such as osteomyelitis, rhinosinusitis, etc. [4, 5]. At the same time, it is known that bacteria structured in biofilm are more resistant to antibiotics [5], which is one of the problems of successful chemotherapy.

Our preliminary studies on the effect of batumin on biofilm formation in representatives of the genus *Staphylococcus* showed that the presence in the medium of 0.125 μg/ml of batumin (half of growth inhibiting concentration) significantly reduced the formation of biofilm in 85% of the studied nasal strains of staphylococci with an initially high level of biofilm formation [6]. The use of atomic force electron microscopy showed that the batumin reduced several times the staphylococci adhesion to the surface, thus inhibiting the early stages of biofilm formation [7].

On the other hand, the role of antimicrobial substances for cells of producer strains in nature is far from being exhausted by their antibiotic activity. Strains of *P. batumici* are isolated from the soil and are rhizosphere

bacteria that interact with plants and compete with different microflora. The formation of biofilm by such bacteria ensures the nutrients exchange with the plant, the synthesis of antimicrobial substances that protect against phytopathogens and many other effects [8].

In connection with the foregoing, the aim of this work was to study the effect of batumin on the biofilm formed by staphylococci, and to study the influence of batumin on the process of biofilm formation by the producer strain *P. batumici*.

Materials and Methods

The object of the study were 16 strains of *Staphylococcus aureus* and *S. epidermidis*, including 5 methicillin-resistant strains (MRSA) isolated at the Institute of Orthopedics and Traumatology of the Academy of Medical Sciences of Ukraine from patients with osteomyelitis. The strains of MRSA are identified by the method suggested by Boutiba-Ben Boubaker [9].

Another object was a typical antibiotic batumin producing strain *Pseudomonas batumici* UCM B-321 from the Ukrainian Collection of Microorganisms (Zabolotny Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine).

Antibiotic batumin was obtained by fermentation of *B. batumici* strain UCM B-321 and purified by preparative chromatography (90% purification degree) [10].

The sensitivity of clinical strains of staphylococci to a broad spectrum of antibiotics was determined using the Kirby-Bauer method, CLSI criteria were used to interpret antibiotic sensitivity. Discs impregnated with the appropriate antibiotics were used: amoxiclav, erythromycin, clindamycin, rifampicin, ciprofloxacin, doxycycline, vancomycin, teicoplanin, amikacin, linezolid, lincomycin (Himedia, India). The minimal inhibiting concentration of the batumin was studied in accordance with CLSI standards on Müller-Hinton agar and Müller-Hinton broth [11].

The biofilm formation study was carried out according to the O'Toole method [12]. In studying the activity of batumin against biofilms formed by *S. aureus* and *S. epidermidis* cultures, biofilms were grown on the bottom of plastic 96-well plates (Thermo Scientific, USA). 0,2 ml of staphylococci broth culture was introduced into the wells. The initial concentration of cells was $5 \cdot 10^7$ cells/ml, cultivation was carried out for 24 hours at t = 37 °C, further the batumin was added in concentrations of 0.1; 0.5 and 5.0 μg/ml, followed by incubation for 24 hours. After that, the liquid contents of the wells were removed, washed three times with phosphate buffer (pH 7.2), dried and stained. The percentage of biofilm inhibition (BI) was calculated as follows [5]:

$$BI\ (\%) = 100 - [\ OD_{batumin}/OD_{control}],$$

where $OD_{batumin}$ is optical density in the holes with the batumin, $OD_{control}$ is optical density in wells without batumin (control).

To study the biofilm formation by *P. batumici* strain UCM B-321, it was grown at t = 28 °C for 48 h in 96-well plates on Luria-Bertani medium (Becton, USA). 0.25 ml of the cell culture suspension ($5 \cdot 10^7$ cells/ml) was introduced into the wells in a nutrient medium, as well as various (1 and 10 μg/ml) concentrations of batumin.

A 0.1% solution of gentian violet ColorGram 2-F (bioMerieux, France) was used as the biofilm dye, and 0.1 ml was added to each culture well. After thirty minutes of contact at room temperature, the dye was washed three times with distilled water and the ink was dissolved with 96% ethyl alcohol. The results were recorded using a Multiskan FC (Thermo Scientific, USA) flatbed photometer at a wavelength of 540 nm.

Results and Discussion

Table 1 shows the results of studying the sensitivity of clinical staphylococcus strains to widely used in medical practice antibiotics.

The obtained data suggest the high resistance of MRSA strains to the studied antibiotics (the growth retardation zones were only 6 mm), which agrees with the literature data [13, 14]. At the same time, all investigated staphylococci strains, including MRSA, regardless of the source of their isolation and sensitivity to different antibiotics, were sensitive to the batumin.

The next stage of our work was to study the ability of clinical isolates of *S. aureus* and *S. epidermidis* to form biofilms (Fig. 1).

All studied strains of staphylococci formed a biofilm with different biomass values from 0.20 to 0.68 units of optical density. Strains that did not synthesize biofilm were not detected.

Further, we determined the MIC of the batumin with its action on staphylococcus cells, both in planktonic culture and in the composition of biofilms (Table 2).

Table 1. **Sensitivity of clinical *Staphylococcus* strains to antibiotics**

Strains	Zones of growth retardation, mm											
	Amoxiclav	Erythromycin	Clindamycin	Rifampicin	Ciprofloxacin	Doxycycline	Vancomycin	Teicoplanin	Amikacin	Linezolid	Lincomycin	Batumin
S. aureus 75(MRSA)	6	6	6	18	6	22	19	17	16	6	6	25
S. aureus 76 (MRSA)	6	6	6	6	6	20	18	20	21	6	26	30
S. aureus 77 (MRSA)	6	6	22	6	6	6	17	20	17	6	20	32
S. aureus 1463(MRSA)	6	6	6	25	20	20	16	19	16	24	28	28
S. aureus 1813(MRSA)	6	6	6	6	6	24	18	22	22	25	6	35
S. aureus 80	22	6	20	31	24	28	18	24	22	25	24	26
S. aureus 175	6	18	22	35	26	6	15	18	Ч	19	30	32
S. aureus 1306	25	10	25	25	28	6	20	18	26	26	26	30
S. aureus 1310	30	16	18	24	22	6	19	22	25	28	22	28
S. aureus 1316	30	30	22	24	6	20	18	22	18	25	22	32
S. aureus 1358	20	26	18	30	6	22	17	22	19	25	20	32
S. aureus 1440	20	6	20	6	30	15	17	18	24	21	28	35
S. aureus 1749	25	28	24	28	30	26	20	20	22	22	25	35
S. epidermidis 151	6	20	28	6	24	17	22	20	22	24	20	30
S. epidermidis 618	6	32	22	22	19	19	20	24	20	20	22	32
S. epidermidis 1803	6	6	26	22	20	17	24	26	25	6	18	28

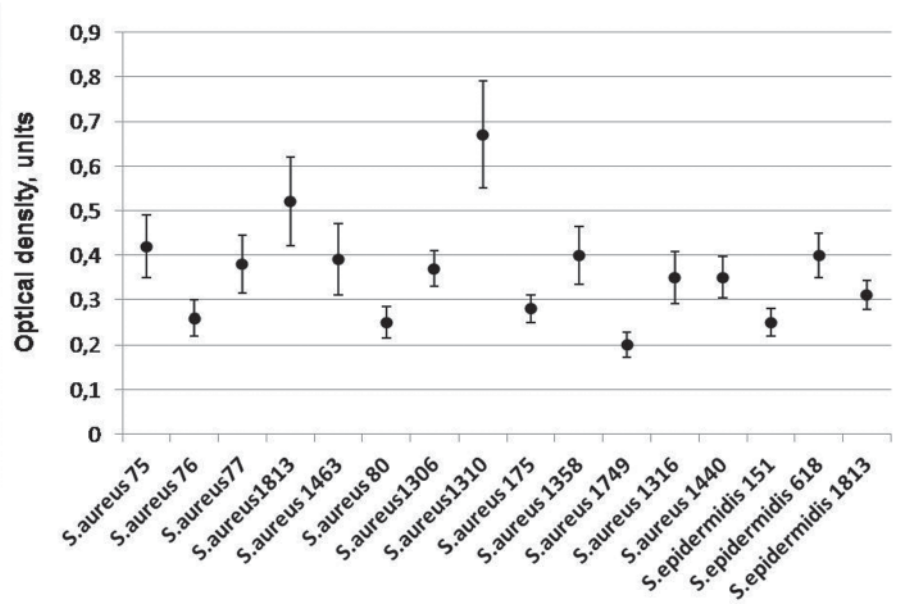

Fig. 1. **Biofilm formation *in vitro* in strains *S. aureus* and *S. epidermidis***

Table 2. **The minimal inhibitory concentration of batumin for plankton cells and *Staphylococcus* biofilms**

Strains	Batumin concentration, μg/ml	
	Plankton cells	Biofilm
S. aureus 75 (MRSA)	0.25	0.5
S. aureus 76 (MRSA)	0.25	0.5
S. aureus 77 (MRSA)	0.25	0.5
S. aureus 1463 (MRSA)	0.25	0.5
S. aureus 1813(MRSA)	0.25	0.5
S. aureus 80	0.25	0.5
S. aureus 175	0.25	0.5
S. aureus 375	0.25	0.5
S. aureus 1306	0.25	0.1
S. aureus 1310	0.25	0.5
S. aureus 1316	0.25	0.5
S. aureus 1358	0.25	0.5
S. aureus 1440	0.25	0.5
S. aureus 1749	0.25	0.5
S. epidermidis 151	0.25	0.1
S. epidermidis 618	0.25	0.5
S. epidermidis 1803	0.25	0.5

For planktonic staphylococcus cells, the MIC of the batumin was 0.25 μg/ml, the MIC for the biofilms of each studied strain was 0.5 μg/ml, except for the strain of *S. aureus* 1306 (MIC = 0.1 μg/ml).

In the course of the work, it has been found that in the presence of batumin the significant changes occurred in the biomass of the microbial community of staphylococci in the formed biofilm (Table 3).

From the results obtained, it can be seen that the decrease in the biomass of the formed biofilm depended on the concentration of the antibiotic, and the intensity of the effect was different in the studied strains. Thus, the batumin concentration of 0.5 μg/ml, which was only twice as high as MIC, caused a decrease in biomass of biofilm from 34.7 to 77.0%. A further increase in the concentration of antibiotic to 5 μg/ml only slightly changed the mass of the biofilm.

According to the literature, the addition of antibiotics such as ampicillin, cefatoxime, levofloxacin, rifampicin in concentrations 50 times higher than MICs causes a decrease in the mass of the formed biofilm up to 30% maximum [15]. Highly active against staphylococci antibiotic mupirocin isolated from bacteria of the genus *Pseudomonas*, reduced biomass of the formed biofilm by 90% in all investigated isolates of *S. aureus* at concentrations 250 times higher than MIC [5].

According to Moreno M.G. et al. [16] the MICs of phosphomycin, rifampicin and levofloxacin when they act on plankton cells were 1–16 μg/ml, and on biofilms — more than 1024 μg/ml. The exception was the antibiotic gentamicin, the MIC of which, when it acts on plankton cells was 4–8 μg/ml, on biofilms — 8–16 μg/ml. Allison et al. [17] found that gentamicin kills persistent cells of *Escherichia coli* and *Staphylococcus aureus*. Persistent cells are a subpopulation in biofilm and are tolerant to antibiotics, thus remain viable when exposed to pharmaceuticals. The author suggests that rifampicin and benzylpenicillin kill most of the cells in the

Table 3. **Batumin effect on formed biofilms biomass of staphylococci**

Strains	Batumin concentration, μg/ml		
	0.1	0.5	5.0
	Decrease in relation to control,%		
S. aureus 75 (MRSA)	47.7	57.2	57.4
S. aureus 76 (MRSA)	31.1	34.7	34.1
S. aureus 77 (MRSA)	57.9	63.2	60.3
S. aureus 1463 (MRSA)	67.7	69.3	68. 2
S. aureus 1813 (MRSA)	75.2	77.0	77.0
S. aureus 80	36.0	40.1	40.0
S. aureus 175	39.3	50.0	49.3
S. aureus 375	38.1	52.4	51.0
S. aureus 1306	56.8	56.8	54.0
S. aureus 1310	64.0	74.3	74.2
S. aureus 1316	25.8	37.2	34.3
S. aureus 1358	65.0	67.5	68.5
*S. aureus*1440	57.2	65.8	62.9
*S. aureus*1749	35.0	60.0	60.1
S. epidermidis 151	48.4	51.5	54.8
S. epidermidis 618	40.2	44.1	44.0
S. epidermidis 1803	50.0	60.3	60.0

biofilm, except persisters, and gentamycin, selectively acting, leads to their complete eradication. Earlier, we showed that the batumin also has a high activity against SSCVs persisters (*S. aureus* small colony variants) [18]. Thus, by the mechanism of action on staphylococcus biofilms the batumin is probably similar to gentamicin.

The obtained results indicate that the batumin penetrates into the formed biofilms of clinical strains of *S. aureus* and *S. epidermidis*, and after 24 hours of action causes a decrease in the biofilm formation process. It should also be noted that the antibiotic significantly reduces the biomass of the biofilm formed in strains with an initially high level of biofilm formation (Table 3). We obtained similar results when studying the action of antibiotic batumin on biofilm developing. The efficacy of batumin at a concentration of 0.125 μg/ml (half of its MIC) was higher for strains with a high biofilm weight [6].

It is known that matrix components play an important role in the process of antibiotics penetration into biofilms. The lipids of the biofilm matrix and bacterial membranes are identical in qualitative composition [19]. When studying the mechanism of batumin action on staphylococci, we found that in this pathogen under the action of antibiotic the significant changes in the fatty acid profile, which leads to an increase in fluidity of membrane lipids and permeability of the membrane in *S. aureus* take place [20]. Based on the similarity of the composition of the biofilm matrix lipids on one side, and the membrane of the bacterial cell on the other side, it can be assumed that the batumin penetrates well into the biofilm of staphylococci and causes significant changes in the biofilm formed, which we observed.

Attention is drawn to the fact that the intensity of changes in biofilms in the presence of batumin differs in different strains of staphylococci, however, these differences do not depend on the sensitivity or resistance of strains to the studied antibiotics (Tables 1 and 3).

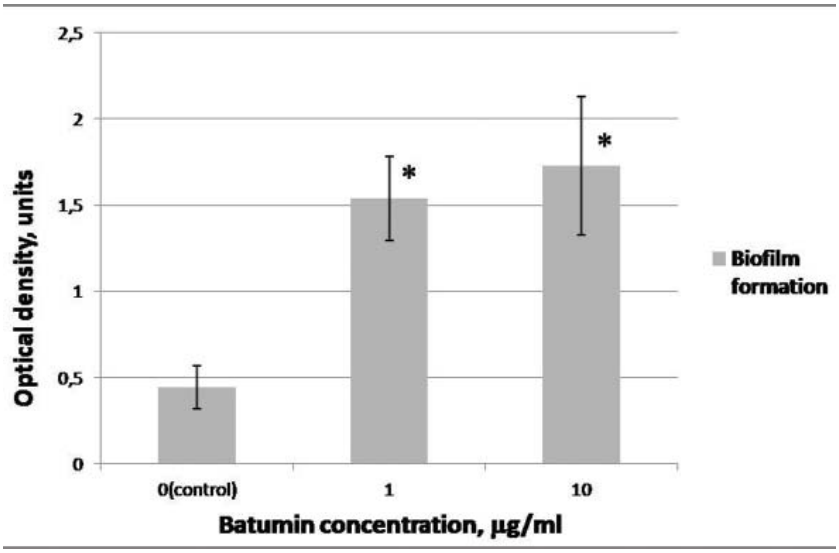

Fig. 2. **Batumin effect on the biofilm formation by strain *P. batumici* UCM B-321:** marked columns (*) are significantly differ from the control at $P \leq 0{,}05$

Apparently, the changes we have detected in the biofilms of staphylococci under the influence of batumin make them unstable, incapable of persistence and dissemination in the human body.

Another stage of our work was the study of the batumin influence on the processes of biofilm formation by the strain-producer *P. batumici* (Fig. 2).

Batumin in concentrations of 1 and 10 µg/ml increased biofilm formation by a culture from 0.442 ± 0.126 units of optical density in the control without antibiotic up to 1.539 ± 0.246 and 1.728 ± 0.402, respectively. It was also found that in the planktonic culture of *P. batumici* B-321 in the presence of 10 µg/ml of batumin, an increase in cell length of the producer by an average of 15% was noted. In control (without batumin), the cell length was 2.34 ± 0.22 × 0.65 ± 0.02 µm; at a concentration of 10 µg/ml of batumen it was 2.69 ± 0.23 × 0.75 ± 0.09 µm. Such an increase in cell length in pseudomonads may be associated with a decrease in the frequency of cell division [21].

In the literature, information is provided on the role of antibiotics, in particular phenazines synthesized by *Pseudomonas* species, in the biofilms formation by pseudomonads. In this case, phenazine antibiotics are able to change the expression of genes associated with cell adhesion and the subsequent formation of biofilms [22, 23]. It is possible that the batumin also plays a certain regulatory and functional role in the processes of biofilm formation by *P. batumici* strain.

Thus, the results of the studies of the batumin influence on biofilm formation processes by *P. batumici* strain suggest that along with high antimicrobial activity, the positive influence of batumin on the biofilm formation enhances the competitiveness of the producer in relation to other inhabitants of the rhizosphere and attest to the importance of this compound for life-support processes in *P. batumici*.

Studies of the batumin effect on the formed biofilm of staphylococci showed a significant decrease in its biomass in *S. aureus* and *S. epidermidis* at antibiotic concentrations of 0.5 µg/ml, which is only twice as high as the MIC of batumin when it acts on plankton cells. The obtained data for the batumin effect on the formed biofilm of staphylococci shed light on the mechanisms of batumin antimicrobial action, based both on the prevention of the formation of *S. aureus* and *S. epidermidis* biofilm, and on the functioning of the biofilm already formed, which makes it possible to consider it as a promising tool in the treatment of infections caused by staphylococci.

REFERENCES

1. *Kiprianova E. A., Klochko V. V., Zelena L. B., Churkina L. N., Avdeeva L. V. Pseudomonas batumici* sp. nov., the antibiotic-producing bacteria isolated from soil of the Caucasus Black Sea coast. *Microbiol. zh.* 2011, 73 (5), 3–8.
2. *Klochko V. V., Kiprianova E. A., Churkina L. N., Avdeeva L. V.* Antimicrobial spectrum of

atibiotic batumin. *Microbiol. zh.* 2008, 70 (5), 41–46. (In Russian).
3. *Churkina L. N., Kiprianova E. A., Bidnenko S. I., Marchenko K. P., Artysyuk E. I.* Antibiotic batumin for diagnostics of staphylococci and treatment of *Staphylococcus aureus* nasal carriage. *Likarska Sprava*. 2009, 1–2, 61–67. (In Russian).
4. *Ernest E. P., Machi A. S., Karolcik B. A., LaSala P. R., Dietz M. J.* Topical adjuvants incompletely remove adherent *Staphylococcus aureus* from implant materials. *J. Orthop. Res.* 2017, 15, 1–6. doi: 10.1002/jor.23804.
5. *Ha K. R., Psaltis A. J., Butcher A. R., Wormald P. J., Tan L. W. In vitro* activity of mupirocin on clinical isolates of *Staphylococcus aureus* and its potential implications in chronic rhinosinusitis. *Laryngoscope*. 2008, 118, 535–540. doi: 10.1097/MLG.0b013e31815bf2e3.
6. *Churkina L. N., Vaneechoutte M., Kiprianova E. A., Perunova N. B., Avdeeva L. V., Bukharin O. V.* Batumin — a selective inhibitor of staphylococci — reduces biofilm formation in methicillin resistant *Staphylococcus aureus*. *Open J. Med. Microbiol.* 2015, 6, 51–59. doi: 10.4236/ojmm.2015.54024.
7. *Bukharin O. V., Perunova N. B., Ivanova E. V., Churkina L. N., Avdeeva L. V., Yaroshenko L. V.* Morpho-functional changes of *Staphylococcus aureus* biofilms under the effect batumin. *J. Microbiol. Epidemiol. Immunobiol.* 2013, 2, 8–12. (In Russian).
8. *Nikolaev Yu. A., Plakunov V. K.* Biofilm — "City of Microbes" or an Analogue of Multicellular Organisms? *Microbiologia.* 2007, 76 (2), 149–163. (In Russian).
9. *Boubaker I., Abbes I., Abdallah B., Mamlouk H., Mahjoubi F., Kammoun A., Hammami Redjeb S.* Evaluation of a cefoxitin disk diffusion test for the routine detection of methicillin-resistant *Staphylococcus aureus*. *Clin. Microbiol. Infect.* 2004, 10, 762–765. doi: 10.1111/j.1469-0691.2004.00919.x.
10. *Klochko V. V.* Biosynthesis and properties of antibiotic batumin. *Biotechnol. acta*. 2014, 7 (6), 46–50. doi: 10.15407/biotech7.06.046.
11. *Wikler M. A., Cockeril F. R., Craig W. A.* Performance standards for antimicrobial susceptibility testing; Fifteenths informational supplement. *Clin. Labor. Stand. Institute*. 2005, 25 (1), 87–151.
12. *O'Toole G., Kaplan H., Kolter R.* Biofilm formation as microbial development. *Ann. Rev. Microbiol.* 2000, 54, 49–79. doi: 10.1146/annurev.micro.54.1.49.
13. *El Aila N. A., Al Laham N. A., Ayesh B. M.* Nasal carriage of methicillin resistant *Staphylococcus aureus* among health care workers at Al Shifa hospital in Gaza Strip. *BMC Infect Dis.* 2017, 17 (1), 28. doi: 10.1186/s12879-016-2139-1.
14. *Churkina L. N., Bidnenko S. I., Lutko O. B., Avdeeva L. V., Babenko L. A.* Study of the efficiency of antistaphylococcal antibiotic batumin against methicillin resistant strains of staphylococci. *Antibiot. Chemother.* 2007, 52 (9–10), 19–23. (In Russian).
15. *Tetz G. V., Artemenko N. K., Tetz V. V.* Effect of DNase and antibiotics on biofilm characteristics. *Antimicrob. Agents Chemother.* 2009, 53 (3), 1204–1209. doi: 10.1128/AAC.00471-08.
16. *Moreno G., Trampuz A., Di Luca M.* Synergistic antibiotic activity against planktonic and biofilmembedded *Streptococcus agalactiae*, *Streptococcus pyogenes* and *Streptococcus oralis*. *J. Antimicrob. Chemother.* 2017, 72 (11), 3085–3092. doi: 10.1093/jac/dkx265.
17. *Allison K. R., Brynildsen M. P., Collins J. J.* Metabolite-enabled eradication of bacterial persisters by aminoglycosides. *Nature*. 2011, 473 (7346), 216–220. doi: 10.1038/nature10069.
18. *Churkina L. N., Bidnenko S. I., Avdeeva L. V., Vaneechoutte M., Makushenko A. S., Lutko O. B., Oserjanskaja N. M.* Characteristics of atypical forms of staphylococci (SCVs), isolated from patients with osteomielitis. *Antibiot. Chemother.* 2011, 55 (5–6), 36–40. (In Russian).
19. *Tetz V. V., Korobov V. P., Artemenko N. K., Lenikina L. M., Panjkova N. V., Tetz G. V.* Extracellular phospholipids of isolated bacterial communities. *Biofilms.* 2004, 1, 149–155.
20. *Lee V. E., O'Neill A. J.* Batumin does not exert its antistaphylococcal effect through inhibition of aminoacyl-tRNA synthetaseInt enzymes. *J. Antimicrob. Agents*. 2016, 49 (1), 121–122.
21. *Steinberger R., Allen A., Hansa H., Holden P.* Elongation correlates with nutrient deprivation in *Pseudomonas aeruginosa* — unsaturates biofilms. *Microb. Ecol.* 2002, 43 (4), 416–423.
22. *Chincholkar S., Thomashow L.* Microbial Phenazines: Biosynthesis, Agriculture and Health. *Springer-Verlag Berlin: Heidelberg*. 2013, 248 p.
23. *Maddula V., Pierson E., Pierson L.* Altering the ratio of phenazines in *Pseudomonas chlororaphis* (*aureofaciens*) strain 30-84: effects on biofilm formation and pathogen inhibition. *J. Bacteriol.* 2008, 190. 2759–2766. doi: 10.1128/JB.01587-07.

ОСОБЛИВОСТІ ДІЇ АНТИБІОТИКА БАТУМІНУ НА ФОРМУВАННЯ БІОПЛІВКИ У *Staphylococcus aureus* І *Pseudomonas batumici*

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Метою роботи було дослідити дію антибіотика батуміну на сформовану стафілококами біоплівку, а також на процес біоплівкоутворення штамом-продуцентом *Pseudomonas batumici*. Мінімальну інгібувальну концентрацію (МІК) батуміну встановлено згідно зі стандартами CLSI Інституту клінічних та лабораторних стандартів. Формування біоплівки вивчали фотометричним методом O'Toole за допомогою планшетного фотометра за довжини хвилі 540 нм. З'ясовано, що батумін проникав у біоплівки стафілококів і знижував біомасу сформованої біоплівки у *Staphylococcus aureus* і *S. epidermidis* за концентрації антибіотика 0,5 мкг/мл, яка лише у два рази перевищує МІК батуміну за дії на планктонні клітини. Зміни біоплівки у присутності батуміну різняться у різних штамів стафілококів, однак ці відмінності не залежать від чутливості або резистентності штамів до антибіотика.

Уперше встановлено здатність штаму-продуцента батуміну до утворення біоплівки і стимуляції цього процесу самим антибіотиком у концентраціях 1 і 10 мкг/мл. Також відзначено, що у планктонній культурі *P. batumici* B-321 за присутності 10 мкг/мл батуміну довжина клітин продуцента збільшувалась у середньому на 15%.

Отримані дані відкривають нові аспекти антимікробної дії батуміну на біоплівку стафілококів, що полягають як у запобіганні формуванню, так і в інгібуванні вже сформованої біоплівки. Це дає підстави розглядати батумін як перспективний засіб лікування стафілококових інфекцій.

Ключові слова: батумін, *staphylococci*, біоплівка.

ОСОБЕННОСТИ ДЕЙСТВИЯ АНТИБИОТИКА БАТУМИНА НА ФОРМИРОВАНИЕ БИОПЛЕНКИ У *Staphylococcus aureus* И *Pseudomonas batumici*

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Целью работы было исследование действия антибиотика батумина на сформированную стафилококками биопленку, а также на процесс биопленкообразования штаммом-продуцентом *Pseudomonas batumici*. Минимальная ингибирующая концентрация (МИК) батумина определена в соответствии со стандартами CLSI Института клинических и лабораторных стандартов. Формирование биопленки изучали фотометрическим методом O'Toole с помощью планшетного фотометра при длине волны 540 нм. Установлено, что батумин проникал в биопленки стафилококков и снижал биомассу сформированной биопленки у *Staphylococcus aureus и S. epidermidis* при концентрации антибиотика 0,5 мкг/мл, которая лишь в два раза превышала МИК батумина при действии на планктонные клетки. Изменения биопленки в присутствии батумина различаются у разных штаммов стафилококков, однако эти различия не зависят от чувствительности или резистентности штаммов к антибиотику.

Впервые установлена способность штамма-продуцента батумина к образованию биопленки и стимуляции этого процесса самим антибиотиком в концентрациях 1 и 10 мкг/мл. Также было отмечено, что в планктонной культуре *P. batumici* B-321 в присутствии 10 мкг/мл батумина длина клеток продуцента увеличилась в среднем на 15%.

Полученные данные открывают новые аспекты антимикробного действия батумина на биопленку стафилококков, которые состоят как в предотвращении формирования биопленки, так и в ингибировании уже сформировавшейся биопленки. Это дает основания рассматривать батумин как перспективное средство лечения стафилококковых инфекций.

Ключевые слова: батумин, *staphylococci*, биопленка.