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ELECTRONIC INFORMATION SYSTEMS FOR MONITORING OF POPULATIONS AND MIGRATIONS OF INSECTS

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The purpose of the work was to analyze existing information systems (IS) of biological objects and to propose the methods for development of such IS for insects on the example of *Noctuidae* (*Lepidoptera*). A detailed analysis of technical information concerning the distributed networked systems, access to computer systems to the common data in electronic IS and the organization of biomedical databases in the Internet was done. The peculiarities of IS' prototypes development for environmental monitoring of the fauna have been discussed, in particular changes in the number of butterflies' populations throughout France (including western and southern departments), the *Noctuidae* (*Lepidoptera*) in the steppe zone of Ukraine (Striltzivskyi Steppe), and the development of such IS for all territory of Ukraine.

The results could be used to develop electronic IS for other biological organisms.

Key words: electronic information systems, bioinformatics, insects *Noctuidae* (*Lepidoptera*).

Peculiarities of biomedical objects important for electronic information systems construction. The works for construction and development of new electronic information systems (ISs) with biomedical databases (DBs) are actual and important in biotechnology as well as in biology and medicine in general. During application of modern information technologies (IT) and computer technologies (CT) in these spheres, it was necessary to keep in mind following peculiarities of biomedical objects that are important for electronic information systems construction [1]. These peculiarities complicated ever the computer processing of biological data, results, and development of technical information systems (tIS) in general. This was, first of all, the problem of the complexity of medical and biological objects, which has several reasons [1].

1. The first reason was an objective one: the complexity of biological organisms by themselves. On the Earth there are millions of species that are characterized by a large variability of individual organisms, populations, and etc. The species have complex chemistry, physiology, developmental cycles and behavior, which are the result of billions years of evolution. There are hundreds, if not thousands of ecosystems, in which many species are associated also with large number of complex relationships.

2. The second reason was a subjective one. Since ecosystems are located at different territories of different countries, they often interfere with the interests of different groups of people: scientific, economic, commercial, others, which influence on the process of biomedical objects studies. During field studies, monitoring, the data obtained in different ecosystems often differ in precision and accuracy, they are made in different meteorological or geographical conditions. Many of such observations were made by

amateurs, but their results are important too. When creating electronic databases all such field data observations and experiments should be formalized, unified and standardized.

If to speak about genetic materials that characterized the certain types of ecosystems, then these obtained scientific data are really accurate and conform to generally accepted standards. Most often these data were the ideal material for electronic DBs and ISs. The ambiguity appeared when an IT professional during his work must match these exact data, for example, with the name of the specie. But there are not exact standards of specie names still; the systematic of biological species is under the revision constantly. Besides of these, the constant evolutionary changes of biological organisms are continuing. Also there is a fact that many species on the Earth have no their names still.

The application of IT / CT in biology was very complicated also because that most of the existing computer methods are developed for certain conditions, certain tasks, limited time periods. In reality, the scientists deal with the evolution (both molecules and species), processes during time intervals in hundreds and thousands of years, with organisms that consist of billions of molecules. There were no standard methods still for the solution of such problems; they needed to be invented. The simple transfer of the methods developed already for technique or for other branches of sciences, a lot of valuable biological information were lost.

A particular problem was the digitization of biological data, on which all modern biological knowledge is based. Up to 2001, even in the United States, where the biological data digitizing process was initiated, it was necessary to digitize information on more than 750 millions of biological species [1]. Consequently, this labor-intensive task needs to be solved all over the world.

Bioogical ISs were designed either for academic purposes — to maximize the accumulation of information about the groups of living organisms, or for the needs of the economy, in particular for biotechnology, for monitoring of polluted areas in industrial centers, and etc. [1–10]. Mathematic methods as well as models that we described in our previous articles and published by other authors also may be used for ISs functioning or to be simulated in result of their functioning [10–81]. A spectrum of mathematic methods were used for the newest biomedical ISs elaboration [1, 11, 75, 77–80, 82–146, 159]. Content for described in this article databases was obtained usually from the results of biological and medical observations and experiments [10, 12–17, 24–44, 47–49, 61, 68, 71–74, 82–90, 94, 104, 106, 109, 111–113, 125–159]. All such technical information systems (tIS) are electronic databases (DB) distributed in networks today [1–11, 25–69, 90–109, 112–120, 159]. Present work was done after the analyzis of approximately 250 current publications in fields of biotechnology, other linked branches of biology and technology, including articles with original authors' works. The newest parts of authors work were supported by patents [12, 160–167].

Biomedical information systems for environmental eco-monitoring. If to monitor the changes that occurred with species populations and their areas under different influences over a period of time, and to record the data about their changes in the electronic databases, then such data can be used for computer modeling of species' populations (areas). If it was registered that areas of these species' distributions reduce, it was necessary to find the reasons of this decrease. If the reason of these area changes was, for example, the damaging anthropogenic impact, then it was necessary to give appropriate recommendations for the neutralization of this impact. Such an algorithm should be put in basis of modern scientifically grounded nature conservation [1, 91].

It was difficult to achieve rather representative geographic survey and the survey in which fauna changes could be traced during a certain time. Such works are quite expensive usually, and the quality of records varies even in the case of accurately executed work. However, because of constant worsening of environmental living conditions, because of constant climate change, then the significance of studying of various bioorganisms' distribution changes are increasing. In our investigations we (Prof. Zoya F. Klyuchko and Dr. Elena M. Klyuchko) have studied such influences on organisms-bioindicators *Noctuidae* (*Lepidoptera*), and a part of our works under the DBs and ISs construction we did with this biological material [1, 9, 42, 135, 136, 139, 140, 144, 156, 157, 159].

An important condition for organization of the local IS network for eco-monitoring was the construction and perfect organization of local networks of biological ISs. They could provide an opportunity for effective organization and cooperation of teams of scientists and other professionals. Nowadays, all local scientific

establishments in Ukraine have already constructed local computer networks, which give users the possibility of more effective group work with the joint use of hardware resources — printers, scanners, modems, faxes, as well as software and information resources, including DBs. As through own local computer, the user can also control the databases using a "database management system" (DBMS) at the local computer network. In general, program-application can be run under the control of DBMS or its kernel, or to be independent one. Most often, the electronic ISs in Ukrainian scientific establishments were based on a ready local computer network of this research institution, and contained only a client-server database at this local network. Later the systems with configuration of such types were transformed into the corporate systems in the Intranet. Such system, unlike the client-server version, is focused not on the data but on information that is available easily to biologist-scientist and combines the benefits of centralized systems for many users of client-server type. However, the separate programs of such systems and contents of some databases — simulation and animation models, etc. — had their value also outside of local ISs, for example, for universities. Therefore in future, the certain content (separate sets of data, partially — the information) from ISs of domestic scientific institutions would be desirable to visualize in the Internet [1], and to make it available in the global network. Such measures allowed both to improve the acquaintance in the world with Ukrainian achievements, and to establish better international cooperation for the further progress of our science and technology. In this article, we will consider a number of specific examples of electronic ISs — domestic and foreign — which could become successfully the prototypes in further IS development for biotechnology.

Analysis of electronic information systems for monitoring of organisms-bioindicators. Analyzing the current published data on developed electronic ISs for organisms-bioindicators monitoring (most of all we are interested in ISs for insect monitoring), it is necessary to remember the following [1].

1. The possibility of usage of electronic ISs for the registration of organisms-bioindicators in Ukraine for our domestic scientific practice, for nature conservation or for works in agriculture. It would be noted that all the elements necessary for large-scale ISs' development and their implementation in Ukraine we have today. The quality standards of each constituent element of such ISs in Ukraine are enough high today and this allows to obtain positive results. However, in our country there is a peculiarity that have to be taken into account when constructing such systems. The peculiarity is that the large-scale system for nature protection, crops protection, and etc. was developed during the USSR period in Ukraine; this system was perfect for its time. This system united numerical professionals in biomedical sciences, it included also numerical "biological research stations" (bio-stations) for nature observations and studying, significant number of nature preserves with relevant scientific units where were accumulated the results of living organisms' observations for decades, including pests of agricultures, and etc. During long decades biological expeditions were financed, the works of individual professionals and amateurs in biological data collections were supported. All this networked and branched structure operated successfully until the 1990s of the XX-th century, and partly it works still. The achievements of these works were in successful management of agriculture, implementation of environmental measures, and so on. For example, the catastrophic mass migrations of locusts (*Locustae*) from the Danube Delta to other territories of Ukraine were stopped in the early 1960s due to successful works of young Ukrainian biologists. Doctors-epidemiologists and biologists stopped numerical mortal epidemics here (human as well as animal), and so on [1, 42].

2. The disadvantage of this monitoring system in Ukraine in XX-th century, from contemporary point of view, was the lack of data digitization, lack of computers, and, consequently, inability to use modern information and computer technologies (ICT). Even today the information about significant part of results is stored as paper versions and, therefore, it is difficultly accessed. Necessary contemporary requests are that the bio-stations or other observation points should be better computerized, which now is financially possible (if to use mostly inexpensive computer models). Consequently, even basing on the developed ready network of domestic bio-stations, it is possible to develop efficient electronic information systems, for example, for tracking of migrations of locusts (*Locustae*), moths (*Noctuidae*, *Lepidoptera*) and other organisms. In domestic practice, probably, it may be advisable to make local systems of personal computers (PCs) of bio-stations as basic elements for future electronic systems for bio-organisms monitoring [1].

3. In process of development of domestic electronic monitoring systems, it is important to adhere one of basic principles of Internet systems. This is the principle of decentralization, which in theory increases the reliability of such ISs: when one node fails, its function performs another similar one. In today's practice in Ukraine, the decentralization principle is very natural and appropriate. Since the individual networks' segments are developed today spontaneously by individual professionals and amateurs in biology, they are actually a networked union of more or less equal elements, and priorities in such networks are not well defined in comparison with similar networks in some foreign countries in Western Europe, America, Australia [1].

Network systems with distributed databases. The networked systems with distributed databases are used successfully in world practice today [1]. Distributed database is constructed from several fragments located in different nodes of the network. They can be managed by different database managing systems (DBMS) from the point of view of programs and users who access the distributed database outside this system; such system can be perceived as a single local database (Figs. 1, 2).

Information about the location of each part of the distributed database and other necessary information was stored in the data dictionary. Such a dictionary can be stored in one of the nodes, or be distributed. In order to ensure correct access to the distributed database, a two-phase protocol for transaction record was used.

System access to common data. The main objects of access in contemporary network electronic ISs can be: the database as a whole, separate tables, records, and etc. [1]. When accessing the common data, DB management tools provide two main access methods: monopoly and collective. As IS objects of access can be the specifications of reports and on-screen forms, requests and programs.

Concept of signal transmission in the development of electronic information systems in biotechnology. Lets investigate the possibility of transferring of some approaches and concepts in radiophysics into the practice of development of networked computer ISs for biotechnology [43]. In process of development of computer systems for biology and medicine, the information side of problem solution, the technical IS details, mathematical justification, and solving of IS implementation problems are considered usually. However, there is another, equally important approach exists also. This is an approach from the point of view of classical radiophysics, when the information presented in the form of signals that pass through the links of the classical chain:

1. Receiver-transmitter →
2. Channel of signal transmission →
3. Receiver-transmitter →
4. Channel of processing (analysis) of observational data (measurements).

Below in present article it will be shown that this sequence, which has already become classical for physical processes, in fact can be accepted in biology and medicine, where it can be accepted for the construction of medical and biological ISs and devices. However, during this scheme use in these spheres, each link will have a different physical meaning (and hence, technical implementation).

An analysis of experience of IS prototype construction for biotechnology and eco-monitoring of fauna. One of interesting examples of modern IS for environmental

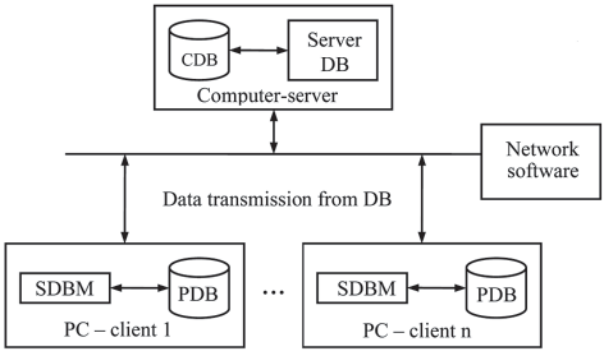

Fig. 1. **The simplest scheme of electronic information system with databases' server [1]**

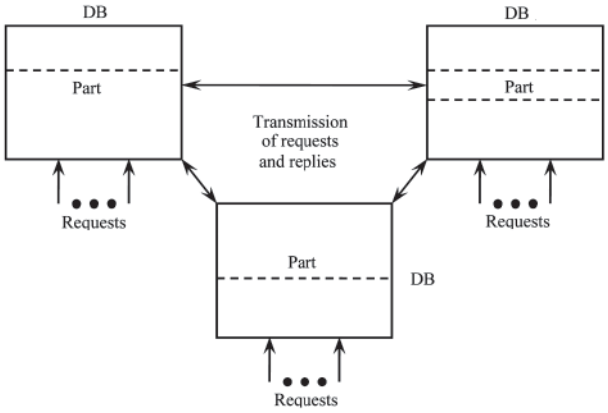

Fig. 2. **Distributed databases of electronic information systems [1]**

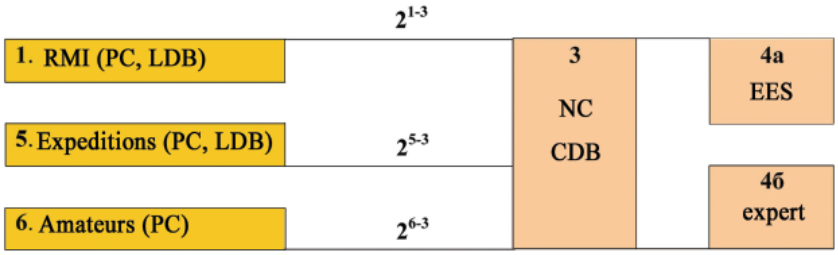

Fig. 3. **Structural scheme of IS for tracing of insects' migrations**

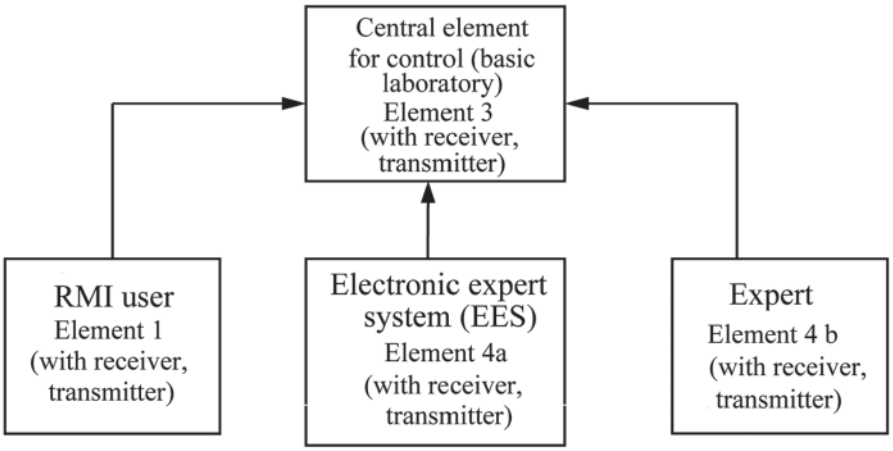

Fig. 4. **Fragment of structural scheme of IS for tracing of insects' migrations**

monitoring and agricultural services was the IS, that had been developed in Australia [1, 15, 43]. This information system consisted on two remotely-spaced radars that were used for insect monitoring (RMI); they both were connected to the node computer (NC) of the basic laboratory (Figs. 3, 4).

Each RMI was equipped [15] with meteorological surveillance devices under the control of portable computers (PCs) that allow receiving and analyzing the data. PCs were connected to node computer (NC) host computer through a modem, and than connected to a public telephone network. PC-NK communications were used to transmit observation data, to perform remote services and to conduct diagnostics. A specially organized automated system was developed to analyze meteorological information and the data about insects' migrations, recorded by the radar. On the base of this analysis the statistical reports and their graphical representations were prepared daily according to information received by radars. The reports and graphs provided the data on the intensity, amplitude, velocity and movement of insect migration directions, orientation, size and frequency of migrant wings, as well as weather conditions at the surface of each point of survey. Such reports were transmitted to NCs and inserted automatically into the Internet pages, which users could see since 12.00 p.m. next day.

This network was used for environmental research in regions where insects' migrations are possible [15], for predicting of mass invasions of such dangerous insect pests like locusts, which can completely destroy the annual harvest. Network technologies applied in this method allow remote radar control through the network, as well as provide the user promptly with insect tracking results in a convenient and easily understandable manner. The RMI's radars were highly specialized, they can provide high-quality and quantitative information on high-altitude insect migrations (above 200 m) in the selected region. Radars worked in vertical sounding modes and detect insects flying over them. There were two types of conducted analysis. The primary type of analysis, called quantitative, was a conical scan, during which the size, shape, direction of movement, orientation and velocity of insect clouds were determined. During the secondary analysis the vibrations of insect wings were detected, their characteristics were determined (frequency of the wings vibration, etc.); these characteristics were the main in the set of parameters, which subsequently determine

the type of migratory insects. The results of the primary and secondary analyzes of both RMIs were recorded on PCs of radar centers. PCs servicing the RMI were connected to the internal network (intranet). Information from them was transmitted to an external Internet networks; there it was stored at server from which the users can visualize it on their own computers through standard browsers. After conducting a series of studies during several years and accumulation of results in the databases, it is possible to determine the frequency and seasonal patterns of migratory insects' movements in different directions, and subsequently to predict the insects' migration. In addition, basing on datasets obtained from radar, one can construct decision making computer system.

Since all elements of this IS (two RMIs, NCs, all PCs, etc.) were connected online, and the described operations were performed by RMI in automatic mode, RMIs can perform the data collection functions remotely and independently from the laboratories that analyze and interpret these data. In the future, the data were transmitted to the centers where the places of control, the centers of scientific expertise, as well as the operating system for managing of pest number control were located. Although certain decisions were made by electronic system, it is also necessary to involve in this scheme an expert-biologist to make sometimes the adjustments. Experts-biologists in this system also participated in the organization of expeditions to different regions insects' collection purposes. Collected information was transmitted to the basic laboratory for scientific interpretation and for the operation of the electronic decision making system. This system was developed to track the number of such insect pests like *Chortoicetes terminifera* and *Helicoerpa punctigera* [1, 43].

Analysis of the structural scheme of electronic information system for monitoring of bioobjects migrations. Let's analyze the described above example in terms of the concept of signaling [1, 43]. An electronic prototype system was a new step in insects monitoring, in controlling of environmental situation at large territories and in agriculture transforming into a science-intensive industry. This system was highly specialized, efficient and technically enough simply implemented. Developing this idea we constructed original IS that differs in some details and solutions from Australian prototype. Its structure and functions are described below, and also at (Figs. 3, 4). Such system would be based on abovementioned set of domestic bio-stations and other previously developed system elements, and it is really important for Ukraine, where agricultural incomes form the significant part of national income.

Element 1. The complex of RMI performed the functions of remote insect detection. These data were transmitted to the RMI intranet where the data were collected in computers' databases. Than the data were processed according to procedures of primary and secondary analysis and transmitted outside. As one could see, the element 1 had a complex structure and can be subdivided into elements by itself (set of PCs, some of their own databases, etc.). Let's consider element 1 as integral, without division into sub-elements for simplification. Then it could be seen as a receiver-transmitter (reception of control signals from the center — from the decision-making system and data transmission to the center). Element 1 was equipped with computer interface that connect it with element 2.

Element 2^{1-3}. It was a channel for signals transmitting from element 2 to the central control element 3. As one could see, this channel was technically implemented as Internet system based on cable networks. The use of a wireless data transmission system was possible too.

Element 3. The central element of control, implemented as a basic laboratory. It also has a complex structure. First, it contains NC, to which the data from RMI (element 1), PC research groups (expeditions, element 5), individual users (item 6) flowed. Secondly, it contained a database for all received data recording. Element 3 was combined with elements 4a — an electronic expert system and 4b — an expert-biologist. Element 3 was also provided with computer interface that connected it with element 2. Thus, element 3 can also be considered as receiver-transmitter (receiving data from element 1 and reversal transmission of control signals from the center and elements 4a, 4b — decision making systems to element 1).

Element 4a. Electronic expert system (EES). Based on the databases of element 3, the system performed a number of analytical operations, for example, to determine the specie of migratory insects and to make decision about the level of danger of this specie. If information from RMI elements 1, 5, 6 was recognized as dangerous one, EES sends corresponding message to element 1 and visualizes it in the Internet, from which each user can find it through standard

browser. As a result of obtaining and/or visualizing information about the dangers in the system, the commands were generated about the preventive and protective measures for environment ecology or agriculture. In accordance with these functions, the element 4a also had an interface for its connection with element 3. In addition, the element 4a also had an interface for its connection (via element 3) with element 4b (expert-biologist) and with elements 5 and 6, from which the data are received also.

Element 4b. In cases of ambiguity, lack of information from element 1, in other non-standard situations it might happen that corresponding decision can not be done by element 4a. Then the system switched the work to a living expert-biologist who was at this time on duty (element 4b). In this case, the functions of data analysis, decision-making and transmission of control signals to element 1 are moved to element 4b. To perform the above functions, element 4b had interfaces for combining it with elements 3, 4a, and elements 1, 5, 6 (via element 3) for obtaining of additional data.

Elements 5 and 6. The part of the data that had to be analyzed when making a decision, comes from the research groups (expeditions, element 5) and individual users, like farmers or amateurs (element 6). Individuals (elements 5, 6) who collected such data and transferred them to element 3 do this via their own computers (PCs) using cable networks or wireless communications (through appropriate interfaces).

Elements 2^{5-3} *and* 2^{6-3}. These elements were the channels for signals transmitting to/from the central control element 3 from/to elements 5 and 6. In fact, they are similar to the above-described channel for signals' transmission from the RMI (element 2^{1-3}). These channels were also implemented through the Internet based on cable networks or needed a wireless data transmission system. In system-prototype, the functions of this channel were carried out also through the Internet site that visualizes data (for example, in form of tables, reports, individual data, and etc.) about the current state of environment.

Organization of biomedical databases in the Internet. During our work under the development of ISs with DBs for biotechnology, we proceeded from the fact that the data in DBs would come from many points (bio-stations, field expeditions, amateurs, and others, Fig. 5); and IS information had to be visualized in the Internet [1].

Therefore, the idea immediately began to evolve from the corporate intranet system construction. Such system united the advantages of centralized systems for many users of client-server type. The following features characterized her: 1) the server generates information suitable for the use, but not the data (for example, in case of DBMS there were database records); 2) during the exchange between client and server, the open standard protocol was used; 3) — system-application was at server, and therefore for user work on client computer it is enough to have a program-navigator (Figs. 1, 2) [1, 43].

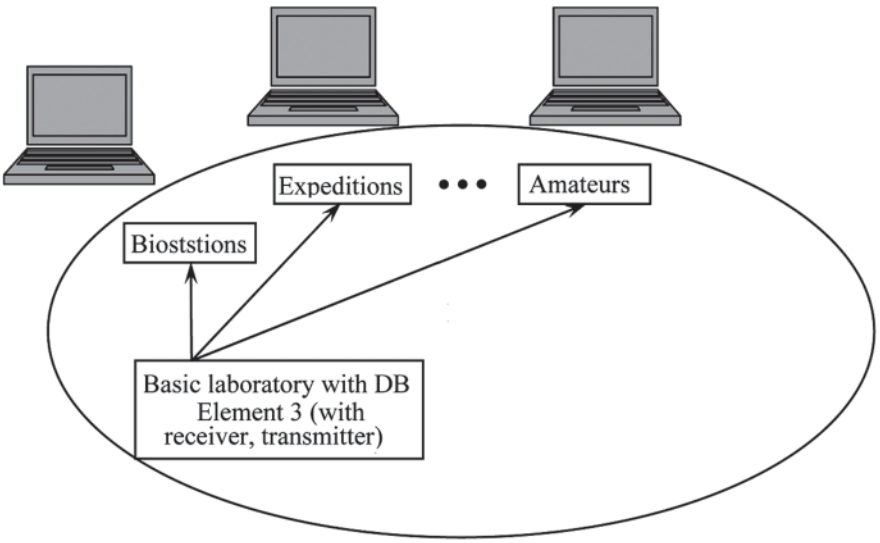

Fig. 5. **Scheme of the part of "EcoIS" system for tracing of insects' migrations**

For DB accessing from client side, the main mean of implementing of interaction mechanisms between Web–client and database server was Java. Additionally, ActiveX control elements can be used. As auxiliary information processing tools on client side JavaScript, Jscript, and VBScript languages are often used to extend the capabilities of declarative HTML language based on the addition of procedural means (but these tools were not used for interaction with database). Programs — scripts were executed on computer Web — browser in interpretation mode. To access database servers from Java-programs have been developed the standard JDBC (Java DataBase Connectivity — database compatibility for Java), based on ODBC concept. The JDBC standard was developed by Sun/JavaSoft companies and it provides universal access to various DBs in Java language.

In access model to database on the server side, the reference to the database server is usually executed by calling of the Web server programs by another programs that are external to them in accordance with conventions of one of CGI and API interfaces. Programs developed in accordance with CGI interface were called CGI scripts. External programs interact with database server using SQL language, for example, directly to a specific server, or using the ODBC driver [1]. External programs were written in programming languages like C, C++, PHP. Access to database on the side of application program server was used during the use of these programs' servers. In this case, the main language of development of distributed applications was Java.

Publication of information in the Internet. The visualization of information from "EcoIS" in the Internet was made using widely spread modern technologies, so this does not require a special description. It is necessary to emphasize only that for such publication it is necessary to solve a number of routine tasks, known to all developers of global network software [1].

1) Building of IS in the Internet basing on the multi-level architecture of the database.

2) Organization of DBMS interconnection on different platforms.

3) Use in the Internet of information from existing local network databases. These tasks appeared when necessary to publish an information from intranets in the global network.

4) Construction of local intranet — networks basing on publication technology.

5) Databases in the Internet. In this case, the local networks were built on Internet principles with the availability to access the global network. It is The use of the databases to organize information and use the SQL language to find the necessary information in the database were recommended.

6) Use of DBMS tools for data security.

7) Standardization of the user interface basing on the use of Web browsers and user-friendly interface design [1].

Above mentioned could be illustrated also at Fig. 1 and 2. The easiest scheme of electronic information system with databases' server is shown on the Fig. 1. The distributed databases of electronic information systems are given in Fig. 2.

Algorithms for environmental monitoring of fauna. The algorithms of eco-monitoring of the fauna may be subdivided naturally into two groups of steps (Fig. 6). [1]. Modern electronic databases with spatial information distribution in the Internet and modern mathematical modeling based on the data from such databases, similar interdisciplinary approaches allow us to investigate at contemporary level how the geographic, ecological and environmental factors acting continuously every day, as well as the these factors that influenced throughout the history, so, how they both effect on the organisms' spread and biodiversity. In addition, such approaches allow us to determine how to preserve better this biodiversity in context of intensive increase of anthropogenic influence. Environmental monitoring of biological organisms to prevent their numbers reduction as a result of various damaging factors — urbanization, man-caused impacts, and etc., are important contemporary tasks. The sequence of steps for such problems solution could be described by an algorithm (Fig. 6) in two steps [1].

Eco-monitoring of bioorganisms of Striltsivskyi Steppe preserve (Ukraine). Electronic databases with spatial distribution of information in the global Internet network, mathematical modeling based on data from such databases, allow us to investigate at the current level how geographic and environmental factors influence on the distribution and number of organisms. Such methods can be used to preserve a healthy environment for people who live in ecologically unfavorable conditions. The generalizations presented in this section are based on original data as the result of long-term monitoring of insects' — moths' (*Noctuidae*, *Lepidoptera*) fauna in Striltsivskyi Steppe preserve (Ukraine, Luhansk region) [1].

Fauna monitoring of different biological species have been conducted in Ukraine during

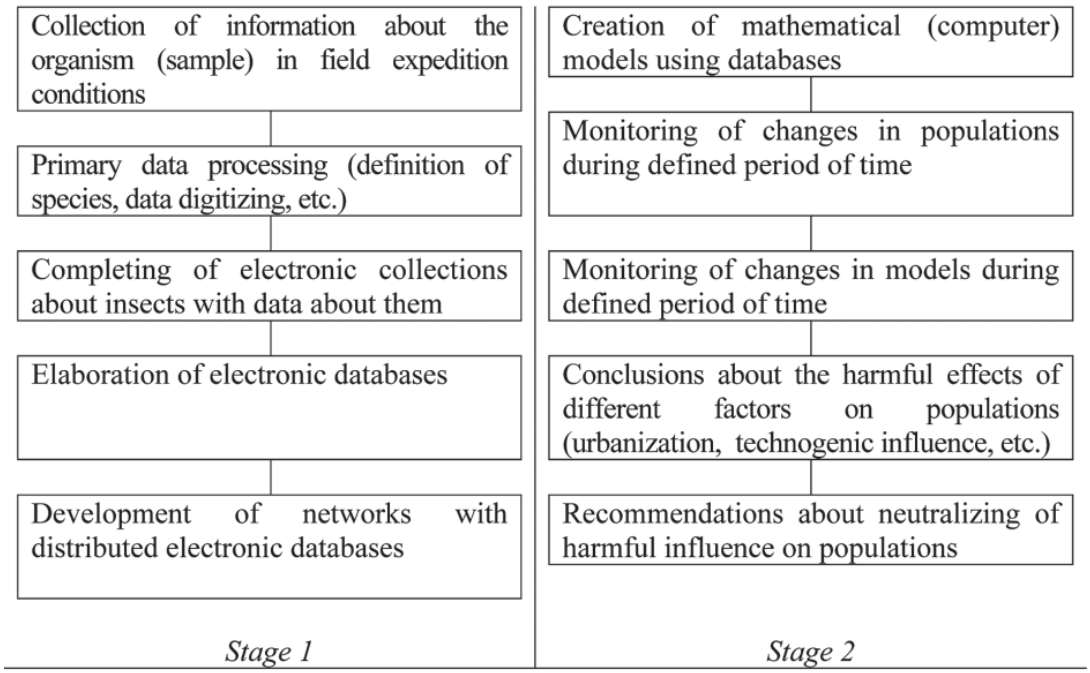

Fig. 6. **Two stages of ecomonitoring data processing using DB construction:**
stage 1 — construction of CBO and electronic DB;
stage 2 — monitoring of populations and areas of the species using the DB based models

the last decades, but such works are irregularly often; during these studies only a few species were monitored sometimes. Some original data of environmental monitoring of fauna of Striltsivskyi Steppe preserve (Luhansk region, Ukraine) by the authors are given in the fragment of the Table.

This *Noctuidae* fauna monitoring has been conducted during more than 40 years (from 1965 to 2007) by Prof. Zoya F. Klyuchko (Shmalgauzen Institute of Zoology of the National Academy of Sciences of Ukraine). During this time there were registered 318 species from 144 genera of *Noctuidae*. Taxonomic analysis reveals that the largest number of species belongs to 17 genera: *Cucullia* (21 species), *Hadena* (13), *Mithymna* (12), *Acronicta* (10), *Catocala* (9) and *Apamea* (9), *Xestia* (8), *Lacanobia* (7), *Euxoa* (7) and *Agrotis* (7), *Eublemma* (6) and *Caradrina* (6), *Lygephila* (5), *Amphipyra* (5), *Sideridis* (5), *Orthosia* (5) and *Noctua* (5). One of the characteristic features of the moths fauna was the large number of genera, that are represented by 1–4 species (127 genera, or 88.2%). The second peculiarity was that composition of moths species' collected in different years varies significantly by their number — 191 specie on 1965 and 260 ones on 2006–2007 (Table). But international experience of such works in other countries evidences that such a picture, however, may be the result of the improvement of methods of observation and registration of insects over time [1].

During the 40 years of monitoring in this preserve, the numbers of meadow-forest species have increased (90 or 28%). This increase was registered both in species number and in frequencies of individual moth samples collecting during the season. At the same time, the number of forest species increased (18 or 5.6%), including the genus *Catocala* (*C. fraxini* L., *C. pacta*, *C. elocata* Esp., *C. hymenaea* Den. & Schiff.). Also the numbers of *Amphipyra* and others increased too (Table); which was an important feature of changes in the local fauna. This fact can be explained by an increase of territories, occupied by forest bushes and trees.

It is necessary to emphasize the significance of obtained original results of long-term monitoring of Striltsivskyi Steppe moths fauna and the huge amount of work done. It should be noted that the accumulation of large arrays of such results is only the first step in the algorithms of contemporary environmental monitoring (but very important step!).

As was noted above in this section when analyzing foreign experience, such reliable information has to be proposed further to

The results of dynamics of changes of *Noctuidae* species quantities, obtained as a result of long-term fauna monitoring by Prof. Klyuchko Z. F. in Striltsivskyi Steppe preserve (Luhansk region, Ukraine)

Name of specie	Number of moths' registered in different years				Distribution in biotopes
	1965	1996–2002	2006	2007	
Eublemma purpurina Den.& Schiff.	31		21	8	Fost
Phytometra viridaria Cl.	30	1	2	2	Fost
Lygephila lusoria L.		3		3	Fost
L. lubrica L.	33	8	3	4	Fost
L. craccae F.	34		3		MeFo
Drasteria caucasica Kol.	28		2	1	St
Euclidia triquetra Den.& Schiff.	464	1	6	1	St
Catocala hymenaea Den.& Schiff.		1	56	26	Fo
Abrostola tripartita Hufn.	12	2	4	2	MeFo
A. asclepiadis Den. & Schiff.	2		26	3	FoSt
Trichoplusia ni Hbn.	2		2	13	FoSt
Macdunnoughia confusa Steph.	70	3	13	12	FoSt
Diachrysia chrysitis L.	80	2	71	11	Eur
D. stenochrysis Warr.	70	3	82	12	Eur
Autographa gamma L.	122	4	5	13	Eur
Phyllophila obliterata Rbr.	100	2	1	6	FoSt
Acontia lucida Hufn.	210	3	9	12	FoSt
A. titania Esp.	70	2	17	14	FoSt
A. trabealis Scop.	986	7	6	32	Eur
Oxycesta geographica F.	1397	5	28	86	St
Acronicta megacephala Den. & Schiff.	4	3	16	10	Eur
Mycteroplus puniceago Bsd.	104		14		FoSt
Tyta luctuosa Den. & Schiff.	210	5	88	79	Evr
Cucullia dracunculi Hbn.	14	4	6	4	FoSt
Calophasia lunula Hufn.	94		5	16	FoSt
Epimecia ustula Frr.	120		4	7	St
Schinia scutosa Den. & Schiff.	186	7	15	16	Evr
Heliothis viriplaca Hufn.	60	1	2	2	Evr
H. maritima Grasl.	143	5	5	22	Evr
Helicoverpa armigera Hbn.		2	8	9	Evr
Periphanes delphinii L.	3		29		St
Cryphia fraudatricula Hbn.		1	5	10	FoSt
Pseudeustrotia candidula Den.&Schiff.	120	4	39	31	Evr
Caradrina wullschlegeli Pueng.	53			1	FoSt
Hoplodrina octogenaria Goeze	15		1	9	MFo
H. blanda Den.& Schiff.	80	1	2	4	MFo
Athetis furvula Hbn.	21		1	8	MFo
Actinotia polyodon Cl.	1		52	6	FoSt
Apamea ferrago Ev.	408		4		St

Note. Designations of species depending on the biotopes of collection: FoSt — forest-steppe; MeFo — meadow-forest; St — steppe; Eur — eurybionts.

digitizing and organizing in the form of databases distributed at IS-based networks.

The table can actually be seen as an example of a printed table, an output of relational database. In fact, it is only a short fragment with dozens of records from hundreds of observations collected by domestic biologists over decades of professional work. All information in it may be in future the subject for processing in accordance with the algorithms described above for relational databases. When structuring the data in the form of such databases, further environmental studies and forecasts become possible [1].

With effective conservation practices, it would be advisable to use such data for the purpose of ecological forecastings, especially because of today's technogenic pressure in Ukraine, when the natural steppe areas are shortening quickly. For the further, the details of such databases would be nice to analyze using series of mathematical methods discussed in publications [1–9]. Using such analytic methods it would be possible to find whether the number of species were changed or not, whether the species' spectrum was changed or not, and etc.). Successful prediction of fauna changes would promote the further use of regression methods for the design of digital maps of species on the base of local databases connected in ISs. Comparing the results of analytical processing using above described novel computer methods and traditional ones one could discover sometimes that output results are different in both cases; and this is important for the implementation of large-scale economic projects linked with monitoring. On Fig. 7 there are some *Noctuidae* (*Lepidoptera*) from Prof. Klyuchko private collection used for our electronic databases construction.

Monitoring of bioorganisms' populations using databases. Data from electronic collections of biological organisms (CBO) are available for further processing [1, 14]. For example, in recent years they have been used to make patterns of species distribution (modeling of areas changes). The influences harmful for population could be studied as on one specie number monitoring during any time period, as well as monitoring of species' populations and their areas of distribution throught the country. For the illustration let's observe an ecological problem solution by modern computer and mathematic methods in different departments of France [1, 14]. The monitoring of species' changes in time was done and the conclusion was made about the presence of anthropogenic influence in studied regions. Two models there were developed, the adequacy of which was compared [14]: 1 — geographic model (GM) based on directly obtained data, and 2 — model based on the data from neighborhood regions (NM — neighbor model). For GM model there were used regional data: latitude, longitude and altitude (above sea level) data; they were called "direct GM data". While the data in framework of the second model (data on the number of species and distribution of species in surrounding regions) were based on the data from the nearby areas according to NM. Both models were done for significant fluctuations of species composition that characterized the rich fauna (68–78%). However, during the application, the NM demonstrated more successful results than GM, where only geographic variables were taken into account. A large amount of the data about species distribution was calculated according to logistic and auto-logistic regression models (222 of 246 species, 90.2%). Auto logistic models were based on information from neighboring regions. It was impossible to perform such analysis for the cases of rare species, when 5 or 6 of them only were registered in one administrative unit (2.4%), or in cases of widespread species that were registered in more than 90% of administrative units (7,3%). It was found that the use of auto-logistic models dominated over the logistics in case of the species' distribution study (with the use of stepwise logical regression); the use of variables, registered in near-located regions helped to creation of 64.5% successful models of species distribution (22.8% models had not such data). The simple measure of proximity (not dependent on distance) C_2 dominated for most models (89 of 246 species, 36.2%); unlike the distance-weight dependent measure (C_1; 77 of 246 species, 31.3%). The models that were developed, appeared to be valuable in detecting of faulty records and fauna losses, in order to fill the "white spots" during the design of fauna maps in regions. Studies demonstrated a significant visible reduction in species' number in the western and northern regions of France. In addition, significant changes in species' number for some administrative units in time period after 1970 were detected, as well as differences in the data that were forecasted and those that were detected in reality. In some regions the probabilities have been calculated for some

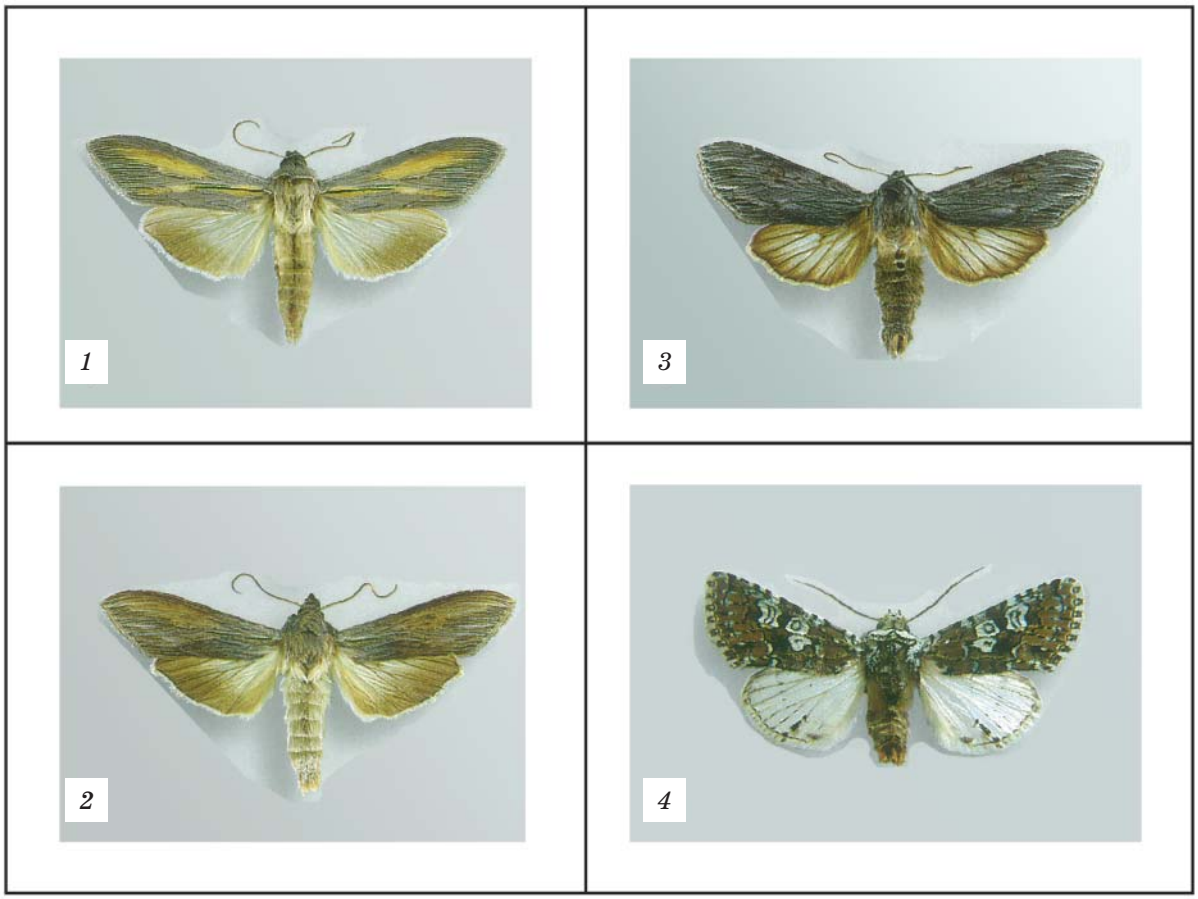

Fig. 7. **Some materials of *Noctuidae* (*Lepidoptera*) used for electronic DB construction in our works** (photos are from private collections of Prof. Klyuchko Z. F. and from her monograph [168]): *1 — Cucullia biornata* Fisher von Waldheim, 1840; *2 — Cucullia umbratica* (Linnaeus, 1758); *3 — Cucullia lucifuga* ([Denis & Schiffermüller], 1774); *4 — Lamprosticta culta* ([Denis & Schiffermüller], 1775)

species; these values were presented and published in the Internet [14].

For stepwise logical regression, two measures of "closest neighbors" were used [14]:

$$C_1 = \left(\sum_{j=1,k} W_{ij} y_j\right) / \sum_{j=1,k} W_{ij\ldots}, \qquad (1)$$

where C_1 is the averaged number of regions that had a weight, among the set of k_i neighboring regions; i and y_j — are the presence or absence of species in area j. The weight given to the area j — $wij = 1/h_{ij}$, where h_{ij} — is Euclidean distance between regions i and j. In the developed scheme k_i = 8 units — the nearest neighboring regions were selected. This number corresponds to the maximum number of neighboring regions for the case where the selected regions-units conditionally had square form, and two times higher than the number recommended for the units of hexagonal form. For some regions the cases of more neighborhoods have been studied. It was found that the distance between neighboring regions in this case exceeded the distances that butterflies can fly throughout their lives, except those species that migrate seasonally over long distances during their lives. Then

$$C_2 = \Sigma_{j\,1,k} \cdot y_j / K_i \ldots, \qquad (2)$$

where C_2 — is simply the proportion of areas in which the specie was registered, among the set of k_i neighboring district i [14].

So, if somebody use the methods of forecasting of changes in the number of species and their territorial distribution, developed by these approaches, they can fill the "white spots" on maps of nature. Such methods also allow more precise identification of regions that should be in the center of special attention. Regression methods allow to elaborate good models for predicting of changes in number of species and their distribution. Most species

have clearly defined distribution areas and borders that can be successfully predicted based on the main geographical variables (region, latitude, longitude, and altitude). Recently, in order to solve biological problems, the regression model was supplemented with auto-logistic functions, which allowed predicting a decrease in the number of species. The comparison procedure in the process of solving the problem of species propagation was further improved, using the possibilities of forecasting by the logistic and auto-logistic model (for nearby geographic points). The result of this work was to obtain the values of the probability of species distribution in different regions of the country [14].

Thus, in present article using the set of examples there were demonstrated that electronic information systems with databases about living objects are really necessary for professionals and amateurs today. We have investigated different examples of IS deeply, some important peculiarities of medical and biological objects that have to be taken into account during biotechnological IS elaboration were discussed. Analysis of electronic ISs for monitoring of organisms (bioindicators) has been done. Necessary technical information was given about: network systems with distributed databases, computer system access to the common data; organization of biomedical databases in the Internet; peculiarities of information publication in the Internet. Also an analysis of IS development for biotechnology and eco-monitoring of fauna and analysis of IS structural scheme for monitoring of bioobjects migrations were done. The data about eco-monitoring of bioorganisms' populations in France and in Ukrainian Striltzivskyi Steppe Preserve were given.

Analyzing examples of electronic systems for insects' monitoring, it is necessary to emphasize the following. If to develop and to apply in Ukraine IS similar to Australian prototype, then it should be noted that all elements that we have distinguished, analyzing the prototype, are present in Ukraine as well, but they are not functionally linked between themselves. There are enough number of bio-stations, preserves with relevant scientific units, highly qualified professionals who could realize such project successfully. The disadvantages of such domestic analog realization, from contemporary point of view are the lack of data digitization, lack of computers, and some others. So, basing on developed network of domestic bio-stations, it is possible to construct an efficient electronic system, similar to prototypes described above.

In the described prototype system from Austraia, one of the basic principles of Internet systems was not realized: the principle of decentralization, which in theory increases the reliability of such ISs: when one node fails, its function performs the similar one. So, for our practical purposes (increase of reliability) some elements of prototype have to be duplicated.

For biomedical ISs successful construction they would be previously theoretically modeled using the concept of signaling from radio physics. The acceptance of this abstraction during the electronic ISs design for biomedical objects should be successful in construction of such ISs analogues.

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ЕЛЕКТРОННІ ІНФОРМАЦІЙНІ СИСТЕМИ ДЛЯ МОНІТОРИНГУ ПОПУЛЯЦІЙ ТА МІГРАЦІЙ КОМАХ

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Метою роботи було проаналізувати наявні інформаційні системи (ІС) біологічних об'єктів та запропонувати методи розроблення такої ІС для комах на прикладі *Noctuidae* (*Lepidoptera*). Проведено детальний аналіз технічної інформації щодо мережевих систем з розподіленими базами даних, доступу комп'ютерних систем до загальних даних в електронних ІС та організації біомедичних баз даних в Інтернеті. Обговорено особливості створення прототипів ІС для екологічного моніторингу фауни, зокрема зміни чисельності популяцій метеликів на всій території Франції (в т.ч. у західних та південних департаментах), совок *Noctuidae* (*Lepidoptera*) у степовій зоні України («Стрільцівський степ»), а також подібної ІС для всієї території України.

Одержані результати можуть бути використані для розроблення електронних ІС для інших біологічних організмів.

Ключові слова: електронні інформаційні системи, біоінформатика, комахи *Noctuidae* (*Lepidoptera*).

ЭЛЕКТРОННЫЕ ИНФОРМАЦИОННЫЕ СИСТЕМЫ ДЛЯ МОНИТОРИНГА ПОПУЛЯЦИЙ И МИГРАЦИЙ НАСЕКОМЫХ

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Цель работы заключалась в том, чтобы проанализировать имеющиеся информационные системы (ИС) биологических объектов и предложить методы разработки такой ИС для насекомых на примере *Noctuidae* (*Lepidoptera*). Проведен детальный анализ технической информации о сетевых системах с распределенными базами данных, доступа компьютерных систем к общим данным в электронных ИС и организации биомедицинских баз данных в Интернете. Обсуждены особенности создания прототипов ИС для экологического мониторинга фауны, в частности изменения численности популяций бабочек по всей территории Франции (в т.ч. в западных и южных департаментах), совок *Noctuidae* (*Lepidoptera*) в степной зоне Украины («Стрельцовская степь»), а также подобной ИС для всей территории Украины.

Полученные результаты могут быть использованы для разработки электронных ИС для других биологических организмов.

Ключевые слова: электронные информационные системы, биоинформатика, насекомые *Noctuidae* (*Lepidoptera*).

EXPERIMENTAL ARTICLES

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CALIX[4]ARENES METHYLENE BISPHOSPHONIC ACIDS EFFECT ON PLASMIN ACTIVITY

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The aim of the work was to study plasmin inhibiting properties of calix[4]arenes functionalized by phosphonic acid residues. The following methods were used: turbidimetry, analysis of enzyme activity using chromogenic substrates, evaluation of caseinolytic activity by tyrosine release from casein, Dixon's method for determining the type and inhibition constant. It was found, that calix[4]arenes C 296, C-425, C-427 and C-145 inhibited fibrin clot lysis by plasmin in dose-dependent manner and inhibition rate is proportional to the number of phosphonic acid residues. C-145 were the most effective plasmin inhibitor (competitive mechanism, K_i = 0,52 μM). However, C-145, as well as C 296, C-425 and C-427, did not affected amidolytic and caseinolytic plasmin activity but inhibited plasminogen activation by streptokinase. Thus, we assume that the mechanism of calyx[4]arene selectivity to fibrinolysis and its ability to obstruct plasminogen-streptokinase interaction is provided by complex formation between calix[4]arene negatively charged phosphonic groups and positively charged amino acids in substrate recognition exosites of plasmin. Calix[4]arene C-145 is effective plasmin fibrinolytic activity inhibitor and perspective for further investigation as antifibrinolytic agent.

Key words: fibrinolysis, plasmin, plasminogen, plasmin inhibitor, plasminogen activation, streptokinase.

Plasminogen overactivation, plasmin hyperactivity and low level of plasmin inhibitors in the circulation lead to wide range of haemorrhagic disorders. Development of effective low molecular weight plasmin inhibitors opens up new prospects in hyperfibrinolysis medication. Calixarenes belong to perspective class of low-toxic compounds due to their ability to form supramolecular complexes with biological molecules.

Fibrinolytic system activation is an appropriate response to blood clotting and the main event that leads to fibrin clot dissolution by proteolysis. Plasminogen and activators from plasma bind to specific sites in fibrin during fibrinogen conversion to fibrin, or to specific receptors on cell surface, and activators cleave proenzyme into plasmin [1].

The key fibrinolytic enzyme plasmin is a trypsin-like serine protease generated from inert precursor plasminogen. Proenzyme molecule consists of N terminal and five kringle domains connected with serine protease domain by activation loop, which is cleaved during plasminogen activation by either tissue-type plasminogen activator (tPA), urokinase-type plasminogen activator (uPA) or factor XIIa complex with callicreine [2].

As result of high level of the activators release into bloodstream or acute decrease of plasmin inhibitors formation excessive activation of fibrinolytic system activation develops and causes hyperfibrinolysis. Elevation of plasmin content in blood leads to hydrolysis of fibrin, fibrinogen, clotting factors and accumulation of fibrinogen/fibrin degradation products, which

detain blood clotting and platelets aggregation, and finally to hard bleeding and hemorrhages. Hyperfibrinolysis accompanies the range of pathologic states like haemophilia, liver diseases, DIC-syndrome, oncological diseases, sepsis, etc. Overactivation of fibrinolytic system has threatening value during extensive wound and burn injuries, surgical operations on heart with artificial blood circulation or on lungs and other parenchymatous organs [3]. Plasmin formation also involved in other physiological and pathological processes including cell metastasis, cell proliferation, angiogenesis, and embryo implantation [1, 3].

Plasmin inhibition is critical in preventing adverse consequences of plasmin overactivation. Aprotinin was widely used as an antifibrinolytic drug before its discontinuation in 2008. Tranexamic acid and ε-aminocaproic acid, lysine analogs that inhibit plasmin by binding to plasmin's kringle domains, are currently used in the clinic. Other developing types of plasmin inhibitors include reactive cyclohexanones, nitrile warheads, peptidomimetics and polypeptides of the Kunitz and Kazal-type, sulfated glycosaminoglycan mimetics that bind to plasmin's catalytic domain [4]. One of the perspective directions of highly specific low molecular weight plasmin inhibitors search is calixarenes. Calixarenes are nanoscale cyclic oligomers with a vase shape produced synthetically by precise cyclo-condensation of substituted phenols and formaldehyde, which possess intramolecular lipophilic cavities formed by aromatic rings of the macrocyclic skeleton, and may be easily functionalized. Calixarenes have low level of toxicity and can form supramolecular complexes with wide range of biopolymers. Promising substances for calixarenes structure modification are bisphosphonates — structural analogues of natural pyrophosphate. These compounds demonstrate versatile bioactivities [5, 6]. Calixarene structure modification by methylene bisphosphonates leads to acquirement of specific bioactivity [7, 8].

In the present study we have estimated the effect of calix[4]arenes derivatives of methylene bisphosphonic acid on plasminogen/plasmin system activity.

Materials and Methods

Materials. Pooled human blood plasma for plasminogen and fibrinogen isolation was purchased from local hospital bank.

Plasminogen with an amino-terminal glutamic acid residue (Glu-plasminogen) was prepared from fresh citrate donor plasma by affinity chromatography using the Lysine-sepharose 4B (Sigma Aldrich, USA) [9]. Glu-plasminogen purity was tested by 11,5% PAGE at pH 3.2.

Plasmin was prepared by activation of Glu-plasminogen with urokinase (HS Medac, Germany), immobilized to BrCN-activated Sepharose 4B (Sigma Aldrich, USA). 1 mg of proenzyme was incubated with 0.5 ml of urokinase-sepharose gel (1250 IU/ml) during 1 hour at 37 °C in 50 mM sodium-phosphate buffer solution pH 7.4 with 25% glycerol. Plasmin was stored in 50 mM sodium-phosphate buffer solution pH 7.4 with 50% glycerol at 20 °C. Activation efficiency was evaluated by plasmin caseinolytic and amidolytic activity and 10% PAGE with SDS in the presence of 2% β-mercaptoethanol.

Fibrinogen was purified from fresh citrate human plasma with 1000 KIU/ml aprotinine (Merckle, Germany) and 20 mM ε-aminocaproic acid (Sigma Aldrich, USA) by fractionation with sodium sulfate. Before fractionation plasma was twice treated by barium sulfate (60 mg/ml) by the method of Smith [10], heated to 25 °C and mixed with 1 M glycine buffer solution pH 9.0 in 1/9 volume ratio. 16% sodium sulfate was added to 5.7% saturation for plasma albumins fraction elimination and the precipitate was removed by centrifugation at 5000 g. Supernatant was slowly mixed with 16% sodium sulfate to 8.5% saturation and centrifuged at 5000 g, precipitate was dissolved in 0.2 M sodium chloride (10/1 part of initial plasma volume). Fibrinogen solution was treated by equal volume of 16% sodium sulfate after addition of 5/1 volume of 0.5 M monopotassium phosphate and centrifuged, precipitate was diluted in 0.15 M sodium chloride (10/1 part of initial plasma volume). After overnight incubation in ice fibrinogen solution was centrifuged at 5000 g for cryofibrinogen elimination and then mixed with equal volume of 16% sodium sulfate. Fibrinogen precipitate was separate by centrifugation at 5000 g, dissolved in 0.15 M sodium chloride and stored at 20 °C.

desAB-fibrin was obtained by dissolving of fibrin clot formed by thrombin (EC 3.4.21.5) — 1 NIH per 1 mg of fibrinogen (Sigma Aldrich, USA) in the presence of 50 mM ε-aminocaproic acid and sodium parahydroxy mercury benzoate (0.35 mg/ml) (Sigma Aldrich, USA) in 20 mM acetic acid as described elsewhere [11].

Calix[4]arenes C145-, C296-, C425-, C427- and 4-hydroxyphenyl methylenbisphosphonic acid were kindly provided by Dr. V. I. Kalchenko

and were synthesized as described in [7]. C-145 is a sodium salt of calix[4]arene 5,11,17,23-tetrakis-methylene bisphosphonic acid, C-296 — 5,17-bis(dihydroxyphosphonylmethynol)-11,23-dibrom-25,26,27,28-tetrapropoxycalix[4]arene, C-425 — 5,17-bis (1-hydroxymethyl-1,1-bisdihydroxyphosphoryl)-25,26-propoxycalix[4]arene, C-427 — 5-(1-hydroxymethyl-1,1-bisdihydrooxyphosphoryl)-17-(1-keto-1-dihydroxyphosphorylmethyl)-25,27-dipropoxycalix[4]arene (Fig. 1). C-145 and 4-hydroxymethylenbisphosphonic acid stock solutions (1 mM) were prepared in working buffers. Stock solutions of calix[4]arenes C-296, C-425 and C427 with concentration 1 mM were prepared in distilled water and then were used for working solutions preparing in working buffers.

All inorganic chemicals, amino acids and acetic acid were purchased from Synbias, Ukraine. PAGE chemicals were purchased from Sigma Aldrich, USA.

Methods. Amidolytic activity assay. Plasmin activity was evaluated by amidolytic activity assay. Assessment of amidolytic activity was performed by optical density changes as result of chromogenic substrate S2251 (H-D-Val-L-Leu-L-Lys-p-nitroaniline, Chromogenix, Sweden) cleavage by plasmin or plasminogen, activated by streptokinase (Kabikinase, Pharmacia, Sweden). The reaction mixture contained 0.03 μM plasmin (or 0.03 μM plasminogen) and 0.3 mM S2251 in 50 mM tris buffer solution, pH 7.4 with 150 mM NaCl. The assay was performed in 96-wells plate at 37 °C. The amidolytic activity was determined by measurement of the absorbance at 405 nm using 96-well plate reader (Multiskan Titertek, Finstruments, Finland).

Fibrinolytic activity assay. For the estimation of fibrinolytic activity of plasmin or plasminogen, activated by tissue type plasminogen activator (Actylise, Boeringher Ingelheim, Germany), streptokinase (Kabikinase, Pharmacia, Sweden), the turbidimetric method was applied as described by Bouvier [12]. The final concentration of desAB-fibrin was 0.6 μM, plasmin — 0.02 μM. The rate of fibrin clot lysis by plasmin was calculated as $V=1/t_{50\%}$. Half-time of clot lysis ($t_{50\%}$) was calculated as period of time from the beginning of clot formation to the point when the turbidity of clot was 1/2 from the maximal.

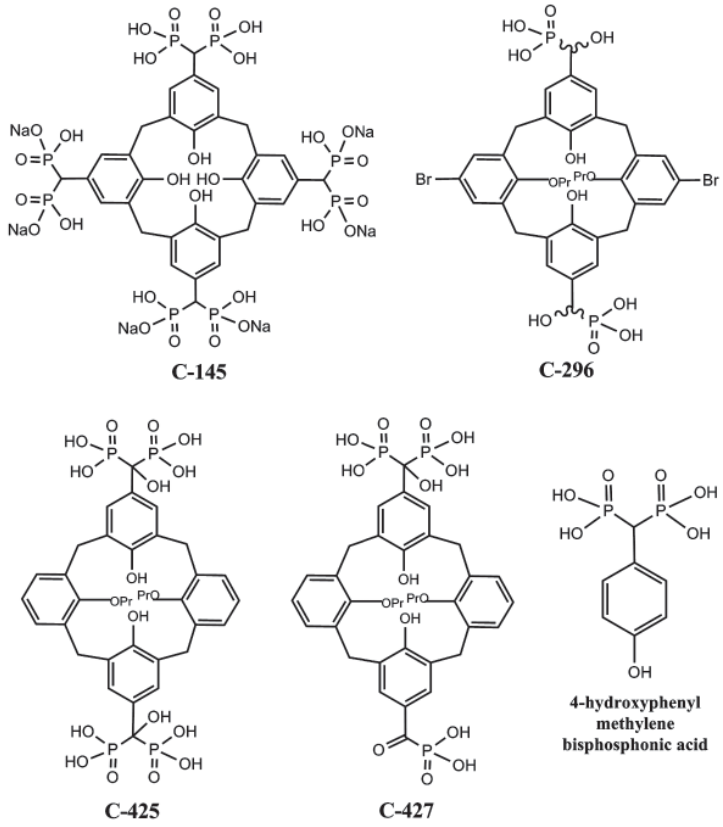

Fig. 1. **Structure of calix[4]arenes C-145, C-296, C-427, C-425 and monomeric molecule 4-hydroxyphenyl methylene bisphosphonic acid**

Caseinolytic activity assay. Casein (Sigma, USA) dissolved in 70 mM PBS pH 7,4 to the final concentration 2% was hydrolyzed by 30 μM plasmin during 30 min at 37 °C. The reaction was terminated by adding of 10% trichloroacetic acid in the 3:2 volume ratio. Precipitated protein was removed by centrifugation at 12,000 rpm and 4 °C for 10 min. The absorbance of the supernatant at 280 nm was measured using a LambdaBio+ spectrophotometer (Perkin Elmer, USA). One unit (U) of enzyme activity was defined as the amount of enzyme required to increase the absorbance at 280 nm by 0.001 AU per minute under the aforementioned assay conditions [13]. Specific caseinolytic activity was calculated as caseinolytic activity per 1 mg of plasmin.

Inhibition constant calculation. For inhibition type determination and inhibition constant calculation Dixon method was applied [14].

Statistical data analysis. Kinetic curves are typical for series of experiment ($n \geq 3$). Data was analyzed using GraphPad Prism 7 software. Enzymes activity is expressed as mean ± SEM. ANOVA Dunnett test was used for P-value assessment where it is applicable. $P < 0.05$ was considered as a level of significance.

Results and Discussion

Calix[4]arenes with various number of phosphonic acid residues inhibit plasmin fibrinolytic activity with different intensity. C-145 — sodium salt of calix[4]arene methylen bisphosphonic acid — demonstrates most effective inhibition of plasmin. At minimal used concentration C-145 (1 μM) decreases clot lysis rate by 46%, whereas maximal (10 μM) totally inactivate plasmin and fibrin is not hydrolyzed. C-425 and C-427 decrease plasmin activity by respectively 1.5% and 17% at 1 μM and by 70% and 77% at 10 μM. C-296 does not affect fibrin clot lysis in concentration range 1 — 5 μM, but at 10 μM decreases lysis rate by 30% (Fig. 2). The data demonstrates that the less phosphonic groups contains calix[4] arene, the less fibrinolytic inhibition activity it has. However, 4-hydroxyphenyl-methylen bisphosphonic acid, which represents 1/4 part of C-145 does not inhibit plasmin fibrinolytic activity at any used concentration. Obviously, full calix[4]arene molecule is necessary for plasmin inhibiting action, probably its spatial "basket" structure provides optimal orientation of negatively charged phosphonic groups.

C-145 has most efficient inhibiting action on fibrinolysis therefore in further we investigated this calix[4]arene interaction with plasminogen/plasmin system proteins.

For the explanation of C-145 action on fibrinolytic activity we have investigated its effect on polymeric fibrin hydrolysis by streptokinase- and tPA-activated plasminogen. The calix[4]arene inhibits fibrin clot lysis by streptokinase- and tPA-activated plasminogen, as well as plasmin, in dose-dependent manner (Fig. 3, *A–C*). At minimal used concentration (1 μM) C-145 decrease the rate of clot lysis by streptokinase- and tPA-activated plasminogen on 40 and 45% respectively (Fig. 3, *D*). At the presence of maximal concentration of C-145 (10 μM) the clot is not hydrolyzed. The same pattern of fibrin clot hydrolysis inhibition for the activated by tPA and streptokinase and plasmin indicates that C-145 interacts with the active enzyme and decline its interaction with physiological substrate — fibrin, resulting in dramatically decreased fibrinolytic activity.

Plasmin in blood circulation catalyzes hydrolysis not only of fibrin, but also of other proteins — fibrinogen, fibronectine, thrombospondine, von Willebrand factor, activates collagenases, induces the generation of bradykinine [1]. Peptide chromogenic substrate assay is used for assessment of non-fibrinolytic hydrolytic activity of plasmin. We investigated amidolytic activity of plasmin against S2251 in the presence of C-145. The

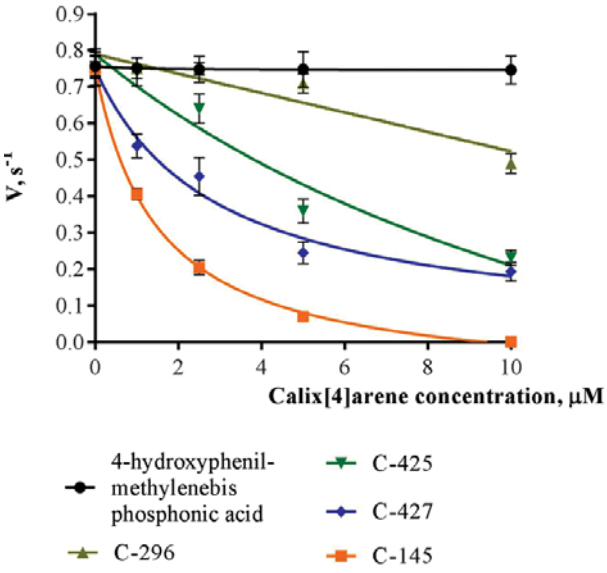

Fig. 2. **Rate of fibrin clot hydrolysis by plasmin in the presence of calix[4]arenes:** reaction rate was calculated as reciprocal value of clot half-lysis time

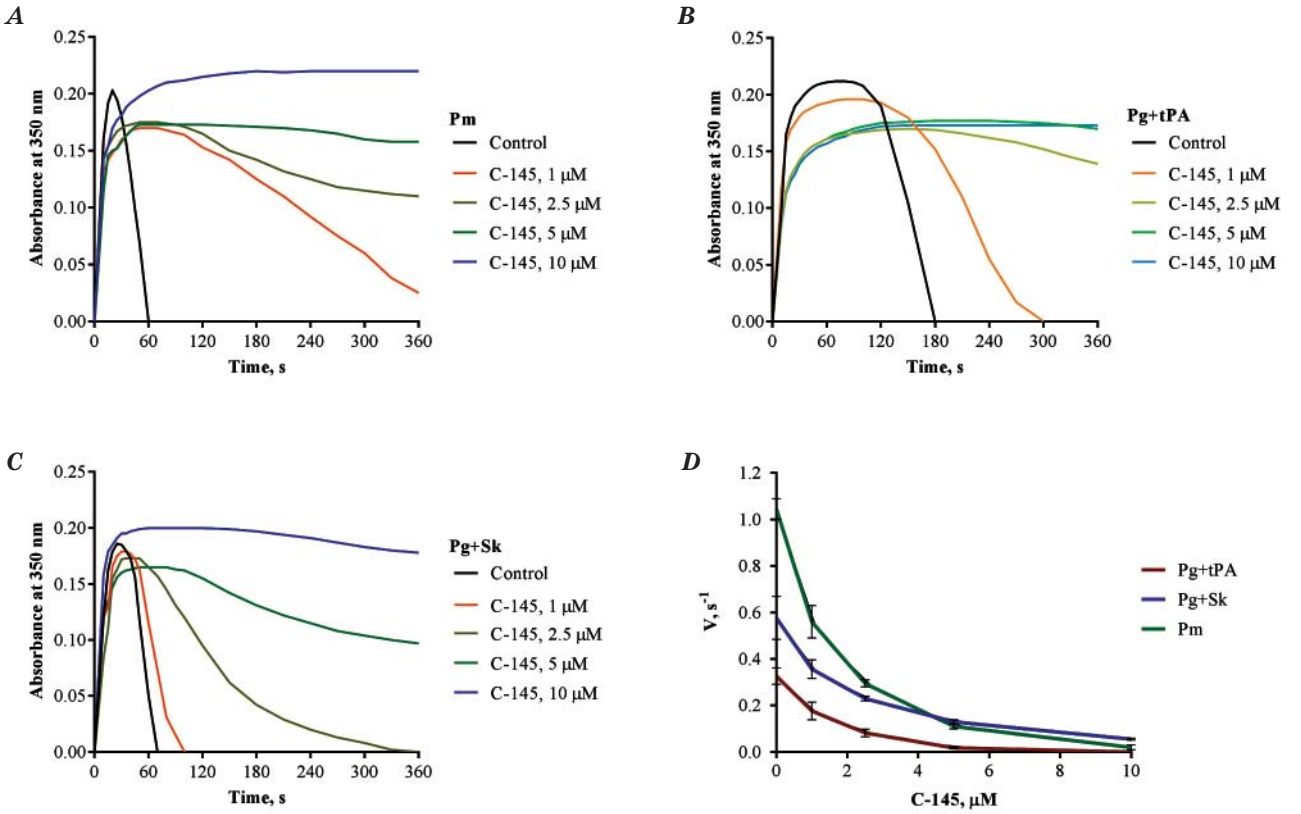

Fig. 3. **desAB fibrin hydrolysis by plasmin and tPA- and streptokinase-activated plasminogen in the presence of calixarene C-145 (1–10 μM):**
A–C — typical kinetic curves; *D* — fibrin hydrolysis reaction rate, represented as reverse value of half-lysis time ($1/t_{50\%}$)

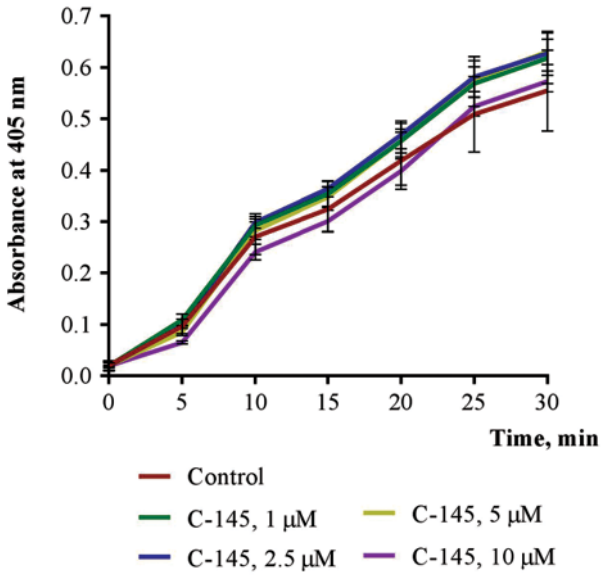

Fig. 4. **Amidolytic activity of plasmin in the presence of calix[4]arene C-145 (concentration range 0–10 μM):**
ANOVA Dunnett test confirms no significant effect of C-145 on chromogenic substrate hydrolysis by the enzyme measured as optical density increase as result of H-D-Val-L-Leu-L-Lys-*p*-nitroaniline formation

calix[4]arene has no effect on chromogenic substrate hydrolysis at any concentration used in the study (Fig. 4).

It should be mentioned that C-296, C-425 and C-427 also do not effect amidolytic activity of plasmin.

Casein is non-specific high molecular weight protein substrate for plasmin and is used for plasmin activity determination. It has linear structure and contains high amount of tyrosine residues. Investigation of casein hydrolysis by plasmin in the presence of C-145 (1–10 μM) have demonstrated that the calix[4] arene does not change plasmin caseinolytic activity.

Due to the absence of inhibiting effect of C-145 on amidolytic and caseinolytic activity of plasmin, the calix[4]arene is probably specific inhibitor of plasmin hydrolytic activity against its physiological substrate fibrin.

To determine the calix[4]arene inhibition constant and inhibition type we have evaluated rate of desAB fibrin lysis by plasmin at different concentration of the inhibitor (0, 1, 2.5, 5 μM) and at two

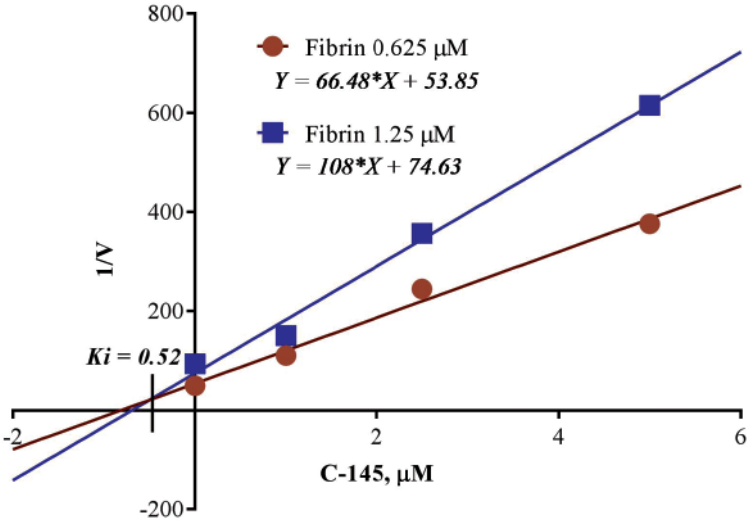

Fig. 5. **Determination of inhibition constant for C-145 delaying of desAB fibrin plasmin-mediated clot lysis using Dixon plot:**
the inhibitor was used in concentration range 0 – 5 μM, substrate — 0.625 and 1.25 μM. K_i is calculated as reverse X coordinate value of lines interception point

concentration of substrate (0.625 and 1.25 μM of fibrin). Obtained data was used for Dixon plots fitting and K_i calculation (Fig. 5). The plot indicates competitive nature of plasmin inhibition by C-145 with inhibition constant K_i = 0.52 μM.

Probably calixarene C-145 forms supramolecular complex with plasmin molecule near the active site and obstructs high molecular weight substrates into the enzyme catalytic pocket, resulting in fibrinolytic, but not amidolytic, activity inhibition.

It is well known, that streptokinase binds to plasminogen kringles 4 and 5 and catalytic domain [15]. Streptokinase binding results in conformational and substrate specificity change of the proenzyme and streptokinase-plasminogen complex do not hydrolyzes fibrin despite of having the proteolytic activity towards plasminogen activation loop [16], as well as towards chromogenic substrate S2251. In catalytic domain of the proenzyme streptokinase occupies positively charged amino acids cluster near active site. We assumed the similar pattern of interaction of streptokinase and C-145 due to the strong negative charge of the calix[4]arene, provided by eight phosphonic groups. To test this hypothesis, we have evaluated the rate of plasminogen activation by streptokinase, using chromogenic substrate S2251. Because of disability of C-145 to inhibit amidolytic activity of plasmin despite of fibrinolysis inhibition, such way is informative for the plasminogen activators activity. As was demonstrated, the calix[4]arene inhibits the proenzyme activation by streptokinase in dose-dependent manner (Fig. 6) probably preventing the interaction between these two proteins. In the presence of 50 μM of C-145 plasmin formation is fully inhibited.

The results demonstrate that calix(4)arene C-145 containing four methylene bisphosphonic acid residues suppresses plasmin fibrinolytic activity, but does not affect the hydrolysis of

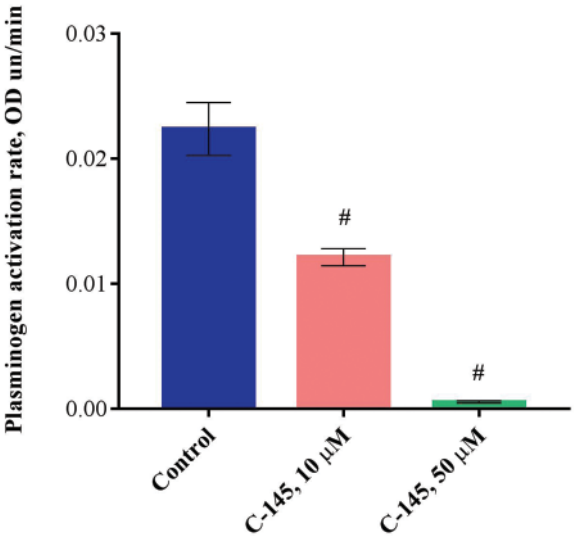

Fig. 6. **Rate of Glu-plasminogen activation by streptokinase in the presence of C-145:**
— $P < 0{,}0001$ compared to control

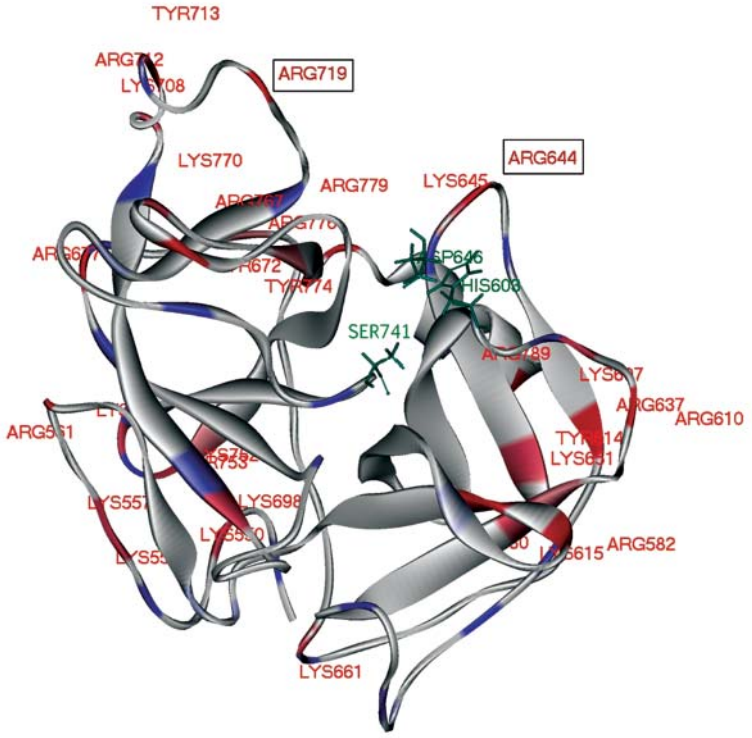

Fig. 7. **Catalytic domain of human plasminogen:**
(figure was generated using Discovery Studio 2016 software and 1DDJ.pdb).
Positively charged amino acids highlighted by red. Amino acids in black boxes are involved in plasminogen-streptokinase interaction.
Catalytic triade (green): His 603, Asp 646, Ser 741

chromogenic peptides and denaturized protein substrates. It indicates that C-145 blocks high molecular weight substrates entering into the enzyme active site. The calix(4)arene structure implies its interaction with positively charged groups. Catalytic domain of plasmin contains 12 arginine and 9 lysine residues clustered near the active site [17] (Fig. 7).

Noncovalent interaction between the calix[4]arene and side chains of these amino acids results in obstruction of plasmin catalytic domain interaction with substrate, which is necessary for the substrate orientation in catalytic pocket. Low molecular weight substrates do not interact with the clusters and reach the catalytic pocket directly, that is why calix[4]arenes demonstrate no inhibition activity towards chromogenic substrate cleavage by plasmin.

Two positively charged amino acids near active site of plasmin — Arg 644 and Arg 719 — are involved in plasminogen-streptokinase complex formation [17]. Blocking of them attenuates streptokinase binding to the proenzyme and plasminogen activation. Prevention of plasminogen activation by streptokinase in the presence of C-145 confirms that phosphonic groups of the calix[4] arene bind to positively charged residues in plasminogen catalytic domain including Arg 644 and Arg 719, obstructing intermolecular interactions.

Calix[4]arenes methylene bisphosphonic acids demonstrate different level of inhibiting action on plasmin fibrinolytic activity. Calix[4] arene C-145 is a most effective plasmin inhibitor due to its structure with four phosphonic acid residues. C-145 delays fibrin clot hydrolysis by plasmin and plasminogen, activated by tissue type activator and streptokinase.

Since C-145 is an effective specific fibrinolysis inhibitor, it is perspective as potential novel pharmaceutical agent for clinical intervention against fibrinolytic system overactivation disorders.

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ВПЛИВ КАЛІКС[4]АРЕНІВ МЕТИЛЕНБІСФОСФОНОВИХ КИСЛОТ НА АКТИВНІСТЬ ПЛАЗМІНУ

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Метою роботи було дослідити інгібувальні властивості калікс[4]аренів, функціоналізованих різною кількістю залишків фосфонової кислоти, стосовно плазміну. Використовували такі методи: турбідиметрію, аналіз ензимної активності за хромогенним субстратом, оцінювання казеїнолітичної активності за вивільненням тирозину з казеїну, метод Діксона для визначення типу та константи інгібування. Було виявлено, що калікс[4]арени С 296, С-425, С-427 та С-145 інгібують лізис фібринового згустку плазміном залежно від дози, і швидкість інгібування пропорційна кількості фосфонових груп. С-145 є найефективнішим інгібітором плазміну (конкурентний механізм, $K_i = 0,52$ мкМ). Проте жоден з досліджених калікс[4]аренів не впливає на амідолітичну та казеїнолітичну активність плазміну, однак інгібує активацію плазміногену стрептокіназою. Ми припускаємо, що механізм селективності калікс[4]аренів до фібринолізу і його здатність перешкоджати взаємодії плазміноген-стрептокінази забезпечується комплексоутворенням між негативно зарядженими фосфоновими групами калікс[4]арену та позитивно зарядженими амінокислотами в екзосайтах активного центру плазміну, що розпізнають субстрат. Таким чином, із досліджених калікс[4]аренів С-145 є найбільш ефективним інгібітором фібринолітичної активності плазміну і перспективним для подальшого вивчення як антифібринолітичний засіб.

Ключові слова: фібриноліз, плазмін, плазміноген, інгібітор плазміну, активація плазміногену, стрептокіназа.

ВЛИЯНИЕ КАЛИКС[4]АРЕНОВ МЕТИЛЕНБИСФОСФОНОВЫХ КИСЛОТ НА АКТИВНОСТЬ ПЛАЗМИНА

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Целью работы было исследование ингибирующих свойств каликс[4]аренов, функционализированных разным количеством остатков фосфоновой кислоты, относительно плазмина. Использовали следующие методы: турбидиметрия, анализ энзимной активности по хромогенным субстратам, оценка казеинолитической активности по высвобождению тирозина из казеина, метод Диксона для определения типа и константы ингибирования. Было обнаружено, что каликс[4]арены С 296, С-425, С-427 и С-145 ингибируют лизис фибринового сгустка плазмином в зависимости от дозы, и скорость ингибирования пропорциональна количеству фосфоновых групп. С-145 является наиболее эффективным ингибитором плазмина (конкурентный механизм, $K_i = 0,52$ мкМ). Однако ни один из исследованных каликс[4]аренов не влияет на амидолитическую и казеинолитическую активность плазмина, но ингибирует активацию плазминогена стрептокиназой. Мы предполагаем, что механизм селективности каликс[4]аренов к фибринолизу и его способность препятствовать взаимодействию плазминоген-стрептокиназы обеспечивается комплексообразованием между отрицательно заряженными фосфоновыми группами каликс[4]арена и положительно заряженными аминокислотами в экзосайтах активного центра плазмина, которые распознают субстрат. Таким образом, из исследованных каликс[4]аренов С-145 является наиболее эффективным ингибитором фибринолитической активности плазмина и перспективен для дальнейшего изучения как антифибринолитическое средство.

Ключевые слова: фибринолиз, плазмин, плазминоген, ингибитор плазмина, активация плазминогена, стрептокиназа.

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DEGRADATION OF FLAVONOIDS BY *Cryptococcus albidus* α-L-RHAMNOSIDASE

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The aim of the work was to investigate the practical using of α-L-rhamnosidase substrate specificity *Cryptococcus albidus*. *p*-Nitrophenyl derivatives of monosaccharides were used to determine the activity and the enzyme specificity. The ability to hydrolyze of natural substrates was evaluated by Davis and high-performance liquid chromatography methods. It was shown that the enzyme exhibited narrow specificity towards the glycon of synthetic substrates and hydrolyzes only *p*-nitrophenyl-α-L-rhamnopyranoside (K_m 4.5 mM) and *p*-nitrophenyl-α-D-glucopyranoside (K_m 10.0 mM). *C. albidus* α-L-rhamnosidase the most active degrades naringin (K_m 0.77 mM), releasing prunin and naringenin. K_m for neohesperidin was 3.3 mM. The efficacy of the naringin hydrolysis in grapefruit and pomelo juice was 98 and 94% in 60 min (40 °C, 2 U/ml). As the result of of green tea and orange juice treatment by α-L-rhamnosidase, there was a decrease in the content of rutin, narirutin and hesperidin, indicating that α-1,2- and α-1,6-linked rhamnose could be cleaved from natural flavonoids. The study shows the possibility of citrus juices and green tea treatment by *C. albidus* α-L-rhamnosidase for the purpose of their taste qualities improvment and obtaining bioavailable flavonoids glucosides.

Key words: *Cryptococcus albidus*, α-L-rhamnosidase, naringin, neohesperidin, rutin, flavonoids, citrus juices, green tea.

Currently, O-glycosyl hydrolases (enzymes able to degrade the glycoside bond) are employed in more and more biotechnological processes, more only to proteases. Most such enzymes are used to treat various plant-derived materials. Among them, an important place belongs to α-L-rhamnosidase (α-L-rhamnoside-rhamnohydrolase — E.C. 3.2.1.40), which splitted off the terminal unreduced α-1,2-, α-1,4- and α-1,6- bound L-rhamnose, present in natural glycoconjugates and synthetic glycosides. Splitting the O-glycoside bond occurs with preservation of the configuration of the anomeric carbon atom (C_1 in the cyclic form of the monosaccharide). Natural substrates for the α-L-rhamnosidases are plant glyscosides, glycolipids, gums, pigments, resins, specific immunopolysaccharides, heteropolysaccharides of bacterial cell walls, saponins, glycoalkaloids, pectins, flavonoids of many plants: grapes, citrus crops, green tea, buckwheat, *Sophora japonica*, *Rosa* sp., *Sorbus* sp., *Prunus* sp., pepper, etc. [1, 2]. Plant bioflavonoids, the most common type of natural polyphenolic compounds, have a special place among possible substrates for α-L-rhamnosidases. Flavonoids incite quite understandable interest, since many studies show their valuable properties for supporting and improving human health; recently, they were used to create certain cardio-vascular drugs and antiviral and immunotropic agents [3]. Yet the bio-accessibility of such flavonoids for humans is rather limited, due to the presence of carbohydrates (rhamnose, glucose and galactose) [4]. The rutinoside component (rhamnose+glucose) of flavonoids in many plant products, was found to hinder their intake in the gut [5]. The strong interest to α-L-rhamnosidase is caused exactly by its ability to split off the terminal rhamnose, thus opening a wide field of possible applications of the enzyme for biodegradation of flavonoids.

The rhamnose binds to glucose at different positions (α-1,2-, α-1,4-, α-1,6 -) in different flavonoid glycosides, and glucose itself has to bind to the aglycone at the third or the seventh carbon. Thus, selective glycosidases

are needed to cleave the glycoside bonds both inside the disaccharide and between the carbohydrate fragment and the flavonoid aglycone. Hence, studying substrate specificity of α-L-rhamnosidase on various substrates is an important stage of developing efficient preparations for the biotransformation of plant polyphenols and their further use. For example, in the pharmaceutical industry, α-L-rhamnosidase is applied to rutin and hesperidin to obtain many medical substances based on the flavonoid glycosides and deglycosylated flavonoids [6]. Using α-L-rhamnosidases in food industry is aimed at improving the quality of drinks (decreasing the bitterness of citrus juices, enhancing the wine aromas) and making food additives (various biopolymers and sweeteners) [7]. As a byproduct of enzymatic hydrolysis of plant glycosides, the rhamnose can be accumulated in large quantities and used afterwards to produce rhamnolipids for cosmetics.

There are reports of a few microbial vigorously α-L-rhamnosidases which have specificity to certain flavonoids. These enzymes of *Aspergillus* and *Penicillium* were highly specific to rutin and naringin [8–11]. The ability to hydrolyze naringin, hesperidin, rutin, narcissin was found for probiotic bacteriae vigorously [12, 13] and bacilli [14]. A few yeast sources were also described of α-L-rhamnosidase with high biotechnologic potential [15].

We isolated the enzyme from yeast *Cryptococcus albidus* and studied some of its physico-chemical, kinetic and catalytic properties [16]. Our current study was aimed at substrate specificity of *C. albidus* α-L-rhamnosidase towards some synthetic and natural substances, and at estimating the enzyme's ability to degrade flavonoids in citrus juices and green tea.

Materials and Methods

α-L-rhamnosidase preparation was obtained from the supernatant of the *C. albidus* cultural liquid by precipitation in ammonium sulfate (to 90% saturation) followed by chromatography on charged and neutral TSK-gels (DEAE-Toyopearl 650-s and Toyopearl HW-60 "Toya Soda" Japan, respectively) [16]. Specific α-L-rhamnosidase activity of the preparation was 12 units/ mg protein.

The glycosidases activities were determined using the synthetic substrates: n-nitrophenyl-α-L-rhamnopyranoside, *n*-nitrophenyl-α- and β-D-galactopyranoside; *n*-nitrophenyl-α- and β-D-glucopyranoside; *n*-nitrophenyl-N-acetyl-β-D-galactopyranoside; *n*-nitrophenyl-N-acetyl-α- and β-D-glucopyranoside; *n*-nitrophenyl-β-D-glucuronide; *n*-nitrophenyl-β-D-xylopyranoside; *n*-nitrophenyl-α-D-mannopyranoside; *n*-nitrophenyl-α-D-fucopyranoside (Sigma-Aldrich, USA).

To assay the glycosidase activity, 0.1 ml of enzyme solution was mixed with 0.2 ml 0.1 M phosphate-citrate buffer (PCB) of pH 5.2 and 0.1 ml 0.01 M solution of the substrate in PCB. The mixture was incubated for 10 min at 37 °C. The reaction was stopped by adding 2 ml 1 M solution of sodium bicarbonate. To control samples, the same components were added, but in reverse. The amount of released *n*-nitrophenol as a result of hydrolysis was determined colorimetrically at 400 nm [17]. One unit of enzyme activity was defined as the amount of the enzyme which hydrolyzed 1 μmol substrate per minute.

The α-L-rhamnosidase activity was followed Davis method[18] using natural substrates (naringin, neohesperidin). Reaction mixture comprised 1 ml 0.05 % naringin or neohesperidin in 0.1 M PCB with pH 5.2 and 1 ml enzyme solution. The mixture was incubated for 60 min at 37 °C. To each aliquot of 0.2 ml, we added 10 ml diethylenglycole and 0.2 ml 4M NaOH. The mixture was kept at room temperature for 10 min, and the intensity of yellow coloring was measured spectrophotometrically at 420 nm. One unit of activity was defined as the amount of enzyme which releases 1 μmol substrate per minute.

Maximal velocity (V_{max}) and Michaelis constant (K_m) were determined by the Lineweaver — Burk plots [19].

Grapefruit, orange and pomelo juices were obtained from fresh fruit. Juice samples were extracted and filtered to exclude seeds and skins. Standard flavonoid solutions (rutin, neohesperidin, naringenin, quercetin, prunin, narirutin, hesperidin) were prepared in concentration of 1 mg in 1 ml 50% ethanol. Enzyme preparation was dissolved in 0.1 M PCB at 1 mg/ml (12 units). 0.1 ml enzyme solution was added to 2 ml juice and incubated at 40 °C for 60 min. Control samples had 0.1 ml 0.1 M PCB+2 ml juice. The reaction was stopped by adding 1 ml anhydrous ethanol to 2 ml of the sample and vigorously mixing. Then, the samples were centrifuged at 10000 rpm for 10 min, the supernatant was filtered and analyzed by high performance liquid chromatography (HPLC).

Green tea leaves were treated in the following way. 1.5 g of tea was boiled for 5 min in 150 ml water with 0.5 ml dimethyl sulfoxide.

Then the samples were processed as above. The control sample had 0.1 ml 0.1 M PCB to 2 ml tea solution.

Quantitative and qualitative flavonoid analysis was done on liquid chromatographer Agilent 1200 HPLC with a diode matrix detector at 280 nm. Samples were injected into column Zorbax SB C18 (2.5×150 mm; 3.5 μm). Thermostat temperature was 27 °C. The calibration curves were built by used the commercial flavonoid preparations (Sigma-Aldrich, USA).

The quantitative and qualitative analysis of flavonoids was carried out in the Center for collective equipment use at the Institute for microbiology and virology of NAS of Ukraine.

All experiments were replicated 5–8 times. Analysis of the data was done using Student's *t*-criterion. The results, presented graphically, were obtained using the Microsoft Exel 2003 software. Values were considered significant at $P < 0.05$.

Results and Discussion

Currently, the enzymatic degradation of carbohydrate-linked flavonoids is considered one of efficient methods to obtain the biologically active substances for pharmaceutics, medicine and food production [2, 3, 6, 7]. Microbial naringinases and α-L-rhamnosidases are able to bioconvert flavonoids safely [8, 9]. There are reports of highly active sources of such enzymes, and their biotechnological potential is studied with regards to substrate specificity [20].

According to literature, various α-L-rhamnosidases are able to hydrolyze the α-1,2, α-1,3-, α-1,4- and α-1,6-glycoside bonds and show higher affinity to plant flavonoids compared to the synthetic analogues [2, 20]. $К_m$ for *n*-nitrophenyl-α-L-rhamnoside is from 0.057 to 2.8 mM, for naringin it is 0.021–1.9 mM, for hesperidin 0.02–1.3 mM, for rutin 0.028–1.44 mM, for quercitrin 0.077–0.89 mM, and for poncirin 0.02–0.93 мМ.

We studied substrate specificity of *C. albidus* α-L-rhamnosidase both for the synthetic *n*-nitrophenyl derivatives of monosaccharides and for the natural flavonoids such as naringin and neohesperidin. α-L-Rhamnosidase of *C. albidus* had higher affinity to naringin and neohesperidin than to synthetic analogues, according to the respective values for $К_m$ and V_{max}/K_m for the hydrolysis of the substances (Table). Such specificity is characteristic for other yeast α-L-rhamnosidases [15, 20]. As to the synthetic derivatives of monosaccharides, it was narrow specific towards glycon; we showed that the enzyme was able to cleave only *n*-nitrophenyl-α-L-rhamnopyranoside and *n*-nitrophenyl-β-D-glucopyranoside (Table).

To evaluate the ability of *C. albidus* α-L-rhamnosidase to biotransform flavonoids we used freshly prepared citrus juices (orange, mandarin, grapefruit and pomelo) as well as green tea. All these drinks have polyphenolic substances which can negatively impact the product taste, or have only limited bioavailability. Thus, large quantities of naringin are the reason why many citrus juices are bitter. They can be removed using naringinases, rutinosidases and α-L-rhamnosidases, which transform naringinin to less bitter prunin and naringenin [1, 7]. The presence of hesperidin and neohesperidin in orange and mandarin juices is the reason why they become cloudy and crystallize. Using the rhamnosidases in their production allows to avoid this and improve the taste. Splitting off the glycon of rutin allows to obtain biologically active isoquercitrin and quercetin, as well as to increase their amount in the product [4, 6, 8–10].

We tested the efficiency of commercial preparations of naringin, neohesperidin, citrus juices and green tea with the *C. albidus* α-L-rhamnosidase. We showed that in all of these cases, the enzyme was able to hydrolyze the flavonoids. α-L-rhamnosidase hydrolysed naringin to prunin and naringenin both in the commercial preparation and in the pomelo and grapefruit juices (Fig. 1, 2). We observed the naringin concentration drop by 98 and 94% of the initial quantity. Prunin production was significantly higher than naringenin production. The latter concentration at the end was 5.4 and 2.5% for grapefruit and pomelo, respectively. Cleaving off of rhamnose and glucose happened gradually as a result of the α-L-rhamnosidase and β-glucosidase activity of the preparation. Also, one-stage cleaving off of the disaccharide rutinoside did not occur. Based on the results of the purification of *C. albidus* α-L-rhamnosidase [16] and the $К_m$ та V_{max}/K_m for the hydrolysis of *n*-nitrophenyl-α-L-rhamnopyranoside and *n*-nitrophenyl-β-D-glucopyranoside, we suppose that this is one of the same enzyme — an α-L-rhamnosidase with nonspecific β-glucosidase activity. Another term for such enzymes is naringinase [7, 14, 20].

Glycoside removal in commercial preparations of naringin and neohesperidin also occurred fairly fast. Thus, after 60 min the flavonoid amount dropped from 500 μg/ml to 20 and 100 μg/ml, respectively. We also noticed that the α-L-rhamnosidase of

Substrate specificity of *C. albidus* α-L-rhamnosidase

Substrate	Bond type	K_m, мМ	V_{max}, µmol/min/mg	V_{max}/K_m
n-nitrophenyl-α-L-rhamnopyranoside	α-1	4.5	15	3.3
n-nitrophenyl-β-D-glucopyranoside	β-1	10	5	0.5
n-nitrophenyl-α-D-galactopyranoside	α-1	–	0	–
n-nitrophenyl-β-D-galactopyranoside	β-1	–	0	–
n-nitrophenyl-N-acetyl-α-D-glucopyranoside	α-1	–	0	–
n-nitrophenyl-N-acetyl-β-D-glucopyranoside	β-1	–	0	–
n-nitrophenyl-N-acetyl-β-D-galactopyranoside	β-1	–	0	–
n-nitrophenyl-β-D-glucuronide	β-1	–	0	–
n-nitrophenyl-α-D-glucopyranoside	α-1	–	0	–
n-nitrophenyl-α-D-fucopyranoside	α-1	–	0	–
n-nitrophenyl-β-D-xylopyranoside	β-1	–	0	–
n-nitrophenyl-α-D-mannopyranoside	α-1	–	0	–
Naringin	α-1.2	0.77	36.0	46.8
Neohesperidin	α-1.2	3.3	10.0	3.1

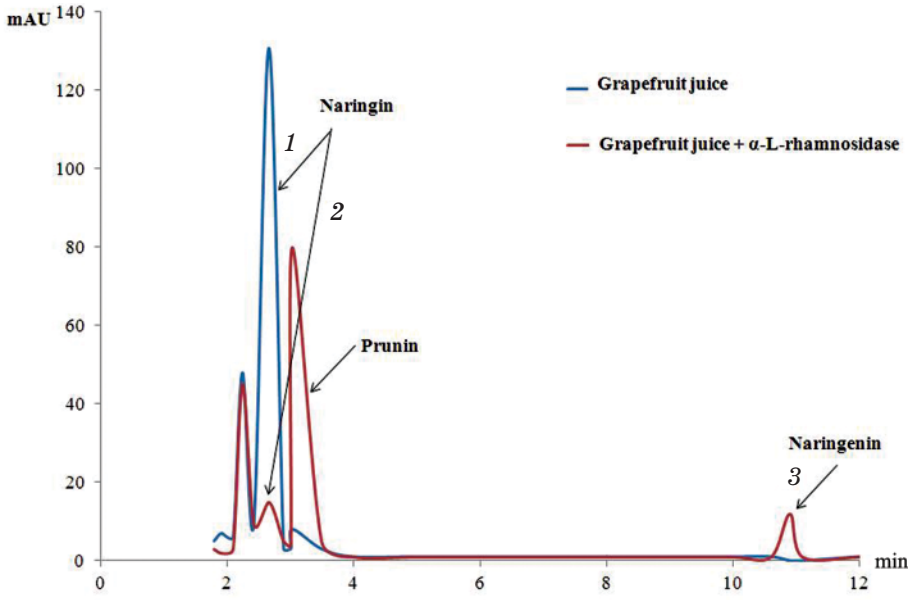

Fig. 1. **Naringin hydrolysis in grapefruit juice by the action of *C. albidus* α-L-rhamnosidase (40 °C, 60 min), determined by HPLC:**
naringin concentration: *1* — 255 µg/ml; *2* — < 5 µg/ml; naringenin concentration *3* — 14 µg/ml

C. albidus actively hydrolyzed narirutin, naringin and hesperidin in orange juice (Fig. 3), similarly to α-L- rhamnosidase of *Aspergillus aculeatus* [9]. We also noted lower amount of rutin in green tea after enzyme treatment (Fig. 4).

Thus, by specificity of action and the ability to cleave off α-bound rhamnose of natural and synthetic substrates, α-L-rhamnosidase of *C. albidus* is close to α-L-rhamnosidases and naringinases of *Penicillium decumbens*, *Aspergillus niger*,

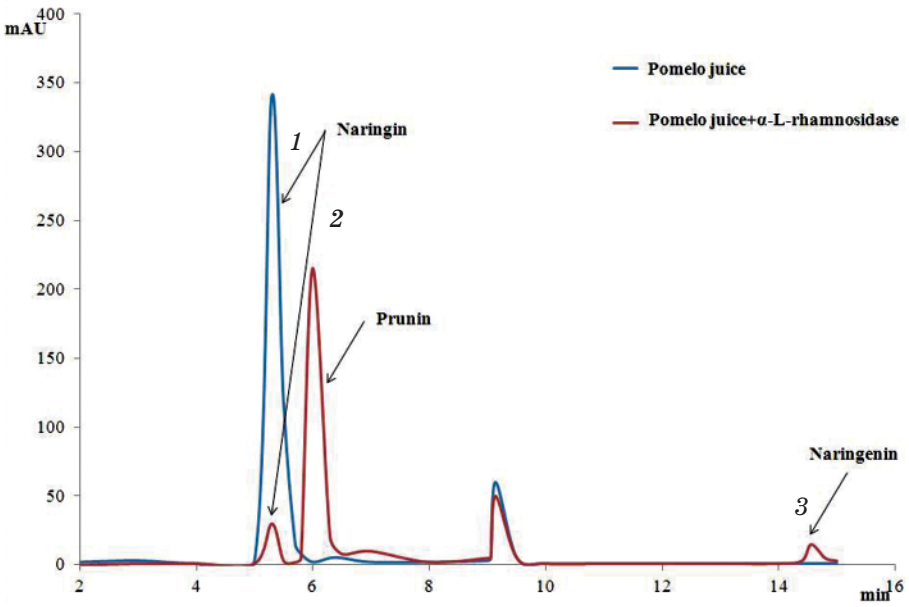

Fig. 2. **Naringin hydrolysis in pomelo juice by the *C. albidus* α-L-rhamnosidase (40 °C, 60 min), determined by HPLC:**
naringin concentration: *1* — 915 μg/ml; *2* — < 60 μg/ml; naringenin concentration: *3* — 24 μg/ml

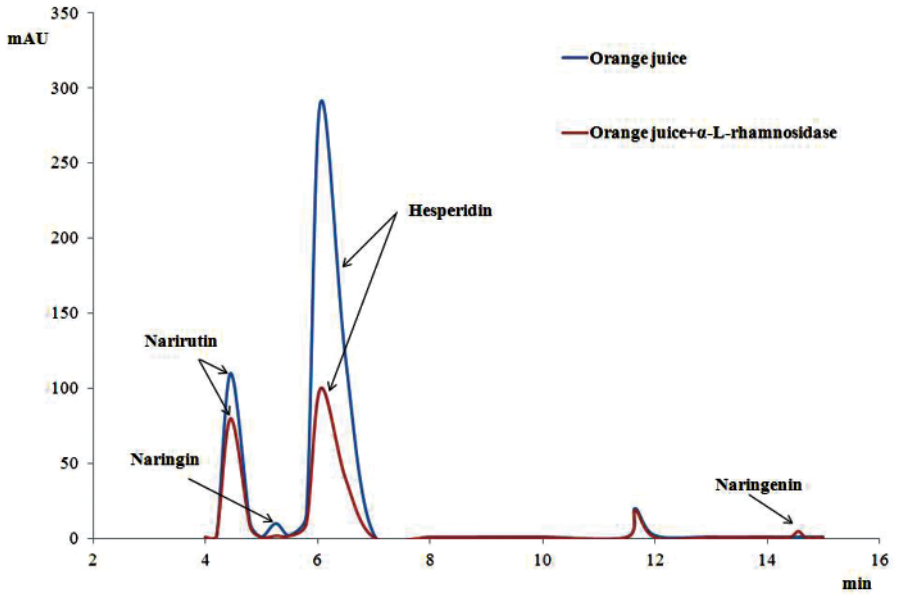

Fig. 3. **Concentration of naringin, naringenin, hesperidin and narirutin in orange juice before and after *C. albidus* α-L-rhamnosidase treatment:**
40 °C, 60 min

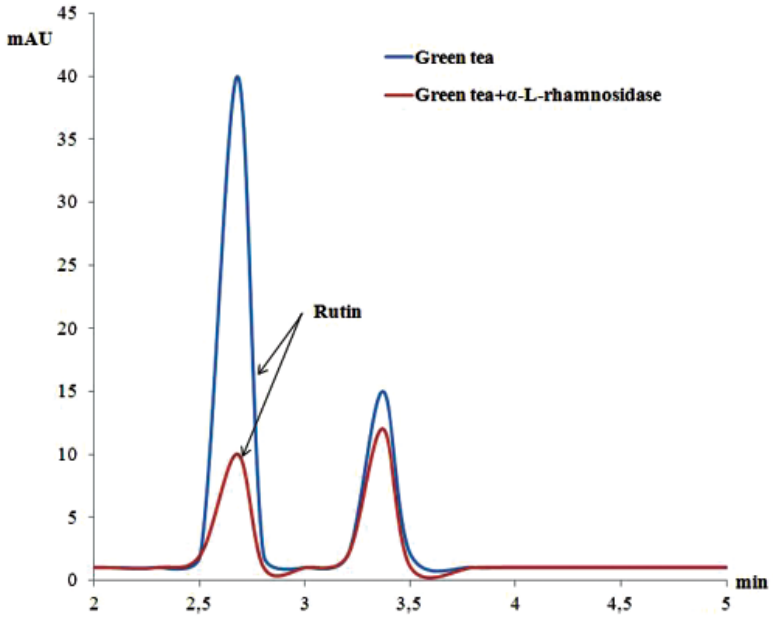

Fig. 4. **Rutin concentration in green tea before and after treatment with *C. albidus* α-L-rhamnosidase:** 40 °C, 60 min, HPLC

Aspergillus aculeatus, *Cryptococcus laurentii* [20], but its activity is 1.5–2 times higher. We also showed that α-L-rhamnosidase of *C. albidus* cleaved the naringin of citrus juices into prunin and naringenin relatively fast. The glycoside removal efficiency was 94 and 98%, respectively. The enzyme showed ability to hydrolyze naringin, neohesperidin, narirutin, hesperidin and rutin, evidence of the high potential of *C. albidus* α-L-rhamnosidase for use in the industries of juice production and to obtain biologically active flavonoids.

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ДЕГРАДАЦІЯ ФЛАВОНОЇДІВ α-L-РАМНОЗИДАЗОЮ *Cryptococcus albidus*

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Метою роботи було дослідити можливості практичного застосування субстратної специфічності α-L-рамнозидази *Cryptococcus albidus*. Для визначення активності та специфічності дії ензиму використовували *n*-нітрофенільні похідні моносахаридів. Здатність гідролізувати природні субстрати оцінювали методами Davis і високоефективної рідинної хроматографії. Встановлено, що ензим виявляє вузьку специфічність щодо глікону синтетичних субстратів, гідролізує тільки *n*-нітрофеніл-α-L-рамнопіранозид ($К_m$ 4,5 мМ) та *n*-нітрофеніл-β-D-глюкопіранозид ($К_m$ 10 мМ). Найефективніше α-L-рамнозидаза *C. albidus* деградувала нарингін ($К_m$ 0,77 мМ), вивільнюючи прунін та нарингенін. $К_m$ для неогесперидину дорівнювала 3,3 мМ. Ефективність гідролізу нарингіну грейпфрутового та помелового соку становила 94 та 98% за 60 хв (40 °С, 2 од/мл). У результаті оброблення зеленого чаю та апельсинового соку відзначалося зменшення вмісту рутину, нарирутину та гесперидину, що свідчить про здатність α-L-рамнозидази відщеплювати α-1,2- та α-1,6-зв'язану рамнозу від природних флавоноїдів. Таким чином, показано ефективність використання α-L-рамнозидази *C. albidus* для гідролізу флавоноїдів цитрусових соків та зеленого чаю з метою поліпшення їхніх смакових властивостей та отримання біодоступних глюкозидів флавоноїдів.

Ключові слова: *Cryptococcus albidus*, α-L-рамнозидаза, нарингін, неогесперидин, рутин, флавоноїди, цитрусові соки, зелений чай.

ДЕГРАДАЦИЯ ФЛАВОНОИДОВ α-L-РАМНОЗИДАЗОЙ *Cryptococcus albidus*

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Целью роботы было исследовать возможности практического применения субстратной специфичности α-L-рамнозидазы *Cryptococcus albidus*. Для определения активности и специфичности действия энзима использовали *n*-нитрофенильные производные моносахаридов. Способность гидролизовать природные субстраты оценивали методами Davis и высокоэффективной жидкостной хроматографии. Установлено, что энзим проявляет узкую специфичность относительно гликона синтетических субстратов и гидролизует только *n*-нитрофенил-α-L-рамнопиранозид ($К_m$ 4,5 мМ) и *n*-нитрофенил-β-D-глюкопиранозид ($К_m$ 10 мМ). Наиболее активно α-L-рамнозидаза *C. albidus* деградирует нарингин ($К_m$ 0,77 мМ), высвобождая прунин и нарингенин. $К_m$ для неогесперидина составила 3,3 мМ. Эффективность гидролиза нарингина в грейпфрутовом и помеловом соке составила 94 и 98% за 60 мин (40 °С, 2 ед/мл). В результате обработки α-L-рамнозидазой зеленого чая и апельсинового сока отмечалось снижение содержания рутина, нарирутина и гесперидина, что свидетельствует о способности отщеплять α-1,2- и α-1,6-связанную рамнозу от природных флавоноидов. Таким образом, показана эффективность использования α-L-рамнозидазы *C. albidus* для обработки цитрусовых соков и зеленого чая с целью улучшения их вкусовых качеств и получения биодоступных глюкозидов флавоноидов.

Ключевые слова: *Cryptococcus albidus*, α-L-рамнозидаза, нарингин, неогесперидин, рутин, флавоноиды, цитрусовые соки, зеленый чай.

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AMPHIBIAN SKIN SECRETIONS: A POTENTIAL SOURCE OF PROTEOLYTIC ENZYMES

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The aim of the work was to study the protein content and proteolytic activity of the skin glands secretions of 10 the most common types of amphibians on the territory of Ukraine such as *B. bombina, B. variegata, B. bufo, B. viridis, R. temporaria, P. ridibundus, P. esculentus, P. fuscus, S. salamandra*, as well as the hybrid of *B. bombina* and *B. variegata* species. It was shown that the skin secretions of the studied amphibians contained a wide range of proteins with a molecular weight in the range from 8 to 150 kDa. By enzyme electrophoresis using gelatin, fibrinogen and collagen as substrates, it was found that they contained proteinases that differ in substrate specificity. It was revealed that the skin glands secretions of *B. bombina, S. salamander* species, as well as the hybrid of *B. bombina* and *B. variegata* species were characterized by the increased protein content with gelatinase and collagen activity.

Key words: amphibians, skin gland secretions, proteolytic activity.

Amphibians are recognized as a rich source of bioactive compounds with valuable biotechnology potential and according to their availability and accessibility they are supposed to be a superior raw material. The molecules derived from amphibian skin secretions have various activities that determine their profound applications in chemical and medical industries. In this regard, one of the main objectives of modern biotechnology is the search for new potential biologically active compounds of natural origin. Since their effectiveness and safety cannot be compared with chemically synthesized compounds, which characterized by a great number of side effects and unpredictable actions, they might have beneficial application in the science and industry.

The promising raw materials that contain different bioactive molecules are various plants, marine invertebrates and reptiles. In the last two decades scientists drew their attention to amphibians as potential objects for biochemical studies and industrial purposes [1]. The great number of researches indicate that the amphibian glandular secretion is a rich source of various molecules with cardiotonic [2, 3,], antidiabetic [4], immunomodulatory [5], antimicrobial [6, 7] and antiviral [8, 9] activities. It has also been established that they have sedative [10] and analgesic effects [11]. Moreover, considering the availability and accessibility of some species of amphibians the further study of the effects of the compounds from their skin secretions is very relevant.

In most of the cases the wide range of the effects of the components of skin secretions are associated with proteolytic activities. Proteolytic enzymes are capable of hydrolyzing peptide bonds in proteins and have great medical and pharmaceutical importance due to their key role in biological processes, such as: in digestion of food proteins, protein turnover, cell division, blood-clotting cascade, signal transduction, processing of polypeptide hormones, apoptosis and the life-cycle of several disease-causing organisms including the replication of retroviruses [12]. Alongside proteases are extensively applied enzymes in

several sectors of industry and biotechnology and numerous research applications require the use of them [1].

Although the territory of Ukraine is dwelled by numerous amphibians, there are a few studies concerning the nature and properties of the biologically active compounds of their skin secretion. So, the purpose of this work is to examine the presence of proteolytic enzymes in the skin secretions of the most common Ukrainian species of amphibians and to evaluate the gelatinolytic, fibrinogenolytic and collagenolytic activities to create a background for further investigations of amphibian secretions and for studying of their pharmaceutical potency.

Materials and Methods

Collection of frog skin secretions

There are a great number of ways of collection of the crude skin secretions that are shown in the studies, but most of them are inhumane. They usually include lethal release of the venoms, when the skin of animals that previously were subjected to decapitation, is removed, dried and ground to powder consistency for further use. Another lethal variant is to place a frog in a flask with anhydrous ether, which stimulates the secretion of the skin poison that is washed from the surface of the animal with deionized water. There are also some modern non-lethal methods, which include the usage of an electric current that causes synchronous release of toxic secretions from the glands or the stimulation of poison release by chemical injection [13]. In our research, we have used safe methodological approach, which allows us to use amphibians the unlimited number of times.

Adult pubescent specimens (both sexes) of *Bombina bombina* ($n = 20$), *Bombina variegata* ($n = 20$), *Bufo bufo* ($n = 15$), *Bufotes viridis* ($n = 10$), *Rana temporaria* ($n = 10$), *Pelophylax ridibundus* ($n = 8$), *Pelophylax esculentus* ($n = 5$), *Pelobates fuscus* ($n = 7$), *Salamandra salamandra* ($n = 2$) and hybrid of *Bombina bombina* and *Bombina variegata* ($n = 5$) were collected outdoors in Kyiv region of Ukraine. The crude skin secretions were obtained by washing the skin with ultrapure water beyond mechanical stimulation of skin glands. Water solutions of skin secretions of all species were centrifuged at 3000 rpm for 15 min to remove debris. The supernatants were lyophilized (TestarLyoQuest) and kept at 4 °C till use.

Samples preparation

The samples of lyophilized skin secretions were resuspended in Tris-buffered saline (TBS), pH 7.4 (30 mg of dried material/ml) and centrifuged at 7000 g for 15 min. Protein concentration in supernatant was assayed by Bradford method [14], using bovine serum albumin as a standard. Samples for sodium dodecyl sulfate polyacrylamaide gel electrophoresis (SDS-PAGE) and for zymography assay were mixed in equal volumes with the standard SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% sucrose, and 0.002% bromophenol blue) without heating.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SDS-PAGE of crude skin secretions was carried out using 4% (w/v) stacking gel and 12% (w/v) separating gel [15]. SDS-PAGE was performed using Mini-Protean Tetra System (Bio Rad, USA) at 19 mA for stacking and 36 mA for separating gels. The volume of sample applied per line was 15 µl. The gels were stained with 2.5% coomassie brilliant blue R-250 in 10% (v/v) ethanol, 10% (v/v) acetic acid, 15% (v/v) isopropanol and the background of the gel was destained with 7% (v/v) acetic acid for 30 min. Apparent molecular weights of proteins were estimated using protein calibration mixture (Bio Rad, USA) containing myosin, β-galactosidase, phosphorylase b, serum albumin, ovalbumin (Mr 97; 66; 45; 31; 21; 14 kDa).

Zymography

Zymography was done according to the method Ostapchenko et al [16]. Separating gel (12% w/v) was polymerized in the presence of gelatin (1 mg/ml), fibrinogen (1 mg/ml) or collagen (1 mg/ml). The volume of samples applied to the gel was 15 µl per line. After electrophoresis, the gels were incubated for 30 min at room temperature on a rotary shaker in 2.5% Triton X-100. Then the gels were washed with distilled water to remove Triton X-100 and incubated in 50 mM Tris-HCl (pH 7.4) at room temperature for 12 hours. The gels were stained with 2.5% coomassie brilliant blue R-250 in 10% (v/v) ethanol, 10% (v/v) acetic acid, 15% (v/v) isopropanol for 30 min and then destained. The digested bands were visualized as the nonstained regions of the zymogram gel.

Calculation of the results

TotalLab 2.04 program was used to analyze the resultant electrophorograms. The

represented electrophorograms and zymograms are typical for the series of the repeated experiments (at least three in each series).

Results and Discussion

Amphibians' glandular skin secretions are a rich source of potent biologically active compounds, many with high potential for therapeutic drug development [17]. While voluminous researches concerning the chemical structure and properties of crude skin secretions have been made all over the world [18], relatively little is known about the protein composition and biological activities of glandular secretion of different families of amphibians that represent the batrahofauna of Ukraine.

Therefore, on the first stage of our work we wanted to get information about the protein composition of glandular skin secretions of the most common Ukrainian species of amphibians. To achieve this the SDS-PAGE analysis was performed. The typical electrophorograms of crude skin secretions are shown on the Fig. 1. The results of electrophoretic protein separation revealed the presence of proteins with molecular weights ranging from 6 to 149 kDa. It indicates that the crude skin secretions of studied amphibian species are characterized by a wide range of proteins with different molecular weights and alongside confirm a diverse protein composition of all studied secretions.

To define the exact molecular weights of identified protein fractions the electrophorograms were analyzed using the TotalLab 2.04 program (Table). It was shown that different representatives of one type of species had similar protein composition. Thus, five common proteins were observed on the electrophoretic profile of crude skin secretion of amphibian species of Bombina family. The differences between these two studied secretions were only in two proteins (31 and 102 kDa), which were present in crude skin secretion of *B. variegata*. Noteworthy is that the protein composition of the hybrid of *B. bombina* and *B. variegata*, was almost identical to *B. variegata*. The protein composition of the representatives of the Bufo family had more pronounced differences. Even though all proteins that were discovered within this family had low molecular weight, their amount varied. Therefore, in *B. bufo* crude skin secretion five proteins ranging from 29 to 72 kDa was identified and nine proteins ranging from 8 to 68 kDa was observed in *B. viridis* skin venom. The protein composition of two representatives of Pelophylax family was also similar, except two proteins with molecular weights 31 kDa and 149 kDa, which were present in *P. ridibundus*. The largest number of proteins that were observed among the studied amphibian species was identified in *R. temporaria*. It was found eleven low molecular proteins ranging from 11 to 64 kDa in its glandular skin secretion. Only three proteins 18, 31 and 46 kDa were observed in *P. fuscus*. It was also identified six proteins in glandular skin secretions of *S. salamander* ranging from 10 to 60 kDa.

The variety of proteins with different molecular weights that are present in the crude skin secretions of studied amphibian species indicates the presence of different molecules

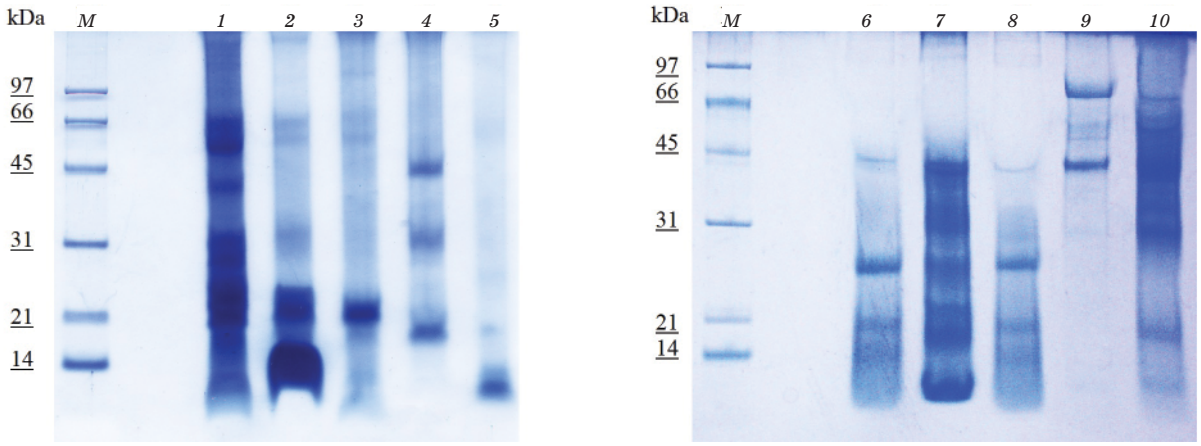

Fig. 1. **Typical electrophoregram of crude skin secretions of studied amphibian species:**
1 — *B. bombina*; *2* — *B. variegata*; *3* — hybrid of *B. bombina* and *B. variegata*; *4* — *B. bufo*; *5* — *B. viridis*; *6* — *R. temporaria*; *7* — *P. ridibundus*; *8* — *P. esculentus*; *9* — *P. fuscus*; *10* — *S. salamander*; *M* — markers of molecular weight

The molecular weights (MW) of proteins that are present in crude skin secretions of studied amphibian species

Amphibian species	MW, kDa
Bombina bombina	7; 14; 20; 27; 42.
Bombina variegata	7; 17; 22; 26; 31; 40; 102.
Hybrid of *B. bombina* and *B. variegata*	6; 13; 19; 27; 29; 41; 99.
Bufo bufo	29; 41; 48; 52; 72.
Bufotes viridis	8; 18; 29; 32; 35; 40; 50; 56; 68.
Rana temporaria	11; 17; 20; 22; 24; 28; 31; 35; 42; 51; 64.
Pelophylax ridibundus	14; 22; 24; 31; 54; 66; 109; 149.
Pelophylax esculentus	11; 18; 22; 54; 69; 115.
Pelobates fuscus	18; 31; 46.
Salamander salamander	10; 19; 26; 30; 38; 60.

that could have biological significance and might be a source of different types of enzymatic activities. The presence of proteins with molecular weight that is lower than 100 kDa especially binds attention, since it is known that most of the proteolytic enzymes have molecular weight up to 100 kDa.

One of the simplest and the most sensitive visual methods of the detection of active proteases in the biological material is zymography method [19]. In this methodological approach the gels polymerize in the presence of the corresponding substrate proteins. And using different polymerized substrates we can identify the presence or absence of proteolytic enzymes.

In this research gelatin, fibrinogen and collagen were used as substrates to evaluate the proteolytic potential of crude skin secretions of studied amphibian species. Our aim was to test the substrate specificity of proteolytic enzymes and to identify the presence of gelatinolytic, fibrinogenolytic and collagenolytic activities.

The typical zymograms of the detection of gelatinolytic, collagenolytic, and fibrinogenolytic activities are shown on Fig. 2. The appearance of the light digested zones of hydrolysis was due to the manifestation of enzymatic activity and indicated the presence of active proteolytic enzymes with the substrate specificity in the studied amphibian crude skin secretions. The active proteins trypsin (24 kDa) and plasmin (84 kDa) were used to identify the approximate molecular weights of active compounds.

Gelatin is considered as a common substrate which helps to study the overall proteolytic activity and usually used to pre-evaluate the presence of active forms of enzymes. According to the results the components of the crude skin secretions of *B. bombina*, *B. variegata*, the hybrid of *B. Bombina* and *B. variegata*, *B. viridis, P. esculentus* and *S. Salamander* had pronounced gelatinolytic activity. Most of the light digested areas corresponds to fractions of proteins with molecular weight up to 70 kDa. Whereas it was not observed expressed gelatinolytic activity in the study of the components of crude skin secretions of other amphibian species.

Collagen and fibrinogen are substrates with high specificity and the small amount of enzymes are capable to hydrolyse them. On the other hand, using these substrates can help us to identify and detail the proteolytic enzymes action.

While studying the zymograms of crude skin secretions with fibrinogen as a substrate, the total fibrinogenolytic activity was insignificant and the hydrolysis zone was identified only in the regions that corresponds to the fractions of proteins with high molecular weight. The light digested zones correspond to the pronounced fibrinogenolytic activity and were revealed on the zymograms of the crude skin secretions of *B. bombina*, *S. salamander* and the hybrid of *B. bombina* and *B. variegate*.

Generally, the true fibrinogenolytic enzymes have a molecular weight in the range from 20 to 60 kDa, but the enzymes with both lower and higher molecular weights are also known [20]. The presence of high molecular weight proteolytic enzymes with specificity to fibrinogen might be due to the inclusion of the enzymes in the complexes.

According to the results of zymography of crude skin secretions using collagen as a substrate, it was revealed the presence active hydrolytic enzymes that are capable to cleave collagen. Moreover, the clearly defined zones of hydrolysis were not detected, since the collagenolytic activity

with different degree of severity was noted throughout the length of the tracks.

Enzymes with collagenolithic activity are very interesting as they have a lot of practical application in medicine, biotechnology and food industry. Based on collagenolithic enzymes several medicines have already been developed to treat wounds, burns and other skin lesions [21]. The new types of collagenolytic enzymes are a promising material for the development of more specialized drugs [22].

Thus, considering the obtained results, we can state that the crude skin secretions of studied amphibian species have a pronounced protease activity with specificity to different substrates. The components of crude skin secretions of *B. bufo* and *R. temporaria* had the least evident gelatinolytic, fibrinogenolytic

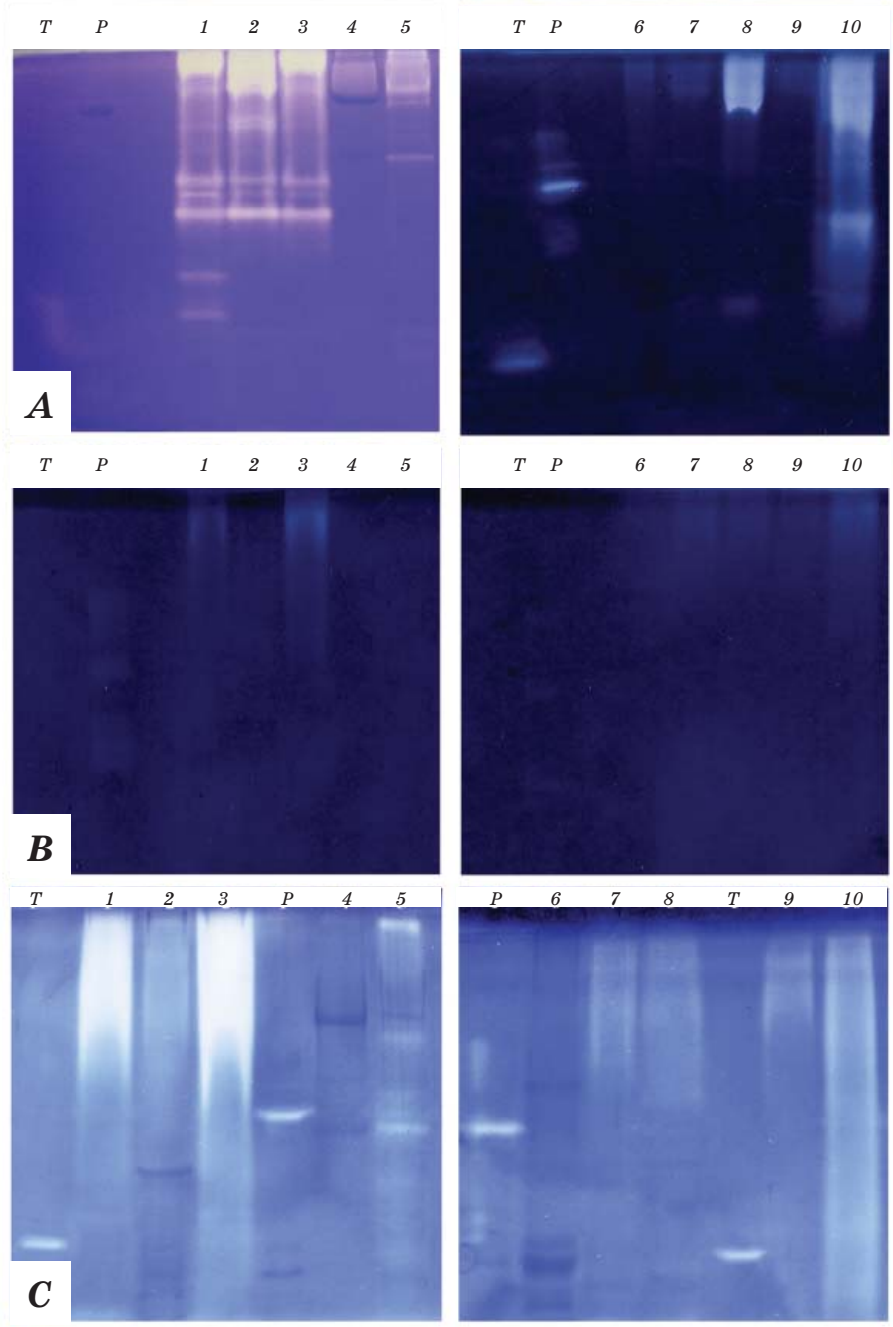

Fig. 2. **Typical zymograms of crude skin secretions using gelatin (*A*), fibrinogen (*B*) and collagen (*C*) as substrates:**
1 — *B. bombina*; *2* — *B. variegata*; *3* — hybrid of *B. bombina* and *B. variegata*; *4* — *B. bufo*; *5* — *B. viridis*; *6* — *R. temporaria*; *7* — *P. ridibundus*; *8* — *P. esculentus*; *9* — *P. fuscus*; *10* — *S. salamander*; *T* — trypsin (24 kDa) and *P* — plasmin (84 kDa)

and collagenolytic activities, whereas the crude skin secretions of *B. bombina*, *S. Salamander* and the hybrid of *B. Bombina* and *B. variegata* characterized by the most expressive activities and, what is worth noting, some zones of hydrolysis on the zymograms with gelatin, fibrinogen and collagen used as substrates coincided. This can be the evidence of the presence of enzymes that simultaneously have two or three kinds of activities. Further investigations concerning the identification of other biologically active compounds in the skin secretions of amphibians and their characterization are required.

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СЕКРЕТИ ШКІРНИХ ЗАЛОЗ АМФІБІЙ — ПОТЕНЦІЙНЕ ДЖЕРЕЛО ПРОТЕОЛІТИЧНИХ ЕНЗИМІВ

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Метою роботи було вивчити вміст протеїнів та протеолітичну активність секретів шкірних залоз 10 найпоширеніших на території України видів амфібій: *B. bombina, B. variegata, B. bufo, B. viridis, R. temporaria, P. ridibundus, P. esculentus, P. fuscus, S. salamandra*, а також гібриду видів *B. bombina* та *B. variegata*. Показано, що секрети шкірних залоз досліджуваних видів містять широкий спектр протеїнів з молекулярною масою від 8 до 150 кДа. Методом ензимелектрофорезу з використанням желатину, фібриногену та колагену як субстратів виявлено, що вони містять протеїнази, які відрізняються за субстратною специфічністю. Встановлено, що секрети шкірних залоз видів *B. bombina*, *S. salamander*, а також гібриду видів *B. bombina* та *B. variegata* характеризуються підвищеним вмістом протеїназ із желатиназною та колагеназною активністю.

Ключові слова: амфібії, секрети шкірних залоз, протеолітична активність.

СЕКРЕТЫ КОЖНЫХ ЖЕЛЕЗ АМФИБИЙ — ПОТЕНЦИАЛЬНЫЙ ИСТОЧНИК ПРОТЕОЛИТИЧЕСКИХ ЭНЗИМОВ

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Целью работы было изучить содержание протеинов и протеолитическую активность секретов кожных желез 10 самых распространенных на территории Украины видов амфибий: *B. bombina, B. variegata, B. bufo, B. viridis, R. temporaria, P. ridibundus, P. esculentus, P. fuscus, S. salamandra*, а также гибрида видов *B. bombina* и *B. variegata*. Показано, что секреты кожных желез исследуемых видов содержат широкий спектр протеинов с молекулярной массой от 8 до 150 кДа. Методом энзимэлектрофореза с использованием желатина, фибриногена и коллагена как субстратов обнаружено, что они содержат протеиназы, которые отличаются по субстратной специфичности. Установлено, что секреты кожных желез видов *B. bombina, S. salamander*, а также гибрида видов *B. bombina* и *B. variegata* характеризуются повышенным содержанием протеиназ с желатиназной и коллагеназной активностью.

Ключевые слова: амфибии, секреты кожных желез, протеолитическая активность.

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ISOLATION OF PURE CULTURES IRON- AND MANGANESE-OXIDIZING BACTERIA FROM RAPID FILTERS

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The aim of the research was the isolation from drinking water the pure cultures of iron- and manganese-oxidizing microorganisms with further assessment of their efficacy to remove these contaminants on rapid filters. To assess the effectiveness selected strains were grown on the solid nutrient medium; the suspension was prepared and was treated to zeolite loading. Ten pure cultures of iron- and manganese-oxidizing bacteria were isolated and identified as 6 genuses: *Siderocapsa, Leptothrix, Sphaerotillus, Galionella, Metallogenium, Hyphomicrobium*. Comparison the efficiency of genuses *Leptothrix, Sphaerotillus, Metallogenium* has shown that under conditions of these experiments *Leptothrix* more effectively removed iron and manganese at low concentrations in model solution.

Key words: iron- and manganese-oxidizing microorganisms, rapid filters, zeolite loading.

Iron compounds related to one of the common components in natural waters in Ukraine. Groundwater with iron is commonly found in almost all regions, sometimes the concentration of iron reaching more than 20–30 mg/dm^3. Iron removal from drinking water in low concentration does not cause difficulties, as long as it does not concern their high concentrations.

Well known that methods (both chemical and biological) for removal iron and manganese compounds differ in the degree of technological reliability, efficiency, ease of use, etc. [1–4]. However, the progressive development of biotechnological processes, and most importantly empower their implementation, does biotechnological methods as one of the most promising areas of water purification compounds of iron and manganese.

The basis of industrial biotechnology processes is accountability biological agent composition, so it is important to get a pure (in species belonging) culture. The manufacturability and efficiency of microorganisms are critical parameters for developing the technology of iron and manganese removal.

That is why the aim of our work was the identification of pure cultures of iron- and manganese-oxidizing microorganisms from drinking water with further assessment of their efficacy to remove these contaminants on rapid filters with zeolite filtration media. Rapid filters are widely used in the practice of water treatment. Usually, they are used for clarification of turbid and colored water after coagulation and settling, with reagent softening, iron removal and in other cases. It works by the principle of volumetric filtration when impurities remain in the pores of the filter throughout the entire volume of the charge as a consequence of the adherence of fine particles to the grains of the filter media.

Materials and Methods

The cultures were isolated from water samples taken on the filters of the water treatment plant in Fastiv (Kyiv region, Ukraine). In raw water, the concentration of iron was 7.27 mg/dm^3.

The isolation of pure cultures was conducted by Drygalski method. Cultures were grown in two nutrient media No.1 and No.2 containing $(NH_4)_2SO_4$, $NaNO_3$, K_2HPO_4, $MgSO_4 \cdot 7H_2O$ — 0.5 g/dm^3 each of the reagents, citric acid — 10 g/dm^3, sucrose — 2 g/dm^3, pancreatic hydrolysate of casein — 1 g/dm^3,

$FeSO_4 \cdot 7H_2O$ — 5.9 g/dm^3 (for iron-oxidizing bacteria) and $MnSO_4 \cdot 5H_2O$ — 4.7 g/dm^3 (for manganese-oxidizing bacteria), agar-agar — 20 g/dm^3, distilled water — 1 dm^3, pH = 6.8 [5]. Capek medium with streptomycin was also used. The same media without iron and manganese were used for evaluating the effectiveness of strains.

Pure cultures were sub-planted for saving to the tubes on the media containing: $MnSO_4$ — 7 mg/dm^3, $(NH_4)_2SO_4$ — 1.5 g/dm^3, KCl — 0.05 g/dm^3, K_2HPO_4 — 0.05 g/dm^3, $Ca(NO_3)_2$ — 0.01 g/dm^3, glucose — 2 mg/dm^3, distilled water — 1 dm^3 [5].

To determine the species affiliation of isolated organisms their morphology, Gram staining, coloring iron and manganese oxides in capsules and covers were studied. The cells were observed under bright field at 1350 magnification (Leica ATC 2000); species affiliation was established by comparison with photos (Bergey's Manual of Systematic Bacteriology).

To determine the iron and manganese oxides in cellular structures cytochemical staining techniques were used: for Fe^{3+} — potassium hexacyanoferrate (II); for Mn^{4+} — benzidine solution. The presence of metal oxides, painted in blue color, found under a light microscope, as well as colonies that grew on the cups.

To assess the effectiveness selected strains were grown on solid nutrient medium; the suspension was prepared and was treated to zeolite loading. The inoculating process was taken place in non-flow mode.

After settling zeolite model solutions prepared on drinking water were passed through columns. Solution containing 1–2 mg/dm^3 Fe(II) and 0.2 mg/dm^3 Mn(II).

During flowing model solutions through columns biomass in 1 g per load, the total microbial count and residual concentration of iron and manganese: Fe(II) — with 2,2-bipyridyl; total manganese content — photo-colorimetric according to GOST 4974-72 (method B) were measured.

Columns with no processing zeolite seeds were controlled for quality of settling load with microorganisms (Output Fe(II) concentration — 1.3 mg/dm^3; output Mn (II) concentration — 0.1 mg/dm^3; Fe(II) removal efficiency — 65%; Mn(II) removal efficiency — 55%, TMN = 180 CCU/dm^3). The accuracy of the experiments was evaluated with common methods of processing of experimental data in chemical technology.

Results and Discussion

Stage 1. Bacterial Cultivation. Growing of bacteria on Petri dishes with selective media No. 1 and No. 2 became noticeable for 4–5 day growth at 25 °C. Specific yellow-orange colonies were observed on a media for iron-oxidizing bacteria; the color of media had been changing from light green to ferruginous during cultivation. Specific brown colonies were observed on a media for manganese-oxidizing bacteria; the color of media had been changing from beige to brown during cultivation. The size and structure of colonies varied on both media.

In the application, Capek's medium with streptomycin development of iron- and manganese oxidizing bacteria was not observed. Accumulation of iron and manganese was identified only on the colonies' surface on a dish, which was insufficient for further work to identify the bacteria.

Stage 2. Identification of Isolated Microorganisms. The next step was the identification of isolated microorganisms with Gram's Method. Iron-oxidizing bacteria are Gram-negative, some strain have no cell wall, Gram-positive are not presented. Based on this principle and on morphology, were identified: spherical, ellipsoidal cells as *Siderocapsa*; cylindrical cells with a sheath as *Leptothrix*; rod-shaped cells as *Sphaerotillus*. Those cells, which are not stained by Gram and took the stalk cells, were assigned to genus *Galionella* (stalk cells a key feature of this genus).

The distinction between *Leptothrix* and *Sphaerotillus* was conducted with coloring iron oxides. It is known that *Leptothrix* accumulates iron oxides in a sheath, which painted in blue with potassium hexacyanoferrate (II); *Sphaerotillus* have a thin cover and hardly accumulate iron oxides, and thus not painted. This principle makes it possible to distinguish between these genuses of bacteria.

The same principle is for manganese-oxidizing bacteria, which grew on the selective medium No. 2. *Metallogenium* was identified by coloring with benzidine. This genus also has specific morphological features — colony in the form of "spider", allowing them fairly easy to detect, among other manganese-oxidizing bacteria. Research under the light microscope colonies on both media was allowed to identify *Hyphomicrobium*, a characteristic feature of which is the formation of the filament.

Ten pure cultures of iron- and manganese-oxidizing microorganisms have been isolated during experimental research. All strains were passaged to a liquid medium for storage.

Stage 3. Evaluate the Effectiveness of Isolates. On solid medium three cultures — *Siderocapsa*, *Galionella*, *Hyphomicrobium* – shown prevented growth: colonies were shallow. Although, the same cultures showed rise growth on the medium without iron, which can cause the ability of these bacteria to remove iron in association with other microorganisms.

Other strains — *Leptothrix, Sphaerotillus, Metallogenium, Siderocapsa, Galionella, Hyphomicrobium* — shown appreciable growth on solid medium: colonies were of yellow-orange color, the nutrient medium had changed color from light green to ferruginous, indicating that oxidation of ferrous iron to ferric. A key feature was that the colonies *Leptothrix, Sphaerotillus, Metallogenium* larger than others were and manifested their growth faster (5–6 days cultivation). Therefore, these cultures were used in further studies.

Table 1 shows the change of iron and manganese after passing model solution with a concentration of 2 mg/dm^3 Fe(II) and 0.2 mg/dm^3 Mn(II) through columns with microorganism *Leptothrix, Sphaerotillus, Metallogenium*.

Change of concentration during the whole filter period (8–48 h) was negligible: the effectiveness of iron removal on loadings with microorganisms lay within the 90–92%; for manganese — 80–90%. Therefore, we can assume that at low concentrations of elements, physical and chemical processes dominate biological. According to the data (Table 1) it can be assumed that iron and manganese at low concentrations were not removed by the biological way.

To identify which culture is the most effective against the removal of iron and manganese, the number of bacteria in 1 cm3 on columns was measured and the average efficiency of removing items each separately was estimated. The data are shown in Table 2.

Based on the data presented in Table 1 the diagrams of the efficiency of iron removal and manganese (Figure) by different genuses of microorganisms have been constructed.

According to bar charts on Figure, *Leptotrix* removes manganese effectively than *Sphaerotillus* and *Metallogenium*. Chemical oxidation prevailed over biological — TMN was low value (300 CCU/dm^3) and almost the same efficiency removal of iron and manganese were observed.

Ten pure cultures of iron- and manganese-oxidizing bacteria were isolated from water samples taken on the filters of the water treatment plant in Fastiv and identified as 6 genuses: *Siderocapsa, Leptothrix, Sphaerotillus, Galionella, Metallogenium, Hyphomicrobium.*

In the experiments with a model solution was shown that in a low concentration of these elements they remove physic-chemical way. The evidence is lack of lag-phase on the beginning of filtration, low data of TMN, practically the same efficiency of removing manganese and iron with three cultures.

While evaluating the effectiveness of isolates by removal of iron and manganese was shown that three cultures — *Siderocapsa*, *Galionella*, *Hyphomicrobium* were weaker and growth slowly than other *Sphaerotillus, Leptothrix, Metallogenium.*

Obtained results on the isolation of iron and manganese-oxidizing bacteria correlate with the work of other authors [6, 7]. In [6] authors

Table 1. **Changing the concentration of iron and manganese**

Time, hours	Output concentration, mg/dm^3		Removal efficiency,%	
	Fe(II)	Mn(II)	Fe(II)	Mn(II)
Leptothrix				
8	0.20*	0.03*	90**	85**
16	0.16*	0.04*	92**	80**
24	0.16*	0.03*	92**	85**
36	0.15*	0.02*	93**	90**
48	0.18*	0.02*	91**	90**
Sphaerotillus				
8	0.18*	0.03*	91**	85**
16	0.17*	0.03*	92**	85**
24	0.18*	0.03*	91**	85**
36	0.17*	0.03*	92**	85**
48	0.18*	0.02*	91**	90**
Metallogenium				
8	0.18*	0.03*	91**	85**
16	0.18*	0.04*	91**	80**
24	0.18*	0.03*	91**	85**
36	0.20*	0.04*	90**	80**
48	0.18*	0.04*	91**	80**

Note: here and in Figure * — $P < 0.05$, in comparison with control: output Fe(II) concentration — 1.3 mg/dm^3; output Mn (II) concentration — 0.1 mg/dm^3;

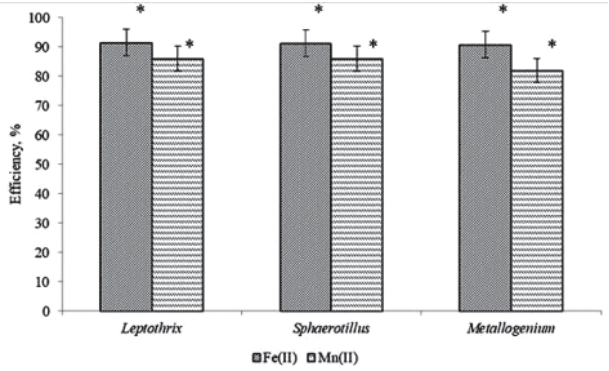
here and in Table 2 ** — $P < 0.05$, in comparison with control: Fe(II) removal efficiency — 65%; Mn(II) removal efficiency — 55%.

Table 2. **Evaluation of removal of iron and manganese with microorganisms**

Genus	TMN, CCU/dm^3	The efficiency of removing Fe(II),%	The efficiency of removing Mn(II),%
Leptothrix	300*	91.5**	86.0**
Sphaerotillus	300*	91.2**	86.0**
Metallogenium	250*	90.8**	82.0**

Note: * — $P < 0.05$, in comparison with control: TMN = 180 CCU/dm^3;

** — $P < 0.05$, in comparison with control: Fe(II) removal efficiency — 65%; Mn(II) removal efficiency — 55%.



Average efficiency of iron and manganese removing

Note: * — $P < 0.05$, in comparison with control: Fe(II) removal efficiency — 65%; Mn(II) removal efficiency — 55%.

isolated iron- and manganese-oxidizing bacteria from the bottom sediments of Lake Baikal and carried them to six genera: *Metallogenim, Lepthotrix, Siderocapsa, Naumaniella, Bacillus* and *Pseudomonas*. Also it was found that cultured ferric bacteria possess high oxidative activity. In [7] it was established that it is possible to inhibit the development of some and the intensive growth of other cultures of *Galionella, Lepthotrix, Metallogenim* depending on the physicochemical composition of water with a relatively constant effect of treatment.

So, comparison the efficiency of genuses *Leptothrix, Sphaerotillus, Metallogenium* has shown that under conditions of these experiments *Leptothrix* effectively removed iron and manganese at low concentrations in solution. In further research is planned to evaluate the effectiveness of iron and manganese removal by mixed cultures and the ability to use biotechnology techniques to improve isolated strains.

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ВИДІЛЕННЯ ЧИСТИХ КУЛЬТУР ЗАЛІЗО- ТА МАРГАНЕЦЬОКИСНЮВАЛЬНИХ БАКТЕРІЙ ІЗ ШВИДКИХ ФІЛЬТРІВ

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Метою дослідження було виділення з питної води чистих культур мікроорганізмів, здатних окиснювати залізо та марганець, з подальшим оцінюванням їхньої ефективності з метою видалення цих речовин на швидких фільтрах. Для оцінювання ефективності вибрані штами вирощували на твердому живильному середовищі, готували суспензію та обробляли нею цеолітове завантаження. Виділено 10 чистих культур мікроорганізмів, здатних окиснювати залізо та марганець, які віднесено до 6 родів: *Siderocapsa, Leptothrix, Sphaerotillus, Galionella, Metallogenium, Hyphomicrobium*. Порівняння ефективності родів *Leptothrix, Sphaerotillus, Metallogenium* показало, що в умовах цих експериментів бактерії роду *Leptothrix* ефективніше видаляють залізо і марганець за низьких концентрацій у модельному розчині.

Ключові слова: мікроорганізми, здатні окиснювати залізо та марганець, швидкі фільтри, цеолітове завантаження.

ВЫДЕЛЕНИЕ ЧИСТЫХ КУЛЬТУР ЖЕЛЕЗО- И МАРГАНЕЦОКИСЛЯЮЩИХ БАКТЕРИЙ ИЗ СКОРЫХ ФИЛЬТРОВ

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Целью исследования было выделение из питьевой воды чистых культур микроорганизмов, способных окислять железо и марганец, с последующей оценкой их эффективности для удаления этих веществ на скорых фильтрах. Для оценки эффективности выбранные штаммы выращивали на твердой питательной среде, подготавливали суспензию и обрабатывали ею цеолитовую загрузку. Выделено 10 чистых культур микроорганизмов, способных окислять железо и марганец, которые отнесены к 6 родам: *Siderocapsa, Leptothrix, Sphaerotillus, Galionella, Metallogenium, Hyphomicrobium*. Сравнение эффективности родов *Leptothrix, Sphaerotillus, Metallogenium* показало, что в условиях данных экспериментов бактерии рода *Leptothrix* более эффективно удаляют железо и марганец при низких концентрациях в модельном растворе.

Ключевые слова: микроорганизмы, окисляющие железо и марганец, скорые фильтры, цеолитовая загрузка.

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THE STRUCTURE AND PROPERTIES OF MICROBIOCENOSIS IN DUMPS OF THE FUEL AND ENERGY COMPLEX OF UKRAINE

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The work aimed to conduct complex chemical and microbiological study of the dumps of the fuel and energy complex of Ukraine. It is established that the qualitative composition of the aboriginal microbiota of the studied technogenic substrates does not to depended on the storage time, because it was determined by the chemical and mineralogical compositions and is mainly represented by the heterotrophic and acidophilic chemolithotrophic bacteria (ACB). It is noted that the number of all groups of microorganisms in dumps increased during long term storage due to internal processes and the impact of external climatic factors. In our experiment the ACB association demonstrated the maximum leaching activity when the divalent iron was as an energy source. It is also noted that the "silicate" bacteria present in the aboriginal consortium and have no leaching activity, significantly increase bioleaching rates by ACB. The results of the study indicate on the formation of resistant specific microbiocenoses in the dumps of the fuel and energy complex that can be used as sources of highly active strains obtaining for use in biotechnological processes of metal extraction.

Key words: aboriginal community, dumps, bioleaching.

The fuel and energy complex (FEC) of Ukraine produces waste products as a result of the coal mining and processing. Accumulating in huge quantities at the territories of industrial complexes, the waste creates an additional burden on the environment. At the same time, the waste of FEC contains, in particular, rare metals in industrial concentrations, which makes it a "man-made deposit", the unconventional raw materials of valuable components [1, 2]. A special aboriginal microbial community is formed under the influence of industrial and natural factors, and later affected by the storage conditions in the studied anthropogenic ecosystems. In long-term storage, the substrates change affecting the structure and composition of the microbiocenosis and the ability of the formed equilibrium systems to destruct. The available literature data suggests that the use of the aboriginal consortium associations in the leaching metals biotechnology is promising due to the effect of syntrophic relationships between individual groups of microorganisms in the community [3, 4]. The microbial biotechnologies should be implemented based on comprehensive studies of the biological and physicochemical properties of the initial solid substrate, the qualitative and quantitative assessment of the indigenous microbial community present in it, the possibility of isolating, selecting and selecting the most promising highly active strains. There is ample evidence of the microbial role in both the formation and destruction processes of geogenic substrates (natural ores and minerals, native sulfur, oil, peat, coal, etc.), accompanied by the bioextraction of useful components [5–7]. Information is limited about the life and biochemical activity of microorganisms in the raw materials of technogenic origin.

The aim of the work was to conduct a comprehensive chemical and biological research of technogenic raw materials produced by the FEC enterprises of Ukraine to establish the structure and properties of their microbiocenosis.

Material and Methods

The objects of research were dumps of the Central concentrating plant (CCP) of the Lviv-Volyn coal basin (LVCB), fly ash and ash from the burning of LVCB coal at Ladyzhinska and Dobrotvorska TPSs, respectively. The samples (more than 60 in total) were taken during 2008–2014 (April to November) on the slopes of the dumps at the 50.0 ± 5.0 cm surface layer.

To identify various physiological groups of native microbial microorganisms, enrichment cultures and specific nutrient media were used: 9K and 9K * for the acidophilic chemolithotrophic bacteria (ACB); Beyerinck for the neutrophilic chemolithotrophic bacteria; 882 for representatives of the genus *Leptospirillum;* 150a for the moderately thermophilic representatives of the genus Acidithiobacillus, such as A. caldus; Gorbenko for the heterotrophic bacteria; Czapek for the filamentous fungi; and A-27 for the "silicate" bacteria (Table 1) [8, 9].

As the energy source, either $FeSO_4 \cdot 7H_2O$ at a concentration of 44.5 g/dm^3, or elemental sulfur or thiosulfate at a concentration of 5.0 g/dm^3 was added to the mineral background of the 9K medium.

The biomass of various representatives of the dump microbiocenosis was accumulated at a ratio of solid (substrate) to liquid (nutrient medium) S: L = 1: 10. In the control experiments sterile substrate was introduced to the nutrient medium. The cultures were incubated at a temperature of 30.0 ± 0.5 °C for mesophilic (MP) and 50.0 ± 0.5 °C for moderately thermophilic (MTP) bacteria, pH 3.0–7.0 for 5 days. The development of microorganisms was evaluated by the presence of the surface film, the change in pH and the appearance of the bacterial suspension. The abundance of representatives of different microbial groups was established by sowing tenfold serial dilutions of the bacterial suspension on agar media of the same composition. The number of spore-forming bacteria was determined after heat treatment at 80.0 ± 0.5 °C for 15 minutes.

The biogeochemical activity of the aboriginal community was judged by the concentration of metals transferred from the solid phase to the culture medium. Selective nutrient media were used as leaching solutions

Table 1. **The composition of nutrient media (g/dm^3) for identification of microbial groups in waste products of the fuel and energy complex of Ukraine**

Mineral components	Culture media [8, 9]						
	A-27	Czapek	9К	9К*	150a	Beyerinck	882
KH_2PO_4			0.50	0.05	0.50		0.027
$(NH_4)_2SO_4$			3.00	0.45	3.00		0.132
$MgSO_4 \cdot 7H_2O$	0.50	0.50	0.50	0.50	0.50		
$MgCl_2 \cdot 6H_2O$						0.10	0.053
NH_4CL						0.10	
KCl		0.50	0.10	0.05	0.10		
$NaNO_3$		3.00					
K_2HPO		1.00					
Na_2HPO_4	2.00					0.20	
$Ca(NO_3)_2$			0.01	0.014	0.01		
$CaCl_2 \cdot 2H_2O$							0.147
$FeCl_3$	0.001	0.01					
$NaHCO_3$						1.00	
$CaCO_3$	1.00						
Quartz	10.00						
Sucrose	5.00	30.00					
Yeast extract				0.02			
pH	7.5–8.0	6.6–7.2	1.0–3.5	1.8–4.0	1.5–3.5	4.5–8.5	2.0–4.0

(Table 1). Sterile waste with a sterile leach solution served as controls. The bioleaching process was carried out by the vat method at a ratio S: L = 1:10, pH — 2.0, 30.0 ± 0.5 °C for MP and 50.0 ± 0.5 °C for MTP bacteria for 7 days. The concentration of metals in solid substrates and solutions was determined by atomic absorption spectroscopy on AAS-1 (Germany) and C-115PK Selmi (Ukraine) devices [10]. The reliability of obtained results was evaluated by the Student's *t*-test with a probability of $P < 0.05$.

Results and Discussion

Table 2 shows the chemical composition of the studied substrates by the main components.

To observe the development of aboriginal associations in substrates, depending on the timing of their accumulation, preliminary microbiological crops were grown on nutrient media selective for acidophilic chemolithotrophic and heterotrophic microorganisms because those are typical representatives of microbiocenosis of geogenic and technogenic origin (Table 3).

The microorganisms were not detected at all or their communities were very poor in fresh substrates, especially heat-treated ash and fly ash. In storage, the communities are formed. In dumps with an acidic environment, the conditions favor the active growth of both chemolithotrophic and heterotrophic microorganisms. In neutral or weakly alkaline ash and fly ash, the heterotrophic component forms an association faster than the chemolithotrophic one, and quantitatively the former is more pronounced, both in comparison with the dumps and in relation to its own chemolithotrophic component. However, generally, stable, numerous aboriginal equilibrium communities form during the storage of the studied technogenic substrates for longer than three years. Then it is possible to isolate active strains for biotechnological developments. For further research, ash and fly ash (storage period 24–36 months), as well as "stale" dumps substrates with different shelf life were chosen: 24–28 months (black) and more than 60 months (red).

The results of microbiological studies are shown in Fig. 1. They indicate the quantitative prevalence of heterotrophic and acidophilic chemolithotrophic bacteria, both mesophilic and moderately thermophilic, in waste products of FEC.

In all the studied substrates, especially with acidic pH (black and red coal preparation waste), ACB dominate which use bivalent iron and sulfur / thiosulfate as an energy source. In the mesophilic association of all substrates that develops on a standard 9K medium with bivalent iron or thiosulfate, a lot of small Gram-negative rod-shaped cells were noted. In the microbial associations of iron and sulfur-oxidizing bacteria, no significant differences in cell morphology were seen in accordance with the available literature data. The number of cells oxidizing bivalent iron and thiosulfate in ferrous dumps, fly ash and ash was 10^4–10^5 cells/g.

In the red "stale" dumps, their number was greater and reached 10^8 and 10^6 cells/g for iron-

Table 2. **The content of main controlled metals in waste products of FEC of Ukraine (g/ton)**

Metal	Minimum industrial content	Ash, Dobrotvorska TPS	Fly ash, Ladyzhinska TPS	Dumps of coal concentrating, CCP "Chervonohradska"	
				Black	Red
Plumbum	18–22	75.0±0.05	120.0±0.1	42.20±0.05	57.92±0.05
Nickel	80–120	110.0±0.1	170.0±0.1	134.2±0.1	132.9±0.1
Cadmium	45,0–55,0	8.5±0.05	7.5±0.05	2.82±0.05	3.63±0.05
Aluminum	$(2.5–5.0)\cdot10^3$	$(105.0±0.1)\cdot10^3$	$(37.5±0.05)\cdot10^3$	$(13.92±0.05)\cdot10^3$	$(8.92±0,05)\cdot10^3$
Cuprum	80–100	92.5±0.1	60.0±0.05	62.18±0.05	78.90±0.05
Manganese	850–1000	1750±0.1	600.0±0.1	317.7±0.1	812.9±0.1
Zinc	65–70	110–180	315.0±0.1	112.5±0.1	130.9±0.1
Germanium	15–20	30–40	45.0±0.05	26.0±0.1	30.0±0.1
Gallium	15–20	30–40	95.0±0.1	15.1±0.1	22.4±0.1

Table 3. **Microbial characteristic of waste products of FEC of Ukraine**

Substrate	Storage time	pH of water extract	Microbial abundance, cell/g			
			Heterotrophic		Acidophilic chemolithotrophic	
			Bacteria	Fungi	Oxidizing Fe(II)	Oxidizing S0
Dumps	Freshly produced	1.8–2.0	100±5	50±2	(5.3±0.4)×102	15±5
Dumps	10–14 months	2.0–2.4	$(3.60\pm0.65)\times10^3$	$(1.50\pm0.25)\times10^2$	$(4.70\pm0.85)\times10^3$	$(1.55\pm0.25)\times10^2$
Dumps	24–28 months	2.6–3.3	$(7.80\pm1.65)\times10^5$	$(8.70\pm0.75)\times10^2$	$(7.50\pm1.56)\times10^5$	$(7.70\pm0.55)\times10^4$
Dumps	≥ 36 months	3.0–3.6	$(5.60\pm1.25)\times10^7$	$(6.70\pm1.35)\times10^3$	$(9.35\pm1.85)\times10^7$	$(4.80\pm0.95)\times10^5$
Dumps	≥ 60 months	3.5–4.5	$(9.30\pm1.85)\times10^7$	$(1.50\pm0.30)\times10^4$	$(3.70\pm0.75)\times10^8$	$(3.95\pm0.75)\times10^6$
Ash	Freshly produced	5.8–6.2	15±3	n/o*	n/o	n/o
Ash	10–14 months	6.0–7.0	$(8.20\pm1.65)\times10^3$	$(2.70\pm0.55)\times10^2$	$(5.80\pm1.15)\times10^2$	$(3.50\pm0.70)\times10^2$
Ash	20–24 months	6.2–7.4	$(7.95\pm1.58)\times10^5$	$(8.75\pm1.75)\times10^2$	$(2.80\pm0.55)\times10^4$	$(4.30\pm0.85)\times10^3$
Ash	24–36 months	6.4–7.8	$(4.70\pm0.96)\times10^7$	$(2.10\pm0.45)\times10^3$	$(3.55\pm0.75)\times10^5$	$(4.55\pm0.95)\times10^4$
Fly ash	Freshly produced	6.4–8.0	9±1	n/o	n/o	n/o
Fly ash	10–14 months	7.0–9.5	$(5.20\pm1.1)\times10^3$	$(4.30\pm0.85)\times10^2$	$(9.70\pm1.95)\times10^2$	$(4.15\pm0.80)\times10^2$
Fly ash	20–24 months	7.8–10.2	$(8.55\pm1.75)\times10^5$	$(2.75\pm0.55)\times10^3$	$(1.25\pm0.25)\times10^3$	$(9.70\pm1.95)\times10^2$
Fly ash	24–36 months	9.0–10.8	$(2.55\pm0.55)\times10^8$	$(7.70\pm1.55)\times10^3$	$(8.70\pm1.75)\times10^4$	$(1.40\pm0.25)\times10^3$

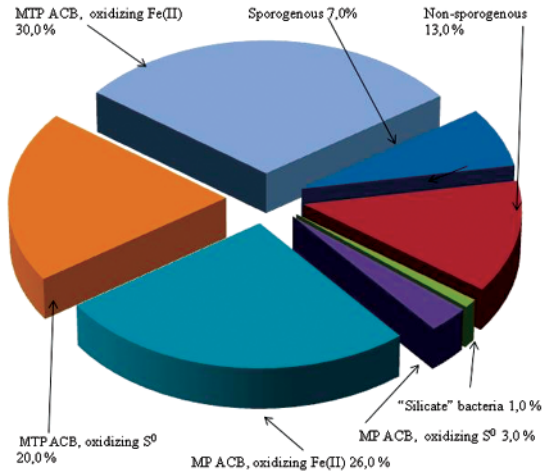
* — not observed.

and sulfur-oxidizing bacteria, respectively. The obtained data suggest the presence of representatives of the genus *Acidithiobacillus* (*A. ferrooxidans* and *A. thiooxidans*), widely distributed in natural sulfide ores in the studied technogenic raw material and actively participating in metal leaching processes [11].

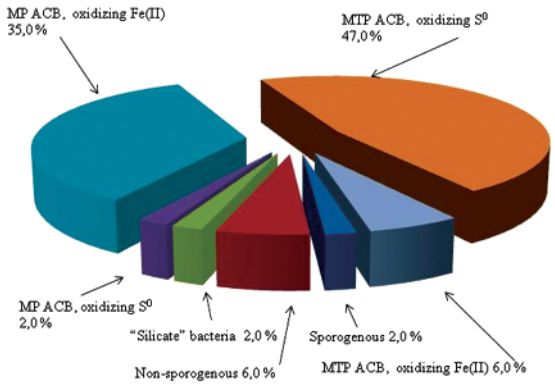
In mesophilic conditions of medium 882, morphologically different cells — spirillus, vibrios, and small curved rods — were observed. Their number in black waste, fly ash, and ash was 10^3–10^4 cell/g, in the red "stale" dumps it was 10^5 cell/g. This suggested the presence of representatives of the genus *Leptospirillum* in the studied microbiocenosis. The genus includes iron chemolithotrophic bacteria [12]. These results are consistent with the available literature data, according to which bacteria of the genus *Leptospirillum* are always present in natural and man-made mineral raw materials and do not make a significant contribution to the bioleaching processes themselves, but in an aboriginous consortium with *A. ferrooxidans* and *A. thiooxidans* they contribute to the efficiency of extraction metals [13].

In mesophilic conditions of Beyerinck's medium, the development of small Gram-negative rods was observed in the amount of 10^2 cell/g (black dumps, fly ash and ash) and 10^4 cell/g in red "stale" dumps. This suggested the presence of neutrophilic thionic bacteria of the genus *Thiobacillus* in the studied microbiocenoses. There is no data on the presence of this group of bacteria in technogenic raw materials, as well as any information about their ability to leach metals. However, it is known that in nature, *Thiobacillus thioparus* in association with *Thiobacillus ferrooxidans* are active agents of corrosion of metallic and non-metallic products. Therefore their presence in biocenoses with quantitative advantage of acidophilic bacteria is quite possible [14].

The microbiocenosis of FEC waste products is formed in widely ranging temperatures during storage. There is also the self-heating and self-ignition phenomena. Thus it was interesting to identify bacteria for which the optimum temperature for growth is 45.0–50.0 °C. An insignificant amount (10^3–10^4 cell/g) of small gram-negative rod-like cells was noted in all substrates in the cumulative culture based on the medium 150a. This can be attributed to the genus *Acidithiobacillus*, in particular *A. caldus*. According to a number of studies, *A. caldus* is always, albeit slightly, present in mineral raw materials and, together with *A. ferrooxidans* and *A. thiooxidans*, contributes to the efficiency of metal extraction [15].



a



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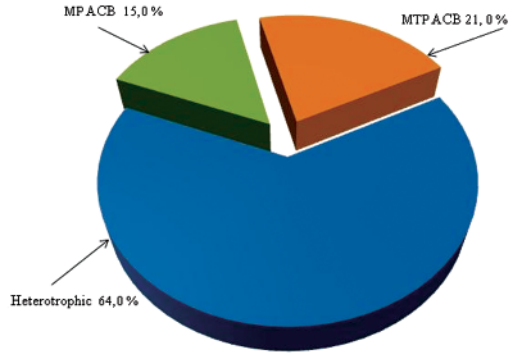

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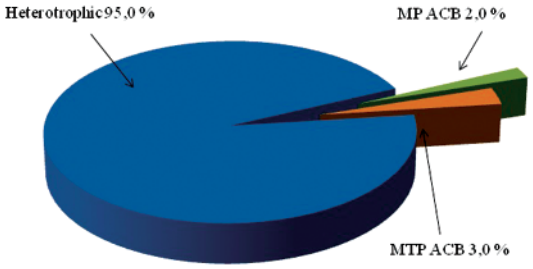


d

Fig. 1. **Quantitative and qualitative content of microbial cenoses:**
a — black dumps of CCP; *b* — red dumps of CCP; *c* — fly ash of Ladyzhinska TPS; *d* — ash of Dobrotvorska TPS

Mixotrophic, moderately thermophilic bacteria were found in all studied waste substrates. In a cumulative culture based on a modified 9K medium with thiosulfate or bivalent iron, an abundant development of Gram-positive short round or cocco-like spore-forming cells was recorded, which can be attributed to the genus *Sulfobacillus*. Their numbers were much higher in red "old" waste and reached almost 10^9 cell/g. This is the maximum quantitative indicator among all the iron- and sulfur-oxidizing bacteria identified in the studied substrates, possibly associated with the processes leading to self-heating of the dumps.

The FEC waste products are bio-inert systems. However, an intensive development of heterotrophic bacteria, both spore-and non-spore-forming (Fig. 1) was observed there, with numerical predominance in ash and fly ash with neutral and weakly alkaline pH (up to 10^8 cell/g), as well as in the "stale" coal enrichment dumps, up to 10^7 cell/g. These results are consistent with available reports on the ability of certain representatives of heterotrophic bacteria to grow in mineral solutions in the presence of trace amounts (μg/dm^3) of organic substrates, as well as the activity of the representatives of the genera *Pseudomonas* and *Bacillus* in the leaching of gold and uranium [16].

The presence of so-called "silicate" bacteria capable of destroying silica and silicates was first established in the heterotrophic component of FEC waste products. In bacterial suspension on nutrient A-27 medium, Gram-positive large rods capable of forming spores were recorded, on agarized A-27 medium they were formed in almost identical round transparent colorless colonies. Their number was in the range of 10^3–10^4 cell/g, reaching a maximum in ash (10^5 cell/g), which may be

due to the increased content of silicon and aluminum in this substrate (Table 1) [17].

Thus, the qualitative composition of the microbiocenoses of the FEC waste products under study does not depend on the storage time, since it is determined by the chemical and mineralogical composition of the substrates. As they accumulate and are stored under the influence of external factors, quantitative differences arise in the microbiocenoses, which are affected by the accumulation time, composition and pH of waste products. When comparing substrates with the same storage time but different pH, it is obvious that in ash (pH 6.4–7.8) and fly ash (pH 9.0–10.8) the conditions are more favorable for the development of heterotrophic microorganisms both in comparison with the black dumps (pH 2.6–3.3), and in relation to the development of own acidophilic chemolithotrophic component (Fig. 1). It should be noted that the abundance of the ACB community in fly ash (amorphous, finely dispersed, with a large specific surface), despite its alkaline pH, is comparable to the coarse ash. This is another important factor for bioleaching associated with the presence of microorganism cells, either free or attached to the surface of solid particles. The presence of defects in the crystal structure and a large specific surface of the substrate contribute to faster growth and development of all possible microorganisms of the consortium [18].

The biotechnological potential of representatives of the consortium of FEC waste products was determined by their ability to create favorable conditions for certain groups of microorganisms to destroy substrates and extract valuable metals from those.

Representatives of acidophilic chemolithotrophic bacteria oxidizing bivalent iron and thiosulfate were the most numerous group of practical interest in the aboriginal consortium of FEC waste products. The results on leaching of metals from FEC waste products by the most numerous groups in the aboriginal consortium, the mesophilic and moderately thermophilic association of ACB, are presented in Fig. 2 and 3 respectively. In the control experiments with sterile substrates and nutrient mediums the leaching of Ge, Ga, Cd, Ni, Cu, Zn, Mn and Al did not exceed 2–4%, the leaching of Pb did not exceed 0,3–0,5%.

Hence, the association of moderately thermophilic bacteria of the studied substrates, regardless of their nature, was distinguished by a higher leaching activity compared to the mesophilic community. However, maximum leaching rates of metals, both rare and heavy, were achieved using bivalent iron as an energy source similarly in mesophilic and moderately thermophilic conditions. This confirms the leading role of *A. ferrooxidans* in the processes of bacterial leaching metals under mesophilic conditions [9, 18, 19]. It is established that the black coal dumps is the most "accessible" to microorganisms; the degree of leaching of almost all registered metals exceeds the similar indicators for other substrates.

The change in the microbial landscape and the number of bacteria was studied during the entire experiment of metal bioleaching of from the black dumps (Fig. 4). Thus, during the first day, the number of bacteria in the mesophilic association did not exceed 4.5×10^2 cell/g. Gram-negative short thin cells prevailed in the stained microscopic preparation, sometimes larger Gram-positive cells with rounded ends were encountered (Fig. 4, *a*). After five days, the number of bacteria increased significantly and reached 7.8×10^7 cell/g. During this period of time, bloated round cells with thickened membrane and unstained contents were recorded in the bacterial suspension (Fig. 4, *b*). Bipolar inclusions were present on the surface of some cells. According to available literature data, those are globules of sulfur resulting from the oxidation of mineral raw materials [20].

The appearance of rounded large cells was also observed during longer-term cultivation of the studied strains (Fig. 4, *c*). After 7 days their total amount in the bacterial suspension decreased to 5.7×10^4 cell/g. This was accompanied by lysis of the cells, a shift in the pH of the leach solution towards neutral values and the beginning of deposition of insoluble Fe^{+3} compounds.

When using the moderately thermophilic association of ACB, the number of bacteria was slightly higher. At the beginning of the experiment it was 5.7×10^4 cell/g, within five days it reached a maximum of 7.3×10^{10} cell/g and at the end of the experiment (after 7 days) decreased to 3.9×10^5 cell/g. This changing pattern of biomass amount is a reflection of the classical phases of growth and development of microorganisms: exponential, stationary, dying off and cell lysis phases [9].

Despite the quantitative advantage of the heterotrophic component in the waste product microbiocenosis (Fig. 1), its leaching activity was insignificant and the extraction of metals into the solution did not exceed 15.0%. Extraction of metals into the solution as a result of the activity of "silicate" bacteria was also

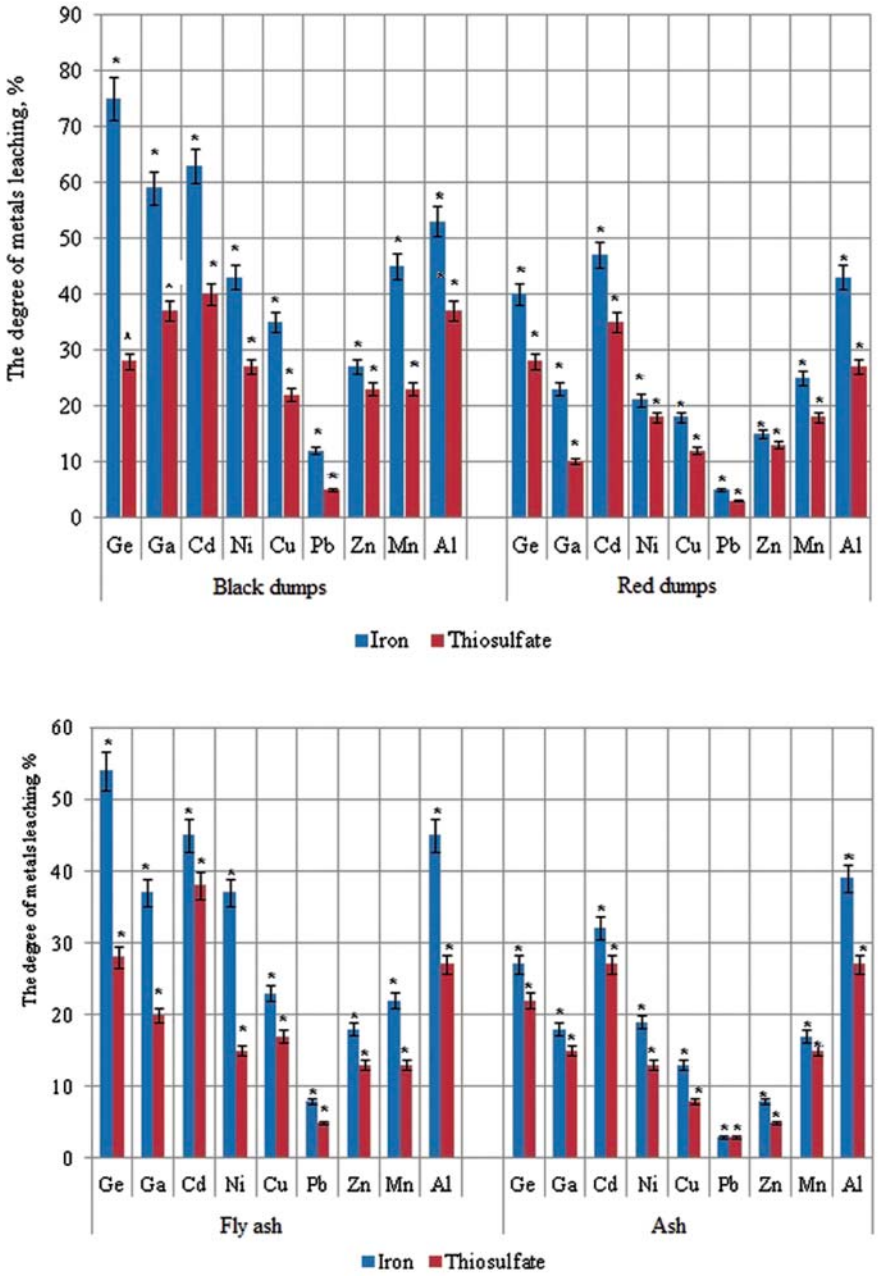


Fig. 2. **Metal leaching by the mesophilic association of acidophilic chemolithotrophic bacteria from the FEC waste products**

Hereinafter: $*P < 0.05$ compared with control

In the control experiments with sterile substrates and nutrient mediums the leaching of Ge, Ga, Cd, Ni, Cu, Zn, Mn and Al did not exceed 2–4%, the leaching of Pb did not exceed 0,3–0,5%

low (it did not exceed 12%). However, it was obvious that the substrates were destroyed and their appearance changed after contacting with the nutrient medium A-27, creating favorable conditions for the growth and activity of "silicate" bacterial consortium (Fig. 5).

The waste products contained a lot of silicon-containing phases (silica, aluminosilicates). Hence our assumption that the association of "silicate" bacteria at the initial stage of processing the FEC dumps due to the destruction of stable crystalline silicate structures can enhance the effectiveness of the further action of the chemolithotrophic component of the consortium in relation to metal recovery. This idea was confirmed (Fig. 6), the results are patented [21].

Thus, the complex chemical and microbiological studies of waste products of FEC of Ukraine showed that the physico-

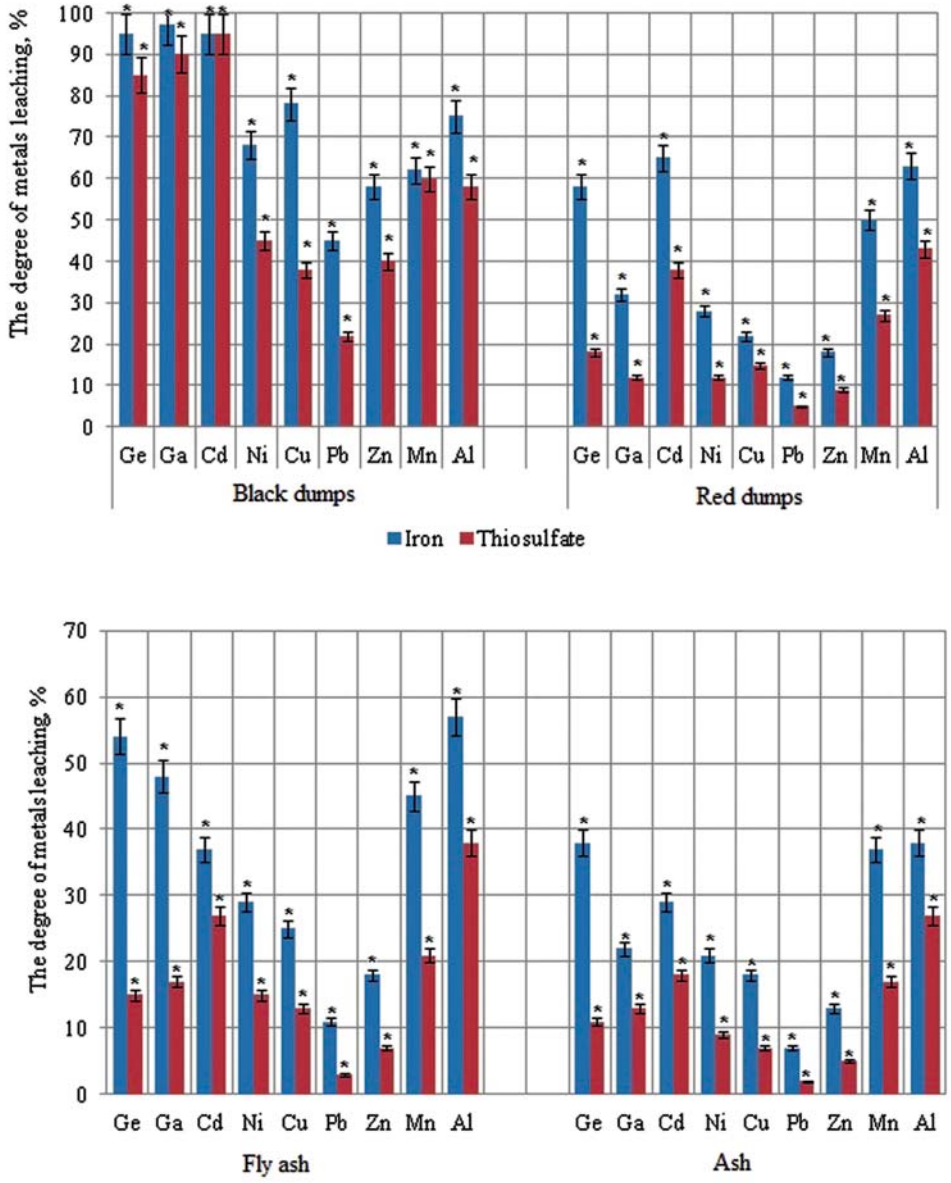
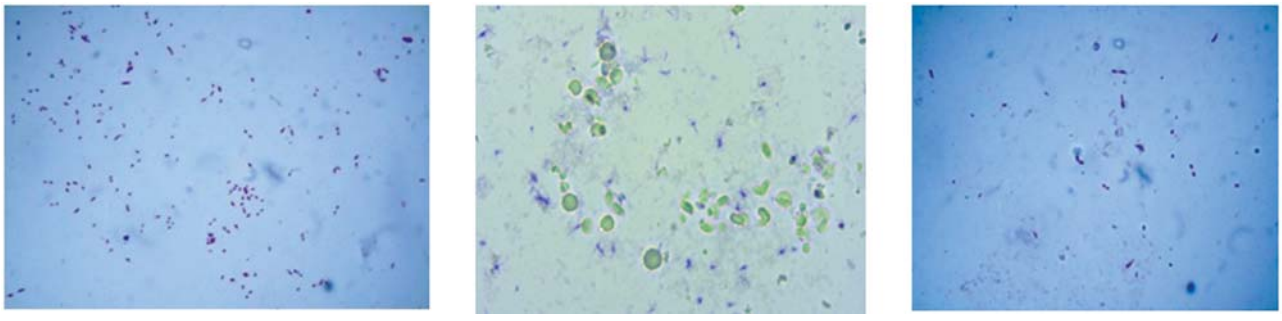


Fig. 3. **Metal leaching by the moderately thermophilic association of acidophilic chemolithotrophic bacteria from the FEC waste products**



a *b* *c*

Fig. 4. **Micrographs of the association of mesophilic ACB in the metal leaching from the black dumps:** at the beginning of the process (*a*), after 5 (*b*) and 7 (*c*) days; ×1000

a *b*

Fig. 5. **Appearance of black waste dumps:**
before (*a*) and after (*b*) bacterial leaching with nutrient medium A-27

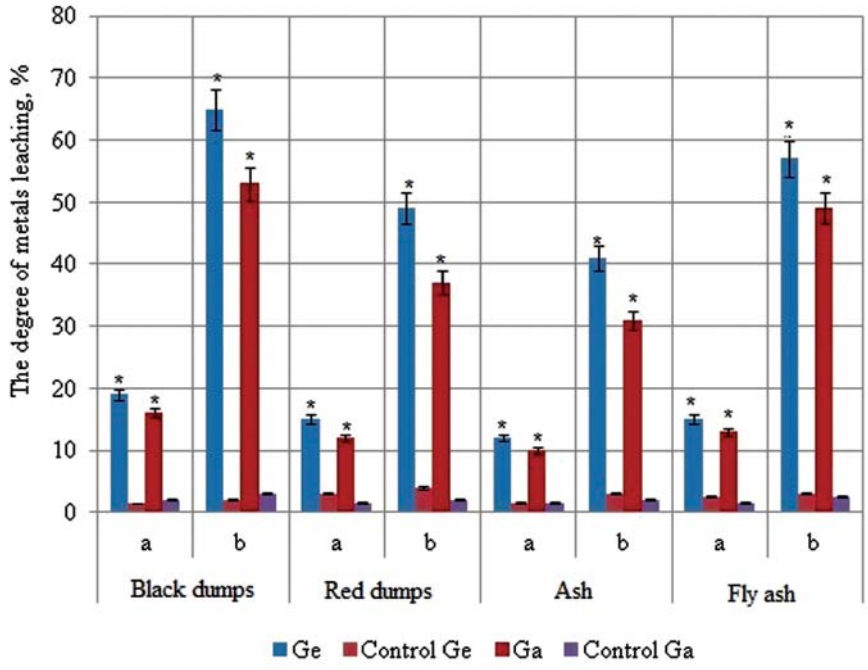


Fig. 6. **Extraction of germanium and gallium from the waste dumps:**
by the mesophilic association of acidophilic chemolithotrophic bacteria (*a*), after pre-treatment with medium A-27 (*b*). Leaching time 24 hours

chemical composition and the conditions of their formation and storage determine the structure of their microbiocenosis. The qualitative composition of the native microbiota does not depend on the storage time. The microbiota is determined by the chemical and mineralogical composition of substrate, and is represented mainly by heterotrophic and acidophilic chemolithotrophic bacteria. An increase in the number of all groups of microorganisms in long-term stored waste products is established, which is a result of internal processes and the influence of external climatic factors. The maximum leaching activity is observed in the ACB associations, especially when using bivalent iron as an energy source. The "silicate" bacteria found in the structure of the aboriginal consortium are not capable of leaching metals on their own. However in combination with ACB they contribute to a significant increase in the bioleaching rates. The research results indicate the formation of stable specific microbiocenoses in FEC waste products. The coenoses can be sources of obtaining highly active strains for use in biotechnological processes of metal extraction.

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СТРУКТУРА ТА ВЛАСТИВОСТІ МІКРОБІОЦЕНОЗІВ ВІДХОДІВ ПАЛИВНО-ЕНЕРГЕТИЧНОГО КОМПЛЕКСУ УКРАЇНИ

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Метою роботи було проведення комплексного хіміко-мікробіологічного дослідження відвальних продуктів паливно-енергетичного комплексу України. Встановлено, що якісний склад аборигенної мікробіоти досліджених техногенних субстратів не залежить від термінів зберігання, оскільки визначається хімічним і мінералогічним складом, і представлений переважно гетеротрофними і ацидофільними хемолітотрофними бактеріями (АХБ). Виявлено зростання чисельності всіх груп мікроорганізмів у відвальних продуктах тривалого зберігання, що є результатом внутрішніх процесів і впливу зовнішніх кліматичних факторів. Показано, що максимальну вилуговувальну активність мають асоціації АХБ, особливо в разі використання двовалентного заліза як джерела енергії. Виявлені в структурі аборигенного консорціуму «силікатні» бактерії, що не здатні вилуговувати метали самостійно, в поєднанні з АХБ сприяють значному підвищенню показників біовилуговування. Результати досліджень свідчать про формування у відвальних продуктах паливно-енергетичного комплексу стійких специфічних мікробіоценозів, що можуть бути джерелами отримання високоактивних штамів для використання в біотехнологічних процесах вилучення металів.

Ключові слова: аборигенне угруповання, відвали, біовилуговування.

СТРУКТУРА И СВОЙСТВА МИКРОБИОЦЕНОЗОВ ОТХОДОВ ТОПЛИВНО-ЭНЕРГЕТИЧЕСКОГО КОМПЛЕКСА УКРАИНЫ

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Целью работы было проведение комплексного химико-микробиологического исследования отвальных продуктов топливно-энергетического комплекса Украины. Установлено, что качественный состав аборигенной микробиоты исследованных техногенных субстратов не зависит от сроков хранения, поскольку определяется химическим и минералогическим составом, и представлен в основном гетеротрофными и ацидофильными хемолитотрофными бактериями (АХБ). Выявлено возрастание численности всех групп микроорганизмов в отвальных продуктах длительного хранения, что является результатом внутренних процессов и воздействия внешних климатических факторов. Показано, что максимальной выщелачивающей активностью обладают ассоциации АХБ, особенно при использовании двухвалентного железа в качестве источника энергии. Обнаруженные в структуре аборигенного консорциума «силикатные» бактерии, не способные выщелачивать металлы самостоятельно, в сочетании с АХБ способствуют значительному повышению показателей биовыщелачивания. Результаты исследований свидетельствуют о формировании в отвальных продуктах топливно-энергетического комплекса устойчивых специфических микробиоценозов, которые могут быть источниками получения высокоактивных штаммов для использования в биотехнологических процесах извлечения металлов.

Ключевые слова: аборигенное сообщество, отвалы, биовыщелачивание.

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Lactobacillus AS PRODUCERS OF EXTRACELLULAR TANNASE

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The aim of the work was to find strains of lactic acid bacteria capable to synthesize extracellular tannase enzyme — the key enzymehe hydrolyzing tannins which are plant food constituent. One of the main product of tannins hydrolyzis is gallic acid- the compound with proven antioxidant and onco-protective. As a result of lactobacteria screening, two biocompatible strains of lactic acid, namely *L. rhamnosus* LB3 and *L. delbrueckii* subsp. *delbrueckii* with a high level of enzyme productivity, were selected. The maximum accumulation of tannase, corresponding to 0.031 ± 0.002 U/ml for *L. rhamnosus* LB3 and 0.03 ± 0.002 U/ml for *L. delbrueckii* subsp.*delbrueckii*, was observed after 48 h of cultivation. Both strains showed rapid growth and performance of tannase in MRS medium in the presence of glucose or lactose as a carbon source. It was shown that gallic acid, which was a necessary component of the medium as a target enzyme inducer, did not affect the accumulation of lactobacilli biomass. The selected strains are of interest as producers of a bicomponent probiotic with antioxidant properties and require further investigation.

Key words: lactobacillus, probiotics, tannase, antioxidants, carbon sources.

Along with the science development new properties and mechanisms for implementing the probiotic biotherapeutic potential are discovered. The study of anticarcinogenic activity of probiotic strains is particularly noteworthy.

The mechanisms of oncoprotective action of lactic acid bacteria, which are widely present in the composition of functional food products and probiotic-containing medications, have not been fully studied, however they are associated with such properties as:

– the ability to modify fecal enzymes, which believed to be involved in carcinogenesis of the colon [1];

– cellular absorption and removal of mutagenic substances or reduction of the mutagenic effect of chemicals, by its transformation [1–5];

– tumor suppression by stimulating the immune response, in a way of increasing the activity of natural killers (NK cells) [3];

– antagonistic activity in relation to pathogenic microorganisms that may have an indirect carcinogenic effect (e.g. *H. pylori*) [6–8];

– induction of apoptosis in myeloid leukemia cells;

– production of substances which induce apoptosis [9];

– biodegradation of natural substances with the formation of antioxidant compounds [10].

The implementation of the last mechanism is well illustrated by the example of the natural polyphenolic compounds of tannins splitting. The main common natural source of tannins is plants. Many types of tannins are found in a range of food products such as tea, coffee etc. The presence of tannins in foods gives it a bitter taste, which makes them less appealing for consumption. In addition, tannins at a certain concentration have toxic, bacteriostatic and carcinogenic properties and irreversibly form compounds with proteins, as well as with other molecules such as starch, cellulose and minerals [10, 11]. Tannase is known to be the main enzyme which is involved in the decomposition of tannins, in particular halo-tannins. It is

produced by a number of microorganisms which belong to fungi and bacteria [12, 13]. However, from that point of view, lactic acid bacteria are the most important among all probiotic cultures.

The high interest in tannase is due to the fact that the main product of tannins hydrolysis is gallic acid, which is known for its antioxidant properties [12, 14, 15].

Modern medical field pays close attention to the substances with antioxidant properties, since it is believed that one of the causes of oncological diseases is oxidative tissue damage. There are reports on the capability of gallic acid to protect human cells from oxidative damage and cause an anti-apoptotic effect, along with a pronounced cytotoxic action in relation to cancer cells [16].

Thus, the data from the literature testifies the promising usage of the drugs based on the tannin-positive bacteria of the genus *Lactobacillus* for both food industry and medical practice, as a source of antioxidant complexes to protect the body from the negative effects of free radicals. Therefore, the search for new rational and effective natural sources of antioxidants among probiotic strains is relevant and feasible.

Materials and Methods

The objects of the research were bacteria strains of the genus *Lactobacillus* from the collection of the Department of Industrial Biotechnology (Faculty of Biotechnology and Biotechnics of the National Technical University of Ukraine "Igor Sikorsky Kyiv Polytechnic Institute") and the strain with proven tannase activity from the collection of Microbial Type Culture Collection and Gene Bank of the Institute of Microbial Technology (Chandigarh, India). A complete list of strains is presented in Table 1.

Incubation conditions. Lactic acid bacteria (LAB) strains were transferred to sterile MRS broth (Man-Rogosa Sharpe, Himedia) and incubated at +37 °C for 24 hours. The medium was previously sterilized at 0.5 MPa during 20 min and the medium pH after sterilization procedure was 6.2–6.6. The investigated *Lactobacillus* cultures were stored at +4 °C in a semi-liquid MRS medium (with 2% of agar).

Tannase activity determination. Extracellular tannase activity of LAB strains was estimated according to previously described technique [17].

Estimation of biomass accumulation. The rate of biomass accomulation was measured after 24 hours and 48 hours of incubation using spectrophotometry (UNICO Spectrophotometer 1201) at wavelengths of 560 nm.

Examination of LAB strains biocompatibility. The biocompatibility of the strains was determined by the method of *in vitro* joint cultivation [18] with minor modifications (strokes and droplets method). The biocompatibility of test cultures was estimated by the sizes of growth zones. Accounting was performed after 24 and 48 hours incubation at +37 °C.

Acid production determination. Acid production by the strains was examined by the titration [19]. The result was estimated by the volume of alkali spent on neutralization of acid in the medium and expressed in degrees Terner (°T).

Statistical Analysis. All experiments were performed in at least three repetitions using appropriate control samples. The digital data obtained during the research was processed by statistical analysis methods with using the Student's *t*-test for small samples at 95–99% levels of significance. Calculation and diagrams construction were done using the computer program Excel (Microsoft Office 2010) [20].

Results and Discussion

10 LAB strains from the microbial collection of the Department of Industrial Biotechnology (Faculty of Biotechnology and Biotechnics, NTUU "Igor Sikorsky Kyiv Polytechnic Institute") were screened for their ability to produce extracellular tannase. The strain *Lactobacillus plantarum MTCC 2621* from the Indian collection of MTCC IMTECH which has proven high-grade tannase activity was chosen as reference microorganism.

As a result of the screening, 3 tannase active strains of the genus *Lactobacillus* were selected: *L. rhamnosus LB3, L. bulgaricus LB51 and L. delbrueckii subsp. delbrueckii* with tannase activity at 48 hours of incubation 0.031 ± 0.002 U/ml, 0.013 ± 0.001 U/ml and 0.03 ± 0.002 U/ml, respectively.

As a result of the data analysis, it was found that the level of enzyme biosynthesis by all selected strains was significantly higher on the second day of incubation in comparison to the estimated tannase activity on the first day After 48 hours of incubation the activity of the reference strain had reached a maximum level of enzymatic activity (0,051 ± 0,002 U/ml), which was 2.29 times higher than the activity on the first 24 hours of growth. Tannase activity on the 48 hour of incubation had

Table 1. ***Lactobacillus*** **strains**

Full name of strain	The sources of origin
L. murinus LE IMB B-7037	Non-commercial dairy products *
L. rhamnosus LB3 IMB B-7038	Non-commercial dairy products *
L. acidophilus (C)	Non-commercial dairy products *
L. rhamnosus (C)	Institute Rossell INC, Canada
L. bulgaricus LB51	Zabolotny Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine
L. delbrueckii subsp. *bulgaricus LB86 ВКПМ-В-5788*	Plant of microbiological synthesis preparations "Enzyme"
L. delbrueckii subsp. *delbrueckii DSM20074*	DSMZ, Germany
L. murinus DSM 20452	DSMZ, Germany
L. plantarum	Lactobacterin *
L. plantarum MTCC 2621	MTCC IMTECH, India

Note: * — the strains were isolated at the department of the industrial biotechnology at the faculty of biotechnology and biotechnics of the National Technical University of Ukraine "Igor Sikorsky Kyiv Polytechnic Institute"; DSMZ — Deutche Summlung von Mikroorganismen und Zellkulturen; MTCC IMTECH — Microbial Type Culture Collection and Gene Bank of Institute of Microbial Technology.

increased for *L. rhamnosus LB3* by 1.63 times; for the strain *L. delbrueckii* subsp. *delbrueckii* by 1.76 times. The level of tannase activity of the strains is presented in Table 2.

Thus, in a result of screening, 3 LAB strains with tannase activity were selected to be considered as potential producers of a new multistrain probiotic with antioxidant action. From the other hand, whenever a multicomponent probiotic product is created, the ability of the strains to exist with each other should be considered.

The microbiota that colonises biological systems and organs of macroorganism is known to be multicomponent. Probiotic strains interact in the own microflora biocenosis of the macroorganism, as well as with other microorganisms from different taxonomic groups. It is known that the ability to inhibit a growth and a reproduction of pathogenic and opportunistic microorganisms is one of the most important criterias for assessing the effectiveness of probiotic strains. An antagonistic activity of the normal microflora is one of the mechanisms of colonization resistance of the macroorganism.

However, it is very important that strains action to be synergistic or at least indifferent in relation to the host organism native microflora. In this regard, investigated strains that demonstrated the ability to synthesize extracellular tannase, as well as the reference strain, have been studied for biocompatibility with each other. The study was conducted using perpendicular strokes and a modified droplet method (common cultivation method) [18].

The results of the study showed that strains *L. rhamnosus LB3* and *L. delbrueckii subsp. delbrueckii* are biocompatible; while strains *L. bulgaricus LB51* and *L. plantarum 2621* do not demonstrate biocompatibility with any of the other investigated strains (Table 3). It worth to be noticed, that both methods of determining the biocompatibility of lactobacillus showed correlative results.

According to the results of biocompatibility test, 2 biocompatible strains (*L. rhamnosus LB3* and *L. delbrueckii subsp. delbrueckii*) were selected for further research as potential components of bi-component probiotic. For the *L. bulgaricus LB51* strain, it was decided not to conduct further studies due to the relatively low tannase activity and incompatibility with other strains. *L. plantarum 2621*, which has the highest tannase activity may possibly have the interest as a mono-probiotic with antioxidant properties.

While creating the technology of a biotechnological product, the focus is primarily put on the development of the technological parameters that provide an increase in the output of the target product. These parameters include optimization of nutrient components

Table 2. **Tannase activity of investigated *Lactobacillus* strains**

LAB Strain	Tannase activity, U/ml			
	24 hours-incubation	% from control*	48 hours incubation	% from control*
L. murinus LE	0	–	0	–
L. rhamnosus LB3	0.019 ± 0.001	79.2	0.031 ± 0.002	60.8
L. acidophilus (C)	0	–	0	–
L. rhamnosus (C)	0	–	0	–
L. bulgaricus LB51	0.009 ± 0.001	37.5	0.013 ± 0.001	25.5
L. delbrueckii subsp. *bulgaricus LB86*	0	–	0	–
L. delbrueckii subsp. *delbrueckii*	0.017 ± 0.001	70.8	0.030 ± 0.002	58.8
L. murinus	0	–	0	–
L. plantarum	0	–	0	–
L. plantarum 2621 (reference strain)	0.024 ± 0.001	100	0.051 ± 0.002	100

*Note:** — the percentage of the level of ectracellular tannase synthesis by the reference strain at the seame time of incubation.

Table 3. **The biocompatibility of tannase active *Lactobacillus* strains**

Strains	*L. rhamnosus LB3*	*L. bulgaricus LB51*	*L. delbrueckii* subsp. *delbrueckii*	*L. plantarum 2621*
L. rhamnosus LB3	++	– / +	+ / +	+ / –
L. bulgaricus LB51	+ / –	++	– / –	– / +
L. delbrueckii subsp. *delbrueckii*	+ / +	– / –	++	– / –
L. plantarum 2621	– / +	+ / –	– / –	++

Note: "+" — growth of the culture; "–" — lack of growth of the culture;
"*/*" — "growth characteristics of the strains located in the left vertical column of the table/ the growth characteristic of the strains located in the horizontal column".

composition of the medium and incubation conditions. It is known that the ingredients composition of the medium and the conditions of incubation are key factors influencing the growth rate of the microorganism-producer and the formation of metabolites. Very often the high cost of microbial preparation production, particuralry biomass obtaining stage, is a major restrictive factor of scaling it up to industrial production. Typically, such components of the medium as the inducer (tannic or gallic acids) and the carbonaceous compounds that directly affect the growth and enzymatic activity of the microorganism-producer, are the most costly for production [11, 21].

Despite of a growing scientific interest to the research of the repression and induction processes of microbial tannase, there still Today, there are still many controversies about the repression and induction of tannase, and the literature data is not full enough, therefore it makes a clear the necessity for thorough research of the mechanisms governing the biosynthesis of tannase.

For a long time, it was believed that the tannase enzyme is possible only in the presence of tannic acid in the medium. However, it was discovered later that the activity of microorganisms-producers has often been manifested also under cultivation on media with different carbon sources — monosaccharides, disaccharides, polysaccharides, even without adding an inductor [22].

It worth to be noticed, that one of the crucial growth factors is the type of the secondary carbon sources adding to the medium, such as

glucose, galactose, mannose, lactose, fructose, etc. Adding these components reduces the lag phase and promotes the growth of the microorganisms. From this point of view, glucose is known as the most considerable compound among the carbon sources. There is the literary evidence that lower glucose concentration contributes to the induction of tanase, whereas higher concentration causes even catabolic repression of the enzyme's biosynthesis [23]. Such a phenomenon can be explained by the emergence of an imbalance in the ratio of carbon and nitrogen in the medium that causes osmotic stress and as a result inhibits the enzyme biosynthesis [24, 25].

As it was mentioned earlier, the medium which is used to produce microbial products does not always ensure the maximum accumulation of biomass. That is very important to be taken into account in the production of probiotics because one of the most important technological parameters of the production of the biotechnological products is the level of accumulation of the target product. In the case of probiotics production, the target product is the biomass of the culture.

Therefore, in order to determine the prospect for the industrial application, the growth analysis of investigated LAB strains was held using the medium MRS (Himedia) and as well as modified MRS medium for microorganisms cultivation. The diagrams with results of the experiment of the growth of strains *L. plantarum 2621*, *L. rhamnosus LB3*, *L. delbrueckii subsp. delbrueckii* are shown in Fig. 1.

The diagram shows that LAB strains *L. rhamnosus LB3* and *L. delbrueckii subsp. delbrueckii* have significantly higher growth intensity compared to the reference strain *L. plantarum 2621*. The obtained data demonstrate that the intensity of biomass accumulation of all investigated strains grown on the industrially produced ready-made MRS medium (Himedia) and on the modified medium prepared from individual components has no statistically significant difference. Such results provide the basis for further research using the modified MRS medium in terms of economic expediency.

Taking into account the existing literature data and the aim of our research, it was decided to study the growth of LAB strains in a modified medium MRS with different carbon-containing components as well as in the absence or adding of an inductor in the medium (gallic acid at a concentration of 2%). Mono and disaccharides, such as glucose, galactose, lactose, fructose and sucrose, at a concentration of 20 g/l, were used as carbon sources. The results of the experiment are presented in the diagrams (Fig. 2–4).

As results of experiment have shown, the rate of biomass accumulation and tannase activity of all investigated strains depends on the type of carbon source contained in nutrient medium. It is important to note that the maximum performance of all investigated microorganisms was demonstrated while using glucose. Glucose has provided both the highest level of biomass accumulation and the

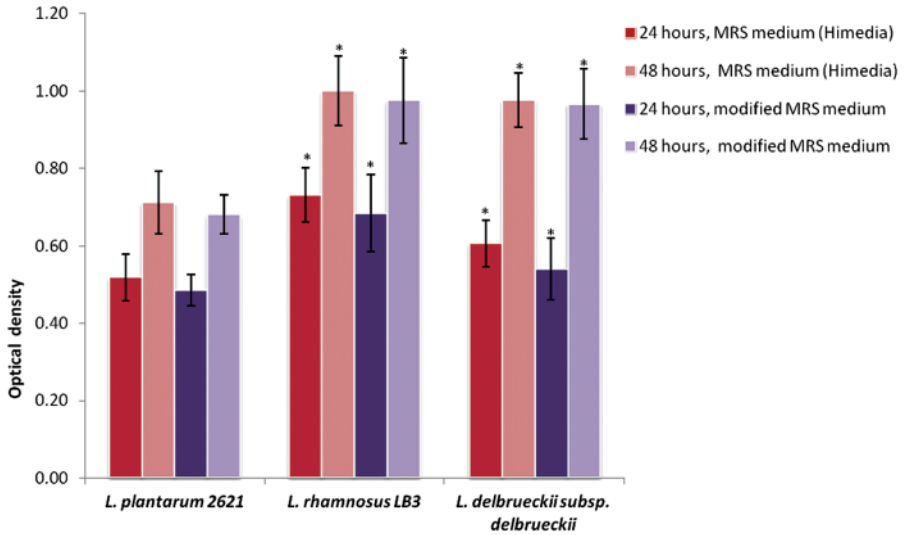

Fig. 1. **Comparative characteristic of growth intensity of the strains with tannase activity on MRS medium (Himedia) and modified MRS medium:**
values represented mean ± SEM. Values for strains *L. rhamnosus* LB3 and *L. delbrueckii* subsp.*delbrueckii* were compared with reference strain *L. plantarum* 2621. *$P < 0.05$ compared to the reference strain on the same medium at the same time of experiment

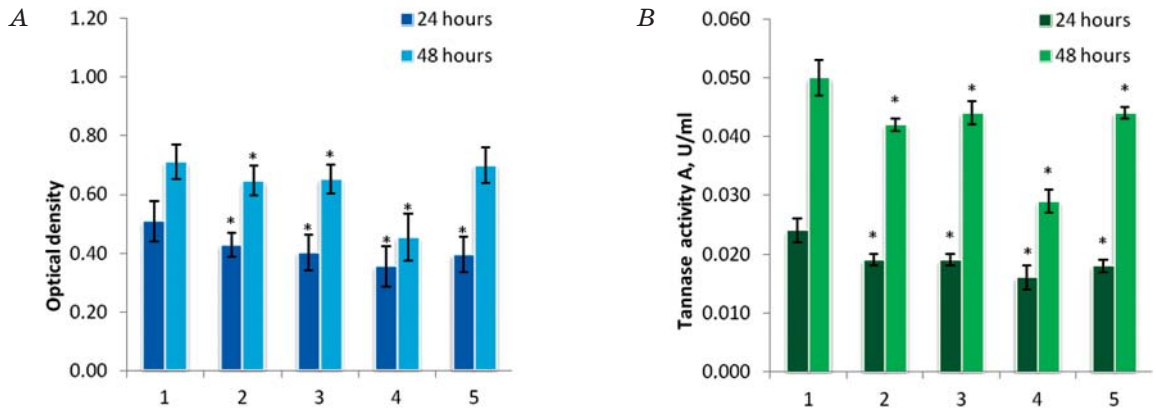

Fig. 2. **The biomass accumulation (*A*) and tannase activity (*B*) of strain *L. plantarum 2621* on the medium with the different carbon sources:**

Here and after: 1 — glucose; *2* — lactose; *3* — galactose; *4* — fructose; *5* — sucrose

*$P < 0.05$ compared to the values for the strain growth on the medium with glucose (1) at the same time of experiment

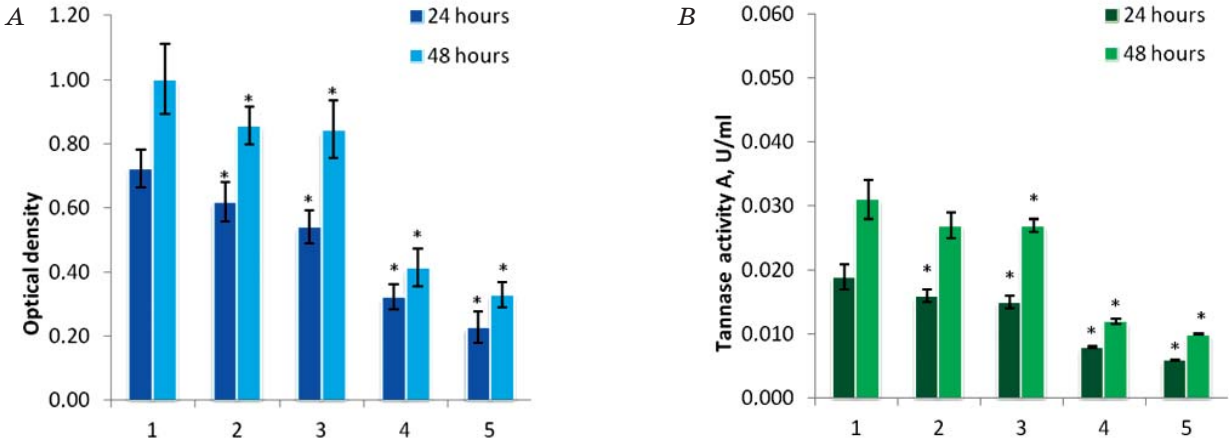

Fig. 3. **The biomass accumulation (*A*) and tannase activity (*B*) of strain *L. rhamnosus* LB3 on the medium with the different carbon sources**

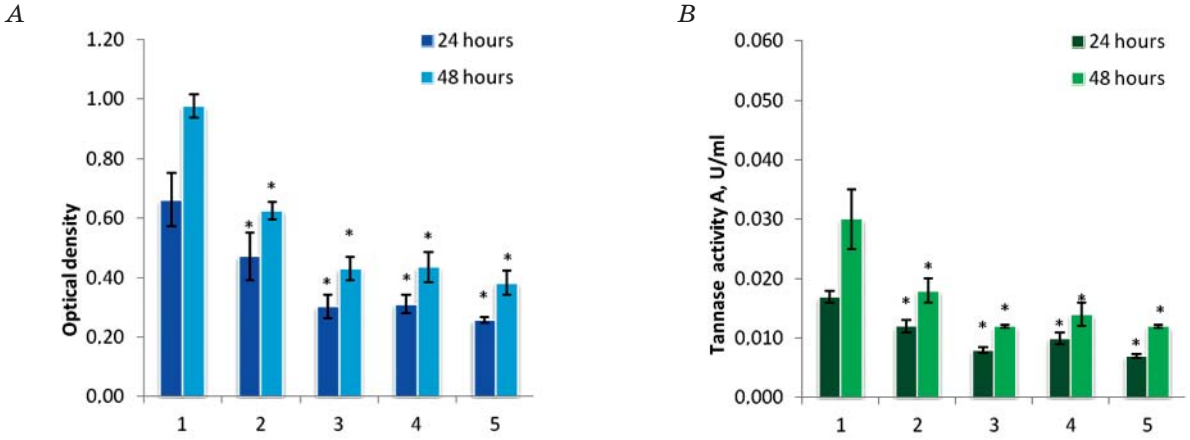

Fig. 4. **The biomass accumulation (*A*) and tannase activity (*B*) of strain *L. delbrueckii* subsp. *delbrueckii* on the medium with the different carbon sources**

Table 4. ***Lactobacillus*** **strain growth rate on medium with different sources of carbon in the presence and absence of gallic acid**

The biomass accumulation of *L. plantarum 2621*, OD										
gallic acid (+/–)	*glucose*		*lactose*		*galactose*		*fructose*		*sucrose*	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
+	0.51± 0.04	0.72± 0.02	0.43± 0.08	0.65± 0.02	0.40± 0.01	0.65± 0.03	0.36± 0.03	0.45± 0.03	0.40± 0.02	0.36± 0.02
–	0.47± 0.02	0.72± 0.03	0.41± 0.04	0.64± 0.03	0.40± 0.02	0.65± 0.03	0.34± 0.02	0.42± 0.02	0.47± 0.03	0.39± 0.05
The biomass accumulation of *L. rhamnosus LB3*, OD										
gallic acid (+/–)	*glucose*		*lactose*		*galactose*		*fructose*		*sucrose*	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
+	0.72± 0.01	1.01± 0.02	0.62± 0.02	0.86± 0.05	0.54± 0.03	0.85± 0.04	0.33± 0.03	0.41± 0.03	0.23± 0.01	0.30± 0.02
–	0.71± 0.03	1.01± 0.03	0.56± 0.07	0.80± 0.02	0.53± 0.04	0.78± 0.04	0.27± 0.02	0.41± 0.03	0.25± 0.04	0.34± 0.03
The biomass accumulation of *L. delbrueckii subsp. delbrueckii*, OD										
gallic acid (+/–)	*glucose*		*lactose*		*galactose*		*fructose*		*sucrose*	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
+	0.67± 0.02	0.97± 0.04	0.48± 0.02	0.62± 0.04	0.30± 0.02	0.43± 0.03	0.38± 0.02	0.44± 0.04	0.26± 0.04	0.38± 0.02
–	0.64± 0.03	0.97± 0.04	0.48± 0.05	0.65± 0.04	0.31± 0.03	0.40± 0.02	0.36± 0.03	0.42± 0.03	0.24± 0.02	0.38± 0.03

maximum level of tannase activity. Lactose may be considered as an alternative source of carbon for *L. rhamnosus LB3* and *L. plantarum 2126*. Biocompatible strains *L. rhamnosus LB3* and *L. delbrueckii subsp. delbrueckii* showed the lowest tannase activity while cultivated on sucrose-containing medium, and strain *L. plantarum 2621* on the medium with fructose.

It is known that the tannase activity of lactobacilli induces by gallic acid, which is one of the tannin decomposition products. However, gallic acid has antioxidant properties and, as the result, could effect on the growth of probiotics microorganisms. The comparative characteristics of growth intensity of the studied microorganisms on the medium with different sources of carbon under the conditions of the presence of gallic acid and in its absence are presented in Table 4.

As can be seen from the results presented in the table, the presence of the inductor did not affect LAB growth (there was no statistically significant difference between values of LAB growth), but during the experiment, none of the examined strains showed tannase activity in its absence. Therefore, the obtained results suggest that for the investigated Lactobacilli strains, the presence of the inductor in the cultivation medium is a prerequisite for the implementation of the tannase activity but, on the other hand, does not affect the accumulation of biomass of bacterial cultures.

Thus, as a result of screening, two tannase-positive and biocompatible strains *L. rhamnosus LB3* and *L. delbrueckii subsp. delbrueckii* were selected and considered as promising microorganisms-producers for creating a new bi-component probiotic with antioxidant properties. Both strains showed the most rapid growth in the presence of glucose in the cultivation medium. At the same time, according to the results of the study lactose can be considered as a carbon source in the composition of nutrient medium because of a pretty high level of biomass accumulation demonstrated by selected lactic acid bacterias. Gallic acid which is essential for tannase enzyme synthesis induction, did not reduce the growth rate of any strain, therefore, it can be used as a component of the medium for probiotic strains cultivating.

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Lactobacillus ЯК ПРОДУЦЕНТИ ПОЗАКЛІТИННОЇ ТАНАЗИ

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Метою роботи був пошук штамів молочнокислих бактерій, здатних синтезувати позаклітинний ензим таназу, необхідний для розщеплення харчових танінів. Одним з продуктів ензиматичної трансформації танінів є галова кислота, яка, в свою чергу, виявляє антиоксидантні та онкопротекторні властивості. У результаті скринінгу відібрано два біосумісні штами молочнокислих бактерій — *L. rhamnosus* LB3 та *L. delbrueckii* subsp. *delbrueckii* з високим рівнем продуктивності ензиму. Максимум накопичення танази, що відповідав рівню 0,031 ± 0,002 U/ml для *L. rhamnosus* LB3 і 0,03 ± 0,002 U/ml для *L. delbrueckii* subsp. *delbrueckii*, спостерігали через 48 год культивування. Для обох штамів показано швидкий ріст та продуктивність танази на середовищі MRS у присутності глюкози або лактози як джерела вуглецю. Виявлено, що галова кислота, яка була необхідним компонентом середовища як індуктор цільового ензиму, не впливала на накопичення біомаси лактобактерій. Відібрані штами становлять інтерес як продуценти двокомпонентного пробіотику з антиоксидантними властивостями та потребують подальшого дослідження.

Ключові слова: лактобактерії, пробіотики, таназа, антиоксидант, джерела вуглецю.

Lactobacillus КАК ПРОДУЦЕНТЫ ВНЕКЛЕТОЧНОЙ ТАННАЗЫ

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Целью работы был поиск штаммов молочнокислых бактерий, способных синтезировать внеклеточную танназу — энзим, необходимый для расщепления пищевых таннинов. Одним из продуктов энзиматической трансформации таннинов является галловая кислота, которая обладает антиоксидантными и онкопротекторными свойствами. В результате скрининга отобраны два биосовместимых штамма молочнокислых бактерий — *L. rhamnosus* LB3 и *L. delbrueckii* subsp. *delbrueckii* с высоким уровнем производительности энзима. Максимум накопления танназы, соответствующий уровню 0,031 ± 0,002 U/ml для *L. rhamnosus* LB3 и 0,03 ± 0,002 U/ml для *L. delbrueckii* subsp. *delbrueckii*, наблюдали через 48 ч культивирования. Для обоих штаммов показаны быстрый рост и производительность танназы на среде MRS в присутствии глюкозы или лактозы в качестве источника углерода. Выявлено, что галловая кислота, которая была необходимым компонентом среды как индуктор целевого энзима, не влияла на накопление биомассы лактобактерий. Отобранные штаммы представляют интерес как продуценты двухкомпонентного пробиотика с антиоксидантными свойствами и требуют дальнейшего исследования.

Ключевые слова: лактобактерии, пробиотики, танназа, антиоксиданты, источники углерода.